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One-step radiosynthesis of 4-[^{18}F]flouro-3-nitro-*N*-2-propyn-1-yl-benzamide ([18F]FNPB): a new stable aromatic porosthetic group for efficient labeling of peptides with fluorine-18

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4-[^{18}F]flouro-3-nitro-*N*-2-propyn-1-yl-benzamide ([^{18}F]FNPB) was developed as a new stable aromatic prosthetic group for more efficient click labeling of peptides. A new aromatic precursor 3,4-dinitro-*N*-2-propyn-1-yl-benzamide was radiofluorinated using [^{18}F]KF/K2.2.2 followed by HPLC purification to obtain the desired product [^{18}F]FNPB. [^{18}F]FNPB was synthesized with a 58% radiochemical yield, a specific activity > 350 GBq/μmol, and radiochemical purity was exceeded 98% in 40 min. The *in vitro* stability studies showed no detectable radiodefluorination over 2 h in mouse plasma. The click labeling yield of three different peptides with [^{18}F]FNPB were all above 87%. The *in vitro* study suggests that [^{18}F]FNPB may be stable *in vivo* and could have general application in labeling peptides with high radiochemical yield for positron emission tomography applications.

Keywords: click chemistry; defluorination; peptides; [^{18}F]FNPB

Introduction

Positron emission tomography (PET) is one of the most important techniques in the field of molecular imaging. With high-resolution and sensitivity, PET can provide quantitative information of physiological, biochemical, and pharmacological processes in living objects.[1–4] Among all the available positron emitting isotopes, ^{18}F ($t_{1/2}$ = 109.8 min) is the ideal and most frequently used PET radiotracers because of its favorable nuclear and chemical properties.[5,6]

An important application of PET imaging is in the field of cancer diagnosis. Because many cancer cells were found to have over expression of certain receptors that regulate their life cycles, peptides-based ligands that target to these receptors were developed and have become a new, promising class of radiopharmaceuticals. [7,8] The combining of ^{18}F purposive sensitivity with the targeting properties of peptides was extensively described for highly specific and early tumor diagnostic and imaging applications. But peptides are not readily amenable to direct fluorination with no-carrier-added [^{18}F]fluoride, direct ^{18}F-labeling of peptides is not a commonly used method.[9] Thus, there have been many ^{18}F-labeling strategies developed including various prosthetic groups[10] and conjugation via acylation,[11,12] amidation,[13] oxime formation,[14,15] alkylation,[16] photochemical reaction, and so on.[17] However, there are still many rooms for improvement because most of these strategies either had poor site-specificity or low radiochemical yield.

Because Sharpless, K. B. first coined of the term 'click chemistry' in 2001,[18] the Cu (I)-catalyzed Huisgen reaction has made a significant impact on bioconjugation processes; it can be performed with defined site-specificity, under mild condition without protective groups, and in high yields.[19] There have been many reports on using click chemistry for labeling of radiopharmaceuticals.[20] The Copper-catalyzed azide-alkyne 1,3-dipolar cycloaddition, also referred as 'the click reaction', was the most commonly used reaction aimed by those alkyne and azide prosthetic groups.[21] However, in most of those designs, ^{18}F is directly attached to an aliphatic carbon.[22–28] The structure-defluorination susceptibility relationship has not yet been fully understood,[29,30] but some aliphatic prosthetic groups were found to be susceptible to significant *in vivo* radiodefluorination.[31,32] It is expected that prosthetic groups on which fluorine is attached to an aromatic carbon would be more stable *in vivo* than those with fluorine attached to aliphatic carbon because of the higher carbon-fluorine strength.[33] Two aromatic prosthetic groups for click labeling have been reported, but the procedure involves multiple steps with radiolabeled materials, or the radiochemical yield for the peptide labeling was rather low.[34,35] In this study, we described one-step radiosynthesis of a new aromatic ^{18}F prosthetic group for more efficient click labeling of peptides with enhanced radiochemical yield.

