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The inverse electron demand Diels–Alder click reaction in radiochemistry‡

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Abstract

The inverse electron-demand Diels-Alder (IEDDA) cycloaddition between 1,2,4,5-tetrazines and strained alkene dienophiles is an emergent variety of catalyst-free ‘click’ chemistry that has the potential to have a transformational impact on the synthesis and development of radiopharmaceuticals. The ligation is selective, rapid, high-yielding, clean, and bioorthogonal and, since its advent in 2008, has been employed in a wide variety of chemical settings. In radiochemistry, the reaction has proven particularly useful with ¹⁸F and has already been utilized to create a number of ¹⁸F-labeled agents, including the PARP1-targeting small molecule ¹⁸F-AZD2281, the $\alpha_v\beta_3$ integrin-targeting peptide ¹⁸F-RGD, and the GLP-1-targeting peptide ¹⁸F-exendin. The inherent flexibility of the ligation has also been applied to the construction of radiometal-based probes, specifically the development of a modular strategy for the synthesis of radioimmunoconjugates that effectively eliminates variability in the construction of these agents. Further, the exceptional speed and biorthogonality of the reaction have made it especially promising in the realm of *in vivo* pretargeted imaging and therapy, and pretargeted imaging strategies based on the isotopes ¹¹¹In, ¹⁸F, and ⁶⁴Cu have already proven capable of producing images with high tumor contrast and low levels of uptake in background, nontarget organs. Ultimately, the characteristics of inverse electron-demand Diels–Alder click chemistry make it almost uniquely well-suited for radiochemistry, and although the field is young, this ligation has the potential to make a tremendous impact on the synthesis, development, and study of novel radiopharmaceuticals.

Keywords

click chemistry; Diels–Alder; tetrazine; norbornene; transcyclooctene; radiochemistry; pretargeting

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Conflict of Interest

The authors did not report any conflict of interest.

Introduction

The intrinsic selectivity and flexibility of click chemistry make it an almost ideal synthetic methodology for the creation of radiopharmaceuticals.^{1,2} Indeed, it is possible that the combination of selectivity, modularity, orthogonality, and rapidity offered by click chemistry can benefit radiochemistry as much as—if not more than—any other chemical discipline.^{3,4}

Without question, the variant of click chemistry most often employed in radiochemistry is the canonical Cu(I)-catalyzed 1,3-dipolar Huisgen cycloaddition between azides and alkynes.¹ Recently, the catalyst-free, strain-promoted cyclo-addition reaction between azides and cycloalkynes pioneered by Bertozzi has also gained traction in the field.^{2,5} Importantly, however, these methodologies are not without limitations. For the former, the requirement of a Cu(I) catalyst can prove a hindrance in many applications, particularly when used in conjugation with sensitive biomolecular vectors, radiometals, or radiometal chelators. The strain-promoted reaction, of course, eliminates the risk of misbehavior by a metallic catalyst; however, in this case, the hydrophobicity of the cycloalkyne starting materials and the relatively sluggish kinetics of the reaction can be problematic in some settings.⁶

