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Automated Preparation of 2-[^{18}F]Fluoropropionate Labelled Peptides using a Flexible, Multi-stage Synthesis Platform (iPHASE Flexlab)

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Abstract

Radiolabelled peptides are vital tools used in positron emission tomography (PET) imaging for the diagnosis of disease, drug discovery and biomedical research. Peptides are typically labelled through conjugation to a radiolabelled prosthetic group, which usually necessitates complex, multi-step procedures, especially for fluorine-18 labelled peptides. Herein, we describe the automated synthesis and formulation of 2-[^{18}F]fluoropropionate labelled RGD-peptides through use of the iPHASE Flexlab as an effective dual-stage radiochemical synthesis module. The fully automated preparation of the monomeric RGD-peptides, [^{18}F]FP-GalactoRGD and [^{18}F]FP-c(RGDy(SO_3)K), was accomplished in under 90 minutes with n.d.c. radiochemical yields ca. 7% from fluoride. Similarly, the automated preparation of the dimeric RGD-peptides, [^{18}F]F-PRGD$_2$ and [^{18}F]FP-E(RGDy(SO_3)K)$_2$, was accomplished in under 105 minutes with n.d.c. yields ca. 4% from fluoride.

Introduction:

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Peptides are attracting increasing interest for their therapeutic applications as a consequence of their favourable properties, which include high target specificity with low toxicity.[1-3] Recent improvements in peptide design, synthesis and analysis have addressed some of the previous limitations of peptides as therapeutics, such as their intrinsic proteolytic instability.[4] The average number of peptide therapeutics entering clinical trials has increased from 1.2% of all drug candidates in the 1970s to 9.7% in the 1990s and upwards to 16.8% in the 2000s.[5] As such, current peptide therapeutics play an important role in the pharmaceutical industry and health care and their utility is expected to expand.

In addition to their therapeutic applications, pharmacologically active peptides have played an increasingly prominent role in clinical diagnosis through the emergence of molecular imaging.[6-9] For example, cyclic RGD-containing pentapeptides bind with high affinity and specificity to $\alpha_v\beta_3$ integrin and have been the focus of intense investigations toward the development of radiotracers for positron emission tomography (PET) imaging applications in the diagnosis and treatment of cancer.[10-15] The large number of labelled cyclic RGD peptides that has been investigated has resulted in this system acting as a de facto model for the development of novel radiolabeling processes.[16] Peptide radiotracers [^{18}F]GalactoRGD **1** and [^{18}F]FPPRGD2 **3** (Figure 1) are RGD peptides currently employed in clinical use or undergoing clinical trials for PET imaging of angiogenesis in cancer.[17-19] Further, we have recently reported the synthesis and evaluation of sulfonated versions of these peptides, **2** and **4**, which show favourable biodistribution.[20] However, the radiochemistry associated with the preparation of fluorine-18 labelled peptides

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such as **1**–**4** is complex, often requiring expert operators and involving multistep radiosynthesis procedures,[21] thereby limiting clinical use of such tracers to facilities with a cyclotron and experienced in-house radiochemists. The full utilization of these valuable clinical agents requires the development of modular, automated radiochemical procedures capable of generating the final radiotracer in a single, programmable module with minimal human intervention. As such, the establishment of an automated radiosynthesis platform is crucial for the preparation of fluorine-18 labelled peptides such as [^{18}F]GalactoRGD **1** and [^{18}F]FPPRGD2 **3**.[22]

Herein, we present the utility of the iPHASE Flexlab automated module for the routine preparation of the fluorine-18 labelled synthon, 4-nitrophenyl 2-[^{18}F]fluoropropionate ([^{18}F]NFP, [^{18}F]**6**) and its subsequent ligation to four different RGD peptides to generate [^{18}F]**1**–**4** (Figure 1).

