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Site-Selective, Late-Stage C-H ^{18}F-Fluorination on Unprotected Peptides for Positron Emission Tomography Imaging

Zheliang Yuan,[†] Matthew B. Nodwell,[†] Hua Yang, Noeen Malik, Helen Merkens, François Bénard, Rainer E. Martin, Paul Schaffer, Robert Britton**

Abstract: Peptides are often ideal ligands for diagnostic molecular imaging due to their ease of synthesis and tuneable targeting properties. However, labelling unmodified peptides with ^{18}F for positron emission tomography (PET) imaging presents a number of challenges. Here we show the combination of photoactivated sodium decatungstate and [^{18}F]-*N*-fluorobenzenesulfonimide effects site-selective ^{18}F-fluorination at the branched position in leucine residues in unprotected and unaltered peptides. This streamlined process provides a means to directly convert native peptides into PET imaging agents under mild aqueous conditions, enabling rapid discovery and development of peptide-based molecular imaging tools.

Positron emission tomography is a functional imaging technique whereby a ligand labelled with a positron-emitting radioisotope (e.g., ^{11}C, ^{18}F, ^{68}Ga) is administered to a patient and its biodistribution is then monitored in real time.[1] Thus, radiotracers that target a specific cellular or metabolic pathway can non-invasively provide information critical to disease diagnosis, status and response to therapy.[2] Among the positron-emitting radioisotopes, ^{18}F is most commonly used in PET imaging based on favourable decay characteristics (97% β^+ decay), low positron energy (0.64 MeV), and a half-life ($t_{1/2}$ = 109.8 min) compatible with radiotracer distribution.[3] Owing to their ease of synthesis, low immunogenicity, tuneable targeting properties and favourable pharmacokinetics, peptides often provide ideal leads for PET ligands.[4,5] However, there is a fundamental incompatibility between the hydrophilic and structurally sensitive nature of peptides and the violent reactivity of [^{18}F]F_2 gas or the high temperature and anhydrous reaction requirements typical for [^{18}F]fluoride ion labelling.[4] Thus, recent efforts have focused on late-stage ^{11}C-labelling ($t_{1/2}$ = 20.4 min) of peptides and include ^{11}C-acylation of lysine residues[6] or ^{11}C-cyanation of peptide-bound arylpalladium complexes.[7] For ^{18}F-fluorination, multi-step labelling approaches are generally required that involve the following key transformations executed in either order: i) incorporation of ^{18}F onto a prosthetic group; and ii) coupling of a prosthetic group to the target peptide.[3,8]

Most commonly, electrophile-containing prosthetic groups such as Michael acceptors[9] or acylating agents[10] are ^{18}F-labelled prior to reaction with a nucleophilic residue on a peptide (e.g., [^{18}F]SFB (**2**),[10] Figure 1). However, the late-stage, aqueous labelling of peptide-bound prosthetic groups by isotopic exchange ([^{18}F]AmBF$_3$[11] or [^{18}F]SiFA[12]), formation of a metal-fluoride complex ([^{18}F]AlF[13]) or via S_NAr[14] reactions are also viable alternatives.[8] Very recently the ^{18}F-trifluoromethylation of cysteine residues[15a] and ^{19}F-trifluoromethylation of tyrosine residues[15b] in unmodified peptides, and ^{18}F-fluorination of C-H bonds in (hetero)arenes[15c] have also been reported.

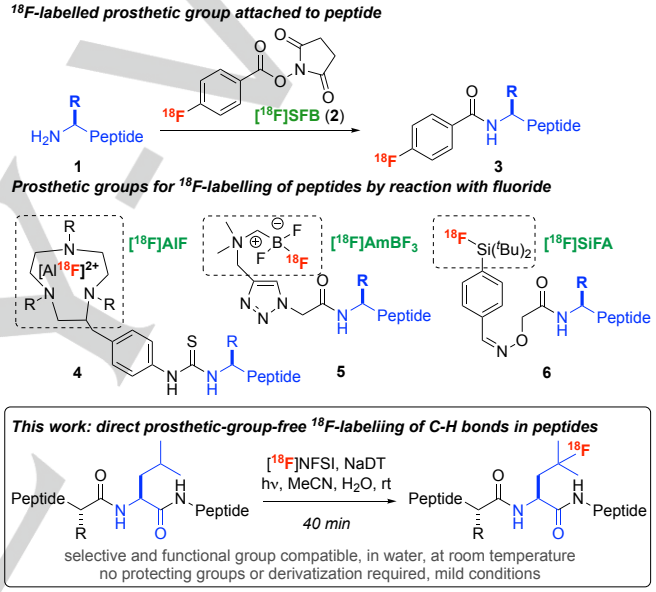

Figure 1. Prosthetic groups commonly used for ^{18}F-labelling of peptides and a prosthetic-group-free ^{18}F-labelling of Leu-containing peptides.

