Chapter 14
Bioorthogonal Reaction for Fluorine-18 Labeling

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Abstract Specific molecular imaging probes including radiopharmaceuticals labeled with positron-emitters, such as fluorine-18 (^{18}F, $t_{1/2}$ = 109.8 min), need to expand their applications of positron emission tomography (PET) molecular imaging study. In recent years, bioorthogonal chemistry such as inverse electron-demand Diels-Alder cycloaddition reactions and strain-promoted alkyne azide cycloaddition (SPAAC) has been regarded as alternative bioorthogonal ligation reactions of bioactive molecules with radiolabeled building blocks. In this chapter, I will introduce an overview of this emerging synthetic strategies based on the catalyst-free SPAAC conjugation reaction and Diels-Alder cycloaddition reactions using tetrazine/*trans*-cyclooctene (TCO) derivatives under physiologically-friendly reaction conditions. I will also introduce that the pretargeting method by the SPAAC reaction for tracking mesoporous silica nanoparticles (MSNs) in in vivo system. This bioorthogonal SPAAC-based pretargeting protocol allow ^{18}F with a short half-life to be used for labeling of the MSNs to obtain their tracking PET images.

14.1 Cu-Catalyzed Click Reaction

Copper (I) catalyzed azide alkyne [3+2] cycloaddition (CuAAC), which referred to as "click chemistry", has received much attention in biomedical area because this click chemistry provided the facile conjugation protocol of biomolecules with a range of functional groups as well as radiolabeling method for the synthesis of molecular imaging probes [1–5]. On the other hand, this CuAAC reaction-based click bioconjugation reaction had limitations in synthetic operation as follows; the frequent undesired interaction of Cu(I) catalyst with biomolecules, such as peptide and antibody, that make this CuAAC reaction blocked or reduce the reaction rate.

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This unexpected interaction can cause the denaturation of biomolecules and the cytotoxicity by residual copper in the bioactive products during this bioconjugation process [6–8].

14.2 Bioorthogonal Reaction

In recent advances in molecular imaging, bioorthogonal ligation reactions played an important role for synthesis of radiolabeled probes [9]. These bioorthogonal reaction s are useful tools to conjugate bioactive compounds with radiolabeled synthon, highly chemoselectively without need for metal catalyst under physiologically friendly condition i.e., in water, aqueous media or even cell media, tolerance of a broad range of functionality [9, 10]. Among various bioorthogonal reactions, the strain promoted alkyne azide cycloaddition (SPAAC) and the inverse Diels-Alder tetrazine click reaction have been widely used for this purpose.

Firstly, the SPAAC reaction, which is referred to as copper-free click chemistry, has acted as a straightforward and fast ligation method for biological applications, as well as an alternative bioorthogonal conjugation reaction of biomolecules with radiolabeled building blocks for the preparation of radiopharmaceuticals [9]. In this regard, many researchers have developed a variety of cyclooctyne building blocks for a variety of applications in molecular imaging [11–20]. Among various cyclooctyne derivatives, aza-dibenzocyclooctyne (ADIBO) or dibenzocyclooctyne (DIBO) compounds have been used widely with azide building blocks to rapidly produce the corresponding triazole derivatives for efficient SPAAC reaction (Fig. 14.1a) [21–23]. The strain promoted inverse electron-demand Diels-Alder cycloaddition reactions of 1,2,4,5-tetrazines are known to be another popular bioconjugation method for molecular imaging applications (Fig. 14.1b). The cycloaddition of a tetrazine with *trans*-cyclooctene (TCO) derivatives to the corresponding cycloocta[*d*]pyridazines can also provide the unusually fast reaction rates in the absence of any catalyst under physiologically friendly condition [24].

