Bioconjugate Chemistry

Review

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Click Chemistry and Radiochemistry: The First 10 Years

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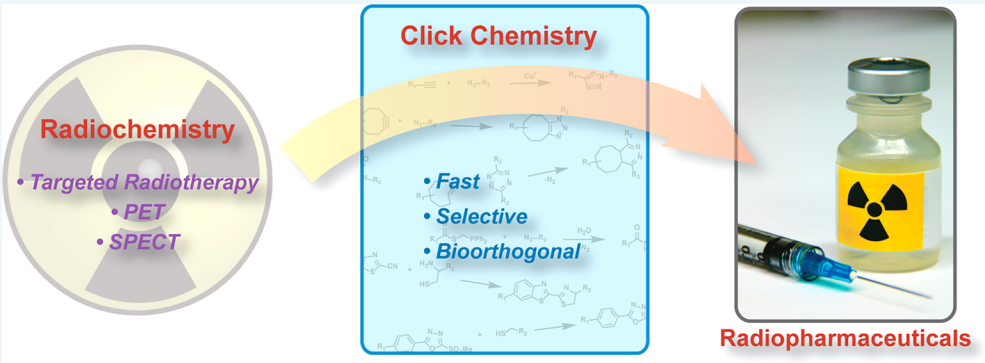

ABSTRACT: The advent of click chemistry has had a profound influence on almost all branches of chemical science. This is particularly true of radiochemistry and the synthesis of agents for positron emission tomography (PET), single photon emission computed tomography (SPECT), and targeted radiotherapy. The selectivity, ease, rapidity, and modularity of click ligations make them nearly ideally suited for the construction of radiotracers, a process that often involves working with biomolecules in aqueous conditions with inexorably decaying radioisotopes. In the following pages, our goal is to provide a broad overview of the first 10 years of research at the intersection of click chemistry and radiochemistry. The discussion will focus on four areas that we believe underscore the critical advantages provided by click chemistry: (i) the use of prosthetic groups for radiolabeling reactions, (ii) the creation of coordination scaffolds for radiometals, (iii) the site-specific radiolabeling of proteins and peptides, and (iv) the development of strategies for in vivo pretargeting. Particular emphasis will be placed on the four most prevalent click reactions—the Cu-catalyzed azide-alkyne cycloaddition (CuAAC), the strain-promoted azide-alkyne cycloaddition (SPAAC), the inverse electron demand Diels-Alder reaction (IEDDA), and the Staudinger ligation—although less well-known click ligations will be discussed as well. Ultimately, it is our hope that this review will not only serve to educate readers but will also act as a springboard, inspiring synthetic chemists and radiochemists alike to harness click chemistry in even more innovative and ambitious ways as we embark upon the second decade of this fruitful collaboration.

INTRODUCTION

A decade and a half have passed since Kolb, Finn, and Sharpless published the landmark review that introduced the concept of click chemistry.[1] In the intervening years, the influence of click chemistry has grown by leaps and bounds. To wit, the number of publications with "click chemistry" in the title has grown from 6 in 2003 to 252 in 2009 to 2014 in 2015![2]

In the words of the original authors, the criteria for a click chemistry ligation are as demanding as they are straightforward:[1]

"The reaction must be modular, wide in scope, give very high yields, generate only inoffensive byproducts that can be removed by non-chromatographic methods, and be stereospecific (but not necessarily enantioselective). The required process characteristics include simple reaction conditions (ideally, the process should be insensitive to oxygen and water), readily availably starting materials and reagents, the use of no solvent or a solvent that is benign (such as water) or easily removed, and simple product isolation."

