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Review Article

Tetrazine–*trans*-cyclooctene ligation: Unveiling the chemistry and applications within the human body

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ABSTRACT

Bioorthogonal reactions have revolutionized chemical biology by enabling selective chemical transformations within living organisms and cells. This review comprehensively explores bioorthogonal chemistry, emphasizing inverse-electron-demand Diels-Alder (IEDDA) reactions between tetrazines and strained dienophiles and their crucial role in chemical biology and various applications within the human body. This highly reactive and selective reaction finds diverse applications, including cleaving antibody-drug conjugates, prodrugs, proteins, peptide antigens, and enzyme substrates. The versatility extends to hydrogel chemistry, which is crucial for biomedical applications, yet it faces challenges in achieving precise cellularization. In situ activation of cytotoxic compounds from injectable biopolymer belongs to the click-activated protodrugs against cancer (CAPAC) platform, an innovative approach to tumor-targeted prodrug delivery and activation. The CAPAC platform, relying on click chemistry between *trans*-cyclooctene (TCO) and tetrazine-modified biopolymers, exhibits modularity across diverse tumor characteristics, presenting a promising approach in anticancer therapeutics. The review highlights the importance of bioorthogonal reactions in developing radiopharmaceuticals for positron emission tomography (PET) imaging and theranostics, offering a promising avenue for diverse therapeutic applications.

1. Introduction

Bioorthogonal reactions are selective chemical reactions that do not interfere with the complex functionality of biological systems [1]. Bioorthogonal chemistry has become a vital tool in chemical biology. It allows organic synthesis within living organisms and cells, functioning *in vivo* without being toxic or disrupting normal physiological responses [2]. These reactions aim to covalently modify biomolecules with non-native functional groups under physiological conditions, facilitating their examination and manipulation [3]. For a reaction to be bioorthogonal, it must function effectively under physiological conditions, generate products selectively, remain unaffected by the biological environment's components, and involve non-natural functional groups while being fast and yielding stable products.

These reactions are relatively new in synthetic chemistry and have led to innovative methods in compound library synthesis, protein engineering, functional proteomics, and the modification of cell surfaces [4]. Moreover, they can be performed in living animals without causing harm, showing promise for noninvasive imaging and therapeutic applications.

One of the most frequently employed bioorthogonal reactions is the copper-catalyzed azide-alkyne cycloaddition (CuAAC) that, thanks to the rapid rate of the reaction between azides (1,3-dipole) and alkynes (dipholarophile), produces 1,2,3-triazole (Fig. 1) [5]. However, the toxicity of copper in biological environments has limited the application of this reaction *in vivo*. To overcome this issue, in 2004, Bertozzi *et al.* developed a novel catalyst-free reaction called strain-promoted azide-alkyne cycloaddition (SPAAC), also known as Cu-free click chemistry [6]. Thanks to its notable selectivity and availability of azide-modified monosaccharide precursors, this reaction is extensively employed for modifying and imaging biomolecules in living cells [7]. Unfortunately, this reaction type has a lower reaction rate than CuAAC [2].

As reagents for biorthogonal reactions, azides have also been reported in the Staudinger ligation with phosphines [8]. The Staudinger ligation exhibits remarkable selectivity and consistently produces its product even in challenging environments like live mice [9]. In fact,

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while azides form a stable triazole linkage in CuAAC and SPAAC reactions, the Staudinger ligation results in a stable amide bond [10]. Nonetheless, the Staudinger ligation has certain drawbacks, including the susceptibility of phosphine reagents to air oxidation and the relatively sluggish kinetics of the reaction [11].

Bioorthogonal reactions include strain-promoted cycloadditions involving nitrones and alkynes and photoclick 1,3-dipolar cycloadditions between tetrazoles and substituted alkenes (Fig. 1) [12]. Thanks to the fast kinetics and ambient reaction conditions, they have a broader application, especially for bioconjugation. However, their use is limited to *in vitro* studies due to the requirement of specific reaction conditions such as acidic pH and a light source in the case of ketones or aldehydes and tetrazoles, respectively [13]. Among various advancements in this field, the inverse electron demand Diels-Alder (IEDDA) reaction between tetrazines and strained dienophiles fulfills the ideal criteria for a biorthogonal reaction. This reaction was first reported in 2008 by Blackman *et al.* [14] and Devaraj *et al.* [15]. This includes rapid kinetics, strong selectivity, physiological reaction conditions in a biological setting, and being free of metal. Unlike typical Diels-Alder reactions, IEDDA occurs between an electron-rich dienophile and an electron-poor diene. Specifically, the diene-tetrazine system reacts with an alkene, forming a strained intermediate that undergoes a *retro*-Diels Alder reaction, releasing N_2, to give a 4,5-dihydropyridazine (Fig. 2). This compound then goes through isomerization to produce the corresponding 1,4-dihydro isomers followed by the formation of the resultant pyridazine product. The kinetics of the reaction is governed by the energy gap between the HOMO and LUMO of the IEDDA partners; the smaller this gap is, the faster the reaction proceeds, with rate constants ranging from 1 to 10^6 M^{-1} s^{-1}. The reactivity of diene and dienophile in IEDDA reactions is influenced by several factors, such as the substituents of the two partners, strain, stereochemical, steric, pH and solvent. All of these factors are discussed extensively in other reviews [13].

Here, we will analyze a particular type of IEDDA reaction between tetrazine (Tz) and *trans*-cyclooctene (TCO), known as Tz–TCO ligation, belonging to the click-to-release reactions, that forms pyridazine through 1,4-dihydropyridazine intermediate [16]. This highly reactive and selective IEDDA pyridazine elimination reaction finds extensive application both *in vivo* and *in vitro*. Its uses span from cleaving TCO-containing antibody-drug conjugates (ADCs) [17], prodrugs [18], proteins [19], and peptide antigens [20], to administering a Tz activator. Additionally, it extends to uncaging fluorogenic compounds and enzyme substrates [21], delivering oligonucleotides into cells [22], labeling cell-specific proteomes [23], and purifying solid-phase synthesized oligonucleotides [24].

Biorthogonal reactions are also widely applied in hydrogel chemistry, representing relevant materials for biology, medicine, and bioengineering applications [3].

These crosslinked polymeric materials are handy for developing injectable protein delivery systems or constructing artificial tissues by allowing precise control over interactions between cells and materials [25,26]. While several studies have demonstrated the formation of hydrogels using click reaction mechanisms, achieving precise in situ

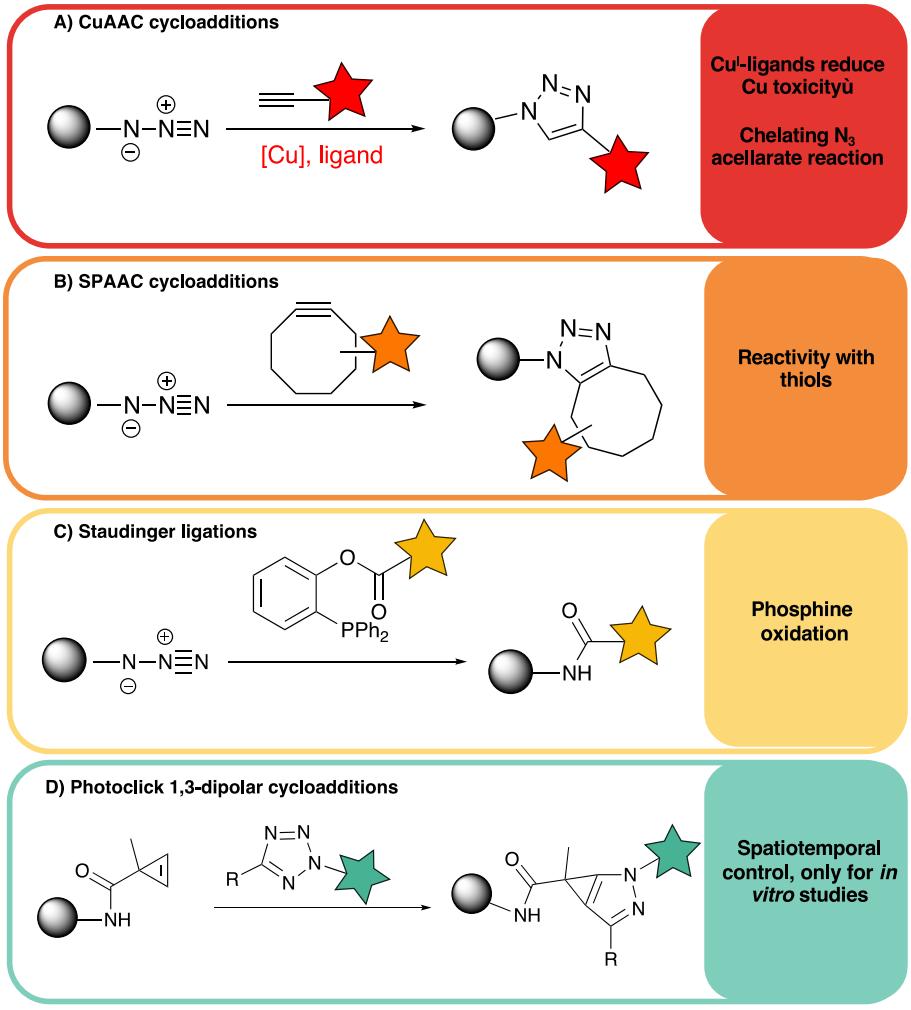


Fig. 1. Reported click-chemistry reactions: A) Copper-catalysed azide-alkyne (CuAAC) cycloadditions; B) Strain-promoted alkyne-azide (SPAAC) cycloadditions; C) Staudinger ligation (azide-phosphine conjugation); D) Click 1,3 dipolar cycloaddition.