The past 5 years have witnessed the rise of a promising click ligation capable of circumventing many of these limitations: the inverse electron demand [4+ 2] Diels–Alder (IEDDA) cycloaddition between a 1,2,4,5-tetrazine and a strained alkene dienophile.^{7–10} Like its click chemistry cousins, this ligation is selective, high-yielding, clean, biocompatible, and bioorthogonal. The reaction proceeds in two steps. The tetrazine and dienophile first undergo an IEDDA cycloaddition to form a tricyclic species with a dinitrogen bridge. This intermediate then undergoes a retro-Diels–Alder reaction driven by the release of dinitrogen to form a stable dihydropyridazine product.^{7,11} The rapidity of the ligation is governed largely by the identity of the two components. The earliest work using the IEDDA reaction utilized derivatives of norbornene as the dienophile; however, norbornenes, though effective, have quickly given way to dienophiles based on transcyclooctene (TCO), which were found to dramatically accelerate the reaction.^{8,11–14} For example, with the same variant of tetrazine [3-(4-benzylamino)-1,2,4,5-tetrazine, Figure 1] the second order reaction rate for the cycloaddition was shown to be $\sim 1\text{--}2\text{ M}^{-1}\text{ s}^{-1}$ with a norbornene-based dienophile compared to $\sim 26,000\text{ M}^{-1}\text{ s}^{-1}$ with a TCO-based reaction partner.^{11,15} Further, a number of different 1,2,4,5-tetrazines have also been tested for their influence on reaction kinetics, revealing rate constants with transcyclooctene ranging from $210\text{ M}^{-1}\text{ s}^{-1}$ to almost $30,000\text{ M}^{-1}\text{ s}^{-1}$.¹⁶ It is perhaps not surprising that in this study aqueous stability and reaction rate were observed to be inversely proportional: the more stable tetrazines generally reacted less rapidly, while the less stable compounds generally reacted more quickly. Ultimately, it becomes clear that one of the chief advantages of the IEDDA cycloaddition lies in its speed. To provide a basis for comparison, the reaction rate of the catalyst-free strain-promoted cycloaddition between an azide and dibenzylcyclooctyne hovers around $2\text{ M}^{-1}\text{ s}^{-1}$, two orders of magnitude *slower* than the *slowest* reported IEDDA reaction between a tetrazine and a transcyclooctene.¹⁵

Although we will address only radiochemical applications of the IEDDA reaction in this mini-review, since its emergence in 2008, the ligation has also been used successfully in a variety of biomedical imaging and bioengineering applications, most notably the creation of reactive markers for antibodies, small molecules, and nanoparticles.^{11–13,17–27}

Applications with ^{18}F

The first radiochemical applications of the IEDDA reaction followed rapidly on the heels of the initial reports on the ligation's immense potential in bioconjugation chemistry.^{7,8,28} Indeed, the use of tetrazines and transcyclooctenes has attracted particular attention in the construction of ^{18}F -based imaging probes, primarily due to the short half-life of the radioisotope ($t_{1/2} = 109.8$ min) and the paramount importance of high specific activity and selectivity in the creation of ^{18}F -labeled radiotracers.²⁹ Furthermore, the effectiveness of the IEDDA ligation under very mild reaction conditions—that is, neutral pH, room temperature—has also proven desirable, for traditional radiofluorination approaches often require high temperatures and nonaqueous solvents, which can in some cases lead to the decomposition of biologically active molecules.

In 2010, Li and coworkers published the first example of the IEDDA reaction in radiochemistry: the design, synthesis, and purification of an ^{18}F -labeled transcyclooctene (^{18}F -TCO) suitable for rapid conjugation with tetrazines.³⁰ In this work, the authors reported radiochemical yields of up to ~70% for the synthesis of ^{18}F -TCO under relatively mild conditions, in addition to rapid, selective, and clean reactions between ^{18}F -TCO and 3,6-diaryl-s-tetrazine click reaction partners. In the same work, efforts to label a 1,2,4,5-tetrazine variant with ^{18}F proved elusive, likely due to the decomposition of the tetrazine during radiofluorination.

Weissleder and coworkers have also reported the use of tetrazines and transcyclooctenes in the design and synthesis of the first ^{18}F -labeled, IEDDA-derived small molecule imaging agent, the PARP-1 targeting ^{18}F -AZD2281.^{31,32} The molecule was shown to effectively target PARP-1 expression *in vivo* in murine models of ovarian and pancreatic cancer, but just as important to the work was the creative application of the IEDDA ligation to the purification of the probe.³³ A common problem in the synthesis of ^{18}F -labeled small molecules is that the low concentration of ^{18}F in the reaction mixture (typically in the low nM to pM range) puts significant strain on the kinetics of the labeling reaction. In many cases, reaction rates can be increased by raising the concentration of cold reactants; however, the subsequent removal of excess cold precursor can be a significant challenge. In this case, the authors circumvented this issue by first reacting ^{18}F -TCO with an excess of a biologically active, tetrazine-labeled small molecule, AZD2281-Tz. Then, without any further purification, the excess AZD2281-Tz was removed by incubating the reaction solution with a TCO-decorated, magnetic scavenger resin that could subsequently be filtered out to yield the ^{18}F -labeled compound in high purity and specific activity (Figure 2).

