Bioorganic & Medicinal Chemistry xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Radiolabeling of RGD peptide and preliminary biological evaluation in mice bearing U87MG tumors

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ARTICLE INFO

Article history:
Received 5 March 2012
Revised 12 April 2012
Accepted 16 April 2012
Available online xxxx

Keywords:
Click reaction
Fluorion-18
Radiolabeling
RGD peptide
Micro-PET imaging

ABSTRACT

2-[^{18}F]Fluoroethyl azide ([^{18}F]FEA) and terminal alkynyl modified propioloyl RGDfK were selected in this study. [^{18}F]FEA was prepared by nucleophilic radiofluorination of 2-azidoethyl 4-toluenesulfonate with radiochemical yield of 71 ± 4% (n = 5, decay-corrected). We assessed the various conditions of the CuAAC reaction between [^{18}F]FEA and propioloyl RGDfK, which included peptide concentration, reaction time, temperature and catalyst dosage. The ^{18}F-labeled-RGD peptide ([^{18}F]F-RGDfK) could be obtained in 60 min by a two-step radiochemical synthesis route, with total radiochemical yield of 60 ± 2% (n = 3, decay-corrected) through click chemistry. [^{18}F]F-RGDfK showed high stability in phosphate buffered saline and new-born calf serum. Micro-PET imaging at 1 h post injection of [^{18}F]F-RGDfK showed medium concentration of radioactivity in tumors while much decreased concentration in tumors in the blocking group. These results showed that [^{18}F]F-RGDfK obtained by click chemistry maintained the affinity and specificity of the RGDfK peptide to integrin $\alpha_v\beta_3$. This study provided useful information for peptide radiofluorination by using click chemistry.

1. Introduction

Positron emission tomography (PET) is considered to be one of the most sensitive molecular imaging techniques.[1–3] In the recent studies, small peptides labeled with positron emitting radionuclides (such as ^{18}F, ^{64}Cu, ^{68}Ga, ^{124}I and so on) have shown great potential as PET probes for their high affinity, in vivo metabolic stability, as well as high target-to-background ratio.[4–9] Fluorine-18, one of the best candidates for labeling bioactive peptides, has favorable physical and nuclear characteristics: suitable half-life ($t_{1/2}$ = 109.8 min) and lower positron energy (0.64 MeV).[10,11]

Click reaction has drawn considerable attention as a powerful modular synthetic approach in organic chemistry, drug discovery, material science and radiopharmaceuticals.[12–15] It is an excellent tool to meet the high synthetic demands of ^{18}F-labeled peptide PET tracers. Up to now, at least eighteen different ^{18}F-labeled alkynes or azides have been studied.[15,16] 2-[^{18}F]fluoroethyl azide([^{18}F]FEA) is a good click reagent for click reaction with peptides.[17]

Integrin $\alpha_v\beta_3$ is highly expressed on activated endothelial cells in neovasculature of tumors.[18,19] It has been reported that RGDfK (Arg-Gly-Asp-D-Phe-Lys) is of high affinity to $\alpha_v\beta_3$ receptor and can specially accumulate in tumors.[20–23] In this study, [^{18}F]FEA was prepared in an efficient way with higher radiochemical yield (RCY) of 71 ± 4% (n = 5, decay-corrected) and higher distillation efficiency of 77 ± 2% (n = 5). We assessed the various conditions of the CuAAC reaction between [^{18}F]FEA and propioloyl RGDfK, including peptide concentration, reaction time, temperature and catalyst dosage, and this study provided useful information for peptide radiofluorination by using click chemistry. We evaluated the labeling compound [^{18}F]F-RGDfK as a PET tracer for imaging $\alpha_v\beta_3$ receptor in the mice bearing U87MG tumors.

2. Results and discussion

2.1. Synthesis

FEA **2** could be obtained by nucleophilic substitution reaction between 2-fluoroethyl tosylate **1** and sodium azide in different solvents, including DMF (bp 153 °C), DMSO (bp 189 °C) and acetonitrile (bp 81 °C).[16,24] Since higher boiling point of solvent was the essential ingredient of the following distillation step, anhydrous DMF and DMSO were preferred in this water sensitive reaction. Precursor compound **5** was prepared by mono-substitution of compound **4** and the yield was highly dependent on the amount of sodium azide and the way how it was added. Dropwise-addition strategy was better than single-shot spreading strategy. Reference compound ^{19}F-RGDfK **3** was prepared by the CuAAC reaction, which could be almost quantitatively performed when excessive FEA was used. Scheme was shown in Scheme 1.

