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Fluorine-18 labelling of a series of potential EGFRvIII targeting peptides with a parallel labelling approach using [^{18}F]FPyME

Charlotte L. Denholt,[a] Bertrand Kuhnast,[b]* Frédéric Dollé,[b] Francoise Hinnen,[b] Paul R. Hansen,[c] Nic Gillings,[a] and Andreas Kjær[a,d]

There is growing interest in the use of radiolabelled peptides as receptor targeting agents for diagnostic imaging of various cancer types using positron emission tomography. In this work, 1-[3-(2-[^{18}F]fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione ([^{18}F]FPyME) has been used for parallel fluorine-18 labelling of PEPHC1, a peptide selective towards the cancer-specific mutation of the epidermal growth factor receptor (EGFRvIII), and a number of truncated and mutated analogues. Conjugation of the peptides with [^{18}F]FPyME was achieved within 10 min in non-decay-corrected radiochemical yields of 30–50%. The high yield of the conjugation reaction combined with its short synthesis time allows the labelling of several peptides from a single batch of [^{18}F]FPyME.

Keywords: targeting peptides; fluorine-18 labelling; [^{18}F]FPyME; EGFRvIII; cancer; PET

Introduction

Peptides as receptor targeting molecules for imaging in cancer research using positron emission tomography (PET) is a field of growing interest.[1–3] Peptides, in contrast to antibodies and larger molecules, are less immunogenic and have better tissue penetration.[4] Peptides are, moreover, cheaper and easier to produce, reinforcing the interest for the development of novel targeting peptides and the associated, rapid and specific radiolabelling protocols.

Of the available positron emitting isotopes suitable for PET imaging, fluorine-18, with a physical half-life of 109.7 min, is probably the most suitable for peptide labelling, as the relative long half-life makes multi-step synthesis approaches possible.[5] Fluorine-18 labelling is usually achieved by conjugation of a labelled prosthetic group with a reactive function on the peptide. The drawback of the fluorine-18 labelling of peptides is that the synthesis of radiolabelled precursors is often time consuming. Therefore, it would be an advantage to label a number of peptides with the same batch of precursor with high yields in order to accelerate the development of new peptide radiopharmaceuticals.

Several fluorine-18-labelled reagents have been described for these conjugation reactions. Most of them were designed for coupling with an amino function of an amino acid residue (*N*-terminal α-NH_2 or lysine ε-NH_2) via acylation. Of these reagents, the activated ester *N*-succinimidyl 4-[^{18}F]fluorobenzoate ([^{18}F]SFB, Figure 1) is the most widely used.[6] Mainly due to the poor regioselectivity observed for the coupling of these reagents with large macromolecules (which often leads to partial loss of the latter's biological properties), considerable attention has been paid in the last decade to the design and development of sulphur-selective fluorine-18 labelled reagents.[7–10] Within this class, one of the latest reported reagents which has proven to be efficient in thioconjugation reactions for labelling of peptides and proteins is 1-[3-(2-[^{18}F]fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione ([^{18}F]FPyME), a [^{18}F]fluoropyridinyl-based maleimide reagent that can be prepared in three steps from cyclotron-produced [^{18}F]fluoride in less than 2 h.[8]

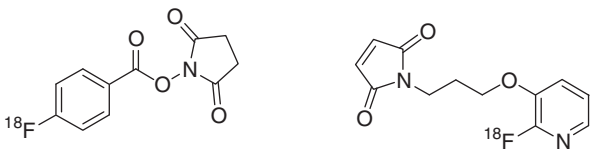


Figure 1. Two examples of [^{18}F]fluorinated reagents for the prosthetic labelling of peptides and proteins.

