Amino Acids (2009) 37:717–724
DOI 10.1007/s00726-008-0200-0

ORIGINAL ARTICLE

Methods for ^{18}F-labeling of RGD peptides: comparison of aminooxy [^{18}F]fluorobenzaldehyde condensation with 'click labeling' using 2-[^{18}F]fluoroethylazide, and *S*-alkylation with [^{18}F]fluoropropanethiol

Matthias Glaser · Magne Solbakken · David R. Turton · Roger Pettitt · Jon Barnett · Joseph Arukwe · Hege Karlsen · Alan Cuthbertson · Sajinder K. Luthra · Erik Årstad

Received: 29 August 2008 / Accepted: 17 October 2008 / Published online: 15 November 2008

Abstract Three strategies for chemoselective labeling of RGD peptides with ^{18}F have been compared. Aminooxy [^{18}F]fluorobenzaldehyde conjugation provided 40 ± 12% decay-corrected radiochemical yield using a fully automated method. An one-pot protocol for 'click labeling' of the RGD scaffold with 2-[^{18}F]fluoroethylazide afforded 47 ± 8% decay-corrected radiochemical yield. Attempted conjugation with 3-[^{18}F]fluoropropanethiol led to extensive decomposition and was therefore found unsuitable for labeling of the RGD peptide investigated. The results suggest that 'click labeling' of RGD peptides provides an attractive alternative to aminooxy aldehyde condensation, however, 2-[^{18}F]-fluoroethylazide may be too small to allow separation of large ^{18}F-labeled RGD peptides from their precursors.

M. Glaser · D. R. Turton · S. K. Luthra · E. Årstad
GE Healthcare, Medical Diagnostics Discovery,
Hammersmith Imanet Ltd, Hammersmith Hospital,
Du Cane Road, London W12 0NN, UK

M. Solbakken · J. Arukwe · H. Karlsen · A. Cuthbertson
GE Healthcare, Medical Diagnostics Discovery,
Nycoveien 2, 0401 Oslo, Norway

R. Pettitt · J. Barnett
GE Healthcare, Medical Diagnostics, The Grove Centre,
Amersham HP7 9LL, UK

Present Address:
E. Årstad (✉)
Institute of Nuclear Medicine, University College London,
University College London Hospitals NHS Trust,
235 Euston Road, London NW1 2BU, UK
e-mail: e.arstad@ucl.ac.uk

Keywords PET · RGD · Angiogenesis · Fluorine-18 · Automation

Introduction

Tumors rely on sprouting of new blood vessels, known as angiogenesis, to support growth and metastasis. In addition to conventional therapies, inhibition of angiogenesis may therefore help delay tumor growth. Bevacizumab (Avastin), an antibody targeting the vascular endothelial growth factor (VEGF), was recently approved for anti-angiogenic therapy, and other drugs are currently in late-stage clinical trials (Folkman 2007; Shahi and Pineda 2008). However, it is still unclear which tumors will respond to treatment, how to optimize drug doses and how to assess response in patients. Non-invasive imaging of tumor vasculature may therefore play an important role in the development of anti-angiogenic therapy, patient stratification and treatment monitoring.

The $\alpha_V\beta_3$ integrin receptor is almost exclusively expressed on proliferating endothelial cells and cancerous tissue, where it regulates angiogenesis, tumor growth and local invasiveness (Moitessier et al. 2004; Van de Wiele et al. 2002; Wester and Kessler 2005). Hence, quantitative imaging of $\alpha_V\beta_3$ integrin populations with positron emission tomography (PET) is of particular interest in conjunction with anti-angiogenic therapy. Quantification of $\alpha_V\beta_3$ integrins is well established in preclinical tumor models using radiolabeled arginine-glycine-aspartic acid (RGD)-containing peptides (Beer et al. 2005; Chen et al. 2004; Haubner et al. 2001, 2004; Poethko et al. 2004a, b; Wu et al. 2007; Zhang et al. 2006) and recently, clinical studies with [^{18}F]galacto-RGD have yielded promising results (Beer et al. 2005, 2008). ^{18}F has close to ideal decay characteristics for PET ($t_{1/2}$ = 110 min, β^+ 0.64 MeV);

however, the existing methods for peptide labeling rely on highly specialized techniques, involve manual handling, and are restricted to a few centers world-wide. Translation to routine clinical applications will therefore depend on the development of automated processes that can support large-scale, routine production.

We recently reported a new class of RGD peptides for PET, which was derived from the single-photon emission computed tomography (SPECT) tracer ^{99m}Tc-NC100692 (Bach-Gansmo et al. 2006; Glaser et al. 2008; Indrevoll et al. 2006). Labeling was achieved with aminooxy aldehyde condensation using [^{18}F]fluorobenzaldehyde ([^{18}F]FB-CHO), [^{18}F]-4-(3-fluoropropoxy)benzaldehyde as well as a polyethylene glycol (PEG)-modified [^{18}F]fluorinated aldehyde (Glaser et al. 2008). Whilst the latter labeling group gave superior biological results in preclinical tumor models, the high radiochemical yields and simplicity of [^{18}F]FB-CHO conjugation made this the reagent of choice for clinical studies (Kenny et al. 2008). Herein, we describe an automated method for clinical production of our own ^{18}F-labeled RGD tracer using the commercially available synthesizer TRACERLab^{TM} FX F-N (GE Healthcare). Using the same peptide scaffold, we also compare the suitability of two alternative chemoselective labeling strategies for automation: alkylation of a chloroacetamide group with 3-[^{18}F]fluoropropanethiol ([^{18}F]FPT) and 'click labeling' of a terminal alkyne with 2-[^{18}F]fluoroethylazide ([^{18}F]FEA) (Chart 1).