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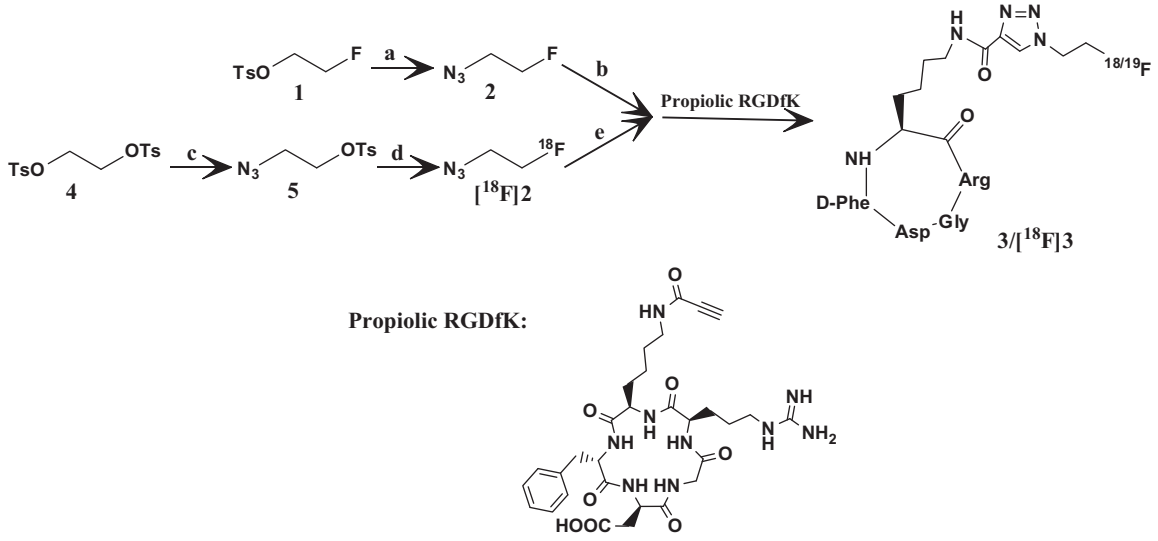
http://dx.doi.org/10.1016/j.bmc.2012.04.037



Scheme 1. Reaction conditions: (a) NaN_3, DMF (anhydrous), room temperature (rt), 10 h, distillation; (b) Propiolic RGDfK, $CuSO_4{\cdot}5H_2O$/sodium ascorbate, PBS (pH 6.0, 0.5 M), rt, 10 h; (c) NaN_3, DMF (anhydrous), rt, 10 h; (d) K[^{18}F]F/$K_{2.2.2}$, CH_3CN (anhydrous), 95 °C, 2 min, distillation; (e) Propiolic RGDfK, $CuSO_4{\cdot}5H_2O$/sodium ascorbate, PBS (pH 6.0, 0.5 M), 10–70 °C, 5–80 min.

2.2. Radiolabeling

The nucleophilic [^{18}F]fluorination of compound **5** could be completed in a sealed vial at 95 °C in anhydrous acetonitrile for 2 min, with labeling efficiency up to 99% (Fig. 1). The synthesis time of [^{18}F]FEA ([**^{18}F**]**2**) could be significantly shortened (compared with literatures[16,17]). According to the distillation units previously described by Marik et al., [25] we improved the units to simplify the distillation process and raise the distillation efficiency of [^{18}F]FEA. The radiochemical purity of [^{18}F]FEA was >98% with total time of approximately 40 min. The distillation efficiency of 77 ± 2% (n = 5) and RCY of 71 ± 4% (n = 5, decay-corrected) were higher than those reported before (63% distillation efficiency and 54% of RCY by Glaser and Årstad[16]). The rapid nucleophilic [^{18}F]fluorination and distillation had also been successful used in the synthesis and purification of 5-[^{18}F]fluoro-1-pentyne in our lab.

