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Fully Automated radiosynthesis of [¹⁸F]Fluoro-C-glyco-c(RDGfC): Exploiting all the abilities of the AllInOne synthesizer[†]

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Abstract

Radiolabelled peptides are crucial tools for biomedical research purposes. Peptides are usually labeled with fluorine-18 via an indirect prosthetic group approach, which requires a complex multistep procedure not easily automatable. However, automation is a compulsory step of paramount importance to produce radiotracers with high reliability and minimal human involvement. Herein, we describe the fully automated radiosynthesis of [¹⁸F]fluoro-C-glyco-c(RDGfC) on an AllInOne (AIO) synthesizer, exploiting for the first time all the abilities of the AIO module. The radiotracer was prepared in high radiochemical purity via a challenging three-steps sequence in 140 minutes with a decay-corrected radiochemical yield of $3.6 \pm 0.4\%$. Presenting a high stability and an improved hydrophilicity, [¹⁸F]fluoro-C-glyco-c(RDGfC) could become a valuable tool in PET imaging for cancer diagnosis. Furthermore, the described automated procedure is modular and could be adapted to other prosthetic groups and/or peptides with minimal changes to afford a large panel of ¹⁸F-radiolabeled peptides.

Introduction

Positron Emission Tomography (PET) is the most sensitive imaging modality providing a specific view of biological processes and allowing an early diagnosis of various diseases.^{1,2} Utilization of PET for molecular imaging relies on the availability of suitable radiotracers.

PET chemistry has therefore evolved to simplify complex chemical and radiochemical processes to aid in the development of new PET radiotracers

Peptides are attracting increasing interest because of their *in vivo* therapeutic efficacy due to their favorable properties, which include high target specificity with low toxicity but also biocompatibility and biodegradability.³⁻⁵ This has prompted researchers to develop radiolabeled peptides for PET.⁶⁻⁸ For example, cyclic arginine-glycine-aspartic acid(RGD)-containing peptides have been the focus of intense investigations toward the development of PET radiotracers in cancer diagnosis because they bind with high specificity and affinity to $\alpha_v\beta_3$ integrin.⁹⁻¹⁵ Several labeled cyclic RGD peptides have been investigated and some of them are nowadays employed in clinical use for PET imaging of angiogenesis in cancer.^{11,12,15-18}

Fluorine-18 (¹⁸F) is a commonly used radioelement for peptide radiolabeling because of its ease of production, low positron energy, high specific activity and appropriate half-life ($t_{1/2} = 109.8$ min) compatible with multistep synthesis.^{19,20} However, ¹⁸F-radiolabeling of biomolecules, *i.e.* peptides, remains a challenge because its incorporation into organic compounds often requires basic conditions and high temperatures, incompatible with their sensitivity. Direct ¹⁸F-fluorination of peptides in mild conditions is reported in few examples.²¹⁻²⁶ The most commonly used strategy for ¹⁸F-fluorination of peptides adopt an indirect approach^{27,28} involving a prosthetic group. In this strategy, a small molecule is easily radiolabeled by [¹⁸F]fluoride and then conjugated in mild conditions with biomolecules of interest. Copper(I) catalyzed azide-alkyne cycloaddition (CuAAC), a click version of the Huisgen cycloaddition,^{27,29-32} is widely applied in this approach. The production of ¹⁸F-labeled peptides requires a multistep radiosynthesis developed by expert radiochemists.

A fully automated radiochemical sequence will enhance reproducibility and reliability, promoting [¹⁸F]labeled peptide translation from pre-clinical to clinical studies. Automation is thus a crucial step in radiochemistry development to produce the final radiotracer with minimal human intervention. However, due to its complexity, fully or almost fully automated ¹⁸F-labeled-peptide radiosynthesis via a prosthetic approach was only scarcely described.³³⁻³⁷ Most of these examples have required some form of manual handling, for reformulation or final purification particularly. Indeed, multistep radiosyntheses are not easily automatable and only few synthesizers have the versatility to implement such sequence. Among the commercially available synthesizers, the AllInOne (AIO) module from Trasis[®] could easily implement a large panel of radiochemical sequences thanks to two heaters and built-in HPLC system with two HPLC column positions.

Herein, we present a challenging fully automated radiosynthesis of [^{18}F]fluoro-*C*-glyco-*c*(RDGfC) on an AllInOne synthesizer. A [^{18}F]fluoro-*C*-glycoside based prosthetic group was used for indirect peptide radiofluorination. It is worthy to note that introduction of glycoside on biomolecules *i.e.* peptides has often been shown to enhance their pharmacokinetic and *in vivo* clearance properties,³⁸⁻⁴⁰ notably in the case of RGD derivatives.^{15,41-43} This is of major interest, in particular for molecular imaging. The subsequent ligation by CuAAC of [^{18}F]fluoro-*C*-glycoside to a cyclic RGD-containing peptide generated [^{18}F]fluoro-*C*-glyco-*c*(RDGfC) which could become an efficient radiotracer in PET imaging for cancer diagnosis. This multistep radiosynthesis exploits for the first time, to the best of our knowledge, all the abilities of the AIO module.

Experimental

Materials and methods

Solvents and liquid reagents were purified and dried according to recommended procedures. Thin layer chromatography (TLC) analyses were performed using standard procedures on Kieselgel 60F254 plates (Merck). Compounds were visualized using UV light (254 nm) and a methanolic solution of sulfuric acid and charred. Column chromatography was performed on silica gel SI 60 (63–200 μm) (Merck). Purification of glycopeptide was achieved by gel filtration on LH20 using water as eluent. Melting points were determined with a Tottoli apparatus and are uncorrected. FTIR spectra were recorded on a Shimadzu IRAffinity-1, ATR PIKE Technologies model GladiAT (cm^{-1}). Optical rotations were measured on an Anton-Paar MCP 300 polarimeter. Analytical High performance liquid chromatography (HPLC) analyses were run on a Waters system (2695eb pump, auto sampler injector, 2998 PDA detector and NaI detector from Berthold [Bad Wildbad, Germany]) controlled by the Empower Software (Orlando, FL, USA). Analyses were performed on a Vydac 218TP C18 (5 μm , 250 \times 4.6 mm) from Grace with ACN/H₂O/TFA mixture (proportions given in brackets) at 1 mL/min. Integrated HPLC system on AllInOne synthesizer was equipped with a radioactivity detector, Smartline UV detector 200 (Knauer, Berlin, Germany) at 254 nm and a HPLC pump P4.1.5 (Knauer, Berlin, Germany). Semi-preparative HPLC were performed on an Alltima C8 endcapped (5 μm , 150 \times 10 mm) from Grace (HPLC n^o1) and on a Vydac 218TP C18 (5 μm , 250 \times 10mm) from Grace (HPLC n^o2) with ACN/H₂O or ACN/H₂O/TFA mixture (proportions given in brackets) at 4.5 or 3 mL/min respectively. ^1H , ^{13}C , and ^{19}F NMR spectra were recorded on a Bruker spectrometer DPX250 (250 MHz, 62.9 MHz and 235 MHz,

