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Rapid and Simple One-Step F-18 Labeling of Peptides

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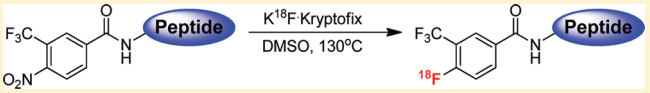
ABSTRACT: Labeling biomolecules with ^{18}F is usually done through coupling with prosthetic groups, which requires several time-consuming radiosynthesis steps and therefore in low labeling yield. In this study, we designed a simple one-step ^{18}F-labeling strategy to replace the conventional complex and the long process of multiple-step radiolabeling procedure. Both monomeric and dimeric cyclic RGD peptides were modified to contain 4-NO_2-3-CF_3 arene as precursors for direct ^{18}F labeling. Binding of the two functionalized peptides to integrin $\alpha_v\beta_3$ was tested *in vitro* using the MDA-MB-435 human breast cell line. The most promising functionalized peptide, the dimeric cyclic RGD, was further evaluated *in vivo* in an orthotopic MDA-MB-435 tumor xenograft model. The use of relatively low amount of precursor (~0.5 μmol) gave reasonable yield, ranging from 7 to 23% (decay corrected, calculated from the start of synthesis) after HPLC purification. Overall reaction time was 40 min, and the specific activity of the labeled peptide was high. Modification of RGD peptides did not significantly change the biological binding affinities of the modified peptides. Small animal PET and biodistribution studies revealed integrin specific tumor uptake and favorable biokinetics. We have developed a novel one-step ^{18}F radiolabeling strategy for peptides that contain a specific arene group, which shortens reaction time and labor significantly, requires low amount of precursor, and results in specific activity of 79 ± 13 GBq/μmol. Successful introduction of 4-fluoro-3-trifluoromethylbenzamide into RGD peptides may be a general strategy applicable to other biologically active peptides and proteins.



INTRODUCTION

Labeling peptides and proteins with $^{18}F-F^-$ is usually done in several radiosynthesis steps using prosthetic groups, which can be attached to the biomolecule, mostly through amino- or thiol-reactive groups via acylation, alkylation, amidation, imidation, oxime, or hydrazone formations.[1−8] The choice of prosthetic group for the labeling should take into account the complexity of its radiosynthesis, which in general requires a number of lengthy radiosynthesis steps and therefore, results in relatively low labeling yield. Multistep synthesis is also not amenable for automation.

One alternative route for labeling bioactive molecules is 1,3-dipolar cycloaddition of terminal alkynes and azides in the presence of Cu(I) as a catalyst, to give the corresponding triazole (click chemistry). ^{18}F-click chemistry has been used in several studies for labeling peptides.[5,7,9−11] However, it requires the preparation of azide or alkyne functional group modified peptides and two radiochemical synthesis steps, and in some cases, it involves volatile ^{18}F-azide synthon.

There have been several attempts to develop a direct labeling of peptides with ^{18}F.[12−15] Höhne et al.[12] showed a direct ^{18}F-fluoride labeling on di-*tert*-butyl silyl functionalized bombesin analogues. Although their peptides were stable in blood, low tumor uptake was observed probably due to the high lipophilicity of the modified bombesin peptides. Becaud et al.[14] reported on the direct one-step labeling of peptides via ^{18}F-fluoride nucleophilic aromatic substitution using trimethylammonium as a leaving group without *in vivo* data. Roehn et al.[15] described the one-step labeling with ^{18}F-fluoride via ring-opening of activated aziridines. However, the ^{18}F-fluoride incorporation yield is rather low. McBride and Laverman et al.[16,17] reported on the chelation of ^{18}F-aluminum fluoride ($Al^{18}F$) by 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) conjugated octreotide.

The introduction of no-carrier-added ^{18}F-fluoride into aromatic compounds is mostly limited to substitution on electron-deficient arenes. Nitro is a known leaving group for aromatic nucleophilic substitution.[18−20] The presence of electron-withdrawing groups, such as cyano, trifluoromethyl, aldehyde, ketone, and nitro, in the ortho or para positions on the aromatic ring, decreases electron density, which allows sufficient activation for nucleophilic substitution.[5,21] Here, we report on a new straightforward one-step labeling strategy with ^{18}F-fluoride by displacing a nitro group in an arene that is activated toward nucleophilic aromatic substitution by an ortho trifluoromethyl group. We applied this new labeling method to cyclic Arg-Gly-Asp (RGD) peptide derivatives containing 4-nitro-3-trifluoromethyl arene as precursors for direct F-18 labeling (Figure 1).

