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Review Article

Bioorthogonal chemistry: implications for pretargeted nuclear (PET/SPECT) imaging and therapy

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Abstract: Due to their rapid and highly selective nature, bioorthogonal chemistry reactions are attracting a significant amount of recent interest in the radiopharmaceutical community. Over the last few years, reactions of this type have found tremendous utility in the construction of new radiopharmaceuticals and as a method of bioconjugation. Furthermore, reports are beginning to emerge in which these reactions are also being applied *in vivo* to facilitate a novel pretargeting strategy for the imaging and therapy of cancer. The successful implementation of such an approach could lead to dramatic improvements in image quality, therapeutic index, and reduced radiation dose to non-target organs and tissues. This review will focus on the potential of various bioorthogonal chemistry reactions to be used successfully in such an approach.

Keywords: Pretargeting, bioorthogonal chemistry, molecular imaging, PET, SPECT, click chemistry

Directly radiolabelled antibodies in imaging and therapy

The success of molecular imaging and therapy depends on the selective accumulation of the diagnostic probe or therapeutic agent at the site of interest. For imaging studies, this ability is required to effectively contrast the target site against surrounding tissues, organs and biological fluids, whilst in therapy it helps confine the toxic agent to the site(s) of disease and prevent off-target effects. In oncology, a multitude of targets have been identified corresponding to the various known hallmarks of cancer [1]. These include elevated rates of glucose metabolism [2], regions of hypoxia [3], changes in pH [4], and a plethora of cellular and chemical biomarkers [5]. This review will focus on the latter category and the development of novel imaging and therapeutic strategies towards such targets based on bioorthogonal chemistry.

Upon identifying a compelling target, several important considerations are required in order to develop the most effective agent. One of these is the selection of an appropriate targeting vector which will both localize at the site of interest and lead to the specific accumulation of the desired 'payload'. When developing an agent against a particular biomarker it is important that the targeting vector has (*i*) high binding affinity for its intended target, (*ii*) high specificity, (*iii*) high signal-to-background ratio, (*iv*) high metabolic stability, and (*v*) low immunogenicity [6, 7].

Since the beginning of the 20th century, antibodies have been considered as suitable vehicles for the delivery of imaging and therapeutic agents, mostly due to their high affinity and specificity [8-15]. Paul Ehrlich first conceived of antibody vectors as 'magic bullets' capable of delivering a payload of toxic agent to antigens associated with certain diseases, thus causing irreversible damage to sites of disease whilst sparing healthy tissue of the toxins deleterious effects [8]. Consequently, a vast array of epitopes (mostly at the cell surface) have been targeted by antibodies for non-invasive imaging and therapeutic applications.

The first examples of radiolabelled antibodies for cancer therapy emerged in the early 1950's [16, 17], although it took roughly two decades before their ability to target human tumour-associated antigens was demonstrated in can-

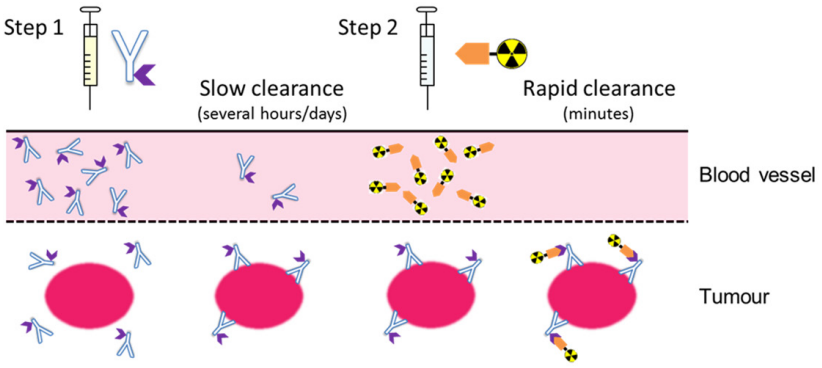


Figure 1. A simplified representation of a two-step pretargeting approach.

cer patients [18]. It was the development of hybridoma technology in 1975 that enabled the relatively facile generation of monoclonal (murine) antibodies (mAbs) in useful quantities [19] and, as a consequence, the number of studies in this area quickly increased. The use of murine antibodies in these applications was found to be problematic due to the provocation of a human anti-murine antibody response [20]. Therefore, significant efforts have been focused on the production of chimeric, humanized, and human monoclonal antibodies which are much less likely to invoke an immune reaction. Currently, the vast majority of approved mAbs are either chimeric or humanized [21].

Despite their attractive properties, antibodies have a few important shortcomings that have prevented their development into the magic bullets that Ehrlich had envisioned. One of the most critical barriers to achieving high tumour-to-blood (T/B) and tumour-to-muscle (T/M) ratios is the slow rate of clearance of antibodies from the blood and non-target tissues due to their high molecular weight [22, 23]. This necessitates the use of radionuclides with correspondingly long radioactive half-lives (*e.g.* ^{89}Zr; $t_{1/2}$ = 78.41 h [24]). Of course, the radiation dose to the patient increases as a function of exposure time and therefore there is a clear incentive to improve the pharmacokinetic properties of antibody constructs. One method of achieving this has involved a variety of lower molecular weight vectors which retain the essential antigen binding pharmacophore yet exhibit more rapid elimination. In decreasing order of size, these include minibodies, diabodies, single chain variable fragments (scFv), and single variable domain fragments (Fv), *etc.*

Pretargeted imaging and therapy: an alternative approach

In the mid-1980s, an alternative strategy referred to as pretargeted radioimmunotherapy (PRIT) was developed to circumvent the issues associated with the prolonged residence times of radiolabelled antibody constructs [25, 26]. In essence, this approach involves the sequential administration of (*i*) a macromolecular (usually antibody-based) targeting vector and (*ii*) a low molecular weight radiolabelled effector species (**Figure 1**). Crucially, the radiolabelled species is administered following a predetermined lag period to allow the antibody sufficient time to accumulate at the target site and for any residual antibody to be cleared from the circulation.