Materials and methods

General

Peptides were synthesised on solid phase using an Applied Biosystems 433A fully automated peptide synthesizer with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) activation of standard Fmoc-amino acids. All peptide syntheses reagents and solvents were purchased from Novabiochem, Bachem and Applied Biosystems. All other reagents were purchased from Acros, Fluka and Sigma-Aldrich. Analytical and semi-preparative HPLC were run on reversed-phase C18 columns using different gradients (solvent A: water/0.1% TFA and solvent B: acetonitrile/0.1% TFA) with UV-detection at 214 and 254 nm using Beckman Gold, Shimadzu (LC-8A pump, SPD-10A UV detector, FRC-10A fraction collector), Waters Delta Prep and Thermo instruments. Mass spectra were recorded on an LCQ quadrupole ion-trap mass spectrometer (Thermo Finnigan Surveyor) using electrospray ionization (ESI). ^{1}H NMR spectra were run at 25°C on a Jeol 500 or a Varian Unity Innova 500 MHz spectrometer equipped with a 5 mm ^{1}H-broadband pfg indirect detection probe.

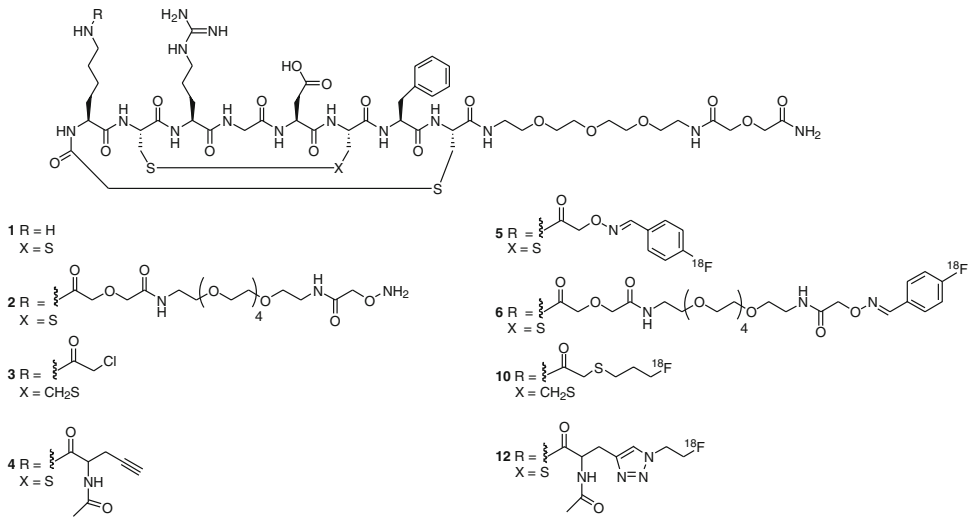
Synthesis of peptide **2**

The synthesis of the aminooxy functionalized peptide **2** has previously been reported (Indrevoll et al. 2006; Solbakken et al. 2006).

Synthesis of peptide **3**

All manual steps were carried out in a nitrogen bubbler apparatus. Fmoc-amino-PEG4-diglycolic acid (Polypure AS) was attached to Rink Amide AM resin (Novabiochem) on a 0.25 mmol scale by standard protocol using an excess of the acid (531 mg, 1.00 mmol), 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (380 mg, 1.00 mmol) and NMM (202 mg, 2.00 mmol). Standard Fmoc-amino acids were coupled on the automated synthesizer to give the sequence

Chart 1 ^{18}F-labeled RGD peptides and precursors



Fmoc-Lys(Boc)-Cys(StBu)-Arg(Pmc)-Gly-Asp(OtBu)-Cys (StBu)-Phe-Cys(Trt)-PEG4-Diglycoloyl-NH-Rink Amide resin. An aliquot of the resin corresponding to 0.12 mmol was treated with a solution of tributylphosphine (1.24 ml, 5.25 mmol) and water (0.2 ml) in DMF (4 ml) for 30 min. Reagents were filtered off and the resin was washed with DMF and dichloromethane. To the resin was added a solution of tetrabutylammonium fluoride (TBAF) (151 mg, 0.490 mmol) in dichloromethane (4 ml). After 60 min reaction time the resin was isolated by filtration and washed with dichloromethane. The Fmoc group was cleaved by treatment with 20% piperidine in DMF (3 × 5 min). A solution of chloroacetic anhydride (103 mg, 0.600 mmol) in DMF (3 ml) was added. After 20 min the resin was filtered off and the washed with DMF and dichloromethane. Treatment with a solution of trifluoroacetic acid, triisopropylsilane and water (95:2.5:2.5 v/v/v) cleaved the peptide from the resin and removed the side-chain protecting groups. After filtration the solution was concentrated under reduced pressure. The crude product was precipitated from diethyl ether, washed with diethyl ether and dried. The material was dissolved in water/acetonitrile (1:1, concentration 1 mg/ml) and the pH was adjusted to 8 by addition of dilute ammonia. After stirring overnight the crude product was isolated by evaporation followed by lyophilisation and subjected to purification by preparative HPLC (column Vydac 218TP1022 C18, 250 × 22 mm, 10 µm; gradient 10–50% B over 40 min; flow rate 10 ml/min) giving 95 mg (60%). To a solution of the peptide (76 mg, 0.060 mmol) in DMF (4 ml) was added chloroacetic anhydride (103 mg, 0.600 mmol). The mixture was stirred overnight and concentrated. The residue was purified by preparative HPLC (conditions as above) yielding 66 mg (82%). HPLC (column Vydac 218TP54 250 × 4.6 mm, 5 µm; gradient 10–50% B over 20 min; flow rate 1.0 ml/min) $t_R = 11.6$ min. MS (ESI) found $m/z = 1348.0$ $(MH)^+$, calcd $m/z = 1348.5$

Synthesis of peptide **4**

To a solution of *N*-acetyl-DL-propargylglycine ethyl ester (186 mg, 1.00 mmol) in ethanol (5 ml) was added 1 M aq. sodium hydroxide solution (1.1 ml, 1.1 mmol). The reaction mixture was stirred for 60 min. After evaporation of ethanol the residual solution was acidified to pH 2 by addition of TFA and subjected to preparative HPLC [column Phenomenex Luna C18(2), 250 × 21.2 mm, 5 µm; isocratic 0% B for 15 min followed by gradient 0–100% B over 10 min, flow 10 ml/min; $t_R = 25.8$ min] giving 75 mg (48%) of the free acid after lyophilisation. LC–MS [column Phenomenex Luna C18(2) 50 × 2 mm, 3 µm; isocratic 0% B; flow rate 0.3 ml/min] $t_R = 2.24$ min; found $m/z = 155.8$ $(MH)^+$, calcd $m/z = 156.1$.