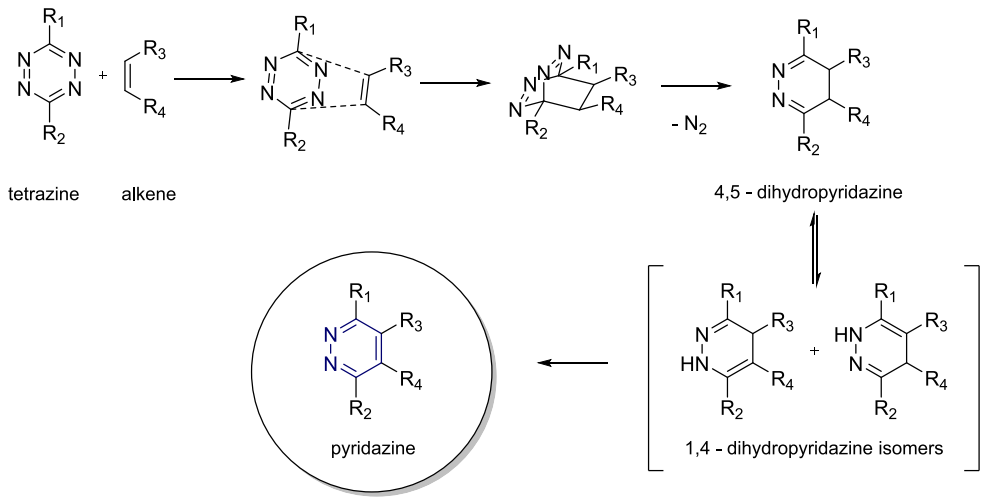


Fig. 2. Mechanism of IEDDA reaction between a generic Tz and alkene.

local cellularization of implanted materials through bioorthogonal methods remains a challenge. This limitation hinders meeting preclinical and clinical research application requirements [27].

These chemoselective cycloaddition reactions are also extensively utilized for the chemical modification and synthesis of radiopharmaceuticals derived from biomolecules for positron emission tomography (PET). The next chapter will highlight these reactions' potential in pretargeted PET imaging, demonstrating their versatility and significance in molecular imaging.

These click chemistry-based reactions have been employed in the CAPAC (Click Activated Protodrugs Against Cancer) platform. This innovative platform relies on a click chemistry-based reaction between a modified attenuated protodrug with *trans*-cyclooctene (TCO) and a biopolymer modified with Tz to elevate the antitumor efficacy while minimizing systemic toxicity. Notably, CAPAC is versatile across varying tumor characteristics in different patients, offering a highly modular approach applicable to a wide range of therapeutics.

Overall, this review focuses on the noteworthy applications of bioorthogonal chemistry in drug discovery. In particular, the tumor-targeted prodrug delivery and activation, radiolabeled peptides, bioorthogonal hydrogels, and CAPAC platform will be discussed.

2. Click to release: A bioorthogonal tool for bond cleavage reaction

Bioorthogonal reactions were initially used to manipulate molecules to label and track biomolecules *in vitro*, taking advantage of precise and selective bond cleavage within the context of living organisms. A limitation arises when attempting to achieve selective bond cleavage *in vivo* [28,29]. In this context, Robillard's research group developed a new type of bond cleavage elimination reaction based on the IEDDA reaction, later named "click to release", that represents a satisfactory system for prodrug activation allowing instantaneous drug elimination and release in a living system [30]. They exploited the fast IEDDA reaction between Tz and TCO, where the final formation of aromatic pyridazine can be achieved by eliminating a leaving group from the vinyl position of TCO or by a double bond shift, depending on the substituent. In this system, the 1,4-dihydropyridazine, obtained from Tz and TCO with a carbamate-linked drug in the allylic position, is converted to a conjugated pyridazine through the formation of an exocyclic double bond followed by the elimination of CO_2 along with the release of the NH_2-substituted drug (Fig. 3). The triggering event seems to be the delocalization of the electron lone pair of NH into the ring that leads to an electronic cascade-

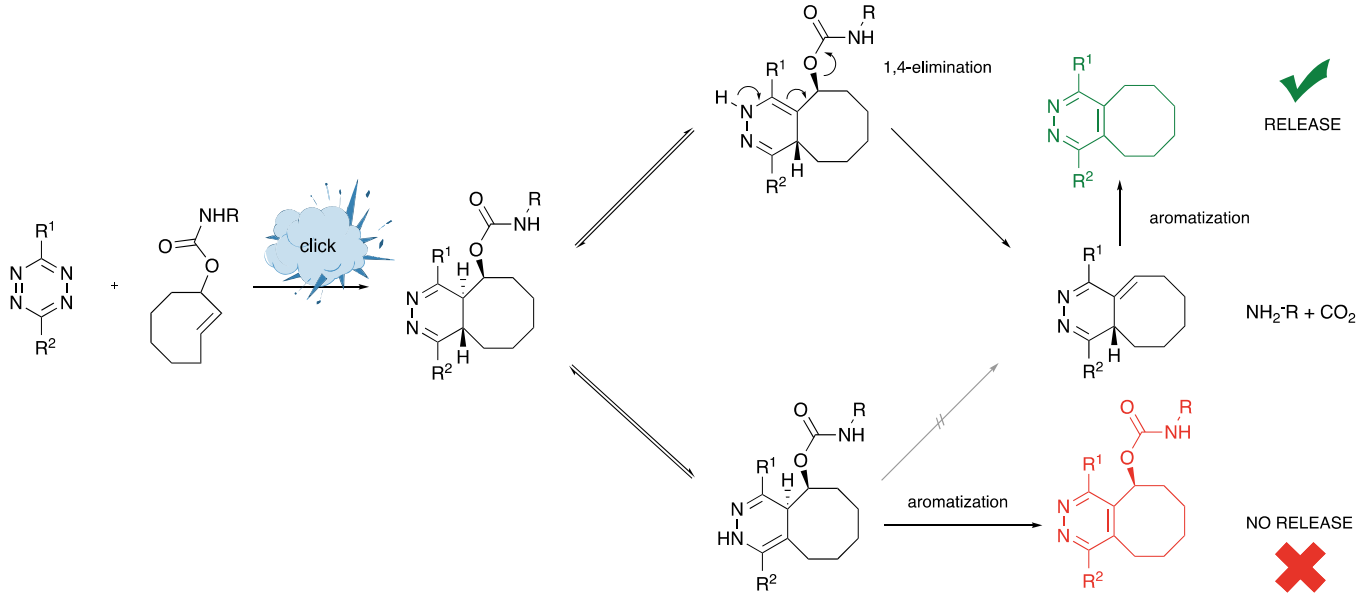


Fig. 3. General scheme of IEDDA reaction between Tz and TCO.

based leading to the drug release. An alternative proposal, although less validated, is that the 4,5-tautomer eliminates the carbamic acid portion, giving rise to the conjugated derivative.

Test reactions with TCO model compounds, (*E*)-cyclooct-2-en-1-yl benzylamine carbamate and (*E*)-cyclooct-2-en-1-yl doxorubicin carbamate, and electron-dense tetrazines (such as 3,6-bis(2-pyridyl)-1,2,4,5-tetrazine, 3-(2-pyridyl)-6-methyl-1,2,4,5-tetrazine, and 3,6-dimethyl-1,2,4,5-tetrazine) have been performed to generate a delivery system for Antibody Drug Conjugate (ADC) (Fig. 4). It was found that IEDDA reaction and subsequent carbamate cleavage can generate multiple interconverting stereoisomers and tautomers with relative abundances controlled by Tz substituents R^1 and R^2. Elimination occurs via carbamic acid release and is influenced by the Tz's nature (*e.g.*, electron density) (bulky substituent on Tz slows the reaction rate). In any case, the drug is eliminated within minutes, yielding 79 % under ambient conditions at micromolar concentrations.

