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¹⁸F-Labeling of Sensitive Biomolecules for Positron Emission Tomography

Hema S. Krishnan^{1,⊥}, Longle Ma^{1,⊥}, Neil Vasdev^{1,*}, and Steven H. Liang^{1,*}

¹Nuclear Medicine and Molecular Imaging, Massachusetts General Hospital & Department of Radiology, Harvard Medical School, Boston, MA, 02114, USA

Abstract

Positron emission tomography (PET) imaging study of fluorine-18 labeled biomolecules is an emerging and rapidly growing area for preclinical and clinical research. The present review focuses on recent advances in radiochemical methods for incorporating fluorine-18 into biomolecules via ‘direct’ or ‘indirect’ bioconjugation. Recently developed prosthetic groups and pre-targeting strategies, as well as representative examples in ¹⁸F-labeling of biomolecules in PET imaging research studies are highlighted.

Keywords

positron emission tomography; fluorine 18; biomolecules; bioconjugation; click chemistry

1. Introduction

Positron emission tomography (PET) is a sensitive imaging modality which enables the visualization and quantification of small molecules and biomolecules in a living subject or animal. PET imaging provides valuable *in vivo* functional information via targeted radiolabeled molecules with high sensitivity complementary to other imaging modalities where anatomic information is the primary focus.^[1] Biomolecules such as nucleotides, peptides, aptamers and antibodies can display excellent binding specificities to targets related to normal and diseased states in neurology, oncology and cardiology.^[2] The spatial and temporal information of biomolecules^[3] *in vivo* are important for research areas such as clinical diagnostic studies,^[4] monitoring biochemical processes,^[5] and evaluation of the pharmacological properties of pharmaceutical candidates in support of drug discovery programs.^[6] Fluorine-18 (¹⁸F, 97% β^+ , $t_{1/2}$ = 109.7 min) is the most commonly used radionuclide for PET due to its ideal chemical, physical and nuclear properties. Longstanding efforts in the development of fluorine-18 incorporation into biomolecules provide strong impetus to translate these ¹⁸F-labeled biomolecules to PET imaging studies in peripheral organs^[7] or in the central nervous system in cases where the blood brain barrier is compromised.^[8] Based on significant advances in the labeling methodologies for syntheses of ¹⁸F-labeled biomolecules, in this article we aim to highlight the recent

* liang.steven@mgh.harvard.edu; vasdev.neil@mgh.harvard.edu; Fax: +1 617-726-6165; Tel: +1 617-726-6107.

⊥ These authors contribute equally to this work.

breakthroughs and provide readers with a general overview of ^{18}F -labeling strategies for biologics, to complement the established literature in this field.^[9] Herein we consider fluorine-18 incorporation into biomolecules as broadly falling into two categories: 1) direct methods where fluorine-18 is incorporated directly to biomolecules of interest through $\text{C-}^{18}\text{F}$, $\text{B-}^{18}\text{F}$ and $\text{Si-}^{18}\text{F}$ bond formation or via chelation with $\text{Al-}^{18}\text{F}$ complexes; and 2) indirect methods where the synthesis of prosthetic groups labeled with fluorine-18 is carried out before conjugation with biomolecules.

2. Direct Methods for ^{18}F -labeling of Biomolecules

Direct methods for ^{18}F -labeling feature direct and one-step incorporation of fluorine-18 into complex biomolecules in the ultimate or penultimate step of the radiosynthesis. The relatively short half-life of fluorine-18 imposes limitations on the time-frame to achieve labeling reactions as well as the methods that can be employed; therefore, it's highly desirable to minimize synthetic steps following the initial incorporation of fluorine-18 and time for the purification procedures and formulation of the final ^{18}F -labeled biomolecules. The direct methods for fluorine-18 incorporation of biomolecules have advantages in this sense and have found usages in a wide range of applications.

2.1 ^{18}F -Radiolabeling Through $\text{C-}^{18}\text{F}$ Bond Formation

One commonly used method for introducing the fluorine-18 radionuclide into complex biomolecules is through nucleophilic substitution reactions between ^{18}F fluoride and biomolecule precursors bearing suitable pre-installed leaving groups. Nucleophilic aromatic ^{18}F fluorination reactions have been extensively studied and aromatic compound bearing a trimethylammonium (TMA) leaving group was first evaluated as a labeling precursor for the synthesis of small molecule radiotracers, including 1-[2-[(4- ^{18}F fluorophenyl)(phenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazine in 1989 by Haka *et al.*^[10] Several electron-withdrawing groups such as trifluoromethyl, cyano, and fluorine have been introduced onto aromatic rings to facilitate nucleophilic aromatic substitution. Subsequently Becaud *et al.* reported a procedure where biomolecule bearing a trimethylammonium (TMA) leaving group is directly replaced by ^{18}F fluoride (Figure 1A).^[11] These reactions were carried out under mild conditions, for example, 50 °C in DMSO, wherein azeotropically dried ^{18}F fluoride in combination with Kryptofix₂₂₂ and K_2CO_3 were reacted with biomolecules bearing TMA group. By employing this method, a series of radiolabeled peptides such as 3-cyano-4- ^{18}F fluorobenzoyl-Val-Ala-Arg-Gly-NH₂ (2), 3-cyano-4- ^{18}F fluorobenzoyl-Ava-Gln-Trp-Ala-Val-Gly-His-FA-Leu-NH₂ (4) were obtained with up to 90% fluorine-18 incorporation from the corresponding TMA precursors **1** and **3**, respectively. In addition, the authors achieved a 24% radiochemical conversion (RCC) for peptides bearing a free amino group in lysine which are otherwise difficult to label using conventional labeling conditions. Peptides containing histidine, tryptophan, and arginine amino acids were also labeled with fluorine-18 via this direct approach. As a commonly used leaving group for nucleophilic aromatic substitution, the nitro group is also found to be useful for ^{18}F fluoride displacement reactions to label biomolecules. Jacobsen *et al.* radiolabeled monomeric and dimeric cyclic RGD peptides by employing precursors containing 4-nitro-3-trifluoromethyl substituted aromatic rings via this direct approach

(Figure 1B).^[12] The [¹⁸F]fluoride displacement reaction on nitro precursors **5** and **7** was conducted in the presence of K₂CO₃ and Kryptofix₂₂₂ in DMSO. Both monomeric peptide **6** and dimeric peptide **8** were isolated in 9-19% (decay corrected) radiochemical yield (RCY) with high molar activity (79 GBq/μmol; 2.1 Ci/μmol).

In addition to the ¹⁸F-aryl bond formation strategy, methods involving aliphatic C–F bond formation have also found their application in incorporating fluorine-18 into biomolecules. Roehn *et al.* described a one-step labeling of peptides via ring-opening of activated aziridines by [¹⁸F]fluoride (Figure 2).^[13] Effects of different activating groups on the aziridine ring were investigated in this study. 2,4,6-Triisopropylphenylsulfone, as shown in the transformation from **9** to **10**, was identified as the optimal activating group to facilitate regioselective aziridine ring-opening by [¹⁸F]fluoride. A model tetrapeptide **11**, octapeptide **12** and a protected thymidine derivative **13** bearing activated aziridine moiety were demonstrated in their work. Tetrapeptide **11** and octapeptide **12** were labeled in 16% and 7% RCCs, respectively, whereas under similar conditions thymidine derivative **13** was prepared in 87% RCC. In general, these approaches for ¹⁸F-labeling require the use of polar organic solvents. Thus it is crucial that the biomolecules are stable to survive polar medium under moderate to high temperature (50–135 °C).

Furthermore, biocatalytic transformation which takes advantage of the naturally occurring fluorinase enzyme in an S_N2 type transhalogenation reaction has also been used for labeling peptides at ambient conditions.^[14] A direct evolution of fluorinase for improved fluorination efficiency was also recently reported.^[15] For instance, arginylglycylaspartic acid (RDG) peptide **14** bound to 5'-chloro-5'-deoxy-2-ethynyladenosine (CIDEA) through a tetraethylene glycol (TEG) linker could be labeled with fluorine-18 for late-stage modification (Figure 3). Specifically, an aqueous solution of [¹⁸F]fluoride, directly from the cyclotron target, was reacted with RDG-TEG-CIDEA in the presence of L-SeMet at room temperature for 30 min to afford the radiolabeled product **15** in 12% RCY (non-decay corrected).

