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Letter

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F-18 Labeled RGD Probes Based on Bioorthogonal Strain-Promoted Click Reaction for PET Imaging

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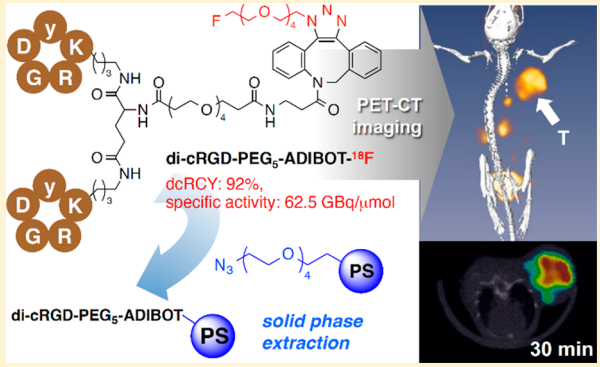
Supporting Information

ABSTRACT: A series of fluorine-substituted monomeric and dimeric cRGD peptide derivatives, such as cRGD-ADIBOT-F (ADIBOT = azadibenzocyclooctatriazole), di-cRGD-ADIBOT-F, cRGD-PEG$_5$-ADIBOT-F, and di-cRGD-PEG$_5$-ADIBOT-F, were prepared by strain-promoted alkyne azide cycloaddition (SPAAC) reaction of the corresponding aza-dibenzocyclooctyne (ADIBO) substituted peptides with a fluorinated azide **3**. Among these cRGD derivatives, di-cRGD-PEG$_5$-ADIBOT-F had the highest binding affinity in a competitive binding assay compared to other derivatives and even the original cRGDyk. On the basis of the *in vitro* study results, di-cRGD-PEG$_5$-ADIBOT-^{18}F was prepared from a SPAAC reaction with ^{18}F-labeled azide and subsequent chemo-orthogonal scavenger-assisted separation without high performance liquid chromatography (HPLC) purification in 92% decay-corrected radiochemical yield (dcRCY) with high specific activity for further *in vivo* positron emission tomography (PET) imaging study. *In vivo* PET imaging study and biodistribution data showed that this radiotracer allowed successful visualization of tumors with good tumor-to-background contrast and significantly higher tumor uptake compared to other major organs.



KEYWORDS: *F-18 labeling method, copper-free click chemistry, fluorine-18, radiopharmaceuticals, RGD peptide, PET molecular imaging, tumor angiogenesis*

Positron emission tomography (PET) is a noninvasive molecular imaging modality and a powerful tool in oncology for examining fundamental biological processes and disease pathologies in living subjects.[1,2] For these purposes, a range of bioactive molecules has been labeled with positron emitting radionuclides to be used as PET radiopharmaceuticals.[3,4] Despite the short half-life of fluorine-18 (F-18, $t_{1/2}$ = 109.8 min), it is the most popular positron-emitter for PET among various radioisotopes owing to its excellent chemical, physiological, and nuclear properties.[3−7] The short half-life of F-18, however, requires rapid, simple and reliable F-18 labeling strategies to obtain new PET molecular imaging probes.[8,9]

Because angiogenesis associated with the integrin $\alpha_v\beta_3$ receptor is a key process in tumor growth and metastasis, PET molecular imaging probes for tumor angiogenesis have potential for early tumor diagnosis and monitoring of the therapeutic effects of various treatments in both preclinical and clinical studies.[10−14] Over the past decade, a series of small peptides based on the Arg-Gly-Asp (RGD) sequence labeled with F-18 have been developed to visualize tumor angiogenesis using PET by targeting the integrin $\alpha_v\beta_3$ receptor in a range of tumor models.[15−17] Moreover, diversely modified multimeric cyclic RGD derivatives have been developed successfully to increase renal excretion and binding affinity for this integrin.[18,19] In particular, F-18 labeled PEGylated or glycosylated dimeric cyclic RGD tracers show good performance in the PET imaging of tumor angiogenesis.[18,20−23] Generally, these F-18 labeled RGD-peptide based probes are prepared using a range of conjugation methods between F-18 labeled prosthetic groups and peptides.[15,24] However, their complicated multistep radiosynthesis has limited their widespread use.[24,25] In particular, because F-18 labeled multimeric RGD peptide tracers frequently show similar retention times to an excess of unlabeled peptide precursors in high performance liquid chromatography (HPLC) purification, their radiosynthesis requires a time-consuming purification process to avoid relatively low specific activity. Specific activity is one of the most important factors for determining PET imaging quality and the toxicity or side-effects of these radiotracers.[3,4,26] In this regard, the development of a more reliable F-18 labeling