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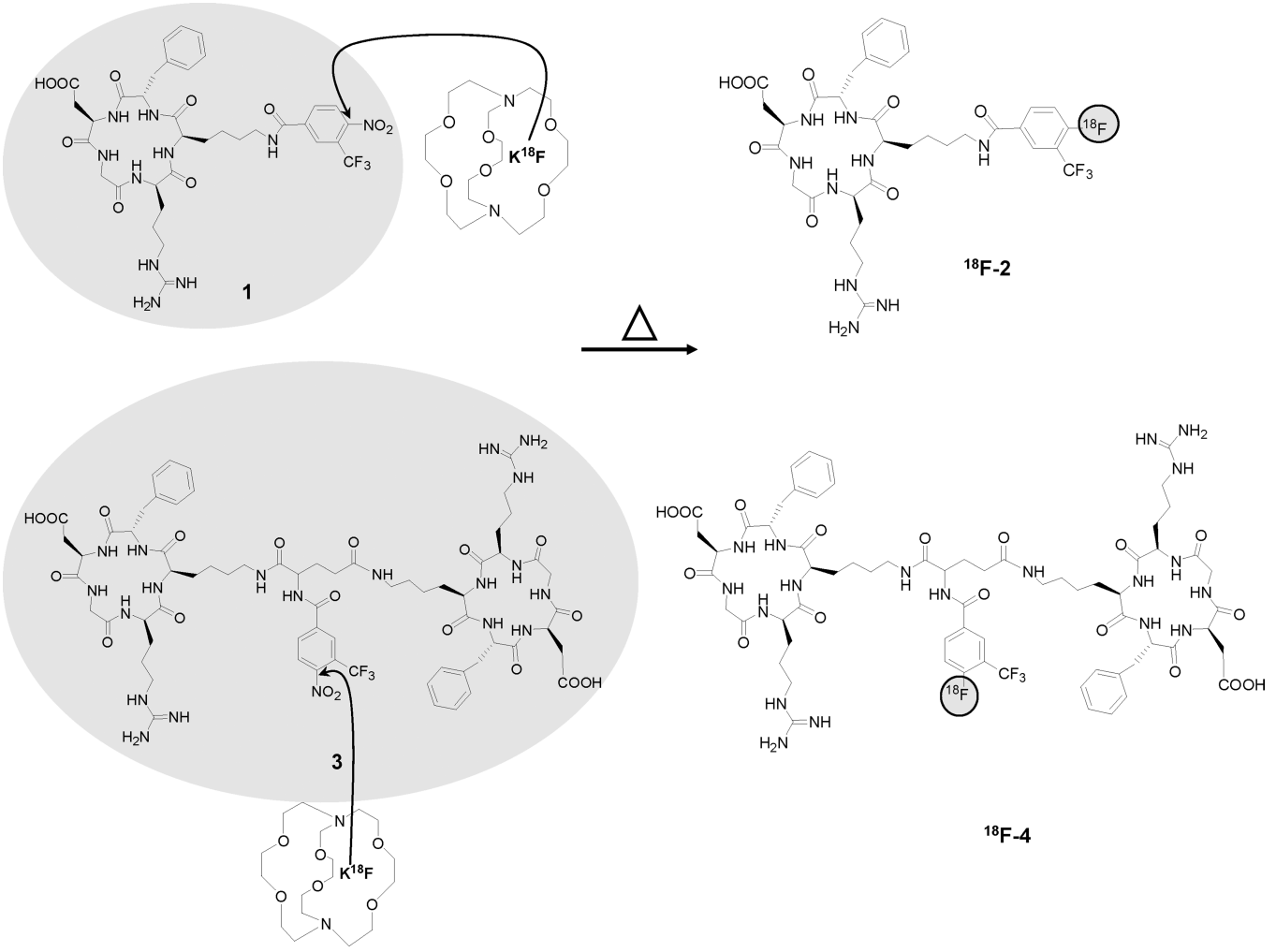

Figure 1. One-step ^{18}F-labeling of RGD peptides.

EXPERIMENTAL PROCEDURES

General. Kryptofix 2.2.2 was purchased from EMD Chemicals (Darmstadt, Germany). All other solvents and chemicals were purchased from Sigma−Aldrich Co. (St. Louis, MO). c(RGDfk) and E[c(RGDfk)$_2$] were purchased from Peptide International (Louisville, KY). ^{18}F-fluoride was obtained from the National Institutes of Health (NIH) Clinical Center (CC) cyclotron facility by irradiation of an ^{18}O-water target by the ^{18}O(p,n)^{18}F nuclear reaction. C_{18} cartridges (Waters Corporation, Milford, MA) were each activated with 5 mL of EtOH and 10 mL of water.

Mass Spectrometry Analysis. LC/MS analysis employed a Waters LC-MS system (Waters, Milford, MA) that included an Acquity UPLC system coupled to the Waters Q-Tof Premier high resolution mass spectrometer. An Acquity BEH Shield RP18 column (150 × 2.1 mm) was employed for chromatography. Elution was achieved with a binary mixture of two components. Solution A was composed of 2 mM ammonium formate, 0.1% formic acid, and 5% acetonitrile (ACN); solution B was composed of 2 mM ammonium formate and 0.1% formic acid in ACN. The elution profile, at 0.35 mL/min, had the following components: initial condition at 100% (v/v) A and 0% B; gradient 0−40% B over 5 min; isocratic elution at 40% B for an additional 5 min; 40−80% B over 2 min; and re-equilibrated with A for an additional 3 min. The retention times for **1** and **3** were 9.8 and 5.8 min, respectively. The retention times for **2** and **4** were 9.2 and 5.4 min, respectively. The injection volume was 10 μL. The entire column eluent was introduced into the Q-Tof mass spectrometer. Ion detection was achieved in ESI mode using a source capillary voltage of 3.5 kV, source temperature of 110 °C, desolvation temperature of 200 °C, cone gas flow of 50 L/H (N_2), and desolvation gas flow of 700 L/H (N_2).