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Materials and methods

All reagents and solvents were purchased from Acros Organics, Sigma-Aldrich, Merck, or Sinopharm Chemical Reagent Co. All chemicals were used as supplied unless stated otherwise. [^{18}F]fluorine was purchased from ShangHai Atom Kexing Pharmaceuticals Co., Ltd.. Nuclear magnetic resonance spectra were recorded on Bruker Avance III 400 MHz (Bruker Corporation, USA) with the corresponding solvent signals as an internal standard. Chemical shift (δ) are reported in parts per million (ppm) relative to tetramethylsilane (δ = 0). Coupling constants (*J*) are given in Hertz (Hz). Splitting patterns are defined by s (singlet), d (doublet), dd (doublet doublet), t (triplet), m (multiplet). Mass spectra were taken on a Q-Tof Premier Mass Spectrometer (Waters Corporation, USA). IR (infrared) spectra were measured with a Bruker Equinox 55 FT-IR Spectrometer (Bruker Corporation, USA). Analytical as well as semipreparative reversed-phase high-performance liquid chromatography was performed on an agilent 1100 system with VWD detector and Flow-Count radio-HPLC detector system (Bioscan. INC.). Semipreparative radio-PHLC was performed using Beckman Coulter Ods C18 Semi-Preparative Column (5 µm, 10 × 250 mm). The flow rate was set at 3 mL/min, with the mobile phase solvent A (0.1% trifluoroacetic acid [TFA] in water) and solvent B (0.1% TFA in acetonitrile). The analytical HPLC was performed using the same gradient system but with a Beckman Coulter Ultrasphere Reversed Phase Column (5 µm, 4.6 × 250 mm). The flow rate was set at 1 mL/min, with the mobile phase solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile). The UV absorbance was monitored at 214 nm.

Synthesis of 3,4-dinitro-*N*-2-propyn-1-yl-benzamide (4)

3,4-Dinitrobenzoic acid (212 mg, 1 mmol) was dissolved in dry dimethylformamide (DMF), 2-propynylamine (55 mg, 1.1 mmol) was added to the solution followed by HOBt (120 mg, 0.88 mmol) and EDC·HCl (316 mg, 1.66 mmol). The resulting solution was stirred at room temperature for 24 h. Ethyl acetate (EA) (40 ml) was added to the reaction mixture after the reaction and washed twice with water. The organic layer was dried with Mg_2SO_4, and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (dichloromethane [DCM]/MeOH = 95:5) to give 135 mg of the desired 3,4-dinitro-*N*-2-propyn-1-yl-benzamide in the form of yellow solid. ESI-MS: *m/z* = 248 (M-H)- for $C_{10}H_7N_3O_5$ calcd. *m/z* = 249.04; HRMS (high resolution mass spectrometry) calculated for $C_{10}H_8N_3O_5$ 250.0464,found 250.0461.^{1}H NMR (400 MHz, DMSO) δ 9.50 (t, *J* = 5.2 Hz, 1H), 8.64 (d, *J* = 1.5 Hz, 1H), 8.39 (dd, *J* = 8.4, 1.6 Hz, 1H), 8.35 (d, *J* = 8.4 Hz, 1H), 4.13 (dd, *J* = 5.4, 2.5 Hz, 2H), 3.32 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ 162.39 (s), 143.43 (s), 141.58 (s), 138.44 (s), 133.39 (s), 126.01 (s), 124.60 (s), 80.33 (s), 73.59 (s), 28.94 (s) (IR spectra see supporting information Figure 1).

Synthesis of 4-[^{19}F]flouro-3-nitro-*N*-2-propyn-1-yl-benzamide ([^{19}F]FNPB, 5)