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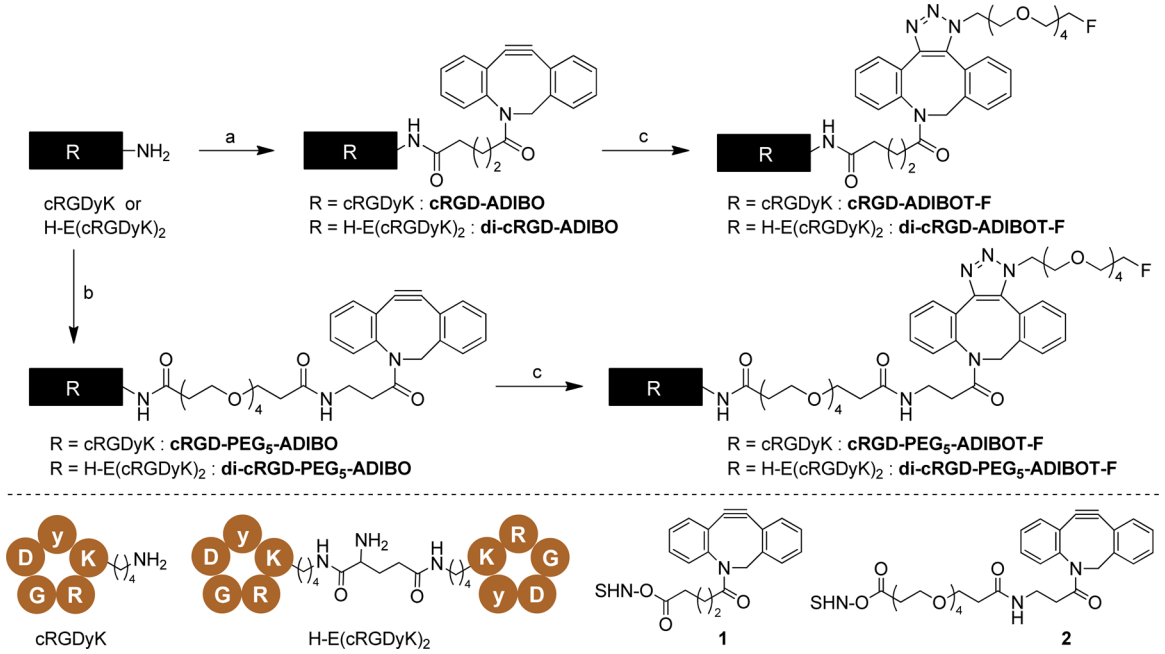
Scheme 1. Preparation of Authentic Nonradioactive cRGD Peptide Derivatives; cRGD-ADIBOT-F, di-cRGD-ADIBOT-F, cRGD-PEG$_5$-ADIBOT-F, and di-cRGD-PEG$_5$-ADIBOT-F[a]



[a]Reagents and Conditions: (a) **1**, DIPEA, DMSO, 25 °C, 12 h; (b) **2**, DIPEA, DMSO, 25 °C, 12 h; (c) fluorohexaethylene glycolic azide (**3**), DMSO, 25 °C, 2 h. NHS = *N*-succinimidyl.

protocol remains an interesting research topic for the preparation of F-18 labeled RGD peptide-based probes.

Recently, copper-free "click chemistry" based on strain-promoted alkyne azide cycloaddition (SPAAC) has been used in radiolabeling studies as a fast and bioorthogonal conjugation method between the radiolabeled prosthetic group and bioactive molecules, such as peptides, protein and nanoparticles.[26−31] In particular, aza-dibenzocyclooctyne (ADIBO) derivatives can act as effective azide acceptors to produce corresponding azadibenzocyclooctatriazoles (ADIBOTs) using the SPAAC reaction.[32−34] In a recent study, we reported that ^{18}F-labeled peptides could be prepared almost quantitatively by the SPAAC conjugation reaction between ADIBO-tethered peptides and a ^{18}F-labeled azide. Moreover, in that radiosynthetic method, ^{18}F-labeled peptides were efficiently purified by solid phase extraction using an azide-substituted resin as an ADIBO precursor-scavenger without an HPLC purification step.[26] This letter introduces the straightforward synthesis of fluorine (including F-18) substituted monomeric and dimeric RGD peptides based on the SPAAC conjugation reaction and reports the results of *in vitro* and *in vivo* evaluation of the PET imaging of integrin expression.

