Click Chemistry in Radiopharmaceutical Chemistry

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Fundamentals

Radiopharmaceuticals often contain radionuclides with very short physical half-lives. As a result, it is often beneficial to incorporate the radionuclide into the agent during the final step of its synthesis in order to ensure that the radiotracer contains as much activity as possible. However, this requirement can present head-scratching synthetic conundrums that radiochemists often struggle to address. One set of chemical transformations that have proven particularly useful in this regard are grouped under the umbrella of 'click chemistry'. Broadly speaking, click chemistry ligations are reactions between two substrates that can selectively react with one another rapidly, cleanly, and quantitatively under very gentle conditions, in essence, molecular puzzle pieces. Recent advances in this area have led to the creation of a versatile library of ligations with wide applicability and improved efficiency, including reactions which can proceed within the complex environment of living organisms. This latter trait is particularly notable, as it presents an attractive approach for the delivery of radionuclides to sites of disease within the human body via the use of complementary click chemistry reaction pairs. This strategy – known as 'pretargeting' – holds several enticing implications for both nuclear imaging and targeted radiotherapy which will be discussed later in this chapter.

Details

The Utility of Click Chemistry Reactions in Radiochemistry

Click chemistry reactions have been applied most effectively to the preparation of ^{18}F-labeled PET imaging agents, including probes based on peptides, proteins, and – to a lesser extent – small molecules [1–8]. Importantly, these reactions offer the ability to circumvent many of the challenges associated with the direct incorporation of the [^{18}F]fluoride anion, including its poor nucleophilicity and limited reactivity in protic environments (see Chaps. 15, 16, and 17) [9–12]. These limitations often necessitate harsh reaction conditions that can damage the vector being radiolabeled and impair its ability to bind to its biological target (this is particularly true for more sensitive peptides and proteins). Consequently, ^{18}F-labeled prosthetic groups containing click chemistry handles (*e.g.* azides or alkynes) have become popular tools for the radiolabeling of more delicate biomolecules [3].

In practical terms, click chemistry-based ^{18}F-fluorinations begin with the radiosynthesis of the ^{18}F-labeled prosthetic group. This step, of course, can be performed under harsh reaction conditions because the sensitive biomolecule is not yet involved. Once the radiolabeled prosthetic group has been created, it is transferred to a vial containing the biomolecule bearing the complementary moiety, and the two undergo their click ligation under much more mild conditions, ultimately producing the desired radiofluorinated molecule (Fig. 1).

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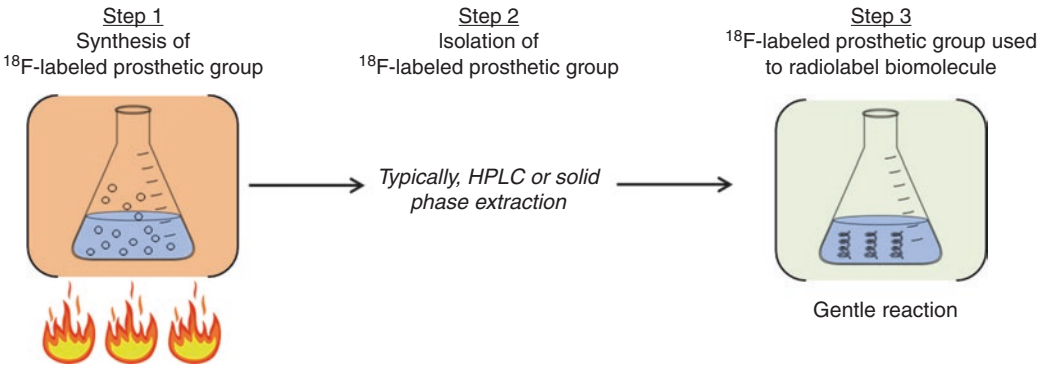

Fig. 1 The general strategy for radiolabeling sensitive biomolecules with ^{18}F-labeled prosthetic groups

While the use of 'clickable' prosthetic groups can yield radiolabeled species which may otherwise be unobtainable, this approach also has some important shortcomings. For example, the synthesis of an ^{18}F-labeled PET imaging agent should ideally be as rapid as possible to combat the inexorably decreasing activity of the decaying radionuclide. This strategy, unfortunately, introduces additional time-consuming steps related to the synthesis and purification of the prosthetic group. A second concern centres on the bulky nature of many clickable prosthetic groups and the linkages formed by the click ligations. The overall impact of incorporating these low molecular weight species onto large proteins (*e.g.* antibodies) can be negligible due to the sheer difference in size. However, these species can – and often do – disrupt the binding properties and biological behaviour of smaller vectors such as peptides and small molecules.

The Archetypal Click Chemistry Reaction

The most popular and widely utilized click chemistry reaction is undoubtedly the Huisgen 1,3-dipolar cycloaddition between an azide and an alkyne which results in the formation of a triazole (Fig. 2a) [13–15]. The earliest version of this reaction – which predates the concept of click chemistry by almost 40 years – was reported in 1963 by its eponym, Rolf Huisgen [14, 15]. In its original form, this reaction has a few critical limitations: it requires elevated temperatures and pressures and also lacks regioselectivity, as it leads to the formation of a mixture of 1,4- and 1,5-substituted triazoles. In the early 2000s, however, this comparatively primitive cycloaddition reaction was revived with the discovery that a copper(I) catalyst allows the reaction to proceed efficiently without heating and leads to the generation of only 1,4-substituted triazoles (Fig. 2b) [13, 16]. The high rate constant of this copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) – which is increased 10^6-fold compared to the uncatalyzed reaction – and the fact that this reaction is 'so insensitive to the usual reaction parameters as to strain credulity' makes it very attractive for radiosyntheses involving short-lived radionuclides [13].

