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REVIEW

Photo-activatable Reagents for Bioorthogonal Ligation Reactions

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Abstract

Light-induced bioorthogonal reactions offer spatiotemporal control over selective biomolecular labeling. This review covers the recent advances in the design of photo-activatable reagents for bioorthogonal conjugation reactions in living systems. These reagents are stable in the absence of light, but transformed into reactive species upon light illumination, which then undergo rapid ligation reactions. The light wavelength has been tuned from ultraviolet to near infrared to enable efficient photo-activation in reactions in deep tissues. The most prominent photo-activatable reagents are presented, including tetrazoles, tetrazines, 9,10-phenanthrenequinone, diarylsydnones, and others. A particular focus is on the strategies for improving reaction kinetics and biocompatibility accomplished through careful molecular engineering. The utilities of these photo-activatable reagents are illustrated through a broad range of biological applications, including in vivo protein labeling, positron emission tomography (PET) imaging, responsive hydrogels, and fluorescence microscopy. The further development and optimization of these biocompatible photo-activatable reagents should lead to new chemical biology strategies for studying biomolecular structure and function in living systems.

Keywords Bioorthogonal reaction · Photoclick chemistry · Tetrazole · Tetrazine · 9,10-phenanthrenequinone · Diarylsydnone · *o*-Nitrobenzyl alcohol · Indanone epoxide

1 Introduction

Bioorthogonal reactions have been used widely in selective modification of biomolecules such as proteins. A key challenge in achieving high bioorthogonality is to find the right balance between reactivity and stability in bioorthogonal reagents, as highly reactive reagents tend to be less stable whereas highly stable

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reagents tend to be less reactive. To identify ideal bioorthogonal reagents with an optimum combination of stability, reactivity, and bioorthogonality (Fig. 1), an attractive strategy centers around the design of photo-activatable reagents that decouple reactivity from stability by masking the reactive intermediates in the stable precursor forms. The stable structures are biocompatible and require light activation before participating in bioorthogonal ligation. This review focuses on the recent advances in photo-activatable bioorthogonal reagents that overcome this barrier and provide spatiotemporal control and enhanced selectivity in biomolecular modification in living systems. Specifically, we discuss the major structural classes of photo-activatable reagents, including tetrazoles, tetrazines, 9,10-phenanthrenequinones, diarylsydnones, and a few others. A multitude of efforts in tuning photo-activation wavelength, increasing reaction kinetics, and improving biocompatibility are presented in some detail. Selected examples in applying these photo-activatable reagents to protein labeling, positron emission tomography (PET) imaging, and designing photo-responsive materials are presented. Since light-triggered click chemistry has been extensively reviewed recently [1, 2], here we will focus on the recent progress in this area since 2016, including the optimization of existing photo-activatable structures and the development of new biocompatible photo-activatable reagents and their use in diverse biological systems.

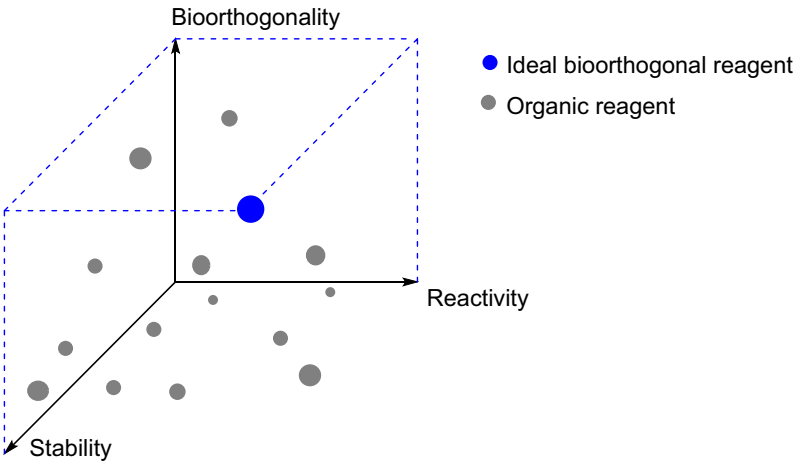

Fig. 1 Depiction of characteristics of bioorthogonal reagents versus organic reagents based on stability, reactivity, and bioorthogonality attributes. An ideal bioorthogonal reagent (blue dot) should possess high stability, high reactivity, and high bioorthogonality. Most organic reagents (gray dots) lack one or more of these desirable attributes. The size of dots depicts the overall reagent fitness

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2 Tetrazoles

In 2007, the Lin group reported a mild, photo-activated tetrazole-based 1,3-dipolar cycloaddition reaction, later on referred to as photoclick chemistry, for facile synthesis of substituted pyrazolines [3]. The compatibility of this reaction with biological systems was examined by replacing the original 450-W mercury lamp with a handheld 302-nm UV lamp and conducting the reaction in an ethanol–water mixture (Fig. 2a). These mild photo-activation conditions allowed rapid deployment of tetrazoles in selective functionalization of model proteins such as green fluorescent protein in pure phosphate-buffered saline [4] as well as an alkene-encoded Z-domain protein inside *Escherichia coli* cells (Fig. 2b) [5].

Since our initial reports, recent efforts in further optimizing the tetrazole photoclick chemistry have focused on increasing the kinetics and selectivity for the cycloaddition reaction, shifting photo-activation wavelength to long-wavelength region including near infrared, and broadening its utility to encompass drug discovery and biological imaging.

2.1 Enhancing Reactivity and Selectivity

The mechanism of the photo-activated 1,3-dipolar cycloaddition with tetrazoles was originally proposed by Huisgen [6]. Upon photo-irradiation, a tetrazole undergoes rapid cycloreversion to release N_2 and produce a highly reactive nitrile imine intermediate, which then reacts with an alkene dipolarophile to generate the pyrazoline adduct (Fig. 3a). In a photo-crystallographic study by the Lin group, after photo-irradiation of a tetrazole–Zn coordination complex at 90 K, the in situ generated nitrile imine was detected in the crystal, validating the presence of this highly reactive intermediate [7]. Owing to their high reactivity, nitrile imines can undergo undesired reactions in aqueous medium such as dimerization, nucleophilic additions, and hydrolysis, especially when strong dipolarophiles are absent. Two strategies have been reported recently to

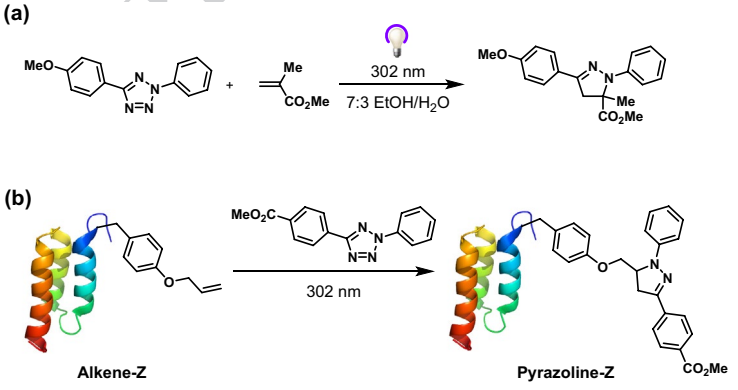

Fig. 2 First reports of tetrazole photoclick chemistry: **a** efficient synthesis of pyrazolines using a 302-nm lamp in a mixed EtOH–H_2O solvent; **b** selective functionalization of Z-domain protein encoding *O*-allyltyrosine inside *E. coli* cells