Scheme 1 presents the synthesis of nonradioactive fluorine-substituted monomeric and dimeric RGD peptide derivatives based on the SPAAC conjugation reaction. Treatment of monomeric cRGDyK and dimeric H-E[c(RGDyK)]$_2$ with ADIBO *N*-succinimidyl ester compound **1** under basic conditions provided the corresponding ADIBO-substituted cRGD compounds, such as cRGD-ADIBO and di-cRGD-ADIBO, respectively. To introduce a polyethylene glycol (PEG) linker between the cRGD peptides and ADIBO moiety, an ADIBO-PEG5 *N*-succinimidyl ester compound **2** was used in the conjugation reaction of cRGDyK to cRGD-PEG$_5$-ADIBO or H-E[c(RGDyK)]$_2$ to di-cRGD-PEG$_5$-ADIBO. The SPAAC reaction of four ADIBO-substituted cRGD peptide precursors with fluorohexaethylene glycolic azide (**3**) at 25 °C within 2 h yielded the corresponding fluorine substituted monomeric and dimeric RGD peptide derivatives, including cRGD-ADIBOT-F, di-cRGD-ADIBOT-F, cRGD-PEG$_5$-ADIBOT-F, and di-cRGD-PEG$_5$-ADIBOT-F. The derivatives were characterized by mass spectrometry.

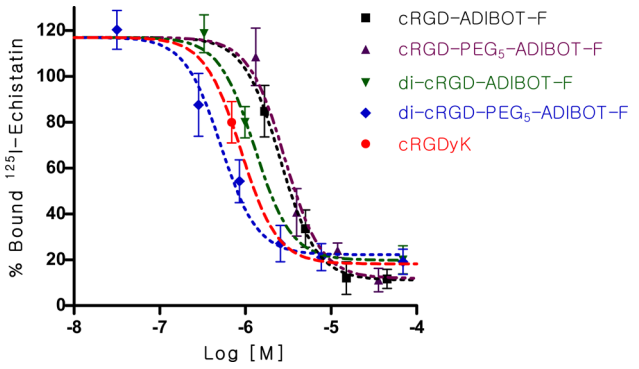


Figure 1. Competitive cell binding assay of cRGD-ADIBOT-F, di-cRGD-ADIBOT-F, cRGD-PEG$_5$-ADIBOT-F, and di-cRGD-PEG$_5$-ADIBOT-F in comparison with unmodified cRGDyK with ^{125}I-echistatin using U87MG cells (n = 3).

The receptor-binding assays for these modified fluorinated cRGD compounds (cRGD-ADIBOT-F, di-cRGD-ADIBOT-F, cRGD-PEG$_5$-ADIBOT-F, and di-cRGD-PEG$_5$-ADIBOT-F) and the unmodified cRGDyk were determined by competitive displacement studies using ^{125}I-echistatin and integrin $\alpha_v\beta_3$-expressing U87MG cells. In this *in vitro* assay, binding of ^{125}I-echistatin to the U87MG cells was inhibited by all of the synthesized fluorinated cRGD peptides in a concentration-dependent manner. As expected, the dimeric cRGD peptides exhibited better binding affinities than the corresponding