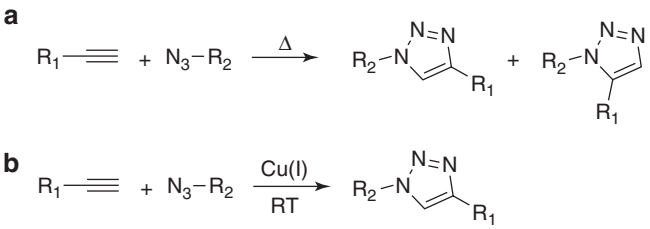

Fig. 2 (**a**) The original Huisgen 1,3-dipolar cycloaddition typically requires heat and/or high pressures and leads to an undesirable mixture of regioisomers. (**b**) Adding a Cu(I) catalyst facilitates an efficient room temperature reaction which only yields the 1,4-substituted triazole

Several examples of ^{18}F-labeled prosthetic groups which undergo CuAAC reactions are provided in Fig. 3. The linear alkyl ^{18}F-labeled alkyne prosthetic groups were among the first developed and have demonstrated promising utility in reactions with azide-bearing small molecules (such as folate receptor-targeting constructs) and peptides (such as A20FMDV2 which targets the integrin $\alpha_v\beta_6$) [4, 5, 17].

Another highly popular ^{18}F-labeled prosthetic group is 2-[^{18}F]fluoroethyl azide ([^{18}F]FEA), which was initially developed in 2007 and has since been used to radiolabel a wide variety of compounds [18–21]. The radiosynthesis of [^{18}F]FEA was originally achieved via the reaction of anhydrous, no-carrier-added K[^{18}F]F/Kryptofix with 2-azidoethyl-4-toluenesulfonate in acetonitrile at 80 °C for 15 min. The recovery of purified [^{18}F]FEA was achieved by distillation at 130 °C, ultimately producing the reagent in a decay-corrected radiochemical yield of 54%. Further refinements in the synthesis and manipulation of [^{18}F]FEA – which can be challenging due to its volatility – have since been reported. These include the simplification of the purification process by incorporating a solid-phase extraction step, a change which has also helped make the radiosynthesis of [^{18}F]FEA compatible with automated synthesis modules [22].

The CuAAC reaction has also been used for the radiolabeling of an alkyne-modified derivative of a Tyr^3-octreotate peptide with a ^{11}C-bearing prosthetic group: [^{11}C]methyl azide ([^{11}C]MeA) [23]. Furthermore, the CuAAC reaction

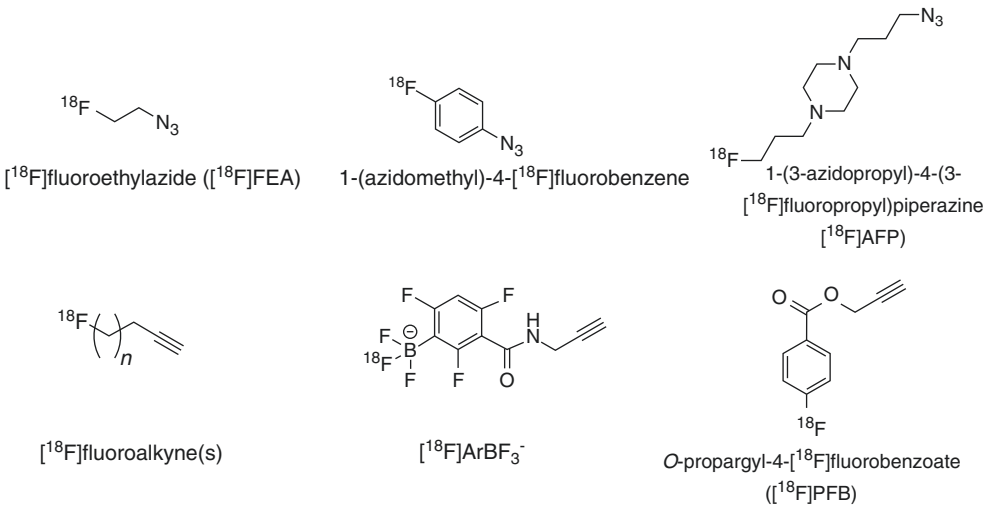

Fig. 3 A selection of commonly used ^{18}F-labeled prosthetic groups for CuAAC-based radiolabelings

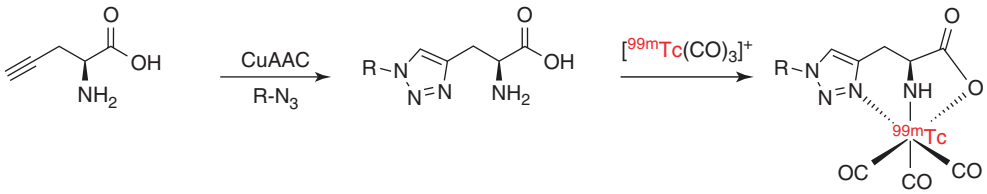

Fig. 4 An example of the 'click-to-chelate' radiolabeling strategy involving ^{99m}Tc(CO)$_3$

has also been elegantly utilized as part of the so-called 'click-to-chelate' strategy for labeling substrates with radiometals such as ^{99m}Tc. In this approach, the click reaction yields a 1,2,3-triazole which forms part of a tridentate scaffold that facilitates the coordination of an M(CO)$_3$ core (Fig. 4). This approach provides a convenient method for radiolabeling compounds with the often mercurial ^{99m}Tc, and – just as importantly – the radiopharmaceuticals created using this strategy have demonstrated high *in vivo* stability [24].

Unfortunately, the 'Cu' in the CuAAC reaction can create several problems. Copper is known to be toxic above certain concentration thresholds, and it is therefore of utmost importance to remove the metal completely from the formulation of the final product. This can be difficult to accomplish, because many peptides and proteins contain amino acids capable of coordinating the metal. The non-specific binding of Cu to proteins and peptides can also adversely affect the ability of the biomolecule to bind its target with high affinity and specificity. Furthermore, a catalyst is an additional reaction parameter which requires thorough optimization and adds complexity to the radiosynthesis protocol.