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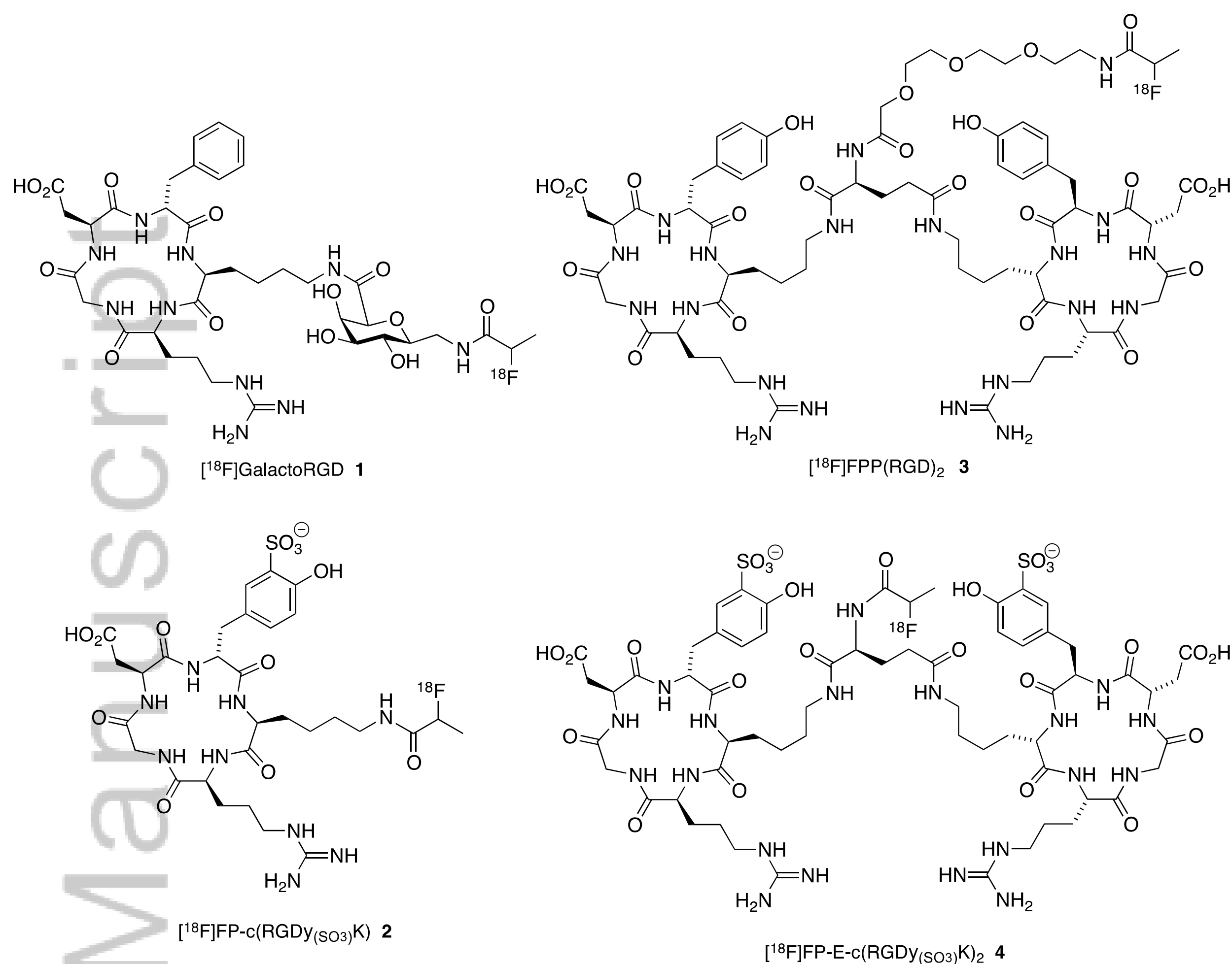

Figure 1: Radiolabelled RGD-peptides [^{18}F]**1**–**4**.

Experimental:

All chemicals were of analytical grade purchased commercially from Sigma-Aldrich and were used without further purification. Fluorine-18 was produced on a GE PETtrace 16.5 MeV cyclotron (Cyclotek Pty. Ltd., Melbourne, Australia) using a high pressure niobium target by proton bombardment [^{18}O(p,n)^{18}F] of 98% ^{18}O enriched [^{18}O]H_2O. Fluorine-18 was isolated on a

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QMA strong anion exchange cartridge (Waters). Reversed phase solid phase extraction (SPE) was performed on 30 mg/mL, 33 μm polymeric reversed phase cartridges (Phenomenex). CRC-15PET dose calibrator (Capintec) was calibrated daily using Cs-137 and Co-57 sources (Isotope Products Laboratories) and used for radioactivity measurements.