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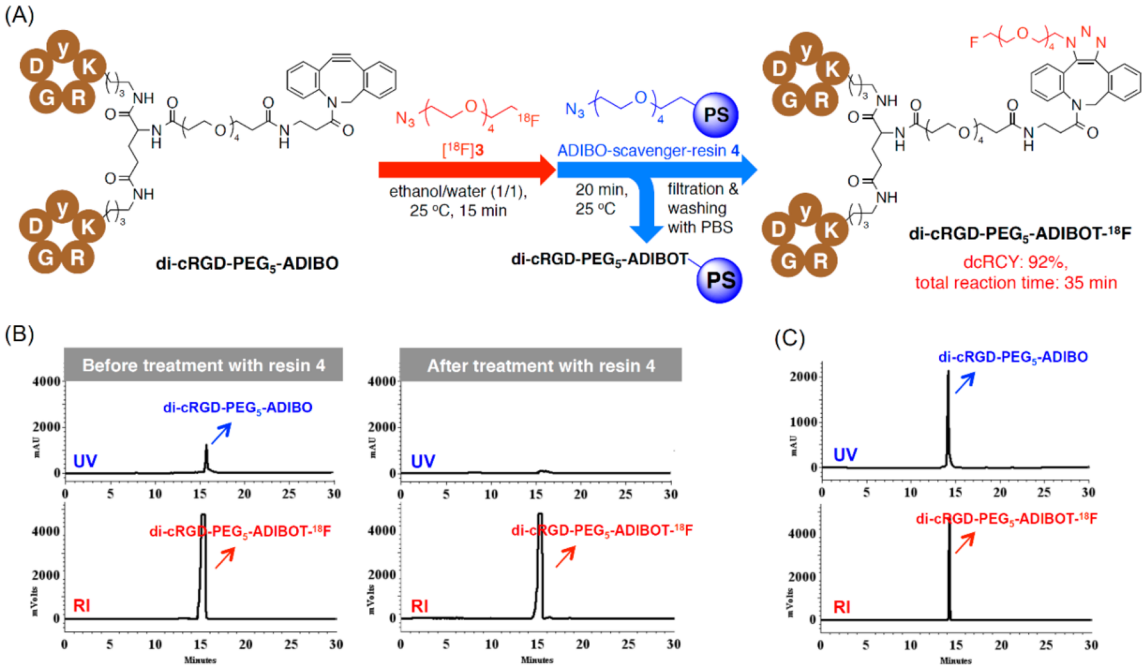

Figure 2. (A) Schematic diagram of the procedure for the radiosynthesis of di-cRGD-PEG$_5$-ADIBOT-^{18}F with [^{18}F]**3** and the subsequent chemo-orthogonal scavenger-assisted separation using a polystyrene-supported azide resin **4**. (B) HPLC chromatograms of the di-cRGD-PEG$_5$-ADIBOT-^{18}F reaction mixture before and after treatment with the ADIBO-precursor scavenger-resin **4**. (C) HPLC chromatograph of di-cRGD-PEG$_5$-ADIBOT-^{18}F coinjected with its nonradioactive di-cRGD-PEG$_5$-ADIBOT-F.

monomeric RGD peptides. In particular, the mini-PEG linker between the cRGDs and ADIBOT-F helped increase their binding affinities. The longer distance between the RGDs and bulk ADIBOT-F moiety by the PEG linker was expected to provide easier accessibility to integrin $\alpha_v\beta_3$. Consequently, di-cRGD-PEG$_5$-ADIBOT-F, which has both dimeric cRGD and the PEG linker, exhibited the highest binding affinity compared to the other RGD derivatives, even the original cRGDyk. These *in vitro* results showed similar patterns to previous binding affinity studies by other research groups.[16−20] On the basis of the *in vitro* study results, di-cRGD-PEG$_5$-ADIBOT-F was selected as the compound of choice for radiosynthesis of di-cRGD-PEG$_5$-ADIBOT-^{18}F and a further *in vivo* PET imaging study.

As depicted in Figure 2A, The SPAAC reaction of the di-cRGD-PEG$_5$-ADIBO precursor (0.87 μmol) as an azide acceptor using ^{18}F-labeled azide [^{18}F]**3** in an aqueous solution proceeded quantitatively within 15 min, affording the ^{18}F-labeled cRGD dimer compound (di-cRGD-PEG$_5$-ADIBOT-^{18}F) with an excess of unreacted di-cRGD-PEG$_5$-ADIBO precursor. This unreacted ADIBO precursor was removed by the subsequent treatment of this crude solution with an azide resin **4** as an ADIBO-scavenger for 20 min, as observed by HPLC analysis of the reaction mixture before and after treatment with resin **4** (Figure 2B). In particular, although the di-cRGD-PEG$_5$-ADIBOT-^{18}F tracer showed a similar retention time to its precursor on HPLC, it was successfully produced with a reasonably high specific activity (62.5 GBq/μmol) in 92% decay-corrected radiochemical yield (dcRCY) within a total reaction time of approximately 35 min without any HPLC purification; rather, resin **4** was merely filtered and washed (total radiosynthetic time of approximately 80 min including the radiosynthesis of ^{18}F-labeled azide [^{18}F]**3** from ^{18}F$^-$). Considering the specific activity of the tracer, at least 0.85 μmol of the precursor could be removed by this chemo-orthogonal solid phase extraction process using resin **4**. This radiosynthetic method was highly efficient for preparation of a ^{18}F-labeled dimeric RGD tracer compared with conventional radiosynthesis.[17,22,35,36] This radiotracer was characterized by HPLC coinjected with its nonradioactive di-cRGD-PEG$_5$-ADIBOT-F (Figure 2C).