Synthesis of 4-Nitro-3-trifluoromethylbenzoyl Chloride. 4-Nitro-3-trifluoromethybenzoic acid (1.06 g, 4.5 mmol) was treated with α,α-dichloromethyl methyl ether (0.6 mL, 6.75 mmol). One milliliter of dichloroethane was added, and the reaction was heated at 50 °C for 4 h. Full conversion to the acid chloride was verified by GC-MS. The solvent and excess reagents were evaporated, and the residual oil was distilled in a Kugelrohr apparatus (140 °C, 1 mm) to give 0.9 g (3.55 mmol) of the desired 4-nitro-3-trifluoromethybenzoyl chloride. The oil solidified upon standing and was used without any additional manipulations. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.04−7.99 (d, 2H), 8.52−8.43 (dd, 2H), 8.57 (d, 2H). GC−MS (CI−CH4) 253.95 (M^+).

Synthesis of 4-Nitro-3-trifluoromethylbenzoyl-c(RGDfk) (1) and 4-Nitro-3-trifluoromethylbenzoyl-E[c(RGDfk)$_2$] (3). The conjugation procedure of 4-nitro-3-trifluoromethy-

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lbenzoyl chloride with monomeric RGD peptide c(RGDfK) and dimeric RGD peptide E[c(RGDfK)]$_2$ was conducted by dissolving the peptide (8−10 mg) in 0.3−0.5 mL of *N*,*N*-dimethylformamide. Then, 1.2 equiv of 4-nitro-3-trifluoromethylbenzoyl chloride was added, followed by the addition of 10 equiv of triethylamine. The reaction mixture was stirred at room temperature for several hours. Purification of **1** and **3** was conducted on a reversed-phase HPLC system using a Higgins C_{18} column (5 μm, 20 × 250 mm). The flow was set at 12 mL/min using two gradient systems; for **1**, starting from 95% of solvent A (0.1% TFA in water) and 5% of solvent B (0.1% TFA in ACN) and increasing to 35% solvent A and 65% solvent B at 32 min. The retention time of **1** under these conditions was 27.1 min. For compound **3**, the gradient started from 95% of solvent A and 5% of solvent B and increasing to 35% solvent A and 65% solvent B at 35 min. The retention time of **3** under these conditions was 26.3 min. The desired functionalized conjugated peptides (compounds **1** and **3**) were collected, and the solvents were removed by lyophlization. The purities of **1** and **3** were determined by injection into analytical HPLC, using a Phenomenex C_{18} column (Luna, 5 μm, 250 × 4.6 mm) at a flow rate of 1 mL/min and a gradient system starting from 70% of solvent A and 30% of solvent B and increasing to 60% solvent A and 40% solvent B at 35 min. The retention times of **1** and **3** on this system were 17.3 and 13.1 min, respectively. LC−MS also confirmed the mass of the conjugated peptides: **1**, 821.37 $[M + H]^+$, calculated, 820.31; **3**, 1535.70 $[M + H]^+$, calculated, 1534.66.

Synthesis of 4-Fluoro-3-trifluoromethylbenzoyl-c(RGDfk) (2) and 4-Fluoro-3-trifluoromethylbenzoyl-E[c(RGDfk)$_2$] (4). Nonradioactive standards for ^{18}**F-2** and ^{18}**F-4** were prepared by the conjugation of c(RGDfk) and E[c(RGDfk)$_2$] with the commercially available 4-fluoro-3-trifluorobenzoyl chloride, under the same conditions as those described above. The conjugation of 4-fluoro-3-trifluorobenzoyl chloride with the peptides was confirmed by LC-MS analysis: **2**, 794.40 $[M + H^+]$, calculated, 793.32; **4**, 1508.90 $[M + H^+]$, calculated, 1507.66. The purities of **2** and **4** were determined by the same analytical HPLC conditions described above for compounds **1** and **3**. The retention times of **2** and **4** on this system were 16.9 and 12.5 min, respectively.

Synthesis of 4-^{18}F-fluoro-3-trifluoromethylbenzoyl-c(RGDfk) (^{18}F-2) and 4-^{18}F-Fluoro-3-trifluoromethylbenzoyl-E[c(RGDfk)$_2$] (^{18}F-4). Reactive ^{18}F-fluoride ion was prepared by adding to 50−100 μL $^{18}F/H_2{}^{18}O$ solution (0.74−2.22 GBq), K_2CO_3 (1.38 mg, 10 μmol), and Kryptofix in 500 μL of ACN (7.52 mg, 20 μmol). Azeotropic removal of water was achieved by adding 500 μL of ACN and evaporating by heating under a stream of argon. Adding a second portion of ACN (500 μL) followed by evaporation further removes the remaining water. The dried $K^{18}F$·Kryptofix 2.2.2 complex was then dissolved in 300 μL of anhydrous dimethylsulfoxide (DMSO) and added to 440−800 μg (~0.5 μmol) of the modified peptides (**1** and **3**) in a screw-cap test tube. The tube was capped, vortexed, and heated in the microwave for 3.5 min at 130 °C.