Clearly, the IEDDA ligation holds promise for the synthesis of small molecule probes. However, we believe that it could prove even more valuable in the synthesis of biomolecular imaging agents, for it represents a labeling technique which occurs rapidly under mild

temperature and pH reaction conditions, is orthogonal to biogenic amino acids, is tolerant of media and buffered aqueous solutions, and proceeds without a reactive catalyst. In 2011, for example, Selvaraj *et al.* created an ^{18}F -labeled variant of the $\alpha_v\beta_3$ -targeting peptide RGD using the IEDDA ligation between ^{18}F -TCO and an RGD precursor that had been modified with a 3,6-di-(2-pyridyl)-s-tetrazine via a simple amide bond formation reaction.³⁴ The resulting ^{18}F -RGD peptide was produced in relatively high specific activity and purity and was shown to be effective at delineating $\alpha_v\beta_3$ -expressing U87MG glioma xenografts *in vivo*.

More recently, Keliher *et al.* have used tetrazine-modified peptides in the design and synthesis of high specific activity probes for GLP-1 receptor imaging.³⁵ To this end, the authors synthesized a bifunctional, maleimide-modified tetrazine which was subsequently incubated with a derivative of the GLP-1-targeting peptide exendin-4 that had been engineered to bear a single cysteine thiol. This conjugation reaction created a tetrazine-bearing peptide which was then rapidly and selectively reacted with ^{18}F -TCO to generate the desired tracer, ^{18}F -exendin-4, in high yield, specific activity, and radiochemical purity. The completed ^{18}F -exendin-4 peptide was successfully shown to target the GLP-1 receptor *in vivo*, displaying specific uptake of up to 2.5 %ID/g in murine models of insulinoma. Finally, and most recently of all, Conti and coworkers have employed bifunctional tetrazines bearing reactive maleimides in the IEDDA-based syntheses of ^{18}F -labeled peptides targeting $\alpha_v\beta_3$, VEGFR, and the GLP-1R.^{36,37}

Applications with radiometals

Although the IEDDA reaction is particularly well-suited to ^{18}F -based chemistry, its radiochemical applications certainly do not end there. Indeed, both the catalyst-free nature of the ligation and its inherent modularity give it the potential to be quite useful with radiometals as well.³⁸ The absence of a Cu(I) catalyst eliminates any risk of competition between the radiometal and the catalyst for the chelator, and the flexibility of the system means that different chelators can be readily and rapidly appended to the same biomolecular vector in order to accommodate the diverse chelation chemistry of various radiometals.

To date, however, there is only one published report employing the IEDDA reaction for the synthesis of radiometal-based agents: a modular strategy for the construction of radiolabeled antibodies geared at eliminating variability in the synthesis of these macromolecular agents.³⁹ In this system, an antibody is first conjugated to norbornene, and this single covalently modified stock can then be clicked with any tetrazine-modified chelator and subsequently radiolabeled. Using a model system employing the HER2-targeting antibody trastuzumab, the positron-emitting radiometals ^{64}Cu and ^{89}Zr , and variants of tetrazine modified with the chelators DOTA and DFO, the authors found that for a given stoichiometry of norbornene to antibody, the final ^{64}Cu -DOTA-labeled and ^{89}Zr -DFO-labeled probes were identical in terms of stability, the number of chelates per antibody, and *in vitro* immunoreactivity. For example, the final ^{64}Cu -DOTA-trastuzumab and ^{89}Zr -DFO-trastuzumab radioimmunoconjugates constructed using an initial construct bearing only three norbornenes per antibody were found to possess identical numbers of chelates per antibody (2.2 ± 0.3 DOTA/mAb and 2.3 ± 0.4 DFO/mAb, respectively), *in vitro* serum

stabilities ($97 \pm 2\%$ and $96 \pm 2\%$), immunoreactive fractions (0.96 ± 0.05 and 0.95 ± 0.05), and specific activities (2.9 ± 0.3 mCi/mg and 3.1 ± 0.2 mCi/mg). Further, both radioimmunoconjugates were shown to selectively and specifically delineate HER2+ BT474 breast cancer xenografts *in vivo*, producing images with high absolute tumor uptake and excellent tumor-to-background contrast.