The drug release rate, measured *in vitro*, was shown to be highly dependent on the surrounding environment [31]. Carlson *et al.* investigated the impact of variations in organic solvent, buffer concentration, and pH on the post-click tautomerization rate, a crucial factor for drug release. The elevated drug release in acidic conditions and higher buffer concentrations suggest a potential advantage for integrating a nearby proton donor at the tautomerization sites. This could lead to an ultra-fast release, depending on the proximity between the solution pH and the pK_a of the protonic acid. In this context, the carboxylic acid plays a role in protonating the adjacent nitrogen in dihydropyridazine, leading to a reaction orientation in two possible directions: one facilitates the formation of the tautomer for rapid release (Fig. 5a), while the other directs the formation of the non-releasing tautomer (Fig. 5b). Studies on the non-releasing tautomer revealed its ability to impede the continuation of the reaction by forming a highly stable intramolecular cyclization product that undergoes slow oxidation. Researchers evaluated the *N*-Me substitution for TCO carbamate to enhance the efficacy of the reaction and prevent the development of dead-end products, as this process excludes tertiary amide cyclization (Fig. 5b,c). This modification effectively increased the reaction yield while avoiding the generation of intramolecular adducts.

The elimination process is more influenced by the formation of the 1,4-dihydropyridazine release tautomer than by the characteristics of the leaving group. To support this observation, Robillard and colleagues showed how the click-to-release reaction between TCO and Tz can be used to cleave carbamate-bound drugs and release drugs from TCO

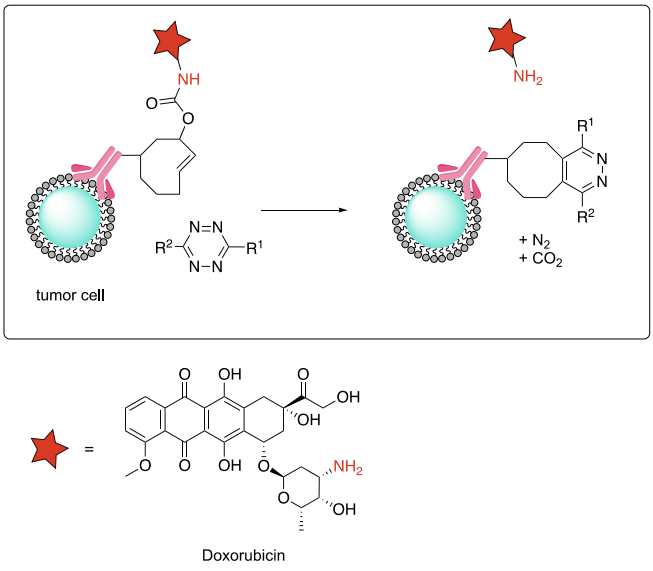


Fig. 4. Release and activation of Doxorubicin from Antibody Drug Conjugate (ADC) by IEDDA click to release-based elimination reaction.

esters, carbonates, and ethers [32]. Mechanistic studies have revealed that the elimination process is primarily governed by the formation of the rapidly eliminating 1,4-dihydropyridazine tautomer and is less influenced by the nature of the leaving group.

Unlike the widely used *p*-aminobenzyloxy linker, which selectively cleaves aromatic but not aliphatic ethers, the aromatic, benzylic, and aliphatic TCO ethers demonstrated efficient cleavage comparable to carbamates, carbonates, and esters. Bioorthogonal ether release was demonstrated using TCO-masked l-tyrosine as a model system. Tyrosinase in serum at 37 °C did not cause any oxidation when incubated with this model. However, the system showed efficient uncoupling when incubated with Tz, as evidenced by the enzymatic oxidation of the released tyrosine to L-dopaquinone. The uncaging of tyrosine was utilized to control cell growth chemically in a tyrosine-free medium. This release system enables the liberation of different molecules like lysine, tyrosine, serine, and glutamic acid, thereby creating novel opportunities for activating proteins and unmasking other biomolecules such as carbohydrates. Click-to-release reactivity could lead to ADC and prodrug approaches with drugs that lack a modifiable amine.

As mentioned above, the reaction's high reactivity enables the payload to be released within seconds in certain instances, making the system suitable for the rapid release of toxic or highly reactive drugs *in vivo*. Despite the well-established understanding of the reactivity of the Tz-TCO system, accurately determining release kinetics at physiologically relevant concentrations remains a challenge.

A recent method, which utilizes TCO-linked 5-[(2-aminoethyl) amino]naphthalene-1-sulfonic acid (EDANS) as fluorophore and 4-[4-(dimethylamino)phenylazo]benzoyl (DABCYL) as a quencher, was reported to assess the capacity of a range of tetrazines to catalyze IEDDA elimination of the pyridazine. So, it could be used as a screening method to choose the Tz with the best substituents for the release system in consideration.

The TCO reagent also influences the kinetics of the reaction; it is known that an increase in strain energy and a non-crown conformation can accelerate the reactivity towards tetrazines [33].

In this regard, Delft *et al.* proved that cyclooctyne-based conjugations can be speeded by introducing a cyclopropane ring linker on TCO [7]. *Cis*-fusion of a cyclopropane forces the TCO ring to adopt a highly strained "half-chair" conformation, making it an excellent click-to-release linker. The downside is that TCO reactivity decreases linker stability *in vivo* due to interaction with copper-containing proteins, increasing the isomerization rate in less reactive *cis*-cyclooctene [34]. *Cis*-cyclooctene is 5/7 orders less reactive than *trans*-cyclooctene toward tetrazines [7,17].

Inverse-electron demand Diels-Alder click reaction has also been used to release H_2S. Hydrogen sulfide (H_2S) is an important biomolecule with high therapeutic potential, and several small molecule H_2S donors have already entered clinical trials [35]. Carbonyl sulfide (COS) represents a novel approach to developing H_2S donors [36]. Bioorthogonal activation of $COSH_2S$ release through the IEDDA click reaction provides a platform for precise temporal control over H_2S release, offering a novel strategy for developing efficient and targeted H_2S donors in biological systems [37]. The IEDDA reaction facilitates the formation of a dihydropyridazine intermediate that undergoes self-immolative decomposition, releasing COS and H_2S in the presence of carbonic anhydrase (CA). Experimental validation confirmed the click reaction and the subsequent gasotransmitter release. Additionally, the system functions efficiently in complex biological environments, including whole blood, highlighting its potential for *in vivo* applications.

3. Radio-and fluorescent-labeling by IEDDA reactions

Pretargeted radioimmunotherapy (PRIT) represents a promising advancement in cancer treatment, particularly for patients with solid tumors and hematologic malignancies [38]. Traditional radioimmunotherapy (RIT) directly couples a radioactive isotope to an

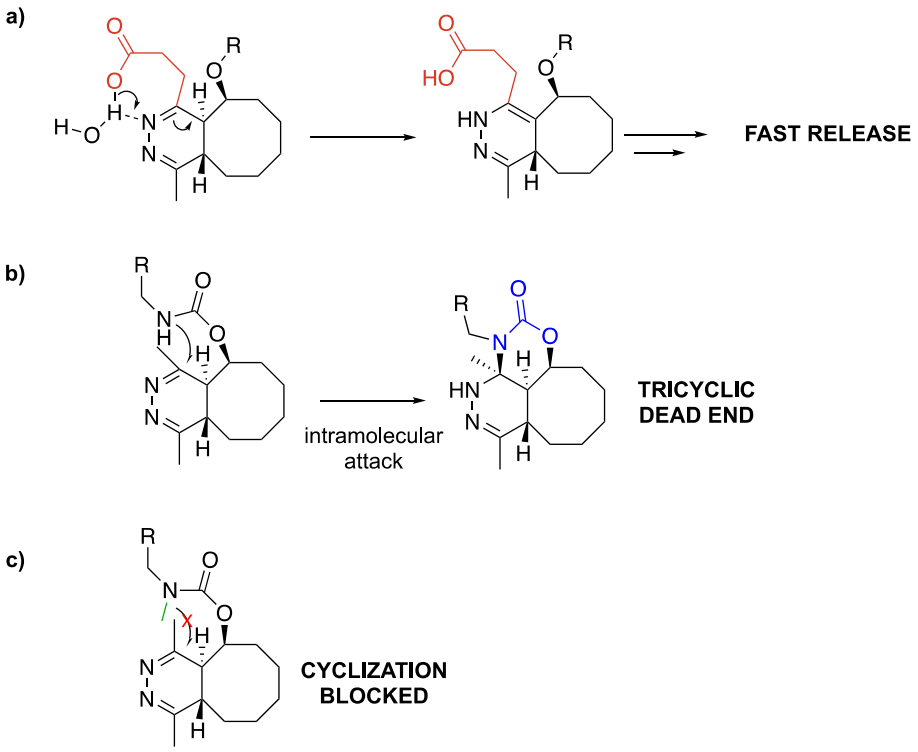


Fig. 5. Various effects that influence the evolution of Tz-TCO reaction.

