Amino Acids (2012) 43:1349–1357
DOI 10.1007/s00726-011-1208-4

ORIGINAL ARTICLE

N-Succinimidyl 4-[^{18}F]-fluoromethylbenzoate-labeled dimeric RGD peptide for imaging tumor integrin expression

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Received: 29 October 2011 / Accepted: 21 December 2011 / Published online: 31 December 2011

Abstract RGD peptides, radiolabeled with ^{18}F, have been used in the clinic for PET imaging of tumor angiogenesis in cancer patients. RGD peptides are typically labeled using a prosthetic group such as *N*-succinimidyl 4-[^{18}F]-fluorobenzoate ([^{18}F]SFB) or 4-nitrophenyl 2-[^{18}F]-fluoropropionate ([^{18}F]NPFP). However, the complex radiosynthetic procedures have impeded their broad application in clinical studies. We previously radiolabeled proteins and peptides with the prosthetic group, *N*-succinimidyl 4-[^{18}F]-fluoromethylbenzoate ([^{18}F]SFMB), which was prepared in a simple one-step procedure. In this study, we labeled a PEGylated cyclic RGD peptide dimer, PEG_3-E[c(RGDyK)]$_2$ (PRGD2), using [^{18}F]SFMB and evaluated for imaging tumor $\alpha v\beta 3$ integrin expression with positron emission tomography (PET). [^{18}F]SFMB was prepared in one step using [^{18}F]fluoride displacement of a nitrobenzenesulfonate leaving group under mild reaction conditions followed by HPLC purification. The ^{18}F-labeled peptide, [^{18}F]FMBPRGD2 was prepared by coupling PRGD2 with [^{18}F]SFMB in pH 8.6 borate buffer and purified with HPLC. The direct labeling on BMBPRGD2 was also attempted. A Siemens Inveon PET was used to image the uptake of the [^{18}F]FMBPRGD2 into a U87MG xenograft mouse model. [^{18}F]FMBPRGD2, was prepared with a 15% overall radiochemical yield (uncorrected) in a total synthesis time of 90 min, which was considerably shorter than the preparation of [^{18}F]SFB- and [^{18}F]NPFP-labeled RGD peptides. The direct labeling, however, was not successful. High quality microPET images using [^{18}F]FMBPRGD2 clearly visualized tumors by 15 min with good target to background ratio. Early tracer accumulation in the bladder suggests fast renal clearance. No obvious bone uptake can be detected even at 4-h time point indicating that fluorine attachment is stable in mice. In conclusion, *N*-succinimidyl 4-[^{18}F]-fluoromethylbenzoate ([^{18}F]SFMB) prosthetic group can be a good alternative for labeling RGD peptides to image $\alpha v\beta 3$ integrin expression and for labeling other peptides.

Keywords Integrin $\alpha v\beta 3$ · RGD peptide dimer · Positron emission tomography · *N*-Succinimidyl 4-[^{18}F]-fluoromethylbenzoate ([^{18}F]SFMB)

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Introduction

Many methods have been developed and improved upon for labeling peptides and proteins with ^{18}F for PET imaging of cancer and other diseases. ^{18}F is a widely used PET radioisotope with proper half-life and energy for peptide-based PET imaging probes. Most methods used in labeling peptides with ^{18}F utilize a prosthetic group approach (Jacobson and Chen 2010). *N*-succinimidyl 4-[^{18}F]-fluorobenzoate ([^{18}F]SFB) (Li et al. 2008; Wu et al. 2007) or 4-nitrophenyl 2-[^{18}F]fluoropropionate ([^{18}F]NPFP) (Liu et al. 2010; Yang et al. 2011b) reacts with amine functionality on the peptide side chains, and *N*-2-(4-[^{18}F]fluorobenzamido)ethylmaleimide ([^{18}F]FBEM) (Kiesewetter et al. 2011; Cai et al. 2006; Gao et al. 2011) is selective for

reaction with the thiol group. The advantage of using a prosthetic group is that the coupling reaction can be performed under mild conditions and hence preserve the integrity of labeled peptides, especially those that are heat and pH sensitive. However, one major drawback of these methods is the time-consuming multiple-step synthetic procedures required for preparation of these radiolabeled prosthetic groups, which has been a deterrent to their widespread clinical applications.

Among the various ^{18}F radiolabeled peptides, [^{18}F]FPPRGD2, a ^{18}F-labeled dimeric RGD peptide with mini-pegylation, has been developed for PET imaging of angiogenesis not only for early detection of cancers, but also for monitoring the treatment response for cancer patients (Liu et al. 2010; Chin et al. 2011; Mittra et al. 2011). This radiotracer was approved by the FDA for use in a clinical trial for imaging tumor angiogenesis by targeting integrin $\alpha v\beta 3$ receptors in tumors and showed very positive results. However, this radiotracer relies on an implementation of an automated radiosynthesis of [^{18}F]NPFP using commercially available synthesis modules. Although this synthesis has been implemented in some centers, the complexity seems to be a limitation to the expansion of use.

There have been two approaches to simplify the radiolabeling of peptides with [^{18}F]fluoride. The first is to develop methods of direct labeling of peptides. The direct labeling of RGD peptides with a pre-attached functional group with a proper leaving group for fluoride displacement was also attempted with limited success. For example, Jacobson et al. (2011) has reported the direct labeling of RGD peptides by replacing nitro group on a highly activated benzene ring at elevated temperature. This procedure required high temperature with microwave heating and resulted in relatively low radiochemical yield. However, the yield was comparable to the overall yield of the multistep procedures via a prosthetic group. The procedure is not likely to be applicable to peptides of low thermal stability. The recent discovery and development of [^{18}F]fluoride-aluminum complex to radiolabel peptides also provided a good alternative for simplifying the labeling procedure (McBride et al. 2009, 2010). In general, labeling peptides that have a pre-attached chelator with radioactive metal ions is a simple procedure. Unfortunately, the current procedure for formation of the Al–F NOTA complex also requires relatively high temperatures. The requirement for heating also reduced the scope of this method.