To ensure the two component parts bind strongly upon interaction at the site of interest, each must be suitably modified with complementary reactive species. In some cases, an additional 'chaser' species is administered prior to the radiolabelled effector, thus creating a 'three-step' approach. The purpose of this chaser species is to assist in the removal of any unbound antibody from the circulation. As a result, improved T/B ratios have been achieved [27].

The successful implementation of a pretargeting approach combines the high target specificity and affinity offered by antibody vectors with the superior pharmacokinetic properties of a low molecular weight compound. As a result, pretargeting strategies can lead to an improvement of imaging contrast at earlier time points following administration of the radiolabelled agent. Furthermore, this approach has been shown to decrease the overall radiation burden to non-target organs and tissues [28].

Whilst the concept of pretargeting has been known for several decades, the number of pretargeting systems has been limited to a few distinct classes. This is mostly due to the inherent difficulties of developing chemical reactions which proceed rapidly within living systems without also reacting with the vast array of

chemical entities that are present often in vast molar excesses.

Conventional pretargeting systems

Bispecific antibodies and radiolabelled haptens

The original pretargeting concept is based on the use of bispecific antibodies which are capable of binding to both a target antigen and a radiolabelled hapten [11, 29-33]. This approach was made possible when Reardan *et al.* developed monoclonal antibodies which were capable of binding radiometal chelates, specifically ^{111}In complexes based on the hexadentate chelator ethylenediamine tetraacetic acid (EDTA) [25]. It was later found that an affinity enhancement effect could be achieved by tethering two of these haptens together *via* a short two amino acid linker [34]. This modification led to improved uptake and retention of the radiolabelled divalent hapten at the tumour site whilst still retaining the essential rapid clearance from the circulation and surrounding tissues.

A notable example of this approach involved an anti-CEA × anti-^{111}In-benzyl-EDTA Fab' × Fab' bispecific mAb and an ^{111}In-EDTA derivative (^{111}In-EOTUBE) as the radiolabelled effector [35]. A clinical trial involving 14 patients with recurrent or metastatic adenocarcinoma of the colon revealed rapid clearance of the radiolabelled species from normal tissues while affording high T/M ratios [35].

Potential limitations of this approach include the practical complexities and high costs involved in the development of bispecific antibodies. Furthermore, a critical aspect of any pretargeted imaging approach is the affinity between the radiolabelled effector species and the antibody vector. Here, the binding interactions between radiolabelled haptens and bispecific antibodies are entirely non-covalent and binding constants greater than ~10^{-10} M are rarely achieved. In an effort to obtain greater binding constants, alternative systems offering much higher affinities such as the biotin-(strept) avidin interaction have been explored.

Biotin-(strept)avidin systems

Shortly after the development of bispecific antibodies for pretargeting, Hnatowich *et al.* reported an alternative strategy exploiting the extremely high binding affinity between biotin and (strept)avidin (K_d = 4×10^{-14} M) [36, 37]. This approach has since been used in various forms which are discussed in depth in several comprehensive reviews [38-42]. The benefits of this approach were clearly demonstrated in a study by Axworthy *et al.* who compared the uptake of a ^{90}Y-radiolabelled biotin in a tumour pretargeted with a streptavidin-modified mAb against a conventional directly radiolabelled antibody [43]. Promisingly, significantly higher T/B ratios were found using the pretargeting method.

Whilst this system shows clear promise, there are a number of limitations to this approach which require consideration. Perhaps most significant is the immunogenic response that occurs following administration of avidin/streptavidin foreign proteins. Another consideration is the presence of endogenous biotin (10^{-7}-10^{-8} M) which could interfere with (strept)avidin pretargeting systems by saturating the biotin binding sites, as well as endogenous biotinidase which mediates the hydrolysis of radiolabelled biotin effector species. Lastly, more so than the other conventional pretargeting strategies discussed herein, it is often necessary to administer a chaser species to remove residual antibody from the circulation prior to the administration of the radiolabelled effector [44-49].

Complementary oligonucleotides

A comparatively more recent approach (also developed by Hnatowich and co-workers) relies on the high affinity interaction between complementary oligomers (such as DNA) [50-59]. Depending largely on the length and the base sequence of the complementary oligomeric chains, this chemical pairing can potentially lead to binding affinities that would rival, or even exceed, that of the biotin-(strept)avidin interaction. This approach can also potentially eliminate some of the inherent limitations of the biotin-(strept)avidin approach. For example, studies in which high doses of single strand DNAs have been repeatedly administered to patients have not revealed any significant immunogenic response or obvious toxicity [60]. Furthermore, unlike the biotin-(strept)avidin approach, the use of complementary oligomers would not be complicated or obstructed by the presence of competing endogenous species. It

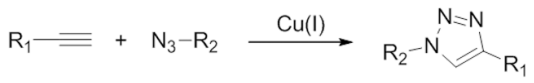


Figure 2. The Huisgen 1,3-dipolar cycloaddition is an extremely efficient click chemistry reaction, however the requirement of a toxic copper(I) catalyst to enhance the reaction rate has prevented its application in living systems.

is important, however, that oligonucleotides are suitably modified to prevent their rapid degradation by nucleases [61]. The most successful oligomers from a pretargeting perspective have been those based on a morpholino backbone (MORFs). These agents have been used in conjunction with a variety of radionuclides for applications in imaging (^{99m}Tc [51-54, 58], ^{111}In [55, 56]) and therapy (^{90}Y [50], ^{188}Re [57]).