After cooling the reaction vial to ambient temperature in a water bath, the vial content was diluted with 10 mL of water and loaded onto an activated C_{18} Sep-Pak cartridge. The cartridge was washed with water (10 mL), and the desired labeled peptide (^{18}**F-2** or ^{18}**F-4**) was eluted with 10 mM HCl in ethanol (1 mL) into a glass test tube. The ethanol was evaporated for 2−3 min under a stream of argon at 60 °C, and then the crude labeled peptide was diluted with 0.1% TFA/H_2O and injected into reversed-phase HPLC using a Phenomenex C_{18} column (Luna, 5 μm, 250 × 10 mm). The flow was set at 4 mL/min using a gradient system starting from 70% of solvent A (0.1% TFA in water) and 30% of solvent B (0.1% TFA in ACN) and increasing to 60% solvent A and 40% solvent B at 35 min. ^{18}**F-2** and ^{18}**F-4** were eluted with retention times of 17 and 12.7 min, respectively. ^{18}**F-2** and ^{18}**F-4** were analyzed using HPLC and compared to nonradioactive standards by coinjection.

MDA-MB-435 Cell Culture. The MDA-MB-435 cell line was purchased from American Type Culture Collection (ATCC) and grown in Leibovitz's L-15 medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS) under a 100% air atmosphere at 37 °C.

Integrin $\alpha_v\beta_3$ Receptor Binding Assay. MDA-MB-435 cells were scraped off and resuspended 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 $MgCl_2$, 1 mM $MnCl_2$, and 0.1% bovine serum albumin (BSA)]. Incubation was conducted in a 96-well plate with a total volume of 200 μL in each well containing 2 × 10^5 cells, 0.02 μCi (0.74 kBq) ^{125}I-echistatin (Perkin-Elmer), and 0−5000 nM of c(RGDfk) or unlabeled **2**, or 0−500 nM of E[c(RGDfk)$_2$] or unlabeled **4**, for 2 h on a shaker at room temperature. After incubation, cells were washed three times with cold phosphate buffered saline (PBS) with 0.1% BSA. Thereafter, the plate was heated to 40 °C and dried. The dried filter membranes were punched off from the wells and collected in polystyrene culture test tubes (12 × 75 mm).

Cell bound radioactivity was measured using a gamma counter (1480 Wizard 3, Perkin-Elmer). The IC_{50} values were calculated by nonlinear regression analysis using the GraphPad Prism computer-fitting program (GraphPad Software, Inc., San Diego, CA). Each data point is a result of the average of duplicate wells.

Tumor Xenograft Model. Athymic nude mice were purchased from Harlan (Frederick, MD) and housed under pathogen free conditions. All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health (NIH) Guide for the Care and Use of Animals, and under protocols approved by the NIH Clinical Center Animal Care and Use Committee (CC/ACUC). The MDA-MB-435 tumor model was generated by orthotopical injection of 5 × 10^6 cells in the left mammary fat pad of female athymic nude mice. The mice were ready for use in 2−3 weeks after tumor inoculation when the tumor size reached 100−300 mm^3.

Small Animal PET Studies. Tumor-bearing mice were anesthetized using isoflurane/O_2 (1.5−2% v/v) and injected with 100 μCi (3.7 MBq) of ^{18}**F-4**. PET scans were performed using an Inveon DPET scanner (Siemens Medical Solutions) at 0.5, 1, and 2 h postinjection (n = 5/group). For blocking experiments, 100 μCi (3.7 MBq) of ^{18}**F-4** were coinjected with 300 μg of c(RGDfk) (n = 5). The images were reconstructed by a two-dimensional ordered subsets expectation maximum (2D-OSEM) algorithm, and no correction was applied for attenuation or scatter. Image analysis was done using ASI Pro VM software.

Biodistribution. Each tumor-bearing mouse was injected 100 μCi (3.7 MBq) of ^{18}**F-4** in a volume of 100 μL phosphate−saline buffer through the tail vein. At 2 h postinjection after the PET scans were completed, blood was drawn from the heart, and the mice were then sacrificed. Liver, muscle, kidneys, intestine, and tumor were removed (n = 5). The organs were wet weighed and assayed for radioactivity using a gamma counter.