[a]*PET & Cyclotron Unit, Copenhagen University Hospital, Rigshospitalet, 2100 Copenhagen, Denmark*

[b]*CEA, Institut d'imagerie biomédicale, Service hospitalier Frédéric Joliot, 91406 Orsay, France*

[c]*Department of Natural Science, University of Copenhagen, 2100 Copenhagen, Denmark*

[d]*Cluster for Molecular Imaging, University of Copenhagen, 2200 Copenhagen, Denmark*

**Correspondence to: Bertrand Kuhnast, CEA, Institut d'imagerie biomédicale, Service Hospitalier Frédéric Joliot, 4 place du Général Leclerc, 91406 Orsay, France.*
E-mail: bertrand.kuhnast@cea.fr

In this study, we investigate the suitability of [^{18}F]FPyME as a reagent for a rapid and efficient radiolabelling of a number of peptides potentially selective towards the cancer-specific mutation of the epidermal growth factor receptor (EGFRvIII). The peptides were developed from an Ala-scan of PEPHC1, an EGFRvIII selective peptide,[11] where the amino acid residues essential for the binding of the peptide to the mutated receptor were identified.[12] Based on the results obtained, eight truncated analogues of PEPHC1 were designed and modified by addition of a cysteine residue at either the *N*- or *C*-terminal to enable labelling with [^{18}F]FPyME.

Results and discussion

PEPHC1 and eight truncated analogues, all containing a cysteine residue (see Table 1), were successfully synthesized using standard solid phase 9-fluorenylmethoxycarbonyl (Fmoc) chemistry[13,14] and purified by using high-performance liquid chromatography (HPLC). Reference-conjugated peptides were synthesized by reaction of the peptides with non-labelled FPyME at ambient temperature for 30 min, with HPLC-determined conversion yields of >95%. The FPyME-conjugated peptides were purified using RP-HPLC and analysed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS; Table 1).

[^{18}F]FPyME was prepared by a slightly modified previously published procedure (Scheme 1).[8] Typically, 5.2–5.8 GBq of HPLC-purified [^{18}F]FPyME could be obtained in about 110–115 min, starting from 37 GBq of [^{18}F]fluoride (28–32% decay-corrected radiochemical yields).

The conjugation of the peptides with [^{18}F]FPyME was carried out in DMSO containing diisopropylethylamine within 10 min at room temperature (Scheme 2). The [^{18}F]FPyME-conjugated peptides were then purified by using preparative HPLC and obtained within another 15 min in non-decay-corrected radiochemical yields of 30–50%, based on starting [^{18}F]FPyME, with specific radioactivities of 74–124 GBq/μmol. Typically, 0.6–0.9 GBq of purified labelled peptide could be obtained for each conjugation reaction using the same [^{18}F]FPyME batch. The radiochemical yields of the fluorine-18 labelling of the peptides were independent of the length of the peptides, suggesting that the method is suitable for labelling both long and short peptides.

Identity of the [^{18}F]peptides was confirmed by co-injection with the corresponding reference peptides onto an analytical HPLC column (Figure 2). Radiochemical purity was >95% for all [^{18}F]peptides.

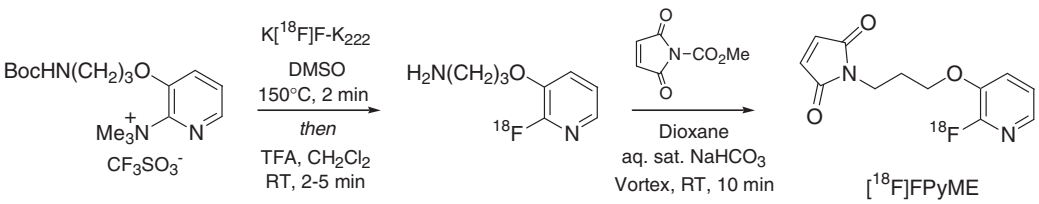
The high yields observed for the preparation of [^{18}F]FPyME allowed for labelling and testing of four different peptides on the same day. In essence, the rapid and efficient conjugation reaction of [^{18}F]FPyME with the cysteine-containing peptides makes possible the parallel labelling of several peptides thus giving access to the preparation of fluorine-18-labelled 'reasonably dimensioned' libraries.