Using bioorthogonal chemistry for pretargeted imaging of cancer

For a chemical reaction to be described as being truly bioorthogonal, it must result in the rapid formation of a covalent bond (even at low concentrations) whilst remaining completely selective against any other chemical species present within a living system. Given the abundance and variety of reactive functional groups within such a biologically and chemically complex environment, this reduces the number of possible reactions to a small selection [62-73]. In addition, it is important that at least one of the bioorthogonal species is small, and that both reacting components exert minimal toxicity [64].

Click chemistry reactions (as defined by Sharpless *et al.* in 2001 [74]) offer important advantages which give them the potential to translate well into an *in vivo* setting and all of the bioorthogonal reactions discussed in this review fall under this umbrella term.

The archetypal and most prominent click chemistry reaction evolved from work started by Rolf Huisgen in 1963 [75-77], although the development of the click chemistry concept itself did not arise until much later [74]. The Huisgen 1,3-dipolar cycloaddition involves the reaction between azide and alkyne starting materials yielding a triazole species (**Figure 2**). By itself, the reaction requires elevated temperatures or pressures and often results in a mixture of regioisomers (specifically, 1,4- and 1,5-substituted triazoles). However, in the early 2000s, the laboratories of both Sharpless and Meldal independently discovered that in the presence of a copper(I) catalyst the reaction proceeds at room temperature, at much higher rates (~10^6-fold), and yields only the 1,4-substituted regioisomer [78, 79]. This click chemistry reaction is particularly attractive as both the alkyne and azide functional groups are easily incorporated into a diverse array of organic species and exhibit high stability under a variety of reaction conditions. As such, the Huisgen 1,3-dipolar cycloaddition has found tremendous utility in the construction of radiopharmaceuticals [80, 81]. In particular, this click chemistry reaction has been used very effectively in the mild preparation of [^{18}F]-radiolabelled peptides [82-84].

Despite having many desirable qualities that would render the Huisgen 1,3-dipolar cycloaddition an attractive choice for a pretargeting strategy, the necessity of a copper(I) catalyst presents a major limitation due to its *in vivo* toxicity. As a result, the comparatively recent development of alternative click chemistry reactions which proceed without the presence of a copper(I) catalyst has attracted a significant amount of interest in the molecular imaging community [85]. The copper-free click chemistry reactions are instead usually driven either by the relief of steric strain (strain-promoted azide-alkyne cycloadditions, or SPAAC [63-68, 72]), or by a so-called inverse electron-demand Diels-Alder mechanism [86] (**Table 1**).

One of the first copper-free bioorthogonal reactions to be evaluated was the Staudinger ligation (**Figure 3**) [87]. This involves the reaction between azide and phosphine functional groups resulting in the formation of an amide bond. There are essentially two forms of this reaction: (*i*) a non-traceless version in which a phosphine oxide moiety remains attached to the final product, and (*ii*) a traceless version in which the phosphine oxide group is eliminated. This ligation and its precise mechanism [88] have been discussed in detail in several excellent review articles [89, 90].

The Staudinger ligation is a reliable and highly selective reaction which has been used successfully for the modification of proteins [91], and the engineering of cell surfaces both *in vitro* [92, 93] and in living animals [94]. It has

Table 1. Rates of reaction for a selection of bioorthogonal reactions

Reaction type	Reacting species	*k* (x 10^{-3} $M^{-1}s^{-1}$)[a]	References
Staudinger (non-traceless)	Phosphine + azide	0.83-3.8	[88, 89]
Staudinger (traceless)	Phosphine + azide	0.12-7.70	[89, 90]
Strain-promoted cycloadditions	Cycloalkyne + azide	0.9-4,000	[93, 99-106, 108, 110, 119, 132, 170-174]
	Cycloalkyne + diazo	2.5-13,500	[174]
	Cycloalkyne + nitrone	1,660-58,800	[105, 115, 116, 136]
IEDDA	Cyclopropene + tetrazine	137-137,000	[140]
	Norbornene + tetrazine	41-20,000	[145, 148, 150, 151, 175]
	TCO + tetrazine	3,100-380,000,000	[152, 166, 175-177]

[a]The reported rates of each reaction are dependent on a number of experimental conditions, including the choice of solvent, pH, temperature, *etc.* For a more precise comparison, refer to the individual references.

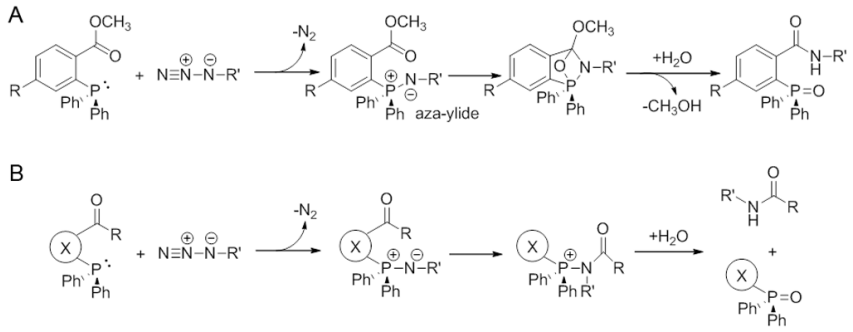


Figure 3. The non-traceless (A) and traceless (B) versions of the Staudinger ligation have been used for *in vivo* bioorthogonal chemistry reactions.