antibody that targets tumor-specific antigens, delivering radiation precisely to cancer cells. While effective, this approach often faces challenges such as off-target toxicity and limited tumor penetration due to the prolonged circulation time of radio-labeled antibodies. PRIT addresses these limitations by separating the targeting and radioactive components into distinct steps. A bispecific antibody or other targeting molecule is initially administered, specifically binding to tumor antigens. After allowing sufficient time for the unbound antibodies to clear from the bloodstream, a radioactive agent designed to bind to the localized targeting molecule is introduced. This sequential administration minimizes systemic exposure to radioactivity and enhances tumor-to-background radiation ratios [39]. PRIT decreases the risk of side effects and enables higher therapeutic doses for more effective tumor eradication. Furthermore, decoupling the targeting and radiolabeling phases allows for short-lived isotopes, which can provide high radiation doses while minimizing long-term radiation exposure.

Recent advancements in bioorthogonal chemistry, such as the inverse electron-demand Diels-Alder (IEDDA) reaction, are suitable for theranostic applications in nuclear imaging and radioimmunotherapy since it is a bioorthogonal and rapid (k_2 30.000 $M^{-1}s^{-1}$) reaction, making it ideal for *in vivo* applications. It allows for specific and efficient ligation of the radioligand to the vector *in vivo*, ensuring precise targeting of cancer cells while minimizing radiation exposure to healthy tissues [40]. IEDDA reaction guarantees that the drug/radiometal remains intact until it reaches its target within the body, enhancing the efficacy and safety of the therapy. It offers high radiochemical yields, purity, and molar activity without further purification, ensuring the integrity of the drug/radiometal for effective theranostic use.

IEDDA is an invaluable bioconjugation technique for radiolabeling larger proteins, making it a versatile and powerful tool in targeted radiotherapy and diagnostics [41]. This approach allows the antibody to reach its target within the body, aiming to reduce radiation doses to healthy tissues and enhance the specificity of PET imaging and radioimmunotherapy applications.

However, this ligation method presents a drawback in biomolecule-based radiopharmaceutical production: developing a mixture containing reduced metastable dihydropyridazines and oxidized cycloadducts [42]. The transformation of the reduced DHPs into stable pyridazines typically involves oxidation, traditionally accomplished through oxidants or photo-irradiated air oxidation. Both methods necessitate additional reagents or extended reaction times, making them incompatible with short-lived radionuclides. Otaru *et al.* [43] developed a mild, swift, and catalyst-free approach for converting DHPs to pyridazines. They used this method to produce Fluorine-18-labeled alkylammoniomethyltrifluoroborate ([^{18}F]AmBF$_3$) tetrazines conjugated to the TCO-TOC analogs at room temperature, enabling rapid synthesis of PET imaging agent candidates. By heating the radiolabeled peptide at 60 °C, the metastable DHPs, produced from IEDDA-based Tz ligation, were oxidized to pyridazines within the timeframe allowed by the physical half-life of Fluorine-18 ($t_{½}$ = 109.8 min).

The IEDDA reaction was also used as a versatile method for conjugating Rituximab, an approved drug for treating hematological malignancies like diffuse large B cell lymphoma [44]. This approach enabled the production of immunoconjugates labeled with radioisotopes and fluorescent dyes, generating Rituximab conjugates with cyanine 5 and 7 tracers, along with the gamma emitter technetium-99 m (99mTc), for both *in vitro* and *in vivo* applications. *In vivo*, conjugation through the TZ-TCO reaction resulted in moderately effective tumor imaging 24 h after TZ-Cy7 administration. The presence of the CD20 antigen was detected in both *in vitro* and *in vivo* assays when a TZ derivative was labeled with 99mTc through a two-step protocol, with CD20 expressed in normal and malignant B cells but not in pre-B hematopoietic stem cells (Fig. 6a).

Catalyst-free inverse electron demand Diels–Alder reactions (IEDDA) with nucleosides like axial 2-TCO are ideal for intracellular DNA labeling [45]. Metabolic labeling of nucleic acids in living cells for real-time tracking of nucleic acid metabolism provides insights into cellular biology and pathogen-host interactions [46]. However, cellular kinase phosphorylation of the modified nucleosides is needed after cellular uptake, and this represents a limitation since triphosphates are not membrane-permeable.

The triphosphate pronucleotide (TriPPPro) approach is introduced to deliver a highly reactive axial 2-TCO-modified 2′-deoxycytidine

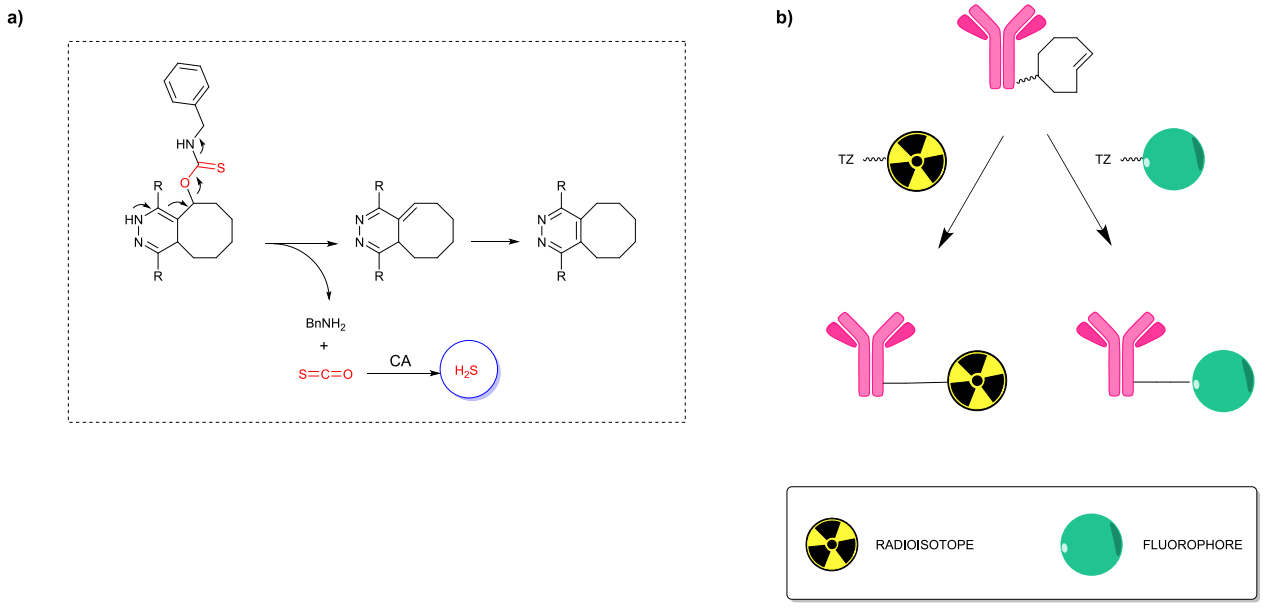


Fig. 6. a) Modular conjugation strategy for in vitro and *in vivo* labeling of Rituximab. TZ = [1,2,4,5]tetrazinyl moiety; b) IEDDA reaction of thiocarbamate-functionalized TCO with tetrazine, generating carbonyl sulfide (COS) and hydrogen sulfide (H_2S).

triphosphate reporter directly into living cells (Fig. 7) [47]. This reporter is incorporated into *de novo* synthesized cellular and viral DNA, allowing labeling with cell-permeable fluorescent dye-Tz conjugates via IEDDA for live-cell imaging of nucleic acids.

The use of biologics in PET imaging is an essential area of radiopharmaceutical development; new automated methods are required to facilitate their production [48]. Allot *et al.* reported an automated radiosynthesis method to produce a fluorine-18 radiolabeled interleukin-2 (IL2) radio-conjugate from a TCO-modified IL2 precursor ([18F]TTCO-IL2) *via* facile IEDDA click chemistry on a single GE FASTLab™ cassette (Fig. 8) [49].