The strength of our strategy, which consists in the *S*-alkylation of the sufhydryl function of a cysteine residue with the maleimide-based reagent [^{18}F]FPyME, is that this reaction could

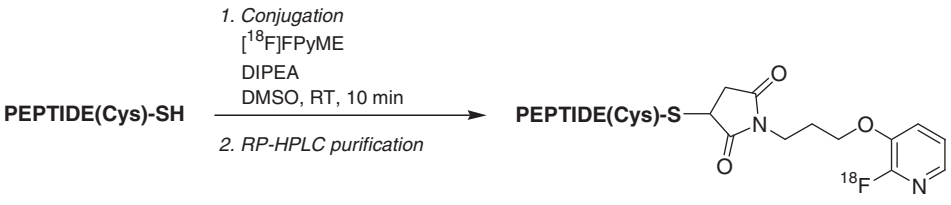
Table 1. Name, sequence and analytical data obtained for the FPyME-conjugated peptides

Peptide name	Peptide sequence	Calculated mass[a] (g/mol)	Found mass[ac] (*m/z*)	Calculated mass[b] (g/mol)	Found mass[bc] (*m/z*)	Retention time[d] (min)	Radiochemical yield[e] (%)	Log *D*[f]
PEPHC1	HFLIIGFMRRALCGA	1728	1730	1953	1951	19.7	52	0.54
PEPCH1a1	CHFLII	769	770	994	993	5.2	51	1.49
PEPHC1a2	HFLIIC	769	770	994	993	5.2	32	1.55
PPEHC1b1	CHFLIIG	826	825	1051	1049	8.8	50	0.66
PEPHC1b2	HFLIIGC	826	827	1051	1052	8.7	43	1.25
PEPHC1c1	CHFLIIGF	973	970	1198	1196	12.2	48	0.97
PEPHC1c2	HFLIIGFC	973	974	1198	1196	12.3	32	1.32
PEPHC1d1	CHFLIIGFM	1105	1106	1330	1332	16.7	31	0.72
PEPHC1d2	HFLIIGFMC	1105	1107	1330	1332	16.8	46	1.06

[a]Mass of non-conjugated peptides.
[b]Mass of conjugated peptides.
[c]MALDI-TOF MS.
[d]HPLC D (see Experimental).
[e]Non-decay-corrected based on the [^{18}F]FPyME radioactivity ($n = 3$–4 for each peptide).
[f]Determined by using the shake-flask method (see Experimental).



Scheme 1. Radiosynthesis of 1-[3-(2-[^{18}F]fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione ([^{18}F]FPyME).



Scheme 2. Fluorine-18 labelling of the peptides using [^{18}F]FPyME.

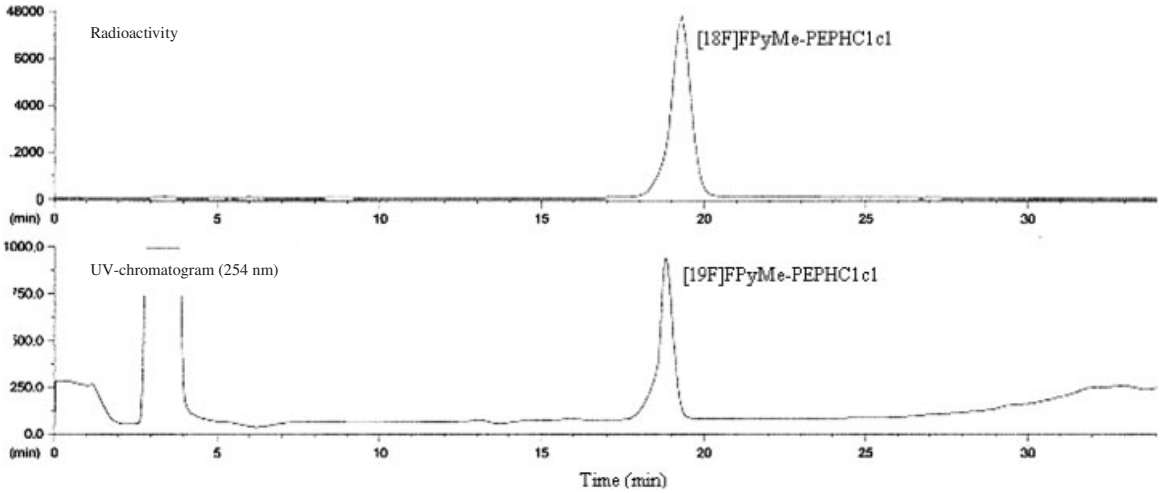


Figure 2. Radio- and UV-chromatograms of [^{18}F]FPyME-PEPHC1c1, after HPLC purification, spiked with [^{19}F]FPyME-PEPHC1c1 (HPLC E, see conditions and equipment in Experimental).