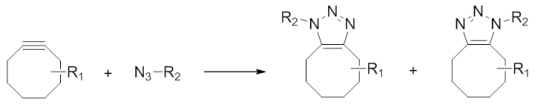


Figure 4. The use of highly strained cyclooctyne derivatives has circumvented the requirement of a toxic copper(I) catalyst and these reactions are therefore more compatible with living organisms compared to the traditional Huisgen 1,3-dipolar cycloaddition.

also been used in combination with fluorophores in *in vitro* studies targeting azide groups present on the surface of live cells [95, 96]. Furthermore, the traceless form of this reaction has also been used to synthesize heterobifunctional linkers to facilitate the construction of radiometal-containing imaging agents [97].

A study conducted by Vugts *et al.* in 2011 explored the possibility of using the Staudinger ligation to facilitate a pretargeted imaging strategy [98]. In this case, an anti-CD44v6 chimeric monoclonal antibody which had been modified with multiple azide functionalities was employed as a targeting vector. A series of phosphine-containing small molecules incorporating radionuclides for imaging ($^{67/68}$Ga, ^{89}Zr, ^{123}I, and ^{177}Lu) and therapeutic applications (^{177}Lu) were then evaluated as secondary agents. Following the administration of a ^{67}Ga-DFO-phosphine agent in non-tumour bearing mice, the presence of Staudinger products in the blood pool was monitored, however no evidence of any ligation was observed. Experiments in serum revealed that the phosphine species is prone to oxidation which renders it unable to undergo reaction with azide groups. Furthermore, the rate of the Staudinger ligation was found to be sub-optimal for *in vivo* bioorthogonal reactions, particularly considering the rapid clearance and elimination of the secondary phosphine agent.

Strain-promoted alkyne-azide (SPAAC) and alkyne-nitrone (SPANC) cycloadditions

In another effort to circumvent the use of a toxic Cu(I) catalyst required for traditional Huisgen-type click chemistry reactions, a related class of azide-alkyne [3 + 2] cycloadditions which are promoted by the relief of steric strain have gained prominence. Mostly, these reactions involve an alkyne moiety within a cyclic 8-membered (cyclooctyne [99]) system which causes the bond angles surrounding the two alkyne sp-hybridised carbon atoms to be severely constrained away from the ideal 180° (**Figure 4**).

A host of cyclooctyne derivatives have been evaluated in terms of their ability to undergo copper-free click chemistry reactions. The purpose of these studies is often focused on enhancing the rate of the cycloaddition reaction by making structural modifications to the cyclooctyne species. For example, it has been found that introducing one or more electron-withdrawing substituents (*e.g.* fluorine [100]) in close proximity to the alkyne group leads to a substantial increase of rates of reaction. Other modifications, such as one [101] or two [102-104] adjacent aryl rings (*e.g.* dibenzocyclooctyne, DBCO), and bicyclic systems (*e.g.* bicyclononynes [105, 106]) have also been found to improve reaction rates yet further. One cyclooctyne derivative 'BARAC' has achieved second order rates of reaction up to 0.96 $M^{-1}s^{-1}$ [104], although a recent study has found evidence that BARAC itself is prone to rearrangement yielding tetracyclic products [107].

Cyclooctyne derivatives can now be purchased from commercial suppliers and this increase in availability has led to the use of this technology in a wide variety of applications [93, 105, 108-123]. Notably, SPAAC reactions have been used in the preparation of fluorine-18 [124-130] and copper-64 [131] radiolabelled compounds for PET imaging applications.

Several studies have now demonstrated the ability of cyclooctyne derivatives to undergo bioorthogonal reactions with azide groups *in vitro* [119, 121, 132], in zebrafish [123], and in live mice [120]. In most of these examples, the azide groups are usually incorporated *via* metabolic glycoengineering.

Given their apparent high selectivity and promising rates of reaction in some cases, it is not surprising that SPAAC reactions have been considered as candidates to facilitate a pretargeting approach [130, 133, 134]. Recently, van den Bosch *et al.* reported an in depth study in which the reaction between a series of ^{177}Lu-containing cyclooctyne derivatives and an azido-functionalised anti-CD20 mAb (Rituximab) was evaluated in non-tumour-bearing mice. In this case, the cyclooctyne species were derived from DIFO and DIBO as both had previously been shown to undergo rapid reactions with benzyl azide [63, 116]. Preliminary *in vitro* studies in phosphate buffered saline and 50% mouse serum indicated sufficient stability within the expected circulation time of these low molecular weight probes. Mice were initially administered the azide-modified mAb and after a lag time of 5 minutes were subsequently injected with the relevant ^{177}Lu-labelled cyclooctyne probe. Evidence of bioorthogonal reaction products was monitored in the blood pool and selected tissues. Unfortunately, this approach was unsuccessful as it was evident that the cyclooctyne probes did not have sufficiently high *in vivo* reactivity towards azides, particularly considering the rapid blood clearance of these agents. Furthermore, in some examples, binding to serum proteins was apparent which, whilst slightly lengthening the circulatory residence times, ultimately limited the availability of these secondary agents and therefore reduced the potential for *in vivo* cycloaddition reactions.