respectively) or Avance III 400 (400 MHz and 100.6 MHz, respectively). For complete assignment of ^1H and ^{13}C signals, two-dimensional ^1H , ^1H COSY and ^1H , ^{13}C correlation spectra were recorded. Chemical shifts (δ) are given in parts per million relative to the solvent residual peak. For clarity, atom numbering starts from the end of the anomeric arm rather than resulting from the IUPAC naming of compounds (cf SI, fig S1). The following abbreviations are used for multiplicity of NMR signals: s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet, b = broad signal and app = apparent multiplicity. The given J values refer to apparent multiplicities and do not represent the true coupling constants. Mass and high resolution mass spectrometry spectra were recorded on a Bruker microTOFQ apparatus and MALDI spectra on a Bruker Ultraflex apparatus. 0.9% Sodium chloride (NaCl) solution for injections was purchased from Fresenius Kabi[®], sterile water for injections (WFI) Macoflex N from Macopharma[®] and sterile product vials from Cis-Bio international[®] (Tec-Elu-5). ^{18}O -enriched water was supplied by CortecNet[®], Sep-Pak[®] QMA-carbonate, silica and Oasis HLB from Waters Corporation[®] and BD luer lock syringes from Dutscher[®]. All the reagents and materials were used as received. No-carrier-added fluoride-18 was produced via the ^{18}O (p,n) ^{18}F nuclear reaction on a PET Trace cyclotron (GE). The bombardment was performed at 10 μA during 5 minutes to provide about 5 GBq of fluoride-18 delivered as a solution in ^{18}O -enriched water (1.6 mL). Radiosynthesis was carried out on an AIO module from Trasis[®].

Radiolabeling precursor and non radioactive prosthetic group reference syntheses

4,8-anhydro-1-azido-1,2,3-trideoxy-5,6,7-tri-*O*-acetyl-D-*glycero*-D-*gulo*-nonitol (**1**), 4,8-anhydro-1-azido-1,2,3-trideoxy-9-*O*-trifluoromethylsulfonate-5,6,7-tri-*O*-acetyl-D-*glycero*-D-*gulo*-nonitol (**2**, radiolabeling precursor), 4,8-anhydro-1-azido-1,2,3,9-tetradeoxy-9-fluoro-5,6,7-tri-*O*-acetyl-D-*glycero*-D-*gulo*-nonitol (**3**) and 4,8-anhydro-1-azido-1,2,3,9-tetradeoxy-9-fluoro-D-*glycero*-D-*gulo*-nonitol (**4**, non radioactive prosthetic group reference) were synthesized according to our previously reported methods.⁹

Glycopeptide synthesis

S-propargyl-c(RGDfC) **5** and cyclo[L-arginylglycyl-L- α -aspartyl-D-phenylalanyl-S-[[1-[1-(4,8-anhydro-5-fluoro-5,1,2,3-trideoxy-D-*glycero*-D-*gulo*-nonitol)]-1*H*-1,2,3-triazol-4-yl]methyl]-L-cysteinyl] **6** were synthesized according to our previously reported methods.^{9,29}

Synthesizer

The AllInOne synthesis module is a very versatile synthesizer equipped with two arrays of eighteen rotary actuators having multiple positions, two heaters and seven radiation detectors. A built-in HPLC system with two HPLC column positions is integrated with UV detector and radioactivity detector. AIO operates with single use cassettes (structured with three-way valve manifolds) and reagent sets, facilitating GMP compliance and preventing cross-contamination. The parts available in bulk allow the assembly of these cassettes for research purpose on which various components can be plugged: spikes (for reagent vials), SPE cartridges, syringes or tubes. All these materials are compatible with acids, bases and organic solvents and are suitable for medical devices. The user-friendly software reproduces the picture of the cassette layout (Figure 1) and shows in real time all the movements of the machine during the radiotracer production.

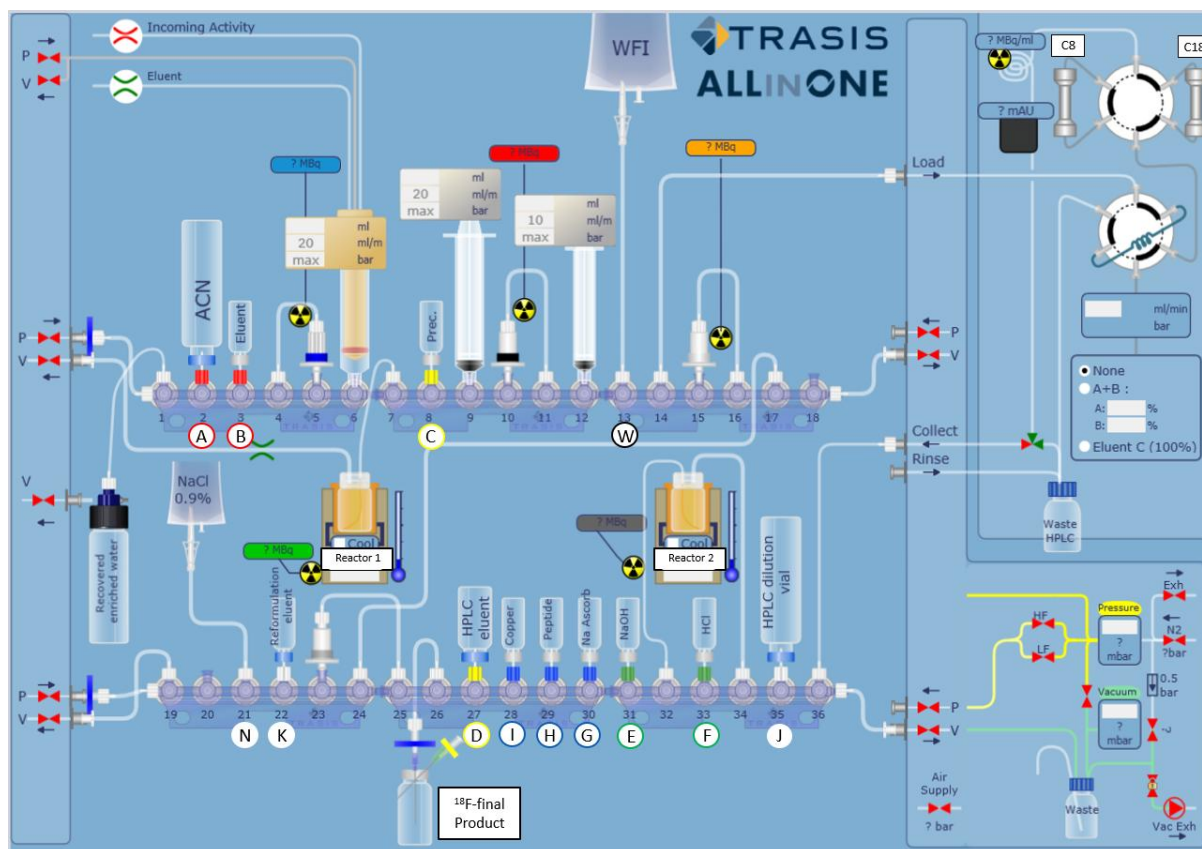


Fig. 1 Schematic representation of the AIO module used for the preparation of [^{18}F]6.