Peptide Synthesis. GalactoRGD **7**, FP-GalactoRGD **1**, miniPEG-E(RGD)$_2$ **9** and FP-P(RGD)$_2$ **3** were synthesized according to our previously reported method.[23] c(RGDy$_{(SO_3)}$K) **8**, FP-c(RGDy$_{(SO_3)}$K) **2**, E(RGDy$_{(SO_3)}$K)$_2$ **10** and FP-E(RGDy$_{(SO_3)}$K)$_2$ **4** were synthesized according to our previously reported method.[20]

Radiochemistry. Radiochemical synthesis was performed on an iPHASE Flexlab radiochemistry module purchased from iPHASE Technologies Pty. Ltd. The schematic diagram for the Flexlab module is shown in Figure 2.

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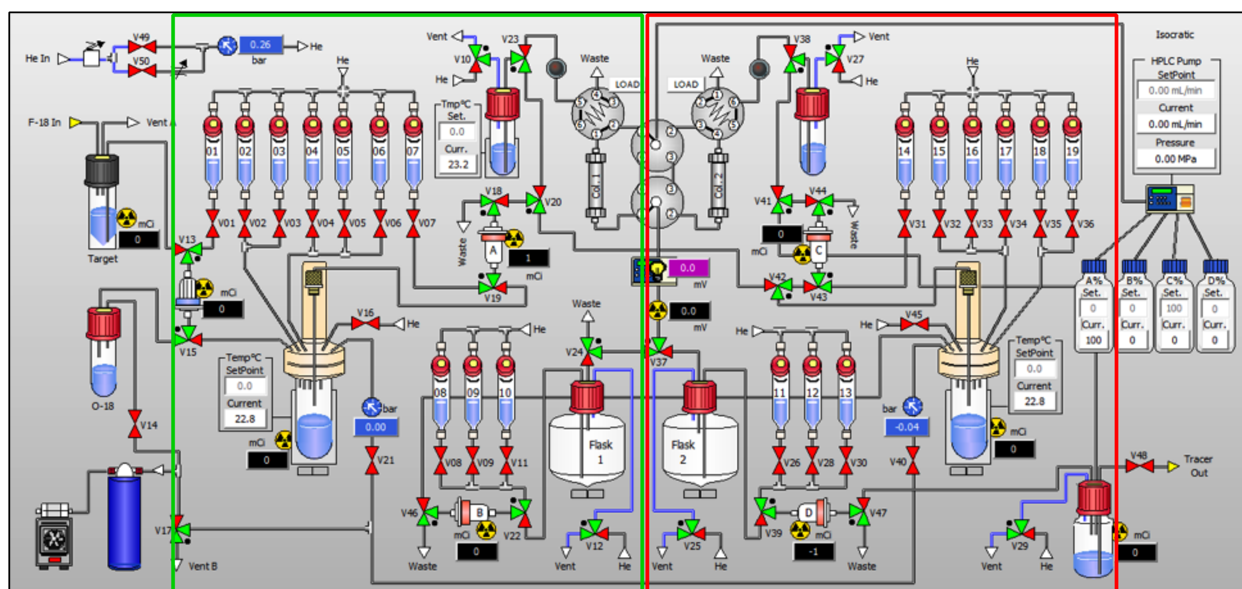

Figure 2: Schematic of the iPHASE Flexlab radiochemistry module. Section within green box represents the first half of the module, which was used to generate [^{18}F]NFP **6**. Section within red box represents the second half of the module which was used to conjugate [^{18}F]NFP **6** to RGD peptides to generate radiolabeled peptide adducts [^{18}F]**1–4**.