It is well known that positron emission tomography (PET) is one of excellent biomedical imaging modality. This PET system has been able to help early detection, characterization, and real time monitoring of disease pathologies. Nowadays, it is also useful for characterizing fundamental biological processes and helping new drug developments [25, 26]. In order to obtain high-quality PET images for this purpose, it is necessary to synthesize specific radiolabeled molecular imaging probes using positron-emitters, such as carbon-11 (^{11}C, $t_{1/2}$ = 20.4 min), nitrogen-13 (^{13}N, $t_{1/2}$ = 10 min), oxygen-15 (^{15}O, $t_{1/2}$ = 2.05 min), copper-64 (^{64}Cu, $t_{1/2}$ = 12.7 h), fluorine-18 (^{18}F, $t_{1/2}$ = 109.8 min) [27, 28]. In particular, ^{18}F is known to be the most prominent positron emitter for PET molecular imaging due to its favorable properties as follow; (i) its minimal steric interference can allow ^{18}F labeled biologically active molecules to maintain favorable interactions with the target proteins or receptors for tracing biological processes; (ii) ^{18}F can be generated easily from a cyclotron through the ^{18}O(p, n)^{18}F nuclear reaction using [^{18}O] water

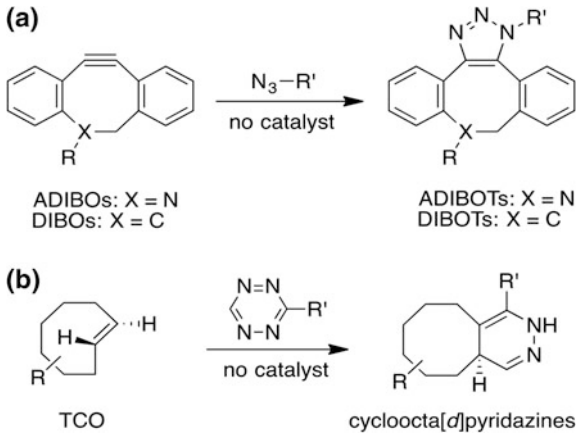

Fig. 14.1 Bioorthogonal reactions without need of catalyst under physiologically friendly condition. **a** Strain promoted alkyne azide cycloaddition (SPAAC) reaction. ADIBO = aza-dibenzocyclooctynes; DIBOs = dibenzocyclooctynes; ADIBOT = aza-dibenzocycloocta-triazoles. **b** Strain promoted inverse electron-demand Diels-Alder cycloaddition reactions. TCO = *trans*-cyclooctenes

targets with proton beams; (iii) a fluorine can form a stable covalent bond with carbon, which can increase its metabolic stability; (iv) fluorine in fluorine-containing compounds can form an unexpected hydrogen bonding with the desired biological target; (v) the half-life of ^{18}F allow ^{18}F labeled molecular imaging probes to be shipped regionally to nearby research sites or hospitals which are not equipped with cyclotron as radionuclide production facility [27–31].

14.3 SPAAC Reaction Based In Vivo ^{18}F-Labeling of Mesoporous Silica Nanoparticles

Since biocompatible nanoparticles which have an optimal size can accumulate in the tumor by passive targeting such as the enhanced permeability and retention (EPR) effects [32–34], they have attracted much interest in therapy and early diagnosis of cancer in broad nanomedicine research field [25–37]. Therefore, the pharmacokinetic studies of the nanoparticles became a crucial topic for investigating the safety of these nanoparticles and their in vivo behavior via real-time tracking using a PET system in the living body. In general, MSNs can show good performance in the biomedical field as the vehicles for drug-delivery [38]. However, because these nanoparticles generally require a long circulation time for homing at the tumor region [37, 38], ^{18}F may be not a suitable radioisotope for the labeling of nanoparticles for PET imaging considering its short half-life ($t_{1/2}$ = 109.8 min) [27–29].

In 2013, Kim et al. firstly reported the pretargeting concept for in vivo covalent ^{18}F labeling reaction of nanoparticles using a bioorthogonal chemistry [39]. In this report, highly efficient MSN pretargeting and PET imaging was successfully done with this short half-life ^{18}F using a rapid and bioorthogonal SPAAC reaction of ADIBO-substituted MSNs with ^{18}F-labeled azide in tumor-bearing mice [39]. Using the strategy of bioorthogonal chemistry, an ADIBO group-tethered PEGylated MSNs (ADIBO–PEG–MSNs) were prepared with such a size of 100–150 nm that MSNs accumulate in the tumor by the EPR effect [40]. To investigate the expected reaction rate in an in vivo system, the model SPAAC reaction was carried out under physiologically similar conditions (pH 7.4 and 36.5 °C) in vitro as shown in Fig. 14.2. The model SPAAC reaction of ADIBO–PEG–MSNs (0.48 mmol of ADIBO portion, 4 mg) with ^{18}F-labeled azide was completed within 15–20 min, and the desired ^{18}F-labeled ADIBOT–PEG–MSNs was produced in almost quantitative radiochemical yield (RCY).