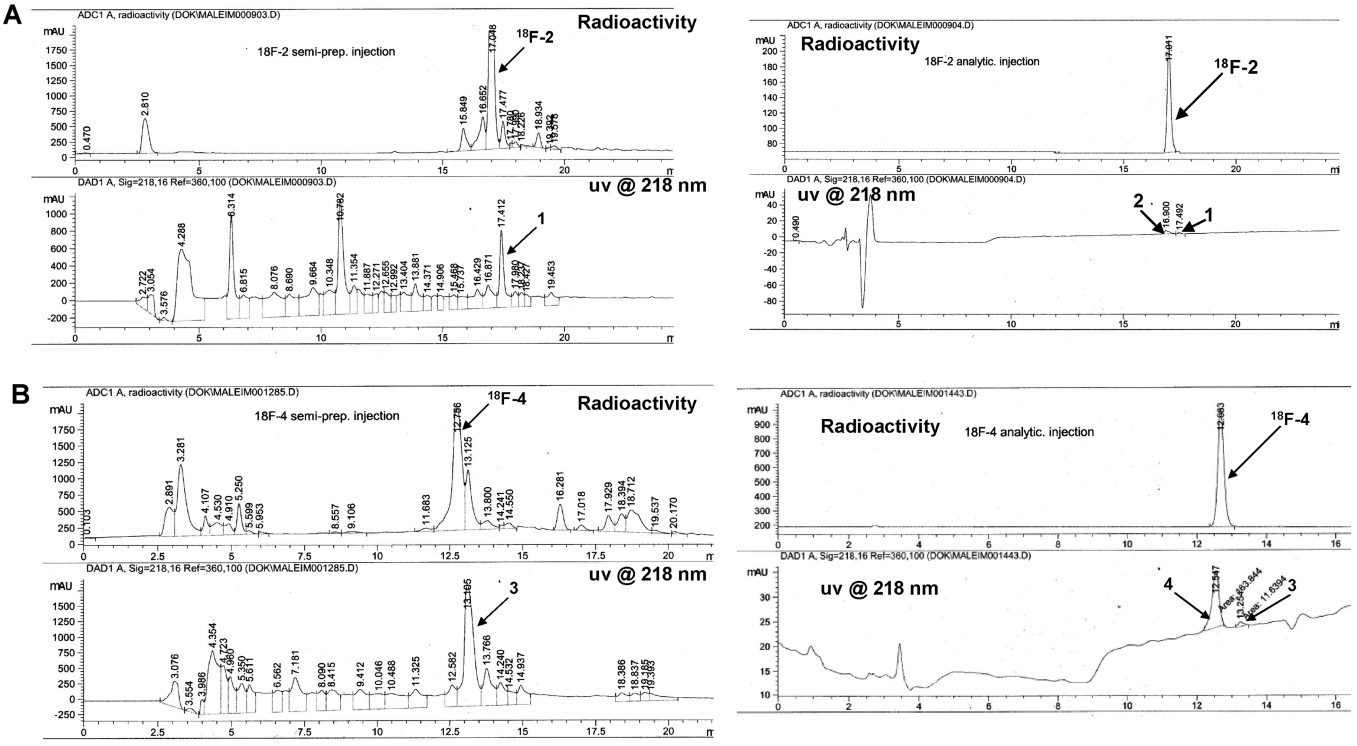

Figure 2. (A) Left: HPLC (UV at 218 nm) and radioactivity chromatograms of ^{18}**F-2** (injection of crude reaction). ^{18}**F-2** was eluted at a retention time of 17.04 min. Right: (A) analytical HPLC (UV at 218 nm) and radioactivity chromatograms of ^{18}**F-2**. (B) Left: HPLC (UV at 218 nm) and radioactivity chromatograms of ^{18}**F-4** (injection of crude reaction). ^{18}**F-4** was eluted at a retention time of 12.75 min. Right: analytical HPLC (UV at 218 nm) and radioactivity chromatograms of ^{18}**F-4**.

Statistical Analysis. Results were expressed as the mean ± SD. Two-tailed paired and unpaired Student's *t* tests were used to test differences within groups and between groups, respectively. *P* values <0.05 were considered statistically significant.

RESULTS

Synthesis of Precursors 1 and 3. 4-Nitro-3-trifluoromethylbenzoyl chloride was synthesized via chlorination of the corresponding acid, using α,α-dichloromethyl methyl ether as a chlorination agent.[22] The conversion to the desired benzoyl chloride was efficient, with a chemical yield of 80%. 4-Nitro-3-trifluoromethylbenzoyl chloride was conjugated to both the RGD monomer c(RGDfK) and dimer E[c(RGDfK)]$_2$ in the presence of triethylamine at room temperature to give **1** and **3**, which were purified on a reversed-phase HPLC system. Eight to ten milligrams of c(RGDfK) and E[c(RGDfK)]$_2$ was used for the conjugation to give functionalized conjugated peptides **1** and **3**, respectively, which were achieved in a reasonable chemical yield of 50−60% and were sufficient for several radioactive runs. The chemical purity of **1** and **3** from the above reaction was greater than 99%, as determined by analytical HPLC and LC-MS analysis.

Synthesis of Standards 2 and 4. The synthesis of unlabeled standards for the fluorination was done in a way similar to that for **1** and **3** using the commercially available 4-fluoro-3-trifluoromethylbenzoyl chloride. The yields of compounds **2** and **4** were slightly lower (42−46%) than those of **1** and **3**. The chemical purity of **2** and **4** was found to be greater than 99% as determined by analytical HPLC and LC-MS analyses.

Synthesis of ^{18}F-2 and ^{18}F-4. ^{18}F-fluoride displacement of the nitro group in **1** and **3** was done rapidly using a microwave device set to a temperature of 130 °C and a low amount of peptide precursors (∼ 0.5 μmol, Figure 1). After ^{18}F-fluoride displacement, the unreacted ^{18}F-fluoride was washed out using an activated C-18 sep-pak cartridge, and the crude labeled peptides were injected into the reversed-phase HPLC system. The incorporation of ^{18}F-fluoride into **1** resulted in one major radioactive peak of the desired labeled peptide ^{18}**F-2** with a retention time of 17 min. ^{18}**F-4** was eluted from the HPLC with a retention time of 12.7 min (Figure 2). The monomeric peptide ^{18}**F-2** was achieved in higher radiochemical yield (19 ± 4%, *n* = 6) than the dimeric peptide ^{18}**F-4** (9 ± 2%, *n* = 4). Both have radiochemical purities greater than 99%, with a high specific activity of 79 ± 13 GBq/μmol (SOS). The overall radiosynthesis time to get ^{18}**F-2** or ^{18}**F-4** formulated and ready for injection was approximately 40 min.