*[^{18}F]NFP ([^{18}F]**6**)* was prepared by transposition of our previously reported method employing the FX_{FN} module[23] to reactor 1 of the Flexlab module. Briefly, anhydrous $K_{222}.K^{+18}F^{-}$ complex (18.5–37.0 GBq) was treated with *p*-nitrophenyl 2-bromopropionate **5** (15.0 mg, 0.05 mmol) in tBuOH:CH_3CN (2 ml, 8:2, vial 3). The reaction was left at 100 °C for 5 min, after which the reaction solvents were reduced to about 0.5 ml at 65 °C. The crude reaction mixture was diluted with 0.1% TFA in 60:40 CH_3CN:H_2O (1.5 ml, vial 4) and transferred into the first HPLC loop loading vial. This step was repeated using 0.1% TFA in 60:40 CH_3CN:H_2O (1.5 ml, vial 6) to transfer any leftover reaction mixture in the vial into the same HPLC loop loading vial. Crude

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[^{18}F]NFP **6** was purified by preparative HPLC 1 (0.1% TFA in 60:40 CH_3CN:H_2O, Macherey-Nagel EP250/16 Nucleosil 100-7 C18 column equipped with EP30/16 guard column, flow rate 6 mL/min). [^{18}F]NFP **6** was collected in the product collection flask containing water (60 ml). [^{18}F]NFP **6** was concentrated on a C18 cartridge and rinsed with water (5 ml, vial 9). Yields were 5.4–8.5 GBq, 26±3% n.d.c., molar activity (MA); 307.1 GBq/μmol. Preparation details for the radiosynthesis of [^{18}F]NFP **6** are illustrated in Table 1.

Table 1: Preparation details for the radiosynthesis of [^{18}F]NFP **6** on the iPHASE Flexlab module.

Position	Reagents or Materials	Quantities
V13–V15	Sep-Pak Light QMA	1
V01	$K_{2.2.2}$/ K_2CO_3 in CH_3CN: H_2O	15 mg $K_{2.2.2}$/3 mg K_2CO_3 in 700:300 μL CH_3CN:H_2O
V03	Ester **5** in tBuOH:CH_3CN	15 mg in 2 ml (1.6:0.4)
V04	0.1% TFA in CH_3CN:H_2O	1.5 ml (0.9:0.6)
V06	0.1% TFA in CH_3CN:H_2O	1.5 ml (0.9:0.6)
V09	H_2O	5 ml
HPLC Flask 1	H_2O	60 ml
V19–V18	Alumina Cartridge	1
V22–V46	C18 SPE Cartridge	1

*[^{18}F]FP-GalactoRGD [^{18}F]**1**.* [^{18}F]NFP **6** (2.2–3.7 GBq) isolated on the C18 cartridge was eluted with a solution of GalactoRGD **7** (1 mg, 1.2 μmol) in DMSO:CH_3CN (2:3, 0.7 ml, vial 8) containing TEA:DMSO (1:20, 20 μL) into reactor 2. CH_3CN was evaporated at room temperature for 10 min. The reaction mixture was then diluted with sterile water (1.5 ml containing 0.1 % TFA, vial 15) and transferred to HPLC loop loading vial 2. The reaction vial

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was further washed with sterile water (1.5 ml containing 0.1 % TFA, vial 16) and transferred to loop vial 2. Crude [^{18}F]**1** was then purified by HPLC 2 (0.1 % TFA in 2–80 % $CH_3CN:H_2O$ over 40 min, column: Luna 5µ C18(2) 100 Å 250×10.0 mm). The fraction containing the product was diluted with sterile water (40 ml, round bottom flask 2) and isolated on C18 SPE cartridge 2. The cartridge was then washed with phosphate buffered saline (PBS) (5 ml, vial 11) and eluted with ethanol (0.6 ml, vial 12). The elution lines were washed with PBS (7 ml, vial 13) and filtered into a sterile vial to obtain formulated [^{18}F]**1** (1.1–1.9 GBq, 52±1 % yield, n.d.c., MA: 122.1 GBq/µmol). The identity of [^{18}F]**1** was confirmed by comobility of the radiolabelled material with the reference standard **1** (Figure 4). Preparation details for the radiosynthesis of [^{18}F]**1** are depicted in Table 2.

Table 2: Preparation details for the radiosynthesis of [^{18}F]**1** on the iPHASE Flexlab module.

