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A fast, simple, and reproducible automated synthesis of [^{18}F]FPyKYNE-c(RGDyK) for $\alpha_v\beta_3$ receptor positron emission tomography imaging

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[^{18}F]FPyKYNE-c(RGDyK) was successfully synthesized by the Cu(I) catalyzed Huisgen 1,3-dipolar cycloaddition of alkynes to azides using [^{18}F]FPyKYNE as a prosthetic group in an overall radiochemical yield of 12%–18% (decay-corrected) and >99.5% chemical and radiochemical purities in 125 min including quality control. This simple, fully automated two-step, two-reactor approach consists of a quick and convenient purification of the prosthetic group using silica gel cartridges and its subsequent use for the labeling of the azido-c(RGDyK) peptide via click chemistry.

Keywords: $\alpha_v\beta_3$ integrin receptors; ^{18}F-labeling; [^{18}F]-FPyKYNE; c(iRGDyK); PET

Introduction

Angiogenesis or the formation of new blood vessels plays a crucial role in tumor vasculature and in various human heart diseases including cardiomyopathy, atherosclerosis, peripheral artery disease, ischemia, inflammation, and atherosclerotic lesions.[1–3] In a cardiovascular setting, angiogenesis is triggered by hypoxia and ischemia, and its major action in tissues is the restoration of perfusion and oxygenation. Among other important biomarkers of angiogenesis, cell surface integrin receptors are of particular interest. Between all of the integrins discovered to date, integrin $\alpha_v\beta_3$ is the most extensively studied. Integrin $\alpha_v\beta_3$ receptors bind to peptides containing the amino acid sequence arginine-glycine-aspartic acid (RGD) present in the extracellular matrix. The goal of pro-angiogenic therapy in the treatment of ischemic diseases is to stimulate the growth of new blood vessels in order to improve tissue vascularization and function.[1–4] However, the lack of a reliable, easily applicable, and noninvasive technique to assess the presence of angiogenesis is a limiting factor in evaluating the therapeutic efficacy of angiogenesis targeted therapies.[1–3]

Several groups have investigated the imaging of integrin $\alpha_v\beta_3$ receptors with RGD containing peptide-based positron emission tomography (PET) tracers.[5–21] Most of these radiolabeled peptides show high affinity and selectivity for the $\alpha_v\beta_3$ integrin receptor *in vitro* and receptor-specific accumulation in tumors *in vivo*. The most evaluated of these radiotracers is ^{18}F-Galacto-RGD[22], which has also been investigated in humans.[19,20] The long, complex, multistep synthetic approach renders their production difficult to bring to a clinical setup. We present here a simple and convenient strategy consisting of a two-step, two-reactor synthesis of an ^{18}F-labeled RGD peptide in a fully automated process using the click chemistry approach. This approach will facilitate the routine production of RGD derivatives for PET imaging research.

Experimental

General methods

2-Nitro-hydroxypyridine, 5-chloropent-1-yne, pentane, diethyl ether, acetonitrile (MeCN), dimethylformamide, dimethyl sulfoxide (DMSO), (+)L-sodium ascorbate, copper sulphate ($CuSO_4$), and Kryptofix®222 (K_{222}: 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8] hexacosane) were purchased from Sigma–Aldrich (Canada Ltd.). Azido-cyclic RGDyK (Azido-cyclic Arg-Gly-Asp-D-Tyr-Lys) was purchased from FutureChem Co. Ltd. (Seoul, South Korea). Solid phase extraction cartridges Chromafix® were purchased from Macherey–Nagel (Duren, Germany) and Sep-Pak® Plus silica cartridges 55–105 μm were acquired from Waters (USA). All other chemicals were purchased from standard commercial sources (VWR or Fisher Scientific) and used without further purification. Semipreparative HPLC for the labeled peptide was performed using a C18-reverse-phase column (Luna, 10 μm,

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250 × 10 mm, Phenomenex, USA) with a radiation detector using acetonitrile/ammonium formate 0.1 M, 25/75, flow rate 5 mL/min, UV at 254 nm. Analytical HPLC was carried out on a C18-reverse-phase column (Luna, 10 μm, 4.6 × 10 mm, Phenomenex, USA) using acetonitrile/ammonium formate 0.1 M, 25/75, flow rate 2 mL/min, UV at 254 nm. Furthermore, two additional reverse-phase analytical columns Luna C18 ODS and SCX (10 μm, 4.6 × 25 mm, Phenomenex, USA) were used for product identification. Nuclear magnetic resonance (NMR) was performed using a 500 MHz Bruker Avance (Bruker BioSpin Ltd., ON, Canada) instrument and mass spectrometry analyzed with a quadrupole time-of-flight (QTOF) spectrophotometer.