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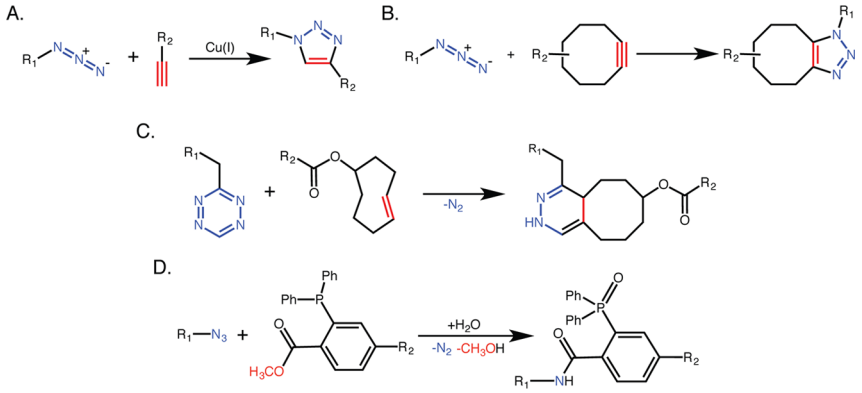

Figure 1. Schematics of the (A) Cu-catalyzed azide−alkyne cycloaddition reaction, (B) the strain-promoted azide−alkyne cycloaddition, (C) the inverse electron demand Diels−Alder cycloaddition, and (D) the Staudinger ligation.

A handful of reactions that satisfy (or, at the very least, come *close* to satisfying) these criteria have been uncovered, including nucleophilic ring opening reactions with epoxides, aziridines, and aziridinium ions; the formation of ureas, oximes, and hydrazones via nonaldol carbonyl chemistry; and oxidative and Michael additions to carbon−carbon double bonds.[3] Yet one particularly powerful reaction has emerged as the canonical click ligation and has proven remarkably useful in myriad applications: the copper-catalyzed [3 + 2] cycloaddition between an azide and a terminal alkyne (Figure 1A).[4,5] More recently, Bertozzi and others have pioneered a subset of click reactions that boast an additional boundary condition: bioorthogonality.[6−9] Bioorthogonal click ligations satisfy all of the requirements of standard click reactions but are also inert within biological systems. Not surprisingly, these reactions are hard to come by, yet a handful (most notably the Staudinger ligation, the strain-promoted azide−alkyne cycloaddition reaction, and the inverse electron demand Diels−Alder cycloaddition) have been developed and proven powerful in the hands of chemical biologists, biochemists, and biomedical scientists (Figure 1B−D).[7,10−16]

Click chemistry has had a paradigm-shifting influence on a wide range of chemical fields, from drug development[17,18] and polymer chemistry[19,20] to chemical biology[21] and nanoscience.[22] However, it is hard to imagine a field that has more to gain from harnessing click chemistry than radiochemistry. The principal reason for this lies in what makes radiochemistry unique: the inexorable physical decay of radioisotopes during synthesis. As a result, radiolabeling reactions—and especially radiolabeling reactions using short-lived isotopes such as ^{11}C ($t_{1/2} \approx 20$ min) and ^{68}Ga ($t_{1/2} \approx 68$ min)—must be rapid and efficient to maximize yield as well as selective and clean to eliminate time-sapping purification steps. Furthermore, the widespread use of biomolecules as targeting vectors has also placed a premium on bioconjugation reactions that are both selective and unencumbered by water. Finally, the proliferation of an ever-growing list of prosthetic groups and radiometal chelators has made modularity a critical feature of radiosynthetic protocols as well. Remarkably, all of these traits can be found in click chemistry ligations.

In light of these benefits, it is somewhat surprising that the first publications describing radiopharmaceuticals synthesized using click chemistry came rather late: a 2006 work from Mindt et al. describing the use of click chemistry to create coordination scaffolds for ^{99m}Tc and a 2007 report from Wuest and co-workers detailing the use of the CuAAC reaction to create an ^{18}F-labeled variant of neurotensin(8−13).[23] Yet in the years since this somewhat belated start, work at the nexus of these two fields has expanded dramatically.[24−27] This growth means that an *exhaustive* review covering every instance in which click chemistry has been applied to nuclear imaging would almost certainly be an *exhausting* read. Instead, in the pages that follow, it is our goal to highlight the most interesting, exciting, and useful points of intersection between click chemistry and nuclear medicine. More specifically, we will focus on the use of click chemistry for (*i*) radiolabeling reactions with prosthetic groups, (ii) the creation of novel chelation architectures, (iii) site-specific bioconjugation, and (iv) in vivo pretargeting. Taken together, we believe that these four areas underscore how the rapidity, efficiency, selectivity, modularity, and bioorthogonality of click chemistry have empowered radiochemists to create innovative agents for imaging and therapy. Ultimately, we sincerely hope that this review not only informs the reader about research at the intersection of chemistry and radiochemistry but also inspires new and seasoned researchers alike to apply this remarkably useful chemical technique to the development radiopharmaceuticals.