The second general approach is to develop simple one-step preparations of prosthetic groups. There have been a large number of prosthetic groups prepared, but most require multiple steps because the relatively harsh conditions for introduction of fluoride are incompatible with the functional groups required for rapid peptide conjugation. The one exception was the preparation of *N*-succinimidyl 4-[^{18}F]fluoromethylbenzoate ([^{18}F]SFMB) (Lang and Eckelman 1994, 1997). The displacement of a benzylic leaving group requires much less strenuous conditions than aromatic nucleophilic substitution. In our previous work, we prepared [^{18}F]SFMB in 30% yield under a room temperature reaction condition and used this agent for labeling proteins and peptides.

The goal of this work was to label RGD peptides either by direct labeling of the functionalized RGD peptide on a benzylic carbon using a pre-functionalized bromomethylbenzoyl prosthetic group or using pre-synthesized [^{18}F]SFMB. The resulting radiotracers were evaluated for microPET imaging of integrin $\alpha v\beta 3$ expression of tumor xenograft models and metabolic stability.

Materials and methods

Cyclic RGD peptides, c(RGDfK), E[c(RGDfK)]$_2$, and PEG$_3$-E[c(RGDyK)]$_2$ (PRGD2) were obtained from Peptides International (Louisville, KY, USA) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mass spectra (MS) were acquired from a Waters Acquity UPLC system coupled with Waters Qtof Premier MS (LC–MS). For purification of modified peptides, preparative reversed-phase HPLC was performed on Waters 600 gradient system with a Waters 996 Photodiode Array (PDA) detector using a Higgins PROTO 300 C$_{18}$ column (5 μm, 250 × 20 mm). For purification of radiolabeled peptides, semi-prep reversed-phase HPLC was performed on a separate Waters 600 gradient system also with a 996 PDA detector plus a Beckman 170 radioisotope detector using Higgins PROTO 300 C$_{18}$ column (5 μm, 250 × 10 mm). Analytical reversed-phase HPLC was performed on a Perkin Elmer Series 200 LC gradient system with a Waters 2784 Dual Absorbance UV detector plus a Bioscan radioisotope detector using a Waters Symmetry column (5 μm, 150 × 3.9 mm). The flow rate was 12 mL/min for preparative column, 5 mL/min for semi-prep column and 1 mL/min for analytical column running the same linear gradient starting from 5% A (0.1% TFA in acetonitrile) and 95% B (0.1% in water) for 5 min and increasing A to 65% at 35 min. For purification of radiolabeled prosthetic group, a separate Perkin Elmer Series 200 LC isocratic system (30% acetonitrile in water with 0.1% TFA) was used with a Knauer 200 UV detector plus a Bioscan radioisotope detector using a Phenomenes Luna C$_{18}$ column (5 μm, 250 × 10.0 mm). Waters C$_{18}$ Sep-Pak Cartridge was used for solid phase extraction of labeled prosthetic group and Varian BOND ELUT C$_{18}$ column (50 mg) was used for solid phase extraction of labeled peptides. F-18 fluoride was obtained from NIH cyclotron facility. *N*-Succinimidyl 4-[^{18}F]fluoromethylbenzoate

([^{18}F]SFMB) was prepared according to a published procedure (Lang and Eckelman 1994).

Preparation of BrPPRGD2 and FPPRGD2

To a 4-mL glass vial containing 21.0 mg of 2-bromopropionic acid in 200 μL of acetonitrile, 22.0 mg of *N*,*N*,*N*′,*N*′-tetramethyl-*O*-9-(*N*-succinimidyl)-uronium tetrafluoroborate (TSTU) and 15 μL of diisopropylethylamine (DIPEA) was added and the mixture was stirred at room temperature for 10 min. At this point, 17.0 mg of PRGD2 and 15 μL DIPEA in 200 μL DMF was added to the mixture and reacted for another 10 min. The reaction was quenched with 40 μL of acetic acid and the reaction mixture was diluted with 1.2 mL of water and injected onto a preparative RP HPLC (12 mL/min) for purification in two separate injections. The peak containing desired product was collected (R_t = 20.6 min) and the solution was frozen over dry-ice and lyophilized overnight to give 13.4 mg of product (72% yield). LC–MS: $[MH]^+$ = 1,673.8352, 1,675.8638 (*m/z*); calc: 1,673.6923, 1,675.6903. FPPRGD2 (Yang et al. 2011a) was prepared with a similar procedure to serve as the reference compound starting from 14.0 mg of PRGD2 and yielded 8.2 mg of product (R_t = 19.7 min, yield 56%). LC–MS: $[MH]^+$ = 1,613.9365 (*m/z*); calc: 1,616.7723.

Preparation of FPc(RGDfK) and BrPc(RGDfK)

To a 4-mL glass vial containing 5.3 mg of 2-fluoropropionic acid in 100 μL of acetonitrile, 10.5 mg TSTU and 8 μL of DIPEA was added and reacted at room temperature. After 20 min, 10.0 mg of c(RGDfK) and 8 μL of DIPEA in 100 μL of DMF was added to the vial and reacted for another 20 min. The reaction was quenched with 20 μL of acetic acid and the reaction mixture was diluted with 0.6 mL of water and injected onto a preparative RP HPLC (12 mL/min) for purification. The peak containing desired product was collected (R_t = 21.2 min) and the solution was frozen over dry-ice and lyophilized overnight to give 3.1 mg of FP-c(RGDfK) (30% yield). LC–MS: $[MH]^+$ = 678.4138 (*m/z*); calc: 678.3375. BrPc(RGDfK) was prepared with a similar procedure using 2-bromopropionic acid instead of 2-fluoropropionic acid starting from 20.0 mg of c(RGDfK) and yielded 7.3 mg of product (R_t = 22.7 min, yield 28%). LC–MS: $[MH]^+$ = 738.3779, 740.3695 (*m/z*); calc: 738.2574, 740.2554.