A solution of *N*-acetyl-DL-propargylglycine (31 mg, 0.20 mmol), 7-azabenzotriazol-1-yloxy-tris-(pyrrolidino) phosphonium hexafluorophosphate (PyAOP) (104 mg, 0.200 mmol) and *N*-methylmorpholine (NMM) (88 µl, 0.80 mmol) in DMF (3 ml) was stirred for 5 min at room temperature followed by addition of peptide NC100717 (Indrevoll et al. 2006) (126 mg, 0.100 mmol) dissolved in DMF (4 ml). The reaction mixture was stirred for 45 min followed by addition of a second portion of peptide NC100717 (132 mg, 0.100 mg) and NMM (44 µl, 0.40 mmol). Stirring was continued for 45 min and the mixture was concentrated in vacuo. The residue was taken up in 10% acetonitrile (100 ml) and subjected to purification by preparative HPLC [column Phenomenex Luna C18(2), 250 × 50 mm, 10 µm; gradient 10–40% B over 60 min; flow rate 50 ml/min; $t_R = 31.3$ min] to give 170 mg (61%) of white solid material after lyophilisation. LC–MS [Phenomenex Luna C18(2) column 50 × 2 mm, 3 µm; gradient 10–40% B over 10 min; flow rate 0.3 ml/min] $t_R = 6.32$ min; found $m/z = 1395.7$ $(MH)^+$, calcd $m/z = 1395.5$.

Synthesis of peptide **6**

The reference compound **6** was prepared as previously described (Indrevoll et al. 2006; Solbakken et al. 2006).

Synthesis of peptide **10**

3-Fluoropropyl triphenylmethylsulfide (Glaser et al. 2004) (1.0 mg, 3.0 µmol) was added to a mixture of trifluoroacetic acid (50 µl), triisopropylsilane (5 µl) and water (5 µl). After 1 min a solution of peptide **3** (2.0 mg, 1.5 µmol) in water (0.6 ml) was added and pH was adjusted to 9.5 by addition of aq. potassium carbonate. The reaction mixture was heated at 70°C for 1 h, cooled to room temperature and subjected to preparative HPLC [column Phenomenex Luna C18(2), 250 × 10 mm, 10 µm; gradient 10–50% B over 30 min; flow rate 5 ml/min] to give 0.72 (34%) mg of peptide **10**. HPLC (column Vydac 218TP54 250 × 4.6 mm, 5 µm; gradient 10–50% B over 20 min, flow rate 1.0 ml/min) $t_R = 13.3$ min. MS (ESI) found $m/z = 1406.3$ $(MH)^+$, calcd $m/z = 1406.5$.

Synthesis of peptide **12**

To a stirring solution of N^{α}-acetyl-[(2-fluoroethyl)-1,2,3-triazol-4-yl)]methylglycine ethyl ester (Glaser and Årstad 2007) (196 mg, 0.719 mmol) in acetonitrile (1 ml) was added a sodium hydroxide solution (1 ml, 1.080 mmol) at 0°C. After 8 h the organic solvent was removed by evaporation at reduced pressure and the crude product extracted in to ethyl acetate (3 × 2 ml) and recrystallized from ethyl

acetate/diethyl ether. White crystals, yield 18 mg (11%). ^{1}H NMR (DMSO-d_6) δ 8.11 (d, $J = 7.8$ Hz, 1H), 7.85 (s 1H), 4.82 (m, 1H), 4.72 (m, 1H), 4.69 (m, 1H), 4.64 (m, 1H), 4.40 (m, 1H), 3.09 (m, 1H), 2.95 (m, 1H), 1.81 (s, 3H) ppm.

A solution of N^{α}-acetyl-[(2-fluoroethyl)-1,2,3-triazol-4-yl)] methylglycine (4.9 mg, 0.020 mmol), 7-azabenzotriazol-1-yloxy-tris-(pyrrolidino)phosphonium hexafluorophosphate (PyAOP) (10.4 mg, 0.020 mmol) and *N*-methylmorpholine (NMM) (8.8 µl, 0.080 mmol) in DMF (1 ml) was stirred for 5 min at room temperature followed by addition of peptide NC100717 (Indrevoll et al. 2006) (38 mg, 0.030 mmol) dissolved in DMF (1 ml). Stirring was continued for 80 min followed by concentration in vacuo. The residue was taken up in 10% acetonitrile (8 ml) and purified by preparative HPLC [column Phenomenex Luna C18(2), 250 × 50 mm, 10 µm; gradient 10–30% B over 60 min; flow rate 50 mL/min; $t_R = 32.8$ min] to give 24 mg (81%) of white solid material after lyophilisation. LC–MS [column Phenomenex Luna C18(2), 20 × 2 mm, 3 µm; gradient 10–30% B over 10 min; flow rate 0.6 ml/min] $t_R = 3.25$ min; found $m/z = 1484.7$ $(MH)^+$, calcd $m/z = 1484.6$.

Radiochemistry

Chemicals including anhydrous solvents were obtained from Sigma-Aldrich (Gillingham, UK). HPLC solvents were purchased from Fisher Scientific (Loughborough, UK). No-carrier-added aqueous [^{18}F]fluoride was obtained from the ^{18}O(p,n)^{18}F reaction (PETTrace cyclotron, GE Medical Systems) by the irradiation of an isotopically enriched [^{18}O] water target using a 16.4 MeV proton beam.