RADIOLABELING WITH PROSTHETIC GROUPS

One of the first reported, and still most extensively employed, applications of click chemistry to radiochemistry lies in the use of "clickable" prosthetic groups for radiolabeling. The ever-increasing use of imaging agents based on biomolecular vectors has put a premium on radiosynthesis strategies that are both mild and selective. Put simply, peptides, proteins, and antibodies should be radiolabeled under aqueous conditions at room temperature to ensure that their structural integrity is preserved, yet critically, many radiolabeling reactions require elevated temperatures, nonaqueous solvents, or (at the very least) pH conditions outside of the physiological norm. This is especially true for ^{18}F-radiofluorination reactions, which often require organic solvents and high temperatures.

Radiolabeled prosthetic groups provide an efficient way to circumvent these issues. Prosthetic groups are radiolabeled reactive small molecules that can be appended to biomolecules under benign conditions. Until recently, the vast majority of prosthetic groups have relied upon reactions with natural amino acids (most notably, couplings between *N*-hydroxysuccinimidyl (NHS) esters and lysines and Michael additions between maleimides and cysteines).[28−30] Yet prosthetic groups of this ilk present a number of problems. Most concerning is the complete loss of regiochemical control during the labeling of a peptide or protein containing more than one lysine or cysteine. This, of course, can only be remedied by yield-sapping separations or the addition of time-consuming protection and deprotection steps.[31]

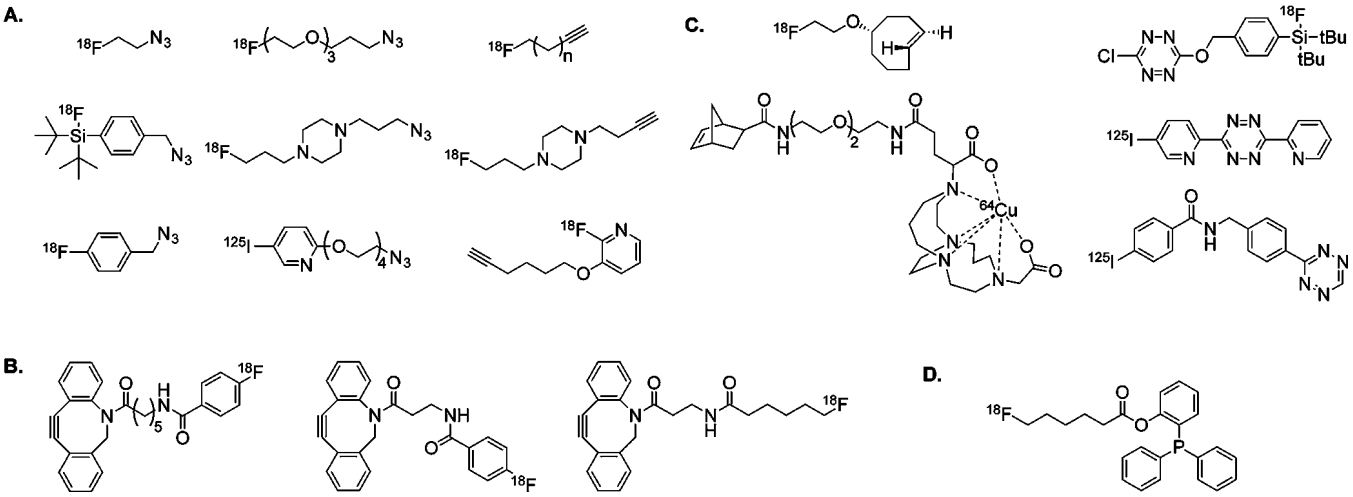

Figure 2. An assortment of radiolabeled prosthetic groups used for the synthesis of radiopharmaceuticals via the (A) copper-catalyzed azide−alkyne cycloaddition, (B) strain-promoted azide−alkyne cycloaddition, (C) inverse electron-demand Diels−Alder cycloaddition, and (D) traceless Staudinger ligation.