2-Nitro-3-pent-4-yn-1-yloxypyridine

The nitro precursor for ^{18}F-labeling was synthesized as previously described by Kuhnast *et al.*[23] The ^{1}H and ^{13}C NMR data matched those reported earlier.[23] EI-MS (*m/z*): calculated for $C_{10}H_{10}KN_2O_3$: 245.033; found 245.030 [M + K].

2-Fluoro-3-pent-4-yn-1-yloxypyridine, (FPyKYNE)

A mixture of potassium fluoride (KF) (0.1 g, 1.74 mmol) and K_{222} (0.6 g, 1.74 mmol) in anhydrous DMSO (5 mL) was stirred at 150°C for 10 min under argon. Subsequently, the 2-nitro-3-pent-4-yn-1-yloxypyridine (0.3 g, 1.45 mmol) was added to the solution. The reaction mixture was left stirring for 1 h. After cooling, water (100 mL) was added and the mixture was extracted with ethyl acetate (100 mL × 3). The organic phases were combined, dried over Na_2SO_4, and the solvent was concentrated under vacuum followed by purification using flash column chromatography with hexanes/ethyl acetate (8.5/1.5) to afford FPyKYNE (0.27 g, 1.49 mmol, 86%) as crystalline oil. The ^{1}H and ^{13}C NMR data matched those reported earlier.[23] ^{19}F-NMR: δ_F: −84.7899 (referenced with respect to trifluoroacetic acid at −76.55 ppm). EI-MS (*m/z*): calculated for $C_{10}H_{10}FNO$ 179.075; found 179.076 [M^+]

FPYKYNE-c(RGDyK)

To a mixture of FPyKYNE (0.001 g, 5.6 μmol) and azido-c(RGDyK) peptide (0.001 g, 1.2 μmol) in 600 μL of DMSO/water (1/1), $CuSO_4$ (1.2 μmol in water) and sodium ascorbate (3.8 μmol in water) were added and this mixture was heated at 40°C. The conversion of the azido-c(RGDyK) to the FPYKYNE-c(RGDyK) was followed by analytical HPLC, with all the azide-peptide (t_R = 3 min) converted to FPyKYNE-c(RGDyK) (t_R = 6.9 min) after 20 min. Purification of FPyKYNE-c(RGDyK) (t_R = 8 min) was achieved by semipreparative HPLC. The identity of FPyKYNE-c(RGDyK) was characterized by electrospray ionization mass spectrometry. Calculated for $C_{35}H_{47}N_{12}O_9$ [M + H]: 958.443; found: 958.446.

[^{18}F]FPyKYNE-c(RGDyK)

The fully automated radiosynthesis and semipreparative HPLC of [^{18}F]FPyKYNE-c(RGDyK) was carried out in a dual reactor TRACERlab® FX N Pro, (GE Healthcare) (Figure 1). [^{18}F]Fluoride was trapped on an anion exchange resin and eluted from the cartridge into reactor-1 with a solution of K_2CO_3 (0.2 mmol) and K_{222} (0.4 mmol) in 95% MeCN in water. The resulting solution was then concentrated to dryness with a stream of N_2 at 110°C under vacuum for 3 min. This procedure was repeated twice to afford the dried K[^{18}F]F-K_{222} complex in reactor-1. The precursor (2-nitro-3-pent-4-yn-1-yloxypyridine) (6.0 mg, 0.03 mmol) in DMSO (600 μL) was added to reactor-1, and the reaction mixture was heated at 155°C for 10 min. The reaction vessel was cooled to 35°C using a stream of air and the mixture transferred onto a column of silica gel cartridges (n = 3) connected in series. Our strategy for the purification of [^{18}F]FPyKyNE is based on the difference in the retention factor (Rf) between the 2-nitro-3-pent-4-yn-1-yloxypyridine used as the precursor (Rf = 0.22) and FPyKYNE (Rf = 0.57) in pentane/ether, 1/1. [^{18}F]FPyKYNE was eluted using 5.5 mL of pentane/ether (1/1) into reactor-2, with the unreacted precursor trapped on silica, and the solvent evaporated under a stream of N_2 at 33°C. A second elution of [^{18}F]FPyKYNE from