A mixture of potassium fluoride dehydrate (15 mg) and Kryptofix [222] (30 mg) in anhydrate acetonitrile (2 mL) was heated at 100°C under a steam of nitrogen for 5 min, subsequently, 2 mL of anhydrate acetonitrile was added three times and evaporated to dryness. A solution of 3,4-dinitro-*N*-2-propyn-1-yl-benzamide (20 mg) in anhydrate acetonitrile was added to the dried mixture, and the reaction mixture was heated at 100 °C for 30 min. After the reaction, the crude product was purified by semipreparative HPLC and the solvent was removed in vacuo. The final product 4-flouro-3-nitro-*N*-2-propyn-1-yl-benzamide (5.4 mg) was obtained in 30% yield. ESI-MS: *m/z* = 221.1 (M-H)- for $C_{10}H_7FN_2O_3$ calcd *m/z* = 222.04; HRMS calculated for $C_{10}H_8N_2O_3F$ 223.0519, found 223.0514,^{1}H NMR (400 MHz, DMSO) δ 9.32 (t, *J* = 5.0 Hz, 1H), 8.65 (dd, *J* = 7.3, 2.2 Hz, 1H), 8.40–8.20 (m, 1H), 7.68 (dt, *J* = 84.6, 42.3 Hz, 1H), 4.10 (dd, *J* = 5.4, 2.5 Hz, 2H), 3.18 (t, *J* = 2.5 Hz, 1H). ^{13}C NMR (101 MHz, DMSO) δ 162.97 (s), 156.32 (d, *J* = 266.3 Hz), 135.25 (s), 130.53 (d, *J* = 3.8 Hz), 125.41 (s), 118.87 (d, *J* = 21.4 Hz), 80.69 (s), 73.28 (s), 28.75 (s). ^{19}F NMR (376 MHz, DMSO) δ −114.74 (s).

General procedure for the synthesis of azido acids

Synthesis of triflyl azide

Sodium azide (4 g, 61.6 mmol) was dissolved in distilled water (10 mL) and then DCM (10 mL) was added. The mixture was cooled on ice bath for 20 min. Triflyl anhydride (2 mL, 12 mmol) was added slowly over 5 min and the mixture was stirred for 2 h. The mixture was extracted with DCM (2 × 8 mL). The organic portions, containing the triflyl azide, were pooled, washed once with saturated Na_2CO_3 (40 mL), and used without further purification.

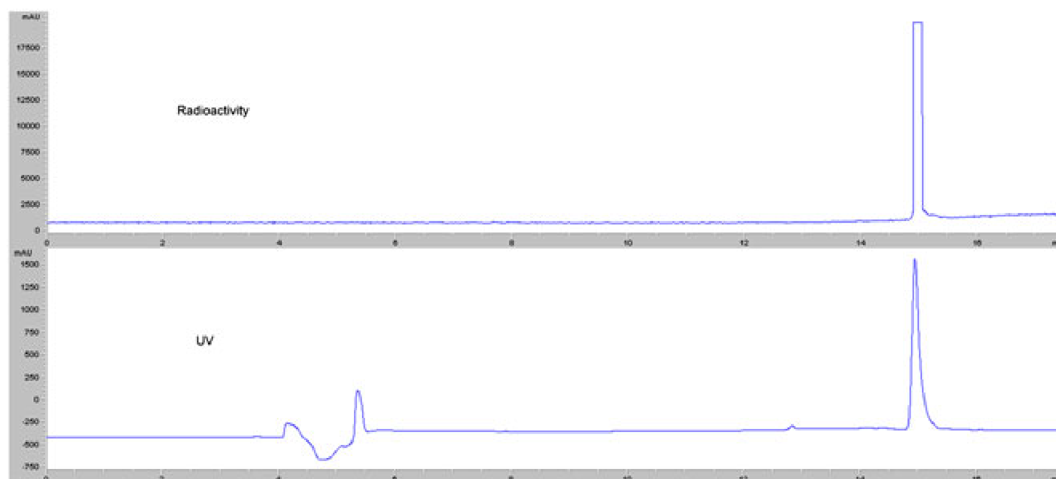

Figure 1. Radio-chromatograms and UV-chromatograms of [18F]FNPB, co-injected with the standard [19F]FNPB(column and conditions see Materials and Methods).