On top of this, both NHS esters and their isothiocyanate cousins are unstable under aqueous conditions, and maleimide−thiol linkages are prone to reversible substitution reactions in vivo.[32]

In response to these limitations, radiochemists have increasingly turned to "clickable" prosthetic groups. Not surprisingly, the canonical CuAAC ligation leads the pack. In this regard, the relative age of the reaction certainly plays a role. Yet another critical advantage of the CuAAC ligation is that its "footprint" — a 1,2,3-triazole ring — is unlikely to perturb the structure or activity of the vector: the heterocycle is both relatively small and a rigid stereoisomer of an amide linkage. At this junction, we would be remiss if we did not mention the CuAAC reaction's lesser-known cousin: the ruthenium-catalyzed azide−alkyne cycloaddition (RuAAC).[33] The RuAAC reaction produces 1,5-disubstituted 1,2,3-triazoles as opposed to the 1,4-disubstituted 1,2,3-triazoles created by the Cu-catalyzed cycloaddition. Even though it is regarded as a "click reaction", the RuAAC ligation requires organic solvents, elevated temperatures, and inert gas atmosphere. Furthermore, the 1,5-disubstituted 1,2,3-triazoles produced by the reaction are—unlike 1,4-disubstituted 1,2,3-triazoles—metabolically active and can be degraded via enzymatic N^3 oxidation to produce highly reactive and potentially toxic metabolites.[34] Given both of these issues, it is not surprising that, to the best of our knowledge, the RuAAC reaction has not been applied to the synthesis of radiopharmaceuticals.

Moving back to the topic at hand, an extensive body of work has emerged on the design, synthesis, and optimization of radiolabeled CuAAC-ready building blocks. Much, although not all, of this work has focused on ^{18}F.[35−38] Indeed, a variety of radiosynthetic methods have been employed to create azide- and alkyne-bearing ^{18}F-labeled prosthetic groups (Figure 2A).[37,39,40] These tools and the CuAAC reaction have been harnessed with great success in the radiolabeling of a wide variety of vectors, including phosphonium ions,[41] peptides,[42−50] oligonucleotides,[39,47] and proteins.[27,47] This application of the CuAAC reaction is not without its flaws, however. These stem primarily from the two reagents needed to facilitate the cycloaddition: Cu(I/II) cations and a sacrificial reductant. The latter, most often ascorbic acid, can inadvertently reduce particularly fragile peptides and proteins.[27] The Cu cations can be even more of a problem. Peptides and proteins (specifically serine, histidine, and arginine residues) can coordinate Cu^{2+} ions, resulting in structural and functional alterations to the peptide.[51] For example, Pretze et al. observed the accidental formation of Cu−peptide complexes following the CuAAC-mediated ligation of an ^{18}F-labeled, alkyne-containing prosthetic group to an azide-bearing SNEW peptide.[45] The coordination of the oxidative Cu(I) species can also lead to dramatic alterations to the chelating amino acid residues, as demonstrated very recently.[52] These issues are compounded even further for radiometal-containing constructs. In these cases, not only can the chelator capture the copper catalyst and prevent the reaction from happening, but residual Cu^{2+} ions can also outcompete the far less abundant radiometal cations for coordination by the chelator.[53] On top of these coordination-related concerns, the presence of Cu^{+} can also increase the likelihood of undesired side reactions such as Glaser couplings or the formation of copper-acetylides.[45,54,55] Some of these issues can be ameliorated through the use of Cu^{+}-stabilizing chelators such as THPTA or *N*-heterocyclic carbene complexes of Cu^{+}; however, these reagents can create their own set of complications.[56−58]