2.2 ¹⁸F-labeling Through B–¹⁸F Bond Formation

Due to the strong bond dissociation energy of B–F bond, boron based fluorination has been used to enable fluorine-18 incorporation into small molecules since the early 1960s.^[16] However, it was not until 2005 that B-¹⁸F chemistry of biomolecules started to gain increased attention.^[17] In 2005, Ting and Perrin *et al.* reported the first example of ¹⁸F-labeling of biomolecules in aqueous conditions based on boron-fluorine chemistry.^[18] In this method, pinacol phenylboronate diester was conjugated to biotin via an amide bond and the biotinylated arylboronic ester **16** was reacted with aqueous [¹⁸F]KHF₂ (Figure 4A) and the resulting ¹⁸F-labeled aryltrifluoroborate **17** precipitated from the reaction medium, thereby greatly facilitating product isolation. The authors also investigated the hydrolytic stability of aryltrifluoroborates at pH=7.5 in aqueous conditions mimicking physiological conditions. It was found that there was no significant release of fluorine-18 from the labeled compounds and they showed considerable stability over 60 min. This pioneering work was further extended by the same group whereby the authors demonstrated that biotinylated

arylboronic ester **18** can be labeled with moderately high molar activity despite adding ^{19}F - KHF_2 as a carrier to facilitate the formation of trifluoroborate **19** (Figure 4B).^[19]

Keller *et al.* reported the ^{18}F -labeling of Marimastat (Figure 4C), a noncovalent MMP inhibitor via the boronic ester approach.^[20] They exploited an arylboronic ester conjugate **20** as a captor for aqueous ^{18}F fluoride for the formation of tracer **21** in a rapid one-step reaction at room temperature. Li and Perrin *et al.* reported the usage of arylborimidine **22** as the precursor for ^{18}F -labeling of dimeric-cylcoRGD (**23**; Figure 4D).^[21] From a distinct mechanistic point of view, ^{18}F trifluoroborate based labeling reagents could also be obtained through isotopic exchange with ^{19}F -trifluoroborates. These authors also conducted a kit-like ^{18}F -labeling strategy for RGD peptide where RGD- ArBF_3 (**24**) was reacted with fluorine-18 in acidic conditions (Figure 5A).^[22] After HPLC purification, the RGD- ^{18}F ArBF₃ (**25**) was isolated in 65% RCY (non-decay corrected) with high molar activity of 518 GBq/ μmol (14 Ci/ μmol).

In line with the isotope exchange strategy, the Perrin laboratory further reported an advanced method by developing a novel zwitterionic organotrifluoroborate **26** which could be readily incorporated into clinically relevant peptides through bioconjugation.^[23] In this method, ^{18}F fluoride drying is not required before reacting with the alkylammoniomethyltrifluoroborate (AMBF₃) precursor, and an aqueous solution of ^{18}F fluoride can be used directly (Figure 5B). The radiolabeling of AMBF₃-rhodamine afforded a dual-mode fluorescent and PET tracer **27** in 25 min with approximately 150 GBq/ μmol (4 Ci/ μmol) molar activity. Recently, Liu, Chen and co-workers also utilized the isotope exchange strategy for the synthesis of ^{18}F -labeled boramino acids to serve as surrogates for amino acids to target amino acid transporters.^[24] The synthesis involves a simple one-step procedure that does not require azeotropic drying of the ^{18}F fluoride or HPLC purification. The representative boramino acids were isolated in good radiochemical yields (>60% RCY, non-decay corrected) with high molar activities greater than 37 GBq/ μmol (1 Ci/ μmol). PET imaging studies with these radiotracers showed high amino acid transporters-mediated tumor uptake and rapid clearance from normal organs and tissues. The B- ^{18}F method for labeling biomolecules necessitates relatively acidic conditions (pH 2-4.5); therefore, stability of the biomolecules must be assessed.

2.3 ^{18}F -labeling Through Si- ^{18}F Bond Formation

^{18}F Fluorosilanes were first reported by Rosenthal *et al.* in 1985 where chlorotrimethylsilane was reacted with ^{18}F fluoride to generate corresponding ^{18}F fluorosilane.^[25] However, subsequent *in vivo* evaluation revealed that ^{18}F fluorotrimethylsilane was prone to undergo fast hydrolysis accompanied by high bond uptake, making it unsuitable as a labeling synthon. There is an unmet need for the development of a ^{18}F -labeled silicon-based synthon with considerable *in vivo* stability. Similar to radiolabeling strategies involving boron-fluorine bond formation, the application of silicon-fluorine bond formation in biomolecule radiolabeling was initially reported by Ting *et al.* where they used an alkyltriethoxysilane conjugated biotin **28** as the precursor for ^{18}F -labeling of **29** (Figure 6A).^[18] Fluorination efficiency was reported to approach 100% but the ^{18}F -labeled tetrafluorosilicate was only moderately stable and slowly

decomposed in aqueous solution. Concurrently, Schirmacher *et al.* reported the syntheses of substituted [^{18}F]fluorosilanes using organochlorosilanes as labeling precursors and an alternative labeling approach employing di-*tert*-butylphenyl fluorosilane, an efficient silicon-based fluoride acceptor (SiFA compound) via ^{18}F - ^{19}F isotope exchange (Figure 6B).^[26] The di-*tert*-butylphenyl based ^{18}F -compounds showed excellent *in vitro* stability in human serum over 60 min. An application of this method is the ^{18}F -labeling of an aminooxy-derivatized Tyr^[3]-octreotate **31** bearing triorganofluorosilane functionality. In this reaction, the authors treated SiFA based precursor **30** with [^{18}F]fluoride in CH_3CN and the fluorine-18 incorporation was determined to be 95%. It is interesting to note that this method is also compatible with aqueous conditions, and high ^{18}F -incorporation (70–90%) could be achieved within 30 min at 95°C (Figure 6B). Mu *et al.* developed a new type of silicon-based precursors for direct ^{18}F -labeling of biomolecules by nucleophilic displacement of an alkoxy, hydroxy, or hydride leaving group (Figure 6C).^[27] Tetrapeptides **32** with pendant dialkylsilanol or dialkylsilane building blocks were treated with [^{18}F]fluoride under acidic conditions to afford the corresponding tracer **33** in *ca.* 50% RCCs from either silanol or silane precursors. Radiolabeling of bombesin derivatives could be achieved using di-*tert*-butyl silyl group as the labeling precursor producing hydrolytically stable ^{18}F -labeled radioligands with 13% RCY (decay corrected) and 62 GBq/ μmol (1.6 Ci/ μmol) molar activity.^[28] Yasui *et al.* reported a resin-supported di-*tert*-butylphenylsilyl chloride system which could enable ^{18}F -labeling of unprotected peptides.^[29] This method allows for efficient release of the radiolabeled product with high molar activity 67–92 GBq/ μmol (1.8–2.5 Ci/ μmol). Mishra *et al.* reported the application of silicon based ^{18}F -labeling of dimeric radioligands by utilizing an isotope exchange strategy. For example, [^{18}F]BMPPSiF (**35**), which is used to image serotonin receptors, was prepared in 52% RCY (decay corrected) with 480 GBq/ μmol (12.9 Ci/ μmol) molar activity from **34** (Figure 6D).^[30] The lipophilic feature of silicon functionalities may increase non-specific binding; therefore, structural modifications including PEGylation and/or zwitterion formation have been developed to reduce the lipophilicity of ^{18}F -Si labeled compounds.^[31] Recently, Niedermoser, Schirmacher and Wangler *et al.* have developed a new precursor for isotope exchange to achieve ^{18}F -labeling of somatostatin receptor analogs (Figure 6E).^[32] In this work, the newly developed SiFAlin-derivatized somatostatin analog **37** was synthesized and radiolabeled with 53% RCY (non-decay corrected) and 44–63 GBq/ μmol (1.3–1.7 Ci/ μmol) molar activity from SiFAlin **36**.