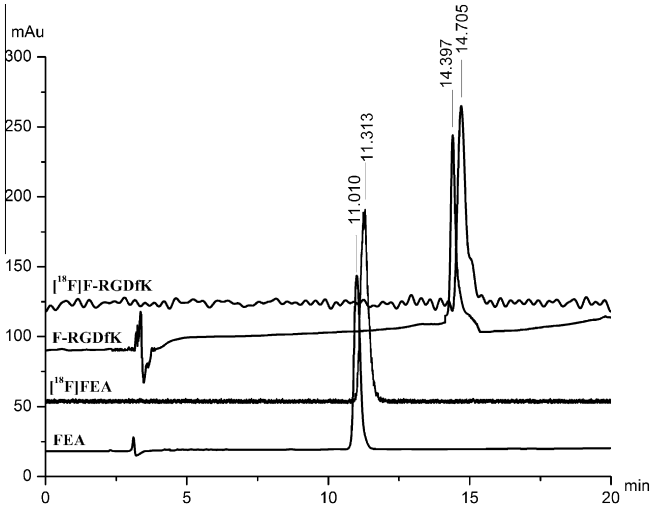


Figure 1. HPLC analysis (UV 220 nm and radioactivity) of ^{18}F-labeled compounds and corresponding ^{19}F-reference compounds. The retention times of [^{18}F]F-RGDfK and F-RGDfK were 14.7 and 14.4 min respectively, and the retention times of [^{18}F]FEA and FEA were 11.3 and 11.0 min, respectively.

2.3. Click reaction

Under the reaction conditions investigated in this study, the labeling efficiency of click reaction ranged from 5% to almost 100%. Peptide concentration, reaction time, temperature and catalyst dosage in the CuAAC reaction between [^{18}F]FEA and propioloyl RGDfK were evaluated. Results were shown in Figure 2.

The concentration of alkynyl precursors played an important role in the CuAAC reaction. When several micro mol of alkynes were used, the CuAAC labeling reaction could almost be completed.[16,25] Nevertheless, when peptide propioloyl RGDfK was added in less amount (0.15–3 μmol, 0.19–3.8 mmol/L), a positive correlation between the RCY and peptide concentration was observed in Figure 2A.

The reaction time and the temperature were another two important parameters of the CuAAC reaction. The curve of click reaction efficiency quickly increased from <10% to ~90% in the first 20 min at 25 °C or 50 °C, and the labeling yields at 50 °C were higher than those at 25 °C in the same reaction time points (Fig. 2B). The longer reaction time might help to improve the labeling yield. However, due to the decay of ^{18}F, extending the reaction time to achieve higher labeling efficiency was not suitable. In order to evaluate the effect of the reaction temperature, radiochemical yields were measured from 15 °C to 75 °C with the reaction time of 15 min. The click reaction efficiency increased from 15 to 50 °C, but decreased at 75 °C, probably due to the stability of peptides (Fig. 2C). Therefore, the reaction temperature was set at 50 °C for investigating other conditions.

Generally, 1:10 Cu/alkyne ratio was suggested in the CuAAC reaction. To achieve reasonable radio click reaction efficiency, we used more copper in our study. One reason would be that the actual chemical concentration of [^{18}F]FEA in radio click reactions was much lower than that in the 'cold' reactions. Results from Figure 2D suggested that there was no need to use more than 7:1 Cu/alkyne mol ratio.

In this study, the four main factors (3.8 mmol/L propioloyl RGDfK, 50 °C, 15 min and 7:1 Cu/alkyne ratio) discussed above were recommended as optimal conditions and RCY of over 90% (n = 3, decay-corrected) of ^{18}F-click-labeling could be obtained reliably (Fig. 1). [^{18}F]F-RGDfK [**^{18}F**]**3** could be synthesized in 60 min through a two-step radiochemical synthesis route with total RCY of 60 ± 2% (n = 3, decay-corrected).