Preparation of BrMBc(RGDfK) and FMBc(RGDfK)

To a 20-mL glass vial containing 20.8 mg of c(RGDfk) and 12.0 mg of *N*-succinimidyl 4-bromomethylbenzoate in 200 μL of DMF, 3 μL of DIPEA and another 3 μL of DIPEA was added after 10 min. The reaction was quenched with 20 μL of acetic acid after another 10 min and the reaction mixture was diluted with 1.4 mL of water and injected onto a preparative RP HPLC (12 mL/min) for purification in two separate injections. The peak containing desired product was collected (R_t = 24.8 min) and frozen over dry-ice and lyophilized overnight to give 8.4 mg of product (30% yield). $[MH]^+$ = 800.3055, 802.3055 (*m/z*); calc: 800.2731, 802.2710. FMBc(RGDfK) was prepared with similar procedure using *N*-succinimidyl 4-fluoromethylbenzoate instead of *N*-succinimidyl 4-bromomethylbenzoate starting from 2.1 mg of c(RGDfK) and yielded 0.5 mg of product (R_t = 22.9 min, yield 19%). LC–MS: $[MH]^+$ = 740.4702 (*m/z*); calc: 740.3532.

Preparation of FMBPRGD2

To a 4-mL glass vial containing 9.0 mg of PRGD2 and 5.0 mg of *N*-succinimidyl 4-fluoromethylbenzoate in 200 μL of DMF, 10 μL of DIPEA was added and stirred at room temperature for 2 h. The reaction was quenched with 15 μL of acetic acid and diluted with 0.6 ml of water. Preparative RP HPLC purification gave 4.5 mg of desired product after lyophilization overnight (R_t = 22.5 min, yield 46%). LC–MS: $[MH]^+$ = 1,675.6169 (*m/z*); calc: 1,675.7780.

Radiochemistry

Preparation of [^{18}F]SFMB

The *N*-succinimidyl 4-[^{18}F]fluoromethylbenzoate ([^{18}F]SFMB) was prepared according to published procedure (Lang and Eckelman 1994, 1997). Briefly, 3 mg of *N*-succinimidyl-4-[(4-nitrobenzensulfonyl)oxymethyl]benzoate in 0.15 mL of acetonitrile was reacted with anhydrous [^{18}F]fluoride containing 2.5 mg of K-222 and 0.5 mg of potassium carbonate to form [^{18}F]SFMB at room temperature in one step. The mixture was diluted with 0.6 ml of HPLC solvent and purified with HPLC on a semi-prep Phenomenex Luna C_{18} column running at 5 mL/min with 30% acetonitrile/water containing 0.1% trifluoroacetic acid. The desired product was collected and trapped on a Waters Sep-Pak Plus C_{18} cartridge and eluted off with 1 mL of methylene chloride to a 1 mL plastic tube.

Preparation of [^{18}F]FMBPRGD2

Methylene chloride in the tube containing [^{18}F]SFMB was removed with argon flow at room temperature and 1.0 mg of PRGD2 in 0.1 mL pH 8.5 0.1 M borate buffer and reacted at room temperature for 10 min. The reaction mixture diluted with 0.7 mL of water containing 15 μL of

acetic acid and injected onto a semi-prep HPLC column. The radioactive peak at retention time of 16.3 min was collected, diluted with 10 mL water, and trapped on a Varian Bond Elut C_{18} column (100 mg). The radioactivity trapped the C_{18} column was eluted with 0.3 mL of 1 mM HCl ethanol solution and the solvent was evaporated with argon flow and product was re-dissolved in normal saline.

Direct labeling of peptides with ^{18}F

About 1.0 mg of pre-labeled peptides were reacted with anhydrous $[^{18}F]F^-$ using different solvents (acetonitrile, DMF, DMSO, and 2-methyl-2-butanol) at different temperatures (80, 105, and 130°C). The reaction mixture was then analyzed with HPLC.

Cell binding assay

The $\alpha v\beta 3$ receptor binding assay was performed to determine binding affinities of the RGD peptide analogs. Briefly, U87 MG cells were cultured in DMEM medium (GIBCO) containing 10% (v/v) fetal bovine serum (GIBCO) supplemented with penicillin (100 μg/ml), streptomycin (100 μg/ml), non-essential amino acids (100 μM) and sodium pyruvate (1 mM) at 37°C with 5% CO_2. When grown to 80% confluence, cells were scraped off and suspended with binding buffer [25 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride (Tris–HCl), pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 1 mM $MnCl_2$, 0.1% bovine serum albumin (BSA)] at a final concentration of 2×10^6 cells/ml. In a 96-well plate, 1×10^5 U87 MG cells/well were incubated with ^{125}I-echistatin (0.02 μCi/well, PerkinElmer, Inc) in binding buffer in the presence of different concentrations of RGD analogs at room temperature for 2 h. After incubation, the plate was washed three times with phosphate buffered saline (PBS) containing 0.1% BSA, and the radioactivity was measured by γ-counting. The IC_{50} values were calculated by nonlinear regression analysis using the GraphPad Prism computer-fitting program (GraphPad Software, Inc., San Diego, CA, USA). Each data point is a result of the average of triplicate wells.

