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Expanding Room for Tetrazine Ligations in the *in vivo* Chemistry Toolbox

Jolita Šeikutė and Neal K. Devaraj*

Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA 92037

Abstract

There is tremendous interest in developing and refining methods to predictably perform chemical reactions within the framework of living systems. Here we review recent advances in applying tetrazine cycloadditions to live cell and *in vivo* chemistry. We highlight new syntheses of the tetrazine and dienophile precursors useful for *in vivo* studies. We briefly overview the use of this reaction in combination with unnatural amino acid technology and discuss applications involving the imaging of glycans on live cells. An emerging area is the use of tetrazine ligations for the development of *in vivo* imaging probes such as those used for positron emission tomography. We summarize recent applications involving tetrazine cycloadditions performed in live mice for pretargeted imaging of cancer cell biomarkers.

Introduction

Bioorthogonal reactions are now routinely utilized for applications in living systems [1]. Among the expanding repertoire of chemistries, *in vivo* chemistry applications of inverse Diels-Alder cycloadditions between tetrazines and strained dienophiles are attracting significant interest, particularly from those interested in performing live cell and animal imaging [2, 3]. Tetrazine ligations benefit from rapid, tuneable kinetics as well as the existence of fluorogenic probes. Biomedical applications of tetrazine cycloadditions were reviewed in 2011 [4]. A recent review has also addressed the implementation of tetrazine ligations to nanomaterial diagnostics [5]. This article will focus on developments in applying tetrazine cycloadditions to *in vivo* chemistry applications over the last two years. Specifically, we will discuss the development of new chemistries, live cell and *in vivo* imaging applications, and finally progress toward the challenging undertaking of reliably performing tetrazine cycloadditions within live animals.

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858-534-9539, ndavaraj@ucsd.edu.

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New synthetic routes to tetrazines and strained dienophiles

Despite the interest in tetrazine ligations, the precursors are not widely available and are oftentimes difficult to synthesize. Although some vendors are slowly making specific tetrazines and dienophiles commercially available, improvements in the methodology would be welcome. Since the structure of 1,2,4,5-tetrazine greatly affects reaction rate and stability, new tetrazines would be useful for tuning these parameters for *in vivo* chemistry applications. Improved tetrazine synthetic routes would also be useful for creating tetrazine fluorescent probes. Hilderbrand and coworkers recently disclosed several synthetic routes to unsymmetric tetrazines with functional group handles (Figure 1a) [6]. These compounds were tested for their reactivity with dienophiles such as norbornene and *trans*-cyclooctene, as well as their stability in biologically relevant media. The latter test revealed several highly stable methyl-terminated tetrazines. These tetrazines can react with *trans*-cyclooctene while avoiding cross-reactivity with cyclooctynes, enabling dual live cell labeling using concurrent tetrazine/*trans*-cyclooctene and azide/cyclooctyne chemistry [7].

The most convenient method to produce tetrazines has been the addition of hydrazine to aromatic nitriles followed by one-pot oxidation of the resulting dihydrotetrazine intermediate. Unfortunately, this convenient strategy is not amenable to unactive nitriles such as alkyl nitriles, limiting the diversity of readily available tetrazines. Recent work from the Devaraj group has uncovered that Lewis acid catalysts, such as zinc and nickel salts, can catalyze the formation of tetrazine from alkyl nitriles, presumably by activating the nitrile to addition by hydrazine (Figure 1b) [8]. A wide variety of symmetric and unsymmetric tetrazines were prepared in one-pot routes from readily available starting materials, several with functional group handles. Unsymmetric dialkyl tetrazines, which are typically some of the most difficult to create, were accessible using this technique, albeit at lowered yield.

Methods to increase the reaction rate between tetrazines and strained dienophiles are extremely interesting for practical applications in complex living systems. Exciting work from the Fox lab has used computation to design a novel class of bicyclic *trans*-cyclooctenes (Figure 1c) [9]. These unique compounds are forced into highly strained conformations leading to much greater reactivity with tetrazine partners compared to the parent *trans*-cyclooctene. However, it should be noted that high concentrations of thiol induced *trans/cis* cycloisomerization. Other groups have observed this isomerization process with *trans*-cyclooctenol, albeit at elevated temperature, and it remains to be seen how this process depends on strain and what impact this has on *in vivo* chemistry applications where thiols are prevalent [10].