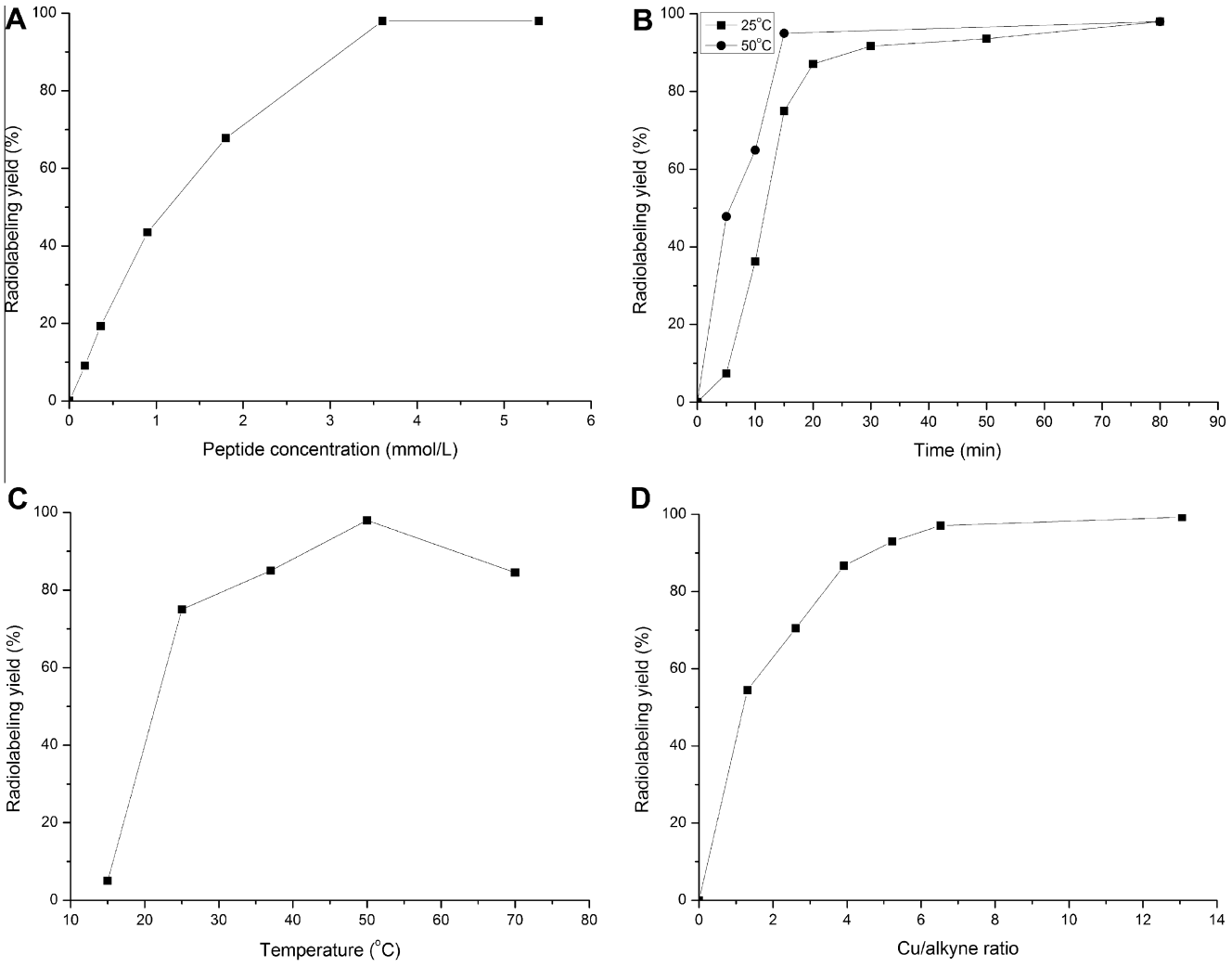


Figure 2. Effect of various click reaction conditions on radiolabeling efficiency of [^{18}F]F-RGDfK. Common conditions: 300 μL PBS (pH 6.0, 0.5 M), 200 μL propiolic RGDfK in *t*-BuOH; 100 μL $CuSO_4{\cdot}5H_2O$ and sodium ascorbate in water; 100 μL [^{18}F]FEA in acetonitrile; nitrogen atmosphere. (A) Effect of peptide concentration (50 °C, 15 min); (B) Effect of reaction time (3.8 mmol/L propiolic RGDfK, 25 °C or 50 °C); (C) Effect of reaction temperature (3.8 mmol/L propiolic RGDfK, 15 min); (D) Effect of Cu/alkyne ratio (1.9 mmol/L propiolic RGDfK, 50 °C, 15 min).

2.4. In vitro stability

After obtaining the radiolabeled compounds [^{18}F]F-RGDfK, we evaluated its in vitro stability. The results were shown in Figure 3. [^{18}F]F-RGDfK was stable in vitro and incubated in new-born calf serum (NBCS) or PBS for 5 h without obvious decreasing of radiochemical purity (>95%).

2.5. Pharmacokinetics

We evaluated the pharmacokinetic blood clearance of [^{18}F]F-RGDfK. As shown in Figure 4, [^{18}F]F-RGDfK was rapidly removed from circulation in the mice (n = 5).

2.6. Micro-PET imaging

From Figure 5A, we found that there was medium accumulation of radioactivity (1.76 ± 0.06%ID/g) in the tumor, but obvious accumulation of radioactivity was detected in the abdominal region. Blocking study showed RGDfK could effectively block the accumulation of [^{18}F]F-RGDfK in the tumor (0.38 ± 0.02%ID/g), and RGDfK could specifically bind to U87MG tumors. These results showed