Preparation of animal tumor models

All animal studies were performed according to the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Clinical Center, NIH. The U87MG tumor model was developed in 5- to 6-week-old female athymic nude mice (Harlan Laboratories) by injection of 5×10^6 cells on the right shoulders. Tumor growth was monitored using caliper measurements of perpendicular axes of the tumor. The mice underwent small-animal PET studies when the tumor volume reached 100–300 mm^3 (3–4 weeks after inoculation).

MicroPET imaging and analysis

PET scans and image analysis were performed using an Inveon microPET scanner (Siemens Medical Solutions). About 3.7 MBq (100 μCi) of ^{18}F-labeled RGD peptide was administered via tail vein injection under isoflurane anesthesia. For blocking study, about 300 μg of unlabeled dimeric RGD peptide $E[c(RGDyK)]_2$ was injected 30 min before the ^{18}F-labeled tracer. Five-minute static PET images were acquired at 30 min, 1 h and 2 h postinjection ($n = 3$ per group). The images were reconstructed using a two-dimensional ordered-subset expectation maximum (2D OSEM) algorithm, and no correction was applied for attenuation or scatter (Bading et al. 2008). For each scan, regions of interest (ROIs) were drawn using vendor software (ASI Pro 5.2.4.0) on decay-corrected whole-body coronal images. The radioactivity concentrations (accumulation) within the tumor, muscle, liver, and kidneys were obtained from mean pixel values within the multiple ROI volume and then converted to MBq per mL. These values were then divided by the administered activity to obtain (assuming a tissue density of 1 g/mL) an image-ROI-derived percent injected dose per gram (%ID/g). Values were expressed as mean ± SD ($n = 3$/group). The dynamic PET data acquisitions were also performed with tail-vein injection of ~3.7 MBq (100 μCi) of tracer under isoflurane anesthesia. During scanning, the body temperature of mice was maintained by a thermostat-controlled thermal heater. PET Images were reconstructed by a 2-dimensional ordered-subsets expectation maximum (OSEM) algorithm and the frame rates were 10×30, 10×90 and 10×240 s.

Biodistribution assay

Immediately after PET imaging, the tumor-bearing mice were killed and dissected. Blood, tumor, major organs, and tissues were collected and wet-weighed. The radioactivity in the wet whole tissue was measured with a γ-counter (Packard). The results were expressed as percentage of injected dose per gram of tissue (%ID/g). Values were expressed as mean ± SD ($n = 4$ per group). The biodistribution study was also performed on normal mice for evaluation of bone uptake.

In vivo stability study

The blood serum and urine samples taken at 30, 60, and 120 min were treated with equal volume of acetonitrile and centrifuged at 6,000 rpm for 10 min. The supernatants

were diluted with three equivalent of water and analyzed with analytical RP HPLC with in-line radioactivity detector. Eluting fractions following injection of serum extract were collected using a fraction collector at 1 mL volume per tube. The samples were counted on a γ counter.

Results

Chemistry

Authentic fluorinated peptide analogs were prepared from commercially obtained c(RGDyK) and PPRGD2 using standard coupling methods. Yields were modest in all cases as HPLC purification was required. The structures of RGD peptides are shown in Fig. 1. The identities of these peptides were confirmed by high resolution LC–MS. The chemical purity of these compounds was over 97% based on the analytical HPLC analysis. For the purpose of attempting direct displacement procedures, we prepared bromo analogs that have the potential for undergoing fluoride displacement reactions. These precursors were prepared in an analogous fashion to the authentic fluoride products.

Radiochemistry

A representative radiolabeling reaction scheme for direct labeling is shown in Fig. 2. Unfortunately, the direct labeling procedures were not successful. Displacement reactions were attempted using K222/K_2CO_3 at various temperatures in different solvents. Under these conditions no radiofluorinated product was observed. A very low yield (< 1%) of the monomeric peptide was obtained when tetraethylammonium bicarbonate (TEABC) was used instead of K222/K_2CO_3.

The radiochemical yield for the prosthetic group, *N*-succinimidyl 4-[^{18}F]fluoromethylbenzoate ([^{18}F]SFMB), was about 20% (uncorrected) after HPLC purification. The HPLC purification provided high specific activity and allowed the coupling reaction to be conducted with low mass of peptide. The yield for coupling of [^{18}F]SFMB with PRGD2 was over 70%. Thus, the overall yield of the radiolabeled peptide was about 15% (uncorrected) based on starting [^{18}F]fluoride (Fig. 3). The total synthesis time was approximately 90 min.

Cell binding assay

Binding affinity was determined by cell binding assays. As shown in Fig. 4, The IC_{50} value for FMBPRGD2 was 205.8 nM, which is close to that of FPPRGD2