Applications in pretargeted imaging

The IEDDA reaction also represents a promising methodology for *in vivo* pretargeting applications. Broadly defined, pretargeted imaging and therapy are methodologies that seek to harness the exquisite tumor-targeting properties of radioimmunoconjugates yet avoid their slow pharmacokinetics and high background doses by decoupling the targeting vector from the radioisotope at the time of injection.^{40,41} To this end, an immunoconjugate capable of binding both an antigen and a radiolabeled hapten is first injected into the patient and allowed to accumulate at the tumor and clear from the blood; then, after a predetermined interval, a radiolabeled, small molecule hapten is administered which travels through the blood, either binding to the immunoconjugate at the tumor or clearing quickly. Using this approach, pretargeting methodologies have the potential to produce high activity uptake in the tumor with extremely low levels of activity uptake in non-target organs; however, this *in vivo* recombination step requires both bioorthogonality and rapidity. These two traits are hallmarks of the IEDDA ligation.

The first report of an IEDDA-based pretargeted imaging methodology was published by Robillard and coworkers in 2010.⁴² Soon thereafter, similar approaches were realized by the groups of Weissleder and Lewis in 2012 and 2013, respectively.^{43,44} Although the specifics differ, each of these systems employs just two components—a transcyclooctene-modified antibody and a radiolabeled tetrazine—and four facile steps (Figure 3):

1. The injection of the mAb-TCO conjugate
2. A localization period during which the antibody accumulates in the tumor and clears from the blood
3. The injection of the radiolabeled tetrazine
4. The *in vivo* click ligation of the two components, followed by the clearance of excess radioligand

The IEDDA reaction offers a number of advantages over other pretargeting strategies. For example, the modularity of the system—both the TCO and tetrazine moieties can be modified with a wide variety of targeting vectors or radioisotopes—presents a notable improvement in cost and versatility over pretargeting systems based on antibody-streptavidin fusion proteins or bispecific antibodies. Further, the selectivity and rapid kinetics of the IEDDA reaction make it more suitable for *in vivo* applications than other catalyst-free click chemistry methodologies. To wit, recently published studies on pretargeted PET imaging strategies based on both the strain-promoted azide-alkyne cycloaddition and the traceless Staudinger ligation suggest neither of these reactions is particularly well-suited for *in vivo* applications.^{6,45}

The first reported methodology employed a TCO-labeled conjugate of the TAG72-targeting antibody CC49 and a tetrazine linked to a ^{111}In -DOTA chelate complex by a PEG chain. By injecting 100 μg of the immunoconjugate and allowing a lag time of 24 hours prior to the injection of the ^{111}In -DOTA-tetrazine radioligand, the authors were able to delineate a colorectal cancer xenograft in a mouse via SPECT with high contrast and low background uptake in non-target organs.⁴² The strategy published by Devaraj, *et al.* used a different approach, employing a ^{18}F -labeled macromolecular, dextran-based tetrazine radioligand in conjunction with a TCO-conjugated A33 antibody to effectively delineate LS174T colorectal cancer xenografts in mice.⁴³ Finally, the most recent example of IEDDA-based pretargeting employed a TCO-modified conjugate of the A33 antibody along with a small molecule ^{64}Cu -NOTA-tetrazine radioligand, a system which produced high contrast PET images of SW1222 colorectal cancer xenografts *in vivo* with extremely low background uptake in non-target organs.⁴⁴ Importantly, this most recent work contained a detailed dosimetric analysis of the pretargeted imaging strategy, illustrating that the IEDDA-based methodology yielded a reduced patient radiation dose compared to traditional directly-labeled radioimmunoconjugates, specifically $\sim 1/4\text{th}$ and $\sim 1/30\text{th}$ that of analogous ^{64}Cu -NOTA-labeled and ^{89}Zr -DFO-labeled antibody constructs. It is important to note that these three strategies also illustrate the degree to which the biodistribution and pharmacokinetics of the tetrazine-based radioligands can be modulated through chemical modifications to optimize *in vivo* behavior. Taken together, these data speak to the tremendous promise of the IEDDA reaction as a tool for pretargeted imaging and therapy.