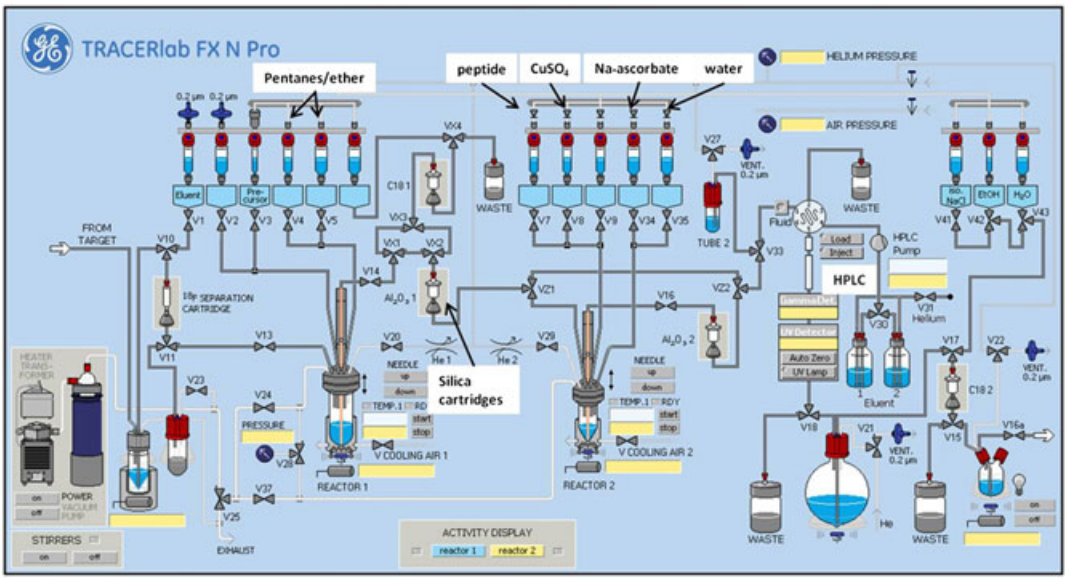


Figure 1. Graphical display of the TRACERlab FX N Pro.

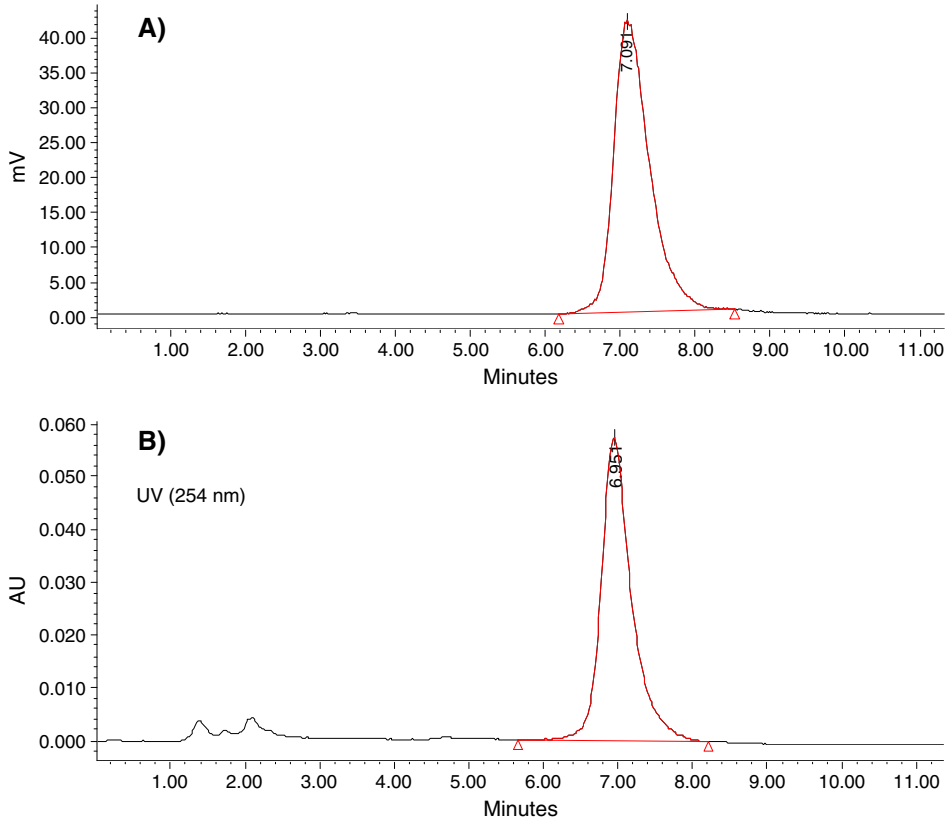
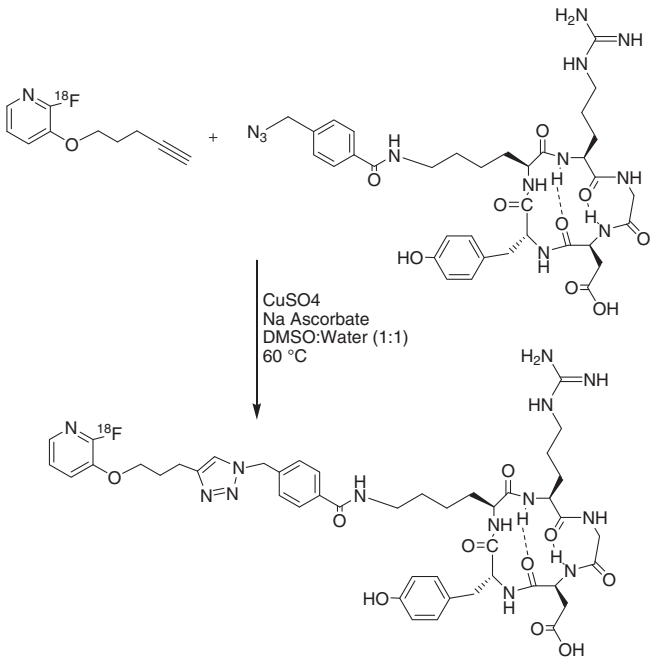