Cassette preparation

Before the activity reception, the module performed automatically the internal and the cassette tests. Columns and eluents for the semi-preparative HPLC were installed for the 1st HPLC (column: Alltima C8, eluent: ACN/H₂O 55/45 v/v) and for the 2nd HPLC (column: Vydac 218TP C18, eluent: ACN/H₂O 17/83 v/v with 0.1% of TFA). The single use cassette was built

from scratch with two arrays of three manifolds. Various components were plugged on the cassette as described Figure 1: TPX spikes, SPE cartridges, luer-lock syringes (BD) and silicone tubing equipped with male or female luer lock connector. Crimped glass vials of 50 mL (vials A, J and D), of 4 mL (vials B, C and K) and of 1.2 mL (vials E, F, G, H and I) were plugged on spikes. All materials were single use and were suitable for medical devices. The list of reagents and materials used for the automated procedure are described in Table 1.

Table 1 Preparation details for the radiosynthesis of [^{18}F]6 on the AIO synthesizer.

Position	Reagents or material	Quantities
P2	Dry acetonitrile (ACN) / Vial A	10 mL
P3	$\text{K}_{222}/\text{K}_2\text{CO}_3$ (5.3 mg/1.3 mg) in ACN/ H_2O (8/2 v/v) / Vial B	1 mL
P4-P5	Sep-Pak® Light QMA-carbonate	1
P6	Plunger (20 mL) / SA2	1
P8	Precursor 2 / Vial C	5 mg
P9	Syringe of 20 mL / SA3	1
P10-P11	Sep-Pak® silica cartridge	1
P12	Syringe of 10 mL / SA4	1
P13	WFI (Water for injection) Bag / Bag W	1
P15-P16	Cation exchange (Chelex®) cartridge	1
P21	0.9% Sodium chloride bag / Bag N	1
P22	Ethanol/ NaCl 0.9% (5/5 v/v) / Vial K	2 mL
P23-P25	Oasis HLB cartridge	1
P27	ACN/ H_2O (55/45 v/v) / Vial D	10 mL
P28	$\text{Cu}(\text{OAc})_2$ in H_2O (3.3 μmol) / Vial I	0.3 mL
P29	Propargylated c(RGDfC) 5 in H_2O (0.5 μmol) / Vial H	0.3 mL
P30	Sodium ascorbate in H_2O (6.6 μmol) / Vial G	0.3 mL
P31	NaOH 1M / Vial E	0.3 mL
P33	HCl 1M / Vial F	0.3 mL
P35	H_2O Dilution Vial / Vial J	10 mL

The precursor 2 (vial C, P8) was diluted with 2 mL of dry ACN (vial A, P2) before activity reception. The module was then ready to receive activity for radiosynthesis.

Radiosynthesis

[¹⁸F]6 was synthesized using an original and fully automated in-house reaction sequence described in Table 2.

Table 2 Reaction sequence for [¹⁸F]6 radiosynthesis

Fluorination of prosthetic group precursor 2
1 - [¹⁸ F]F ⁻ trapping on QMA carbonate cartridge 2 - [¹⁸ F]F ⁻ elution with eluent 3 - Azeotropic evaporation at 110°C for 10 min to give dry K[¹⁸ F]F-K ₂₂₂ complex 4 - Addition of precursor 2 into the reactor 1 5 - [¹⁸ F]Radiofluorination at 95°C for 5 min 6 - Pre-purification on silica cartridge
Purification of [¹⁸F]3 on 1st semi-preparative HPLC
1 - Injection of crude product on semi-preparative HPLC 2 - Collection of [¹⁸ F]3 into the reactor 2
Preparation of ¹⁸F-fluoro-C-glycoside based prosthetic group [¹⁸F]4
1 - Evaporation of mobile phase 2 - Saponification with 1M NaOH at 30°C for 9 min 3 - Neutralization with 1M HCl
Preparation of the [¹⁸F]F-C-glyco-c(RGDfC) [¹⁸F]6
1 - Addition of Na-ascorbate 2 - Addition of propargylated peptide 5 3 - Addition of Cu(OAc) ₂ 4 - Coupling at 55°C for 25 min
Purification of the glycopeptide [¹⁸F]6
1 - Injection of crude product on 2 nd semi-preparative HPLC 2 - Collection of [¹⁸ F]6 into the dilution vial
Formulation of [¹⁸F]6
1 - Trapping of [¹⁸ F]6 on Oasis HLB cartridge 2 - Washing of the cartridge with WFI 3 - Elution with ethanol/NaCl 0.9% mixture then with NaCl 0.9% 4 - Recovering of [¹⁸ F]6 into the final product vial

Preparation of K[¹⁸F]F-K₂₂₂ complex. ¹⁸F-fluoride (~ 5 GBq) in [¹⁸O]water was recovered in the AIO synthesizer *via* the plunger (SA2, P6, *vacuum* -200 mbar) by suction and passed through a Sep-Pak[®] light QMA-carbonate cartridge (P5-P4), where [¹⁸F]fluoride was trapped and [¹⁸O]water was collected for recycling (P1). The QMA-carbonate cartridge was then flushed with nitrogen gas flow during 40 seconds by combination of positive and negative pressure (pressure 400 mbar; *vacuum* -300 mbar). The trapped ¹⁸F-fluoride was eluted by suction from the QMA-carbonate cartridge into the reactor 1 (P7, *vacuum* -350 mbar) with the

K_{222}/K_2CO_3 solution (vial B, P3, previously pressurized at 500 mbar). Solvent was removed under a stream of nitrogen gas flow (combination of pressure 15 to 500 mbar and *vacuum* -850 mbar) at 110°C during 10 minutes to give the dried $K[^{18}F]F-K_{222}$ complex. The reactor was then cooled at 75°C to perform the ^{18}F -radiolabeling.