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Synthesis of azido-Leu

Leucine (786 mg, 6 mmol) was combined with K_2CO_3 (1189 mg, 9 mmol) and $CuSO_4{\cdot}5H_2O$ (15.6 mg, 60 μmol) in a distilled water (20 mL) and methanol (MeOH, 40 mL). The triflyl azide in CH_2Cl_2 (DCM, 25 mL) was added and the mixture was stirred at ambient temperature and pressure for 36 h. Subsequently, the organic solvents were removed under reduced pressure and the aqueous slurry was diluted with water (100 mL). This was acidified to pH 6 with conc. HCl and diluted with phosphate buffer (250 mM, pH 6.2, 100 mL) and extracted with EA (4 × 20 mL) to remove sulfonamide by-product. The aqueous phase was then acidified to pH 2 with conc. HCl. The product was obtained from another round of EA extractions (3 × 20 mL). These EA extracts were combined, dried (on $MgSO_4$), and evaporated to dryness giving 753-mg pale yellow oils in 80% yield with no need for further purification.[36]

Synthesis of 6-azidohexanoic acid

6-Azidohexanoic acid was synthesized according to the literature procedure with slight modification[37]; briefly, sodium azide was added to a solution of 6-bromohexanoic acid (1.5 g, 7.7 mmol) in DMF (5 mL), and the mixture was stirred and heated at 85°C for 18 h. The crude reaction mixture was diluted in DCM, and this solution washed with 0.1 N aq HCl. The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure to give 6-azidohexanoic acid (1.0 g, 82%) as an oil, which was used without further purification.

General procedure for the synthesis of peptides

Peptides were synthesized using standard solid-phase peptide synthesis strategy on resin (standard coupling procedure: 5 equiv amino acid, 5 equiv HBTU, 6 equiv HOBt, 6 equiv DIPEA; Fmoc-cleavage: 20% piperidine in DMF). The peptide was cleaved from the resin by incubation with a mixture of TFA/triisopropylsilane/H_2O (94:3:3) for 2 h, precipitated in diethyl ether, and purified by semipreparative HPLC.

Synthesis of azido-LARLLT ***(7)***

Azido-leucine-alanine-arginine-leucine-leucine-threonine acid (azido-LARLLT) was obtained as white powder. ESI-MS: $m/z = 712.3$ (M + H) + for $C_{31}H_{57}N_{11}O_8$ calcd $m/z = 711.4$.

Synthesis of 6-azidohexanoic acid-LARLLT ***(8)***

6-Azidohexanoic acid conjugated-leucine-alanine-arginine-leucine-leucine-threonine acid (6-azidohexanoic acid-LARLLT) was obtained as white powder. ESI-MS: $m/z = 825.8$ (M + H) + for $C_{31}H_{57}N_{11}O_8$ calcd $m/z = 825.0$.

Synthesis of 6-azidohexanoic acid-cRGDfK ***(9)***

6-Azidohexanoic acid conjugated-cyclic arginine-glycine-aspartic-phenylalanine-lysine acid (6-azidohexanoic acid-cRGDfK) was obtained as white powder. ESI-MS: $m/z = 743.8$ (M + H) + for $C_{31}H_{57}N_{11}O_8$ calcd $m/z = 742.8$.

General procedure for the synthesis of [^{19}F]fluoro-triazole-peptide

To a solution of 10-mg peptide and 3.4-mg [^{19}F]FNPB in MeOH/H_2O(1:1, 1600 μL), 1.05 mg $CuSO_4$ and sodium 2.52 mg ascorbate was added. The mixture was stirred vigorously at room temperature for 20 min, and the reaction was then quenched and purified by semipreparative HPLC.

Synthesis of [^{19}F]fluoro-triazole-7

The [^{19}F]fluoro-triazole-7 was obtained as white powder (9 mg, 69%). MS m/z: $[M + H]^+ = 934.9$ for $C_{41}H_{64}FN_{13}O_{11}$ calcd $m/z = 933.48$.

Synthesis of [^{19}F]fluoro-triazole-8

The [^{19}F]fluoro-triazole-8 was obtained as white powder (9 mg, 71%). MS m/z: $[M + H]^+ = 1047.7$ for $C_{41}H_{64}FN_{13}O_{11}$ calcd $m/z = 1046.57$.