Strain-Promoted Azide-Alkyne Cycloadditions

Efforts to circumvent this need for a Cu(I) catalyst led to the discovery that building ring strain into the alkyne moiety can promote click chemistry ligations with azide-bearing species in the absence of a catalyst (Fig. 5a) [25–29]. The origin of this steric strain lies in the 'preferences' of the two sp-hybridized alkyne carbon atoms. Under most circumstances, these two atoms would adopt 180° bond angles; however, in a cyclooctyne ring, for example, they are forced to adopt bond angles closer to 163°, creating an associated ring strain of ~18 kcal/mol [30]. The relief of this ring strain is a potent driving force behind this catalyst-free reaction. These strain-promoted [3 + 2] azide-alkyne cycloaddition (SPAAC) reactions – part of a growing group of 'copper-free click' ligations – have now been widely utilized in the preparation of agents for both nuclear imaging and targeted radiotherapy [31, 32].

Compared to analogous CuAAC ligations which typically exhibit rate constants in the range of 10–200 M^{-1} s^{-1}, the reactions between the first generation of these cyclooctynes and azides had relatively modest rate constants (Fig. 5b) [33]. For example, the reaction of the cyclooctyne 'OCT' (see Fig. 5b) with benzyl azide has a second-order rate constant of just 0.0024 M^{-1} s^{-1}. However, it was soon realized that the introduction of electron-withdrawing substituents such as fluorines to positions proximal to the alkyne can lead to substantial increases in reaction rates [34]. To wit, monofluorinated (MOFO) and difluorinated (DIFO) cyclooctynes have yielded SPAAC rate constants of 0.0043 and 0.076 M^{-1} s^{-1}, respectively, under comparable reaction conditions. Other chemical modifications – including the introduction of delocalized aromatic systems into the greater structure of the cycloalkyne – have led to even faster reaction rates [27, 35–39]. Dibenzocyclooctyne (DBCO) and biarylcyclooctyne (BARAC) have achieved rate constants of 0.31 and

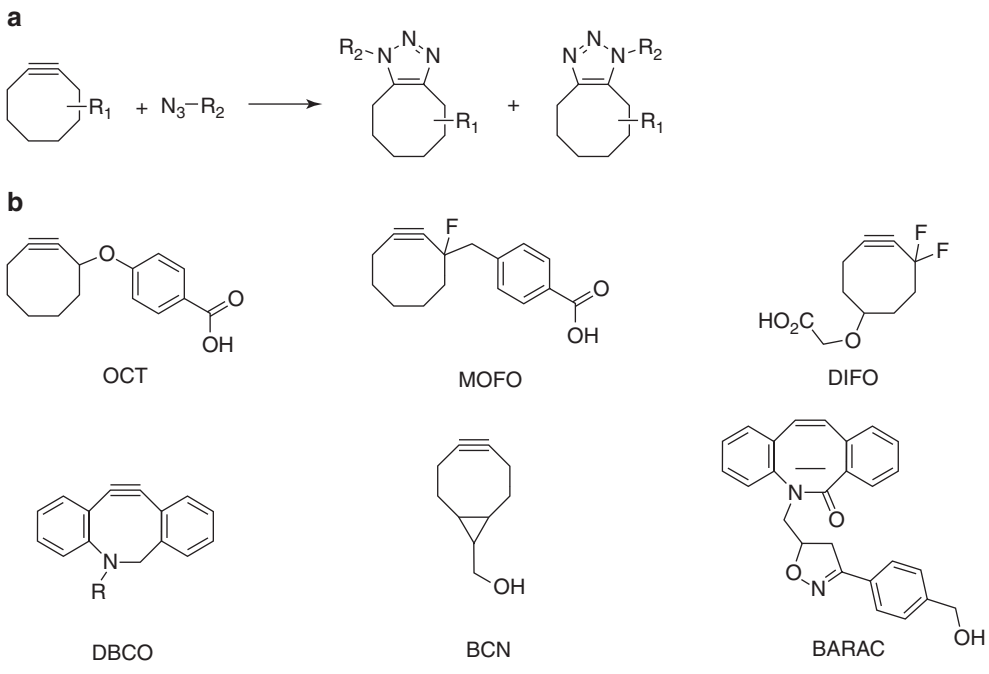

Fig. 5 (**a**) The strain-promoted [3 + 2] azide-alkyne cycloaddition (SPAAC) reaction is an excellent example of a copper-free click chemistry reaction; (**b**) a selection of cyclooctynes commonly used for SPAAC reactions

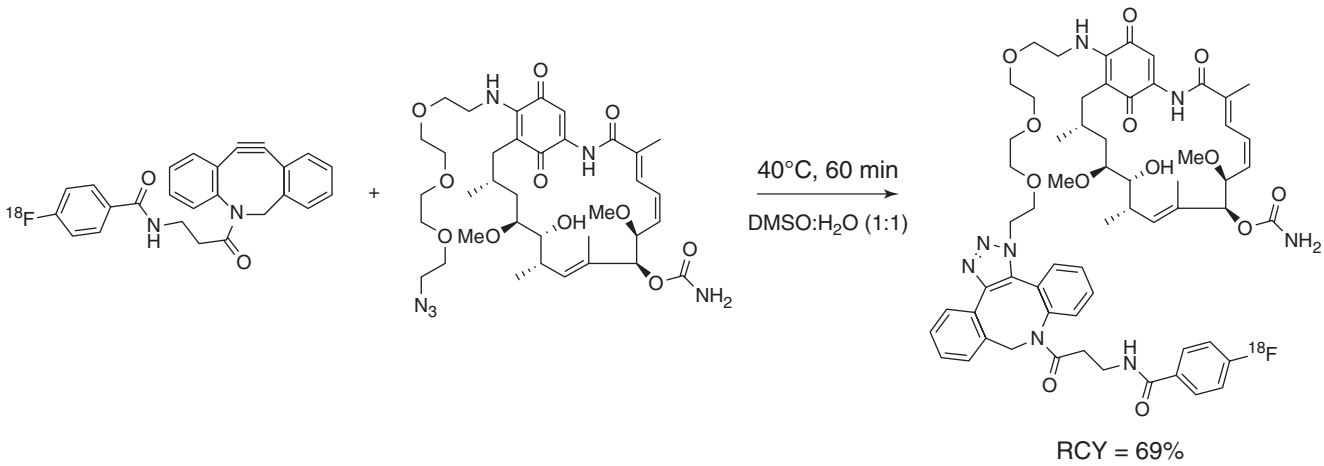

Fig. 6 The radiofluorinated dibenzocyclooctyne – [^{18}F]FB-DBCO – developed by Bouvet *et al.* has been used successfully as a prosthetic group for radiolabeling a variety of azide-containing compounds, including the complex natural product geldanamycin [40]