As results of the tumor targeting ability of MSNs by the EPR effect and the fast rate of SPAAC reaction under physiologically similar conditions, a further MSNs-based pretargeting and later covalent ^{18}F labeling via SPAAC was performed in the living body (Fig. 14.3a). As shown in PET-CT images in Fig. 14.3c, ^{18}F-labeled azide, even though it has no targeting capability to the tumor tissue, exhibited high tumor uptake in the mice pretreated with ADIBO–PEG–MSNs 24 h earlier via in situ synthesis of ^{18}F-labeled ADIBO–PEG–MSNs by in vivo SPAAC reaction within 2 h after injection of the ^{18}F-labeled azide. In contrast, the PET-CT images of the non-pretargeted mice, to which only the ^{18}F-labeled azide was administered, showed transient initial ^{18}F uptake with lower signal intensity, washing out soon via kidneys (Fig. 14.3b). This bioorthogonal SPAAC reaction-based pretargeting protocol could provide a feasibility that the nanoparticles were given first and wait until they are localized in the target region and with later administration of the tracer ^{18}F-azide to track them using PET. Later injected ^{18}F-azide is bound to the nanoparticles via an in situ ^{18}F labeling reaction on site in the tumor in a living body. More recently, this SPAAC reaction-based pretargeting protocol was applied in tracking in vivo other macrobiomolecules such as peptides or antibodies while using PET with short half-life radioisotope.

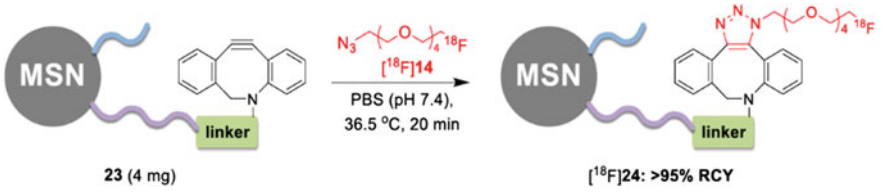

Fig. 14.2 Formation of ^{18}F-labeled ADIBOT-PEG-MSNs under physiologically similar conditions (pH 7.4, 36.5 °C) in PBS by SPAAC reaction of ADIBO-PEG-MSNs with ^{18}F-labeled azide. Adapted from [39] with permission

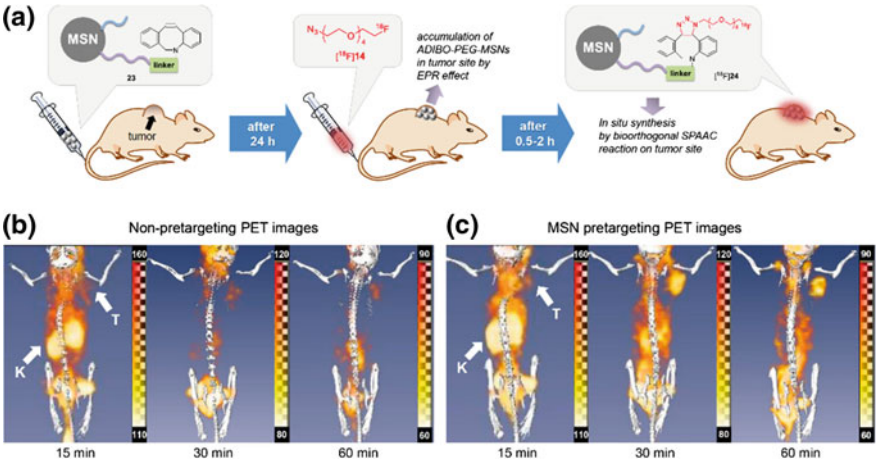

Fig. 14.3 **a** Bioorthogonal SPAAC reaction for the in situ synthesis of ^{18}F-labeled MSNs in a living specimen for the MSNs pretargeting PET-imaging in tumor mice model. **b** PET-CT images of ^{18}F-labeled azide in a U87MG tumor-xenograft mouse administrated only ^{18}F-labeled azide alone (non-pretargeted). **c** PET-CT images in a U87MG tumor-xenograft mouse pre-treated ADIBO-PEG-MSNs 24 h earlier (pretargeted procedure) at 15, 30, and 60 min after injection of ^{18}F-labeled azide. T = tumor, K = kidneys. Adapted from [40] with permission

14.4 General Radiolabeling Procedure with ^{18}F for Peptides

Over the past few decades, the development of the combinatorial chemistry and phage display technology allowed to discover novel bioactive peptide sequences In particular, the phage display has provided about 160 sequences of tumor targeting peptides [41]. Based on high expectation to use these new peptide sequences as the molecular imaging probe or new drug candidates, development of peptide radiolabeling reactions with various radioisotopes came to be an important research topic for nuclear molecular imaging, diagnosis, and therapy [42].