Conclusions: challenges and future directions

Despite its rise to prominence only 5 years ago, the inverse electron demand Diels–Alder cycloaddition reaction has already left a sizeable imprint on the biomedical imaging community. The IEDDA reaction between tetrazines and transcyclooctenes has been employed in a wide variety of imaging applications, ranging from fluorescence imaging with targeted small molecules to magnetic resonance imaging with nanoparticulate vectors. However, we strongly believe that the most significant impact of this ligation may lie in radiopharmaceutical chemistry, for its singular combination of selectivity, orthogonality, and rapidity make the IEDDA reaction almost ideally suited for radiochemical applications. Indeed, as we have discussed here, the IEDDA reaction has already been effectively employed in the development of ^{18}F -labeled small molecules, ^{18}F -labeled peptides, radiometal-labeled antibodies, and—perhaps most strikingly—a handful of *in vivo* pretargeted imaging applications in which the click ligation occurs not in a test tube but rather at the tumor (Table 1).

Yet despite these successes, the field is unquestionably young, so a number of important obstacles remain. For example, the syntheses of the tetrazine and transcyclooctene precursors are somewhat cumbersome, and while these components will soon become commercially available, improved synthetic methods would no doubt make the technology less expensive and more widely accessible to the radiochemistry community. And further, while chelator-modified tetrazines and ^{18}F -TCO have proven to be very valuable synthons, the synthesis of an ^{18}F -labeled variant of tetrazine—which, to date, has eluded researchers—would be an especially useful development. Along these lines, however, the *very* recent

publication of the synthesis of ^{11}C -modified tetrazines by Kristensen and coworkers is an exciting result, particularly in the context of short-lived tracers and pretargeted imaging.⁴⁶

As the field progresses, these hurdles and others must certainly be addressed, but it is also important to note a few of the myriad exciting unexplored avenues for the IEDDA reaction in radiochemistry. For example, in the literature to date, all of the peptides and proteins bearing reactive IEDDA groups were generated through the use of secondary, chemically selective conjugation agents (*e.g.* activated esters or maleimides). These methods have proven effective, but the need to incorporate the bioorthogonal group *after* peptide synthesis adds an additional synthetic step, reduces yield, and creates an added layer of complexity. If, on the other hand, future applications could make full use of the biological and chemical orthogonality of tetrazines by incorporating them into peptides during automated solid-phase synthesis rather than after synthesis, it would open the door for the rapid construction of libraries of potential imaging agents and, in turn, the development and optimization of more effective targeted probes. Finally, another significant untapped application of the IEDDA reaction—in particular, the pretargeted *in vivo* ligation—lies in the delivery of therapeutic radioisotopes such as ^{177}Lu , ^{90}Y , ^{225}Ac , and $^{186/188}\text{Re}$.^{47,48} Although each possesses significant clinical potential, these isotopes can cause dose-limiting toxicity in the bone marrow or renal system when delivered using traditional targeting vectors. Because of their modularity and flexibility, however, pretargeted delivery systems based on the *in vivo* IEDDA reaction could be precisely tuned to maximize activity uptake in the tumor while concomitantly accelerating clearance from the body in a manner that minimizes non-target uptake and tissue damage, thereby producing higher therapeutic ratios and better treatment outcomes.