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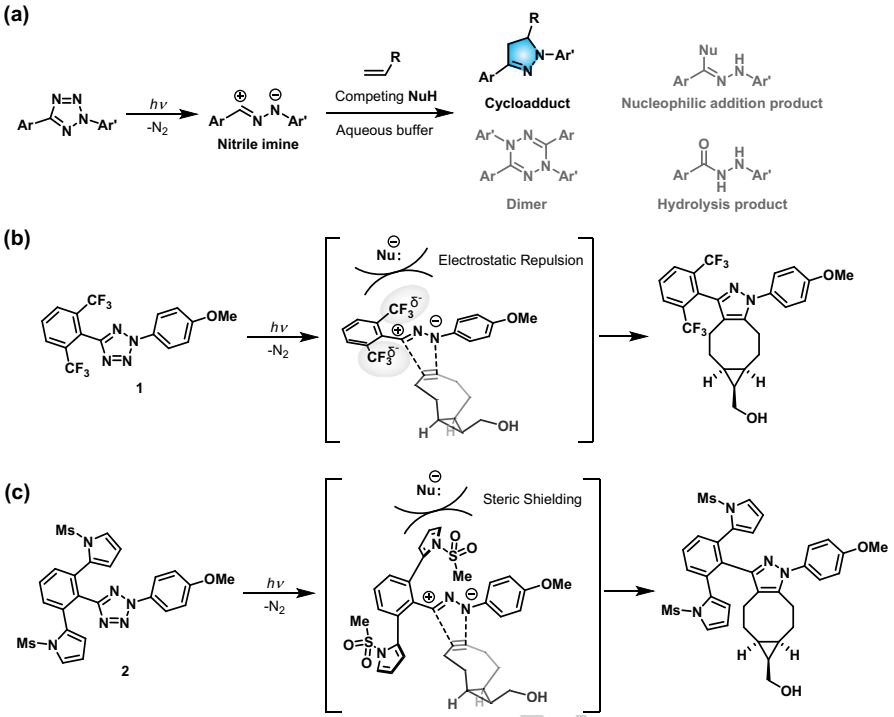

Fig. 3 Optimizing photo-activatable tetrazole reagents: **a** reactivity profile of nitrile imine—reactive intermediate of tetrazole photoclick chemistry; **b** *ortho*-CF_3 induced electrostatic sheltering effect for faster tetrazole photoclick reaction; **c** *ortho*-*N*-mesylpyrrole induced steric shielding effect for faster tetrazole photoclick reaction

mitigate these side reactions. One involves adding two -CF_3 groups, which carry partial negative charges on fluorine atoms, to the *C*-phenyl ring (**1**) to provide electrostatic repulsion against an incoming nucleophile (Fig. 3b) [8]. Using this design, Yu and coworkers observed significantly higher selectivity for and faster cycloaddition reaction with bicyclo[6.1.0]nonyne (BCN), a strained alkyne and outstanding dipolarophile. The reported k_2 value in acetonitrile was 9.7×10^5 M^{-1} s^{-1}, one of the fastest bioorthogonal ligation reactions known to date. The other strategy reported by the Lin group involves the introduction of steric hindrance proximal to the developing nitrile imine [9]. Bulky groups such as *N*-mesylpyrrole (**2**) have been designed to block the approach of nucleophiles, decreasing the undesired side reactions (Fig. 3c). The sterically shielded tetrazole offered one the fastest tetrazole–BCN ligation reactions with a measured k_2 value of $39{,}200 \pm 4{,}600$ M^{-1} s^{-1}.

2.2 Tuning Photo-activation Wavelength

While tetrazole photoclick chemistry offers significant advantages such as fast kinetics and exquisite spatiotemporal control, its extension to in vivo animal

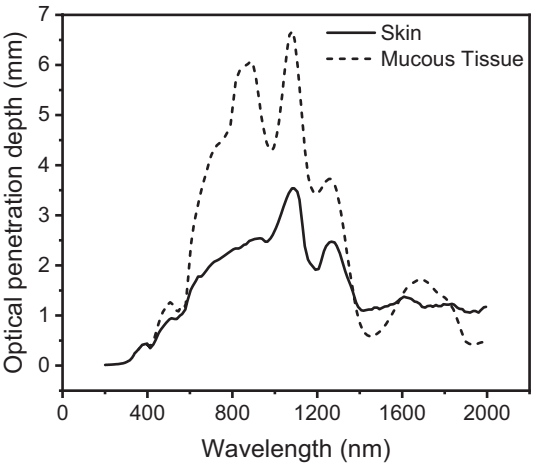

Fig. 4 Wavelength-dependent penetration of light for two tissue types. Data were taken from Ref. [10]

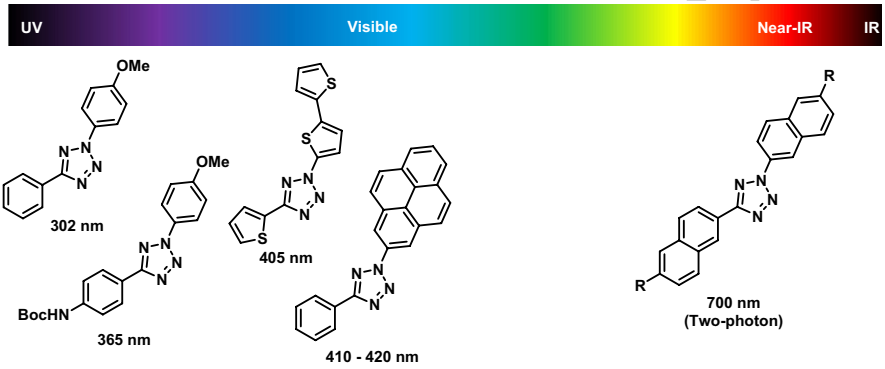

Fig. 5 Structures of photo-activatable tetrazoles at different wavelengths of electromagnetic spectrum

studies remains a challenge as light usually cannot penetrate deep into tissues. In addition, short-wavelength light causes phototoxicity to cells and animals. However, near-infrared light in 650–1350 nm range shows improved tissue and skin penetration of a few millimeters [10] (Fig. 4). Thus, tetrazoles that are photo-activatable by visible or near-infrared light are highly desired for in vivo applications.

One strategy to tune photo-activation wavelength involves "scaffold hopping" in which the tetrazole core was inserted into an extended aromatic system [11], e.g., oligothiophenes with absorption maxima of 405 nm [12] or 2,5-dinaphthyltetrazole responsive to femtosecond near-infrared two-photon activation [13] (Fig. 5). Barner-Kowollik and colleagues accomplished the same goal using a pyrene-substituted tetrazole [14] (Fig. 5). Moreover, they showed that nanoparticles can be used to upconvert a femtosecond near-infrared two-photon laser to a short-wavelength light, which in turn activate the pyrene-substituted tetrazole [15].

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An alternative strategy in harnessing the intrinsic high reactivity of nitrile imines is to mask them in stable tautomeric form without the need for photo-activation. To this end, Lin and coworkers recently reported a new class of bioorthogonal reagents called hydrazonyl sultones (**3**) (Fig. 6) [16]. The spontaneous tautomerization of hydrazonyl sultones in biological buffer generates the reactive nitrile imines, which then react with a BCN-lysine-encoded glucagon receptor on mammalian cell surface (Fig. 6). Interestingly, hydrazonyl sultones exhibited microenvironment-dependent bioorthogonal labeling of BCN-lysine-encoded nanobodies, a phenomenon not observed previously with the photo-activatable tetrazoles.

2.3 Expanded Use of Tetrazoles in Biological Systems

2.3.1 2-Aryl-5-Carboxytetrazole as a Photo-affinity Label

Taking advantage of the substituent effect in determining the reactivity profile of nitrile imines, the Lin group reported the development of 2-aryl-5-carboxytetrazole (ACT) as a novel class of photo-affinity labels for identification of the protein targets of Dasatinib, an inhibitor of Src family kinases and Bcr-Abl, and JQ-1, an inhibitor of bromodomains. The ACT-based probes showed robust photo-crosslinking reactivity with their known targets. Notably, ACT provides a unique target capture mechanism in which the photogenerated carboxy-nitrile imine intermediate reacts with a carboxylate close to the target's active site to form a stable adduct (Fig. 7). A drawback of ACT-based photo-affinity label is that, when a carboxylate is not present close to the ligand, the photo adduct cannot be formed, leading to a loss of positive identification. However, this shortcoming can be overcome by increasing the linker length to permit a wider search of the surface nucleophiles. Besides drug target identification, ACT has been genetically encoded into protein structure for studying transient protein–protein interactions [17, 18].