Automated production of [^{18}F]**6** using TracerLab $FX_{F\text{-}N}$

[^{18}F]**6** was produced on a TracerLab $FX_{F\text{-}N}$ module (GE Medical systems) using manual control of the HPLC pump. The HPLC system comprised of a HPLC pre-column (4 mm h × 3 mm ID, Part No. AJO-4287, Phenomenex), a C18 HPLC column (100 mm × 10 mm, Part No. 00D-4252-N0, Luna, Phenomenex) running a mobile phase of water/ethanol/phosphoric acid (72/28/0.1 % v/v/v) at 3 ml/min.

The [^{18}F]fluoride containing water (1.0–1.5 ml, 10.3–14.6 GBq) was transferred into the reactor vial containing a mixture of Kryptofix® (5.5 mg, 14.6 µmol), potassium carbonate (0.77 mg, 5.6 µmol), acetonitrile (0.44 ml), and water (0.11 ml).

After the removal of water under reduced pressure at 90°C, acetonitrile (1.5 ml) was added and evaporated under reduced pressure. This procedure was repeated twice, with 4 min drying time after the first addition of acetonitrile and 1 min drying time after the second addition. The total time for azeotropic of [^{18}F]fluoride was 12 min. Following cooling to 40°C, a solution of 4-*N,N,N*-trimethylanilium aldehyde trifluoromethanesulfonate (**7**) (3.0 mg, 9.4 µmol) in dimethylsulfoxide (1.0 ml) was added. The stirring mixture was heated for 5 minutes at 90°C, diluted with water (1 ml), and transferred onto a C18-SepPak cartridge. After washing with water (1 ml), the radioactive product was flushed back into the reactor vessel with acetonitrile (1 ml). A solution of the deprotected aminooxy peptide precursor **2** (1.5 mg, 0.83 µmol) in buffer (1.0 ml, sodium citrate/phosphate buffer, pH 2.6) was added and the mixture heated with stirring at 70°C for 15 min. A stream of nitrogen gas was applied for 5 min to remove the bulk of acetonitrile. After diluting with water (3 ml), the labeled peptide [^{18}F]**6** was purified by preparative HPLC. The isolated peptide [^{18}F]**6** was mixed with phosphate buffered saline (12 ml) and sterile filtered (Pall 13 mm Acrodisc with HT Tyffryn membrane).

Preparation of [^{18}F]**12**

Method A (solution phase catalysis)

A solution of copper(II) sulfate pentahydrate (0.72 mg, 2.88 µmol, 50 µl) in a conical vial (1 ml Wheaton) was purged with nitrogen (10 ml/min) for one minute. A solution of sodium ascorbate (0.58 mg, 2.93 µmol) in potassium carbonate buffer (50 µl, pH 8.0, 50 mM) was added. To the resulting suspension a solution of the alkyne peptide precursor **4** (2 mg, 1.43 µmol) in DMF (25 µl) and potassium carbonate buffer (25 µl, pH 8.0, 50 mM) was added, followed by [^{18}F]FEA (60–370 MBq, Glaser and Årstad 2007) in acetonitrile (50 µl). The reaction mixture was heated for 30 min at 80°C, diluted with water (100 µl, 0.1 % TFA), and the resulting mixture purified by preparative HPLC (Solvent A: water/0.1% TFA, solvent B: acetonitrile 0.1%/ TFA, column: Phenomenex Onyx Monolithic C18 100 × 10 mm, flow: 4.0 ml/min, gradient: 5–80% B in 15 min, $\lambda = 216$ nm). The radiochemical purity was >99% and the decay-corrected radiochemical yield 70 ± 5% ($n = 3$).

Method B (solid phase catalysis)

The peptide precursor **4** (0.5 mg, 0.36 µmol) was dissolved in sodium phosphate buffer (50 µl, pH 6.0, 50 mM), mixed with copper powder (200 mg, −40 mesh) and [^{18}F]FEA (60–370 MBq) in acetonitrile (25 µl). After incubating for 15 min at room temperature the reaction mixture was quenched with HPLC mobile phase (water/5% acetonitrile/0.1% TFA), passed through a syringe filter (Acrodisk PTFE, 0.45 µm) and injected into the preparative HPLC.

The radiochemical purity was >99% and the decay-corrected radiochemical yield 42 ± 14% ($n = 3$).

Method C (one-pot solution phase catalysis)

A conical vial (Wheaton, 1 ml) containing a magnetic stirring bar was charged with potassium bicarbonate (0.72 mg, 7.2 µmol) in water (50 µl), Kryptofix® (5 mg, 13.3 µmol), acetonitrile (0.5 ml) and ^{18}F-fluoride containing water (60–740 MBq, 0.2–0.5 ml). The water was removed by azeotropic distillation at 100°C using a stream of nitrogen (50 ml/min) with repeated additions of anhydrous acetonitrile (3 × 0.5 ml). The vessel was cooled to room temperature. After addition of 2-azidoethyl touluenesulfonic ester (0.26 µl, 1.3 µmol) in anhydrous dimethylsulfoxide (0.2 ml), the solution was stirred at 80°C (5 min). Copper(II) sulfate pentahydrate (0.72 mg, 2.88 µmol) in water (10 µl) was mixed with sodium ascorbate (0.58 mg, 2.93 µmol) in water (10 µl) under a nitrogen atmosphere and transferred into a solution of the alkyne precursor **4** (2 mg, 1.43 µmol) in dimethylformamide (25 µl). The resulting suspension was added to the vial containing [^{18}F]FEA (prepared as above). After stirring for 5 min at 80°C, the reaction was quenched by adding an aqueous solution of trifluoroacetic acid (0.1 % v/v, 0.2 ml). [^{18}F]**12** was isolated by preparative HPLC (gradient: 2 min 5% B, then 5 → 80% B in 15 min using the system described above). The radiochemical purity was >99% and the decay-corrected radiochemical yield 47 ± 8 % ($n = 3$). The total synthesis time was 105 min after end of bombardment.