While the impact of prosthetic groups on radiopharmaceutical discovery has been profound, advanced knowledge of structure-activity relationships is required to identify a suitable site for prosthetic group attachment.[8,16] Additionally, this approach can require complex protecting group strategies, multi-step syntheses and face challenges with chemoselectivity. Prosthetic groups also impact peptide structure, and can negatively affect biological properties that include binding affinity and target specificity, as well as pharmacokinetic behaviour.[8,17] Thus, an ideal scenario would involve the direct and selective ^{18}F-fluorination of a single C-H bond in a native peptide under rapid, mild, and aqueous conditions. While important contributions towards the selective ^{18}F- and ^{19}F-fluorination of C-H bonds in peptides have been made,[18] amine and carboxylic acid protecting groups are mandatory. To the best of our knowledge the only direct C-H ^{18}F-fluorination of an unprotected peptide was carried out on a cyclic RGD peptide using [^{18}F]AcOF, and the extreme reactivity of this reagent resulted in a mixture of fluorinated products.[19] Here we show that a range of unprotected and unaltered peptides can be directly labelled

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with ^{18}F using an inexpensive photocatalyst and apparatus under mild aqueous reaction conditions (Figure 1, inset).

Our group has reported the direct ^{18}F-fluorination of several branched aliphatic amino acids[20] via a reaction involving photoactivated sodium decatungstate (NaDT) as a hydrogen atom abstracting agent[21,22] in combination with the fluorine atom donor [^{18}F]-*N*-fluorobenzenesulfonimide ([^{18}F]NFSI).[23,24] Inspired by this advance, we considered the feasibility of using leucine (Leu (L)) as a native fluorination site in peptides. An examination of the literature revealed that Leu is uniquely susceptible to C-H abstraction[25-27] and most commonly forms a carbon centered radical at the branched (isopropyl) position. In our hands, subjecting various amino acids (e.g., Glu (E), Lys (K), Arg (R), Phe (F)) to NaDT fluorination returned unreacted amino acid without degradation. Encouraged by these results, we prepared a series of Leu-containing dipeptides to test the compatibility and kinetics of peptide fluorination. As detailed in Figure 2, the selectivity of this reaction for the branched position of Leu residues and compatibility with charged, polar and hydrophobic amino acid residues is striking. For example, the charged amino acids Arg, Lys, His (H) and Asp (D) all proved to be fully compatible with both the photocatalyst and NFSI, and the respective dipeptides underwent rapid and clean fluorination. Likewise, dipeptides containing the amino acids Ser (S), Thr (T), Asn (N) and Gln (Q) fluorinated cleanly, though the Thr-containing dipeptide partially degraded after extended (6 h) reaction times. Among the hydrophobic amino acids, dipeptides incorporating Gly, Ala, Val (V), Ile (I), Pro (P) and Phe were all compatible. Notably, the branched position in Val and Ile also eventually underwent fluorination, however, in both cases the amount of difluorinated peptide was only ~5% after 30 min. In the case of the Tyr (Y)-containing dipeptide, the fluorination proceeded smoothly, albeit at a decreased rate, while Trp (W) proved to be incompatible. Reaction of a Met (M)-containing dipeptide resulted in coincident oxidation at sulfur by NFSI and afforded the corresponding sulfoxide fluorinated on the Leu residue, while fluorination of a Cys (C)-containing dipeptide led to a mixture of oxidation products. Considering, however, that Cys, Met, and Trp are the rarest amino acids, their incompatibility should not significantly impact the utility of this peptide labelling strategy.