^{18}F -radiolabeling of the labeling precursor 2. The triflated precursor **2** (Vial C, P8) was pressurized (pressure 500 mbar) and was sucked into the reactor 1 (P7, *vacuum* -800 mbar) containing the dried $K[^{18}F]F-K_{222}$ complex. Labeling was performed at 95°C for 5 min in a closed reactor thanks to pinch valves. After cooling at 30°C, the reaction mixture was passed through a Sep-pak[®] plus silica 690 mg cartridge (P10-P11), to remove unreacted ^{18}F -fluoride, and then recovered into SA4 (P12) containing 1.5 mL of WFI (Bag W). The reactor 1 and silica cartridge were rinsed with 1 mL of dry acetonitrile (Vial A, P2) taken with syringe 3 (P9). The eluted solution was recovered into syringe 4 (P12). HPLC loop was preconditioned with 8 mL of HPLC eluent (vial D, P27) via syringe 3 (P9). Crude product in SA4 was transferred into the injection loop of the semi-preparative HPLC and was purified on the first HPLC column (Alltima C8, solvent: ACN/H₂O 55/45, 4.5 mL/min). $[^{18}F]$ **3** was collected ($t_R \sim 7.5$ min) during approximately 40 seconds and recovered into the reactor 2 (P32-P34).

Preparation of ^{18}F -fluoro-C-glycoside based prosthetic group $[^{18}F]$ **4.** HPLC solvent (~3 mL, reactor 2) was evaporated first gently by heating 15 min at 80°C under low pressure (*vacuum* -300 to -1000 mbar), and then under nitrogen gas flow during 1 minute by combination of positive and negative pressure (pressure 250 mbar; *vacuum* -500 mbar). After complete evaporation and cooling at 30°C, saponification of the ^{18}F -glycoside $[^{18}F]$ **3** was performed for 9 min at 30°C with an aqueous NaOH 1M solution (vial E, P31) added by suction (*vacuum* -500 mbar) after pressurization of the vial (pressure 500 mbar). Aqueous HCl 1M solution (vial F, P33) was then added by the same way to neutralize the solution containing $[^{18}F]$ fluoro-C-glycoside based prosthetic group $[^{18}F]$ **4**.

Preparation of the $[^{18}F]F$ -C-glyco-c(RGDfC) $[^{18}F]$ **6.** To $[^{18}F]$ fluoro prosthetic group $[^{18}F]$ **4** (reactor 2, ~850 MBq) were added by suction (*vacuum* -500 mbar), after pressurization of each vial (pressure 500 mbar), sodium ascorbate (vial G, P30), propargylated peptide **5** (vial H, P29) and $Cu(OAc)_2$ (vial I, P28). The solution was heated at 55°C during 25 min under a gentle nitrogen gas flow (pressure 150 mbar; *vacuum* -500

mbar). No solvent evaporation occurred at this step. The elution lines and SA4 were washed with WFI (10 mL, Bag W). The reaction mixture was then diluted with 3 mL of WFI (Bag W, taken with SA4), the solution was passed through a cartridge pre-charged with Chelex[®] 100 resin (V15-V16) to remove copper, and was recovered in syringe 4 (P12). Crude product in SA4 was transferred into the injection loop of the semi-preparative HPLC and was purified on the 2nd HPLC column (Vydac 218TP C18, solvent: ACN/H₂O 17/83 v/v with 0.1% of TFA, 3 mL/min). The collected peak of [¹⁸F]6 (*t_R* ~ 15.0 min) was recovered into the dilution vial containing 10 mL of WFI (vial J, P35). To reformulate [¹⁸F]6, the pressurized mixture (pressure 500 mbar) was trapped on an Oasis HLB cartridge (P25-P23, *vacuum* -100 mbar) by suction. The cartridge was flushed with nitrogen gas flow during 10 s by combination of positive and negative pressure (pressure 500 mbar; *vacuum* -300 mbar) and then washed with 7.5 mL of WFI (Bag W, taken with SA4). The product was eluted from the Oasis HLB cartridge with 0.7 mL of ethanol/NaCl 0.9% 50/50 v/v solution (vial K, P22) taken by SA4 (P12) and recovered into final product vial (P26). The cartridge was rinsed with 1,8 mL of NaCl 0.9% (Bag N, taken with SA4) pushed into final product vial (P26), and then flushed by nitrogen gas flow (pressure 500 mbar) during 10 seconds. [¹⁸F]6 was produced with a decay corrected (d.c.) radiochemical yield of 3.6 ± 0.4% (n = 12).

Quality control

An aliquot of final product [¹⁸F]fluoro-C-glyco-c(RGDfC) [¹⁸F]6 was injected on analytical HPLC (20 µL) for chemical and radiochemical purities determination. A second injection with the corresponding reference compound **6** confirmed the identity of [¹⁸F]6. A Vydac 218TP C18 column was used in isocratic conditions; eluent: ACN/H₂O 17/83 v/v with 0.1% of TFA, flow: 1.0 mL/min and UV detection (230 nm). Molar activity (*A_m*) was checked on analytical HPLC using a standard curve relating mass to UV absorbance. Radiochemical stabilities of the entire preparation of [¹⁸F]6 were tested by HPLC at regular intervals during 120 min. The pH of the solution was determined by applying a drop onto a pH indicator strip and comparing the result with the provided scale.

Partition coefficient (log*D*_{7.4}) measurements

Approximately 5.6 MBq of [¹⁸F]6 was diluted in 2 mL of phosphate-buffered saline (pH 7.4, PBS). Five aliquots from this sample (50 µL) were distributed into five separate Eppendorf tubes containing 500 µL of 1-octanol and 450 µL of PBS. Samples were vortexed for 1 min at

room temperature and then centrifuged for 5 minutes at 4000 rpm. From each sample, 100 μ L samples of both the organic and the PBS layer were transferred into plastic tubes, and the samples were counted with a gamma counter (2470 WIZARD[®], PerkinElmer).

***In vitro* stability in serum**

An aliquot of [¹⁸F]**6** (5.5 MBq each) was added to human serum (200 μ L, 5 samples) and incubated at 37 °C. Samples were collected at various interval times (0, 10, 30, 60 and 120 min) and precipitated using acetonitrile (200 μ L). The samples were centrifuged at 4000 rpm for 5 min, and the supernatant layer was then analyzed by radio-HPLC (column: Vydac 218TP C18 eluted with ACN/H₂O 17/83 v/v with 0.1% of TFA at 1.0 mL/min). The experiments were performed in triplicate.