2.4 ^{18}F -Radiolabeling Through Al- ^{18}F Complex Chelation

The recent development of ^{18}F -labeling through chelation has greatly enriched the landscape of biomolecule modification for PET imaging. This strategy takes advantage of the fast kinetics of chelation chemistry and can potentially eliminate the need for HPLC purification. McBride *et al.* reported a method where fluorine-18 forms an aluminum- ^{18}F (Al- ^{18}F) complex, which was then bound to a chelating group attached to a peptide, forming a stable Al- ^{18}F -chelate-peptide complex in a one-pot process (Figure 7).^[33] In their initial report, the authors found that an Al- ^{18}F complex could bind tightly to a 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) chelating ligand. The NOTA ligand contains three nitrogen atoms and three carboxylic acid functional groups which serve as a hexadentate ligand for the Al- ^{18}F complex. As a closely relevant labeling strategy, DOTA (1,4,7,10-tetraazacyclododecane-

N,N',N'',N'''-tetraacetic acid) has also been widely used for gallium-68 labeling of biomolecules through similar chelation chemistry. However, there are several considerations when choosing Al-¹⁸F]F or gallium-68 based peptide radiolabeling: 1) Fluorine-18 ($t_{1/2}$ = 109.7 min) has a longer half-life than gallium-68 ($t_{1/2}$ = 67.7 min) and can enable longer imaging times and shipment of the tracers to multiple sites; 2) Fluorine-18 has higher positron yield than gallium-68 (96.86% vs. 88.88%), which generally results in better imaging resolution;^[34] 3) Gallium-68 is generator-produced while fluorine-18 is generated in a cyclotron, thereby facilitating widespread use of ⁶⁸Ga. The peptide IMP449 **38** bearing the NOTA ligand was treated with a pre-formed Al-¹⁸F aqueous solution and approximately 60% of Al-¹⁸F was incorporated into the peptide **39** and the molar activity was determined to be 48 GBq/ μ mol (1.3 Ci/ μ mol). Following this work, the same group continued to develop several structural variations **40–42** of the NOTA ligand.^[35] Peptides were labeled using this method, for example, IMP467 was prepared in 87% RCY (non-decay corrected) with high molar activity up to 115 GBq/ μ mol (3.1 Ci/ μ mol) within 30 min without chromatographic purification.

The Al-¹⁸F chelation approach has enriched the landscape for biomolecule radiolabeling and there are numerous applications in literature utilizing this strategy. For example, Lang *et. al.* reported the ¹⁸F-labeling of dimeric cyclic RGDyK peptide in 5–25% RCY (non-decay corrected), which was used for targeting the $\alpha\beta_3$ integrin receptor.^[36] Laverman *et. al.* disclosed their findings with the NOTA ligand in the ¹⁸F-labeling of NOTA-octreotide (IMP466; **43**).^[37] These authors investigated the effects of temperature, buffer solutions and solvent on the efficiency of the radiolabeling. The RCYs were *ca.* 45% (non-decay corrected) when aqueous buffers or CH₃CN/buffer was used as the solvent. In sodium acetate buffer, the molar activity was in the range of 23–32 GBq/ μ mol (0.6–0.7 Ci/ μ mol). The use of chelation chemistry allows the radiotracer to be prepared without the need of HPLC purification, while maintaining reasonable molar activities. More recently, Chen and co-workers have used the NOTA chelation method to prepare a folate-NOTA-Al¹⁸F radiotracer for the diagnosis of folate-receptor (FR) expressing cancers.^[38] In this work, the desired ¹⁸F-labeled tracer was prepared with a decay corrected RCY of 19% and 69 GBq/ μ mol (1.9 Ci/ μ mol) molar activity. Since most Al-¹⁸F methods which use the NOTA or NODA chelators require elevated reaction temperatures (100–120 °C), it imposes limitations for ¹⁸F-labeling of heat-sensitive biomolecules. In 2016, Cleeren *et. al.* developed a new polydentate ligand **44** for the complexation of Al-¹⁸F at 40 °C.^[39] This new Al-¹⁸F ligand was used for radiolabeling of peptide Glu-NH-CO-NH-Lys(Ahx)L3 in 25% RCY (non-decay corrected) and 27 GBq/ μ mol (729 mCi/ μ mol) molar activity. Similar to the B-¹⁸F method, ¹⁸F-labeling of biomolecules via Al-¹⁸F complex takes place in relatively acidic medium and special care is necessary for acid sensitive molecules.

3. Indirect Methods for ¹⁸F-Radiolabeling of Biomolecules

As discussed in the previous section, direct nucleophilic fluorination is usually carried out under relatively harsh conditions for biomolecules, such as low pH conditions (pH 4–5) and high temperatures (100°C). These non-physiological conditions are not well tolerated by many biomolecules and are subject to increased likelihood of hydrolysis and/or degradation during radiolabeling. One attempt to circumvent this problem is to utilize

indirect methods, which typically involve the utilization of ^{18}F -labeled prosthetic groups that contain orthogonally reactive functionalities to conjugate with biomolecules. Thus, such radiolabeling takes place over several steps whereby the prosthetic group is attached to a biomolecule via conjugation reactions, including alkylation, amidation, acylation, imidation, oxime or hydrazone bond formation and glycosylation.^[40] In addition to traditional prosthetic groups, several prosthetic groups have been developed that allow the employment of 'click chemistry' strategy. In the following sections, we aim to highlight recent examples for the ^{18}F -labeling of sensitive biomolecules utilizing traditional or 'click chemistry' prosthetic groups. It is worth mentioning that the choice and site of introduction of prosthetic groups are critical factors as these variables can alter the pharmacological and physiological properties of the biomolecule. Therefore, pharmacological testing of non-radioactive analogs must be carried out to ensure that no significant changes to target specificity and selectivity are observed.

3.1 ^{18}F -Radiolabeling via Amino-selective Prosthetic Groups

Prosthetic groups are usually bifunctional small molecules that can be modified at one position with fluorine-18 and the second functional group allows for conjugation with the active site in an unlabeled biomolecule, such as an amino or a thiol group, under suitably mild conditions. The most common amino-selective fluorine-18 prosthetic group to date for such bioconjugation is *N*-succinimidyl-4- ^{18}F fluorobenzoate (^{18}F SFB, **45**; Figure 8).^[41] ^{18}F SFB is an acylating agent that conjugates to biomolecules via a reactive amine group, such as the primary amino group in lysine. Its synthesis was first reported by Zalutsky and Vaidyanathan in 1992 via a multi-step process that resulted in 25% decay corrected RCY (synthesis time 100 min).^{41a} Following this synthesis, these authors applied ^{18}F SFB to the radiolabeling of a monoclonal antibody F(ab')₂ fragment and obtained the radiotracer in 40-60% RCY (decay corrected) in 20 min. Other syntheses of ^{18}F SFB have since been reported that have improved the RCY up to 55% (decay corrected).^{41a, 42} Carboxylic acids and related derivatives including 4- ^{18}F fluorobenzoic acid (**46**),^[43] *N*-succinimidyl *o*-(di-*tert*-butyl ^{18}F fluorosilyl)-benzoate (^{18}F SiFB, **47**)^[44] and 6- ^{18}F fluoronicotinic acid tetrafluorophenyl ester (^{18}F F-Py-TFP, **48**)^[45] are also used as amine-reactive prosthetic groups. It is noteworthy that the ^{18}F F-Py-TFP prosthetic group can be prepared in one step from the trimethylammonium precursor^[46] and it has been utilized in the fluorine-18 labeling of albumin and c(RGDfK).^[47]

3- ^{18}F Fluoropropanesulfonyl chloride **49** was reported by Li *et. al.* for conjugation with amino groups in biomolecules via a sulfonamide bond.^[48] An improved synthesis of this prosthetic group was reported and conjugation with model peptides showed that high RCYs could be achieved with this group under neutral conditions.^[49] In addition, 4-nitrophenyl-2- ^{18}F fluoropropionate (^{18}F NFP, **50**) has also been employed as an efficient agent to acylate amines. This approach has been utilized to label an RGD (Arg-Gly-Asp) sequence to target $\alpha_v\beta_3$ integrins in cancer patients.^[50] Other examples involving photoconjugation of 3-azido-5-nitrobenzyl- ^{18}F fluoride^[51] and 4-(^{18}F fluoromethyl)phenyl isothiocyanate are also reported for the fluorine-18 labeling of oligonucleotides.^[52]

Bioconjugation reactions featuring oximes formation have also drawn extensive attention in the field and are widely used for labeling aminoxy-functionalized peptides with fluorine-18. A variety of ^{18}F -containing aldehydes/hemi-acetal prosthetic groups such as 6-(2- ^{18}F fluorohexyloxy)nicotinaldehyde (**51**),^[53] 4-formylbenzene-1-sulfonyl ^{18}F fluoride (**52**),^[54] 3-formyl-2,4,6-trimethylbenzene-1-sulfonyl ^{18}F fluoride (**53**),^[54] 2-(2-(2- ^{18}F fluoroethoxy)ethoxy)acetaldehyde (**54**),^[55] 4- ^{18}F fluorobenzaldehyde (^{18}F FBA, **55**),^{55b} *p*-(di-*tert*-butyl ^{18}F fluorosilyl)benzaldehyde (^{18}F SiFA-A, **56**),^[56] 2-deoxy-2- ^{18}F fluoroglucose (^{18}F FDG, **57**)^[57] and 5-deoxy-5- ^{18}F fluororibose (^{18}F FDR, **58**)^[58] have been developed for ^{18}F -labeling of biomolecules (Figure 9).