In light of the limitations of the CuAAC ligation, researchers have turned to a handful of "second generation" click reactions that are both bioorthogonal and catalyst-free. The most obvious place to start is the strain-promoted azide−alkyne cycloaddition (SPAAC). The SPAAC reaction is an azide−alkyne cycloaddition in which ring strain built into a cyclic alkyne—often a dibenzocyclooctyne (DBCO)—drives the reaction and eliminates the need for a catalyst.[59,60] Campbell-Verduyn et al. were among the first to use this approach for radiochemistry, creating a series of ^{18}F-labeled bombesin derivatives via the reaction of a DBCO-modified peptide with an array of ^{18}F-bearing, azide-containing prosthetic groups.[61] Following a similar strategy, another laboratory modified a series of $\alpha_v\beta_3$-targeting RGD peptides with DBCO and radiolabeled them using an [^{18}F]fluoro−PEG_4−azide prosthetic group.[50,62] In a creative twist, the authors scavenged excess unlabeled peptide using an azide-grafted resin, allowing them to achieve specific activities of up to 62.5 GBq/μmol. Critically, all of the ^{18}F-labeled peptides bore biological affinity comparable to their unlabeled cousins and were shown to be effective for the visualization $\alpha_v\beta_3$-expressing U87MG xenografts (Figure 3). Of course, radiolabeling via the SPAAC reaction goes both ways: several laboratories have created ^{18}F-labeled cyclooctynes for the radiofluorination of azide-modified small molecules, sugars, and peptides (Figure 2B).[63−65]

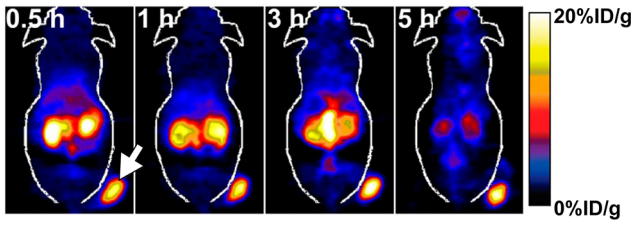

Figure 3. Coronal PET images of a NOD/SCID mouse bearing a GLP-1R-positive insulinoma xenograft (white arrow) collected 0.5, 1, 3, and 5 h after the injection of an ^{18}F-labeled Exendin-4 radiotracer synthesized using a "clickable" prosthetic group. Adapted and reprinted with permission from Wu et al., copyright 2013 by the Society of Nuclear Medicine and Molecular Imaging, Inc.

The SPAAC reaction has also been used for radioiodinations and radiometalations. Choi et al., for example, used a DBCO-bearing cRGD peptide and a prosthetic group composed of a PEG_4–azide moiety grafted to an ^{125}I-labeled pyridine to create an ^{125}I-labeled cRGD.[66] Evans et al. labeled an azide-modified DOTA with ^{68}Ga for the radiometalation of several DBCO-modified peptides.[53] Likewise, the Anderson group has conjugated DIBO-bearing copper chelators to an azide-modified cetuximab antibody and an azide-bearing somatostatin analogue.[67,68]

Despite its utility, the SPAAC ligation has one critical limitation: its dibenzocyclooctatriazole "footprint". The work of Hausner and co-workers provides a particularly useful cautionary example.[69] Here, the authors radiolabeled an azide-modified A20FMDV2-peptide using an ^{18}F-labeled variant of DBCO. While in vitro experiments confirmed that the ^{18}F-labeled peptide retained its affinity and specificity for $\alpha_v\beta_6$-expressing cells, in vivo imaging suggested that the bulky and hydrophobic benzocyclooctatriazole footprint introduced by the SPAAC ligation led to dramatic changes in the pharmacokinetics of the tracer and significantly impaired its uptake in $\alpha_v\beta_6$-expressing xenografts.