The large size of tetrazines and strained dienophiles such as *trans*-cyclooctene, cyclooctyne, and norbornene has been a roadblock in the broader application of tetrazine ligations. For instance, an important implementation of bioorthogonal chemistry involves introducing a reactive handle into a metabolite analog which is then incorporated into a biopolymer through various enzymatic pathways [1]. These pathways can be highly sensitive to the size and nature of the handle. Furthermore, an important consideration for *in vivo* chemistry is how large hydrophobic functional groups can influence the pharmacokinetics when appended to proteins and small molecules.

The use of smaller dienophiles, such as cyclopropenes, as tetrazine reactive partners offers a possible solution. However, the development of cyclopropene mini-tags required balancing the rate of reaction, which depends heavily on the substitution pattern, and the poor stability of unsubstituted cyclopropenes. Considering these requirements, the Devaraj group introduced two 1-methyl-cyclopropene mini-tags as fast and stable dienophiles for reaction with tetrazines (Figure 1d) [10]. These tags are capable of rapidly reacting with tetrazines, elicited fluorogenic responses with quenched tetrazine-fluorophores, and were shown to be suitable for live-cell labeling of lipid analogs. Subsequent work has further utilized the small size and high reactivity of cyclopropenes as coupling agents [11, 12]. However, methyl-cyclopropenes react significantly slower with tetrazines compared to *trans*-cyclooctenes, and so may be most suited for live cell applications where larger concentrations of the labeling agent can be utilized.

Live-Cell imaging through uptake of unnatural metabolites

There have been several recent examples utilizing tetrazine cycloadditions for live-cell imaging of small molecules that are biosynthetically incorporated into biopolymers such as proteins and glycans [11, 13, 14]. Several groups demonstrated the ability to genetically encode dienophiles or tetrazines using site-specific unnatural amino acid insertion [15–17]. Incorporation of norbornene, cyclooctyne, or *trans*-cyclooctene readily takes place using a variety of tRNA synthetase and modified tRNA pairs. Dienophile-tagged proteins could be imaged in live prokaryotic and eukaryotic cells, both extra- and intracellularly (Figure 2). Intracellular imaging was aided by the use of fluorogenic tetrazine dyes [18]. Indeed tetrazine reactions benefit from the ability to routinely perform the reaction in the cytoplasm. Mehl and coworkers incorporated an unnatural tetrazine amino acid [19]. Remarkably, incorporation of a tetrazine ~12 Å from the chromophore of a green fluorescent protein (GFP) resulted in 11-fold quenching of GFP fluorescence, similar to the quenching effect observed with synthetic tetrazine-fluorophore dyes. Reaction with *trans*- recovered GFP fluorescence by eliminating the tetrazine chromophore.

A relatively new application of tetrazine cycloadditions has been their use in imaging glycans that have metabolically incorporated unnatural sugars containing dienophile tags. The Prescher group elegantly utilized the 3-carbamoyl substituted 1-methyl-cyclopropene tag to introduce modified neuramic acids on glycans and reveal them through tetrazine cycloadditions [11]. This work nicely demonstrated the potential of methyl-cyclopropenes to undergo tetrazine cycloaddition in parallel with azide-alkyne bioorthogonal chemistry, a feature that was suggested based on density functional theory calculations [20]. The Devaraj group recently demonstrated the ability of methylcyclopropene modified *N*-acetyl mannosamines to be used in metabolic imaging, an application that is highly sensitive to the steric bulk of appended bioorthogonal probes [21]. Unstrained vinyl groups have also been reported to be useful for metabolic glycan imaging using highly reactive tetrazines [22]. However, it should be noted that the kinetics of this reaction are much slower compared to tetrazine reactions with cyclic strained alkenes. Finally, Leeper and coworkers have developed a novel tetrazine/isonitrile coupling reaction to label and image glycans on live cells [23].

Molecular imaging probes

Tetrazine ligations have been well utilized in developing new probes for *in vivo* imaging applications. In particular, radionuclide imaging probe development for use in positron emission tomography (PET) and single-photon emission computed tomography (SPECT) has greatly benefited from the rapid kinetics of tetrazine ligations. Radiochemistry is challenging due to the finite half-lives of popular radiolabels such as fluorine-18 (~110 minutes) and carbon-11 (~20 minutes) and the need to work with materials in trace amounts. These realities often impede the development of new probes. It is therefore not surprising that radiochemists have quickly adopted rapid and high-yielding tetrazine cycloadditions. Typical strategies involve devising a rapid and efficient synthesis of a radiolabelled dienophile or tetrazine that serves as a universal reactive agent for appending the radiolabel to a wide variety of targeting ligands possessing the complementary reactive group. The result is a kit-like approach to the development of new imaging probes.