The process achieved a decay-corrected radiochemical yield of 19.8 ± 2.6 % in 110 min from the start of synthesis, with a molar activity of 132.3 ± 14.6 GBq μmol^{-1}. *In vitro*, [^{18}F]TTCO-IL2 uptake correlated with the differential receptor expression (CD25, CD122, CD132). The automated method demonstrated potential adaptability for the radiosynthesis of any TCO-modified protein via Inverse Electron Demand Diels-Alder chemistry.

IEDDA conjugation with Tz was also used for synthesizing and radiometal labeling of well-known chelators. Benzyl-1,2,4,5-tetrazine-comprising((1,4,7,10-tetraazacyclododecane-4,7,10-triyl)triacetic acid-1-glutaric acid) (DOTA − GA) and ((1,4,7-triazacyclononane-4,7-diyl)-diacetic acid-1-glutaric acid) (NODA − GA) chelators were synthesized and labeled with radiometal $^{68}Ga^{3+}$ and $^{64}Cu^{2+}$ [50]. While the secondary labeling precursors were obtained successfully, subsequent reactions with TCO-modified peptides resulted in unexpected side product formation, limiting overall yields and molar activities. In contrast, one-step radiolabeling protocols provided high yields, purities, and molar activities for the target radiopeptides. The study suggests limitations in the two-step labeling approach for TCO-modified peptides with radiometal-labeled chelator-tetrazines.

By utilizing radiolabeled *trans*-cyclooctenes (TCOs) or tetrazines that can cross the blood–brain barrier, PRIT enables the visualization of biomolecules within the brain using positron emission tomography (PET)[51]. Studies have shown that PRIT can improve pharmacokinetic properties, increase tumor-to-tissue ratios, and reduce off-target toxicities compared to conventional radioimmunotherapy (RIT).

The use of tetrazine ligation as a bioorthogonal chemistry tool has also revolutionized cell biology research of subcellular structures such as mitochondria and microtubules. IEDDA reaction proved effective in the fluorescent imaging using fluorescent probes into organelles to detect local distribution of H_2O_2 [52]. Novel Tz-fused probe (TP) was developed with a rapid click reaction capability and sensitivity to H_2O_2 (Fig. 9) [53]. TP Fluorescent probes were designed with organelle-targeted functions, and TP's turn-on fluorescence, quenched by the Tz part upon H_2O_2 treatment, was restored only after a click reaction with

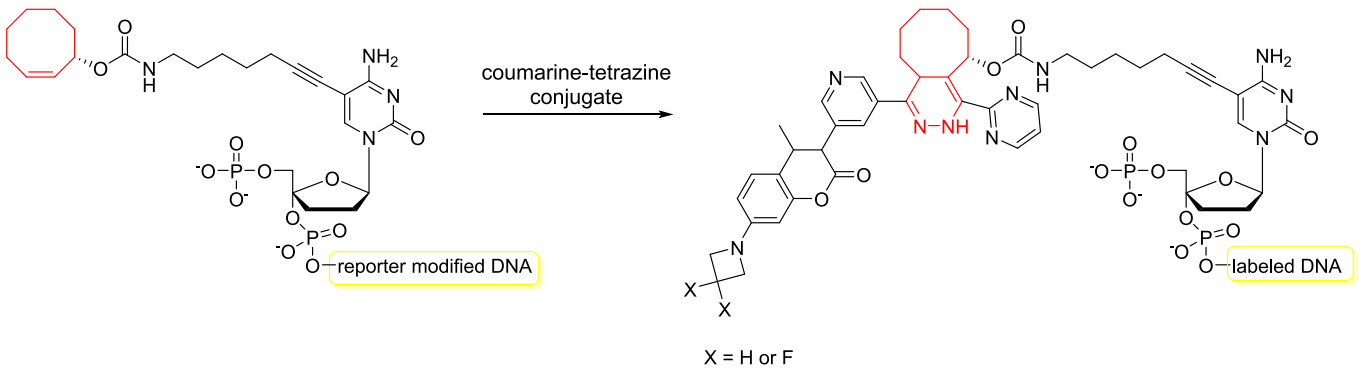


Fig. 7. The TriPPPro delivery system enables the intracellular release of the active nucleoside triphosphates and facilitates the *in vivo* incorporation of the reporter nucleoside into *de novo* synthesized DNA.

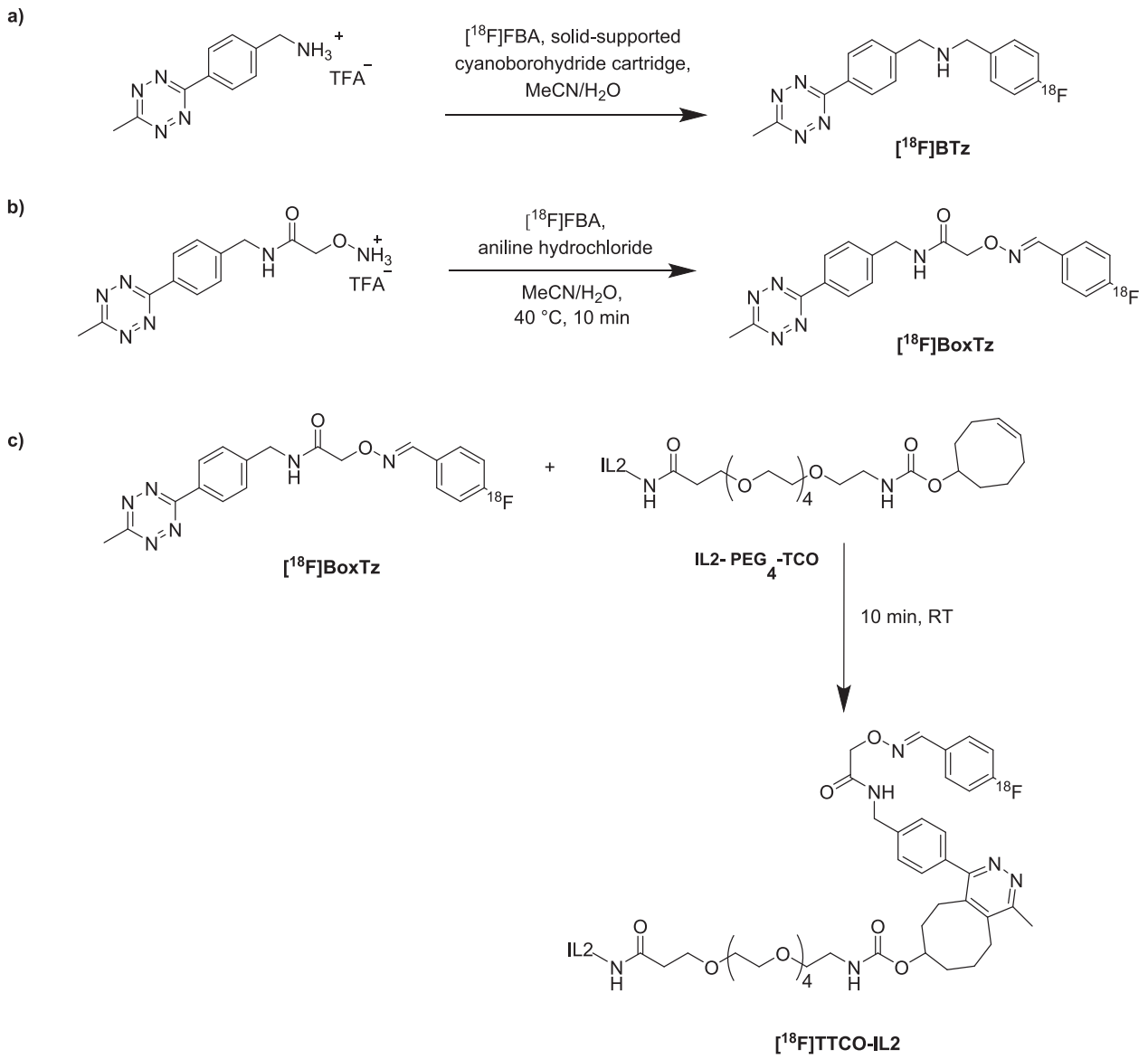


Fig. 8. Two radiosynthetic routes to ^{18}F-tetrazines: a) *N*-(4-[^{18}F]fluorobenzyl)-1-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanamine ([^{18}F] FB-Tz) and b) E-2-(((4-[^{18}F]fluorobenzylidene)amino)oxy)-*N*-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)acetamide ([^{18}F]FBoxTz). c) Reaction between [^{18}F]FBoxTz and TCO-PEG$_4$-IL2 to produce the radioconjugate [^{18}F]TTCO-IL2.

dienophiles. In practical applications, cells were labeled with triphenylphosphorus-tagged norbornene (TPP-NB) for mitochondria, followed by TP introduction to trigger the click reaction, resulting in the in situ construction of probe P1 as a local H_2O_2 sensor. Similarly, probe P2 was constructed in lysosomes (Fig. 10). This modular assembling strategy with double turn-on features demonstrated high flexibility and anti-interference performance, suggesting potential applications in biological studies.