The inverse electron demand Diels–Alder (IEDDA) cycloaddition between tetrazine (Tz) and a dienophile, most commonly *trans*-cyclooctene (TCO) but also norbornene (NB), has also provided fertile ground for the development of prosthetic groups. Like the SPAAC ligation, the IEDDA reaction is bioorthogonal and proceeds without a catalyst. The principal advantage of the IEDDA ligation is its extraordinary speed (vide infra), which makes it particularly well suited for applications with short-lived radioisotopes. In 2010, the laboratories of Fox and Conti reported the first ^{18}F-labeled TCO (Figure 2C).[70] This prosthetic group was used for the rapid (t < 5 min) radiolabeling of a range of tetrazine-bearing peptides, including RGD and the GLP agonist Exendin.[71−73] The ^{18}F-labeled Exendin proved particularly promising, enabling the PET imaging of GLP-1R-positive insulinoma xenografts in mice. The same ^{18}F–TCO was also used to great effect by Weissleder and co-workers for labeling a Tz-bearing analog of the PARP1 inhibitor AZD2281. In this work, however, the authors added a creative wrinkle: removing unlabeled AZD2281–Tz using a TCO-coated magnetic resin.[74,75] Finally, a number of ^{18}F-labeled tetrazines have also been synthesized, but the in vivo use of radiopharmaceuticals created using these moieties has thus far remained somewhat sparing.[76,77]

The utility of the IEDDA reaction extends beyond radiofluorination.[53] To wit, a handful of radioiodinated tetrazine constructs have been successfully developed (Figure 2C). Albu et al., for example, synthesized an ^{125}I-labeled tetrazine and conjugated this building block to a TCO-modified anti-VEGFR2 antibody.[78] Interestingly, in vivo studies using this tracer revealed an additional benefit of this approach: the ^{125}I-labeled antibody proved to be more than 10-fold more stable to deiodination over 48 h compared to traditionally radioiodinated analogs. More recently, Choi et al. used a similar strategy for the radiolabeling of both a cRGD peptide and human serum albumin (HSA).[79] The ^{125}I-labeled HSA displayed impressive in vivo behavior, with a deiodination rate reduced by 50-fold compared to constructs created via traditional radioiodination. In 2011, Zeglis et al. employed the IEDDA reaction to create a modular strategy for the bioconjugation of a trastuzumab–TCO immunoconjugate with Tz–desferrioxamine (for $^{89}Zr^{4+}$) and Tz–DOTA (for $^{64}Cu^{2+}$).[80] More recently, Kumar and co-workers harnessed the IEDDA reaction to circumvent the incompatibility of antibodies with the high temperatures required to radiolabel the CB-TE2A-1C chelator with ^{64}Cu.[81] To this end, the authors modified the chelator with a norbornene moiety and grafted tetrazine onto an anti-PSMA antibody (YPSMA). After radiolabeling of the chelator-NB building block with ^{64}Cu at 85 °C, the ^{64}Cu–CB-TE2A1C-NB synthon was attached to YPSMA–Tz under mild conditions, and the ^{64}Cu-labeled radioimmunoconjugate was successfully deployed for the PET imaging of PSMA-expressing tumors in a murine model of prostate cancer.

Although the rapidity of the IEDDA reaction provides a marked improvement over the sluggish SPAAC ligation, it fails to solve one of the latter's major issues: a bulky, hydrophobic footprint. As we have discussed, the SPAAC reaction leaves a benzocyclooctatriazole moiety in its wake. The IEDDA ligation creates an equally large footprint: a bicyclic [6.4.0] ring system. Both structures have the potential to interfere with the biological activity and pharmacokinetics of vectors, particularly small molecules and short peptides. The traceless version of the Staudinger ligation offers an exciting alternative (Figure 4A). This ligation relies on an initial reaction between a phosphine-based moiety and an azide followed by a rearrangement that produces a simple amide linkage. Along these lines, the radiolabeling of peptides with ^{18}F has been achieved via the reaction between (diphenylphosphanyl)methanethiol thioester-bearing peptides and an ^{18}F-labeled azide as well as that between a radiolabeled 2-(diphenylphosphanyl)phenol ester with an azide-bearing peptide (Figure 2D).[82−84] Unfortunately, however, the traceless Staudinger ligation requires high temperatures (90–130 °C) to achieve speeds that are compatible with short-lived isotopes. This undoubtedly limits its utility with fragile small molecules, peptides, and proteins; however, we are optimistic about the potential applications of this elegant transformation with longer-lived isotopes.