Position	Reagents or Materials	Quantities
V08	GalactoRGD **7** in $DMSO:CH_3CN$ containing TEA	1 mg peptide in 320:400 µL $DMSO:CH_3CN$ containing 1 µL of TEA
V11	7.4 PBS buffer	5 ml
V12	Ethanol	0.6 ml
V13	7.4 PBS buffer	7 ml
V15	0.1% TFA H_2O	1.5 ml
V16	0.1% TFA H_2O	1.5 ml
HPLC Flask 2	H_2O	40 ml
V39–V47	C18 SPE Cartridge	1

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*[^{18}F]FP-c(RGDy$_{(SO_3)}$K) [^{18}F]**2***. [^{18}F]**2** (2.1–2.7 GBq, 49±2% yield n.d.c., MA: 144.3±9.6 GBq/µmol) was prepared from precursor **8** in a manner analogous to the radiosynthesis of [^{18}F]**1**. The identity of [^{18}F]**2** was confirmed by comobility of the radiolabelled material with the reference standard **2** (Figure S2).

*[^{18}F]F-PRGD$_2$ [^{18}F]**3***. [^{18}F]NFP **6** was eluted with dichloromethane (DCM) (1 ml, vial 8) into reactor 2 via a sodium sulfate cartridge to remove trace amounts of water. The DCM was evaporated at room temperature for 3 min under vacuum and a stream of helium gas. Residual DCM was further evaporated at 50 °C for 2 min. MiniPEG(RGDyK)$_2$ **9** (1 mg, 0.6 µmol) containing TEA (15 µL, 0.11 µmol) in DMSO (0.35 ml, vial 18) was charged into reactor 2. The reaction was heated to 70 °C for 5 min then was heated under vacuum for 5 min at 70 °C. The reaction mixture was subsequently diluted with sterile water (1.5 ml containing 0.1 % TFA, vial 15) and transferred to HPLC loop loading vial 2. The reaction vial was washed with sterile water (1.5 ml containing 0.1 % TFA, vial 16) and transferred to HPLC loop loading vial 2. The crude peptide [^{18}F]**3** was purified by HPLC 2 (0.1 % TFA in 10–80 % $CH_3CN:H_2O$ over 40 min, column: Luna 5µ C18(2) 100 Å 250×10.0 mm). The fraction containing the product was diluted with sterile water (40 ml, HPLC flask 2) and isolated on the C18 SPE cartridge 2. The cartridge was then washed with PBS (5 ml, vial 11) and eluted with ethanol (0.6 ml, vial 12). The elution lines were washed with PBS (7 ml, vial 13) and filtered into a sterile vial, to obtain [^{18}F]**3** (488–704 MBq, 22±1% yield n.d.c., MA: 255.3 GBq/µmol). The identity of [^{18}F]**3** was confirmed by

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comobility of the radiolabelled material with the reference standard **3** (Figure S3). Preparation details for the radiosynthesis of [^{18}F]**3** are depicted in Table 3.

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Table 3: Preparation details for the radiosynthesis of [^{18}F]**3** on the iPHASE Flexlab module.

Position	Reagents or Materials	Quantities
V08	DCM	1 ml
V11	7.4 PBS buffer	5 ml
V12	Ethanol	0.6 ml
V13	7.4 PBS buffer	7 ml
V15	0.1% TFA H_2O	1.5 ml
V16	0.1% TFA H_2O	1.5 ml
V18	miniPEG(RGDyK)$_2$ **9** in DMSO containing TEA	1 mg peptide in 350 µL DMSO containing 15 µL TEA
HPLC Flask 2	H_2O	40 ml
V46–Reactor 2	Sodium sulfate cartridge	1
V39–V47	C18 SPE Cartridge	1

*[^{18}F]FP-E(RGDy$_{(SO_3)}$K)$_2$, [^{18}F]**4***. [^{18}F]**4** (622–740 MBq, 17±2% yield n.d.c., MA: 125.8 GBq/µmol) was prepared from precursor **10** in a manner analogous to the radiosynthesis of [^{18}F]**3**. The identity of [^{18}F]**4** was determined by comobility of the radiolabelled material with the fully characterized reference standard **4** (Figure S4).