Synthesis of [^{19}F]fluoro-triazole-9

The [^{19}F]fluoro-triazole-9 was obtained as white powder (10 mg, 76%). MS m/z: $[M + H]^+ = 965.5$ for $C_{41}H_{64}FN_{13}O_{11}$ calcd $m/z = 965.00$.

Radiochemistry

Synthesis of [^{18}F]FNPB

No-carrier-added [^{18}F]fluoride was produced by proton bombardment of enriched ^{18}O-water via the $^{18}O(p, n)^{18}F$ reaction, and it was then trapped on an SEP-PAK LIGHT QMA (Waters). The [^{18}F]fluoride was eluted from the cartridge into a reaction vessel using a solution of $K_{2.2.2.}/K_2CO_3$ solution (2-mL 96/4 MeCN/water, 9.5-mg $K_{2.2.2.}$, 1.7-mg K_2CO_3). The elute was dried at 100°C under a gentle stream of nitrogen gas. Then compound **4** in anhydrate acetonitrile (1 mL) was added, and the mixture was heated at 90°C for 10 min. The reaction was then quenched and purified by semipreparative HPLC using a gradient of 10 → 60% solvent B over 20 min. The retention time of 18.6 min peak was collected and evaporated to dryness for the next click labeling step. Co-injection was performed by analytical HPLC using a gradient of 10 → 50% solvent B over 10 min.

***In vitro* stability studies**

The stability of [^{18}F]FNPB was investigated in mouse plasma at various incubation times (0 min, 30 min, 60 min, and 120 min) and 37°C. PBS was used as a control. After incubation, plasma proteins were precipitated with acetonitrile and centrifuged for 10 min at 16500 rpm and 4°C. The PBS (Phosphate buffered saline) control was diluted with the same volume of acetonitrile. The supernatants and the PBS control were analyzed by analytic HPLC.

General procedure for the synthesis of [^{18}F]fluoro-triazole-peptides

A total of 0.5-mg peptide was added to the reactor vial, followed by a solution of [^{18}F]FNPB in MeOH/H_2O (50:50, 1 mL). Copper sulfate and sodium ascorbate were added to the aforementioned solution, and the mixture was stirred vigorously at room temperature for 15 min. The reaction was then quenched and analyzed by HPLC.

Result and discussion

We first tried to use 4-fluorophenylacetylene as a click labeling prosthetic group, but our attempts to convert 4-nitrophenylacetylene into 4-[^{18}F]fluorophenylacetylene using [^{18}F]KF/$K_{2.2.2.}$ resulted in no radiolabeled product. In order to improve the fluoride

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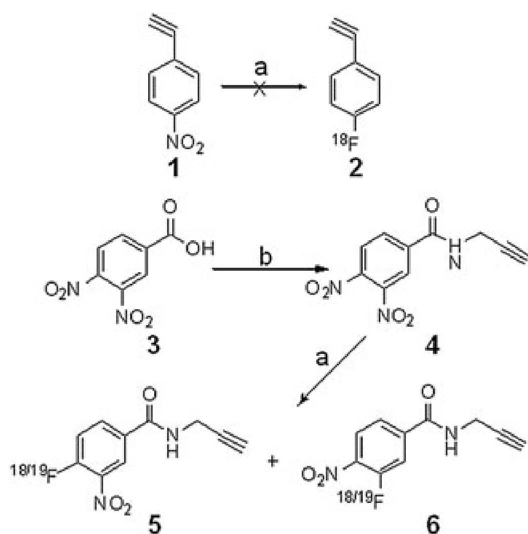
substitution efficiency, we studied the reports by Sun and DiMagno; they suggested that the aromatic ring should bear at least one electron-withdrawing group that is more potent than the ester group in order for the substitution of nitro group by fluoride.[38,39] Therefore, we designed compound **4** as a precursor for ^{18}F-labeling. As shown in Scheme 1, compound **4** was obtained by using acylation of 3,4-dinitrobenzoic acid with 2-propynylamine in 63% yield.