Results and discussion

Non radioactive syntheses

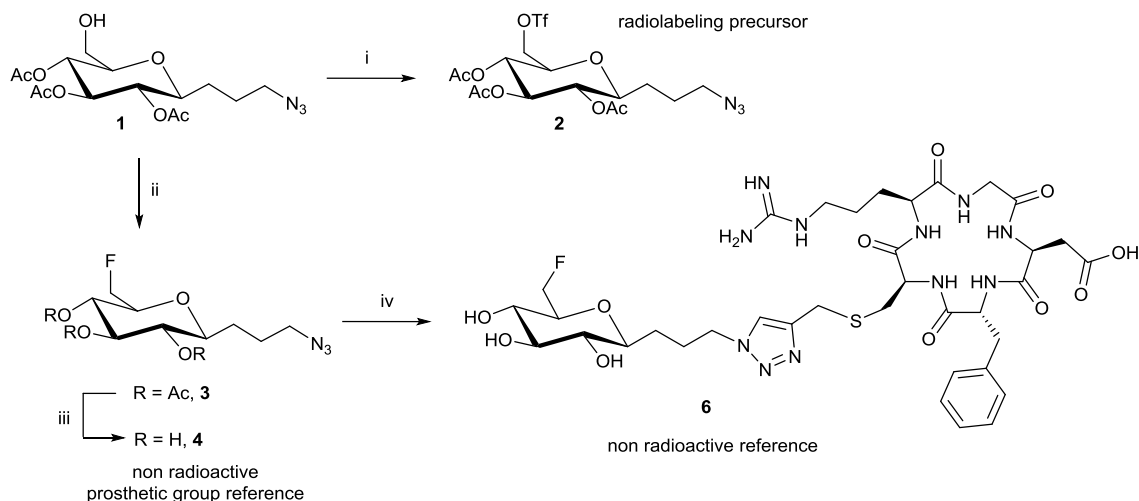
The synthesis of *C*-glycoside based prosthetic group, *i.e.* non-radioactive reference and the corresponding radiolabeling precursor was first investigated. *C*-glycosidic compounds, thanks to their robust C-C link at pseudo-anomeric position, are resistant to acidic and enzymatic hydrolysis and are thus stable *in vivo*.^{9,44,45}

A *C*-glucoside derivative in beta configuration was designed with a spacer-arm bearing an azido group, necessary for the CuAAC. A three carbons spacer-arm has shown a good flexibility and steric arrangement in the final *C*-glyco-c(RGDfC), conferring an affinity in the same range than native c(RGDfC) toward $\alpha_v\beta_3$ integrin.⁹ This is of particular importance, because the introduction of prosthetic group on biomolecules can sometimes generate an affinity decrease.

Radiolabeling precursor **2** was easily obtained from **1** by reacting triflic anhydride with pyridine as base in 80% yield (Scheme 1).⁹ Triflate was chosen as leaving group for its high reactivity. Triflated compounds could be less stable compared to tosylated compounds for example, but **2** was stable several weeks and even months at -20°C. The synthesis of the non-radioactive reference of *C*-glycoside based prosthetic group **4** was carried out starting from alcohol **1** in two steps, by fluorination with DAST followed by Zemplén deprotection (Scheme 1).⁹

S-propargylation of the cysteine residue of c(RGDfC) was performed according to our described method²⁹ allowing to work with non protected peptides and regioselectively providing the S-propargyl-peptide derivative **5**. Non-radioactive reference fluoro-*C*-glyco-

c(RGDfC) **6** was prepared from **5** and C-glycoside reference **4** via CuAAC in good yield (69%).⁹



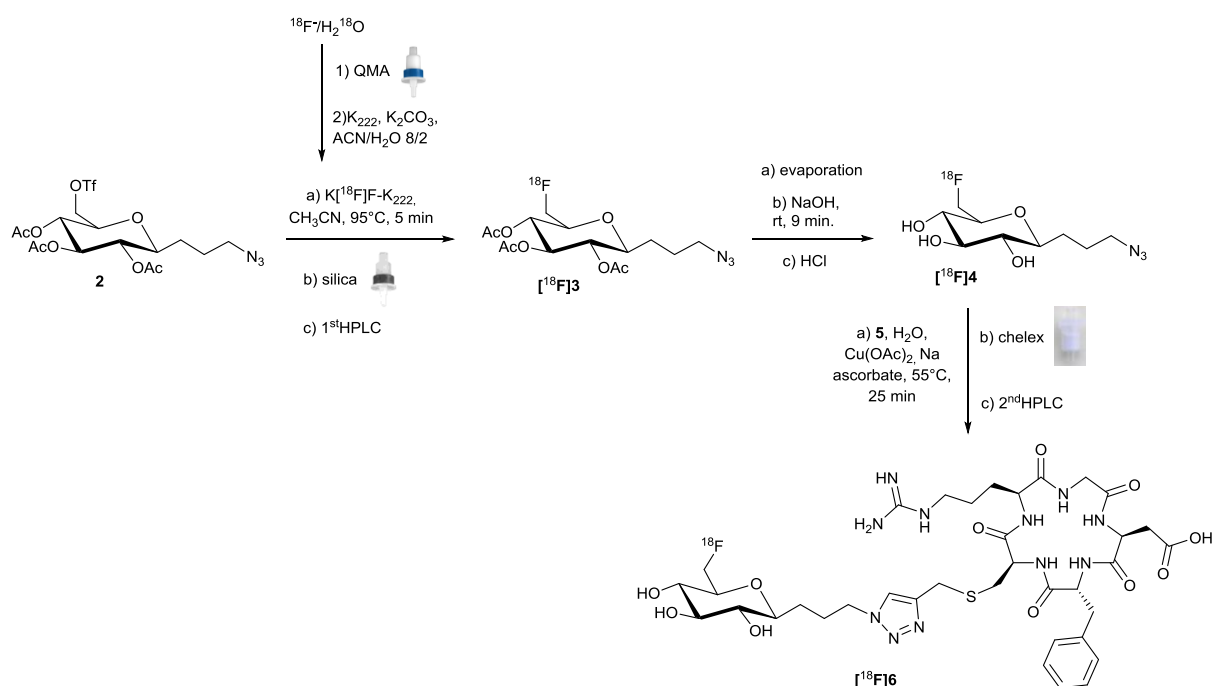
Scheme 1 Synthesis of radiolabeling precursor **2**, and non radioactive references **4** and **6**. Reagents and conditions: i) F_2O , pyridine, -25°C to rt, 25 min, 80%; ii) DAST, diglyme, 110°C , 8 min, 61%; iii) Na^+ , dry MeOH, 25°C , 1h, quantitative; iv) **5**, H_2O , $\text{Cu}(\text{OAc})_2$, Na ascorbate, rt, 2h, 69%.

Radiochemistry development and automation

The development of the radiosynthesis of [^{18}F]**6** required a multistep radiosynthesis, more precisely a two-pot three-steps synthesis. The AIO equipped with two reactors, two HPLC column positions and multiple possibilities of vial arrangements thanks to two arrays of 18 valves allows such radiosynthesis. Nevertheless, implementation of all the steps of this synthesis on the AIO was challenging and we have exploited, for the first time to the best of our knowledge, all the abilities of this module.