Lee *et al.* have also recently utilised SPAAC-based bioorthogonal chemistry for *in vivo* pretargeting using, in this case, fluorine-18 for PET imaging [134]. Here, rather than use an antibody as the primary targeting agent, the authors employed mesoporous silica nanoparticles (MSNs; 100-150 nm) which were expected to accumulate in tumours *via* the enhanced permeability and retention (EPR) effect. The MSNs were PEGylated and then modified with DBCO (~0.12 mmole of DBCO per gram of product, DBCO-PEG-MSN). These nanoparticles were administered intravenously to mice bearing U87 MG tumour xenografts and, after a lag time of 24 h, the secondary agent ([^{18}F]fluoropentaethylene glycolic azide) was subsequently injected. Promisingly, PET-CT images acquired between 0.25-2 h p.i. revealed accumulation of radioactivity at the tumour site while control experiments performed without prior injection of the DBCO-PEG-MSNs exhibited substantially lower tumour uptake. This observation was supported by data obtained from *ex vivo* biodistribution experiments which showed an improved tumour-to-blood ratio using the pretargeting approach. These experiments provide a good indication that bioorthogonal chemistry reactions based on SPAAC can be used successfully to facilitate *in vivo* pretargeting for imaging and therapeutic applications.

A few studies focused on achieving faster rates of reaction have found that cyclooctyne derivatives also undergo rapid strain-promoted cycloaddition reactions with nitrone species (SPANC)

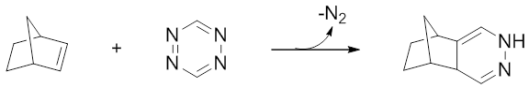


Figure 5. The norbornene-tetrazine ligation has been used successfully in the radiosynthesis of PET imaging agents based on peptide and antibody constructs. The rate of reaction between these species (and derivatives thereof) appears to be too slow to facilitate a pretargeting strategy.

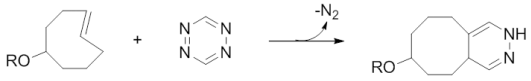


Figure 6. The TCO-tetrazine ligation has been the most successful bioorthogonal chemistry reaction for implementing a pretargeting strategy due to its extremely high rates of reaction.

[135]. The reaction between BARAC and one cyclic nitrone derivative yielded a second-order rate constant of 47.3 $M^{-1}s^{-1}$, representing a 47-fold increase compared to the equivalent reaction involving benzyl azide [136]. To the best of the authors knowledge this class of reactions has not yet been evaluated in a pretargeting strategy, although it holds clear promise particularly as cyclic nitrones can be readily conjugated to amine and carboxylic acid functional groups and have already been used successfully to functionalise extracellular targets on live cancer cells [115].

Inverse electron-demand Diels-Alder cycloadditions

In an opposite fashion to the traditional Diels-Alder reaction, inverse electron-demand Diels-Alder (IEDDA) cycloaddition reactions involve the use of an electron-rich dieneophile and an electron-deficient diene [86]. The reaction is formally accepted as a [4 + 2] cycloaddition, however it is still not clear whether it is a truly concerted mechanism. In the early 1990s, Sauer *et al.* demonstrated that the rates of reaction between electron deficient tetrazines and a variety of dienophiles were extremely fast [137], and consequently tetrazines have been commonly employed as dienes in these reactions.

From a bioorthogonal chemistry perspective, the two most prominent dienophiles which have so far been explored are norbornene and *trans*-cyclooctene (TCO) [138], although recently other species such as cyclopropene [139, 140] and terminal alkenes [141] have also shown promise in this area.

The norbornene-tetrazine ligation was first reported in the 1980s [137, 142] and has seen a resurgence of interest in recent years (**Figure 5**). Whilst this reaction leads to the formation of multiple isomers, it otherwise meets the criteria of a click chemistry reaction and is often placed in this category. In particular, this ligation has been shown to be very rapid, modular in scope, and high yielding, and has consequently been used very effectively in a variety of applications [143-148], including the preparation of various radiopharmaceutical agents. In 2011, Zeglis *et al.* demonstrated that this ligation can be applied as an effective tool for bioconjugation in their development of radiometallated antibody constructs [149]. Here, the authors synthesized both ^{64}Cu-NOTA- and ^{89}Zr-DFO-containing norbornene derivatives which were able to rapidly conjugate to a tetrazine-modified antibody under mild reaction conditions. In a more recent example, Knight *et al.* synthesised an [^{18}F]-containing norbornene prosthetic group ([^{18}F]NFB) which was used successfully to radiolabel a bombesin peptide-derivative, also under mild reaction conditions [150].

This reaction has also been used by Devaraj *et al.* in *in vitro* pretargeting experiments involving SKBR3 human breast cancer cells [151]. In this case, norbornene-modified trastuzumab (Herceptin) was used to pretarget Her2/neu growth factor receptors at the cell surface. A near-infrared fluorescent VT68-tetrazine species was then used as a secondary labelling agent. Rapid and highly selective labelling was observed which highlights the utility of this bioorthogonal reaction.

In addition, experiments performed in whole mouse blood have further demonstrated the ability of this reaction to proceed efficiently even in complex biological environments [150]. This reaction has therefore been considered as a candidate to facilitate a pretargeted imaging strategy, however it should be noted that the rate of this reaction (~2 $M^{-1}s^{-1}$) appears to be sub-optimal for *in vivo* pretargeting applications.