almost be considered as a 'click' reaction. The conjugation yields are high and the reaction times are short. The conjugation is also highly chemoselective as the maleimide-based reagent will selectively react with the sulfhydryl function even in the presence of other nucleophilic functions such as amines, carboxylates or alcohols. In addition, maleimides are less sensitive than active esters (like [^{18}F]SFB) to the often aqueous basic conjugation conditions required.

Moreover, due to the highly selective conjugation of [^{18}F]FPyME, the radiochemical yield of PEPHC1 was very similar to the yields of the smaller peptides, although concentration of PEPHC1 is 2–3 times lower than the smaller peptides. This has been reported to be an issue when other labelling methods are used, with a tendency to reduced radiochemical yields for larger peptides.[15,16]

The lipophilicity (log *D*, see Table 1) of each [^{18}F]peptide was measured using the shake-flask method.[17] All peptides had log*D* between 0.5 and 1.5, indicating potential brain uptake.[18]

Experimental

General

Chemicals

TentaGel S RAM resin (loading 0.24 meq/g) was purchased from RAPP Polymers (Tübingen, Germany); 1-hydroxybenzotriazole, Fmoc-protected amino acids, acetonitrile (MeCN), *N,N′*-diisopropylcarbodiimide (DIPCDI), thioanisol and α-cyano-*p*-hydroxycinnamic acid were purchased from Sigma Aldrich (Hamburg, Germany); piperidine and triisopropylsilane (TIS) were purchased from Fluka (Buchs, Switzerland); adrenocorticotropic hormone (ACTH) and substance *P* were purchased from Sigma (St Louis, USA); trifluoroacetic acid (TFA) was purchased from Merck (Schuchardt, Germany). All other starting chemicals were purchased from standard commercial sources (Sigma, Aldrich or Fluka) and used without further purification.

FPyME (1-[3-(2-fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione) was synthesized in three steps from (3-hydroxypropyl)carbamic acid *tert*-butyl ester and 2-fluoro-3-hydroxypyridine, as previously described.[8,19,20] ^{1}H NMR (CD_2Cl_2, 298.0 K): δ: 7.69 (bd, J = 3.0 Hz, 1H); 7.27 (*t*, J = 9.0 Hz, 1H); 7.11 (dd, J = 9.0 & 3.0 Hz, 1H); 6.69 (s, 2H); 4.04 (*t*, J = 6.0 Hz, 2H); 3.72 (*t*, J = 6.0 Hz, 2H); 2.11 (q^5, J = 6.0 Hz, 2H). ^{13}C NMR (CD_2Cl_2, 298.0K): δ: 171.2 (2 × C); 154.1 (d, $J^1_{F\text{-}C}$ = 235 Hz, C); 142.4 (d, $J^2_{F\text{-}C}$ = 25 Hz, C); 137.7 (d, $J^3_{F\text{-}C}$ = 13 Hz, CH); 134.5 (2 × CH); 123.2 (CH); 122.2 (CH); 67.5 (CH_2); 35.4 (CH_2); 28.4 (CH_2). MS (DCI/NH_4^+) $C_{12}H_{11}F_1N_2O_3$: 251 [$M+H^+$].