Glaser *et al.* reported the radiosynthesis of ^{18}F fluciclatide by reacting ^{18}F FBA (**55**) with hydroxyl amine derived-RGD peptide in ammonium acetate buffer at 70°C. The desired ^{18}F fluciclatide (**60**) was obtained in 23% RCY (decay corrected, synthesis time 3h) with 76–170 GBq/ μmol (2.0–4.6 Ci/ μmol) molar activity from precursor **59** (Figure 10).^{55b} Alternatively, Schirmacher and co-workers disclosed a SiFA-based aldehyde prosthetic group ^{18}F SiFA-A (**56**) which takes the advantage of $^{19}\text{F}/^{18}\text{F}$ isotope exchange chemistry. In this work, the authors were able to label the *N*-terminal amino-oxy derived peptide Tyr^[3] octreotate **62** at room temperature with 50% RCY (decay corrected, synthesis time 40 min) from hydroxylamino peptide **61**.^[56] Furthermore, Inkster *et al.* developed several sulfonyl based bifunctional fluorine-18 attached prosthetic groups for fluorine-18 labeling of biomolecules.^[54]

These bifunctional prosthetic groups include 4-formyl-, 3-formyl-, 4-maleimido- and 4-oxylalkynyl-arylsulfonyl ^{18}F fluorides and they can be conveniently prepared from the corresponding sulfonyl chlorides with Cs ^{18}F F/Cs₂CO₃. The authors have demonstrated the utility of sulfonyl ^{18}F fluoride **53** in the fluorine-18 labeling of a bombesin analogue **64** via oxime linkage with 35% RCY (decay corrected, synthesis time 105 min) from precursor **63**. Subsequently, Matesic and co-workers investigated the suitability of various aryl sulfonyl ^{18}F fluorides under microfluidic fluorine-18 labeling conditions.^[59] Moreover, Fiel *et al.* reported the use of magnetic droplet microfluidics platform to prepare sulfonyl fluoride prosthetic group for fluorine-18 labeling of biomolecules.^[60]

^{18}F FDG, the most commonly used fluorine-18 radiotracer, could also be employed as a prosthetic group for biomolecule labeling through oxime formation (Figure 11). Hultsch *et al.* reported the fluorine-18 labeling of aminoxyacetyl-conjugated cyclic RGD peptide c(RGDfK)(Aoa-Boc) **65** with ^{18}F FDG in DMSO/water at 120°C for 20 min to achieve the desired ^{18}F FDG-RGD (**66**) in 56-93% RCYs (decay corrected, synthesis time *ca.* 70 min).^[57] As a closely related prosthetic group, ^{18}F FDR was also found to be efficient in bioconjugation reactions with peptides. For example, Li and co-workers reported the labeling of glutathione attached alkoxyamine **68** with ^{18}F FDR in more than 95% RCC from precursor **67**.^[58] In addition, hydrazine derived peptides could be conjugated with ^{18}F FBA to enable fluorine-18 labeling through hydrazone formation. This strategy has been demonstrated in the fluorine-18 labeling of RGD peptides.^[61]

3.2 Fluorine-18 Radiolabeling via Thiol-selective Prosthetic Groups

While [^{18}F]SFB could also be employed in a cysteine (cys) thiol acylation to afford fluorine-18 labeled peptides through conjugation with the 4-fluorobenzoyl moiety,^[62] alternative strategies amino-targeting prosthetic groups. For example, *N*-(4-fluorobenzyl)-2-bromoacetamide ([^{18}F]FBBA, **69**),^[63] and 2-bromo-*N*-[3-(2-[^{18}F]fluoropyridin-3-yloxy)propyl]acetamide ([^{18}F]FPyBrA, **70**)^[64] were used in thiol-alkylation reactions of α -bromo ketone derivatives (Figure 12A). In addition, fluorine-18 labeled maleimide analogues have been developed for reaction with thiols.^[65] Maleimides undergo chemoselective reactions with thiol groups via Michael addition, and are utilized to radiolabel peptides that contain a cysteine or methionine residue. Figure 12B depicts a recent collection of ^{18}F -labeled maleimides from a variety of reliable labeling methodologies that demonstrated the diversity of these thiol-selective prosthetic groups. Maleimide based prosthetic groups such as 4-((4-[^{18}F]fluorobenzylidene) aminooxybutyl) maleimide ([^{18}F]FBAM, **71**),^[66] 1-[3-(2-[^{18}F]fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione ([^{18}F]FPyME, **72**),^[67] *N*-[2-(4-[^{18}F]fluorobenzamido)ethyl]maleimide ([^{18}F]FBEM, **73**),^[68] [^{18}F]FDG-maleimidehexyloxime ([^{18}F]FDG-MHO, **74**),^{40a} *N*-5-[^{18}F]fluoropentylmaleimide ([^{18}F]FPenM, **75**),^[69] *N*-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)-6-[^{18}F]fluoronicotinamide ([^{18}F]FNEM, **76**),^[70] [^{18}F]SiFApMaleimide (**77**),^[71] 4-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)benzenesulfonyl fluoride (**78**)^[54] and [^{18}F]fluorobutyl ethacrynic amide ([^{18}F]FBuEA, **79**)^[72] have been used to couple with biomolecules via thiol-selective Michael additions (Figure 12B).

Other fluorine-18 labeling methods which involve sulfur-containing fluorine-18 prosthetic groups have also been developed for thiolation reactions, including disulfide formation. For example, [^{18}F]fluorothiols **80**,^[73] [^{18}F]SiFA-SH (**81**)^[74] are developed as radiosynthons for peptide labeling. 3,4,5-Tri-*O*-acetyl-2-[^{18}F]fluoro-2-deoxy-*D*-glucopyranosyl 1-phenylthiosulfonate (Ac₃-[^{18}F]FGlc-PTS, **82**)^[75] and thiolated-[^{18}F]FDG **83**^[76] are used for fluorine-18 glycosylation of peptides (Figure 13).

Recently Jacobsen *et al.* disclosed a novel approach to produce dimeric [^{18}F]RGD-tetrafluorobenzene peptide by employing hexafluorobenzene (HFB) as the prosthetic group.^[77] Specifically [^{18}F]HFB (**85**) was synthesized by fluorine exchange of hexafluorobenzene with K[^{18}F]F/K₂₂₂, and the resulting [^{18}F]HFB was engaged in a thiol-substitution reaction in the presence of a thiolated c(RGDfK) peptide **84**, TRIS base and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) at room temperature to produce RGD-[^{18}F]TFB-RGD (**86**) in 40% RCY (non-decay corrected) with 0.5 GBq/ μmol (15 mCi/ μmol) molar activity (Figure 14A). There is also a recent progress to address the issue that the reversible nature of maleimide-thiol linkage could sometimes induce the loss of the radiolabeled prosthetic group *in vivo*. Chiotellis *et al.* reported the synthesis of a new prosthetic group based on a fluorine-18 labeled methylsulfone derivative, [^{18}F]FPOS (**87**).^[78] This approach has been exploited to label an affibody, Z_{HER2:2395}-Cys, in PBS buffer at 50°C to produce the fluorine-18 labeled Z_{HER2:2395}-Cys **88** in *ca.* 40% RCC with 17 GBq/ μmol (459 mCi/ μmol) molar activity (Figure 14B). One unique feature of this strategy is that the resulting conjugates are more stable under physiological conditions in comparison to the corresponding analogues obtained from maleimide-based reactions. The

tumor targeting properties of the resulting fluorine-18 labeled radiotracer was studied *in vivo* by PET imaging in CD1 nude mice bearing Her2-positive SCOV3 xenografts. The PET scans showed high accumulation of the radiotracer in the tumor with no [¹⁸F]fluoride leaching in the bones.