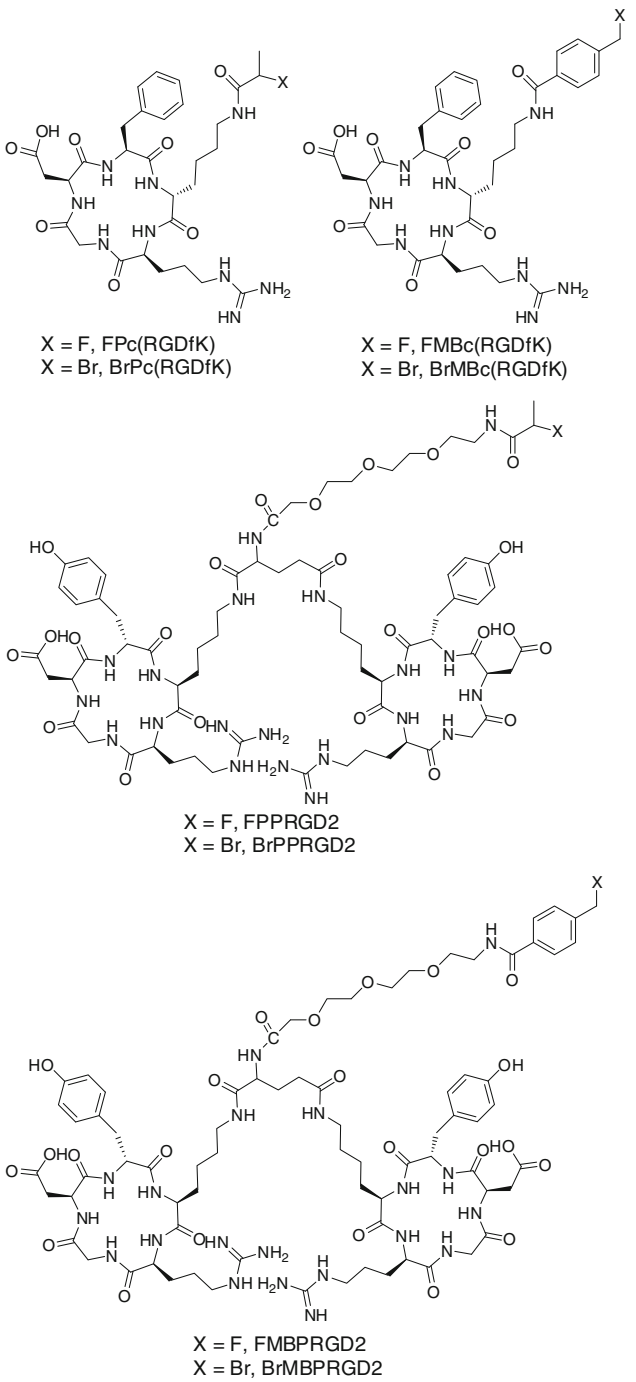

Fig. 1 Chemical structures of RGD peptides

(IC_{50} = 175.4 nM) and is somewhat higher than that of E[c(RGDfK)]$_2$ (IC_{50} = 91.4 nM).

MicroPET imaging

Representative coronal microPET images of U87MG tumor-bearing mice at 30, 60, and 120 min after intravenous (i.v.) injection of 3.7 MBq (100 μCi) of [^{18}F]FMBPRGD2 are

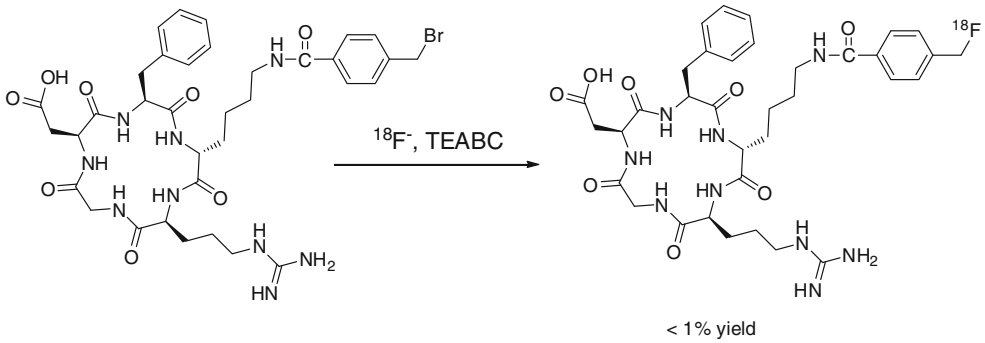

Fig. 2 Direct labeling of c(RDGyK) with prosthetic group pre-attached on the lysine ε-amine group

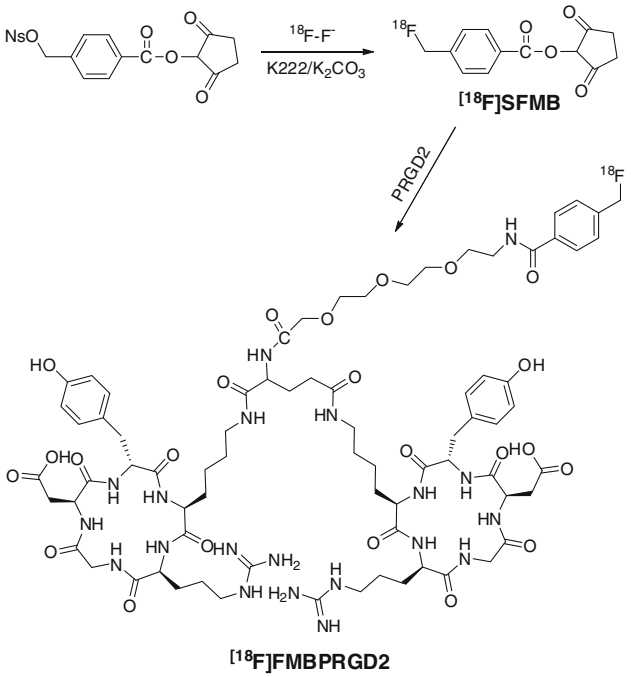

Fig. 3 Synthetic scheme for [^{18}F]FMBPRGD2 starting from *N*-succinimidyl-4-[(4-nitrobenzensulfonyl)oxymethyl]benzoate