Experiment to investigate the nature of the radioactive side product from the preparation of [^{18}F]12 (one-pot solution phase catalysis)

A solution of copper(II) sulfate pentahydrate (3.6 mg, 14.4 µmol, 50 µl) in a conical vial (1 ml Wheaton) was purged with nitrogen (10 ml/min) for 1 min. A solution of sodium ascorbate (2.9 mg, 14.6 µmol) dissolved in sodium phosphate buffer (50 µl, pH 6.0, 50 mM) was added. To the resulting suspension a solution of alkyne peptide precursor **4** (2 mg, 1.43 µmol) in DMF (25 µl) and sodium phosphate buffer (25 µl, pH 6.0, 50 mM) were added, followed by [^{18}F]FEA (40 MBq) in acetonitrile (50 µl). The reaction mixture was heated for 30 min at 80°C, diluted with water (200 µl, 0.1% TFA), and injected into the preparative HPLC. Two products were isolated (product 1, $t_R = 7{:}47$ min and product 2, $t_R = 8{:}14$ min). Product 2 (100 µl) was mixed with aqueous ammonia (28%, 50 µl). The mixture was left at room temperature for one minute and re-analyzed using the preparative HPLC system. The chromatogram showed a main peak corresponding to [^{18}F]**12** ($t_R = 7{:}49$ min, 92%).

Results and discussion

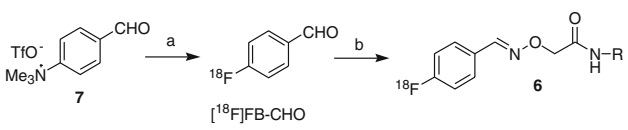
Peptide synthesis

The cyclic RGD peptide **1** (Chart 1) was prepared as previously described (Indrevoll et al. 2006). An aminooxy functionalized PEG linker was incorporated to provide the precursor **2** for conjugation with [^{18}F]FB-CHO. For *S*-alkylation with [^{18}F]FPT the peptide **3** was modified with a chloroacetamide group, and a methylene spacer was inserted into the disulfide bridge to prevent ring opening during the conjugation step. Propargyl glycine was incorporated to provide the precursor **4** for 'click labeling' with [^{18}F]FEA.

Aminooxy [^{18}F]fluorobenzaldehyde condensation

Aminooxy groups rapidly condense with aldehyde and ketone carbonyls to form stable oximes. The reaction is highly efficient, tolerates water as the reaction medium, and can be effected over a wide pH range. Oxime formation with [^{18}F]FB-CHO has been established as a superior method for ^{18}F-labeling of aminooxy-containing peptides (Poethko et al. 2004a, b; Schottelius et al. 2004). The method lends itself well to automation as the prosthetic labeling group can be obtained in good yields in a single step. Following cartridge purification peptide conjugation can be achieved in near quantitative yields under mild conditions, short reaction times, and with low precursor concentrations.

We have previously reported the radiosynthesis of RGD peptide [^{18}F]**5** (Glaser et al. 2008). Here we employed the PEG-modified analog **2** as a precursor and automated the labeling protocol of peptide [^{18}F]**6** (Scheme 1) using TRACERlab^{TM} FX F-N (GE Healthcare). The automated radiosynthesis involved the following steps: (1) drying of aqueous [^{18}F]fluoride, (2) reaction with 4-formyl-*N,N,N*-trimethylanilinium trifluoromethanesulfonate (**7**) at 90°C for 5 min to produce [^{18}F]FB-CHO, (3) cartridge purification and elution of the radioactive product back into the reaction vial, (4) addition of the aminooxy peptide precursor **2** (1.5 mg, 0.83 µmol) followed by heating at 70°C for 15 min, (5) concentration of the reaction mixture, (6) HPLC purification of the labeled peptide [^{18}F]**6**, (7) formulation and (8) sterile filtration. The method was fully



Scheme 1 Formation of [^{18}F]FB-CHO and conjugation with the aminooxy functionalized RGD peptide **2**. (**a**) [^{18}F]KF-222/K_2CO_3, DMSO, 15 min at 90°C; (**b**) **2**, pH 2.6, 15 min at 70°C

automated with the exception of the HPLC pump, which was manually controlled. The total synthesis time was 69–74 min, providing the RGD peptide [^{18}F]**6** in 40 ± 12% (n = 10, decay-corrected) radiochemical yield, with a specific activity of 173.0 ± 52.4 GBq/μmol (n = 10). Starting from 10.3–14.6 GBq of ^{18}F-fluoride, the ^{18}F-labeled RGD peptide [^{18}F]**6** was obtained in 2.3–5.3 GBq. Full details are provided in the experimental part.

S-alkylation with 3-[^{18}F]fluoropropanethiol ([^{18}F]FPT)

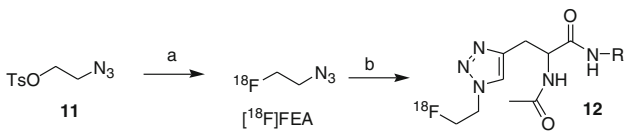
In an earlier study, we found that ^{18}F-labeled alkylthiols can be conjugated to chloroacetyl-modified peptides in good yields (Glaser et al. 2004). Of the reagents investigated, [^{18}F]FPT (Scheme 2) was found to give the best results. Whilst the radiosynthesis of this reagent requires the use of a trityl protecting group, we anticipated that deprotection could be achieved as an integrated part of the cartridge purification step, and that the method therefore should be amenable to automation using a similar protocol to that described for [^{18}F]FB-CHO. Unfortunately, when the chloroacetyl-modified peptide precursor **3** was subjected to the labeling conditions previously reported (aqueous ammonia, 10 min at 80°C), a multitude of radioactive products was produced. Further investigation indicated poor stability of the peptide precursor **3** under alkaline conditions, and complete degradation was observed within 30 min at 80°C. We also observed decomposition of [^{18}F]FPT, however, this was effectively prevented by addition of tris(2-carboxyethyl)phosphine as a stabilizer. It is conceivable that the alkylation reaction can be achieved under milder conditions than those investigated here; however, given the moderate reaction kinetics, the need for a basic reaction medium, and the poor stability of the unprotected prosthetic group, we conclude that *S*-alkylation with [^{18}F]FPT is unsuitable for the synthesis of RGD peptide [^{18}F]**10**.