Fluorine-18 labeled *N*-terminal cysteine-bearing peptides were synthesized by the coupling of a labeled cyanobenzothiazole **89** with cysteine and cysteine-terminated peptides in good RCY under mild conditions (Figure 15).^[79] The fluorine-18 containing cyanobenzothiazole **89** was synthesized from the corresponding tosylate precursor in 20% RCY (decay corrected). A fluorine-18 labeled dimeric cyclic RGD peptide (**91**, 8% RCY, non-decay corrected) from precursor **90** and fluorine-18 labeled RLuc8 protein (**93**, 12% RCY, decay corrected) from **92** were successfully synthesized via this methodology in a site-specific manner. Inkster *et. al.* recently reported the synthesis of a fluorine-18 labeled pyridyl prosthetic group with a cyanobenzothiazole moiety **94** to selectively label cysteine terminated peptides and proteins.^{79b} This reagent was used to label a cyclic RGD peptide with a terminal cysteine group to obtain the purified radiotracer with a decay corrected RCY of 7% (synthesis time *ca.* 120 min).

3.3 Fluorine-18 Radiolabeling via C_{sp2}-X Bond (X = C or N) Formation with Prosthetic Groups

Gao and co-workers recently reported a new prosthetic group, 4-[¹⁸F]fluorophenylboronic acid (**95**, Figure 16A), which was coupled via the Suzuki-Miyaura coupling reaction with peptides and proteins in the presence of a palladium catalyst.^[80] It is important to note here that the biomolecule had to be modified to contain an aryl iodide at the desired radiolabeling site in order for the reaction to proceed. The decay corrected RCYs obtained by this procedure are moderate (~30%) and the prosthetic group is also synthesized, albeit in low RCY over three steps.

In 2010, Ban *et. al.* reported their seminal work on the click-like reaction for tyrosine bioconjugation under aqueous conditions.^[81] In this study, a reactive electrophile 4-phenyl-3H-1,2,4-triazole-3,5(4H)-dione (PTAD) was employed to participate in an ene-type reaction with the phenol moiety of tyrosine residues. This site-specific bioconjugation method has been applied to a wide range of biomolecules. For example, Hu and co-workers take advantage of the tyrosine-selective transformation to the synthesis of a well-defined conjugate anti-candidiasis vaccine glycosylated at tyrosines.^[82] Ban *et. al.* developed the PEGylated PTAD and used this novel agent for the selective PEGylation of chymotrypsinogen and antibody-drug candidate.^[83] Subsequently, Nilo *et. al.* focused on tyrosine-directed conjugation of Group B Streptococcus capsular polysaccharides to streptococcal pilus protein.^[84] In this context, Jessica *et. al.* and Al-Momani *et. al.* developed 4-(4-[¹⁸F]fluorophenyl)-1,2,4-triazole-3,5-dione ([¹⁸F]PTAD, **96**)^[85] and 4-(*p*-([¹⁸F]fluorosulfonyl)phenyl)-1,2,4-triazoline-3,5-dione ([¹⁸F]FS-PTAD, **97**)^[86] respectively, as prosthetic groups, which could be used for selective conjugation with tyrosine residues **98** to afford tyrosine-selective adduct **99** in biomolecules (Figure 16B).

3.4 Cu-Catalyzed Alkyne-Azide Cycloaddition

First described in 1893,^[87] the Huisgen 1,3-dipolar cycloaddition reaction between an alkyne and an azide received new attention as the copper-catalyzed variant in 2002.^[88] Independently reported by the groups of Sharpless^[89] and Meldal,^[90] the copper catalyzed alkyne-azide cycloaddition (CuAAC) yields the 1,4-regioisomer exclusively and can be performed at ambient temperatures. As a result of these favorable properties, the CuAAC has been extensively used in diverse fields of chemistry, including biochemistry and radiochemistry. The resulting 1,2,3-triazole linker is stable *in vivo* and is considered to be a bioisostere to peptide bonds, which has led to the utilization of the CuAAC reaction in the synthesis of several biomolecules.^[91] In seeking to develop radiolabeled biomolecules for targeted imaging, several reports have been published detailing the design, synthesis and application of radiolabeled prosthetic groups for CuAAC with the radiolabel both in the alkyne moiety and the azide moiety.

The first application of CuAAC in fluorine-18 labeling of biomolecules was reported in 2006 by Marik and Sutcliffe who synthesized three fluorine-18 containing alkynes **100** of varying chain lengths that were coupled with azide-functionalized peptides (Figure 17).^[92] The radiolabeled alkynes were synthesized from the tosylate precursors and purified by co-distillation with CH₃CN. The corresponding radiotracers, including a folic acid derivative, were obtained in 10-20 mins after reaction at room temperature in good RCYs (30-97%, non-decay corrected) using solid phase extraction (SPE).^[93] The volatility of the fluorine-18 labeled aliphatic alkyne moieties led to the discovery of fluorine-18 labeled PEGylated alkynes **101** and **102** (Figure 17).^[94] In contrast with the aliphatic alkynes, these compounds are non-volatile, thermally stable and the corresponding bioconjugates show higher blood clearance. The radiolabeling of various biomolecules, including deoxythymidine (97%, non-decay corrected RCY) and RGD peptide dimer (55%) have been reported utilizing fluorine-18 labeled PEGylated alkynes in two-step, one-pot synthesis.^[95]

[¹⁸F]Fluoroaryl alkynyl precursors have also been used to radiolabel biomolecules. A wide variety of reactions available to radiolabel aryl compounds in combination with improved metabolic stability of C_{sp2}-F bond make these building blocks favorable for radiolabeling of biomolecules. The first [¹⁸F]fluoroaryl alkynyl precursor is a sulfonamide based alkyne 4-[¹⁸F]fluoro-*N*-(prop-2-ynyl)benzamide ([¹⁸F]FSA; **103**) prepared from trimethylammonium precursor **107**.^[96] [¹⁸F]FSA was then applied in the radiolabeling of an azide functionalized neurotensin, human serum albumin (HSA, **108**, Figure 18A),^[97] phosphopeptide and an Loligonucleotide.^[98] First synthesized in 2008, pyridine-based fluorine-18 alkyne precursors **104** have been utilized to successfully radiolabel a DNA analog (25% decay corrected RCY, synthesis time *ca.* 270 min) and peptides (5-20%).^[99] Li *et. al.* reported the synthesis of a novel fluorine-18 labeled aromatic alkyne, 4-[¹⁸F]fluoro-3-nitro-*N*-2-prop-1-ynylbenzamide ([¹⁸F]FNBP, **105**) as a stable prosthetic group for efficient click labeling of peptides.^[100] [¹⁸F]FNBP was synthesized in 58% overall yield and *in vitro* studies showed no radiodefluorination in mouse plasma over 2h, suggesting stability *in vivo* as well. A novel approach was described by using an alkyne functionalized fluorine-18 labeled trifluoroborate building block **106** as the coupling partner to azido containing biomolecules such as bombesin and RGD peptide (Figure 18B).^[101] As stated earlier,

arylboronate **109** can capture [^{18}F]fluoride easily in aqueous media and subsequent copper-mediated coupling with the azido moieties resulted in the synthesis of the radiotracers **110** in 30-40% non-decay corrected RCY with high molar activities up to 444 GBq/ μmol (12 Ci/ μmol).

Although a large array of azides have been utilized in the synthesis of non-radiolabeled biomolecules, only a few of them have been applied for PET imaging studies. Fluorine-18 labeled fluoroethylazide ([^{18}F]FEA, **111**) is the first aliphatic azide synthesized in good yields and in most cases is obtained by co-distillation with CH_3CN (Figure 19A).^[102] [^{18}F]FEA has been used widely in the synthesis of fluorine-18 containing radiotracers such as fluorine-18 deoxyuridine (**112**),¹⁰³ ^{18}F -MitoPhos_01 (**113**),¹⁰⁴ ^{18}F -ICMT-11 (**114**),¹⁰⁴⁻¹⁰⁵ ^{18}F -RGDFK (**115**)^[106 and 18] ^{18}F -RGD-PEG analog (**116**)^[107] (Figure 19B). It is also worth noting that ^{18}F -labeling with [^{18}F]FEA has been shown to result in poor yields in some cases. This may be attributed to the reduction of [^{18}F]FEA to ^{18}F -fluoroethylamine in the presence of copper wire in acidic conditions as reported by Glaser *et. al.*^[108] Despite its high volatility, [^{18}F]FEA is one of the most widely used building blocks for fluorine-18 labeling via CuAAC. Other representative aliphatic azides include [^{18}F]fluorobutylazide (**117**, Figure 19C),^[109] a pyrazine derivative [^{18}F]AFP (**118**),^[110] [^{18}F]fluoro-PEG₃-azide (**119**),^[111] a glucose derivative [^{18}F]FDG- β -Az (**120**)^[112] and an [^{18}F]SiFA derivative (**121**).^[113]