[3-(3-*tert*-Butoxycarbonylaminopropoxy)-pyridin-2-yl]-trimethylammonium trifluoromethanesulfonate, as precursor for fluorine-18-labelling, was synthesized in two steps from commercially available (3-hydroxypropyl)carbamic acid *tert*-butyl ester and 2-dimethylamino-3-hydroxypyridine, as previously described.[8,19] ^{1}H NMR (CD_2Cl_2, 298 K): δ: 8.10 (bd, J = 3.3 Hz, 1H); 7.66 (d, J = 8.1 Hz, 1H); 7.60 (dd, J = 6.1 & 4.2 Hz, 1H); 4.31 (*t*, J = 6.3 Hz, 2H); 3.71 (s, 9H); 3.31 (q, J = 6.3 Hz, 2H); 2.12 (q^5, J = 6.3 Hz, 2H); 1.38 (s, 9H). ^{13}C NMR (CD_2C1_2, 298 K): δ: 156.6 (C); 147.7 (C); 142.6 (C); 139.0 (CH); 129.0 (CH); 124.6 (CH); 121.2 (q, J = 319 Hz, CF_3); 79.3 (C); 68.1 (CH_2); 54.8 (3 × CH_3); 37.5 (CH_2); 30.0 (CH_2); 28.4 (3 × CH_3).

No-carrier-added aqueous [^{18}F]fluoride ion was produced via the [^{18}O(p,n)^{18}F] nuclear reaction by irradiation of a 2-ml [^{18}O]water (>97%-enriched, CortecNet, Paris, France) target on an IBA Cyclone-18/9 cyclotron (18 MeV proton beam) and was transferred to the appropriate hot cell.

Target hardware: Commercial, 2-ml, two-port, stainless steel target holder equipped with a domed-end niobium cylinder insert. *Target to hot cell liquid-transfer system*: Sixty metre PTFE line (0.8 mm internal diameter; 1/16 inch external diameter), 2.0 bar helium drive pressure, transfer time 3–6 min. Typical production of [^{18}F]Fluoride ion at the end of bombardment for a 20 μA, 30 min (10 μA.h) irradiation: 750–800 mCi (27.7–29.6 GBq).

High-performance liquid chromatography

HPLC A (for purification of starting peptides): performed on a preparative Vydac C_{18}-reverse-phase column (10–15 μm, 22 × 250 mm, VYDAC, Hesperia, CA, USA). Solvents and conditions: solvent A: 90% H_2O, 10% MeCN, 0.1% TFA, solvent B: 10% H_2O, 90% MeCN, 0.1% TFA; linear gradient over 30 min 0–100% B, flow rate 2.5 ml/min, absorption detection $\lambda = 254$ nm. HPLC B (for purification of non-labelled conjugated peptides): performed on a analytical Jupiter 4 m Proteo column (250 × 4.6 mm). Solvents and conditions: solvent A: 90% H_2O, 10% MeCN, 0.1% TFA, solvent B: 10% H_2O, 90% MeCN, 0.1% TFA, linear gradient 30–100% B over 25 min, flow rate 1.5 ml/min, absorption detection $\lambda = 254$ nm. HPLC C (for purification of [^{18}F]FPyME): performed on semi-preparative SiO_2 Zorbax® Rx-SIL (5 μm, 250 × 9.4 mm; Hewlett Packard). Solvents and conditions: isocratic elution with heptane/EtOAc: 60/40 (v/v), flow rate 5 ml/min, absorbance detection $\lambda = 254$ nm, radioactive detection using a Geiger-Müller detector. HPLC D (for purification of labelled conjugated peptides): performed on a semi-preparative C18 μBondapak® (10 μm, 300 × 7.8 mm, Waters). Solvents and conditions: solvent A: 90% H_2O, 10% MeCN, 0.1% TFA, solvent B: 10% H_2O, 90% MeCN, 0.1% TFA, linear gradient 20–50% B over 20 min, flow rate 4 ml/min, absorbance detection $\lambda = 254$ nm, radioactive detection using a Geiger-Müller detector. HPLC E (for quality control of labelled conjugated peptides): performed on a semi-preparative C18 μBondapak® (10 μm, 300 × 7.8 mm, Waters). Solvents and conditions: solvent A: 90% H_2O, 10% MeCN, 0.1% TFA, solvent B: 10% H_2O, 90% MeCN, 0.1% TFA, linear gradient 20–50% B over 20 min, flow rate 4 ml/min; absorbance detection at $\lambda = 254$ nm, radioactive detection using a Packard Flow One Scintillation Analyzer equipped with a positron-dedicated cell for radioactivity monitoring.