'Click labeling' with 2-[^{18}F]fluoroethylazide ([^{18}F]FEA)

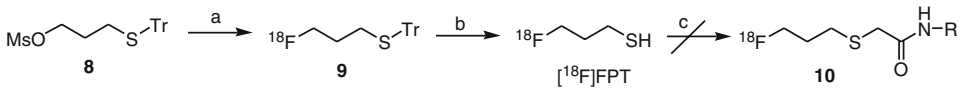
'Click chemistry' refers to reactions that are particularly high yielding and resource efficient. In particular, the term is used to describe the Cu(I)-catalyzed cycloaddition of terminal alkynes and azides (Kolb et al. 2001; Rostovtsev et al. 2002). We recently developed [^{18}F]FEA as a prosthetic labeling group (Scheme 3) and demonstrated that 'click labeling' can be achieved with a range of functionalized alkynes in high radiochemical yields (Glaser and Årstad 2007).

In this study we aimed to develop an efficient radiosynthesis of the RGD peptide [^{18}F]**12** that was amenable to automation. Initially, [^{18}F]FEA was purified by distillation and peptide conjugation was optimized for the Cu^{2+}/ascorbate catalytic system, typically with heating to 80°C for 5 min. When investigating the effect of pH on the cycloaddition of [^{18}F]FEA and the RGD precursor **4** (2.0 mg, 1.43 μmol) we found that slightly basic conditions (pH 8.0) were optimal. However, these conditions led to formation of a side product as well as the expected product [^{18}F]**12** (Fig. 1). We speculated that this may be due to formation of a copper adduct, and following HPLC purification and treatment of the side product with aqueous ammonia, we were able to retrieve the desired product [^{18}F]**12** (Fig. 2). Attempting to prevent adduct formation in situ, we added various copper chelators such as propargyl amine, EDTA, and TBTA (Chan et al. 2004) to the reaction mixture, all of which failed to block the side reaction. However, copper adduct formation was significantly reduced by reducing the excess of copper(II) sulfate and sodium ascorbate relative to the RGD peptide **4** from ten to twofold (Fig. 3). Under these conditions, the RGD peptide [^{18}F]**12** was isolated in 70 ± 5% (n = 3) radiochemical yield (decay-corrected from [^{18}F]FEA) with >99% radiochemical purity.

Substituting the solution phase catalyst with copper powder lead to excellent conversion of the RGD precursor **4** (0.5 mg, 0.36 μmol) to the labeled peptide [^{18}F]**12** within 15 min at room temperature (Fig. 4). Despite its superior catalytic properties, the advantages of copper powder were offset by poor recovery of the product [^{18}F]**12** (42 ± 14%



Scheme 3 Formation of [^{18}F]FEA and copper catalyzed cycloaddition with the alkyne functionalized RGD peptide **4**. (**a**) [^{18}F]KF-222/K_2CO_3, MeCN, 15 min at 80°C, distillation, or [^{18}F]KF-222/$KHCO_3$, DMSO, 5 min at 80°C; (**b**) **4**, $CuSO_4$/Na-ascorbate, pH 8.0, 5 min at 80°C, or **4**, copper powder, 15 min at room temperature



Scheme 2 Formation of [^{18}F]FPT and conjugation with the chloroacetyl functionalized peptide **3**. (**a**) [^{18}F]KF-222/K_2CO_3, DMSO, 15 min at 80°C, C18-SepPak; (**b**) TFA/TIS/H_2O (5/1/1 v/v/v) 10 min at 80°C; (**c**) **3**, NH_4OH, 30 min at 80°C

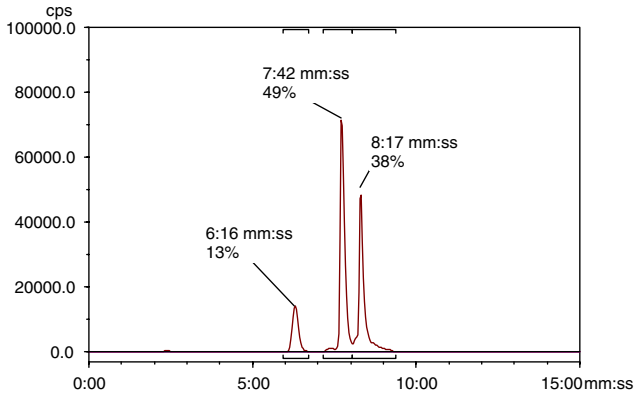


Fig. 1 Preparative radio-HPLC of reaction mixture showing [^{18}F]FEA (t_R = 6:16 min), [^{18}F]**12** (t_R = 7:42 min) and the side product (t_R = 8:17 min)

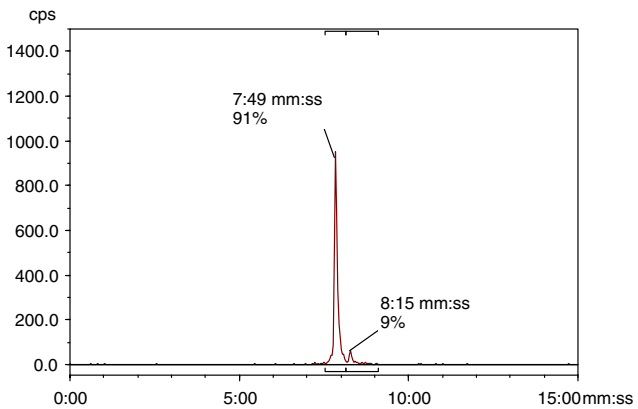


Fig. 2 Radio-HPLC analysis of side product after treatment with NH_4OH showing conversion to product [^{18}F]**12** (t_R = 7:49 min)