4. Latest pioneering studies and uses of the CAPAC platform

Recently, IEDDA chemistry was used for an *in vivo* tumor pre-targeting approach thanks to the stability and biocompatibility of this type of biorthogonal rection. Bioconjugation reaction can occur in situ at the picomolar level, with a favorable pharmacokinetic profile enhanced by conjugation of chemical groups to macromolecules, like in ADCs [30]. Local prodrug activation using biorthogonal reaction allows cytotoxic concentration of drugs to be achieved *in vivo* and lowers the side effects of systemic administration. In this context, CAPAC platform shows significant potential as a highly efficient targeted delivery strategy for diverse therapeutics [54]. CAPAC platform consists of cytotoxic drugs locally activated by the combination of systemically administered protodrugs and a locally injected biopolymer, where a protodrug is a chemically modified form of the active drug with reduced toxicity (Fig. 11). The approach primarily relies on passive targeting through the enhanced permeability and retention (EPR) effect, introducing artificial clickable receptors on cancer cell plasma membranes [55]. These receptors can be activated using complementary clickable species like prodrugs. Natural systems lack clickable groups, so this strategy offers an effective targeting method. In contrast, traditional targeted delivery systems rely on biomarkers that can vary between patients or tumor types, and these biomarkers may be present on both cancerous and healthy cells, potentially causing harm to healthy cells as well. Considering this, Royzen *et al.* employed this strategy to treat soft tissue Sarcoma [56]. They injected a biocompatible hydrogel modified with Tz in the near vicinity of the tumor and later administered intravenously the prodrug containing a TCO moiety. Drug activation is achieved quickly and exclusively at the hydrogel injection site when bioorthogonal agents

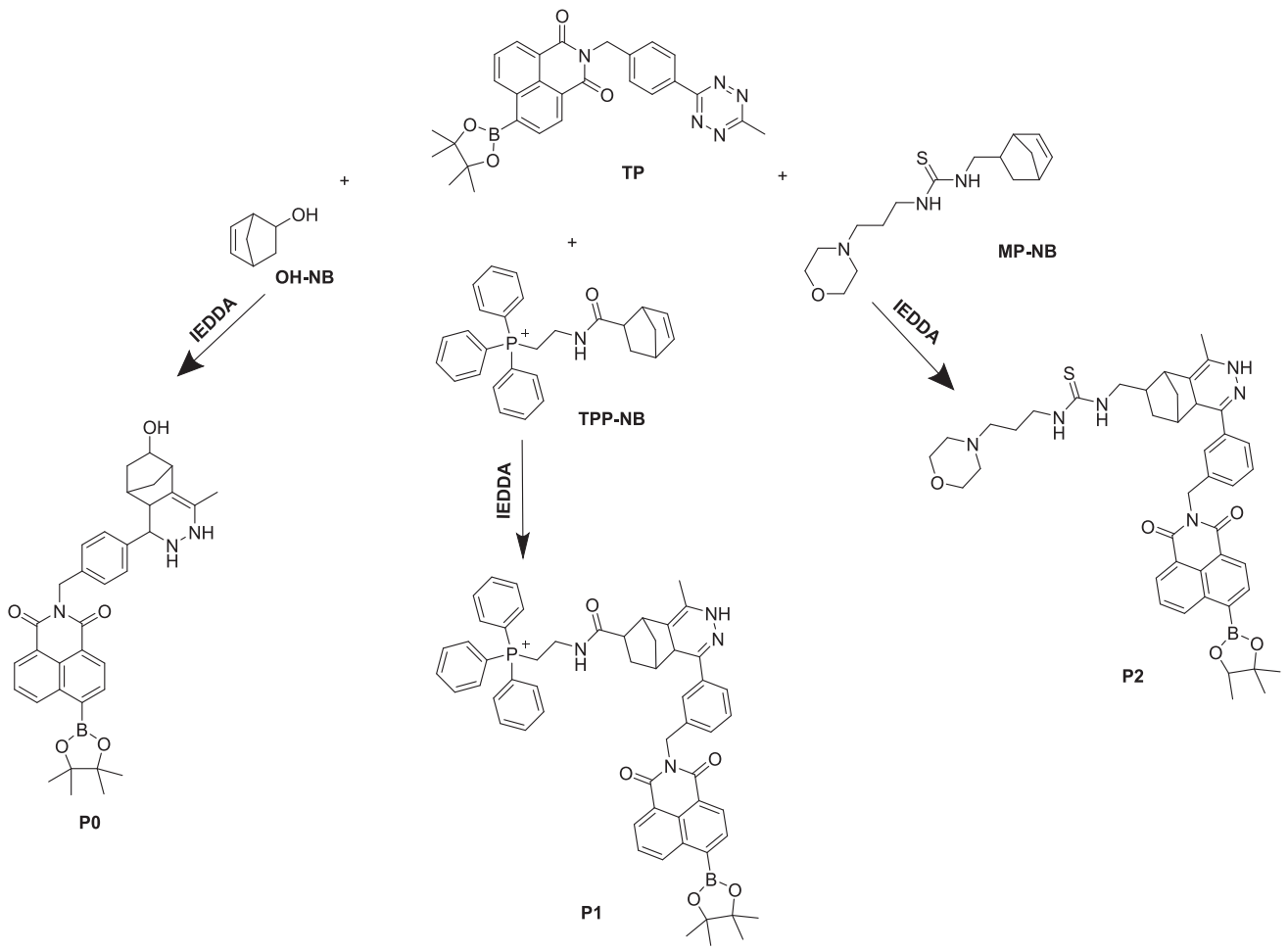


Fig. 9. IEDDA reactions between TP and OH-NB, TPP-NB, and MP-NB led to reaction products P0, P1, and P2.

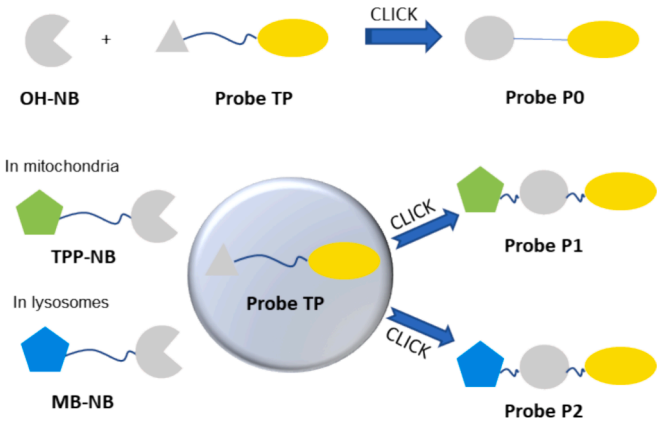


Fig. 10. Strategy to construct fluorescent probes P1 and P2 for H_2O_2 detection in mitochondria and lysosomes.

(TCO and Tz) react, thus excluding enzymatic processes or interaction with tumor antigens. This makes the strategy applicable to the tumor's genetic profile, bypassing possible resistance due to antigen mutations.

This innovative approach allows spatiotemporal control over drug release and activation. The same approach of local prodrug activation has been used to administer antibiotics against the harsh *Staphylococcus aureus* infection [18]. The most relevant consideration is that the approach of local drug activation is functional for multiple dose administrations, with the kinetics of drug transformation remaining unchanged due to the presence of numerous Tz groups on the polymer surface. The CAPAC approach is independent of biomarkers or tumor characteristics, placing it as a highly applicable therapy against a broad panel of solid tumor types. Several animal studies of the platform showed safety and dose efficacies in reducing systemic cytotoxic exposure. TCO-modified protodrug of Doxorubicin, SQP33, was administered in cumulative doses in animals with SQL70 biopolymer, composed of sodium hyaluronate modified with Tz [57]. Given the safety, efficacy, and ease of treatment in 2020, the FDA approved the first-in-human Phase I clinical trial of SQ3370 in patients with advanced solid tumors, in which the biopolymer is administered by *peri*/intratumoral injection to evaluate the safety, tolerability, and preliminary activity of SQ3370. SQ3370 is well-tolerated at 5.9 times the maximum dose of conventional Doxorubicin. It induces a systemic antitumor response against injected and non-injected lesions, a potential benefit for patients with micro-metastatic lesions [58]. The trial, NCT04106492 on ClinicalTrials.gov, has an estimated primary completion date in 2024 [59]. The sponsor, Shasqi Inc., is also rapidly advancing several antigen-targeted combinations toward clinical trials [60].