Figure 2. (A) Radioactivity and (B) UV (254 nm) chromatograms of [18 F]FPyKYNEc(RGDyK).

the cartridges was performed with 3 mL of the pentane/ether solution into reactor-2. After complete evaporation of the solvent in reactor-2, azido-c(RGDyK) (0.5 mg, 2.8 μmol), $CuSO_4$ (4 μmol), sodium ascorbate (12 μmol) were added to a final volume of 1 mL in a mixture of DMSO/water (1/1). The reaction mixture was left reacting at 65°C for 20 min and subsequently purified by semipreparative HPLC. The fraction containing [^{18} F]FPyKYNE-c(RGDyK) peptide was collected. The solvent was evaporated under vacuum at 80°C and reformulated in saline using an in-house rotary evaporator system. Analytical HPLC was performed to determine the chemical and radiochemical purities of [^{18} F]FPyKYNE-c(RGDyK), as well as for the determination of the specific activity using the reference compound FPyKYNE.

Results and discussion

FPyKYNE-c(RGDyK) peptide (standard) was synthesized via 'click chemistry' reaction between the azido-c(RGDyK) peptide and FPyKYNE. [^{18} F]FPyKyNE was prepared via aromatic nucleophilic substitution in reactor-1 with a modification performed to the purification method. Instead of utilizing HPLC, a rapid purification was performed using three silica gel cartridges connected in series, acting as a flash column chromatography for ease of separation. Elution of pure [^{18} F]FPyKyNE was performed using a 50% mixture of pentane and ether into reactor-2. These low boiling points solvents can be easily evaporated. On average, 100–200 mCi was obtained after evaporation of the eluent in about 20–25 min (20%–35% decay-corrected radiochemical yield). Subsequent labeling of the azido-c(RGDyK) peptide with [^{18} F]FPyKYNE was carried out in a mixture of DMSO/water in the presence of $CuSO_4$ and sodium ascorbate (Figure 2). [^{18} F]FPyKYNE-c(RGDyK) was purified by reverse-phase semipreparative HPLC and obtained in 12%–18% radiochemical yield (decay-corrected from



Scheme 1. 18 F-Labeling of the azido-c(RGDyK) with [18 F]FPyKYNE via click Chemistry.

end of beam; $n = 6$) with a total preparation time of 125 min, including synthesis and quality control. Typically, 45–60 mCi of purified labeled peptide with specific activity of 980–2000 mCi/μmol were obtained with >99% radiochemical purity (Scheme 1). The identity of the [^{18}F]FPyKYNE-c(RGDyK) peptide was confirmed by co-injection with the reference FPyKYNE-c(RGDyK) peptide in three different analytical reverse-phase HPLC columns.

Conclusion

A fast, reproducible and simple two-step automated method was successfully developed for the preparation of [^{18}F]FPyKYNE-c(RGDyK) peptide. Our strategy includes rapid purification of the prosthetic group [^{18}F]FPyKYNE and its subsequent attachment to an azido-c(RGDyK) derivative via 'click chemistry' in high yield, radiochemical and chemical purities. The straightforward synthesis of the novel [^{18}F]FPyKYNE-c(RGDyK) can be brought to a routine clinical setup using the approach presented here.

Acknowledgements

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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