Cold labeling experiments by using [^{19}F]KF/$K_{2.2.2}$ indicated that there would be two reaction products: [^{19}F]FNPB and 3-[^{19}F]fluoro-4-nitro-*N*-2-propyn-1-yl-benzamide **(6)**. But we can control the yield of the undesirable byproduct compound **6** by lowering the reaction temperature.

For the radiolabeling experiment according to Scheme 1, the resulted [^{18}F]FNPB was purified by semipreparative HPLC. The fraction that peaked at around 18.6 min was identified as the desirable product based on its co-injection with the ^{19}F reference compound (Figure 1). They were collected and dried for the next click labeling step. The radiochemical yields as well as the radiochemical purity were reported in Table 1.

With the increase of reaction temperature, the yield of ^{18}F-labeling would improve (Table 1). But because we found that at temperatures above 100°C, the yield of the byproduct **6** would also increase dramatically. So we select the reaction condition of 90°C and 10 min. It should be noted that there would be some lose during the HPLC purification, in a typical experiment, 166 MBq of [^{18}F]FNPB with a specific activity > 350 GBq/μmol could be obtained from 370 MBq [^{18}F]fluoride in about 40 min. The stability of [^{18}F]FNPB was investigated in mouse plasma at 37°C and analyzed by radio-HPLC. Plasma studies with [^{18}F]FNPB revealed no detectable radiodefluorination over 2 h.



Scheme 1. Synthesis of FNPB. Reagents and conditions: (a) [18/19F]KF, Kryptofix, 90°C, 10 min.; (b) 2-Propynylamine, HOBt, EDC.HCl, rt., 24 h.

Table 1. Different reaction temperatures of 4-[^{18}F]flouro-3-nitro-*N*-2-propyn-1-yl-benzamide were studied

Temperature	Yield (RCY)[1] (%)	RCP[2] (%)
85	67.3	98
90	82.5	98
95	83.7	98
100	88.6	98

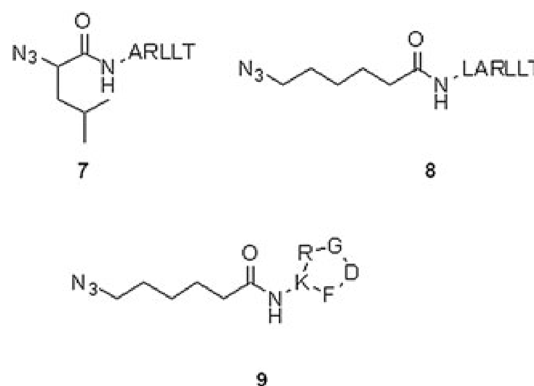
N ≥ 3
[1]The radiochemical yield was determined on the basis of the HPLC analysis.
[2]Radiochemical purity was analyzed by radio-HPLC.

The [^{18}F]FNPB precursor obtained was then used to label peptide probes containing the azide group. We here explored the radiolabeling of the D4 peptide (Leu-Ala-Arg-Leu-Leu-Thr) and cRGDfK. The D4 peptide was identified as an EGFR targeting ligand, and cRGDfK is a widely used ligand for $\alpha_v\beta_3$ integrin.[40,41] We modified the peptides with azido-Leu or 6-Azidohexanoic acid to facilitate click labeling. The azido-peptides were synthesized by standard solid-phase peptide synthesis strategy on resin (Scheme 2).

The click labeling of the peptides with [^{19}F]FNPB was evaluated using Cu^{2+}/sodium ascorbate as catalysts, and the resulted ^{19}F-peptides were purified by HPLC and confirmed by MS. For the radiolabeling experiments using [^{18}F]FNPB (Table 2), the reaction was performed in methanol and water (50:50) at room temperature for 15 min under vigorous mixing. The reaction products were again purified by the semipreparative HPLC to obtain ^{18}F-labeled peptide probes.