Several groups have introduced the popular PET radioisotope fluorine-18 into dienophiles such as *trans*-cyclooctene and norbornene [24–26]. Introducing fluorine-18 onto dienophiles instead of tetrazines is preferred due to the fact that fluorine-18 introduction typically requires harsh labeling conditions, under which reactive tetrazines degrade [25]. *In vivo* applications of fluorine-18 dienophiles include imaging integrins and PARP1 inhibitors [25, 27, 28]. For the latter application, the authors used a clever approach to clear unreacted tetrazine from fluorine-18 labeled inhibitor by using a *trans*-cyclooctene modified magnetic bead as a scavenger. *Trans*-cyclooctenes have also been labeled with the rapidly decaying PET isotope carbon-11 [29].

Though less commonly used, metal positron emitting radionuclides are gaining attention for their longer half-lives and the mild conditions required to introduce them onto chelating agents. Recent work has expanded the use of tetrazine cycloadditions to the modular preparation of long-lived PET radioisotopes using zirconium-89 and copper-64 (Figure 3) [30]. Unlike previous reports utilizing fluorine-18, this work focused on radiolabeling the tetrazine functional group. This element of novelty was made possible since the chemistry required to chelate these metals is very mild, and under these conditions reactive tetrazines remain stable.

Performing tetrazine cycloadditions in live animals

Perhaps the most challenging application of tetrazine cycloadditions is performing the reaction in live animals. The initial reports of utilizing tetrazine cycloadditions for live-cell imaging suggested use of the reaction for pretargeted imaging of monoclonal antibodies and demonstrated the proof-of-concept *in vitro* [2, 31, 32]. Pretargeted imaging with tetrazine cycloadditions involves injecting a *trans*-cyclooctene modified monoclonal antibody and allowing this antibody to bind to the target and for the excess to clear, usually over a period of 24 hours. After clearance, a secondary tetrazine imaging agent is delivered, allowed to react with the pretargeted antibody, clear, and imaged. Since the secondary tetrazine chaser agent clears from the blood much faster than the monoclonal antibody, short-lived radionuclides such as fluorine-18 can be potentially used to image antibody distributions, an

application that would otherwise be impossible with antibodies directly modified with fluorine-18. Furthermore, such techniques can eventually find application in radioimmunotherapy, where systemic delivery of cytotoxic slowly-clearing radioimmunoconjugates is clinically problematic [33]. Since tetrazines have limited serum stability, the antibodies, which must reside *in vivo* for longer than 24 hours, are typically modified by the more stable *trans*-cyclooctene dienophile. The chaser, which by design persists for a short time in the blood stream, is modified by the tetrazine.

A team led by Dr. Robillard quickly followed *in vitro* pretargeting reports with a demonstration that tetrazine cycloadditions would be appropriate for *in vivo* pretargeted imaging [34]. The team injected *trans*-cyclooctene modified anti-TAG72 monoclonal antibodies into mice bearing colon cancer xenografts. After waiting for antibody clearance, they next injected a tetrazine chaser bearing the SPECT imaging agent indium-111. This small molecule was rapidly cleared, and imaging and analysis took place after three hours. Remarkable SPECT images demonstrating high tumor-to-background ratios were observed. However, the use of the long-lived radioisotope indium-111 (half-life 2.8 days) obviated the need for multistep imaging, as one could alternatively inject indium-111 modified monoclonal antibodies and wait for clearance before imaging.

Recently, Devaraj et. al. reported the use of short-lived fluorine-18 probes for pretargeted PET imaging [35]. This report detailed a strategy to achieve efficient *in vivo* chemistry by carefully considering both the bioorthogonal reaction rate and the rate of secondary clearance. Computational modeling followed by experiments was used to better understand how these two parameters combine to influence the *in vivo* reaction yield. As one might expect, the reaction rate required to achieve highly efficient *in vivo* chemistry is lowered if the secondary agent is retained longer in the bloodstream. Since pretargeted imaging requires that the secondary agent clears over several hours, a balance was met by developing tetrazine imaging probes on oligomeric dextran scaffolds. Pretargeted PET imaging was demonstrated using fluorine-18 dextran chasers. Mice bearing A33 overexpressing LS174T colon cancer xenografts were injected with *trans*-cyclooctene modified anti-A33 monoclonal antibodies. After waiting 24 hours for clearance, a fluorine-18 dextran was injected and after 3 hours, and analysis was performed by PET imaging. Significant uptake of fluorine-18 enabled visualization of the tumors (Figure 4a). This was much greater than control experiments with antibodies lacking *trans*-cyclooctene, and was further verified using autoradiography of tumor tissue slices. Autoradiography also demonstrated that the fluorine-18 dextran chaser showed good colocalization with the *trans*-cyclooctene monoclonal antibody, which was visualized using an appended near-infrared fluorophore (Figure 4b). Finally, biomarker specificity was demonstrated by injecting mice that were implanted simultaneously with LS174T and A431 tumors, the latter having been shown to not express the A33 antigen. Significant uptake was observed in the LS174T tumor with minimal background in the A431 tumor (Figure 4c,d). These results are impressive because of the utilized short-lived fluorine-18 radionuclide to observe antibody and thus biomarker distributions. However, the secondary agents need refinement as background uptake, particularly from liver accumulation, needs to be lowered before clinical utilization.