Finally, a handful of other, less-well-known click ligations have made sparing yet interesting appearances in the literature of prosthetic groups. In 2012, Zlatopolskiy et al. reported the formation of a reactive nitrone from ^{18}F-fluorobenzaldehyde and phenylhydroxylamine.[85] The authors showed that this ^{18}F-labeled nitrone could undergo a [3 + 2] cycloaddition with a maleimide, resulting in quantitative conversion in less than 15 min at 80 °C (Figure 4B). It must be said, however, these reaction conditions leave much to be desired when it comes to labeling biomolecules. Later the same year, the same group probed the potential of cycloaddition reactions between nitriloxides and dipolarophiles (Figure 4C).[86] An ^{18}F-labeled nitriloxide was synthesized from ^{18}F-*p*-fluorobenzaldehyde and reacted with a series of dipolarophiles, producing quantitative conversions in <10 min at 40 °C. However, these reactions were performed in alcohol, and no data was presented regarding the feasibility of this

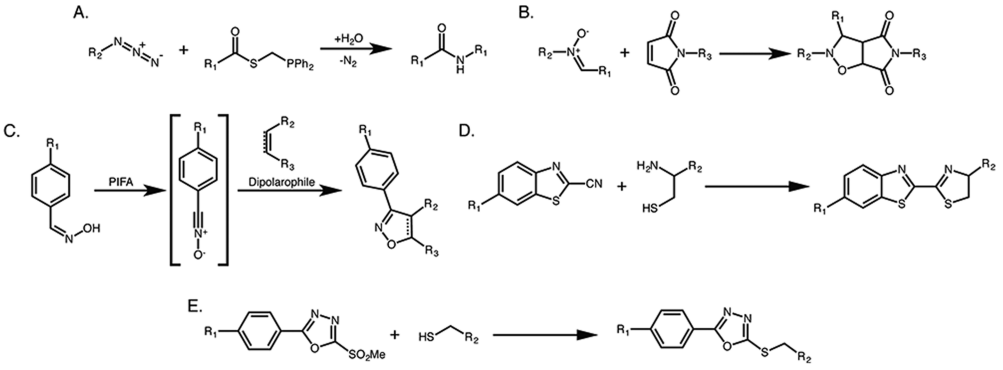

Figure 4. Schematics of an assortment of click chemistry ligations (beyond those depicted in Figure 1) used for prosthetic group radiolabelings: (A) traceless Staudinger ligation, (B) nitrone−alkene cycloaddition, (C) nitrile−oxide cycloaddition, (D) 1,2-aminothiol−cyanobenzothiazole condensation, and (E) phenyloxadiazole methylsulfone−thiol conjugation.

transformation under aqueous conditions. Recently, other groups have harnessed the reactivity of 2-cyanobenzothiazoles toward 1,2-aminothiols to radiolabel peptides and proteins containing *N*-terminal cysteines (Figure 4D).[87,88] To this end, ^{18}F-labeled 2-cyanobenzothiazoles were synthesized and appended to RGD and diRGD peptides bearing *N*-terminal cysteines as well as a genetically engineered variant of luciferase with a cysteine at the N-terminus. Lastly, just this year, Chiotellis et al. have explored phenyloxadiazole methylsulfones (PODS) as more stable alternatives to maleimides for conjugations with thiols (Figure 4E).[89] In this work, an ^{18}F-labeled PODS was used to radiolabel both a cysteine-bearing peptide and a cysteine-modified affibody, and the resulting constructs were used to HER2-positive tumors in a mouse model of breast cancer.