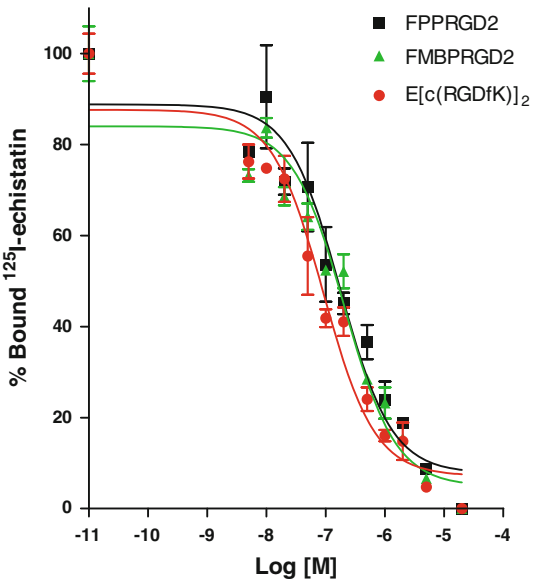

Fig. 4 U87MG cell binding assay of RGD peptides with ^{125}I-echistatin as the radioligand ($n = 3$)

shown in Fig. 5a. The U87MG tumors, as early as 30 min after injection of tracer, were clearly visible with high contrast in relation to the contralateral background. The receptor specificity of the tracer accumulation was confirmed by a blocking study, in which about 300 μg of unlabeled dimeric RGD peptide E[c(RGDfK)]$_2$ was injected 30 min before the tracer injection (Fig. 5b). The tumor uptake of [^{18}F]FMBPRGD2 was determined to be 4.19 ± 0.13, 3.33 ± 0.37 and 2.40 ± 0.30%ID/g at 30, 60 and 120 min p.i., respectively (Fig. 5c). Following a blocking dose, the tumor uptake of [^{18}F]FMBPRGD2 was significantly lower ($p < 0.01$) than that of unblocked tumors at all time points tested (0.81 ± 0.004 vs. 4.19 ± 0.13%ID/g at 30 min, 0.45 ± 0.003 vs. 3.33 ± 0.37%ID/g at 60 min, and 0.33 ± 0.006 vs. 2.4 ± 0.3%ID/g at 120 min). The kidneys also showed relatively very high uptake of [^{18}F]FMBPR GD2, which is consistent with the known predominant renal-urinary clearance routes of this type of peptides. There was no obvious bone uptakes in the images.

Representative coronal images of a dynamic scan are shown in Fig. 6a and the time–activity curves are shown in Fig. 6b. The blood clearance of the tracer was very fast and the majority of activity was taken up by kidneys. Clearance from non-specific compartments was apparent by 15 min. The binding potential for the tumors were calculated to be 1.7–2.5 based on a two-compartment model (Zhang et al. 2006). The tumor-to-tissue ratio are shown in Fig. 6c.

Biodistribution studies

The results of biodistribution of [^{18}F]FMBPRGD2 are shown in Fig. 7. The results were consistent with PET imaging data and the tumor uptake measured by direct tissue sampling and gamma-counting was 2.54 ± 0.48%ID/g at 2-h time point. The tracer accumulations in the kidneys, liver and bone were 4.97 ± 0.3, 1.29 ± 0.3, 0.44 ± 0.1%ID/g, respectively, at 2-h p.i. Biodistribution study also confirmed

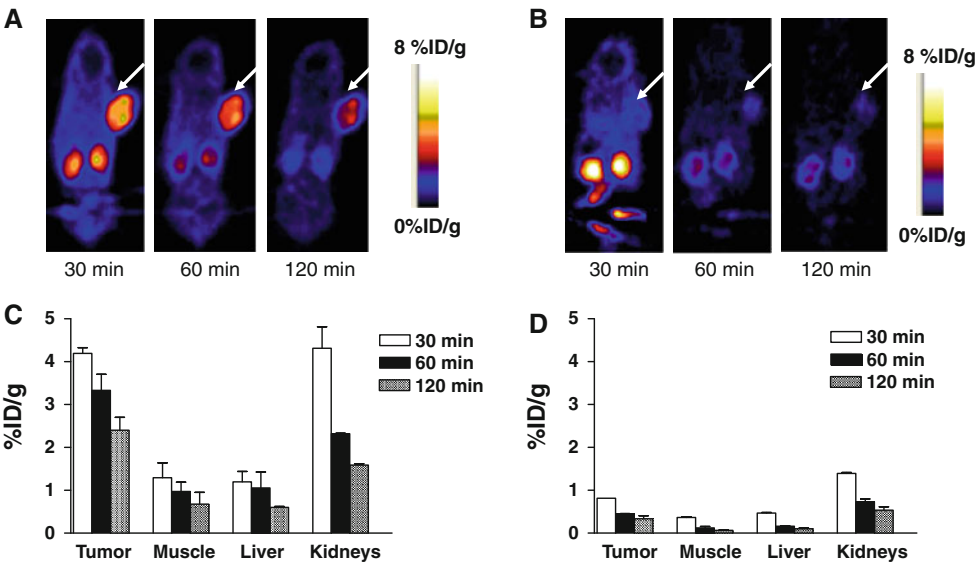

Fig. 5 In vivo PET imaging of U87MG xenografted mice by [^{18}F]FMBPRGD2. **a** Decay-corrected whole-body coronal microPET images of U87MG tumor-bearing mice at 30, 60, and 120 min after injection of 3.7 MBq of [^{18}F]FMBPRGD2 and **b** with blocking dose. Tumors are indicated by *arrows*. **c** Quantification of [^{18}F]FMBPRGD2 in U87MG tumor, liver, kidneys and muscle. ROIs are shown as mean %ID/g ± SD and **d** with blocking dose. The uptakes in the tumor and all organs tested were significantly blocked in the presence of non-radiolabeled RGD peptide ($p < 0.01$)