0.96 M^{-1} s^{-1}, respectively, in reactions with benzyl azide and similar aliphatic azides.

One of the earliest applications of the SPAAC in radiopharmaceutical chemistry is summarized in Fig. 6. In this example, an ^{18}F-labeled prosthetic group derived from DBCO – [^{18}F]-FB-DBCO – was used for the radiolabeling of the complex natural product geldanamycin [40]. The click reaction preceded efficiently under relatively mild reaction conditions (40 °C) and resulted in a radiochemical yield of 69% after 60 min, clearly underscoring the radiochemical value of the SPAAC reaction.

It is also important of course to acknowledge the limitations of SPAAC reactions. Perhaps not surprisingly, the most significant drawback of the ligation is the hydrophobicity of both the ring-strained cycloalkyne reagent and the product of the click ligation. This unfortunate (and often unavoidable) characteristic can often present difficulties during radiosynthesis – for example, the need for solubilizing agents which can be difficult to remove – and can also have a detrimental impact on the *in vivo* pharmacokinetic and pharmacodynamic profiles of the resulting radiopharmaceuticals [41].

The Inverse Electron-Demand Diels-AlderCycloaddition Reaction

Another class of click chemistry reactions which represent an evolutionary progression from the CuAAC ligations discussed earlier are the inverse electron-demand Diels-Alder (IEDDA) [4 + 2] cycloadditions between an electron-rich dienophile – such as norbornene or *trans*-cyclooctene (TCO) – and an electron-deficient diene (*e.g.* tetrazine) [42–44]. This concerted cycloaddition initially forms a highly strained bicyclic adduct that subsequently forms a dihydropyridazine through the loss of $N_{2(g)}$. It is thought that this product may be oxidized further to form an aromatic pyridazine [42].

The importance of the IEDDA reactions stems mostly from their extremely high rate constants – up to $k = 380{,}000\ M^{-1}\ s^{-1}$ – which are achieved without the need for a catalyst [45–49]. Furthermore, IEDDA ligations often exhibit high chemoselectivity in the presence of other functional groups, including other click chemistry reagents such as azides and alkynes [48, 50, 51]. Due to these favourable attributes, IEDDA reactions have attracted substantial attention from radiochemists and have been successfully employed in a variety of applications relating to radiosynthetic methodology [52–57], protein engineering [58, 59], and *in vivo* click chemistry [60–76]. For example, the IEDDA ligation has demonstrated great utility as the basis of ^{18}F-labeled prosthetic groups, particularly for the radiolabeling of sensitive peptides and proteins. One such prosthetic group based on a norbornene dienophile – [^{18}F]NFB (Fig. 7) – has been employed for the mild radiofluorination of a metabolically stabilized, tetrazine-modified bombesin peptide (TT-BBN) [77]. After a reaction time of 20 min, this approach afforded the desired ^{18}F-labeled peptide with a radiochemical yield of 50%. Notably, this method has been shown to be superior to the more conventional approach of using the primary amine-reactive acylation agent 4-succinimidyl-[^{18}F]fluorobenzoate ([^{18}F]SFB), as the IEDDA-based approach affords comparable radiochemical yields while using fivefold less peptide.

The IEDDA ligation has also been used to facilitate a modular strategy for the radiolabeling antibodies with positron-emitting radiometals. For example, the anti-HER2 antibody trastuzumab was modified with norbornene, and the resulting construct was then reacted with tetrazine-modified versions of DOTA and DFO for the subsequent coordination of copper-64 and zirconium-89, respectively. Importantly, given the sensitive nature of many antibodies, the norbornene conjugation and radiolabeling steps can be performed under gentle reaction conditions, thus eliminating any threat to the immunoreactivity of the antibody [78].

The dienophile TCO has now also been widely utilized as the basis for ^{18}F-labeled prosthetic groups, producing constructs which have demonstrated excellent versatility and efficacy (Fig. 8a). One of these prosthetic groups – [^{18}F]TCO (Fig. 8b) – has been utilized for the preparation of radiofluorinated derivatives of exendin-4 (which targets the glucagon-like protein-1 [GLP-1] receptor) and the cyclic peptide RGD (which targets the $\alpha_v\beta_3$ integrin) [55, 79]. [^{18}F]TCO has also been used in the synthesis of a small molecule PET radiotracer based on the PARP1 inhibitor, AZD2281 (see Fig. 8b) [53]. In this case, the IEDDA cycloaddition afforded a high decay-corrected radiochemical yield of 59.6 ± 5.0% after a reaction time of merely 3 minutes (at room temperature). Interestingly, the use of this relatively bulky prosthetic group had only a slightly detrimental