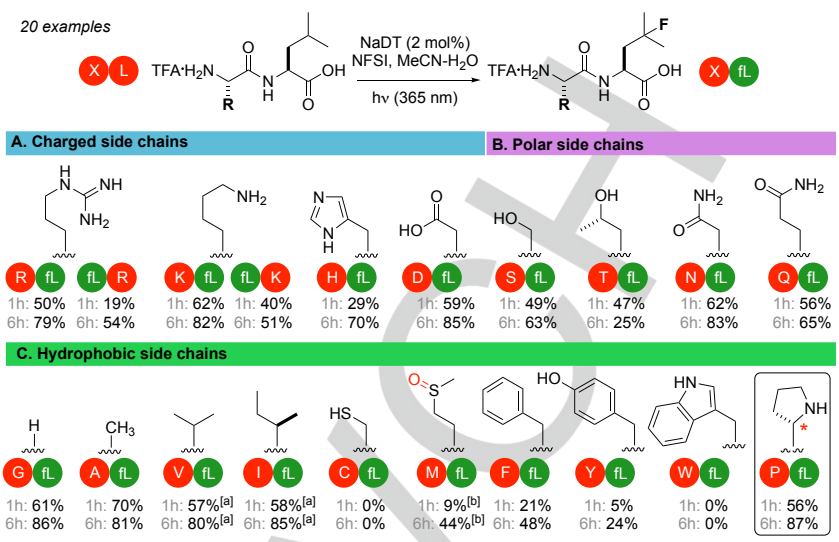

Figure 2. NaDT-promoted fluorination of Leu-containing dipeptides. Conversion at 1h (determined by analysis of NMR spectra) and reaction yield after 6 h are reported. [a] Product accompanied by fluorination at a secondary site; [b] accompanied by formation of sulfoxide or sulfonic acid. Note for proline (P) the * denotes the carbon adjacent to the amide carbonyl. fLR and fLK refer to dipeptides with Leu at the N-terminus. fL = 4-fluoroleucine.

To further examine the scope of this process, several tetrapeptides containing Leu residues at various positions were prepared and their fluorination examined. As highlighted in Figure 3, Leu residues in GLKA, EKLA and GKLK, and C-terminal Leu residues in KSGL, PAKL and AEFL were all fluorinated selectively in yields varying from ~10-30% after 2 h. Considering the decreased yield for AEFfL (**12**) (12% after 2 h), we examined fluorination of positional isomers of this tetrapeptide in an effort to deconvolute the influence of Leu positioning and amino acid compatibility. As indicated, while the *N*-terminal Leu isomer LAEF underwent clean fluorination, production of AEfLF (**14**) and EfLAF (**15**) were accompanied by degradation. Established pathways for peptide hydrolysis by polyoxometalates notwithstanding,[28] we speculated that here C-H abstraction on Leu could be followed by intramolecular abstraction of a benzylic hydrogen atom on a proximal Phe residue and subsequent fragmentation; a process known to occur between Leu residues in short peptides.[29] Notably, when Phe was replaced with Gly (i.e., AELG), the resulting tetrapeptide fluorinated in much improved yield (**18**: 41% yield after 2 h). Two additional isomeric tetrapeptides consisting of ARLF or LRAF were also fluorinated and again degradation was only observed when Leu and Phe residues were proximal (ARLF) and not when separated (LRAF). We also examined fluorination of the hexapeptide FALGEA-NH_2, a ligand for the cancer-specific receptor EGFRvIII,[30] and were pleased to see that fluorination occurred cleanly on the Leu residue. Likewise, fluorination of the pseudopeptide matrix metalloproteinase inhibitor marimastat,[31] the inhibitor of NAAG peptidase ZJ-43 (**21**),[32] as well as two structural analogues of ZJ-43 (**22** and **23**) occurred on the Leu residue.

Prior to examining the direct ^{18}F-labelling of peptides, we explored the use of three unique 365 nm photochemical reactor configurations [33] that comprised i) a borosilicate glass tube (5 mm diameter) immersed in a custom photochemical reactor (8 x 9 W UV curing lamps), ii) a narrow bore PTFE tube wrapped around a BLB lamp, and iii) a microreactor glass chip placed on a transilluminator (see Supplementary Figures 1-3 for details). As summarized in Figure 3 (inset), fluorination of the tetrapeptide AELG was significantly improved in both the PTFE tube reactor and microreactor. In fact, after one hour this reaction was near complete in the microreactor (86% conversion). Likewise, fluorination of the dipeptides HL and DL reached 82% and 98% conversion, respectively, after 1 h in the

microrector. Here, a combination of increased surface area and light intensity clearly benefits Leu fluorination.[33]