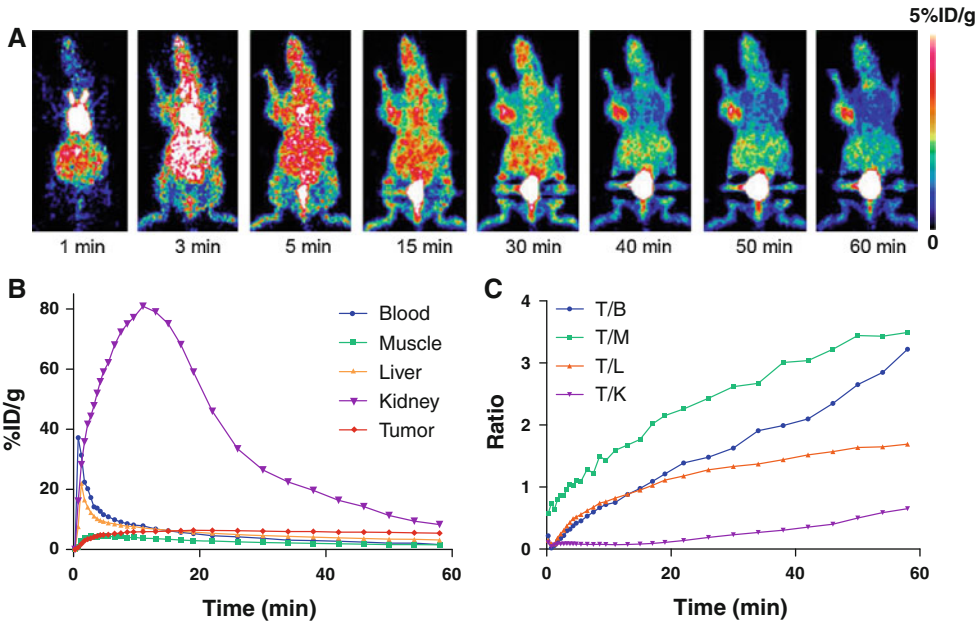

Fig. 6 **a** Representative coronal images at different time points of a dynamic scan. **b** Time–activity curves for tumor, blood and major organs. **c** Tumor-to-normal tissue ratio

negligible defluorination as reflected by the low bone uptake over time. In the blocking experiment, U87MG tumor showed significantly decreased accumulation of [^{18}F]FMBPRGD2 with an uptake value of 0.39 ± 0.15%ID/g ($p < 0.001$). The blocking dose also resulted in decreased uptake in all other tissues sampled.

In vivo stability

Blood and urine were collected 2 h after i.v. injection of [^{18}F]FMBPRGD2. The samples were treated with acetonitrile and the supernatants were analyzed by HPLC (Fig. 8). Based on these analyses, [^{18}F]FMBPRGD2 appears to be very stable. The 2-h urine sample showed very minor metabolite peaks. For all samples >95% of activity was still the parent compound and no [^{18}F]fluoride was detected.

Discussion

The initial intent of this study was to simplify the labeling procedure for making [^{18}F]FPPRGD2 which has been extensively used laboratory for imaging $\alpha v\beta 3$ integrin expression in tumor models. The published procedure for the radiochemical synthesis of [^{18}F]-FPPRGD2 required a 3-h tedious synthesis procedure. This discouraged frequent studies and assimilation by other research groups. In previously published work from our laboratory, Jacobson et al. (2011) demonstrated the successful direct aromatic

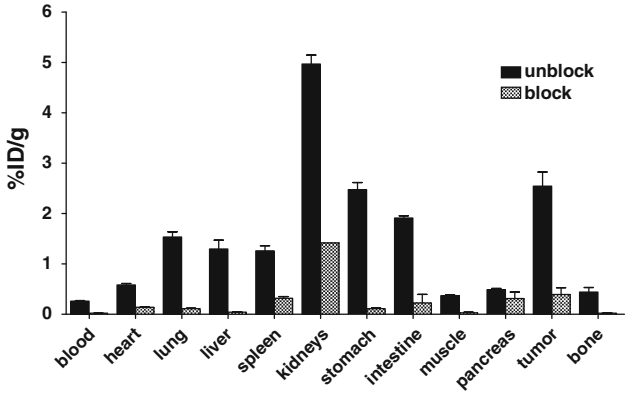

Fig. 7 Ex vivo biodistribution of [^{18}F]FMBPRGD2 (3.7 MBq per mouse) in U87MG tumor-bearing nude mice at 2-h time points after microPET scans with or without unlabeled RGD peptide as blocking agent. *Columns* mean %ID/g ($n = 4$ per group); *bars* SD

fluorination of RGD peptides by [^{18}F]fluoride attack on 4-nitro-3-trifluoromethyl benzoate functionalized analogs and found that yields were better with RGDfK derivatives than RGDyK derivatives and that monomeric RGD provided better yields than dimeric RGD.

We hypothesize that a suitably activated aliphatic position could also be substituted by fluoride even in a peptide structure. The preparation of a suitable precursor would require the ability to functionalize the peptide without subsequent reaction of the electrophilic leaving group. We pre-attached 2-bromopropionyl to PRGD2 and conducted direct labeling on this compound, but no desired compound was formed under a variety of conditions. Because of the earlier observations of Jacobson et al., we then focused our direct fluorination study on monomeric c(RGDfK). In addition to the 2-bromopropionyl functional group), we attached a potentially more reactive 4-bromomethylbenzoyl to the ε-amino group of lysine residue of c(RGDfK). We observed the formation of the desired radiolabeled product only in the case of 2-bromomethylbenzoyl derivative with tetraethyl ammonium bicarbonate (TEAB) as the base. The yield, however, was very low.