Recently, CAPAC was developed as a protodrug of monomethyl auristatin E. This potent antimitotic agent inhibits tubulin polymerization, activated by tumor-targeting agents, to release the payload exclusively at tumor sites [61]. Clickable groups have also been introduced in polysaccharides (PSA) to perform PSA-based bioconjugation inside living cells [55]. Clickable PSA is a potent and adaptable toolkit for biomaterials researchers, playing an increasingly pivotal role in the biomedical field. Notably, bio-click reactions involving PSA have been utilized in crafting sophisticated drug delivery systems and injectable

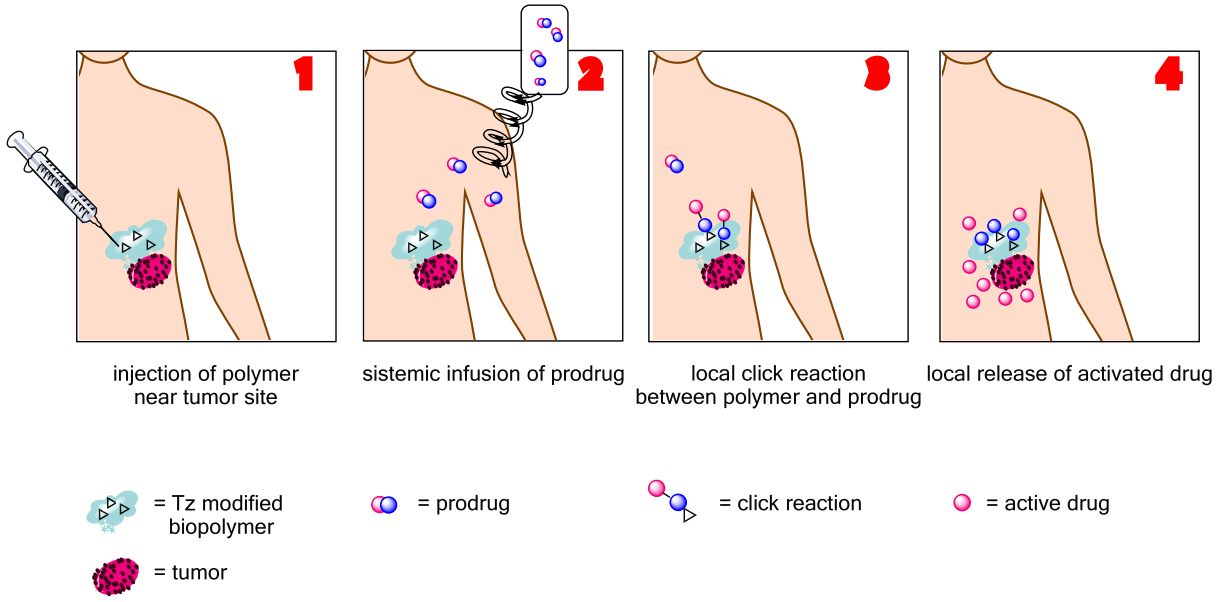
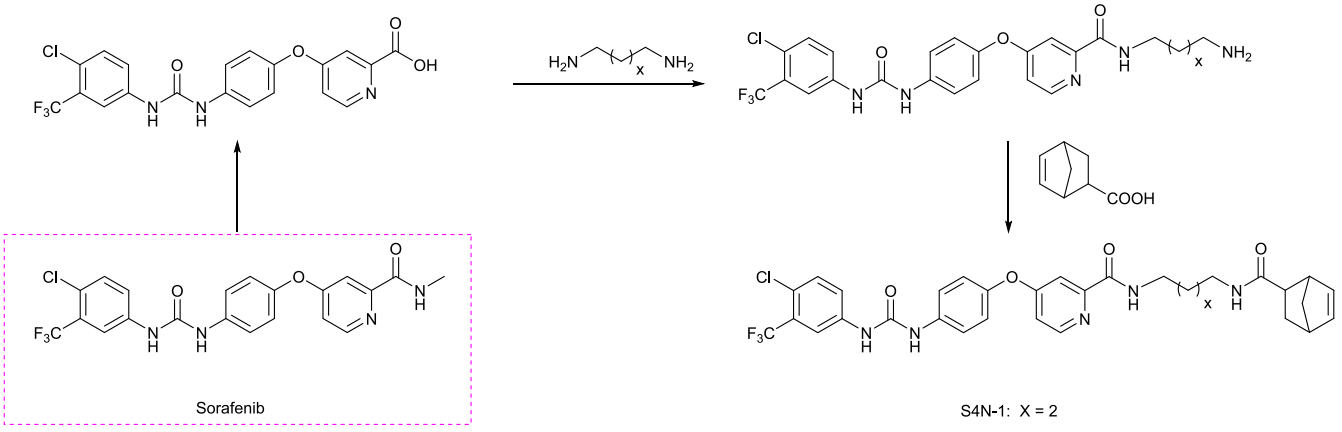


Fig. 11. CAPAC platform mechanism: 1) biopolymer, consisting of a Tz-modified form of sodium hyaluronate, is locally injected at the tumor site; 2) protodrug is infused systemically; 3) protodrug is locally captured by biopolymer through a click reaction between Tz (yellow star) and TCO (blue dot) moieties, followed by 4) rearrangement and release of active drug (green dots).

hydrogels for minimally invasive applications.

In line with the targeted cancer therapy in 2023, another general cancer-targeting platform has been developed by building artificial receptors on cancer cell surfaces *via* a chemical remodeling of cell surface glycans [62]. A Tz-functionalized chemical receptor has been designed and installed on the cancer cell surface as an overexpressed biomarker through metabolic glycan engineering.

Another significant accomplishment is using bioorthogonal click and release to activate PROTACs (proteolysis targeting chimera) from their prodrugs and transport them to tumor sites [63]. PROTACs is a technology that utilizes the ubiquitin–proteasome system (UPS) in cells to specifically degrade target proteins and consists of three parts: a target



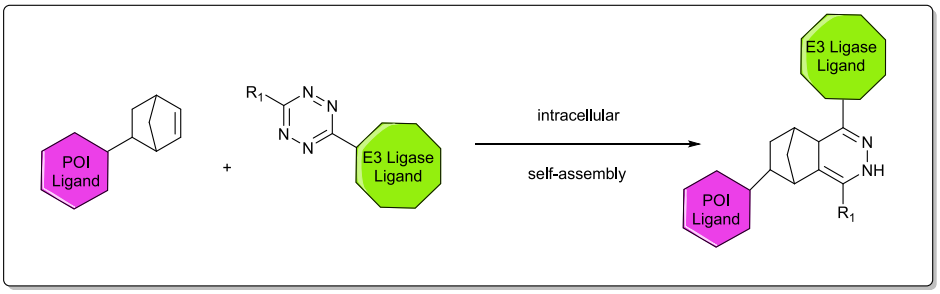


Fig. 12. Synthesis of S4N and self-assembly of PROTAC.

protein–ligand, an E3 ligase ligand, and a linker that connects the two unities [64]. Thanks to the drug-free approach, PROTACs have emerged as a novel and promising therapeutic strategy. However, systemic degradation of proteins leads to toxicity that can limit its utilization [63]. In this context, Tz-modified RGD peptide (c(RGDyK)-Tz) targeting integrin $\alpha v\beta_3$ in cancer cells was used as click-release PROTAC prodrugs to achieve targeted degradation of proteins of interest (POIs). In this way, selective activation of PROTAC prodrugs in an integrin $\alpha v\beta_3$-dependent manner results in the degradation of POIs, specifically in cancer cells. Utilizing a Tz-modified RGD peptide (c(RGDyK)-Tz) targeting integrin $\alpha v\beta_3$ in cancer cells, the click-release PROTAC prodrugs achieve targeted degradation of proteins of interest (POIs).

Investigation into the formation of potent PROTACs through spontaneous bio-orthogonal reactions in living cells also involved the use of a new E3 ubiquitin ligase ligand featuring Tz (E3L-Tz) and target protein ligands incorporating norbornene (TPL-Nb) [65]. Notably, the norbornene-containing precursor (S4N-1) (Fig. 12) demonstrated superior efficacy, efficiently degrading VEGFR-2, PDGFR-β, and EphB4. These findings highlight the potential of a highly specific bioorthogonal reaction-driven intracellular self-assembly strategy for enhancing PROTAC degradation activity in living cells.