Another fluorine-18 azido building block that has been studied is 4-[^{18}F]fluorobenzylazide (**123**), the first azido aryl- ^{18}F click agent used in PET imaging studies. This agent was initially synthesized via a multi-step process in 75 min with 34% RCY (decay corrected),^[114] or in a single-step reaction from the respective diaryliodonium salt using a microfluidic device over 94-188s in 35-45% RCCs.^[115] 4-[^{18}F]Fluorobenzylazide (**123**) has since been used for the fluorine-18 labeling of peptides and siRNA derivatives.^[114, 116] Our laboratory recently reported the one-step synthesis of 4-[^{18}F]fluorobenzylazide (**123**) by reaction of [^{18}F]fluoride with a novel spirocyclic iodonium(III) ylide precursor **122**. The 4-[^{18}F]fluorobenzyl azide (**123**) was isolated with high molar activities of 129.5-148 GBq/ μmol (3.5-4 Ci/ μmol) in ca. 30% RCY (non-decay corrected, synthesis time 45 min) and applied in the fluorine-18 labeling of single-stranded DNA (ssDNA) aptamers.¹¹⁷ In the same report, a novel ortho-stabilized fluorine-18 azido building block **125** was synthesized from ylide precursor **124** that overcomes the challenges posed by previously used fluorine-18 labeled azido compounds (Figure 20).¹¹⁸ The radiolabeling of ssDNA aptamers **126**, including TsC (49% RCY based on 4-[^{18}F]fluorobenzylazide) and sgc8 (48% RCY) were demonstrated, along with the SPAAC reaction of this prosthetic group with a phenylalanine derivative. A similar reaction with [^{18}F]SFB yielded the labeled ssDNA aptamer TsC in only 1.5% RCY (based on [^{18}F]SFB). PET imaging studies of the fluorine-18 containing sgc8-aptamer tracer was performed in mice bearing HCT116 tumors to map expression of the protein PTK-7. The images showed modest tumor uptake of $0.71 \pm 0.07\%$ ID/g at various time points, high tumor-to-muscle ratio of 3.54 ± 0.7 (1h) and 4.15 ± 0.26 (2h), and exhibited rapid renal clearance with minimal [^{18}F]fluoride leaching.

Fluorine-18 incorporation to silicon under mild conditions has been discussed earlier, and this method was exploited in the synthesis of a silicon- ^{18}F based azide **128** from precursor

127 (Figure 21).^[119] Subsequent fluorine-18 labeling of a model peptide was carried out at ambient temperature and the resulting radiotracer **129** was obtained in 75% RCY (decay corrected, based on the fluorine-18 azide). Unfortunately, the radiotracer was shown to undergo hydrolytic cleavage of the Si-¹⁸F bond ($t_{1/2} < 10$ min) in water (pH=7.5) and acidic buffers (pH <4) due to the lack of stability afforded by the isopropyl groups on the silicon.

3.5 Strain-Promoted Azide Alkyne Click Reaction

First demonstrated by Blomquist and co-workers, the strain-promoted alkyne-azide cycloaddition (SPAAC) reaction is a variant of the traditional Huisgen cycloaddition.^[120] It takes advantage of the ring-strain of a cyclooctyne ring, due to its bent triple bond, which then reacts in the absence of any catalyst with an azide to afford the 1,2,3-triazole. Like the copper-mediated Huisgen cycloaddition reaction, the SPAAC is also bioorthogonal and has been employed extensively in the synthesis of biomolecules.^[121] It also overcomes one of the major drawbacks of the CuAAC coupling reaction, *i.e.* the use of copper, which can complicate separation protocols due to its ability to chelate to biomolecules. Two approaches can be considered for its utilization in the radiolabeling of biomolecules – one wherein the radiolabel is on the cyclooctyne moiety and the other where the radiolabel is on the azide. Bouvet *et al.* synthesized a fluorine-18 labeled azadibenzocyclooctyne (ADIBO) agent **131** from the commonly used prosthetic group [¹⁸F]SFB (85% decay corrected RCY, based on [¹⁸F]SFB) and amine **130**, which upon reaction with the corresponding azide terminated biomolecules yielded the radiotracers **132-134** in 69-98% RCCs (Figure 22).^[122] The *in vivo* stability of the fluorine-18 labeled alkyne was studied in mice and showed that 60% of the tracer was intact after 1 hour of injection. Carpenter *et al.* described the synthesis of another fluorine-18 labeled ADIBO agent with six carbon linker in high efficiency, wherein the two possible regioisomers of the SPAAC were observed.^[123]

Arumugan *et al.* reported the reaction between *N*-terminal-azido-modified peptides with a novel fluorine-18 ADIBO prosthetic group **135** to produce the tracer **136** via SPAAC in 21% overall yield in a fully automated manner.^[122] The reaction was also carried out in ethanol as solvent, thereby allowing for the development of a “kit-like” peptide labeling approach (Figure 23A). Hausner and co-workers reported the synthesis of an fluorine-18 labeled radiotracer that selectively targets the integrin $\alpha_v\beta_6$ -receptor.^[123] An azide-terminated peptide containing a PEG₇ spacer, A20FMDV2 was coupled with an [¹⁸F]fluorobenzyl-ADIBO building block **137** to yield the desired radiotracer **138** which was then subjected to *in vivo* imaging studies in mice (Figure 23B). Even though the synthesized peptides have been shown to possess good binding to the integrin $\alpha_v\beta_6$ receptors, in this case, the radiotracer showed no significant binding to the target. This dramatic change in the pharmacokinetics may be attributed to the increased lipophilicity of the radiotracer due to the presence of the dibenzocyclooctyltriazole moiety.^[124]

Other groups have also reported the radiolabeling of biomolecules using SPAAC wherein the fluorine-18 label is applied to the azide and the biomolecule has been modified to contain an alkyne. Campbell-Verduyn and co-workers reported the labeling of bombesin derivatives **139** containing an ADIBO group with various azide terminated fluorine-18 containing prosthetic groups, such as [¹⁸F]fluorobenzyl azide (**123**) and [¹⁸F]fluorobutylazide (**117**).

The corresponding radiotracers **140** and **141** were obtained in 19-37% RCYs based on the fluorine-18 azide precursors (Figure 24).^[109] ^[18F]Fluoroethylazide and ^[18F]fluoroPEG_x azides have also been utilized as precursors to ADIBO and dibenzocyclooctyne containing biomolecules. Interestingly, Sachin *et. al.* concurrently devised a method to radiolabel and formulate fluorine-18 labeled peptides as directly injectable solutions at the end of synthesis.^[125] This was achieved using a scavenger azide that was attached to a resin that successfully removed all traces of the remaining alkyne containing biomolecule without necessitating any HPLC purification.

3.6 Staudinger Ligation

The Staudinger reaction was first reported in 1919 as the reaction between an azide and a phosphine resulting in an iminophosphorane or aza-ylide that rapidly hydrolyzes to yield the primary amine and the corresponding phosphine oxide.^[126] The reaction between the phosphine and azide takes place rapidly at room temperature with high yields of the product. Only one application of the non-traceless Staudinger ligation for fluorine-18 labeling of biomolecules has been reported to date. ^[18F]FDG derivatives for PET imaging of cancers were synthesized by the reaction between a fluorine-18 labeled phosphane and an azide containing sugar or amino acid.^[127] In contrast, several fluorine-18 labeled biomolecules have been synthesized utilizing the traceless variant of the Staudinger ligation. The first among these was reported by Mamat *et. al.* in 2009.^[128] In this work, fluorine-18 labeled phosphines were synthesized in three steps starting from *para*-substituted benzoyl chloride or benzoic acid, via Pd-catalyzed cross-coupling with diphenylphosphine, followed by reaction with 3-^[18F]fluoroethyl tosylate. The labeled phosphine was treated with several azide terminated carbohydrates to yield the desired radiotracer in *ca.* 20% decay corrected RCY. Following this, Pretze *et. al.*^[129] performed a one-step synthesis of the fluorine-18 labeled phosphine prosthetic group **143** based on aliphatic phosphine derivatives containing a 5-carbon long aliphatic chain terminated with a tosyl group as the precursor (**142**; Figure 25). The ligation between the 6-^[18F]fluorohexanoyl phosphane moiety **143** and azides (**144-146**) provided the products **147-149** with decay corrected RCYs of *ca.* 12-31% at the end of synthesis.