Spectroscopy

NMR spectra were recorded on a Bruker AMX (300 MHz) apparatus using the hydrogenated residue of the deuterated solvent (CD_2Cl_2, $\delta = 5.32$ ppm) as internal standards for ^{1}H NMR as well as the deuterated solvent (CD_2Cl_2, $\delta = 53.8$ ppm) as internal standards for ^{13}C NMR. The chemical shifts are reported in ppm, downfield from TMS. The mass spectra were measured on a Nermag R10-10 apparatus spectrometer. MALDI-TOF-MS was performed on a VG Tof Spec E Fisons instrument (Fisons Instruments, Beverly, CA, USA), using α-cyano-*p*-hydroxycinnamic acid as matrix. Substance *P* and ACTH were used as calibrants.

Chemistry

Peptide synthesis

All peptides were synthesized using standard solid phase 9-Fmoc chemistry.[13,14] The stepwise synthesis was carried out using TentaGel RAM (50 mg, loading 0.24 mmol/g). Activation of the Fmoc amino acids and formation of peptide bonds were carried out using DIPCDI and *N*-hydroxybenzotriazole. All Fmoc amino acids and coupling reagents were used in fourfold excess. Fmoc deprotection was accomplished by treatment with 20% piperidine in *N*-methyl-2-pyrrolidone for 10 min at ambient temperature. The peptides were detached from the solid support and permanent side chain protection groups removed by treatment with TFA/Water/TIS/Thioanisol (90:5:2.5:2.5: v/v) for 2 h at room temperature. All peptides were purified by using HPLC (HPLC A) and identified using MALDI-TOF-MS (see Table 1).

Peptide conjugation with FPyME

Conjugation with FPyME was performed as follows: FPyME (5 eq. in DMSO) was added to 1 mg of peptide dissolved in a mixture of 495 μl DMSO and 5 μl DIEPA. The reaction mixture was left standing at ambient temperature for 30 min. The conjugated peptide was purified using HPLC (HPLC B) and identified using MALDI-TOF-MS (see Table 1).

Radiochemistry

Synthesis of 1-[3-(2-[^{18}F]fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione ([^{18}F]FPyME)

Radiosyntheses using fluorine-18, including the HPLC purifications, were performed in a 7.5-cm-lead shielded cell using a computer assisted Zymate robot system (Zymark Corporation, USA).

Fluorine-18 (half-life: 109.8 min) as [^{18}F]fluoride ion was isolated by passing the irradiated [^{18}O]water target, using helium pressure (1.5–2.0 bar), through an anion exchange resin (Sep-pak® Light Waters Accell™ Plus QMA cartridge (OH^- form, generated from the Cl^- form by washing with aq. 1 M $NaHCO_3$ (2 ml) and rinsed with water (20 ml) and CH_3CN (10 ml)). Helium was passed through the column to maximally extract [^{18}O]water. The [^{18}F]fluoride ion was then eluted from the resin, using an aq. K_2CO_3 solution (1.0 ml of a 4.5 mg/ml solution), into a Vacutainer® tube containing Kryptofix®222 (K_{222}: 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane, 12.0–15.0 mg). The resulting solution was then gently concentrated to dryness at 145–150°C under a nitrogen stream for 10 min to give no-carrier-added K[^{18}F]F-K_{222} complex as a white semi-solid residue.

DMSO (600 μl) containing [3-(3-*tert*-butoxycarbonylaminopropoxy)-pyridin-2-yl]-trimethylammonium trifluoromethanesulfonate (4.0 mg) was added to the Vacutainer® tube containing the dried K[^{18}F]F-K_{222} complex. The tube (not sealed) was then thoroughly vortexed (15 s) and placed in a heating block (at 145°C, for 2 min) without stirring the contents. The reaction vessel was then cooled using an ice-water bath, and the contents diluted with water (1 ml) and transferred to a reservoir on top of a C18 cartridge (PrepSep™ R-C18, Fisher Scientific, activated with EtOH (2 ml) and then rinsed with water (10 ml)). The reaction vessel was rinsed twice with water (1 ml), which was also transferred and added to the diluted reaction mixture on the reservoir. After addition of another 2 ml of water, the whole solution was passed through the C18 cartridge. The cartridge was washed with water (1 ml) and partially dried for 0.5 min by applying a nitrogen stream. The intermediate [3-(2-[^{18}F]fluoropyridin-3-yloxy)propyl]carbamic acid *tert*-butyl ester was eluted from the cartridge with CH_2Cl_2 (3 ml) into a