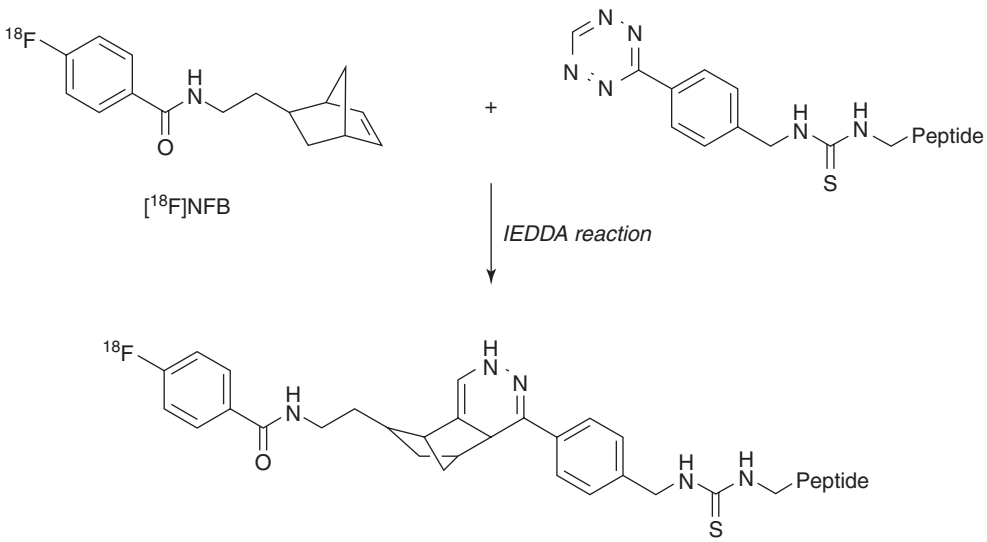

Fig. 7 (**a**) The IEDDA reaction between the norbornene-based prosthetic group [^{18}F]NFB and tetrazine-modified peptides results in the rapid and clean formation of the radiofluorinated peptide under mild reaction conditions

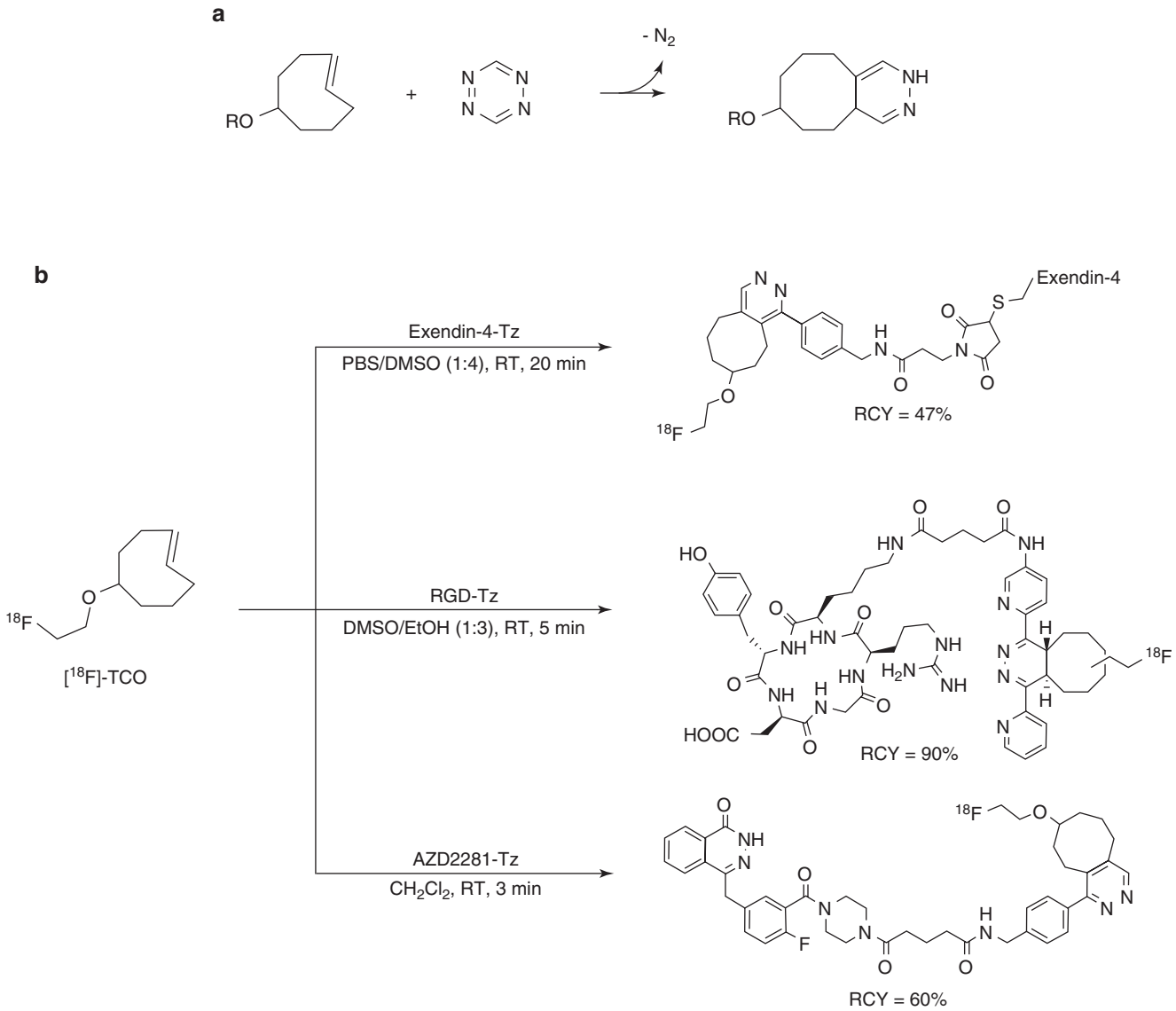

Fig. 8 (**a**) The IEDDA cycloaddition reaction between TCO and tetrazine (Tz) species is a catalyst-free click chemistry reaction that has a high rate constant and excellent chemoselectivity. (**b**) [^{18}F]-TCO is a highly versatile prosthetic group that has been successfully utilized for the synthesis of a diverse array of PET imaging agents, including examples based on exendin-4 [79], the cyclic peptide RGD [55], and the small molecule PARP inhibitor AZD2281 [53]

impact on the ability of [^{18}F]AZD2281 to bind to the PARP1 enzyme compared to the parent compound, AZD2281 (IC_{50} values of ~18 and 5 nM, respectively).