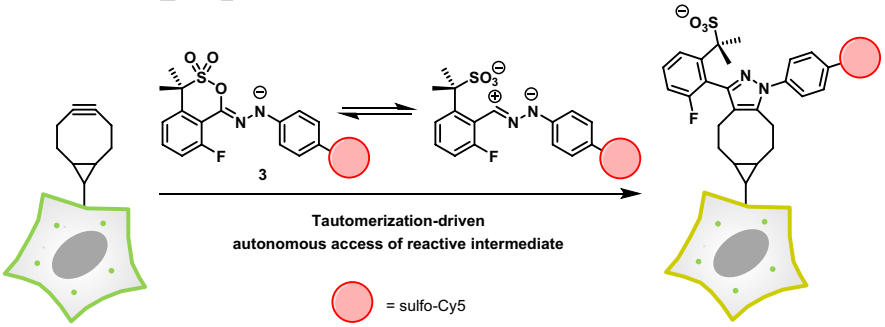

Fig. 6 A fast and light-independent 1,3-dipolar cycloaddition reaction between hydrazonyl sultone (**3**) and BCN on mammalian cell surface

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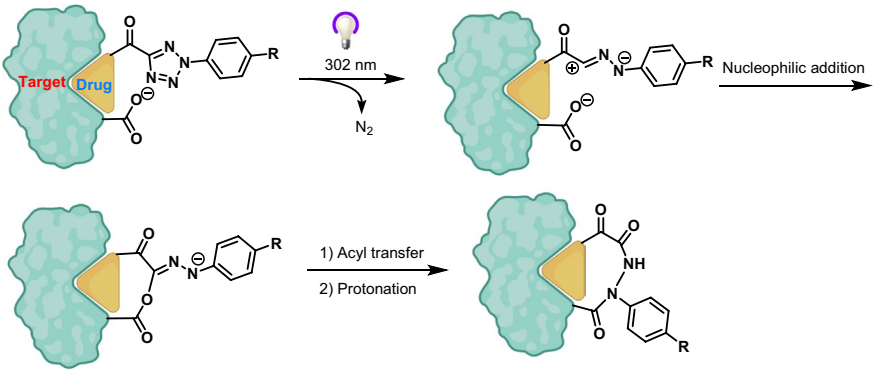

Fig. 7 A proposed mechanism of ligand-dependent photo-crosslinking with a target protein by 2-aryl-5-carboxytetrazole

2.3.2 Fluorescence Sensors of Protein Conformations

The tetrazole photoclick reaction produces an environmentally sensitive pyrazoline fluorophore, with enhanced fluorescence in nonpolar solvents. The use of tetrazoles to generate fluorescence sensors to track protein conformational change was reported recently by the Lin group [9]. In the experiment, glutamine-binding protein (QBP) was selected as a model because its active site closes following glutamine binding, leading to a reduction in local polarity. A QBP mutant encoding a spirohexene-lysine (SphK) at the active reacted with a water-soluble sterically shielded tetrazole to generate a fluorescent pyrazoline probe. This fluorescence sensor then permitted quantitative analysis of the binding between glutamine and QBP in a fluorescence-based titration experiment [19].

3 Tetrazines

Since its initial reports in 2008 [20, 21], tetrazine ligation has gained a widespread adoption in chemical biology and used in diverse biological contexts including drug delivery, nuclear medicine, imaging, and proteomics. There were no reports of photo-activatable tetrazines for bioorthogonal ligation reactions until 2016. The recent development of light-triggered tetrazine ligation has centered on three pivotal aspects: (1) establishing a proof of concept, (2) enhancing biocompatibility by curbing oxidative damage, and (3) fine-tuning cell permeability of photo-activatable tetrazines to permit intracellular applications in living systems. These advances have laid the foundation for using this highly efficient, spatiotemporally controlled ligation reaction to address various biomedical problems.

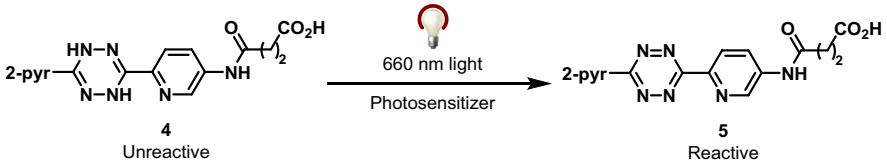

Fig. 8 Highly reactive tetrazine **5** is generated through photo-oxidation of 1,4-dihydrotetrazine **4** using a red light (660 nm) in the presence of a photosensitizer

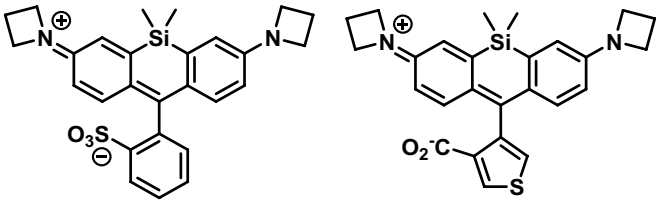

Fig. 9 Far-red Si–rhodamine dyes as photosensitizers for enhanced biocompatibility

3.1 Photo-activatable Tetrazines

The tetrazine synthesis typically begins with the preparation of 1,4-dihydrotetrazine (DHTz), which are oxidized to give the desired tetrazine. The Fox group reported that, when air was used as the oxidant, methylene blue acted as a catalyst to convert DHTz **4** to tetrazine **5** efficiently along with illumination of a red light (Fig. 8) [22]. This photo-oxidation procedure generates highly reactive tetrazines for robust ligation reactions with a range of dienophiles, e.g., *trans*-cyclooctene (TCO), highlighting the role of light as a precision modulator of bioorthogonal reactions.

3.2 Enhancing Biocompatibility

3.2.1 Biocompatible Photosensitizers

While methylene blue was effective in catalyzing the photooxidation of DHTz, it is nonetheless phototoxic to cells. To solve this problem, the Fox group repurposed silicon–rhodamine (SiR) dyes such as **6** and **7** traditionally used in fluorescence imaging to serve as photocatalysts [23] (Fig. 9). Notably, the SR dyes showed broad compatibility with biological systems. One application involved the use of SiR in conjunction with far-red light to crosslink the DHTz/TCO-modified hyaluronic acids (HA) to form hydrogel matrices from liquid cellular suspension for 3D cell culture. Another remarkable application involved the creation of

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hydrogel materials in live mice by subcutaneous injection of a solution of SiR catalyst, HA–DHTz, and HA–TCO, followed by brief irradiation with 660 nm far-red light, illustrating its broad biocompatibility, including animals.

3.2.2 Photocaged Dihydrotetrazines

As an alternative approach, Devaraj and coworkers reported a visible light-induced generation of tetrazine from a stable photocaged DHTz precursor [24]. Upon exposure to a blue light, the photo protecting group is removed, releasing the DHTz anion, which then undergoes spontaneous oxidation to generate the reactive tetrazine (Fig. 10). The resulting tetrazine then reacts with a suitable dienophile in an inverse electron-demand Diels–Alder reaction. The photo-decaging efficiency was quantitative, and the conditions were mild enough to permit a light-triggered ligation in live mammalian cells.

3.3 Optimizing Cell Permeability

Since dihydrotetrazines exhibit poor cell permeability, their initial uses were in the extracellular environment. To address this limitation, Fox and coworkers recently reported spatiotemporally controlled labeling of biomolecules inside living cells through catalytic activation of bioorthogonal chemistry with light (CABL) [25]. Specifically, they synthesized 6-(2-pyridyl)-dihydrotetrazine-3-carboxamide **9** that is cell permeable and suitable for intracellular applications. Upon SiR-catalyzed photo-oxidation at 660 nm, the in situ generated tetrazine showed fast reaction kinetics toward a strained TCO, with the rate constant exceeding 10^6 M^{-1} s^{-1} (Fig. 11). The utility of this CABL strategy was illustrated by organelle-specific labeling of a protein of interest inside nucleus, mitochondria, cytoskeleton, and cytoplasm. The study also demonstrated that CABL increased the efficiency of traditional tetrazine ligation reactions in mammalian cells by restoring the reactivity of 3-aryl-6-methyl-tetrazines that had been partially reduced to DHTz.