To explore the ^{18}F-fluorination of unprotected peptides we further optimized the synthesis of [^{18}F]NFSI reported by Luthra and Gouverneur,[23] and ultimately, [^{18}F]NFSI was produced as a solution in MeCN with a radiochemical purity >90%. An aqueous mixture of the peptide and NaDT catalyst were then added to an MeCN solution of [^{18}F]NFSI (no azeotropic drying required) and the solution was irradiated for 40 min in a narrow-bore PTFE tube or in a microreactor. As summarized in Figure 4, we observed excellent radiochemical conversion (RCC) in the ^{18}F-fluorination of dipeptides, producing the ^{18}F-labelled dipeptides **24** – **27** with no detectable epimerization or fluorination at other sites. The radiochemical yield (RCY, decay corrected from [^{18}F]NFSI) for these processes were comparable or better than those reported previously by us for the radiofluorination of branched aliphatic amino acids.[20] We next demonstrated the radiofluorination on a small series of Leu-containing tetrapeptides, and in each case the RCC was >25% and ^{18}F-labelled tetrapeptides **28**–**30** were isolated in RCYs ranging from 16 to 35%. The EGFRvIII-targeting peptide FALGEA-NH_2[30] was also directly radiofluorinated providing [^{18}F]FAfLGEA-NH_2 (**31**) (RCC ~10%) after ~80 min total radiosynthesis time. This last result compares well to previous labelling studies of FALGEA-NH_2 that involved coupling with the [^{18}F]fluorobenzoic acid ([^{18}F]FBA) prosthetic group and proceeded with RCYs ranging from of 2.6-9.8% (decay corrected) after a 3 h radiosynthesis.[34] Finally, the NAAG peptidase inhibitor ZJ-43[32] was directly fluorinated to provide **32**, and fluorination of a homologated analogue afforded the ^{18}F-labelled peptide **33**. We note that these are the only examples of prostethic group free ^{18}F-fluorination within this important family of prostate cancer imaging agents.[35] Importantly, analysis of the biodistribution of [^{18}F]ZJ-43 (**32**) in healthy mice showed low bone accumulation (1.7% ID/g) suggesting that the peptidic [^{18}F]fluoroleucine fragment is not susceptible to the same degradation pathways of [^{18}F]fluoroleucine itself, which displays significant bone accumulation (11.9% ID/g).[20] While the specific activitiy (SA) of ^{18}F-labelled peptides **24**–**33** produced in this study ranged from 1.3–5.3 MBq μmol^{-1}, significant improvements in SA can be achieved by lengthening the proton irradiation time on [^{18}O]O_2. Moroever, [^{18}F]F_2 with SAs up to 55 GBq μmol^{-1} can be prepared using a process described by Bergman and Solin,[36] and [^{18}F]NFSI produced via this method has a SA of 10.3 GBq μmol^{-1}.[37]

In summary, we have developed a versatile and direct, aqueous approach for radiofluorination of unprotected Leu-containing peptides that does not rely on prosthetic groups. This unique approach, successfully demonstrated on 10 peptides, is amenable to radiopharmaceutical production with mild reaction conditions, a simple fluid path and column-based purification.