CREATING COORDINATION SCAFFOLDS

The use of click chemistry to create radiometal chelation architectures provides one of the best examples of the unique modularity conferred by this synthetic approach.[90,91] Easily the best known of these methods, dubbed "click-to-chelate" by its inventors, was introduced in 2006 by Mindt et al. (Figure 5).[92−94] This strategy employs the Cu^{I}-catalyzed azide−

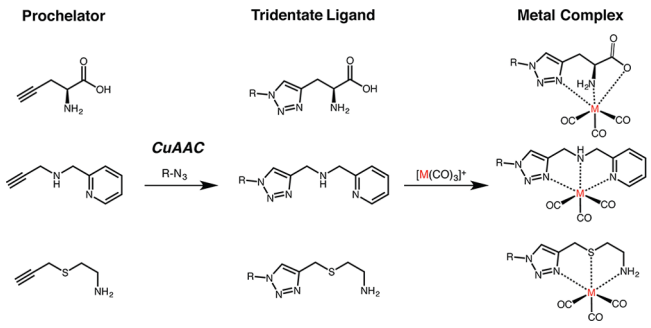

Figure 5. "Click-to-chelate" approach: a variety of prochelators exhibiting electron-donating groups undergo the Cu^{I}-catalyzed azide−alkyne cycloaddition with an azide to from a tridentate ligand that can coordinate an organometallic $[M(CO)_3]^+$ synthon.

alkyne cycloaddition (CuAAC) reaction to attach small molecule "pro-chelators" to peptides and small molecules. However, the 1,2,3-triazole produced by the click ligation becomes far more than just a simple link between the subunits of the construct. Indeed, the heterocycle forms an integral part of a tripodal coordination scaffold capable of the rapid chelation of $[M(CO)_3]^+$ synthons, in which M can be the γ-emitting radiometal ^{99m}Tc ($t_{1/2}$ = 6.01 h) or the β-emitting radiometal ^{188}Re ($t_{1/2}$ = 16.98 h). In this way, "click-to-chelate" facilitates the creation of a chelator *and* its subsequent radiometalation in a rapid, robust, and reproducible one-pot reaction. This is particularly important given the mercurial coordination chemistry of ^{99m}Tc.

In their initial proof-of-concept report, the authors created seven different tripodal scaffolds—including N_3, N_2S, and N_2O ligand architectures—using a series of azide-modified small molecules. Subsequent labeling with $M(CO_3)$ [M = Re, ^{99m}Tc] synthons resulted in a series of highly stable, low-spin d^6-complexes despite differences in the size, molecular charge, and hydrophilicity of the prochelator.[92−95] The creation of a ^{99m}Tc-labeled variant of folate using "click-to-chelate" provides an excellent example of the approach (Figure 6). The 1,2,3-triazole ring formed in the first phase of the reaction between the azide-bearing folate construct (**1**) and the alkyne-modified amino acid (**2**) not only connects the pro-chelator to the folate vector but *also* serves as an essential part of the N_2O coordination scaffold for the $[^{99m}Tc(CO)_3]^+$ moiety. The incubation of the chelator-bearing construct with $[^{99m}Tc(CO)_3(H_2O)_3]^+$ reproducibly yields ^{99m}Tc-labeled folate (**3**) in high yield and specific activity.[92]

In subsequent work, this technique was applied to peptides as well as an array of other biologically active small molecules such as sugars, nucleosides, and steroids.[96−100] Fernandez et al., for example, developed a ^{99m}Tc-labeled glucose derivative as an imaging probe for glucose metabolism.[97] Similarly, Struthers et al. developed an elegant one-pot "click-to-chelate" synthesis of a ^{99m}Tc-labeled thymidine analogue as a SPECT surrogate for the clinically successful proliferation marker ^{18}F−FLT.[98] Taken together, this work clearly demonstrates that ^{99m}Tc-labeled tracers created using the "click-to-chelate" methodology demonstrate in vivo behavior that is comparable, and in some cases superior, to the current "gold standard" chelators for $[^{99m}Tc(CO)_3]^+$: *Nτ*-derivatized histidine and *Nα*-acetylated histidine. Indeed, studies using ^{99m}Tc-labeled folate revealed that the click-to-chelate approach furnished compounds in purities and radiochemical yields equal to those achieved using traditional radiolabeling techniques. Furthermore, in this work, the click-to-chelate approach did not alter biodistribution patterns or pharmacodynamic parameters such as receptor affinities and selectivities. Finally, the superiority of the click-to-chelate methodology becomes most obvious in the context of synthetically challenging molecules. In the case of the azide-modified

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