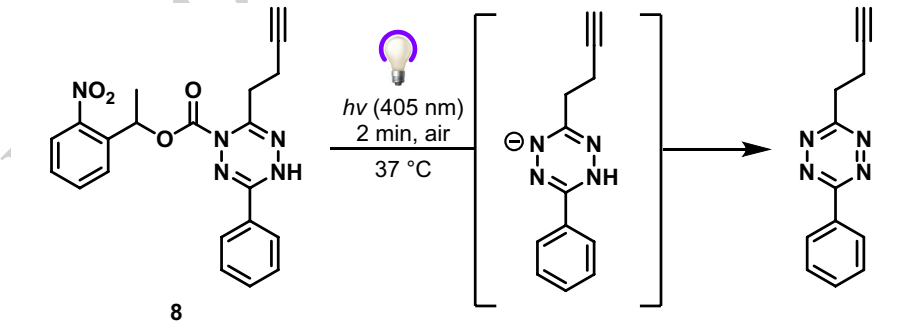

Fig. 10 Photogeneration of tetrazine from the photocaged dihydrotetrazine **8** after 2-min irradiation with an LED light (405 nm, 18 W) in open air at 37 ℃

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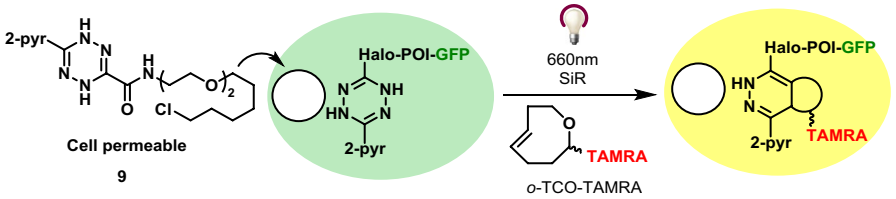

Fig. 11 Cell permeable dihydrotetrazine **9** for intracellular SiR dye-catalyzed photo-oxidation to generate a reactive tetrazine for robust ligation with a TAMRA-conjugated *o*-TCO inside HeLa cells

4 9,10-Phenanthrenequinones

The photocycloaddition of 9,10-phenanthrenequinone (PQ) with electron-rich alkenes (ERA) to form fluorescent cycloadducts was first reported by Zhang and coworkers in 2018 [26]. This reaction is notable for its fast kinetics and high biocompatibility, making it a valuable tool for bioconjugation and material science. During the investigation of the substituent effect on PQs and ERA, enamines stood out as outstanding reactants with significantly higher reaction rates. The PQ-mediated photo-ligation has been applied to address a number of biological problems, including ultrafast flow synthesis of ^{18}F-positron emission tomography (PET) tracers, and mutually exclusive orthogonal protein labeling. In addition, improved synthesis of PQ analogs has led to new PQ/ERA reactant pairs with dramatically increased reaction kinetics and photoreaction quantum yields (Fig. 12). AQ2

4.1 Flow Synthesis of PET Tracers

PET tracers are crucial for noninvasive medical imaging and staging of diseases in the clinic. A general rule for synthesizing radiopharmaceuticals is that the synthesis time should not exceed three half-lives of the radionuclides; for ^{18}F-based PET tracers ($t_{1/2}$ = 109.8 min), the synthesis time should ideally be less than 2 h [27]. Recently, Feringa and colleagues reported the use of PQ-mediated photo-ligation for flow synthesis of ^{18}F-PET tracers [28]. The advantages of their synthesis include high functional group tolerance, fast radiolabeling, and high radiochemical conversions. Using easily accessible PQ derivatives and ^{18}F-labeled vinyl ether **10** as reactants and a flow photo-microreactor, a number of clinically valuable ^{18}F-PET tracers were prepared in 60 s in high yields, including **11** for detecting prostate-specific membrane antigen and **12** derived from vancomycin for detection of bacterial infection.

4.2 Mutually Orthogonal Protein Labeling

While PQ favors electron-rich alkenes such as vinyl ether (VE) in its photocycloaddition reactions, it is unreactive toward alkynes including terminal alkyne and internal strained alkynes. Therefore, PQ-mediated photocycloaddition is mutually orthogonal to the azide-alkyne click reaction and tetrazole photoclick chemistry.

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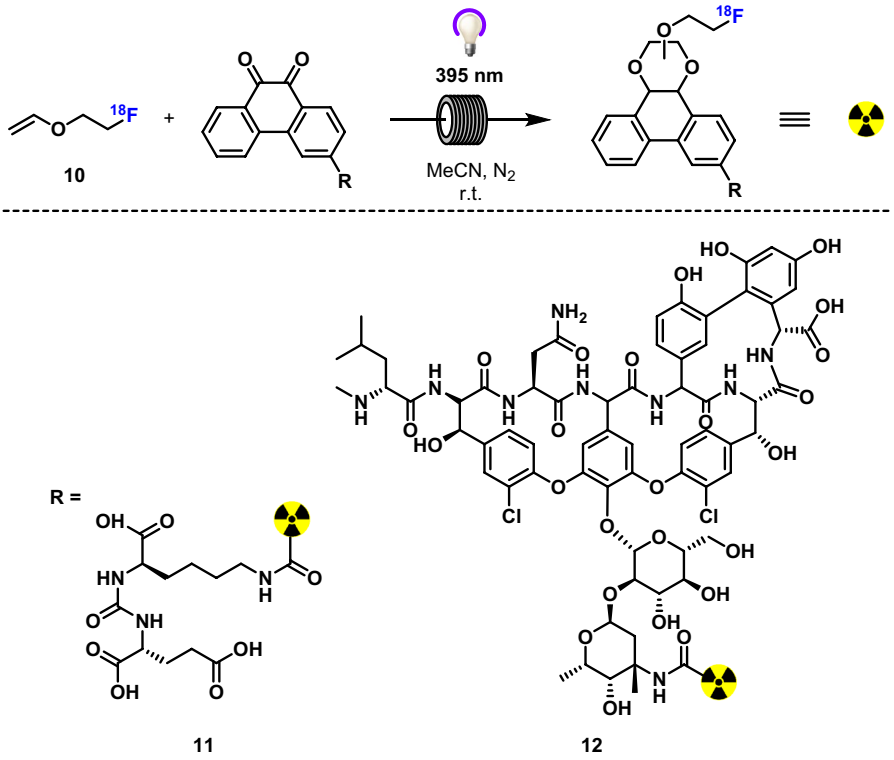

Fig. 12 Ultrafast flow synthesis of ^{18}F-PET tracers via PQ-mediated photocycloaddition

This unique feature was demonstrated through selective orthogonal labeling of a mixture of VE-tagged BSA (**16**), azide-tagged lysozyme (**17**), and monomethyl fumarate-modified lysozyme (**18**) using their cognate reaction partner [26] (Fig. 13).

4.3 Improving PQ Reactivity

In an effort to further improve the efficiency of PQ-mediated photocycloaddition, Feringa and colleagues recently reported the discovery of a new PQ/ERA pair through a systematic investigation of the substituent effect [29]. Leveraging on the commercially available 3-bromophenanthrene-9,10-dione (**19**) and phenyl boronic acids (**20**), the research team synthesized a large panel of PQ analogs through Suzuki–Miyaura cross-coupling and evaluated their reactivities toward various electron-rich alkenes (Fig. 14). When cyclic enamines such as **21** were used as ERA, the reaction rate rose to 634 M^{-1} s^{-1}, more than 5400 times faster than the original photocycloaddition reaction. The new reactant pairs also produced high photoreaction quantum yields (up to 0.65), high fluorescence quantum yields (up to 0.97) of the photo adducts, and multicolor emission output.