The quantity of catalysts was optimized. We observed that the reaction time could be dramatically shortened and the yield increased in the presence of higher concentration of Cu^{2+}. Figure 2 plotted the effect of Cu^{2+} concentration on the reaction yield. At low Cu^{2+} concentrations (≤0.1 equiv.), there was hardly any click labeling product found. At high Cu^{2+} concentrations (~0.6 equiv of $CuSO_4$), the radiolabeling yield was 88% within 15 min.

The radiolabeling yield of different peptide structures (8 and 9) using the same procedure all resulted in high yield of close to 90% (Table 2). The high radiolabeling efficiency was quite significant as compared with about 37% reported by Vaidyanathan G.[35] In addition, we did not observed the crucial effect of peptide concentration as reported by many other studies.[4,35] Although a concentration of 12.5 mg/mL is in the range typically used for peptide labeling reactions with [^{18}F]SFB as the most commonly employed prosthetic group, we could obtain as high as 80% radiochemical yield using a relatively low peptide concentration of 0.5 mg/mL. The final ^{18}F-labeled peptides can be obtained after semipreparative HPLC (see supporting information Figure 2, 3, 4).



Scheme 2. The structure of the different peptides

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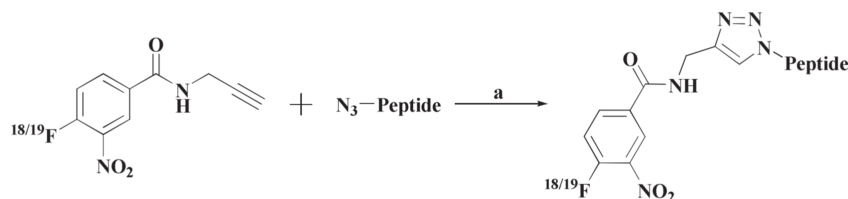
Table 2. Click labeling of different azido-peptides with [^{18}F]FNPB



Entry	Azido-peptide	Product	Yield (%)[2]
1	7	[^{18}F]fluoro-triazole-7	93
2	8	[^{18}F]fluoro-triazole-8	92
3	9	[^{18}F]fluoro-triazole-9	87

[1]Reaction conditions: 0.5-mg peptide, [^{18}F]FNPB, $CuSO_4{\cdot}5H_2O$ (0.6 equiv), sodium ascorbate (1.8 equiv), Methanol/H_2O = 50:50, rt., 15 min.
[2]The yield was determined on the basis of the HPLC analysis.

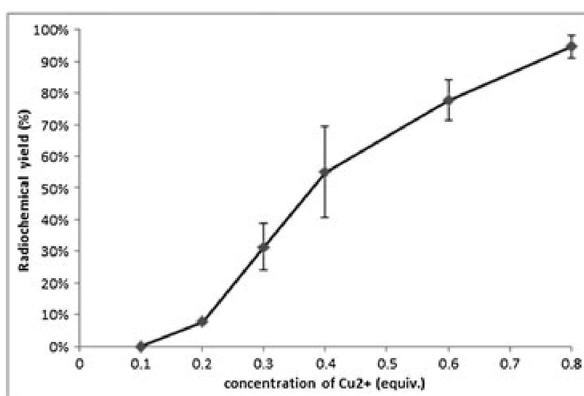


Figure 2. Influence of the concentration of Cu2+ on the click chemistry reaction between 7 and [18F]FNPB ($n{\geq}3$).

We will be looking into the *in vivo* stability and PET imaging properties of these peptide probes in further studies.

The current process required HPLC purification twice, which was somewhat time-consuming. We are trying to utilize the quaternary ammonium salt as the prosthetic group in order to separate the [^{18}F]FNPB by use of SEP-PAK C_{18} cartridges (Waters Corporation, USA).

Conclusions

We have developed a one-step procedure for radiosynthesis of [^{18}F]FNPB as a useful prosthetic group for radio-labeling of peptides via click chemistry with high radiochemical yield. With ^{18}F attached to an aryl sp2 carbon, the structure was shown to be stable in plasma. We hope it will have general applications in labeling peptides with high radiochemical yield for PET imaging studies.

Acknowledgements

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Conflict of Interest

The authors declare that they have no conflict of interest.

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