The related TCO-tetrazine ligation proceeds *via* a similar inverse electron-demand Diels-Alder

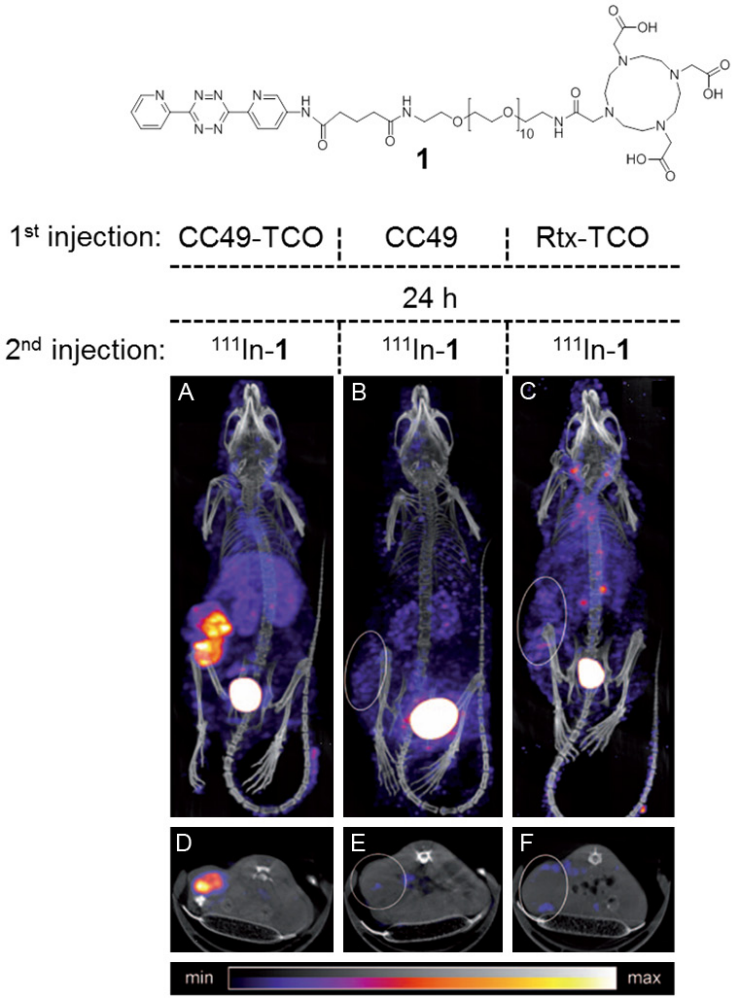


Figure 7. In the first example of *in vivo* pretargeting using the TCO-tetrazine ligation, Rossin *et al.* used the indium-111 complex of **1** (top) to obtain clearly distinguishable SPECT images of colon cancer xenografts. Tumour-bearing mice were injected with (A) CC49-TCO (100 μg) and after 24 h were subsequently injected with [^{111}In]-**1** (25 equiv. to CC49; 3.4 equiv. to TCO); (B) CC49 (100 μg) followed 24 h later with [^{111}In]-**1** (identical amount as in (A)); and (C) Rtx-TCO (100 μg) followed 24 h later by [^{111}In]-**1** (same amount as in (A)); (D-F) single transverse planar images intersecting the tumours in (A-C). Images acquired at 3 h p.i. Rossin R, Verkerk PR, van den Bosch SM, Vulders RCM, Verel I, Lub J, Robillard MS, In Vivo Chemistry for Pretargeted Tumor Imaging in Live Mice, Angew Chem Int Ed. 2010; 122: 3447-3450. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

mechanism (**Figure 6**), although it is more rapid than the norbornene-tetrazine ligation by several orders of magnitude. Reaction rates of up to 3.8×10^5 $M^{-1}s^{-1}$ have been determined [152], making this among the most rapid bioorthogonal copper-free click chemistry reactions. As a result, the TCO-tetrazine ligation was quickly adopted by the radiopharmaceutical community and has been used in the preparation of radiolabelled small molecules [153] (including PARP inhibitors [154, 155]), peptides (*e.g.* cyclic-RGD peptide antagonists of $\alpha_v\beta_3$ [156, 157], Exendin-4 [158]), and proteins (*e.g.* VEGF [157]).

There are now several reports in which the TCO-tetrazine ligation has been used to facilitate an *in vitro* pretargeting strategy [132, 159-166]. In each of these examples, it is the TCO species which is coupled to the targeting vector, and a fluorescently-tagged tetrazine species is employed as the secondary agent. Epitopes at the cell surface [132, 159, 161, 163, 165] and in intracellular regions [160-164] have been successfully targeted using this strategy.

In their landmark study, Rossin *et al.* were first to demonstrate the utility of bioorthogonal chemistry as a pretargeting strategy in live mice bearing colon cancer xenografts [167]. In this example, the antigen TAG72 was selected as a model target due to its limited degree of internalisation (which would otherwise render it inaccessible) and its resistance to shedding. TAG72 also has clinical relevance as it is overexpressed in a variety of cancer cell lines. As a targeting vector, an anti-TAG72 mAb (CC49) was functionalised with an average of 7.4 TCO species using conventional NHS-based conjugation chemistry. A complementary dipyridyl tetrazine derivative was selected based on its high reactivity with TCO and good stability. This tetrazine species was modified with a DOTA chelating agent to facilitate radiolabelling with the SPECT radionuclide ^{111}In (^{111}In-**1**; **Figure 7**). Preliminary *in vitro* experiments conducted in PBS revealed that the bioorthogonal reaction between CC49-TCO and ^{111}In-**1** proceeded with a second order rate constant of 13,090±80 $M^{-1}s^{-1}$. In their *in vivo* experiments, a lag time of 1 day was used between