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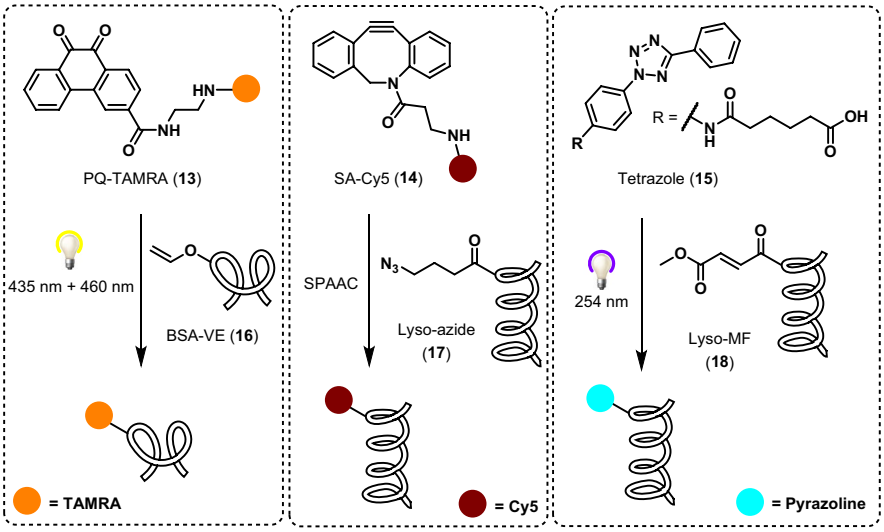

Fig. 13 PQ-mediated photocycloaddition is orthogonal to azide-alkyne click reaction and tetrazole photoclick chemistry

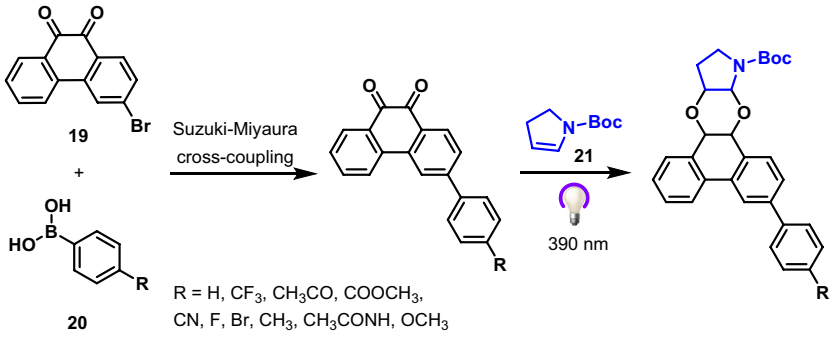

Fig. 14 Synthesis of PQ analogs and electron-rich alkenes for accelerated photocycloaddition reactions

5 Diarylsydnones

Building on the pioneering work by Krauch [30], Yu and colleagues reported in 2018 a photoinduced diarylsydnone (DASyd)-mediated cycloaddition as a new bioorthogonal ligation reaction [31]. The mechanism of the reaction involves internal ring closure, CO_2 expulsion, and ring opening to generate highly reactive *N*-phenyl nitrile imine (**22**), which then reacts with electron-deficient alkenes such as diethyl fumarate to form fluorescent pyrazoline adducts (**23**) (Fig. 15a). While DASyd and tetrazoles share the nitrile imine intermediate, DASyd can be activated by longer wavelength UV light (311–371 nm) compared with 2,5-diaryltetrazoles. A systematic study of substituted DASyd provided several insights: (1) DASyd

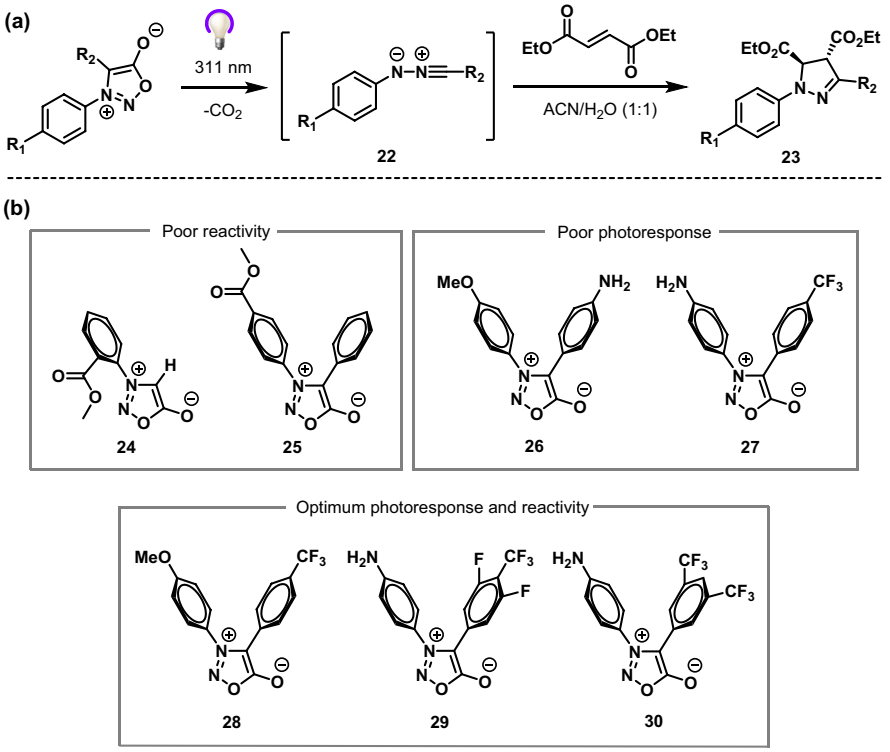

Fig. 15 Photoinduced diarylsydnone-mediated cycloaddition reaction: **a** mechanism; **b** structures of diarylsydnone analogs and their disparate reactivity and photoresponse

with electron-deficient *N*-phenyl rings (**24, 25**) showed poor reactivity toward alkenes; (2) DASyd with *p*-amino group at the *N*- or *C*-phenyl ring (**26, 27**) gave very poor photoresponse despite their strong absorption at 371 nm; and (3) DASyd with electron-rich -phenyl and electron-deficient *C*-phenyl rings (**28–30**) gave optimal photoresponse and reactivity (Fig. 15b). As expected, the reactions also exhibited significant fluorescence turn-on effect, with turn-on ratios ranging from 122-fold to 321-fold.

Following the initial report, this photo-activatable DASyd reagents have recently been employed for peptide photo-stapling/cyclization [32] as well as unprecedented ligation with dibenzo[*b*,*f*] [1, 4, 5] thiadiazepine (DBTD) under visible light [33].

5.1 DASyd-Mediated Peptide Photoclick Stapling

Yu and colleagues reported the synthesis and evaluation of a series of photo-activatable β-diarylsydnone-L-alanine (DASA) [32]. DASA exhibited excellent photoreactivity in cycloaddition reactions with alkenes under biocompatible conditions, and the resulting pyrazolines gave strong turn-on fluorescence. When DASA was

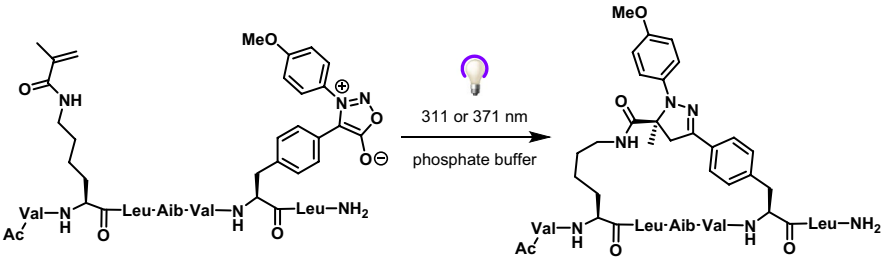

Fig. 16 Photoclick stapling of a linear peptide carrying DASyd and alkene side chains at i, $i+4$ positions

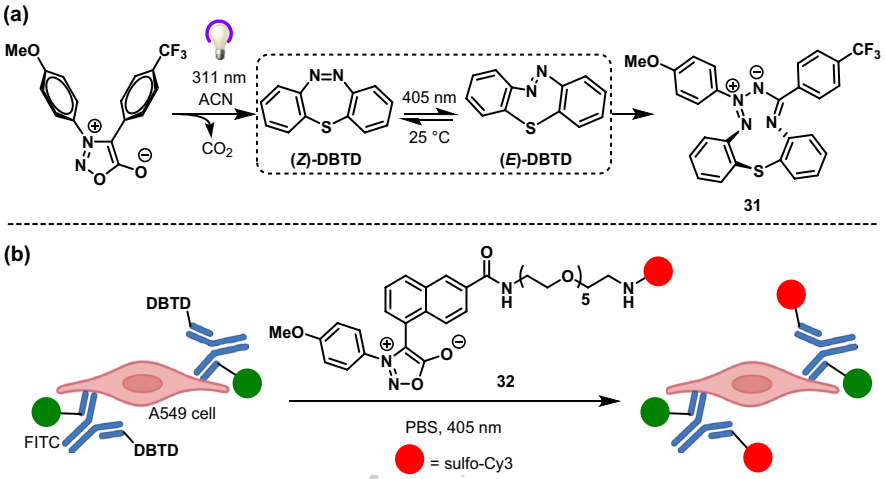

Fig. 17 Photoclick ligation between DASyd and DBTD: **a** Reaction scheme; **b** Visible-light-triggered photoclick ligation of DASyd-Cy3 with a DBTD-modified antibody on A549 cell surface

incorporated into a linear peptide containing a methacrylamide-modified lysine, upon UV irradiation the peptide underwent photo macrocyclization/cycloaddition between the DASyd and the alkene side chains in phosphate buffer (Fig. 16). Interestingly, the fluorescent cyclic peptide showed enhanced permeability into A549 cells compared with the linear peptide.