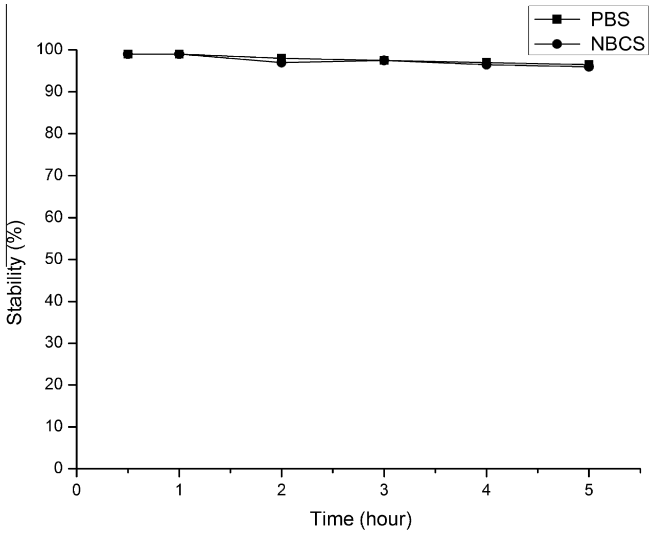


Figure 3. In vitro stability of [^{18}F]F-RGDfK.

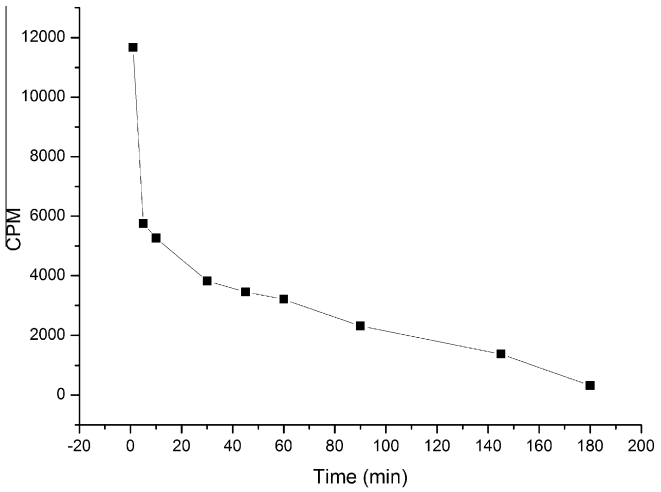


Figure 4. Blood radioactivity (CPM)-time curve of [^{18}F]F-RGDfK. CPM, count per minute.

that [^{18}F]F-RGDfK obtained by click chemistry maintained the affinity and specificity of the RGDfK peptide to integrin $\alpha_v\beta_3$. The further biological evaluation of [^{18}F]F-RGDfK in mice bearing U87MG tumors is under way and will be reported in due course.

3. Conclusion

Efforts were made to improve the synthesis and purification of the interesting agent [^{18}F]FEA and its click labeling of the model peptide—propioloyl RGDfK. With optimized radiosynthesis conditions and distillation units, [^{18}F]FEA was prepared with distillation efficiency of 77 ± 2% (n = 5) and RCY of 71 ± 4% (n = 5, decay-corrected). [^{18}F]F-RGDfK was obtained with RCY of 60 ± 2% (n = 3, decay-corrected) in 60 min. 'Click' reaction was successfully applied in the synthesis of ^{18}F-labeling reaction of peptide. Compared to other synthesis approaches, the CuAAC reaction provided a more general and efficient method for the ^{18}F-labeling of peptides. The major factors influencing the CuAAC reaction—peptide concentration, reaction time, temperature and catalyst dosage—were optimized to achieve higher RCY and shorter synthesis time. However, more experiments are needed in future study to reduce the addition of peptides and optimalize distillation units for its automation.

Radiolabeled compound [^{18}F]F-RGDfK was stable in NBCS and PBS, and could rapidly clear from blood of the mice. Results from the micro-PET imaging indicated that [^{18}F]F-RGDfK could specifically accumulate in the U87MG tumors and [^{18}F]F-RGDfK obtained by click chemistry maintained the affinity and specificity of the RGDfK peptide to integrin $\alpha_v\beta_3$.

4. Experimental section

4.1. General

Propioloyl RGDfK was provided by Suzhou ChinaTech Peptide Co., Ltd. [^{18}F$^-$] was purchased from Shanghai Atom Kexing Pharmaceuticals Co., Ltd. The other reagents and solvents in the study were purchased from Sigma–Aldrich Co., TCI Co., J&K Scientific Ltd or Sinopharm Co., and used as supplied unless stated otherwise. Radioactive samples from in vitro experiments were measured with a