The fully automated process involved a two-pot three-step reaction: radiolabeling of the precursor **2**, deprotection to afford the prosthetic group [^{18}F]**4** and coupling with the cyclic RGD-containing peptide to give [^{18}F]fluoro-C-glyco-c(RGDfC) [^{18}F]**6** (Scheme 2). This strategy involved two HPLC purification procedures and a formulation step.

The different steps were symbolized with different luer lock ring colours on the layout (Figure 1). Vials with red luer lock rings were used for $\text{K}[^{18}\text{F}]\text{F-K}_{222}$ preparation, yellow luer lock rings for radiofluorination, green luer lock rings for deprotection, blue luer lock rings for coupling by CuAAC and white luer lock rings for reformulation.



Scheme 2 General strategy for $[^{18}\text{F}]\text{6}$ radiosynthesis

The non-carrier added ambitious three-step radiosynthesis of $[^{18}\text{F}]\text{F-C-glyco-c(RGDfC)}$ started with classical preparation of $\text{K}[^{18}\text{F}]\text{F-K}_{222}$ complex. The first step was the ^{18}F -radiolabeling of precursor **2** in the reactor 1. By taking advantage of our previous work on glycoside based prosthetic groups,^{9,10} in order to obtain a high radiolabeling rate and a better repeatability, different parameters such as the amount of precursor, the reaction duration and the temperature were screened upstream. The following conditions were found to be optimal for the ^{18}F -radiofluorination: 5 mg of precursor in 2 mL of dry acetonitrile and reaction of 5 min at 95°C. Before HPLC purification, a pre-purification of the mixture was performed by trapping unreacted ^{18}F -fluoride on a silica SPE cartridge. The eluted solution was then purified by semi-preparative HPLC to remove the precursor and potential related impurities. Developments made with non radioactive derivatives have shown good separation of the radiolabeling precursor **2** ($t_{\text{R}} \sim 16$ min) from the fluorinated acetylated C-glycoside **3** ($t_{\text{R}} \sim 7.5$ min) on a C8 analytical column with acetonitrile/water 55/45 v/v as eluent at a flow rate of 1.0 mL/min. Purification of $[^{18}\text{F}]\text{fluoro-C-glycoside } [^{18}\text{F}]\text{3}$ was performed using these conditions by semi-preparative HPLC at a flow rate of 4.5 mL/min (Figure 2). This HPLC purification step is time consuming but could not be avoided. Indeed, precursor of radiolabeling **2** can not be separated from radiofluorinated compound $[^{18}\text{F}]\text{3}$ by simple SPE cartridge purification.

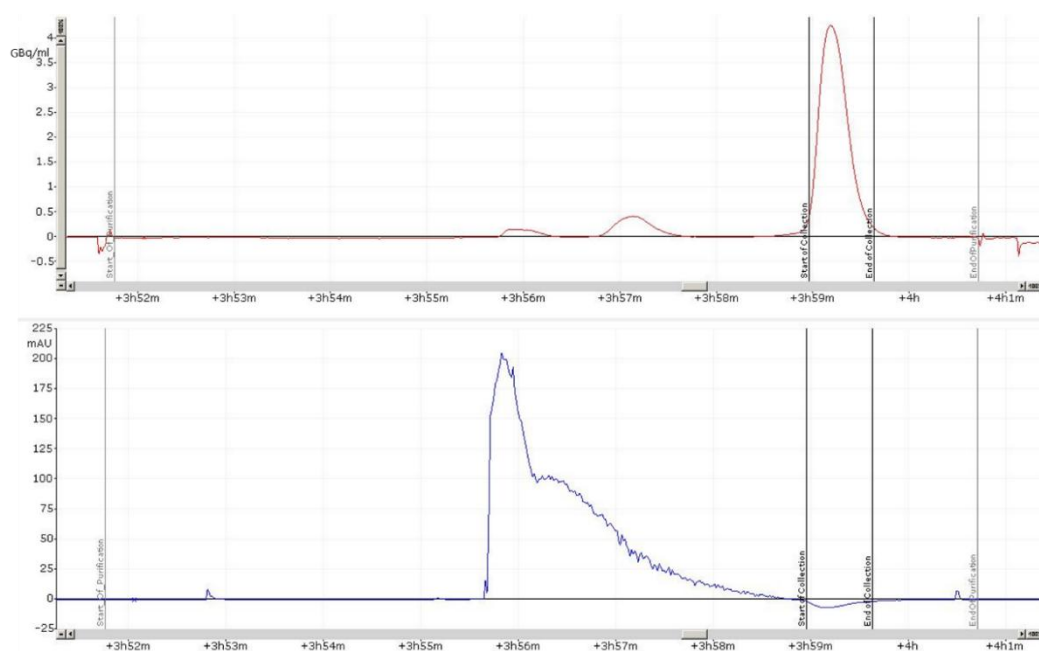


Fig. 2 Semi-preparative HPLC purification of [^{18}F]**3**. ($t_R \sim 7.5$ min, Alltima C8, ACN/ H_2O 55/45 v/v, 4.5 mL/min). Radioactivity (top) and UV (254 nm, bottom) traces are shown.

The HPLC chromatogram showed one major peak corresponding to [^{18}F]**3** ($t_R \sim 7.5$ min, identical to the non radioactive reference). Two others peaks ($t_R \sim 4.2$ min and $t_R \sim 5.4$ min) can also be distinguished on the chromatogram corresponding to partial deprotection of the peracetylated derivative. Indeed, despite careful optimization of the radiolabeling step, partial saponification occurred due to the basic conditions. [^{18}F]**3** was recovered into the reactor 2 and HPLC solvent was evaporated.

The second step of the radiosynthesis *i.e.* deprotection by saponification of [^{18}F]**3**, was performed at 30°C during 9 min using an aqueous solution of 1M NaOH affording C-glycoside based prosthetic group [^{18}F]**4** in quantitative yield. Neutralization with aqueous HCl was required to subsequently perform CuAAC which does not occur in highly basic medium.

The third step of the radiosynthesis, performed in the same reactor, was the CuAAC between radiolabeled prosthetic group [^{18}F]**4** and propargylated c(RGDfC) **5**. This reaction was performed by introducing sodium ascorbate and copper(II) acetate diluted in water. Optimized conditions for this reaction were 0.5 μmol of peptide, 6.6 μmol of sodium ascorbate and 3.3 μmol of $\text{Cu}(\text{OAc})_2$ in about 1.5 mL of water at 55°C for 25 min. Pre-purification of ^{18}F -labeled C-glyco-c(RGDfC) [^{18}F]**6** was performed by slow filtration on a Chelex[®] 100 resin-loaded cartridge to remove copper. Development of optimal HPLC conditions was carried out with non radioactive standards and good separation between prosthetic group **4**, propargylated

c(RGDfC) **5** and C-glyco-c(RGDfC) **6** was shown on a C18 analytical column with acetonitrile/water 17/83 v/v containing 0.1% TFA as eluent at a flow rate of 1.0 mL/min. Purification and isolation of [^{18}F]fluoro-C-glyco-c(RGDfC) [^{18}F]**6** ($t_R \sim 15.0$ min) were performed by semi-preparative HPLC using these optimum conditions with a flow rate of 3 mL/min. As illustrated on figure 3, unreacted prosthetic group [^{18}F]**4** ($t_R \sim 7.5$ min) was also detected and peak integration of compounds [^{18}F]**4** and [^{18}F]**6** indicates an average yield of 44% for the click coupling reaction.