The versatility of these reactions has also been demonstrated by a recent study in which IEDDA chemistry was used to construct a novel bispecific protein (Bs-F(ab)$_2$) [59]. Here, two Fab fragments – one targeting the epidermal growth factor receptor (EGFR) and the other targeting CD105 – were modified with either TCO or tetrazine, thereby facilitating the efficient cross-coupling of the two constructs. The resulting Bs-F(ab)$_2$ was then modified with NOTA via the reaction of an NHS-bearing variant of the chelator with the ε-amino groups of the lysines of the construct and subsequently radiolabeled with ^{64}Cu. This innovative approach highlights the value of IEDDA reactions for applications at the intersection of protein engineering and radiopharmaceutical chemistry.

In Vivo Applications of Click Chemistry

Rejuvenating an Old Concept: Pretargeted Imaging and Therapy

Click chemistry reactions have recently revitalized a concept known as 'pretargeting'[71, 80–82]. The objective of pretar-

geting is to overcome the principal disadvantage of using radiolabeled antibodies to target biomarkers of disease: their slow clearance from the blood. While antibodies can be superb targeting vectors due to their exquisite specificity and affinity for their antigens, their slow blood clearance is a major disadvantage. From a practical perspective, it means that – in the context of imaging – PET/SPECT scans usually have to be performed 5–7 days after the administration of the radioimmunoconjugate in order to obtain images with satisfactorily high tumor-to-background activity contrast ratios [83–88]. For therapeutic studies (and, to a lesser extent, imaging studies), the protracted biological half-life of directly radiolabeled antibodies also results in a high radiation burden to the patient [89]. Pretargeting represents an alternative approach with the potential to overcome all of these problems.

In its simplest form, pretargeting is performed using a two-step approach (Fig. 9). The first step is the injection of the antibody – the 'primary agent' – which is then permitted sufficient time to accumulate within the target tissue and clear from circulation. This lag interval usually lasts for several days and is followed by the administration of the 'secondary agent', typically a radiolabeled small molecule with a rapid pharmacokinetic profile. Upon encountering one another *in vivo*, the primary and secondary agents rapidly and selectively combine, leading to the accumulation of the radionuclide in the target tissue. Due to the rapid clearance of the secondary agent from the blood, high tumor-to-background contrast ratios can be obtained at much earlier time points compared to directly radiolabeled antibodies. In addition – and just as importantly – pretargeting drastically reduces the overall radiation burden to the patient. Thus, in essence, pretargeting combines the favourable targeting properties of antibodies with the superior pharmacokinetic profiles of small molecules while simultaneously skirting the limitations of both constructs.

It is critical to note that the successful implementation of this approach requires that both the primary and secondary agents are modified with complementary reactive groups which are capable of binding together rapidly and selectively in the complex *in vivo* biological milieu. A variety of approaches have been applied in this endeavour. The original strategy involved the use of bispecific antibodies with the ability to bind both a target antigen and a radiolabeled hapten species (typically, a derivative of ethylenediaminetetraacetic acid [EDTA]). Another popular strategy has exploited the exceptionally high-binding affinity between biotin and (strept)avidin (4×10^{-14} M) [90, 91]. In this case, an antibody conjugated to the protein (strept)avidin is typically employed as the primary agent, and a radiolabeled biotinylated species is used as the secondary agent. Other more recent approaches have involved the use of complementary oligonucleotides [92–101] and enzymatic reactions (*e.g.* HaloTag) [102, 103]. While some of these approaches have yielded promising results in clinical trials, they are often hampered by their inherent limitations, which include difficulties in the production of the (often expensive) primary antibody species, immunogenicity, competing endogenous species (such as biotin), and the metabolism of the secondary agents. As a result of these confounding factors, pretargeting has so far

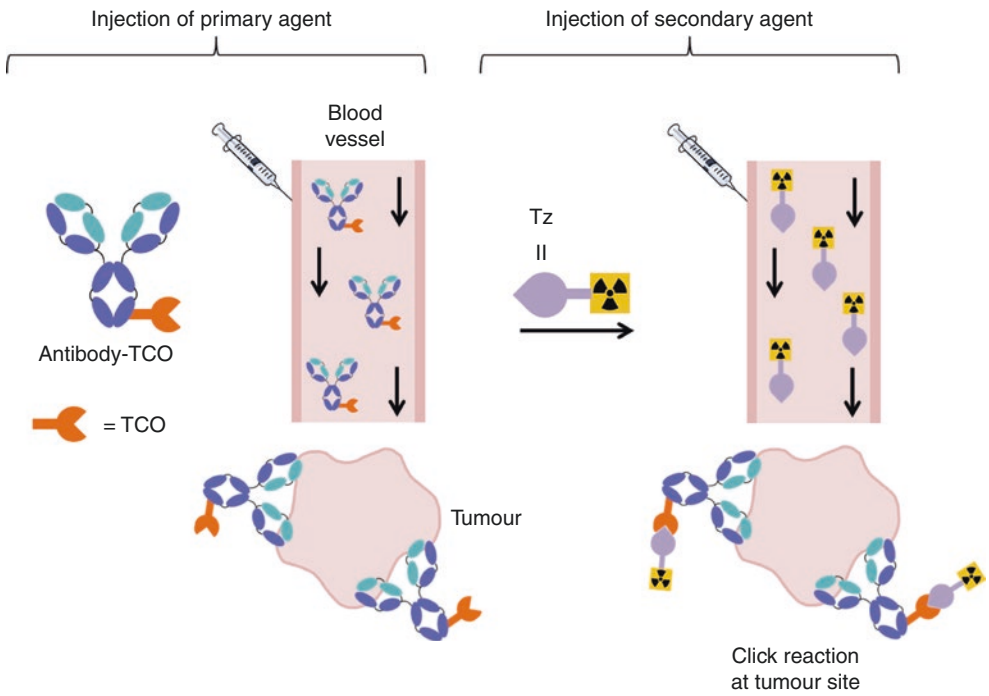

Fig. 9 Simplified schematic of a two-step pretargeting strategy based on IEDDA click chemistry

had a negligible impact on clinical practice despite over 30 years of development.