Results and discussion:

The iPHASE Flexlab provides the versatility of two HPLC columns, dual reactors and formulation capability and 19 reagent vials incorporated in one module. Our aim was to utilize

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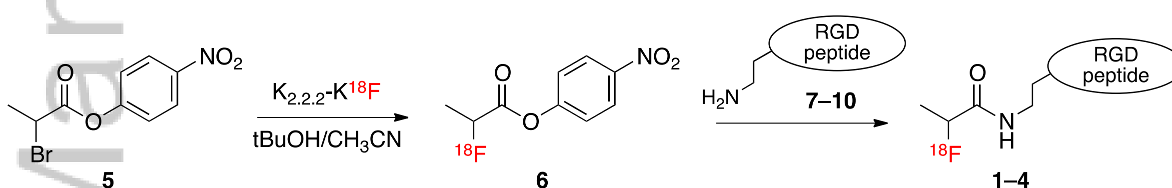
this flexible automated module in the 2-step radiochemical synthesis of four RGD peptides [^{18}F]**1**–**4** (Figure 1).

We have previously reported the improved one-step radiosynthesis of 4-nitrophenyl 2-[^{18}F]fluoropropionate ([^{18}F]NFP, **6**) using the TRACERlab FX_{FN} module.[23] Using the first part of the Flexlab module (vials 1–10, see Figure 2), the radiosynthesis of [^{18}F]NFP **6** was undertaken according to our previously reported method (Scheme 1). Pleasingly, the radiosynthesis of [^{18}F]NFP **6** was translated to the Flexlab in comparable yields (26±3% n.d.c. from free fluoride). [^{18}F]NFP **6** produced was subsequently concentrated on a C18 SPE cartridge for further chemical manipulations.



Scheme 1: The one-step radiosynthesis of [^{18}F]NFP **6** and subsequent conjugation to peptides **7–10**.

Due to the susceptibility of [^{18}F]NFP **6** to hydrolysis we sought to minimize any manipulations before conjugation to the peptides. Accordingly, [^{18}F]NFP **6** was eluted from the cartridge into reactor-2 with a solution of the RGD peptide (GalactoRGD **7** or c(RGDy(SO_3)K) **8**) in DMSO (320 μL) containing triethylamine (TEA) (1 μL), followed by a flush with CH_3CN (400 μL). The

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CH_3CN was removed under vacuum and a stream of nitrogen. Employing this direct elution of [^{18}F]NFP **6** with solutions of RGD peptides in DMSO/CH_3CN generated the [^{18}F]**1** and [^{18}F]**2** peptides as the major radiochemical products. After HPLC purification, isolation, formulation and sterile filtration [^{18}F]**1** was obtained in good radiochemical yield (52±1% (n=3) n.d.c.) and high molar activity (MA 116.5±1.5 GBq/µmol (n=3)) from [^{18}F]NFP **6**, 7% from free [^{18}F]fluoride (Figure 3). Similarly, [^{18}F]**2** was obtained in 49±2% yield (n=3) n.d.c. from [^{18}F]NFP **6**, 7% from free [^{18}F]fluoride, MA 144.3±9.6 GBq/µmol (n=3) (Figures S1,S2). The radiosynthesis and preparation of formulated and sterilized [^{18}F]**1** and[^{18}F]**2** was accomplished in approximately 90 minutes. The overall preparation time and number of radiochemical steps required are significantly reduced compared with reported methods for the preparation of [^{18}F]**1** and [^{18}F]**2** (Table 4).