Conclusions

Tetrazine cycloadditions are increasingly applied to *in vivo* chemistry applications. The rapid rate of reaction even in the absence of a catalyst has enabled their use particularly in imaging small molecules in living cells or animals. Performing tetrazine ligations in live animals will advance as reaction rates are improved while preserving stability and specificity, and as the pharmacokinetic parameters of the reactive agents are optimized to maximize delivery of imaging agents and therapeutics to target cells while avoiding non-specific background uptake. Although these challenges should not be underestimated, we are hopeful that an improved understanding and further development of the synthetic chemistry of tetrazine and dienophile precursors will enrich and broaden future applications.

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Highlights

- overview of new tetrazine and dienophile syntheses useful for chemistry in living systems
- review of live-cell imaging applications
- tetrazine applications related to the development of in vivo imaging probes
- examples of tetrazine cycloadditions performed in live animals

Figure 1.

New developments in the synthesis of tetrazines and dienophiles suitable for chemistry in living systems. a) Synthesis of a stable dialkyl substituted tetrazine from an imidate ester and amidine. b) A wide variety of tetrazines can be synthesized directly from alkyl nitriles using Lewis acid metal catalysts. c) Development of a highly strained bicyclic *trans*-cyclooctene that reacts with tetrazines at unprecedented rate constants. d) Synthesis of 1-methyl-3-substituted cyclopropene “mini-tags” which are stable to decomposition and react readily with tetrazines. Figures adapted from references 6, 8, 9, and 10.

Figure 2.

Live-cell imaging of proteins using site-specific incorporation of unnatural dienophile amino acids followed by reaction with tetrazine fluorophores. a) Surface localized EGFP containing a single dienophile unnatural amino acid is rapidly reacted with a tetrazine appended to carboxytetramethylrhodamine (TAMRA), leading to surface staining and excellent colocalization between the TAMRA and EGFP signals. b) This technology can also be used to reveal intracellular proteins. A nuclear protein fused to the fluorescent protein mCherry, is genetically programmed to incorporate a reactive dienophile amino acid. This is revealed in live cells using a fluorogenic fluorescein-tetrazine probe. Figure used with permission from reference 13.

Figure 3.

Modular synthesis of *in vivo* molecular imaging agents. Monoclonal antibodies can be decorated with dienophiles such as norbornenes and modified using a tetrazine chelating agent (Tz-DOTA). After modification, the conjugate can be readily metallated with either zirconium-89 or copper-64, two long-lived positron emitting radionuclides useful for PET imaging. Alternatively, the dienophile modified antibodies can be reacted with tetrazines chelates already containing metal ions. Figure used with permission from reference 30.

Figure 4.

Performing tetrazine cycloadditions *in vivo* for detecting human colon carcinoma biomarkers. a) PET/CT fusion of LS174T tumor xenograft labeled using either *trans*-cyclooctene (TCO) monoclonal antibodies (MAB TCO) or control unlabeled antibodies (MAB) followed by a fluorine-18 modified tetrazine polymer. Arrows indicate location of the tumor xenograft. Bladder has been omitted for clarity. b) Imaging using autoradiography (left side) and fluorescence reflectance (right side) of 1 mm LS174T tumor slices after targeting with fluorescent *trans*-cyclooctene monoclonal antibody and fluorine-18 modified tetrazine polymer. c) PET/CT fusion of mouse bearing A431 (non-A33 expressing) and LS174T (A33 overexpressing) tumors after targeting with anti-A33 TCO monoclonal antibodies followed by fluorine-18 modified tetrazine polymer. Arrows indicate location of tumors and the liver has been omitted for clarity. d) Autoradiography of representative 1 mm LS174T or A431 tumor slices after multistep targeting. Figure used with permission from reference 35.