Click chemistry-mediated pretargeting – specifically pretargeting based on the TCO/tetrazine reaction – is well-placed to overcome the limitations of its predecessors and has yielded highly promising *in vivo* preclinical data in a variety cancer models [71, 80–82]. While even the fastest click chemistry reactions are slower than many biomolecular interactions, the IEDDA ligation offers many advantages in the context of pretargeting, including (*i*) low immunogenicity, (*ii*) high modularity (*i.e.* it can be readily adapted to virtually any combination of antibody and radionuclide), (*iii*) synthetic simplicity (*iv*) bioorthogonality, and (*v*) the availability of commercially available and inexpensive reagents.

First demonstrated in 2010, IEDDA-based pretargeted imaging has now been demonstrated in a variety of preclinical settings with very promising results. The majority of studies have probed cell surface receptors which meet several critical – yet relatively uncommon – criteria, most notably a high level of persistence on the surface of cells. It is also particularly important that the antibody primary agent is not internalized into the cell, as this would prevent its interaction with the secondary agent. So far, only a handful of suitable targets have been identified and used in 'proof-of-principle' studies to validate and optimize this approach to pretargeting. Suitable targets include TAG-72, A33, CEA, and CA19.9. Interestingly, CA19.9 would not appear to strictly abide by these criteria, as it is known to readily shed into circulation; however, IEDDA-based pretargeting has successfully overcome this specific adversity, underscoring the promise of the methodology.

In the vast majority of IEDDA-based pretargeting studies, it is the TCO species rather than the tetrazine which is attached to the antibody. This is mainly due to the superior *in vivo* stability of the TCO compared to tetrazine (these issues are discussed in more detail in the section on 'Early Obstacles and Innovative Solutions' and 'The Future'). Accordingly, the secondary agent is usually a radiolabeled variant of tetrazine. Several secondary agents have been developed in recent years, including constructs containing radionuclides for both imaging (*e.g.* ^{18}F [60, 64], ^{64}Cu [63, 65–67], ^{68}Ga [60], and ^{111}In [69, 104]) and therapy (*e.g.* ^{177}Lu [61, 69, 70, 72, 105]). Many of these reports demonstrate that while tumour uptake values (%ID/g) are invariably lower than what can be achieved using directly radiolabeled antibodies, pretargeting often yields improved activity concentration ratios (such as tumor-to-blood and tumor-to-muscle) at much earlier times after the administration of the radioligand (hours rather than days) [67]. Notably, this strategy has also revealed more favourable dosimetry in mouse models [67].

The promising preclinical data from IEDDA-based pretargeted imaging experiments suggests that both same-day clinical scans and vastly reduced radiation doses to patients are realistic prospects. This reinvigorated imaging strategy is now poised for evaluation in clinical trials and, if successful, has the potential to make a more substantial clinical impact than its predecessors.

Early Obstacles and Innovative Solutions

The recent development of IEDDA-based pretargeting has encountered several early obstacles which, if left unaddressed, would have tempered its successful application. However, as a result of focused investigations, these issues have been identified and have been overcome through innovative, chemistry-based solutions. In this sub-section, we will discuss some of the most important challenges faced by IEDDA-based pretargeting.

Stability of the Tetrazine Reagent Molecular species which are primed with high reactive potential – such as the protagonists of the TCO/tetrazine reaction – are often prone to rapid decomposition. This is undesirable for several (fairly obvious) reasons: First, compounds which will be administered to patients and those which are prepared in accordance with good manufacturing practice (GMP) standards should have a long shelf life. The decomposition of the IEDDA precursor reagents may interfere with the preparation of the primary and secondary agents by reducing reaction yields and producing unwanted side products that would require purification. Second, compounds with short shelf lives would also require more frequent chemical manufacturing at additional expense. And last, the rapid decomposition of these species within the patient would prevent the ligation of the primary and secondary agents, thereby rendering the entire strategy futile.

The stability and reactivity of a wide variety of tetrazine agents have now been evaluated [46]. These data clearly illustrate that the tetrazines with the highest rate constants in reactions with either norbornene or TCO species are often the least stable and therefore do not necessarily represent the best candidates for *in vivo* applications. While a wide array of tetrazines have been evaluated, only two – shown in Fig. 10 – have been widely studied in preclinical pretargeting experiments. These reagents have been selected as they each represent a compromise between stability and reactivity.

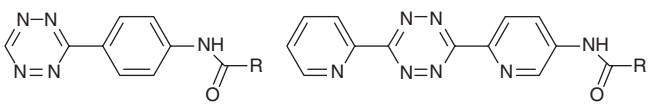

Fig. 10 The two tetrazine species which have been most widely utilized for *in vivo* pretargeting experiments

Stability and Reactivity of the *Trans*-Cyclooctene Reagent As the TCO-modified antibody can require days or weeks to reach its optimal biodistribution *in vivo*, it is crucial that the TCO species is resistant to degradation within this timeframe. Otherwise, of course, its degradation would prevent the ligation with its tetrazine reaction partner. In the first reports of preclinical IEDDA-based pretargeted imaging, the selected TCO species had a suboptimal ability to withstand degradation in mice ($t_{1/2}$~2.6 days). While SPECT images with promising tumour contrast have been obtained, the more protracted circulatory half-life of antibodies in humans necessitates a more stable version of TCO which would allow imaging at much later times.

This issue was addressed in 2013 in an elegant study which identified that this loss of reactivity was caused by the isomerism of TCO to the comparatively unreactive *cis*-cyclooctene (CCO) [73]. Based on the assumption that endogenous metals were responsible, a thorough investigation of several metal-containing proteins led to the discovery that copper-bound proteins were largely to blame. This observation informed rational chemical modifications designed to obstruct this degradation process. Specifically, shortening the PEG linker between the TCO and the antibody leads to an increase in steric hindrance around the TCO which in turn reduces the likelihood of copper-induced isomerization. Another important advancement has been the recognition that the higher energy axial isomer of TCO undergoes a considerably faster reaction with tetrazines compared to its equatorial cousin (k_2 = ~27 × 10^4 versus ~2.7 × 10^4 M^{-1} s^{-1}, respectively). Applying these two improvements in preclinical experiments has enabled the acquisition of exceptional SPECT images following a lag period of 3 days. SPECT images acquired at much later time points – up to 3 days after the injection of the secondary agent – revealed that the radioactivity concentrations within the tumour were not reduced by this added delay, suggesting that the product of the click chemistry ligation is itself resistant to *in vivo* degradation.