^{18}F labeled peptides can be prepared through two synthetic pathways as shown in Fig. 14.4. Firstly, the direct ^{18}F labeling procedure can be applied to produce ^{18}F labeled peptides by radiofluorination with no-carrier-added (n.c.a.) ^{18}F. This direct labeling reaction requires the harsh strong basic reaction conditions in organic solvent, which is known to be not suitable for maintain the integrity of these peptides molecules. As a result, the direct radiofluorination reaction was hardly used to incorporate ^{18}F atom into the peptides, as the bioactive peptides are sure to be denatured or decomposed under this direct ^{18}F labeling scheme (Fig. 14.4a) [43, 44]. Secondly, the other ^{18}F labeling of peptides is an indirect labeling method with stable ^{18}F labeled building blocks (referred to prosthetic groups or synthons) by a conjugation reaction. This indirect synthetic pathway is generally suitable to synthesize ^{18}F-labeled peptides via the conjugation reactions between the peptides and

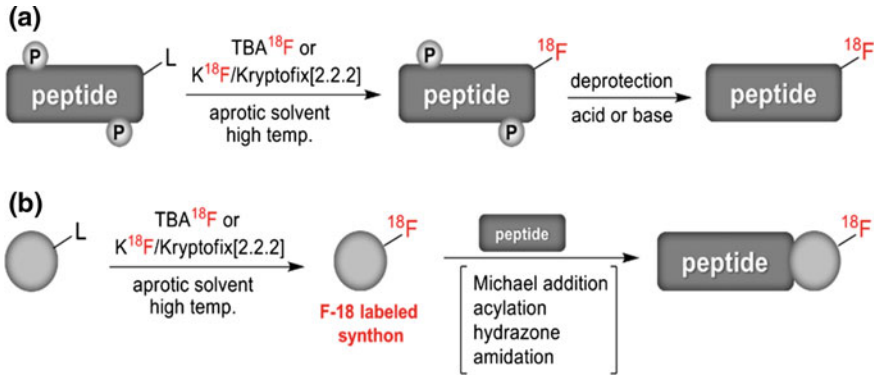

Fig. 14.4 Synthetic pathway for the synthesis of ^{18}F labeled peptides. **a** Direct ^{18}F labeling procedure by the radiofluorination with n.c.a. fluoride-18. **b** Indirect ^{18}F labeling procedure by a conjugation reaction using ^{18}F labeled synthon

^{18}F labeled prosthetic groups, such as amidation, acylation, alkylation, or hydrazone formation reaction with reactive amino- or thiol groups (Fig. 14.4b) [43, 44].

However, the reactive reagents or organic solvents for the preparation of ^{18}F labeled peptides by these indirect radiolabeling methods still lead to the denaturation of peptides. In addition, the half-life of ^{18}F is occasionally too short to complete these two step indirect radiolabeling processes. To accomplish the labeling or conjugation reaction with the ^{18}F labeled building blocks within a reasonable reaction time and with sufficient RCY, excess amounts of peptide precursor should have been used. As a result, trace quantities of ^{18}F labeled peptides should be separated and isolated from the remaining excess precursors, other non-labeled molecules and chemical reagents after the reaction. High molar specific activity is needed to ascertain imaging quality of PET and reduce the toxicity or side-effects of the collateral administration of unlabeled peptides (Fig. 14.5a). High performance liquid chromatography (HPLC) is the most popular purification process and is well-known to take long time [27, 28]. This time-consuming purification process using HPLC as well as the complicated synthetic procedures cannot frequently provide the satisfactory product to be used for imaging in preparation of the peptide radiotracer with reasonable RCY or/and molar activity [43–45].