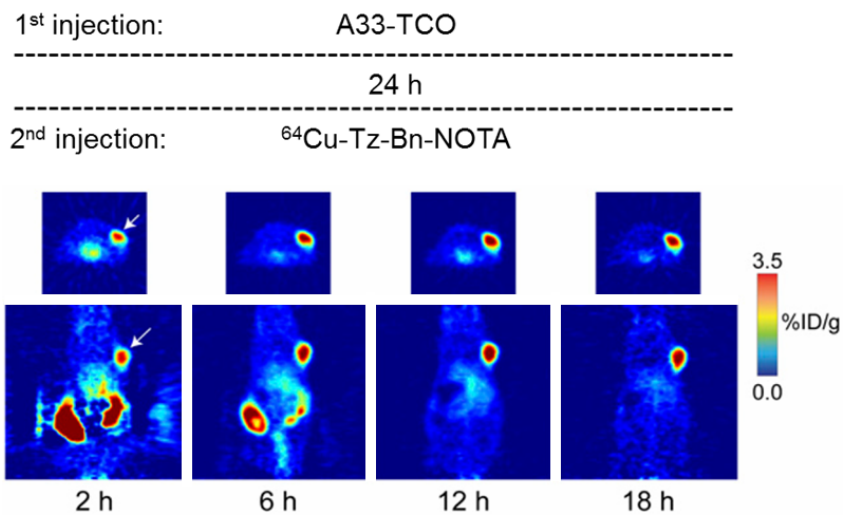


Figure 8. Zeglis *et al.* successfully employed the TCO-tetrazine ligation for *in vivo* pretargeted PET imaging of SW1222 tumour xenografts (white arrow; transverse [top] and coronal [bottom] images) in mice. A33-TCO (100 μg) was administered *via* tail vein injection and after a lag time of 24 h were then administered ^{64}Cu-Tz-Bn-NOTA (10.2-12.0 MBq [275-325 μCi], 1.2-1.4 μg, for 2.5-2.8 Tz-to-A33 ratio). Reprinted by permission of SNMMI from: Zeglis BM, Sevak KK, Reiner T, et al. A Pretargeted PET Imaging Strategy Based on Bioorthogonal Diels-Alder Click Chemistry. J Nucl Med. 2013; 54(8): 1389-1396. **Figure 4**.

the administration of the TCO-mAb construct and the radiolabelled tetrazine derivative. Promisingly, a rapid reaction between the two complementary bioorthogonal species occurred *in vivo* and SPECT/CT images acquired at 3 h p.i. revealed high contrast images of the tumour xenografts, yielding a T/M ratio of 13:1 (**Figure 7**). Importantly, only low levels of radioactivity were observed in the blood and non-target tissues (including the liver) at 24 h p.i. which were attributed to small amounts of TCO-CC49 which remained circulating in the blood pool.

Shortly after, Devaraj *et al.* reported a study which was focused on further elucidating the parameters linked to the *in vivo* behaviour of the TCO-tetrazine ligation [168]. In this case, the secondary agents were based on dextran scaffolds (10 kDa and 40 kDa) modified with multiple tetrazine species. In non-tumour-bearing mice, the longer residence times of these high molecular weight constructs proved beneficial in achieving higher *in vivo* labelling efficiencies in the blood pool. This study also included pretargeting experiments aimed at imaging the A33 glycoprotein in LS174T tumour-bearing mice. The A33 glycoprotein was selected as a suitable target due to its high persistence at the cell surface and practical half-life (>2 days). A33 is also overexpressed in >95% of all human colorectal cancers, including the LS174T cell line, and therefore represents a target with high clinical relevance. The targeting vector in this case was an anti-A33 mAb modified with ~3 TCO moieties as well as a near-infrared VT680 fluorophore to enable the acquisition of complementary optical imaging data. For the acquisition of high contrast PET images, an [^{18}F]-containing 10 kDa dextran construct ([^{18}F]PMT10) containing multiple tetrazine species was administered as the secondary agent. In a similar manner to the previous study, a lag time of 24 h was used between the primary and secondary agent, and PET/CT images were acquired at 3 h p.i. of the radiolabelled probe. While T/M or T/B ratios were not reported, the A33 tumours were clearly contrasted against the surrounding tissues and had substantially higher uptake on comparison to control tumours which did not express the A33 antigen.

In another example, Zeglis *et al.* used the TCO-tetrazine ligation to successfully implement a pretargeted imaging strategy in mice bearing SW122 colorectal tumour xenografts (**Figure 8**) [28]. This group also used an anti-A33 mAb (this time modified with approximately 5 TCO moieties per mAb) and importantly found no significant detrimental effect on the overall immunoreactivity. Here, the secondary agent comprised of a tetrazine group modified with a NOTA chelator for labelling with the PET radiometal copper-64. In common with the two previous studies, a lag time of 24 h was used and, in this case, PET images were acquired at various time points between 2-18 h. This group compared their findings against the directly labelled construct, ^{64}Cu-NOTA-A33, as well as the comparable ^{89}Zr-labelled antibody (in which