Re-routing Excretion Pathways by Chemical Design The lipophilic character of most tetrazine-containing secondary agents promotes excretion *via* the hepatobiliary system. Not surprisingly, this is a problem in cases in which malignant tissue is located within the abdominal region, as sites of interest may be obscured, thus complicating or even preventing the reliable quantification and interpretation of images. This is particularly problematic when considering that the cell surface receptors which represent the most promising candidates for pretargeted imaging and therapy – such as A33 and CEA – are primarily markers of colorectal cancers.

Most of the tetrazine-based secondary agents developed to date have suffered from this shortcoming. However, in recent years, some promising progress has been made in this area. A notable example involving a ^{64}Cu-labeled tetrazine nicely demonstrates the importance of coordination chemistry in determining the overall biodistribution and pharmacokinetic properties of radiometal-containing imaging agents [66]. This study found that when copper-64 was complexed by the macrocyclic chelator NOTA, the overall molecular charge of −1 contributed to the unfavorable excretion of the radioligand through the gut. Yet replacing NOTA with the neutral cryptand chelator SarAr results in an overall electronic charge of +2, which greatly increased the hydrophilicity of the compound and helped to direct its excretion to the renal system. Modifications to the chemical structures of other secondary agents containing non-metal radionuclides such as ^{18}F have also helped re-route the excretion of these radioligands towards renal clearance [75].

The Future

Looking ahead, there are many attractive prospects for click chemistry reactions within radiopharmaceutical chemistry. A particularly exciting area is the development of click chemistry-based prosthetic groups for attaching radionuclides to biomolecular vectors. Existing methods of bioconjugation often rely on the random attachment of radionuclides to functional groups which are prevalent in proteins and peptides (*e.g.* the ε-amino group of lysine residues). This approach – while facile and reliable – invariably leads to a heterogeneous and poorly characterized mixture of products, each with slightly different (and potentially compromised) pharmacokinetic and binding properties. Encouragingly, several bioconjugation methods are being developed which offer considerable improvements over these existing approaches. One such strategy involves the removal of the terminal galactose residues of the heavy chain glycans of the antibody using *β*-1,4-galactosidase and then incorporating azide-modified *N*-acetylgalactosamine monosaccharides into the glycans using a promiscuous galactosyltransferase. These moieties can then be used as handles for the subsequent site-selective SPAAC-mediated attachment of cyclooctyne-modified chelators or prosthetic groups [63, 106, 107]. Other similar chemoenzymatic methods have also shown promise in this area [108]. These site-selective bioconjugation methods will facilitate the highly controlled attachment of a fixed number of cargoes to well-defined and carefully selected sites on antibody-based agents. Given the recent resurgence in antibody-based therapeutics – and their companion diagnostic imaging agents, as well – these site-selective bioconjugation methods will undoubtedly become the preferred option for the synthesis of radioimmunoconjugates.

Given the important advantages offered by pretargeting strategies, it is somewhat surprising that only a small handful

of cancer-associated cell surface receptors have been the subject of investigation in this area. Clearly, these receptors have been selected based on highly restrictive criteria which exclude many of the most clinically relevant antigens (described in the section on 'Rejuvenating an Old Concept: Pretargeted Imaging and Therapy'). Unfortunately, in cases where the antibody has been shown to internalize rapidly – such as the anti-HER2 antibody trastuzumab – these strategies have not been as successful. However, in order to widen the scope of pretargeting strategies beyond this handful of markers, it will be of great importance to identify additional suitable targets which also have high clinical value.

Lastly, the recent development of a new drug delivery strategy referred to as 'click-to-release' is also poised to make an important impact in the coming years [109, 110]. In essence, this approach involves the controlled release of a drug which is initially bound to TCO and is cleaved during the IEDDA reaction with a tetrazine-based secondary agent. From a mechanistic standpoint, the drug is attached at the allylic position of TCO by a carbamate linker and is ejected (with CO_2 as an additional side product) via an electron cascade mechanism when the 1,4-dihydropyridazine intermediate converts to a pyridazine which then subsequently rearranges to an aromatic pyridazine. While there are many alternative (usually enzymatic) systems which allow for the cleavage of drugs bound to targeting vectors, the click-to-release mechanism is advantageous because it does not rely on the assistance of intracellular enzymes and can therefore be targeted against extracellular epitopes and matrix constituents. It is also worth noting that while doxorubicin has been utilized as a model drug in these early investigations, it is easy to envision this approach applied to other chemotherapeutics as well. Another attractive prospect using this chemical technology is the incorporation of a radiolabeled tetrazine into this approach, an alteration which could enable the *in vivo* monitoring of drug release and possibly even facilitate combined chemo- and radiotherapy.

The Bottom Line

Over the last several years, advancements in radiochemistry have yielded an abundance of synthetic methodologies which can now be used to generate radiopharmaceuticals previously considered too complex and time-consuming to synthesize. The incorporation of click chemistry reactions into the radiochemistry toolbox is a particularly notable advancement. Click chemistry offers a mild, rapid, and high-yielding approach for the radiolabeling of heat-sensitive biomacromolecules such as proteins and antibodies. The use of click chemistry to radiolabel smaller vectors such as peptides and small molecules requires more careful consideration, as the comparatively bulky groups created by click chemistry ligations are more likely to affect the ligand-binding properties and *in vivo* behaviour of these constructs. Lastly, the IEDDA cycloaddition is well-placed to facilitate *in vivo* pretargeted imaging and therapy. Continual refinements to the underlying chemistry have resulted in a highly optimized technology which is now poised for evaluation in patients.

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