14.5 Chemically Orthogonal Scavenger-Assisted ^{18}F Labelling Method

It is well-known that bioorthogonal click conjugation reactions such as the SPAAC reaction and the inverse electron-demand Diels-Alder cycloaddition reactions can work well in aqueous media without chemical reagents or any catalysts [11, 24]. This fact can allow the ^{18}F labeled peptides or other bioactive molecules to be

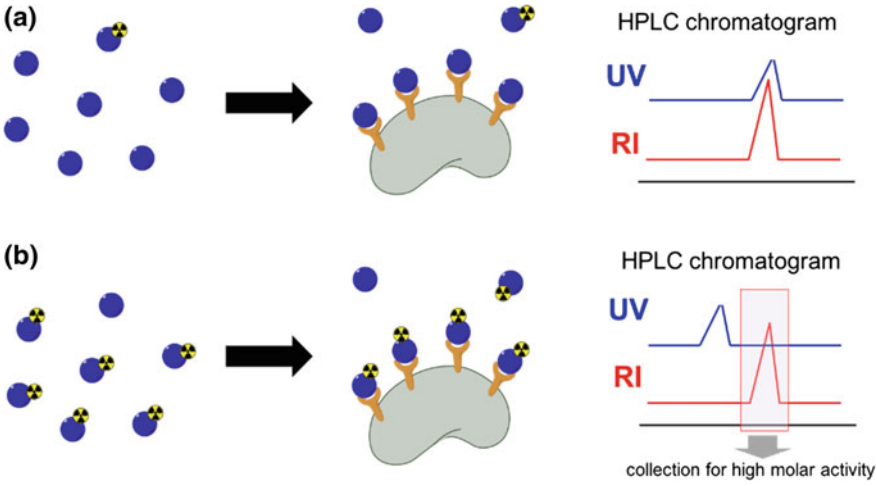

Fig. 14.5 Concept of the molar activity of radiolabeled molecular imaging probes for targeting. **a** Low molar activity of radiotracer: bad image quality with high toxicity. **b** High molar activity of radiotracer: good image quality with low toxicity

obtained by these bioorthogonal click conjugation reactions under physiologically friendly reaction conditions (such as room temperature, pH 7 and water solvent) to avoid the decomposition and denaturation of these bio-molecules. In addition, the ^{18}F labeling procedure became simple for easy handling. In 2011, Weissleder et al. firstly reported a chemically orthogonal scavenger-assisted high-performance ^{18}F labeling protocol using the inverse electron-demand Diels-Alder cycloaddition reactions to synthesize ^{18}F labeled PARP1 inhibitor derivative (Fig. 14.6a) [46]. Using this protocol, ^{18}F labeled PARP1 inhibitor was prepared from the tetrazine-tethered precursor in 59.6% RCY within 3 min, by the bioorthogonal tetrazine-*trans*-cyclooctene ligation reaction with an ^{18}F labeled TCO synthon. Moreover, the excess non-labeled tetrazine-tethered precursor could be removed using a magnetic TCO scavenger resin and they could increase its molar activity. In 2012, it was reported that the bioorthogonal SPAAC based ^{18}F peptide labeling reaction could provide various ^{18}F labeled peptide tracers under physiologically-friendly reaction conditions without HPLC purification process. According to this protocol, the SPAAC reaction of ADIBO-tethered cRGD peptide was reacted with the ^{18}F labeled azide synthon in aqueous media and reaction completed within 15 min to afford an ^{18}F-labeled cRGD peptide quantitatively [47]. Based on chemically orthogonal scavenger-assisted purification-concept above mentioned, a subsequent treatment of the mixture solution with the polystyrene-supported azide scavenger resin for 20 min could allow remove the excess cold ADIBO–cRGD precursors (Fig. 14.6b). Just after filtration and washing using a PBS, the ^{18}F labeled cRGD peptide could be synthesized in a 92% RCY within the total reaction time of 35 min. What is more appealing was that this RGD radiotracer was obtained