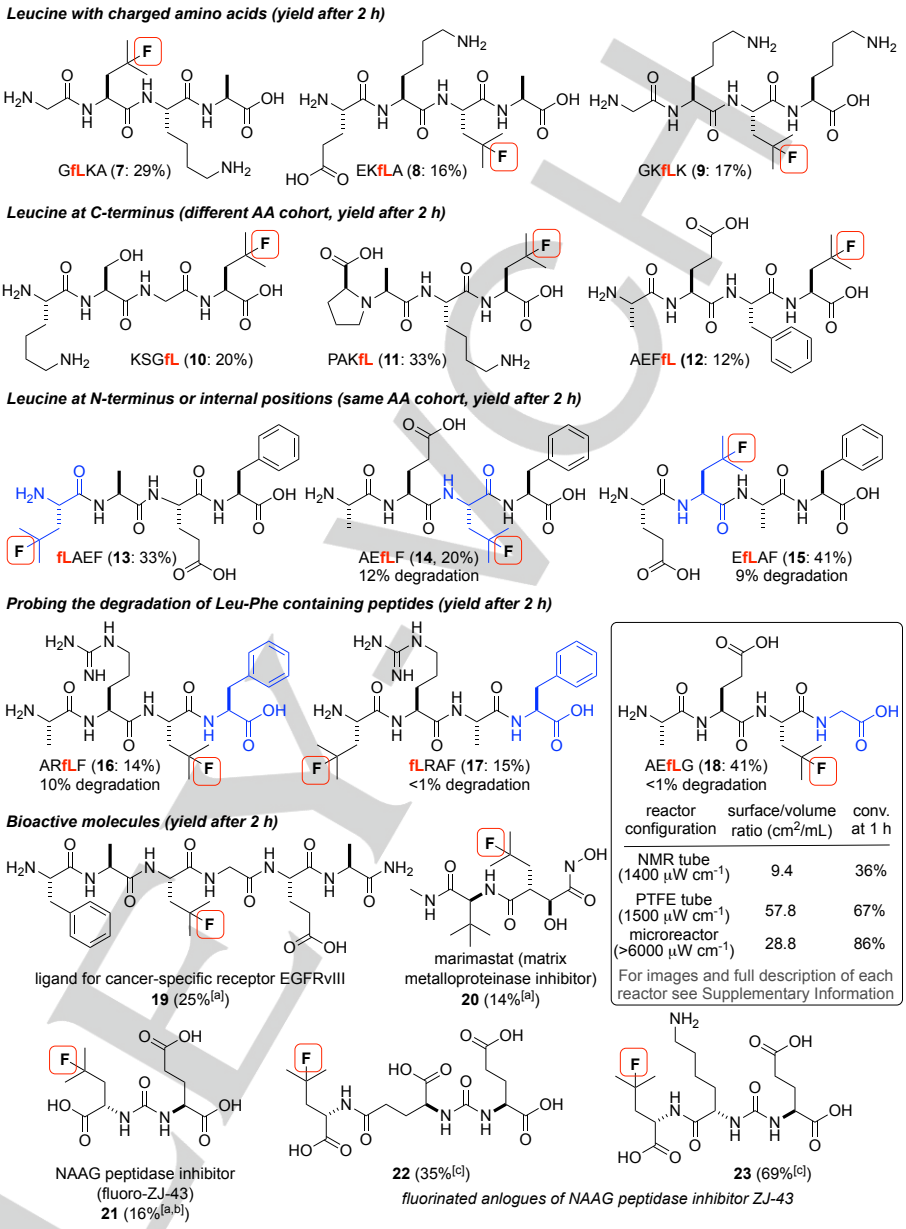

Figure 3. Fluorination of Leu-containing tetrapeptides. Yields were determined using the ERETIC method[38] after 2 hours of irradiation in a borosilicate glass NMR tube immersed in a custom photochemical reactor (Supplementary Figure 1). Degradation refers to the formation of other fluoroleucine-containing products. [a] Reaction in a PTFE tube reactor system (Supplementary Figure 2); [b] reaction time = 3 h; [c] Yield determined by analysis of ^{1}H NMR spectra with internal standard after 6 h.

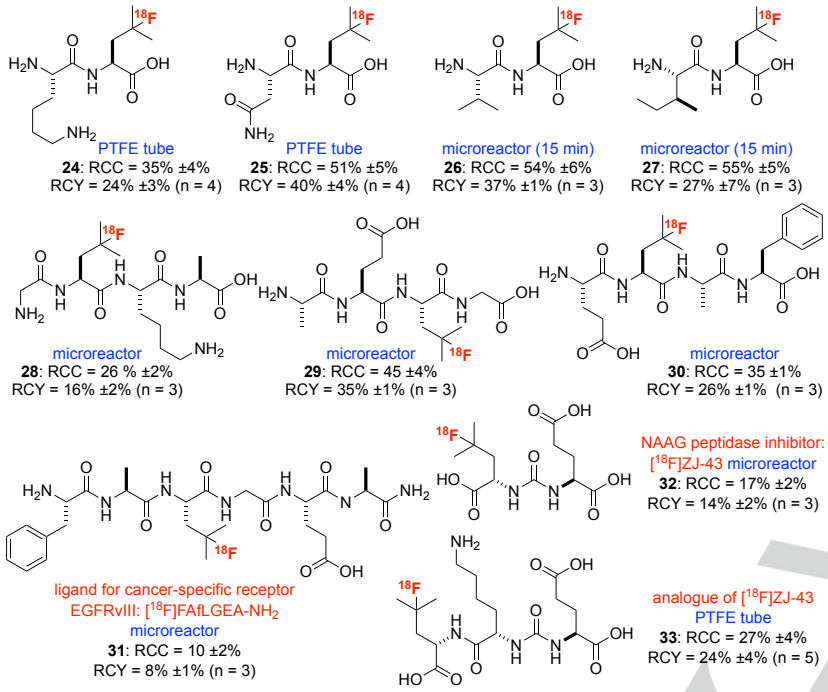

Figure 4. Direct site-selective ^{18}F-fluorination of unprotected peptides. Reaction conditions: peptide·TFA, NaDT (2 mol%), [^{18}F]NFSI, MeCN-H_2O, hν (365 nm), 40 min. See Supplementary Information for full experimental details.

The apparatus used to rapidly afford a number of model fluorinated peptides consists of inexpensive components and the process is amenable to automation. Overall, NaDT-mediated fluorination of peptides represents a convenient and facile method to rapidly accelerate the discovery process for radiolabelled peptides as imaging agents, or as a tool to facilitate the discovery of peptide-based drugs.

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