It is of note that attempts to perform this direct fluorination using an oil bath at 130 °C for 20 min with the same small amount of conjugated peptides were also successful and gave similar radiochemical yields.

Competitive Binding Assay with Radiolabeled ^{125}I-Echistatin. The affinity of **2** and **4** for integrin $\alpha_v\beta_3$ was tested using the human breast carcinoma cell line MDA-MB-435, which is known to express a medium level of integrin $\alpha_v\beta_3$. Binding affinities of the modified-RGD peptides, **2** and **4**, were compared with the nonmodified RGD peptides, c(RGDfk) and E[c(RGDfk)$_2$]. The IC$_{50}$ values of **2** and **4** binding to MDA-MB-435 cells were 119 nM and 63 nM, which were comparable with those of c(RGDfk) (67 nM) and E[c(RGDfk)$_2$] (33 nM), respectively (Figure 3).

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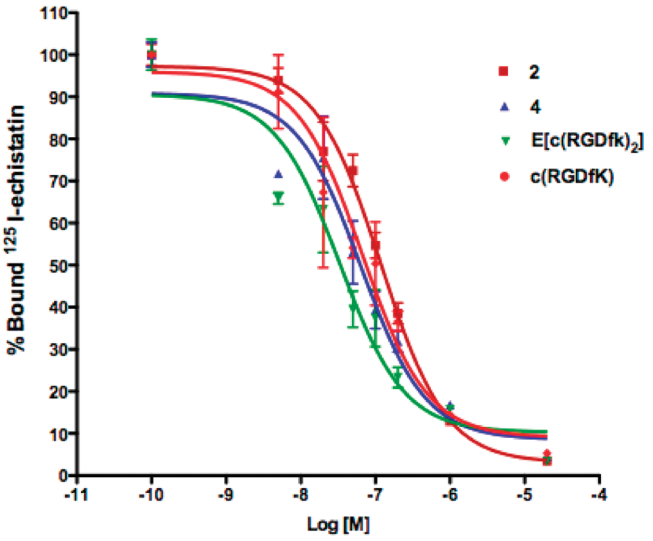

Figure 3. Competitive binding assay of RGD-modified peptides (**2** and **4**) in comparison to the nonmodified peptides (c(RGDfK) and E[c(RGDfK)]$_2$) with ^{125}I-echistatin using MDA-MB-435 cells.

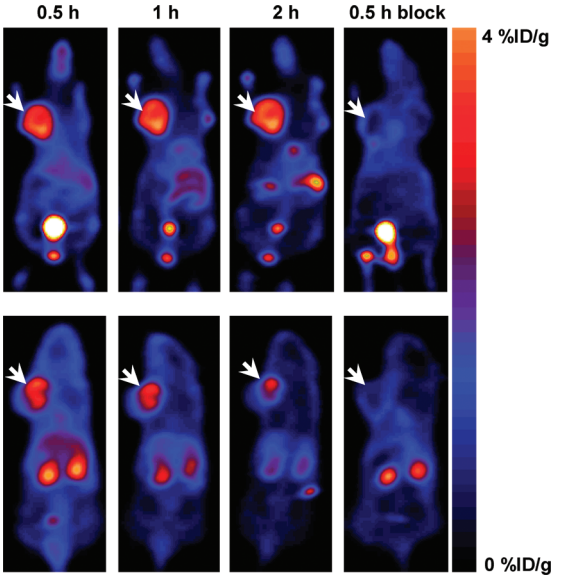

Figure 4. Representative PET images of an athymic nude mouse bearing an orthotopic MDA-MB-435 tumor on the left mammary fat pad, at 0.5, 1, and 2 h postinjection of 100 μCi (3.7 MBq) of ^{18}**F-4**, or coinjection with 300 μg of c(RGDfK). Upper row, ventral slices; arrows indicate the MDA-MB-435 tumor. Lower row: dorsal slices.

PET Imaging and Biodistribution. Since compound **4** showed higher affinity for integrin $\alpha_v\beta_3$ in the binding assay than compound **2**, it was further evaluated *in vivo* in orthotopic MDA-MB-435 tumor mice. PET images of ^{18}**F-4** were acquired at 0.5, 1, and 2 h postinjection (Figure 4). ^{18}**F-4** had initial high tumor uptake (3.8 ± 0.16%ID/g) and good tumor-to-background contrast at 0.5 h post injection, which was slightly increased at 2 h time point (4.43 ± 0.6%ID/g, Figures 4 and 5A). At 0.5 h postinjection, ^{18}**F-4** uptake in metabolic organs such as the liver and intestine was low (2.5 ± 0.3 and 0.87 ± 0.09%ID/g, respectively, Figures 4 and 5A). At 1 h postinjection, ^{18}**F-4** uptake in the liver decreased to 1.67 ± 0.34%ID/g; however, the uptake in the intestine increased (2.43 ± 0.04% ID/g, Figures 4 and 5A). At 2 h postinjection, the uptake in the intestine increased to 4.19 ± 1.1%ID/g (Figure 5A), and some uptake in the gallbladder was detected (Figure 4).