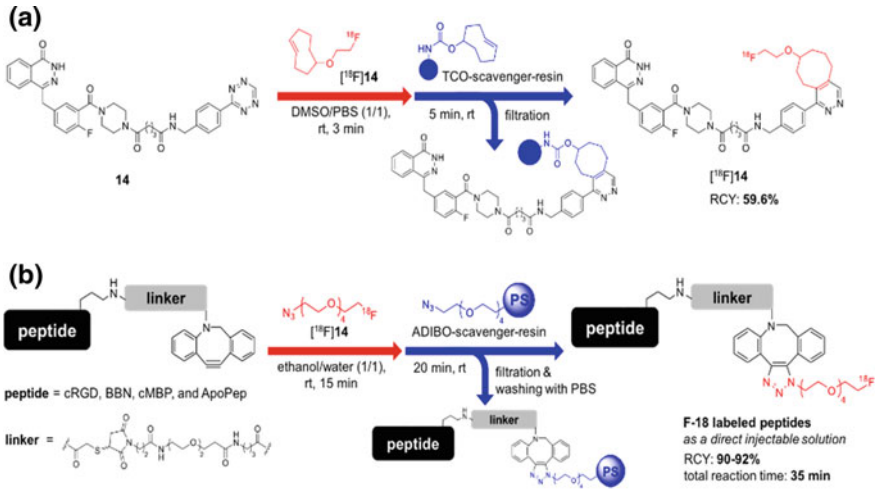

Fig. 14.6 Overview of chemically orthogonal scavenger-assisted ^{18}F labelling protocol platform. **a** The bioorthogonal tetrazine-TCO ligation based ^{18}F labeling and purification procedure for synthesis of ^{18}F labeled PARP1 inhibitor derivative. **b** The SPAAC based synthesis of various ^{18}F labeled peptides with ^{18}F labeled azide synthons and subsequent chemo-orthogonal purification using a polystyrene-supported azide resin. Adapted from [46] with permission

as a directly injectable solution for PET molecular imaging study without HPLC purification and formulation process. More recently, Kim et al. reported that ^{18}F labeled di-cRGD peptide, which was prepared by this ^{18}F peptide labeling protocol, could visualize successfully the tumor in vivo on PET imaging study [48].

In particular, the high throughput synthesis of various ^{18}F labeled peptide tracers can be achieved efficiently by this ^{18}F labeling protocol platform [47]. With only once production of ^{18}F labeled azide synthon, three different peptides such as bombesin (BBN), c-Met binding peptide (cMBP), and apoptosis targeting peptide (ApoPep) could be radiolabeled simultaneously from the corresponding ADIBO-substituted peptide precursors via this SPAAC based ligation reaction and subsequent scavenger-assisted separation process in 90–92% of RCYs (molar activities of 55–45 GBq/μmol).

14.6 Bioorthogonal Chemistry for ^{18}F Peptide Labeling

The catalyst-free bioorthogonal chemistry have been applied to peptide labeling with ^{18}F as a chemo-orthogonal conjugation of bioactive peptides with the ^{18}F-labeled building blocks. For this purpose, two synthetic approaches were generally used such as the SPAAC and the tetrazine-TCO Diels-Alder cycloaddition. The ^{18}F peptide labeling based on SPAAC approach focused on the