5-ml reaction vial containing TFA (0.1 ml). Two 1 ml quantities of CH_2Cl_2 were used to wash the cartridge and to completely transfer the fluorine-18-labelled ester. The resulting CH_2Cl_2/TFA solution (50/1, v/v) was concentrated to dryness (at 65–75°C under a gentle nitrogen stream for 4–6 min) giving the corresponding amine (3-(2-[^{18}F]fluoropyridin-3-yloxy)-propylamine). This residue was first redissolved in CH_2Cl_2 (2 ml) and concentrated again to dryness to minimize TFA presence (at 65–75°C under a gentle nitrogen stream for another 2–3 min), then redissolved in dioxane (0.250 ml) containing *N*-methoxycarbonylmaleimide (25 mg). Aq. sat. $NaHCO_3$ (0.75 ml) was then added to the solution and the vessel was gently vortexed for 10 min at room temperature. The reaction mixture was diluted with 1 ml of aq. 1M HCl and transferred onto a C18 Sep-pak cartridge (PrepSep^{TM} R-C18; Fisher Scientific, activated as described above and loaded with water (5 ml)). The vessel was rinsed twice with water (1 ml), which was also transferred and added to the diluted reaction mixture on top of the cartridge. The solution was then passed through the cartridge. The cartridge was washed with water (3 ml) and partially dried for 0.5 min by applying a nitrogen stream. Crude [^{18}F]FPyME was eluted from the cartridge with CH_2Cl_2 (3 ml) into a 5-ml vial. Two 1 ml quantities of CH_2Cl_2 were used to wash the cartridge and to completely transfer the [^{18}F]FPyME. The resulting solution was concentrated to a volume of 1.0–1.5 ml (at 65–75°C under a gentle nitrogen stream for 2–4 min) and purified by using HPLC (HPLC C, R_t : 10.0–10.5 min) to give radiochemically pure [^{18}F]FPyME.

Peptide conjugations with [^{18}F]FPyME

To 100 µl of DMSO containing HPLC-purified [^{18}F]FPyME (HPLC-solvents having been removed by concentration to dryness at 65–75°C under a gentle nitrogen stream) was added the peptide (1 mg) dissolved in a mixture of DMSO (495 µl) and DIEPA (5 µl). The reaction mixture was left standing at ambient temperature for 10 min and subsequently purified using preparative HPLC (HPLC D R_t: see Table 1). The fractions containing the radiochemically pure [^{18}F]peptide were collected and evaporated to dryness at 65°C under vacuum, followed by reformulation in 5% DMSO in PBS buffer. In cases of two or more consecutive preparations of [^{18}F]peptides, the initial batch of HPLC-purified [^{18}F]FPyME was retaken in a sufficient volume of DMSO and simply divided in as many portions as peptides to label before conjugation and purification.

Measurement of log *D*

The log *D* of the ^{18}F-labelled peptides was determined by using the shake-flask method.[17] A mixture of 50 µl peptide solution and 450 µl phosphate buffer pH = 7.4 (PBS) was added to 500 µl *n*-octanol. To thoroughly bring in contact the two immiscible phases, the mixture was vortexed for 3 min. Following 2 min of centrifugation (3000 rpm), an aliquot of 100 µl of each phase was measured in a well counter. Log *D* was calculated by: log $D = \log(cpm_{octanol}/cpm_{PBS})$.

Conclusion

The high conjugation yields and short coupling times of the [^{18}F]FPyME labelling method allow for the labelling in one single set of experiments a series of bioactive peptides using a parallel synthetic approach.

Acknowledgements

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