In sum, we believe that in the years to come, the IEDDA cycloaddition reaction will become a cornerstone in the development of radiopharmaceuticals for imaging and therapy, ultimately crossing the bridge between preclinical and clinical research and directly benefitting patients at the bedside.

Biographies

Professor Thomas Reiner received his PhD in 2009 from the Technical University of Munich, Germany, where he was trained as an organometallic chemist. After graduation, he accepted a position as a post-doctoral fellow at the Center for Systems Biology at Massachusetts General Hospital under the mentorship of Ralph Weissleder and was promoted to Instructor at Harvard Medical School in 2011. The following year, he transferred to Memorial Sloan-Kettering Cancer Center, where he and his group are specializing in the development of bioorthogonally labeled tracers and imaging agents.

Brian Zeglis received his BS from Yale University and his PhD from the California Institute of Technology, where he worked on the synthesis and development of DNA-binding metal complexes under the mentorship of Professor Jacqueline K. Barton. In 2009, Brian moved to Memorial Sloan-Kettering Cancer Center, where he works as an NIH NRSA post-doctoral fellow in the laboratory of Professor Jason S. Lewis. Currently, his research is focused on the application of bioorthogonal chemistry to the design and synthesis of ^{64}Cu -based and ^{89}Zr -based PET radiopharmaceuticals.

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Figure 1.

The inverse electron demand Diels–Alder cycloaddition between a 1,2,4,5-tetrazine and a strained transcyclooctene dienophile.

Figure 2.

(A) Schematic of the synthesis of an ^{18}F -labeled small molecule probe using IEDDA-assisted conjugation and purification. ^{18}F -labeled TCO and AZD2281-Tz were first combined and incubated; then, a magnetic, TCO-modified scavenger resin was added, incubated, and filtered from the solution; and finally, the purified ^{18}F -AZD2281 was reconstituted and brought into an injectable volume; (B) Synthesis and structure of ^{18}F -AZD2281 (only one isomer shown); (C) Radioactivity (red) and absorption (black) traces of the ^{18}F -AZD2281 reaction mixture before and after purification with the magnetic, TCO-modified scavenger resin (*modified and reprinted with permission from ref. 33*).

Figure 3.

(A) General schematic of an IEDDA-based *in vivo* pretargeting system; (B) SPECT/CT image of a LS174T colorectal cancer xenograft pretargeted *in vivo* with a TCO-modified CC49 antibody and a ^{111}In -DOTA-PEG-tetrazine radioligand (*reprinted with permission from ref. 42*); (C) PET/CT image of a LS174T colorectal cancer xenograft pretargeted *in vivo* with a TCO-modified A33 antibody and a macromolecular ^{18}F -dextran-tetrazine radioligand (*reprinted with permission from ref. 43*); (D) PET image of a SW1222 colorectal cancer xenograft pretargeted *in vivo* with a TCO-modified A33 antibody and a small molecule ^{64}Cu -NOTA-tetrazine radioligand (*reprinted with permission from ref. 44*).

Table 1

Summary of the radiochemical applications of IEDDA

Radiolabeled precursor	Type of system	Target	Isotope	Reference
Transcyclooctene	–	–	^{18}F	30
	Small molecule	PARP-1	^{18}F	31
	Small molecule	PARP-1	^{18}F	32
	Small molecule	PARP-1	^{18}F	33
	Peptide	$\alpha_v\beta_3$	^{18}F	34
	Peptide	GLP-1	^{18}F	35
	Peptide	$\alpha_v\beta_3/\text{VEGFR}$	^{18}F	37
	Peptide	GLP-1	^{18}F	36
Tetrazine	–	–	^{11}C	46
	Antibody	Her2/ <i>neu</i>	^{64}Cu , ^{89}Zr	39
	Pretargeting (antibody/small molecule)	Tag72	^{111}In	42
	Pretargeting (antibody/dextran)	A33	^{18}F	43
	Pretargeting (antibody/small molecule)	A33	^{64}Cu	44