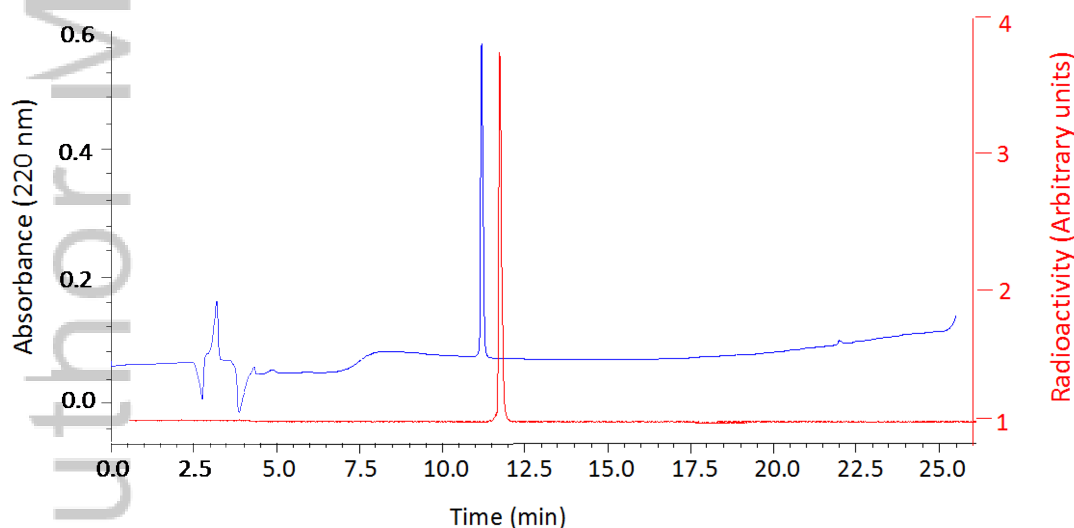

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Figure 3: HPLC chromatogram for the co-injection of cold reference standard **1** with purified and formulated [^{18}F]**1**. Red chromatogram (offset) indicates radioactivity monitored by a scintillation detector. Blue chromatogram represents UV absorbance at 220 nm. Analytical method run from 5–100% CH_3CN containing 0.1% TFA at 1 mL/min over 20 min on a Phenomenex, Jupiter 4 µm Proteo 90 Å column (250×4.6 mm).

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Table 4: Comparison of Flexlab method for the radiosynthesis of [^{18}F]**1** and [^{18}F]**3** with reported procedures.[24-26]

Peptide	Preparation method	Preparation time (min)	Radiochemical steps	Automation
1	Haubner et. al.[24]	180–218	4	none
1	this work	84–90	2	full
3	several methods[25,26]	170–180	4	customized*
3	this work	94–105	2	full

*Automation customized using a modified commercial radiosynthesis module (TRACERlab FXFN) in conjugation with a custom self-built module.

Our previous experience with the labeling of the dimeric RGD peptides **9** and **10** suggested conjugation was less effective than for the monomeric RGD peptides **7** and **8** and required heat.[20,23] As such, we adopted a slightly different procedure for the preparation of radioabeled dimeric RGD peptides [^{18}F]**3** and [^{18}F]**4**. To avoid hydrolysis of the activated ester of [^{18}F]NFP **6** under heating, it was eluted with the volatile solvent DCM and residual water from the C18 SPE cartridge was removed by passage over an anhydrous sodium sulfate cartridge. The resulting dry [^{18}F]NFP **6** was then treated with a solution of peptide **9** and TEA in DMSO and heated at 70°C for 5 min. The crude peptide was purified, formulated and sterilized as described for the monomeric RGD peptides to afford [^{18}F]**3** (22±1% yield (n=3) n.d.c. from [^{18}F]NFP **6**, 4% from free fluoride; MA 255.3±2.5 GBq/µmol (n=3), Figure S3). Similarly, [^{18}F]**4** (17±2 % yield (n=3) n.d.c. from [^{18}F]NFP **6**, 3% from free fluoride; MA125.8±1.5 GBq/µmol (n=3)) was obtained

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from peptide **10** (Figure S4). The radiosynthesis and preparation of formulated and sterilized [^{18}F]**3** and [^{18}F]**4** was accomplished in under 105 minutes (Table 5).

Table 5. Comparison of Flexlab method for the radiosynthesis of [^{18}F]**2** and [^{18}F]**4** with reported procedure.[20]

Peptide	Preparation method	Preparation time (min)	Radiochemical steps	Automation
2	Haskali et. al.[20]	~ 90	2	none
2	this method	84–90	2	full
4	Haskali et. al.[20]	~ 105	2	none
4	this method	94–105	2	full

In conclusion, automated two-stage radiochemical synthesis of several RGD peptides was achieved using the iPHASE flexlab dual reactor module. The radiolabelled peptides were prepared in good radiochemical yield and molar activity, with minimal handling and operator exposure.

Supporting Information.

HPLC, UV and scintillation traces of purified [^{18}F] **1**–**4**.

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Abbreviations

RGD, arginyl-glycyl-aspartyl-containing peptide; NFP, 4-nitrophenyl 2-fluoropropionate; FP, 2-fluoropropionyl; PEG, polyethylene glycol; PET, positron emission tomography.

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