In a proof-of-concept approach, the highly reactive *trans*-cyclooctene was introduced into the well-characterized PROTAC molecule MZ1, resulting in the bioorthogonally activatable prodrug (BT-PROTAC) [66]. Unlike MZ1, BT-PROTAC alone does not degrade BRD4 protein [67]. However, it can be activated by the potent Tz compound *in vitro*. Despite BT-PROTAC showing a degradation efficiency 100 times lower than MZ1 in targeting BRD4, its antitumor activity was nearly equivalent to MZ1 (Fig. 13). This innovative strategy suggests a versatile alternative for designing BT-PROTACs targeting various proteins.

CAPAC platform is thus a valid and practical approach that demonstrates the potential of Tz-TCO click chemistry in determining precise control of space–time drug activation.

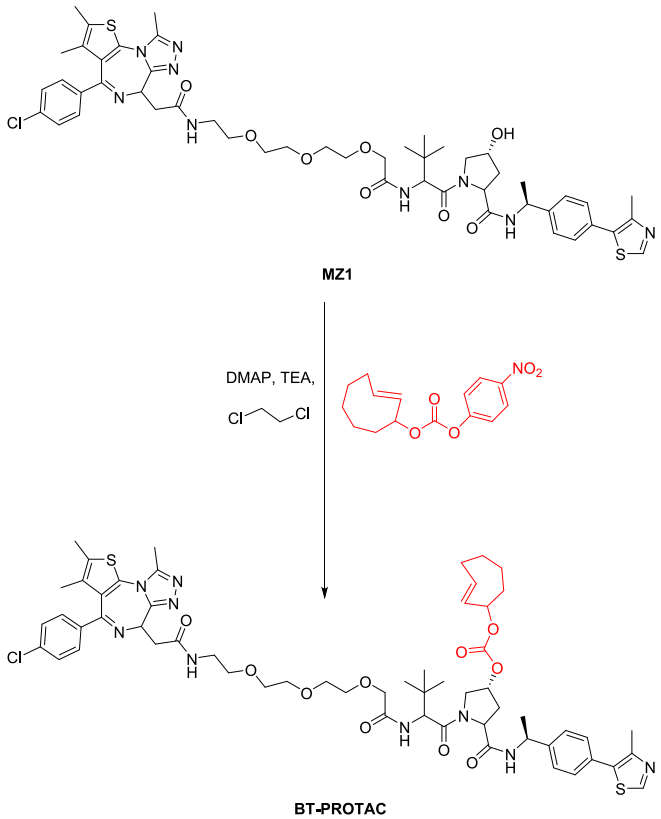


Fig. 13. Synthesis of BT-PROTAC.

5. Biotechnological applications of the reaction (targeted release)

Gansevoort *et al.* designed and developed pro-regenerative biomaterials that could find employment beyond skin wound healing, drug screening, or in articular cartilage and muscle tissue engineering [68].

Pro-regenerative biomaterials were obtained by functionalizing one side of bis-*N*-hydroxysuccinimide TCO with human epidermal growth factor (hEGF), a regulator during wound healing, and the other side with either type I collagen scaffolds or bovine serum albumin (BSA), as carrier proteins. The click reaction between TCO and Tz releases the bioactive compound. The coupling of hEGF-TCO and hEGF release following exposure to Tz was demonstrated through mass spectrometry. The hEGF-TCO complex ability to ligate to collagen scaffolds and hEGF release were also explored by SDS-PAGE and Western blot. However, these assays did not allow the researchers to exclude the interference of the non-specific binding of collagen to hEGF. Analogous consideration resulted from hEGF-TCO-BSA to the same experimental protocol.

Recently, a cancer-targeting platform was developed with a bifunctional aim to enable local prodrug activation that reflects in more effective and safe cancer therapy [69]. The approach consisted of building artificial receptors on the surface of cancer cells. Specifically, through the chemical remodeling of cell surface glycans, a Tz mannosamine (Ac4ManNTz) was deposited on the cancer cell surface overexpressed endogenous biomarker for targeted anticancer drug delivery. Indeed, the Tz-labeled cancer cells locally activate TCO-caged prodrugs and release drugs through a Tz-TCO click-release reaction. Ac4ManNTz was incorporated into the MDA-MB-231 cell surface, and the Tz-labeled cancer cells' capability to locally activate TCO-caged prodrugs doxorubicin and ARV771 was monitored by ultra-performance liquid chromatography (UPLC). In this way, the prodrug consumption and the release of parent drug doxorubicin upon reaction with Tz was monitored and compared to the prodrug itself that in the same experimental conditions (incubated with Ac4ManNTz in PBS buffer at 37 °C) did not liberate doxorubicin for at least 24 h while it was detected in a percentage up to 90 % from TCO-Dox treatment.

A fluorogenic near-infrared probe, activatable by bioorthogonal chemistry and imaging tumors in mice by caging hemicyanine with TCO (single-walled carbon nanotubes) SWCNTs, was developed as a diagnostic tool and carrier to deliver chemotherapeutic drugs [70]. The tumor-pretargeted strategy consisted of accumulating Tz-modified SWCNTs (TZ@SWCNTs) in tumor tissues due to the EPR effect. Then, the TCO-carbamate-containing chemotherapeutic prodrug or diagnostic probe systemically administered will be activated bioorthogonally *in situ*. This activation strategy enables active drug/probe localization and selectivity for tumor sites over normal tissues. Moreover, the bioorthogonally applicable fluorogenic NIR probe was tested for real-time *in vivo* imaging in living cells with an instantaneous turn-on signal in mitochondria without washing. In addition, the activatable NIR imaging featured tumor selectivity over normal tissues in this circumstance. It gave in-depth imaging with a high signal-to-noise ratio in a real-time and non-destructive manner.

The click-to-release reactions have been found for lysosome-targeted Tz for organelle-specific deprotection reaction. In particular, Ligthart *et al.* investigated TCO deprotection as a reaction to control the biological activity of ligands for invariant natural killer T cells in the lysosome, highlighting the processing pathway in antigen-presenting cells [71]. The selective lysosomal deprotection was achieved by using as reagent the (2-aminoethyl)-morpholine group, which is protonated at lysosomal pH-values and therefore accumulates in compartments with pH $\leq$ 4.5, linked to 3-(pyridin-3-yl)-6-aminoethyl-1,2,4,5-TZ. The bifunctional TCO was conjugated to a DABCYL quencher, and the cell-permeable BODIPY fluorophore as a quenched fluorophore.

As mentioned, bioorthogonal ligation methods show versatile applications in biotechnology and materials science for the post-functionalization and immobilization of biomolecules.

6. Conclusions

In the last ten years, bioorthogonal chemistry has played a crucial role in shaping key aspects of compound design for targeted drug discovery and has contributed to expanding our understanding of challenging targets in biology. Various research teams have concentrated on uncovering reactions with enhanced biocompatibility and accelerated reaction rates, anticipating their pivotal role in future therapeutic advancements.

The IEDDA reaction is a hallmark of bioorthogonal chemistry, showcasing remarkable application versatility. Its role is exemplified in developing prodrugs, pro-regenerative biomaterials, and cancer-targeting platforms, underscoring its potential impact across diverse domains such as regenerative medicine, cancer therapy, and diagnostic imaging. Notably, the IEDDA reaction facilitates *in vivo* tumor pre-targeting, representing a promising strategy with considerable potential in pursuing innovative cancer treatment approaches. This review accentuates the impactful applications of bioorthogonal chemistry in drug discovery, encompassing tumor-targeted prodrug delivery, radiolabeled peptides, bioorthogonal hydrogels, and the innovative CAPAC platform. The collective insights contribute to the ongoing advancement of bioorthogonal chemistry in multifaceted biomedical applications.

These innovative approaches, involving the precise design and controlled release of bioactive compounds, offer promising avenues for advancing targeted drug delivery, tissue engineering, and real-time *in vivo* imaging. The applications of bioorthogonal chemistry, particularly in radioimaging and theranostic fields, profoundly influence the biomedical research arena. PRIT applications promise to augment the efficacy of cancer treatment, all while mitigating adverse effects, for increasingly precise and personalized therapies in the future. As we witness the evolution of bioorthogonal chemistry, it is evident that these innovative strategies are expanding the scope of biomedical applications and paving the way for a new era in personalized and targeted medicine.

CRediT authorship contribution statement

Elisabetta Grazia Tomarchio: Conceptualization. **Rita Turnaturi:** Writing – original draft. **Erika Saccullo:** Writing – original draft. **Vincenzo Patamia:** Writing – original draft. **Giuseppe Floresta:** Writing – original draft. **Chiara Zagni:** Writing – review & editing, Writing – original draft, Supervision. **Antonio Rescifina:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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