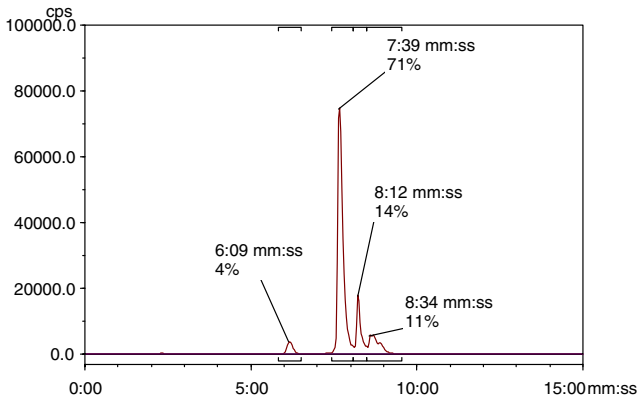


Fig. 3 Preparative radio-HPLC of [^{18}F]**12** (t_R[^{18}F]FEA = 6:09 min, t_R [^{18}F]**12** = 7:42 min) under optimized reaction conditions using Cu^{2+}/ascorbate catalysis

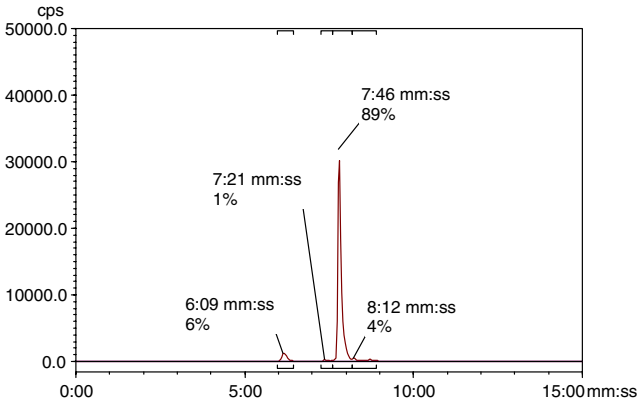


Fig. 4 Preparative radio-HPLC of [^{18}F]**12** (t_R [^{18}F]FEA = 6:09 min, t_R [^{18}F]**12** = 7:46 min) using copper powder catalysis

isolated r.c.y., $n = 5$, decay-corrected, >99% radiochemical purity), due to further losses during filtration (27 ± 9%, $n = 3$, decay-corrected). However, the results suggest that copper powder should be highly suitable for non-conventional systems, such as microfluidic reactors, where the catalyst can be immobilized.

Distillation is frequently used for preparation of ^{11}C-labeled compounds, but much less so for ^{18}F-labeling. Seeking to avoid distillation, we investigated the possibility of reacting [^{18}F]FEA with the RGD peptide **4** without an intermediate purification step. The azide precursor **11** (1.3 μmol) was reacted with fluoride in DMSO (5 min, 80°C). Subsequent addition of the RGD precursor **4** (2.0 mg, 1.43 μmol), copper sulfate and sodium ascorbate led to near complete consumption of [^{18}F]FEA within 5 min at 80°C. HPLC purification of the reaction mixture provided the ^{18}F-labeled peptide [^{18}F]**12** in 47 ± 8% radiochemical yield ($n = 3$, decay-corrected,) with a total synthesis time of 105 min. Unfortunately, the unlabeled alkyne precursor **4** was too similar in size and polarity to allow HPLC separation from the ^{18}F-labeled RGD peptide [^{18}F]**12**, preventing us from measuring the specific activity.

Summary and conclusions

A fully automated method has been developed for labeling of an aminooxy functionalized RGD peptide with [^{18}F]fluorobenzaldehyde using TRACERlab^{TM} FX F-N. 'Click labeling' of a structurally similar alkyne functionalized RGD peptide with 2-[^{18}F]-fluoroethylazide provided comparable radiochemical yields without the need to purify the prosthetic labeling group. Whilst the results suggest that 'click labeling' of RGD peptides provides an attractive alternative to aminooxy aldehyde condensation, 2-[^{18}F]-fluoroethylazide may be too small to allow separation of large ^{18}F-labeled RGD peptides from their precursors.

Acknowledgments We thank Bruce Mackay, Colin Steel, and the Cyclotron Team at Hammersmith Imanet for producing fluorine-18. We are also grateful to Sue Champion, GE Healthcare MDX Research, for providing 4-*N,N,N*-trimethylanilium aldehyde trifluoromethanesulfonate.

References

Bach-Gansmo T, Danielsson R, Saracco A, Wilczek B, Bogsrud TV, Fangberget A, Tangerud A, Tobin D (2006) Integrin receptor imaging of breast cancer: a proof-of-concept study to evaluate Tc-99 m-NC100692. J Nucl Med 47:1434–1439

Beer AJ, Haubner R, Goebel M, Luderschmidt S, Spilker ME, Wester H-J, Weber WA, Schwaiger M (2005) Biodistribution and pharmacokinetics of the $\alpha_v\beta_3$-selective tracer ^{18}F-Galacto-RGD in cancer patients. J Nucl Med 46:1333–1341

Beer AJ, Niemeyer M, Carlsen J, Sarbia M, Nahrig J, Watzlowik P, Wester HJ, Harbeck N, Schwaiger M (2008) Patterns of $\alpha_v\beta_3$ expression in primary and metastatic human breast cancer as shown by F-18-galacto-RGD PET. J Nucl Med 49:255–259. doi:10.2967/jnumed.107.045526

Chan TR, Hilgraf R, Sharpless KB, Fokin VV (2004) Polytriazoles as copper(I)-stabilizing ligands in catalysis. Org Lett 6:2853–2855. doi:10.1021/ol0493094

Chen XY, Park R, Hou YP, Khankaldyyan V, Gonzales-Gomez I, Tohme M, Bading JR, Laug WE, Conti PS (2004) MicroPET imaging of brain tumor angiogenesis with ^{18}F-labeled PEGylated RGD peptide. Eur J Nucl Med Mol Imaging 31:1081–1089. doi:10.1007/s00259-003-1452-2