5.2 DBTD-Mediated Photoclick Ligation

Diarylsydnone-dibenzo[*b*,*f*][1,4,5]thiadiazepine (DBTD) possesses an azobenzene constrained in a dibenzo-annulated seven-membered ring, which undergoes $Z \rightarrow E$ isomerization upon irradiation at 405 nm. Yu and coworkers discovered that (*E*)-DBTD could serve as an excellent dipolarophile for the DASyd-mediated photoclick ligation, yielding a macrocyclic azimine imine **31** (Fig. 17a) [33]. The second-order rate constants of photoclick ligation reactions involving (*Z*)-DBTD and (*E*)-DBTD were determined by NMR to be $2.4 \pm 0.24 \times 10^4$ M^{-1} s^{-1} and $1.6 \pm 0.16 \times 10^5$ M^{-1}

s^{-1}, respectively. The enhanced reactivity observed with (*E*)-DBTD is reminiscent of TCO used in tetrazine ligation reaction. DBTD can also mediate photoclick ligation with Cy3-conjugated DASyd **32** on A549 cell surface pretreated with cetuximab-DBTD after photo-irradiation with a 405-nm LED array (Fig. 17b).

In another application, Yu and colleagues designed amphiphilic amino acids containing a photo-switchable cyclic azobenzene (**33**–**37**) [34]. These amino acids self-assemble into supramolecular structures that insert into the phospholipid membrane. Upon UV irradiation, the azobenzene underwent *cis*-to-*trans* isomerization to generate reactive *trans*-DBDAA, which then participated rapid photoclick ligation reactions with diaryltetrazole or diarylsydnone embedded in the membrane (Fig. 18). It was noteworthy that the photoclick reaction yield was higher in the lipid membrane. Interestingly, when a crown ether was incorporated into DBDAA and inserted into the liposomes, a photo-switchable ion channel was generated, which can be subsequently disrupted using the photoclick chemistry.

6 Other Photo-activatable Bioorthogonal Reagents

6.1 *o*-Nitrobenzyl Alcohols

In 2011, Arumugam and Popik reported a light-induced hetero-Diels–Alder cycloaddition as a facile photoclick reaction [35]. Photo-irradiation of 3-hydroxy-2-naphthalenemethanol **38** at 365 nm generates a reactive *o*-naphthoquinone methide (oNQM, **39**), which subsequently undergoes rapid cycloaddition with electron-rich vinyl ether **40** to form stable benzochroman cycloadducts (**41**–**44**) in excellent

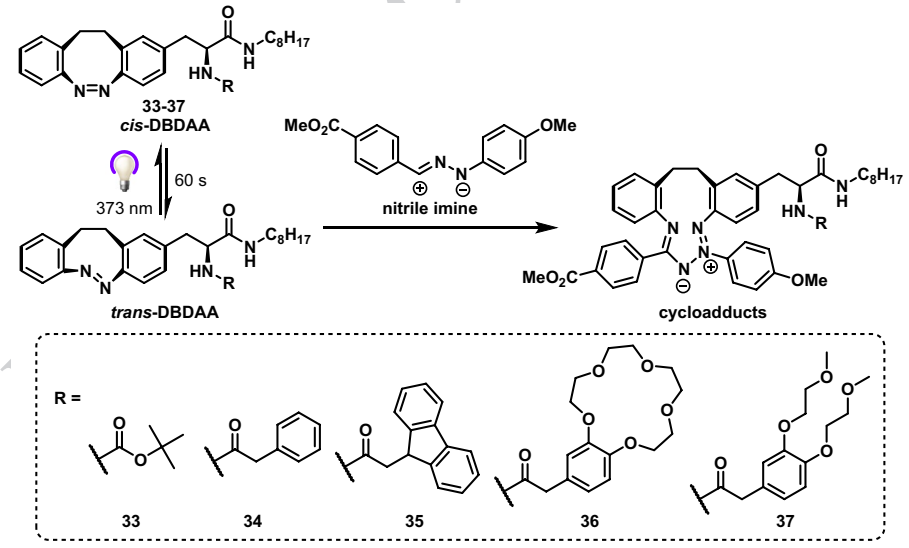

Fig. 18 Membrane-active β-DBDA-L-alanine (DBDAA) analogs and their photoclick ligation with DASyds in lipid membrane

yields (Fig. 19a). The advantages of this photoclick reaction include fast kinetics, chemo-selectivity for electron-rich olefins, and spatiotemporal control through light activation. Besides, after cycloaddition the excess oNQM rapidly reverts to the starting material through hydration.

Inspired by this pioneering work, Chen and colleagues developed a light-induced primary amines and *o*-nitrobenzyl alcohols cyclization (PANAC) as a versatile

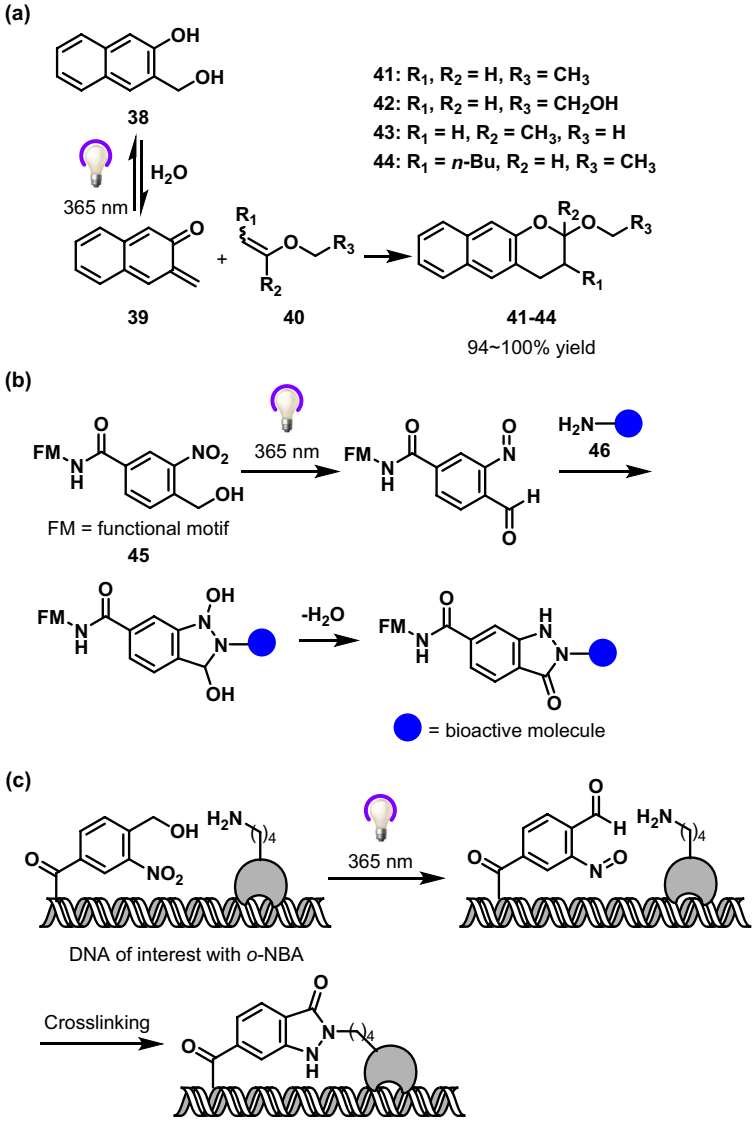

Fig. 19 **a** Photo-activatable 3-(hydroxymethyl)-2-naphthol and its reaction with vinyl ether. **b** Photo-activatable *o*-nitrobenzyl alcohol and its reaction with primary amine. **c** Global profiling of DNA–protein interactions through light-induced lysine-enabled crosslinking (LIKE-XL) with *o*-nitrobenzyl alcohol

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photoclick reaction for bioconjugation [36]. The *o*-nitrobenzyl alcohol (*o*-NBA) group was incorporated into diverse molecules (**45**) and subjected to UV irradiation to induce a selective reaction with proximal primary amines (**46**) to form stable indazolone heterocycles (Fig. 19b). The versatility of this ^{o}-NBA-based photoclick reaction was shown through rapid functionalization of small molecules, peptides, proteins, and even live cells. The key attributes of this reaction include spatiotemporal control, excellent chemo-selectivity for primary amines, fast kinetics, and biocompatibility. A wide range of applications were presented, including late-stage functionalization of drugs, lysine-specific peptide labeling, protein modification, kinase profiling in cells, and organelle-targeted labeling. Overall, this PANAC procedure provides another powerful photoclick chemistry tool for selective bioconjugation in chemical biology where spatiotemporal control is desired.