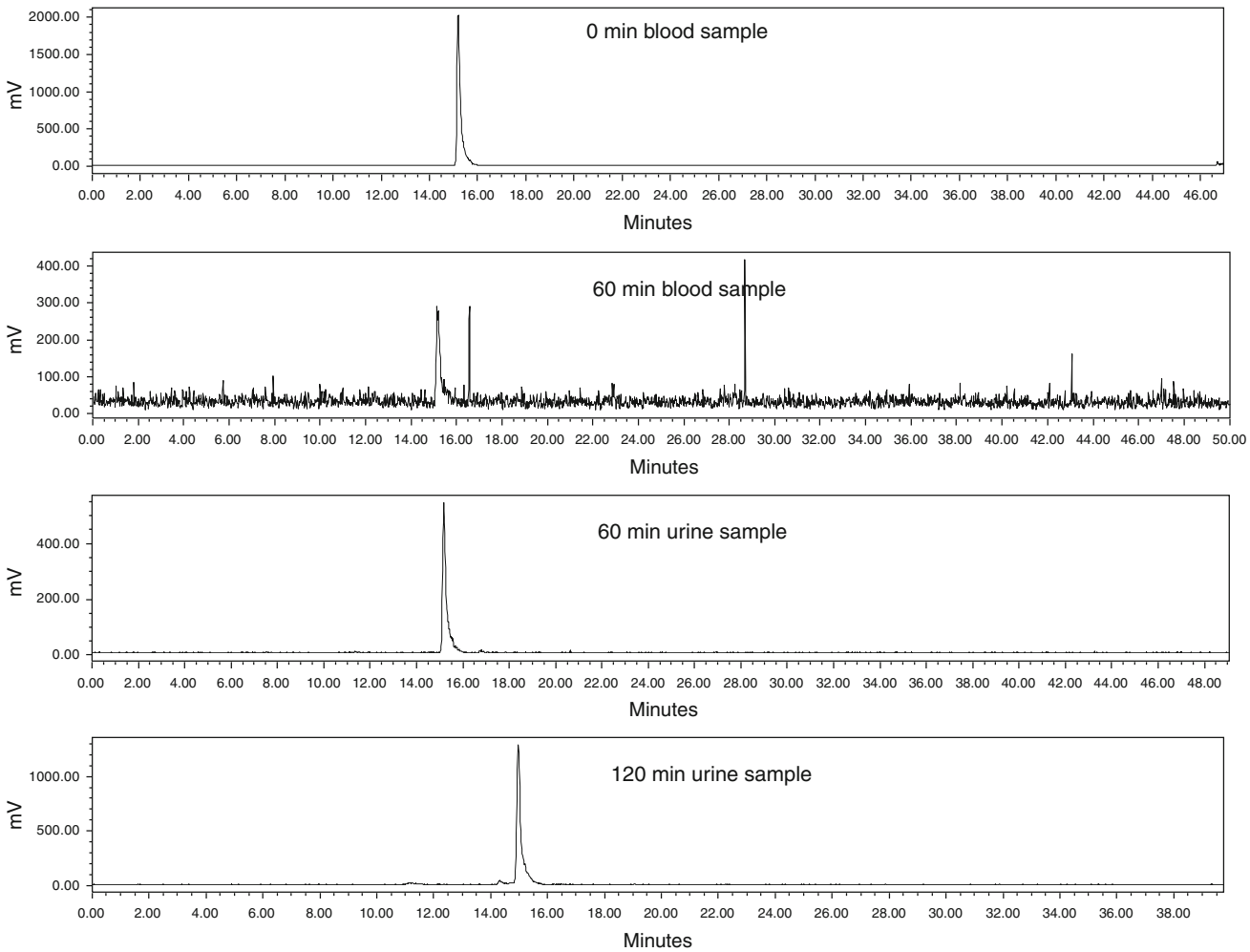

Fig. 8 HPLC chromatograms of urine and blood samples. The narrow peaks in the 60 min serum sample are noise artifacts due to the low amount of radioactivity counts in the blood

As an alternative, we reverted to our original prosthetic group route and prepared *N*-succinimidyl 4-[^{18}F]-fluoromethylbenzoate ([^{18}F]SFMB). The total synthesis time for the preparation of this prosthetic group followed by peptide coupling was about 90 min, including HPLC purifications. This synthesis time was considerably shorter than the preparation of [^{18}F]SFB- and [^{18}F]NPFP-labeled RGD peptides. The prosthetic group was purified by HPLC in order to minimize the amount of peptide precursor required for the conjugation reaction. The overall yield was also comparable or somewhat higher than the other reported methods of ^{18}F-labaled RGD peptide dimers.

The dimeric RGD peptide tracer [^{18}F]FMBPRGD2 was evaluated by PET imaging studies and compared to the results with those of [^{18}F]FPPRGD2 already in literature (Liu et al. 2010). [^{18}F]FMBPRGD2 behaved quite similarly to that of [^{18}F]FPPRGD2 in imaging $\alpha v\beta 3$ integrin positive tumors. The tumor accumulation could be blocked upon co-injection of unlabeled dimeric RGD peptide. [^{18}F]FMBPRGD2 displayed high in vivo stability as the radioactivity in both serum and urine samples were entirely intact even at 60 min post-injection. The bone uptakes of [^{18}F]FMBPRGD2 and [^{18}F]FPPRGD2 were also similar, suggesting that there was no appreciable metabolic defluorination in mice. Further study is warranted to determine if defluorination will occur in other species.

Conclusion

We successfully synthesized a novel ^{18}F-labeled dimeric RGD peptide tracer [^{18}F]FMBPRGD2 that are comparable to [^{18}F]FPPRGD2 in terms of integrin-specific uptake and in vivo kinetics but with much shorter synthesis time and more amenable for automation. *N*-succinimidyl 4-[^{18}F]-fluoromethylbenzoate ([^{18}F]SFMB) can be a good alternative to *N*-succinimidyl 4-[^{18}F]-fluorobenzoate ([^{18}F]SFB) and 4-nitrophenyl 2-[^{18}F]-fluoropropionate ([^{18}F]NPFP) for labeling RGD peptides to image $\alpha v\beta 3$ integrin expression and for labeling other peptides and biologically active molecules.

Acknowledgments This work was supported by the intramural programs of the National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, the International Cooperative Program of the National Science Foundation of China (NSFC) (81028009), and the International Cooperation Project of the Chinese Ministry of Science and Technology (2009DFB30040).

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