Folkman J (2007) Opinion—angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov 6:273–286. doi:10.1038/nrd2115

Glaser M, Karlsen H, Solbakken M, Arukwe JM, Brady F, Luthra SK, Cuthbertson A (2004) ^{18}F-Fluorothiols: a new approach to label peptides chemoselectively as potential tracers for positron emission tomography. Bioconjug Chem 15:1447–1453. doi:10.1021/bc0498774

Glaser M, Årstad E (2007) "Click labeling" with 2-[^{18}F]fluoroethylazide for positron emission tomography. Bioconjug Chem 18:989–993. doi:10.1021/bc060301j

Glaser M, Morrison M, Solbakken M, Arukwe J, Karlsen H, Wiggen U, Champion S, Kindberg GM, Cuthbertson A (2008) Radiosynthesis and biodistribution of cyclic RGD peptides conjugated with novel [^{18}F]fluorinated aldehyde-containing prosthetic groups. Bioconjug Chem 19:951–957. doi:10.1021/bc700472w

Haubner R, Wester H-J, Weber WA, Mang C, Ziegler SI, Goodman SL, Senekowitsch-Schmidtke R, Kessler H, Schwaiger M (2001) Noninvasive imaging of a_vb_3 integrin expression using ^{18}F-labeled RGD-containing glycopeptide and positron emission tomography. Cancer Res 61:1781–1785

Haubner R, Kuhnast B, Mang C, Weber WA, Kessler H, Wester H-J, Schwaiger M (2004) [^{18}F]Galacto-RGD: synthesis, radiolabeling, metabolic stability, and radiation dose estimates. Bioconjug Chem 15:61–69. doi:10.1021/bc034170n

Indrevoll B, Kindberg GM, Solbakken M, Bjurgert E, Johansen JH, Karlsen H, Mendizabal M, Cuthbertson A (2006) NC-100717: a versatile RGD peptide scaffold for angiogenesis imaging. Bioorg Med Chem Lett 16:6190–6193. doi:10.1016/j.bmcl.2006.09.033

Kenny LM, Coombes RC, Oulie I, Contractor KB, Miller M, Spinks TJ, McParland B, Cohen PS, Hui A-M, Palmieri C, Osman S, Glaser M, Turton D, Al-Nahhas A, Aboagye EO (2008) Phase I trial of the positron-emitting Arg-Gly-Asp (RGD) peptide radioligand ^{18}F-AH111585 in breast cancer patients. J Nucl Med 49:879–886. doi:10.2967/jnumed.107.049452

Kolb HC, Finn MG, Sharpless KB (2001) Click chemistry: diverse chemical function from a few good reactions. Angew Chem Int Ed 40:2004–2021. doi:10.1002/1521-3773(20010601)40:11<2004::AID-ANIE2004>3.0.CO;2-5

Moitessier N, Henry C, Maigret B, Chapleur Y (2004) Combining pharmacophore search, automated docking, and molecular dynamics simulations as a novel strategy for flexible docking: proof of concept: docking of arginine-glycine-aspartic acid compounds into the $\alpha_V\beta_3$ binding site. J Med Chem 47:4178–4187. doi:10.1021/jm0311386

Poethko T, Schottelius M, Thumshirn G, Hersel U, Herz M, Henriksen G, Kessler H, Schwaiger M, Wester H-J (2004a) Two-step methodology for high-yield routine radiohalogenation of peptides: ^{18}F-labeled RGD and octreotide analogs. J Nucl Med 45:892–902

Poethko T, Schottelius M, Thumshirn G, Herz M, Haubner R, Henriksen G, Kessler H, Schwaiger M, Wester H-J (2004b) Chemoselective pre-conjugate radiohalogenation of unprotected mono- and multimeric peptides via oxime formation. Radiochim Acta 92:317–327. doi:10.1524/ract.92.4.317.35591

Rostovtsev VV, Green LG, Fokin VV, Sharpless KB (2002) A stepwise Huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. Angew Chem Int Ed 41:2596–2599. doi:10.1002/1521-3773(20020715)41:14<2596::AID-ANIE2596>3.0.CO;2-4

Schottelius M, Poethko T, Herz M, Reubi J-C, Kessler H, Schwaiger M, Wester H-J (2004) First ^{18}F-labeled tracer suitable for routine clinical imaging of sst receptor-expressing tumors using positron emission tomography. Clin Cancer Res 10:3593–3606. doi:10.1158/1078-0432.CCR-03-0359

Shahi P, Pineda I (2008) Tumoral angiogenesis: review of the literature. Cancer Invest 26:104–108. doi:10.1080/07357900701662509

Solbakken M, Arbo B, Cuthbertson A, Gibson A (2006) Diagnostic compounds. WO 2006/030291A2

Van de Wiele C, Oltenfreiter R, de Winter O, Signore A, Slegers G, Dierckx RA (2002) Tumour angiogenesis pathways: related clinical issues and implications for nuclear medicine imaging. Eur J Nucl Med 29:699–709. doi:10.1007/s00259-002-0783-8

Wester HJ, Kessler H (2005) Molecular targeting with peptides or peptide-polymer conjugates: just a question of size? J Nucl Med 46:1940–1945

Wu Z, Li Z-B, Chen K, Cai W, He L, Chin FT, Li F, Chen X (2007) MicroPET of tumor integrin $\alpha_v\beta_3$ expression using ^{18}F-labeled PEGylated tetrameric RGD peptide (^{18}F-FPRGD4). J Nucl Med 49:1536–1544. doi:10.2967/jnumed.107.040816

Zhang X, Xiong Z, Wu Y, Cai W, Tseng JR, Gambhir SS, Chen X (2006) Quantitative PET imaging of tumor integrin $\alpha_v\beta_3$ expression with ^{18}F-FRGD2. J Nucl Med 47:113–121