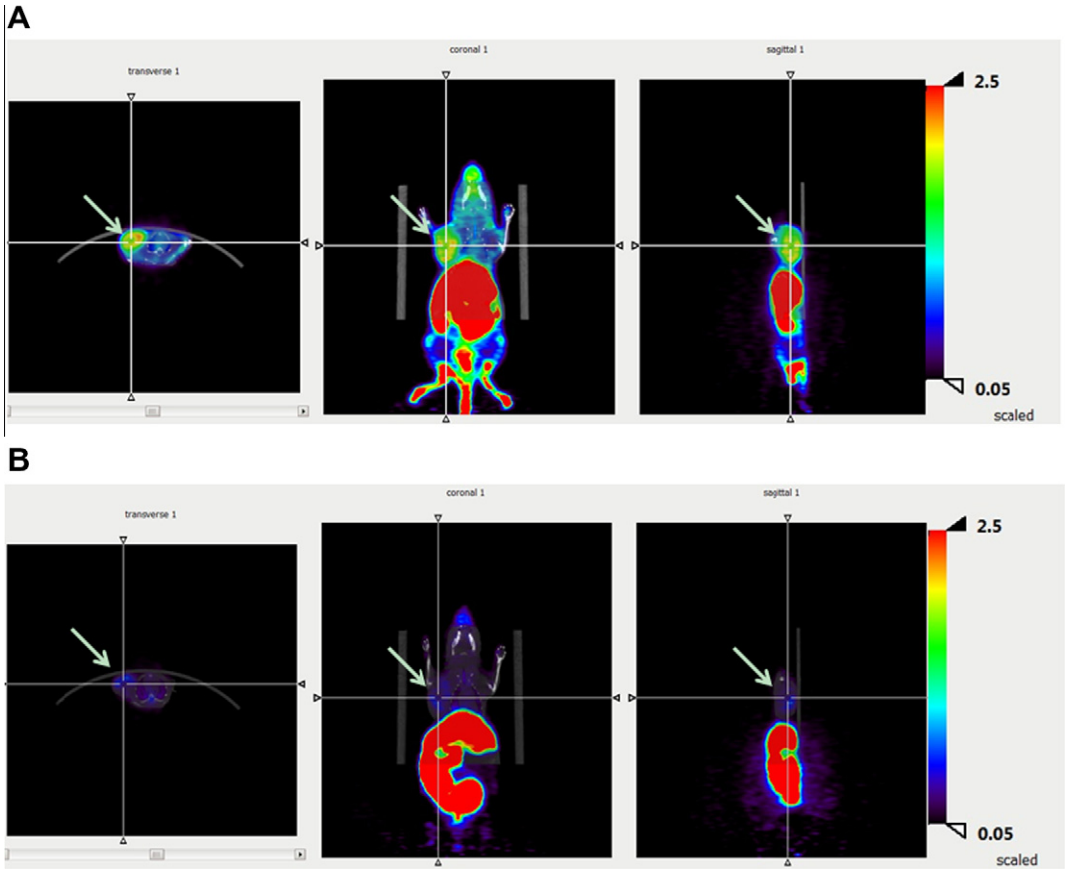


Figure 5. Micro-PET imaging of the mice bearing U87MG tumors at (A) 1 h post injection of 130 μCi of [^{18}F]F-RGDfK; (B) 1 h post injection of 130 μCi of [^{18}F]F-RGDfK and 1 mg RGDfK. The location of the tumor was indicated by an arrow.

γ-counter (SN-697, Shanghai Rihuan Photoelectronic Instrument Co., Ltd.). Micro-PET imaging was performed on an Inveon MM Platform (Siemens Ltd.) with a computer-controlled bed and 8.5 cm transaxial and 5.7 cm axial fields of view (FOV).

The ^{1}H NMR spectra was recorded on a Bruker 400 or 300 MHz instrument with the corresponding solvent signals as an internal standard. Chemical shifts (δ) were given in ppm relative to tetramethylsilane (0.00 ppm). Values of the coupling constant, *J*, were given in hertz (Hz); the following abbreviations were used for multiplicity of NMR signals: singlet (s), doublet (d), triplet (t). The chemical shifts of complex multiplets were given as the range of their occurrence. ESI-MS test was done by GL Biochem (Shanghai, China).

Analytical as well as semi-preparative high-performance liquid chromatography (HPLC) was achieved using an Agilent 1100 chromatography system with a variable-wavelength UV detector and a radiodetector connected in series. Analytical HPLC was performed with a Zorbax C18 column (4.6 × 250 mm) (eluents: 0.1% TFA (v/v) in water and 0.1% TFA (v/v) acetonitrile; gradient: 0–20 min, 95→50% water; 1 mL/min; λ = 220 nm). Semi-preparative HPLC was performed with a Waters C18 column (7.3 × 300 mm), using the same solvent system as analysis with a flow rate of 2 mL/min.