Blocking studies were done by coinjection of ^{18}**F-4** with an excess (0.5 μmol) of monomeric c(RGDfk). ^{18}**F-4** tumor uptake was reduced by 82% (Figures 4 and 5B) at 0.5 h, which suggested that the ^{18}**F-4** uptake in the tumor is due to specific binding to integrin $\alpha_v\beta_3$. The inhibition of ^{18}**F-4** uptake in other organs and tissue expressing low level of integrin $\alpha_v\beta_3$ was also found, which was consistent with previously reported observations.[2,23−25]

In addition to the PET scans, biodistribution of ^{18}**F-4** by organ dissection was performed at 2 h postinjection. The results were achieved by dissection of the organs and counting using a gamma counter (Figure 5C). Similar to the PET scan data, the uptake in the tumor was 4.57 ± 0.67%ID/g, and the uptake in the intestine was relatively high (5.04 ± 0.39%ID/g) at 2 h postinjection. Low uptake (0.68 ± 0.28%ID/g) was detected in the bone (Figure 5C).

DISCUSSION

^{18}**F-2** was achieved in higher radiochemical yield than ^{18}**F-4**. Direct fluorination on monomeric RGD peptide **1**, to yield ^{18}**F-2**, gave lesser radioactive byproduct than fluorination on the dimeric RGD, **3**, to yield ^{18}**F-4** (Figure 2). Future analysis of the byproduct might be beneficial for finding ways to eliminate the byproduct and to give better yields.

The ^{18}F-fluoride displacement reaction was conducted at relatively high temperature (130 °C), which resulted in slight decomposition of **1** and **3**, as detected by UV at 218 nm (Figures 2a and b) but had minimal effect on the labeling efficiency. Attempts to perform the labeling at other temperatures such as 120 and 145 °C did not result in a significant difference in the labeling efficiency as detected by radio-TLC and HPLC (data not shown).

One downside of labeling **1** and **3** is the difficulty of separating the nitro containing precursors (**1** and **3**) from the desired ^{18}F-labeled products (Figure 2). The specific activity of the final product is thus related to the amount of precursor and radioactivity used for the reaction.

In order to verify that the introduction of substituted arene into the peptide does not affect its biological behavior *in vitro*, the nonradioactive standards **2** and **4** were tested for binding to integrin $\alpha_v\beta_3$ expressing MDA-MB-435 cells in a competitive binding assay against ^{125}I-echistatin. Modification of RGD peptides did not significantly change the biological binding affinities in comparison to the RGD monomer c(RGDfK) and dimer E[c(RGDfK)]$_2$ (Figure 3).

^{18}**F-4** has higher integrin binding affinity than ^{18}**F-3** and was further evaluated for its usability as a PET imaging agent by injection into MDA-MB-435 tumor-bearing mice. Biodistribution of ^{18}**F-4** was analyzed at 0.5, 1, and 2 h postinjection using PET scans of live animals. At the 0.5 h time point, ^{18}**F-4** showed a very clear image with high tumor to background contrast (Figure 4), high tumor uptake (3.81 ± 0.16%ID/g), and low accumulation in metabolic organs such as the liver and intestine. The tumor uptake was elevated at 1 and 2 h postinjection (Figure 5A). However, accumulation of ^{18}**F-4** was higher in the intestine at 2 h postinjection (Figures 4 and 5A), suggesting a hepatobiliary clearance of ^{18}**F-4**. The ^{18}F-labeled RGD peptides are metabolically stable with little to no defluorination as evidenced by very low bone uptake at the 2 h time point