In a recent application, Chen and coworkers developed a light-induced lysine-enabled crosslinking (LIKE-XL) strategy for global profiling of DNA–protein interactions (Fig. 19c) [37]. The photo-activatable *o*-NBA was incorporated into DNA probes at specific sites, which upon 365-nm photo-irradiation induced covalent crosslinking with proximal lysine residues on the protein binding partners. Using this approach, the weak and transient DNA-binding transcription factors were captured that are typically lost using traditional noncovalent pulldown methods. Notably, the LIKE-XL strategy enabled the discovery of DNA binding sites of low-affinity transcription factors that were previously unknown as well as time-resolved analysis of DNA-binding dynamics of transcription factors in response to drug treatment.

6.2 Photo-activatable Indanone Epoxides

Recently, Yu and colleagues reported a novel photoinduced bioorthogonal ligation reaction based on a photo-switchable oxidopyrylium ylide dipole [38]. Upon irradiation at 365 nm, 2,3-diaryl indenone epoxide (DIO) undergoes ring expansion to generate a reactive oxidopyrylium ylide (PY) (Fig. 20). This photochemical transformation is fully reversible as the oxidopyrylium ylide can be switched back to the inert DIO form using 520 nm light. Importantly, the oxidopyrylium ylide reacts rapidly with strained alkenes and alkynes such as TCO and BCN and TCO via [5 + 2] cycloaddition to afford cycloadduct **47** (Fig. 20). The second-order rate constants of the cycloadditions were exceedingly high, with k_2 values up to 1.9×10^5 M^{-1} s^{-1}. By

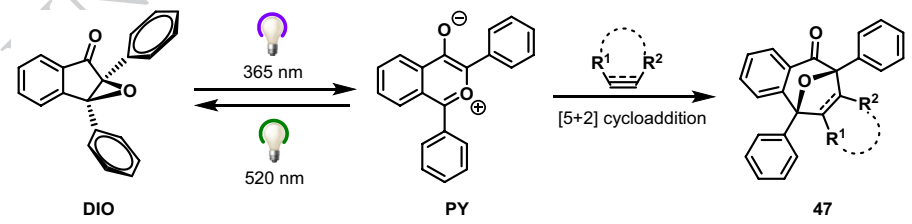

Fig. 20 Light-triggered click reaction between 2,3-disubstituted indanone and ring-strained dipolarophiles

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toggling photo-switching wavelength between 365 and 520 nm, the reactivity of this masked 1,5-dipole could be temporally controlled, allowing on-demand labeling as well as reagent recycling.

One major advantage of oxidopyrylium ylide-mediated photo-switchable ligation reaction is that the spatial distribution of the reactive intermediate can be controlled precisely through an interlaced dual-wavelength stimulation. As a result, this photo switchable system showed greater chemo-selectivity in complex environments, recyclability of the unreacted precursor, and, most notably, spatial confinement of the reactive intermediate during bioconjugation reactions. These unique attributes were illustrated through submicron patterning on substrate surfaces as well as spatially resolved, site-specific labeling on living cell membranes while minimizing off-target labeling due to diffusion of the reactive intermediate [38] (Fig. 21).

6.3 Photo-activatable Phosphines

Staudinger–Bertozzi ligation was the first bioorthogonal reaction reported in the literature [39]. In 2016, Carrico and coworkers reported a photocaged phosphine reagent for light-activated Staudinger–Bertozzi ligation [40]. A 4,5-dimethoxy-2-nitrobenzyl (DMNB)-caged phosphine was prepared to mask its reactivity toward the azide, which can be released at a desired time after photo-irradiation with 350 nm light. The resulting phosphine then reacts with the azide reporter in biological system, rendering a spatially controlled fluorescent labeling. In a fixed-cell study, spatially resolved UV irradiation induced local uncaging and generated the reactive phosphine, which selectively labeled azido-glycan on cell surface (Fig. 22a).

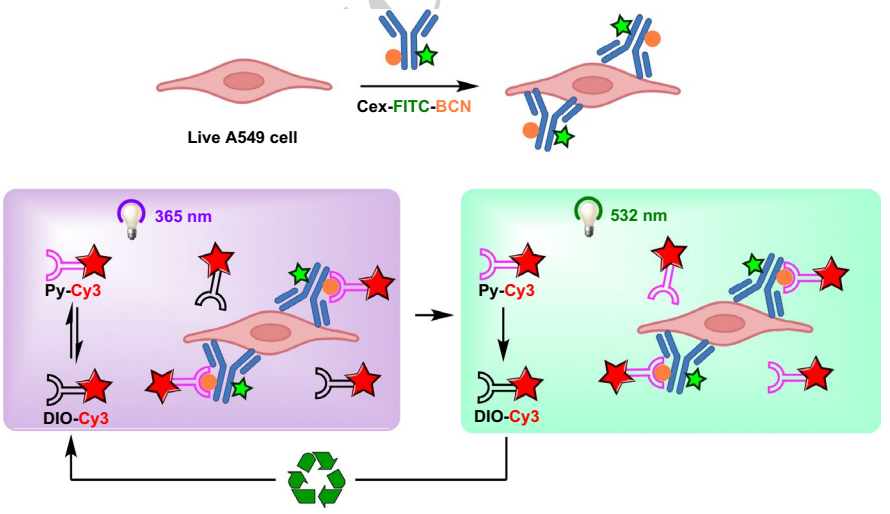

Fig. 21 Spatially controlled bioorthogonal labeling of A549 cells using a photo-activatable DIO-Cy3 reagent. A549 cells were treated with cetuximab–FITC–BCN first followed by sequential photo-irradiation with 365 nm and 532 nm light to activate and recycle the DIO–Cy3 reagent, respectively

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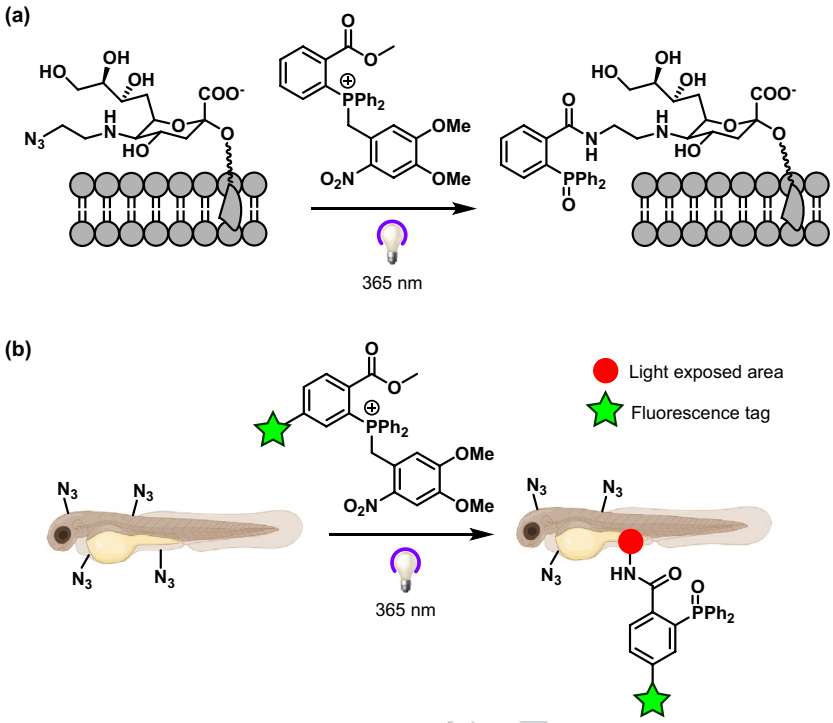

Fig. 22 Spatially controlled modification of azido-glycans using a photocaged phosphine reagent: **a** Glycoprotein modification on fixed mammalian cell surface; **b** Fluorescent tagging of azido-glycans in zebrafish metabolically labeled with GalNAz at one-cell stage

Moreover, photocaged phosphine also enabled spatially controlled labeling in vivo, as illustrated by regiospecific fluorescence tagging of zebrafish larvae injected with an azido sugar and irradiated in the jaw region (Fig. 22b). This photocaged phosphine offers a modular platform for achieving spatiotemporal control over Staudinger–Bertozzi ligation in advanced biomolecular labeling.