4.2. Chemistry

4.2.1. Compound 2-fluoroethyl azide (FEA) (2)

The preparation of FEA was referred to Glaser and Årstad[16] Sodium azide (1.3 g, 16.2 mmol) was added to a solution of **1** (3.6 g, 14.9 mmol) in **DMF** (anhydrous), and the mixture was stirred at rt for 10 h. Then the reaction mixture was filtered and the filtrate was distilled. FEA was obtained as pungent volatile colorless liquid (azeotrope with 5% DMF, 0.9 g, 10.0 mmol, 68.4% yield, bp 96 °C). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.567 (dt, 2H, CH_2-F, $^2J_{FH}$ = 47 Hz, $^2J_{HH}$ = 4.5 Hz); 3.494 (dt, 2H, CH_2-CH_2-F, $^3J_{FH}$ = 27 Hz, $^2J_{HH}$ = 4.5 Hz). Analysis was performed on HPLC (t_R = 11.0 min).

4.2.2. Compound 2-azidoethyl 4-toluenesulfonate (5)

The preparation of compound **5** was referred to Lundt et al.[26] Ethylene glycol ditosylate **4** (3.7 g, 10 mmol) was dissolved in 20 mL anhydrous DMF, to which sodium azide (0.65 g, 10 mmol) was added in small portions. After stirring for 10 h at rt, the reaction mixture was diluted with water and extracted with ethyl acetate. The organic layer was dried and the solvent was removed in vacuum. The residue was purified by column chromatography with dichloromethane and ethyl acetate as eluate. The compound **5** was obtained as colorless or light yellow oil (0.7 g, 30% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.819(d, 2H, aromatic, *J* = 8.4 Hz); 7.368(d, 2H, aromatic, *J* = 8.4 Hz); 4.162(t, 2H, CH_2–O, *J* = 5.2 Hz); 3.484(t, 2H, CH_2–N_3, *J* = 5.2 Hz); 2.014(s, 3H, CH_3).

4.2.3. Reference compound F-rgdfk (3)

The desired non-radioactive reference compound was synthesized according to the method reported by Glaser et al.[16,27] Propioloyl RGDfK (0.5 mg, 7.63 μmol in 200 μL PBS, pH 6.0, 0.5 M), copper (II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$) (aq 0.4 M, 50 μL), sodium ascorbate (aq 1.2 M, 50 μL) and FEA (5 μL in 200 μL acetonitrile) were sequentially mixed under nitrogen. The mixture was stirred at rt for 10 h, then purified by semi-preparative HPLC (t_R = 15.6 min). The reference compound F-RGDfK was obtained by lyophilization. ESI-MS: *m*/*z* 745.5 for $[MH]^+$ ($C_{32}H_{45}FN_{12}O_8$, calculated molecular weight [MW] = 744.7).

4.2.4. [^{18}F]FEA ([^{18}F]2)

[^{18}F]Fluoride was produced via the $^{18}O(p, n)^{18}F$ nuclear reaction with an enriched $[^{18}O]H_2O$ target which was bombarded by 16.5 MeV proton using a cyclotron (IBA Cyclone-30). [^{18}F]FEA was synthesized by nucleophilic [^{18}F]fluorination of precursor compound **5**.[16] [^{18}F]Fluoride was fixed on a QMA cartridge (an ion exchange ^{18}F Trap and Release Column) and eluted with a mixed solution of Kryptofix$_{2.2.2.}$ (14.4 mg, 38.3 μmol), potassium carbonate (3 mg, 21.6 μmol), water (40 μL) and acetonitrile (960 μL). The solvent was removed by heating at 95 °C under a stream of nitrogen (100 mL/min). Afterward, acetonitrile (0.5 mL) was added and removed by distillation. This procedure was repeated twice. After cooling to rt, a solution of compound **5** (5 mg, 25 μmol) in anhydrous acetonitrile (400 μL) was added. The reaction mixture was heated at 95 °C for 2 min, and labeling efficiency (98 ± 1%, *n* = 5) was measured through analytical radio-HPLC. [^{18}F]FEA was purified by distillation at 95 °C under a flow of nitrogen (15 mL/min to 20 mL/min for 10 min) using a home-made distillation units. About 400 μL acetonitrile solution of [^{18}F]FEA was collected with distillation efficiency of 77 ± 2% (*n* = 5) and RCY of 71 ± 4% (*n* = 5, decay-corrected). Analysis was performed on HPLC (t_R = 11.3 min).