The common prosthetic group, ^[18F]FEA (**112**) was used as the azide component by Carroll *et. al.*^[130] Several amino acid derivatives were functionalized with diarylphosphine based thioesters **150** and the corresponding radiotracers **151** and **152** were obtained in >95% RCC, based on conversion from ^[18F]FEA (Figure 26). While the Staudinger ligation does not require a metal catalyst and resulting radiotracers can be purified with ease, its major limitation is the relatively slow kinetics ($2.0 \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$)^[131] of the reaction, which is often accelerated by high reaction temperature.

3.7 Inverse Electron Demand Diels-Alder Click Reactions

The inverse electron demand Diels-Alder reaction (IEDDA) between 1,2,4,5-tetrazines with strained alkenes, such as *trans*-cyclooctene (TCO), cyclooctyne and norbornene has found several applications as a bioorthogonal reaction.^[132] The extremely rapid reaction kinetics ($k_{\text{app}} = 2000 \text{ M}^{-1} \text{ s}^{-1}$ in 9:1 MeOH/water) for the reaction between tetrazine and TCO has enabled the development of several prosthetic groups with short-lived radioisotopes for

radiolabeling. In addition to its fast rate, the IEDDA proceeds in the absence of a metal catalyst, and shows an increase in reaction rate in the presence of water in the reaction medium. This is particularly attractive in the synthesis of biomolecules. Mechanistically, the reaction proceeds via an IEDDA reaction followed by a retro Diels-Alder reaction to eliminate nitrogen gas as the only by-product. While norbornene has also been used as a dienophile in this reaction, higher reaction rates are achieved when TCO is used, and is likely attributed to the high ring-strain of TCO.^[132] In 2010, Li, Fox and co-workers demonstrated the utility of this coupling reaction to radiolabel biomolecules.^[133] Their initial attempt to radiolabel a tetrazine fragment with fluorine-18 failed due to the instability of the labeled tetrazine under the direct radiofluorination conditions. Thus, a fluorine-18 labeled TCO derivative **154** was synthesized from the nosylate precursor **153** (Figure 27A) and coupled with a sample tetrazine molecule in the initial reaction. The success of the trial reaction led to the radiolabeling of a cyclic RGD peptide **155** with a tetrazine-RGD conjugate and **154** under ambient conditions in less than 5 minutes yielding the radiotracer **156** in >95% RCC (Figure 27B).^[134] In comparison, reactions of the RGD peptide in higher concentration with [¹⁸F]SFB yielded only 30% RCC by HPLC analysis. Small animal PET imaging studies with this radiotracer in a U87MG xenograft model showed a tumor uptake of 4.4% ID/g after 1h. Blocking experiments showed specific binding to integrin $\alpha_v\beta_3$. Using a similar strategy, other RGD peptides and the GLP agonist exendin were radiolabeled and used in subsequent PET imaging studies.

The efficiency of the tetrazine-click reaction was demonstrated by Weissleder and co-workers in the synthesis of fluorine-18 labeled PARP1 inhibitors by both direct fluorination of a tosyl precursor **159** and indirect labeling by tetrazine ligation of **157** with a suitably substituted tetrazine.^[135] By modifying the structure of the PARP1 inhibitor with the addition of a tetrazine moiety in the side chain, they were able to successfully obtain a suitable radiotracer in 60% decay corrected RCY by coupling with the fluorine-18 labeled cyclooctene [¹⁸F]**154**. In contrast, direct ¹⁸F-labeling of a derivative of the PARP1 inhibitor **160** resulted in low (1%) radiochemical yield (Figure 28).

In 2015, Li and Fox *et. al.* developed a fluorine-18 labeling system for PET probe construction based on a CF₃-substituted diphenyl-s-tetrazine derivative of biomolecules.^[136] In this work, c(RGDyK) was tagged by a fluorine-18 labeled TCO via reacting with the tetrazine moiety. The resulting tracer has shown improved metabolic stability. The [¹⁸F]TCO derivative was further improved by the same groups to radiolabel a cyclic RGD-conjugate **162** with a TCOPEG analog **161** to improve solubility and *in vivo* distribution of the resulting probe (Figure 29).^[137] The tracer **163** was obtained in 99% RCY relative to the fluorine-18 TCO derivative **161** with high molar activity of 33.7 ± 7.4 GBq/ μ mol (0.91 ± 0.20 Ci/ μ mol). PET imaging of human U87MG tumor bearing mice showed good tumor uptake of $5.3 \pm 0.2\%$ ID/g at 1h post injection of the radiotracer and increased to $8.9 \pm 0.5\%$ ID/g at 2h.

Likewise, Li *et. al.* developed a bicyclononyne-based prosthetic group for the fluorine-18 labeling of oligonucleotide (Figure 30).^[138] In this approach, fluorine-18 bound tobicyclo[6.1.0]nonyne ([¹⁸F]BCN, **164**) participated in an IEDDA reaction with a tetrazine-bound microRNA 165 to afford ¹⁸F-anti-microRAN 166 resulting in nearly quantitative

RCC in 3 min and molar activity of 52–61 GBq/μmol (1.4–1.6 mCi/μmol). A tetrazine moiety was also labeled with ¹⁸F recently, for example, Pieve and co-workers reported a unique approach where they conjugated the ZHER3:8698 affibody molecule with the NOTA-A118F ligand bearing a tetrazine functional group.¹³⁹ The resulting fluorine-18 labeled bioconjugate could be used to target human epidermal growth factor receptor 3 (HER3). It is worth noting that structural rearrangement of adduct dihydropyridazine and aromatization, as identified by chromatography, could occur during IEDDA reactions.^[137, 139-140]

Most recently, the tetrazine-click reaction has been investigated for *in vivo* bioorthogonal processes (*in vivo* pre-targeting), although no clinically-relevant radiotracer has yet been discovered for this application.^{9h} Towards this end, several research groups are working on radiolabeling the tetrazine moiety with fluorine-18 since these molecules, when suitably modified, have been shown to maintain their reactivity *in vivo*. The first of these was reported by Devaraj *et al.* where they showed that *in vivo* tetrazine/TCO [4+2]cycloaddition was enabled by polymer modified tetrazines (PMTs); fluorine-18 labeled PMT was synthesized and applied in LS174T tumor xenograft model.^[141] Denk *et al.* reported a novel synthesis of a low-molecular-weight fluorine-18 labeled tetrazine **168** (from tosylate **167**) that was successfully coupled with a TCO moiety to yield the targeted radiotracer in decay corrected RCYs up to 18%.^[142] The coupling reaction was also carried out *in vivo* and dynamic PET imaging showed no radiodefluorination. In 2015, a fluorine-18 labeled TCO was successfully coupled with a tetrazine terminated VHH (the variable domain of a camelid heavy-chain only antibody) in a rapid, and site-specific manner and used for the PET imaging of inflammation in xenogenic and syngenic tumor models with high specificity.^[143] In another example, our colleagues reported the synthesis of fluorine-18 labeled antibodies by the cycloaddition of a tetrazine-^{[18}F]FDG adduct **170** (from precursor **169**) with TCO functionalized proteins in 25% RCY (non-decay corrected).^[144] Subsequently, our laboratory reported the synthesis of another fluorine-18 labeled tetrazine moiety that was synthesized by the reaction between ^{[18}F]SFB and a suitable tetrazine precursor **171**. The fluorine-18 labeled tetrazine moiety **172** was then coupled to a TCO group containing biomolecule to produce fluorine-18 labeled anti-Class-II and anti-CD11b dimers that were applied in preclinical PET imaging studies (Figure 31).^[145]

Several groups are also working on development of fluorine-18 labeled tetrazine derivatives for the synthesis of fluorine-18 labeled biomolecules with favorable pharmacokinetics. Schirmacher and co-workers developed an ^{[18}F]SiFA-tetrazine derivative **173** that reacted with a model cyclooctene to yield the fluorine-18 labeled cycloaddition product in 78% isolated RCY.^[113] Keinänen *et al.* reported the synthesis of a fluorine-18 labeled tetrazine derived from fluorine-18 labeled ribose **174** that was shown to have favorable pharmacokinetics for *in vivo* biorthogonal IEDDA reactions with suitably substituted cyclooctenes. The labeled tetrazine showed excellent enzymatic stability in mouse serum and minimal radiodefluorination.^[146] Another interesting report was summarized by Meyer *et al.* wherein they reported the coupling reaction between ^{[18}F]NOTA labeled tetrazine **175** and TCO-bearing antibodies to yield the radiotracer in *ca.* 60% RCY (decay corrected,

synthesis time 108 min) for the visualization of CA19.9-expressing BxPC3 pancreatic cancer xenografts.^[147]