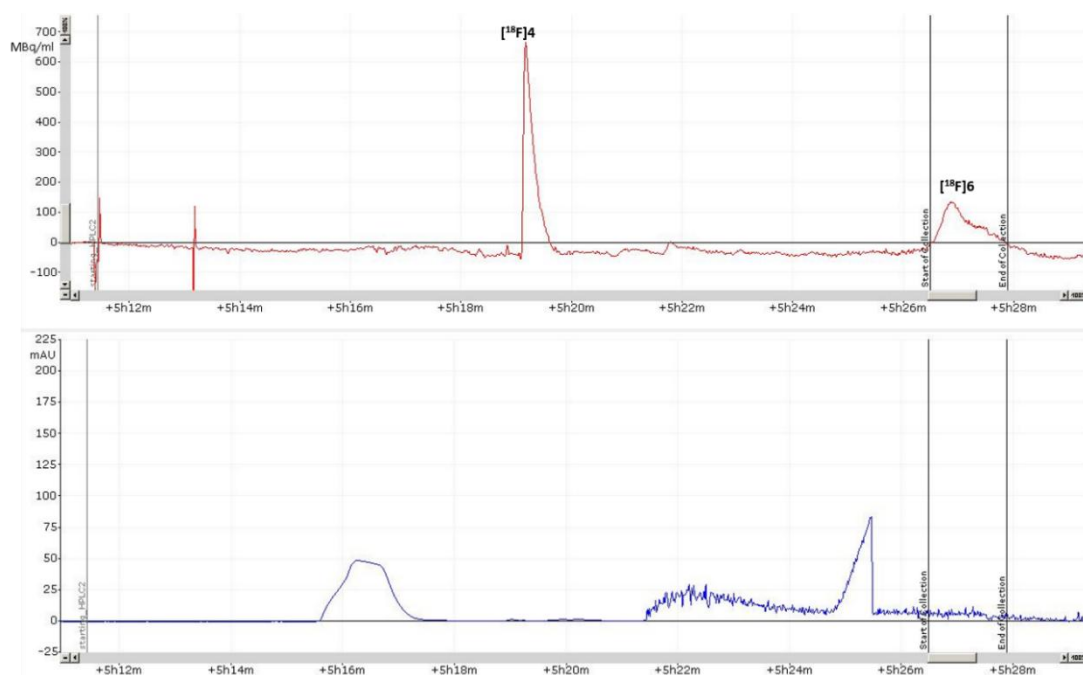


Fig. 3 Semi-preparative HPLC purification of [^{18}F]**6** ($t_R \sim 15.0$ min, Vydac 218TP C18, ACN/H₂O 17/83 v/v with 0.1% of TFA, 3 mL/min). Radioactivity (top) and UV (254 nm, bottom) traces are shown.

The [^{18}F]**6** product fraction was collected in a vial containing 10 mL of WFI to facilitate the Oasis HLB fixation of the glycopeptide. The reformulation of the product was performed by pushing this mixture through an Oasis HLB cartridge to trap the glycopeptide. The cartridge was then rinsed with WFI and flushed with nitrogen gas flow. [^{18}F]**6** was eluted with 0.7 mL of an ethanol/NaCl 0.9% 50/50 v/v solution and recovered into a sterile dose vial. The Oasis HLB Sep-Pak[®] was washed with 1.8 mL of 0.9% NaCl and transferred into the same sterile dose vial.

Typical preparation run started with 5 GBq and produced in average 73 MBq of 1.5 mL [^{18}F]**6** solution in approximately 140 minutes starting from activity reception. A satisfactory and reproducible overall decay corrected radiochemical yield of $3.6 \pm 0.4\%$ ($n = 12$) was obtained.

This radiosynthesis is the first fully automated production of [^{18}F]radiolabeled peptide *via* a prosthetic approach in a three-step two-pot method including two HPLC purification steps. Indeed, to date, automated batch production of [^{18}F]radiolabeled peptides *via* a prosthetic strategy has been scarcely reported due to its complexity. To our knowledge, the first example was proposed by Thonon et al.³⁶ using the FASTLab™ GE synthesis module. [^{18}F]SFB was synthesized *via* a one- or two-pot method and was purified by a tC18 cartridge. The [^{18}F]SFB was then coupled to RGD and the final radiotracer was purified by HPLC. [^{18}F]FPRGD₂ was thus produced in 130 minutes in $13 \pm 3\%$ decay corrected yield. However, this production was not fully automated as the RDG addition was manually carried out. Valdivia et al.³⁴ reported the automated radiosynthesis of [^{18}F]FPyKYNE-c(RGDyK) performed in a dual reactor TRACERlab FX N Pro, (GE Healthcare). After semi-preparative HPLC, the radiotracer was manually reformulated. [^{18}F]FPyKYNE-c(RGDyK) was obtained *via* this semi-automated method in 12-18% decay corrected radiochemical yield in 125 minutes. More recently, Haskali et al.³³ described the fully automated radiosyntheses (including two HPLC purifications and reformulation) of [^{18}F]FP- GalactoRGD, [^{18}F]FP- PRGD₂ and the sulfonated versions of these peptides. The 2- [^{18}F]fluoropropionate labeled RGD- peptides were obtained *via* a two-pot method in 90 to 105 minutes with non-decay corrected radiochemical yields of 4 to 7%. These radiosyntheses, including the two HPLC purifications (performed on the iPHASE Flexlab fix-part synthesizer) were fully automated, similarly to our sequence performed on the AIO. It is noteworthy that the AIO operates with single use cassettes, facilitating GMP compliance and preventing cross-contamination. Davis et al.³⁵ reported a two-pot automated production of three radiolabeled peptides (YGGFL, cRGDyK, and Pyr-QKLGNGWAVGHLM) *via* coupling of 6- [^{18}F]fluoronicotinyl-2,3,5,6-tetrafluorophenyl ester ([^{18}F]FPy-TFP) as prosthetic group. [^{18}F]FPy-TFP was purified with an Oasis MCX-Plus cartridge and the final purification of ^{18}F -radiolabeled peptides was not automated. The ELIXYS FLEX/CHEM® module allowed the semi-automated preparation of [^{18}F]FPy-peptides in 13-26% decay corrected radiochemical yields in 97 ± 3 minutes. Allott et al.³⁷ proposed an improved fully automated radiosynthesis of [^{18}F]FET- β AG-TOCA using the GE FASTlab™ platform with a non-decay corrected radiochemical yield of $16.7 \pm 0.6\%$. This elegant one-pot procedure cleverly uses SPE cartridges for purification of prosthetic group (2- [^{18}F]fluoroethylazide) and also for CuAAC coupling (HLB SPE cartridge). HPLC purification with biocompatible mobile phase enabled the bypass of reformulation step but was not yet optimal in terms of purity and A_m .