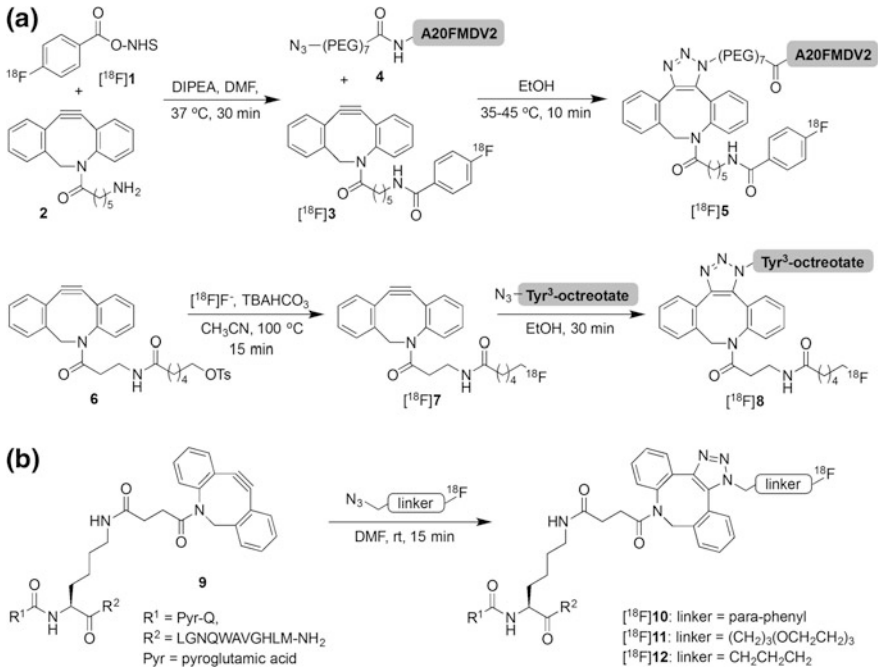

Fig. 14.7 ^{18}F peptides labeling. **a** SPAAC reaction of ^{18}F labeled ADIBO-synthon with azide-tagged peptides. **b** SPAAC reaction of ^{18}F-labeled azides building blocks with the ADIBO-functionalized peptide

development of the conjugation between ^{18}F labeled cyclooctyne building blocks and azido-tagged peptides [9]. For example, ^{18}F labeled A20FMDV2, which is an integrin $\alpha_v\beta_6$-specific peptide, was prepared using this SPAAC based protocol by Sutcliffe et al. [49]. In this report, the acylation of an ADIBO derivative **2** using *N*-succinimidyl-4-[^{18}F]fluorobenzoate proceeded at 37 °C for 30 min in DMF, giving an ^{18}F labeled ADIBO synthon. Then, its SPAAC reaction with azide-tagged A20FMDV2 peptide provided the desired ^{18}F labeled peptide in 11.9% RCY with a molar activity of 68 ± 25 GBq/μmol after HPLC purification process (Fig. 14.7a, up) [50]. An ^{18}F labeled ADIBO building block could be obtained directly in 65% RCY using the nucleophilic displacement reaction of the corresponding tosylate **6** with ^{18}F at 100 °C for 15 min in CH_3CN [49]. Then, ^{18}F labeled Tyr^3-octreotate peptide was produced in 95% RCY by treatment of the corresponding azide-substituted precursor for 30 min in ethanol (Fig. 14.7a, down) [51].

The other SPAAC based labeling approach was reported by Campbell-Verduyn et al. In this approach, an ^{18}F labeled azides were used as radiolabeled building blocks for the SPAAC conjugation reaction with ADIBO functionalized peptide. An ADIBO substituted lys[3] -bombesin peptide was synthesized for this purpose. Then, the ^{18}F labeled bombesin peptide derivatives were produced in 19–37% RCY by the SPAAC conjugation of a bombesin peptide with three different ^{18}F-labeled

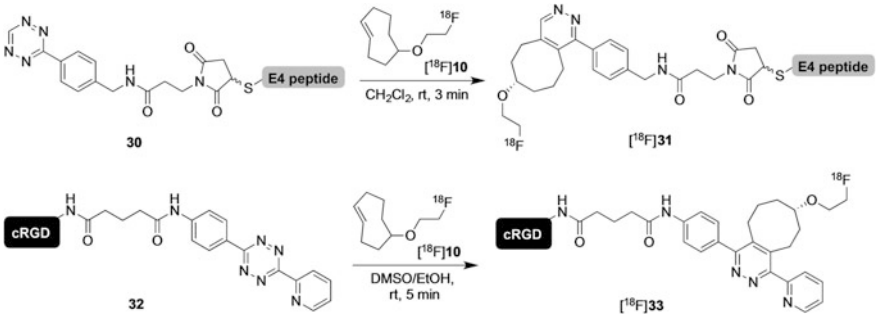

Fig. 14.8 Bioorthogonal tetrazine-TCO Diels-Alder cycloaddition for ^{18}F labeling of peptides

azide synthons at ambient temperature within 15 min (Fig. 14.7b). In the aspect of RCY and reaction step, this protocol employing using the ^{18}F labeled azide synthons is generally known to be more efficient than those using ^{18}F labeled cyclooctyne synthons for the ^{18}F labeling [52].

The bioorthogonal tetrazine-TCO Diels-Alder cycloaddition is also extremely efficient conjugation reaction for ^{18}F labeling of peptides. It have been reported that ^{18}F labeled cRGD [^{18}F]**x** and exendin-4-affine peptide radiotracer could be prepared in excellent RCYs by this cycloaddition reaction of a ^{18}F labeled TCO synthon with the corresponding tetrazine-substituted peptides as shown in Fig. 14.8 [53, 54].

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