4.2.5. [^{18}F]F-RGDfK ([^{18}F]3)

$CuSO_4{\cdot}5H_2O$/sodium ascorbate were chosen as the catalysts for click reaction between [^{18}F]FEA and propioloyl RGDfK. 0.2 mg (3 μmol) propioloyl RGDfK in 300 μL PBS (pH 6.0) and 200 μL *t*-BuOH, $CuSO_4{\cdot}5H_2O$ (aq 0.4 M, 50 μL) and sodium ascorbate (aq. 1.2 M, 50 μL) were mixed under nitrogen. Then 2-[^{18}F]fluoroethylazide in 200 μL acetonitrile was transferred to the mixture and heated at 50 °C for 15 min. Analysis was performed on HPLC (t_R = 14.7 min). After cooling, the reaction mixture was diluted with water, and [^{18}F]F-RGDfK was isolated via semi-preparative radio-HPLC (t_R = 15.9 min). Under optimized conditions [^{18}F]F-RGDfK could be prepared in 60 min with total RCY of 60 ± 2% (*n* = 3, decay-corrected at the end of synthesis) and radiochemical purity of more than 98%.

4.3. Distillation units

[^{18}F]FEA could be efficiently isolated by distillation using a home-made unit (Fig. 6). Five small vials (vial I, II, III, IV and V) were connected subsequently by silicon tubes. Vial I (containing 2 mL acetonitrile) was used to observe the rate of N_2 flow. Vial II (distilling vial) was heated and equipped with a long tube (>600 mm) to connect vial IV (gathering vial). The long tube and vial IV were both placed in an ice-water bath to ensure the condensation of the vapor from vial II. Exhaust from vial IV was transferred to vial V (containing 2 mL acetonitrile and a venting outlet). After distilled for 10–15 min, [^{18}F]FEA was collected in about 400 μL acetonitrile solution.

4.4. In vitro stability

In vitro stability of [^{18}F]F-RGDfK was studied by measuring the radiochemical purity using radio-HPLC at different time intervals after preparation. 100 μL of [^{18}F]F-RGDfK (about 100 μCi) was added to an EP tube containing 1 mL PBS, and incubated at 37 °C. The radiochemical purity was measured at 30 min, 1, 2, 3, 4 and 5 h. The same procedure was applied to the experiment using NBCS. Before analyzed by HPLC, [^{18}F]F-RGDfK in NBCS was filtered by 0.22 μm membrane.

4.5. Pharmacokinetics

The pharmacokinetics was determined in normal healthy Kunming mice (4 weeks old). Each mouse received 100 μL of [^{18}F]F-RGDfK (about 20 μCi) via the tail vein (*n* = 5). At the designated times (1, 5, 10, 15, 30, 45, 60, 90, 145 and 180 min), the blood of each mouse was immediately collected via the tail and detected in a γ-counter.

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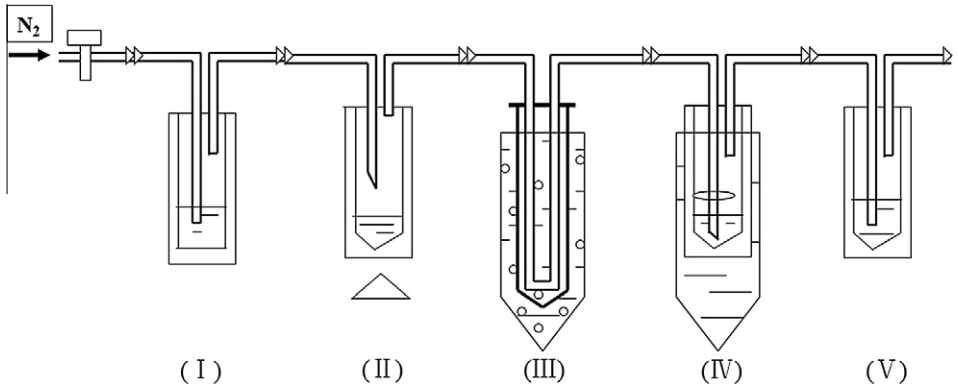

Figure 6. Distillation unit used for separation and purification of [^{18}F]FEA.

4.6. Micro-PET imaging

We made use of micro-PET to evaluate tumor-targeting of [^{18}F]F-RGDfK in the mice bearing U87MG tumors. Xenograft tumors were generated by subcutaneously injecting 5×10^6 U87MG cells in 100 μL PBS into the right fore-limb of athymic male mice (4 weeks old). Experiments were performed when tumors reached 6–8 mm in diameter. We injected 130 μCi of [^{18}F]F-RGDfK into the mouse via the tail vein. In the blocking group, we injected 130 μCi of [^{18}F]F-RGDfK and 1 mg of RGDfK into the mouse via the tail vein. Ten minutes static images were acquired at 1 h post injection in both groups.

Acknowledgment

This work was financially supported by National Nature Science Foundation of China (No.10875163).

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