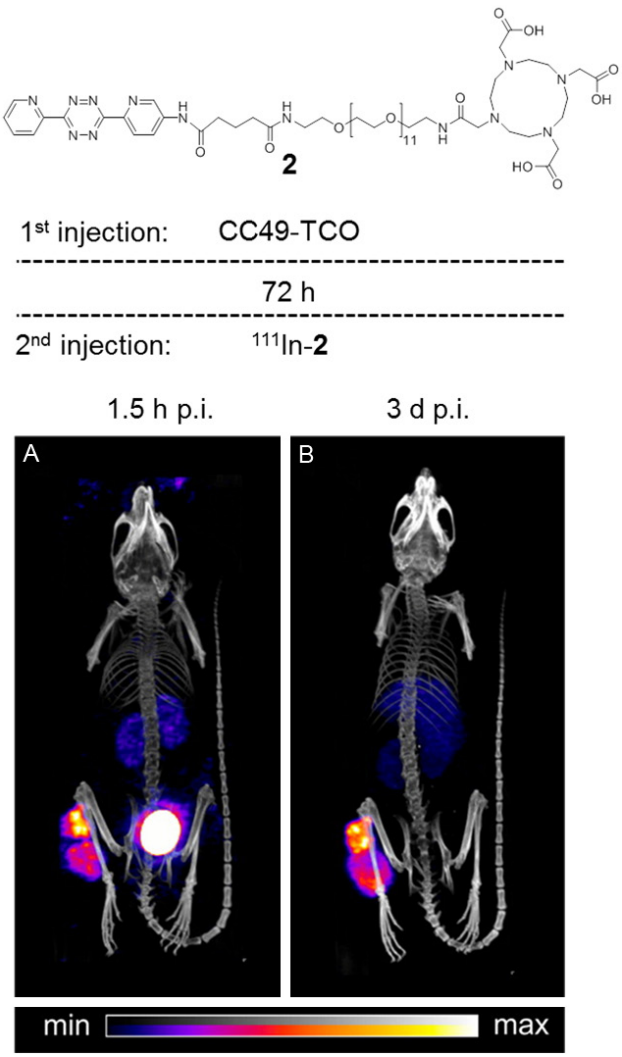


Figure 9. Rossin *et al.* significantly improved the *in vivo* stability and reactivity of the TCO-tetrazine ligation by increasing the steric hindrance of the TCO tag. An LS174T tumour-bearing mouse was administered CC49-TCO (100 μg) and after a lag-time of 72 h was then administered the ^{111}In-labelled tetrazine secondary agent (**2**). SPECT/CT images were acquired 1.5 h (A) and 3 days (B) after injection of the tetrazine species. Adapted with permission from Rossin R, van den Bosch SM, ten Hoeve W, Carvelli M, Versteegen RM, Lub J, and Robillard MS, Highly Reactive trans-Cyclooctene Tags with Improved Stability for Diels-Alder Chemistry in Living Systems. Bioconjugate Chem. 2013; 24(7): 1210-1217. Copyright 2013 American Chemical Society.

a DFO chelating agent was used). It was found that whilst the directly labelled antibody constructs resulted in higher overall tumour uptake at 12 h and 24 h p.i., the pretargeting approach yielded greater T/M ratios. Furthermore, it was also found that as a result of the faster clearance rate of the radiolabelled secondary agent, the overall radiation burden to off-target tissues and organs was substantially reduced.

A major concern regarding the application of TCO in a pretargeting strategy has been its sub-optimal *in vivo* stability. Rossin *et al.* recently addressed this issue and were able to determine that protein-bound copper likely deactivates TCO by promoting its conversion to the comparatively unreactive *cis*-cyclooctene isomer (CCO) [169]. By shortening the distance between the TCO moiety and the lysine residue to which it binds, an increase in steric hindrance on the TCO tag was found to obstruct this deactivation. As a result, the *in vivo* stability of TCO in mice was greatly improved. In addition, the authors also reported significantly improved rates of reaction for TCO derivatives substituted in axial rather than equatorial positions with bulky linking groups (~10-fold higher). *In vivo* pretargeting experiments were performed (**Figure 9**) using a very similar experimental design compared to their previous study [167], however in this instance the more stable TCO-derivative allowed a longer three-day lag period prior to the addition of the ^{111}In-labelled tetrazine secondary agent (^{111}In-**2**; **Figure 9**). The extended lag period resulted in reduced background signal in the circulation and, promisingly, a similar degree of tumour uptake which substantiates the improved *in vivo* stability of this TCO species. Furthermore, after 3 days the radioactivity in the tumour was unchanged which indicates that the bioorthogonal reaction product is extremely stable *in vivo*.

Summary and future perspectives

Pretargeting is a more complex approach compared with the use of directly radiolabelled imaging or therapeutic agents and it will require careful optimisation in order to be successfully translated into a clinical setting. Ultimately, the incentive to undertake this costly endeavour will come from the demonstrable proof that this approach is superior to the use of directly radiolabelled macromolecules. To date, a small number of clinical trials involving conventional pretargeting strategies have shown such promise, despite suffering from a few inherent shortcomings.

Bioorthogonal chemistry has the potential to circumvent many of the limitations of its predecessors and has shown significant promise in a

few pioneering preclinical studies. From a selection of potential candidates, only one bioorthogonal chemistry reaction has been used successfully to enable pretargeting of specific cancer biomarkers, namely the TCO-tetrazine ligation. However, as bioorthogonal reactions continue to be refined through innovative chemical design, this number is likely to increase, yielding chemical pairings with improved bioavailability, bioorthogonality, metabolic stability, and rates of reaction.

It is also worth noting that many of the most compelling biomarkers of cancer exist within the intracellular (and, indeed, intranuclear) compartments of tumour cells. This presents a significant challenge to the imaging community as these targets are much less accessible, particularly to the types of macromolecular targeting vectors that are involved in a pretargeting strategy. Therefore, research efforts should also be focussed on enhancing the cell permeability of these constructs. So far, only a handful of publications have addressed this.

In summary, the continual improvement of bioorthogonal chemistry reactions over recent years has facilitated an alternative pretargeting strategy which is demonstrating much promise for both imaging and therapy of cancer. This approach offers key advantages over more conventional strategies which will undoubtedly increase its potential for translation into a clinical setting.

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Disclosure of conflict of interest

None.

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