As described above, HPLC purification of the prosthetic group could be circumvented by a simple cartridge purification. This technique is very time saving but is not applicable to every type of molecules. Only precursors and corresponding fluorinated compounds with very different polarities or solubilities can be separated by cartridge. In our case, the sugar-based prosthetic group was chosen for its hydrophilicity properties despite the purification inconvenience. Indeed, the triflated precursor **2** and the resulting fluorinated molecule [^{18}F]**3** have close solubilities and polarities, explaining the compulsory HPLC purification. One pot fluorination and deprotection were first envisaged, however, the acetate removal lead to inseparable mixture of polyhydroxylated compounds (very close t_R). For this reason, it is mandatory to achieve the first HPLC purification of [^{18}F]**3** prior to acetate deprotection. Therefore, the following reactions (deprotection and CuAAC coupling) were both performed in reactor 2. This three-step synthesis including a deprotection step thus requires a two-pot sequence with two HPLC purifications. Consequently, this complex radiosynthesis ensures a maximal adaptability to a large panel of peptides and prosthetic groups.

Quality control and physico-chemical data

Quality control of [^{18}F]**6** was performed on an aliquot of the final batch. The prepared radiotracer solution was clear and colourless with a pH around 7. Chemical and radiochemical purities were determined by analytical HPLC with a C18 column using radioactive and UV detection (Figure 4). UV chromatogram showed no by-products demonstrating good chemical purity. The identity of [^{18}F]**6** ($t_R = 7.8$ min) was confirmed by a second injection with the authentic non radioactive standard. The radiochemical purity was determined to be over 98%. Molar activity can not be determined due to the poor UV absorbance of **6** (detection limit: 2 $\mu\text{g}/\text{mL}$), indicating that A_m is higher than 20.8 GBq/ μmol .

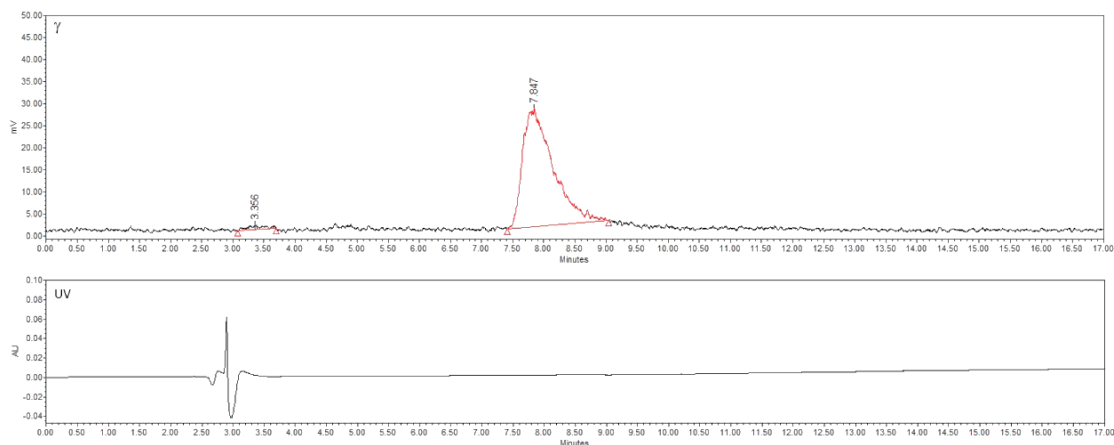


Fig. 4. Analytical HPLC profile of [^{18}F]**6**. Retention time of [^{18}F]**6**: 7.8 min; radiochemical purity of [^{18}F]**6** was $\geq 98\%$. Conditions: Vydac 218TP C18 column eluted with ACN/ H_2O

17/83 (v/v) with 0.1% of TFA in isocratic conditions, with a flow rate of 1.0 mL/min. Radioactivity (top) and UV (230 nm, bottom) traces are shown.

The stability of [^{18}F]6 has been checked on the entire preparation and we did not observe any degradation during a period of 120 min. *In vitro* stability of [^{18}F]6 was also determined in human serum at 37°C by radioactive HPLC detection. Analyses of acetonitrile serum extracts at different incubation time points (0, 10, 30, 60 and 120 min) demonstrated a high *in vitro* stability of [^{18}F]6.⁹

Experimental $\log D_{7.4}$ (n-octanol/PBS pH 7.4 partition coefficient) of [^{18}F]6 was measured using the shake-flask method and a value of -2.62 was found. It is worthy to note that this shows an enhanced hydrophilicity certainly promoted by the glycoside based prosthetic group providing an improvement of peptide biodistribution. This value is in the same range of the gold standard ^{18}F -galacto-RGD,^{15,46} also bearing a carbohydrate moiety. Previous work of our group demonstrated that [^{18}F]fluoro-C-glyco-c(RGDfC) has an affinity toward $\alpha_v\beta_3$ integrin in the same range than native c(RGDfC).⁹ The improved hydrophilicity combined with adequate *in vitro* stability and high affinity toward $\alpha_v\beta_3$ integrin make [^{18}F]6 suitable in PET imaging for cancer diagnosis.

Conclusion

The radiosynthesis of [^{18}F]fluoro-C-glyco-RGD conjugate has been fully automated on an AllInOne synthesizer via a prosthetic group approach. [^{18}F]fluoro-C-glyco-c(RGDfC) was obtained with an ambitious two-pots three-steps procedure with high radiochemical purity ($\geq 98\%$) and satisfactory and reliable decay-corrected radiochemical yield of $3.6 \pm 0.4\%$. The radiotracer shows an enhanced hydrophilicity, surely promoted by the C-glycoside prosthetic group, and appears therefore as a promising tool in PET imaging for cancer diagnosis. For the first time, all the abilities of the AIO synthesizer were exploited in this challenging fully automated multistep radiosynthesis. The described automation is modular and its implementation could be easily adapted to other prosthetic groups and/or peptides to afford a large panel of ^{18}F -radiolabeled peptides. Moreover, this fully automated method without any manual handling should contribute to the development of automation of complex radiosyntheses in the future.

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Conflict of Interest

There are no conflicts to declare.

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