3.8 Nitrone or Nitrile Oxide-based [3+2] Cycloaddition

In addition to the extensively studied CuAAC and SPAAC, less known bioorthogonal methodologies have also been utilized to label biomolecules with fluorine-18. Zlatopolsky and co-workers in 2012 reported the radiofluorination of maleimides via [3+2] nitrone-alkene cycloaddition. Fluorine-18 labeled C-(4-fluorophenyl)-*N*-phenyl nitrone reacts with various maleimides to produce corresponding isoxazolidines.^[148] Similarly, these authors also investigated reaction of suitable dipolarophiles with 4-^[18F]fluorobenzonitrile oxide **177** (prepared from ^[18F]FBA and oxime **176**) and *N*-hydroxy-4-^[18F]fluorobenzimidoyl chloride to yield the corresponding labeled [3+2] cycloaddition product **178** (Figure 33).^[149] RCCs >95% were obtained in some cases with sample small molecules. The methodology was extended to synthesize labeled model dipeptides **179-181** in *ca.*50% RCCs.

4. Summary and Outlook

This review provides an overview of the various methodologies developed to radiolabel biomolecules with fluorine-18. With respect to the radiolabeling of biomolecules, however, we still have a way to go as many of the current methods have stalled and very few tracers developed have advanced to clinical trials, in part, due to various limitations such as low molar activities, non-physiological reaction conditions, and large lipophilic residual footprints to name a few. Innovative chemistry such as the use of 'click chemistry'-type reactions *in vitro* or *in vivo* provides an opportunity to solve many of these issues and have streamlined the synthesis of these biomolecules with the recent advances of site-specific chemical modifications of biomolecules.^[150] We believe that future work should not only aim to develop new chemistry methodologies that allow for facile and selective radiolabeling of biomolecules by addressing the limitations of current methods, but also to apply existing chemistry and radiolabeled biomolecules to ultimately improve clinical outcomes and to accelerate clinical translation of radiolabeled biomolecules.

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Biographies

Hema Krishnan studied synthetic chemistry and methodology development for organofluorination, at the University of Southern California (PhD, 2015). Her methodology was applied to synthesize fluorinated analogues of the anti-malarial drug, Artemisinin™. As a Research Fellow in the Radiochemistry Program at Massachusetts General Hospital and at Harvard Medical School, her current research interests include the development of medicinal chemistry libraries, new methodologies for radiolabeling and PET radiotracer development for neurodegenerative diseases.

Longle Ma studied chemistry (B.Sc.2010) at Nankai University, China. He performed his graduate studies in Chemistry at Rutgers University and obtained his Ph.D. in 2015. His doctoral research focused on the development of redox-neutral C–H bond functionalization methodologies for cyclic amines. As a Research Fellow in the Radiochemistry Program at Massachusetts General Hospital and at Harvard Medical School, he has been studying carbon-11 and fluorine-18 radiochemistry and neuroimaging using positron emission tomography since 2015.

Neil Vasdev obtained undergraduate degrees and a PhD in Radiochemistry from McMaster University, followed by a NSERC postdoctoral fellowship at Berkeley National Laboratories. In 2004, he started his independent research career at the University of Toronto and CAMH. In 2011, he was recruited as an Associate Professor at Harvard Medical School and is the Director of Radiochemistry at Massachusetts General Hospital. He has developed a radiopharmaceutical chemistry research program aimed at synthesizing new radiotracers and translating these for human imaging studies.

Steven H. Liang obtained his BSc at Tianjin University in 2003, followed by his PhD in Chemistry with Professor Marco Ciufolini in the University of British Columbia in 2010. Then he started as a NSERC fellow with Professor EJ Corey at Harvard University. In 2012, Dr. Liang accepted an Instructor position in the Department of Radiology, Harvard Medical School and Massachusetts General Hospital. In 2013, he was promoted to Assistant Professor. Steven's research ranges from PET imaging biomarker development to novel radiochemistry methods with ^{11}C and ^{18}F .

Figure 1. Direct ^{18}F -Labeling Through C- ^{18}F Bond Formation. Radiochemical conversion, RCC; radiochemical yield, RCY.

Figure 2.
Direct ^{18}F -Labeling Through Aziridine Ring-Opening and C- ^{18}F Bond Formation.

Figure 3.
Biocatalytic [^{18}F]Fluorination of Peptide Using Fluorinase.

Figure 4.
Direct ^{18}F -Labeling Through B- ^{18}F Bond Formation.

Figure 5. Direct ^{18}F -Labeling Through ^{19}F - ^{18}F Isotope Exchange

Figure 6.
Direct ^{18}F -Labeling Through Si- ^{18}F Bond Formation.

Figure 7.
Direct ¹⁸F-Labeling Through Al-¹⁸F Chelation.

Figure 8. Examples of NH_2 -selective ^{18}F -Prosthetic Groups

Figure 9.
Fluorine-18 Containing Prosthetic Groups for Oxime Formation.

Figure 10. Fluorine-18 labeling of Biomolecules involving Oxime Formation

Figure 11.
Fluorine-18 labeling of Biomolecules using $[^{18}\text{F}]\text{Fluorosugars}$.

BrCC(=O)NCc1ccc(F)cc1

69
[¹⁸F]FBB

O=C1C=CC(=O)N1O/C=C/c2ccc(F)cc2

71
[¹⁸F]FBAM

Chemistry. Author manuscript; available in PMC 2017 November 08.

Figure 13. Fluorine-18 Labeling Methods Involving Sulfur-containing Prosthetic Groups

A. ^{18}F -Radiolabeling of RGD using ^{18}F HFB **B. ^{18}F -Radiolabeling of Z_{HER2:2395}-Cys-SH using ^{18}F FPOS** **Figure 14. Recent Thiol-Selective Fluorine-18 Radiolabeling**

Figure 15. Cyanobenzothiazole Prosthetic Groups for Fluorine-18 labeling of Cysteine-containing Biomolecules.

Figure 16.
Fluorine-18 Labeling via X-C_{sp2}Bond Formation with Prosthetic Groups.

Figure 17.
Fluorine-18 Labeled Alkyne Building Blocks.

Figure 18.

Fluorine-18 Labeling of Biomolecules via CuAAC Between Azide Containing Biomolecules and Labeled Alkynes.

Figure 19.

A) Synthesis of $[^{18}\text{F}]\text{FEA}$; B) Examples of Radiotracers Synthesized with $[^{18}\text{F}]\text{FEA}$ Building Block; C) Some Representative Fluorine-18 labeled Azides in Click Chemistry.

Figure 20.
Fluorine-18 Radiolabeling of Biomolecules via Spirocyclic Iodonium Ylides.

Figure 21.
Silicon-¹⁸F Based Building Block for Biomolecule Labeling via CuAAC.

Figure 22. Fluorine-18 Labeled ADIBO Prosthetic Groups for SPAAC

Figure 23.
Fluorine-18 Labeling of Azide-Terminated Peptide Containing PEG Linker via SPAAC.

Figure 24.
Alkyne-ADIBO Building Block for Fluorine-18 Labeling of Biomolecules.

Figure 25.
Traceless Staudinger Ligation to Radiolabel Biomolecules.

Figure 27.

A) Synthesis of Fluorine-18 Labeled TCO, $[^{18}\text{F}]\mathbf{16}$. B) Fluorine-18 Labeling of Cyclic-RGD peptide via Tetrazine Ligation.

Figure 28.
Comparison of Fluorine-18 Labeling by Tetrazine Ligation and Conventional Labeling Strategies.

Figure 29.

A Fluorine-18 labeled PEG-TCO Prosthetic Group with Improved Pharmacokinetics.

Figure 30.
Bioconjugation through Tetrazine-Alkyne IEDDA Reactions.

Figure 31.
Synthesis of Fluorine-18 Labeled Tetrazine Building Blocks.

Figure 32.
Radiolabeling of TCO-terminated Biomolecules *in vivo* with Fluorine-18 Labeled Tetrazine Building Blocks.

Figure 33.
[3+2] Cycloaddition of Dipolarophiles with 4-[¹⁸F]Fluorobenzonitrile Oxide.