7 Conclusions and Outlook

Light-triggered bioorthogonal reactions have attracted significant interest in recent years, owing to their spatiotemporal control over bioconjugation. To take advantage of this unique property, diverse photo-activatable bioorthogonal reagents have been developed that generate reactive intermediates upon light activation at wavelengths spanning UV to near-infrared range. The most prominent reagents in this class include tetrazoles, tetrazines, phenanthrenequinones, diarylsydnones, and other photo-responsive compounds. Through rational molecular design based on physical organic principles and systematic structural modification, the photophysical properties, stability, reactivity, and bioorthogonality of these reagents have been optimized

for maximum efficiency in light-triggered bioorthogonal ligation reactions. A broad range of biological applications have been demonstrated using these photo-activatable reagents, including biomolecular labeling, drug delivery, radiotracer synthesis, photo-responsive biomaterials, and fluorescence imaging.

Moving forward, several frontiers remain to be explored before the full potential of light-controlled bioorthogonal chemistry is realized. Extending photo-activation wavelengths closer to the more transparent near-infrared window would entail deeper tissue penetration and reduced phototoxicity. New near-infrared-sensitive photo-caging groups and photocatalyst systems merit further exploration to improve the precision of spatiotemporal control. Detailed kinetic studies of photoclick reactions in living systems may be warranted to identify the rate-limiting steps and improve the overall reaction kinetics. Combining photo-activatable reagents with antibody-based pretargeting strategies could lead to practical advances in the applications of photoclick reactions in medical imaging. Other high-impact applications such as therapeutic delivery, immunotherapy, and single-cell omics analysis will further drive the progress of bioorthogonal photoclick reactions. We envision that further development of advanced, biocompatible photo-activatable reagents and their innovative use are poised to transform chemical biology research and clinical practice in the future. AQ3

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Declarations

Conflict of Interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

References

1. Kumar GS, Lin Q (2021) Chem Rev 121:6991
2. Fairbanks BD, Macdougall LJ, Mavila S, Sinha J, Kirkpatrick BE, Anseth KS, Bowman CN (2021) Chem Rev 121:6915
3. Wang Y, Rivera Vera CI, Lin Q (2007) Org Lett 9:4155
4. Song W, Wang Y, Qu J, Madden MM, Lin Q (2008) Angew Chem Int Ed 47:2832
5. Song W, Wang Y, Qu J, Lin Q (2008) J Am Chem Soc 130:9654
6. Clovis JS, Eckell A, Huisgen R, Sustmann R (1967) Chem Ber 100:60
7. Zheng S-L, Wang Y, Yu Z, Lin Q, Coppens P (2009) J Am Chem Soc 131:18036
8. Jiang S, Wu X, Liu H, Deng J, Zhang X, Yao Z, Zheng Y, Li B, Yu Z (2020) ChemPhotoChem 4:327
9. An P, Lewandowski TM, Erbay TG, Liu P, Lin Q (2018) J Am Chem Soc 140:4860
10. Bashkatov AN, Genina EA, Kochubey VI, Tuchin VV (2005) J Phys D: Appl Phys 38:2543
11. Yu Z, Ho LY, Wang Z, Lin Q (2011) Bioorg Med Chem Lett 21:5033
12. An P, Yu Z, Lin Q (2013) Chem Comm 49:9920
13. Yu Z, Ohulchanskyy TY, An P, Prasad PN, Lin Q (2013) J Am Chem Soc 135:16766
14. Lederhose P, Wüst KNR, Barner-Kowollik C, Blinco JP (2016) Chem Comm 52:5928
15. Lederhose P, Chen Z, Müller R, Blinco JP, Wu S, Barner-Kowollik C (2016) Angew Chem Int Ed 55:12195
16. Fang M, Kumar GS, Racioppi S, Zhang H, Rabb JD, Zurek E, Lin Q (2023) J Am Chem Soc 145:9959

Journal : SmallExtended 41061	Article No : 447	Pages : 21	MS Code : 447	Dispatch : 6-12-2023

17. Tian Y, Jacinto MP, Zeng Y, Yu Z, Qu J, Liu WR, Lin Q (2017) J Am Chem Soc 139:6078
18. Tian Y, Lin Q (2018) Chem Comm 54:4449
19. Yu Z, Ho LY, Lin Q (2011) J Am Chem Soc 133:11912
20. Blackman ML, Royzen M, Fox JM (2008) J Am Chem Soc 130:13518
21. Devaraj NK, Weissleder R, Hilderbrand SA (2008) Bioconjugate Chem 19:2297
22. Zhang H, Trout WS, Liu S, Andrade GA, Hudson DA, Scinto SL, Dicker KT, Li Y, Lazouski N, Rosenthal J, Thorpe C, Jia X, Fox JM (2016) J Am Chem Soc 138:5978
23. Wang C, Zhang H, Zhang T, Zou X, Wang H, Rosenberger JE, Vannam R, Trout WS, Grimm JB, Lavis LD, Thorpe C, Jia X, Li Z, Fox JM (2021) J Am Chem Soc 143:10793
24. Liu L, Zhang D, Johnson M, Devaraj NK (2022) Nat Chem 14:1078
25. Jemas A, Xie Y, Pigga JE, Caplan JL, Am Ende CW, Fox JM (2022) J Am Chem Soc 144:1647
26. Li J, Kong H, Huang L, Cheng B, Qin K, Zheng M, Yan Z, Zhang Y (2018) J Am Chem Soc 140:14542
27. Cole EL, Stewart MN, Littich R, Hoareau R, Scott PJ (2014) Curr Top Med Chem 14:875
28. Fu Y, Helbert H, Simeth NA, Crespi S, Spoelstra GB, van Dijl JM, van Oosten M, Nazario LR, van der Born D, Luurtsema G, Szymanski W, Elsinga PH, Feringa BL (2021) J Am Chem Soc 143:10041
29. Fu Y, Simeth NA, Toyoda R, Brilmayer R, Szymanski W, Feringa BL (2023) Angew Chem Int Ed 62:e202218203
30. Krauch CH, Kuhls J, Piek HJ (1966) Tet Lett 7:4043
31. Zhang L, Zhang X, Yao Z, Jiang S, Deng J, Li B, Yu Z (2018) J Am Chem Soc 140:7390
32. Yao Z, Wu X, Zhang X, Xiong Q, Jiang S, Yu Z (2019) Org Biomol Chem 17:6777
33. Gao J, Xiong Q, Wu X, Deng J, Zhang X, Zhao X, Deng P, Yu Z (2020) Commun Chem 3:29
34. Wu X, Deng J, Guo G, Zheng Y, Xiong Q, Zheng T, Zhao X, Yu Z (2021) Chem Eur J 27:11957
35. Arumugam S, Popik VV (2011) J Am Chem Soc 133:5573
36. Guo A-D, Wei D, Nie H-J, Hu H, Peng C, Li S-T, Yan K-N, Zhou B-S, Feng L, Fang C, Tan M, Huang R, Chen X-H (2020) Nat Comm 11:5472
37. Guo A-D, Yan K-N, Hu H, Zhai L, Hu T-F, Su H, Chi Y, Zha J, Xu Y, Zhao D, Lu X, Xu Y-J, Zhang J, Tan M, Chen X-H (2023) Nat Chem 15:803
38. Xie X, Hu F, Zhou Y, Liu Z, Shen X, Fu J, Zhao X, Yu Z (2023) Angew Chem Int Ed 62:e202300034
39. Saxon E, Bertozzi CR (2000) Science 287:2007
40. Shah L, Laughlin ST, Carrico IS (2016) J Am Chem Soc 138:5186

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