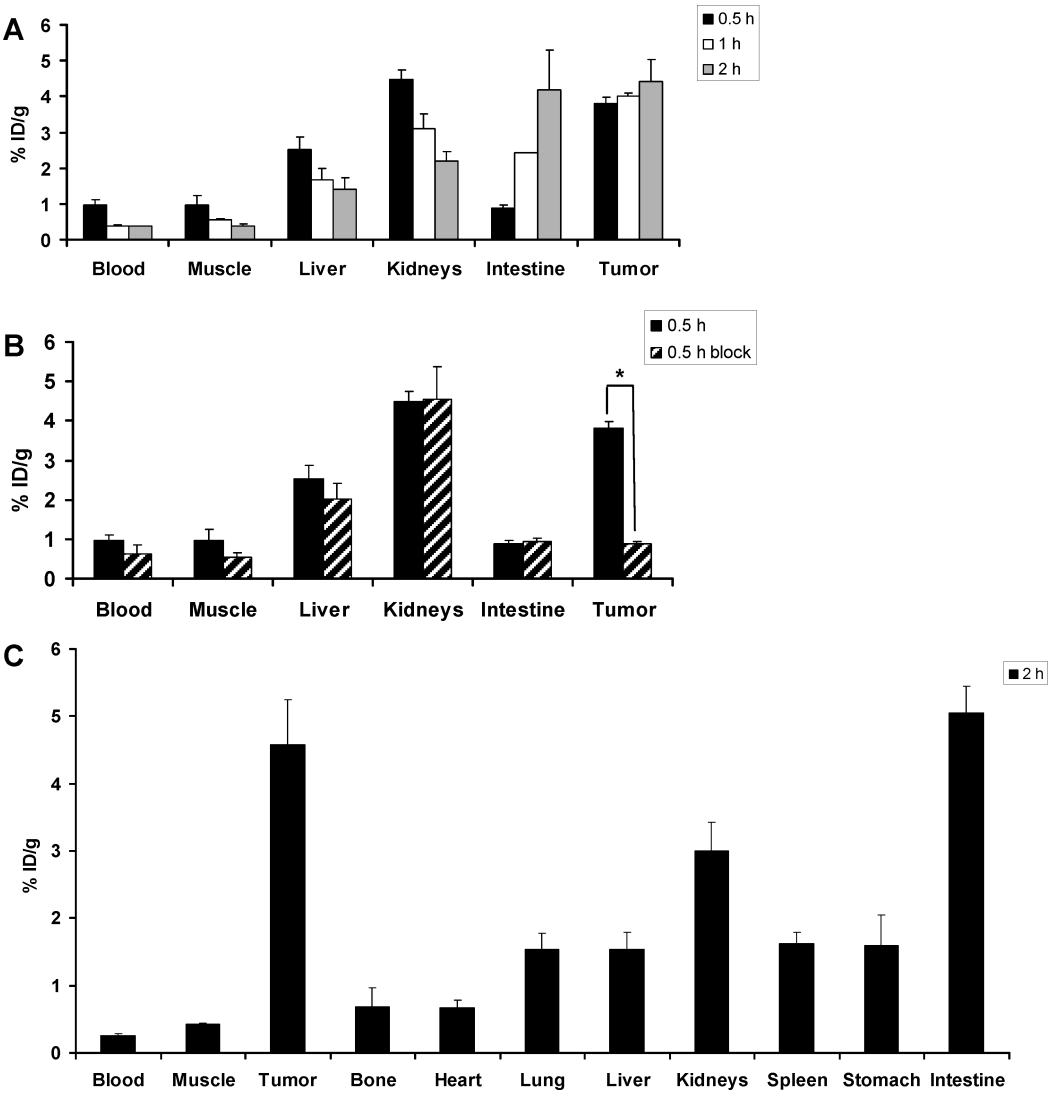

Figure 5. (A) Biodistribution of ^{18}**F-4** in MDA-MB-435 tumor-bearing mice at 0.5, 1, and 2 h postinjection of the labeled peptide. (B) Biodistribution of mice either injected with ^{18}**F-4** or coinjected with ^{18}**F-4** with nonradioactive c(RGDfK) (300 μg), 0.5 h postinjection. Results are calculated from PET scans and are shown as averages of 5 mice ± SD *P < 0.01. (C) Bioditribution of ^{18}**F-4** (calculated from gamma counting of dissected organs) in MDA-MB-435 tumor-bearing mice at 2 h postinjection of the labeled peptide. Results are shown as averages of 5 mice ± SD.

(Figure 5C). Co-injection of ^{18}**F-4** with c(RGDfK) effectively blocked the uptake in the tumor (Figures 4 and 5A), which implies specific binding of ^{18}**F-4** to integrin $\alpha_v\beta_3$. The PET quantification (Figure 5A) was consistent with the results obtained from direct tissue sampling (Figure 5C).

The goal of this study was to develop a robust one-step F-18 labeling method for the dimeric RGD peptide that can be applied for clinical translation. On the basis of our previous experience, E[c(RGDyK)]$_2$ is more hydrophilic and thus has more rapid renal clearance and improved imaging quality than E[c(RGDfK)]$_2$. However, attempts to label E[c(RGDyK)]$_2$ using this method did not succeed, most likely due to two possible reasons: (1) the OH group of Tyr may undergo deprotonation. This may interfere with the labeling reaction by capturing the fluoride ion, preventing nucleophilic substitution of the nitro group. (2) E[(RGDyK)]$_2$ is not very stable under this condition, and thus the precursor may have been partially decomposed. In general, tyrosine containing peptides may not be suitable substrates for this type of fluorination.

Therefore, we applied this labeling method to RGDfk derivatives. Introduction of 4-fluoro-3-trifluoromethylbenzamide into cyclic RGD peptide did not affect the biological properties in vitro or in vivo. The PET imaging and biodistribution results described here are comparable to the previously reported results of the RGDyk dimers.[2,25,26] Nevertheless, the other reported procedures require several radiosynthesis steps, have significantly longer reaction time, and have similar radiochemical yield. The complex and long process of multiple-step radiolabeling reassured the value of developing an alternative one-step route for labeling. The simplicity of this labeling method makes it amenable for automation, and the high PET image quality of one-step ^{18}F-radiolabeled RGD dimer makes it attractive for clinical translation.

CONCLUSIONS

We have developed a novel and rapid one-step F-18 radiolabeling strategy for peptides that contain a specific arene group

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activated for nucleophilic aromatic substitution with $^{18}F-F^-$. This method significantly shortens the reaction and overall synthesis time, requires low amounts of the precursor, and provides acceptable yields of the high specific activity product. The success of this procedure for RGD peptide labeling can be generalized to other thermally stable peptides and proteins.

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