After radiolabeling, *in vivo* evaluation of the di-cRGD-PEG$_5$-ADIBOT-^{18}F tracer was performed on U87MG tumor bearing mice using a micro-PET-CT system. Figure 3A shows the representative three-dimensional reconstruction, coronal, and transverse images at 30, 60, 90, and 120 min intervals after intravenous injection of the radiotracer (1.8 MBq). This radiotracer was used to visualize the tumor region successfully with good tumor-to-background contrast within 30 min postinjection. Most activity in other organs and nontargeted tissues was cleared within 1 h after injection. In a mouse model coinjected with radiotracer and nonradiolabeled di-cRGD-PEG$_5$-ADIBOT-F (10 mg/kg), tumor uptake was negligible, demonstrating the specificity of di-cRGD-PEG$_5$-ADIBOT-^{18}F for *in vivo* imaging of the integrin $\alpha_v\beta_3$ expressing tumor using the micro-PET-CT system, as shown in Figure 3B. The results indicated that the di-cRGD-PEG$_5$-ADIBOT-^{18}F produced by the chemo-orthogonal F-18 labeling protocol can act as a promising molecular PET imaging probe for cancer diagnosis.

To further confirm the relationships derived from PET imaging results, the tissue distribution of di-cRGD-PEG$_5$-ADIBOT-^{18}F with and without a blocking dose was evaluated in the U87MG tumor bearing mice immediately after PET imaging (Figure 4A). The tumor uptake values of di-cRGD-PEG$_5$-ADIBOT-^{18}F at 30, 60, and 90 min postinjection (p.i.) were 6.3 ± 0.1, 2.2 ± 0.3, and 1.2 ± 0.1%ID/g, respectively (n = 4), showing significantly higher tumor uptake compared to the other major organs, including the liver, spleen, blood, and muscle. In particular, our radiotracer exhibited higher tumor uptake compared (in particular, at 30 min p.i.) with previous

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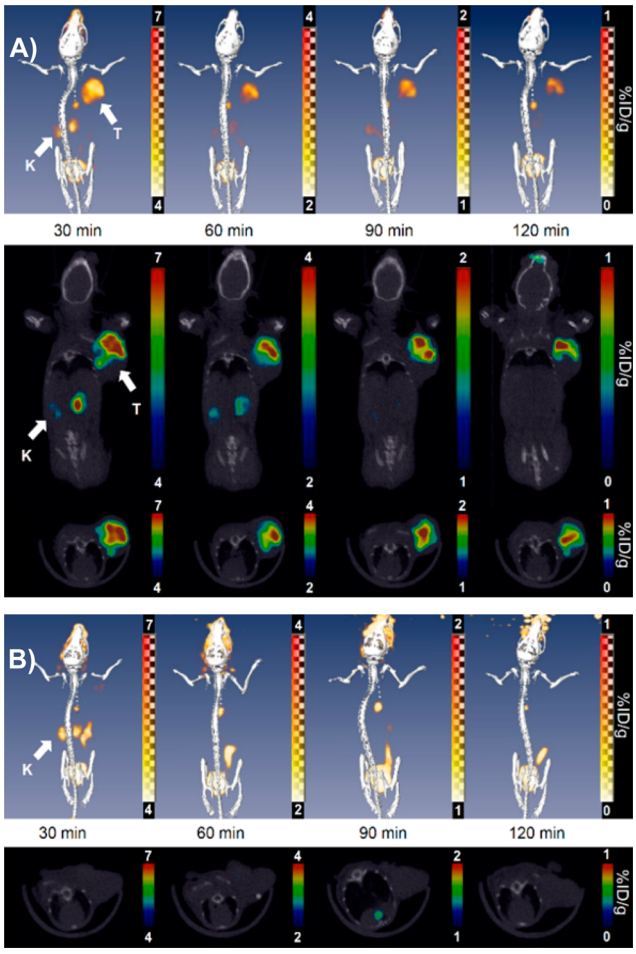

Figure 3. (A) *In vivo* evaluation of di-cRGD-PEG$_5$-ADIBOT-^{18}F; three-dimensional (3D) reconstruction (upper), coronal (middle), and transverse section (lower) combined PET-CT images of the U87MG tumor-bearing mice at 30, 60, 90, and 120 min postinjection of di-cRGD-PEG$_5$-ADIBOT-^{18}F (1.8 MBq). (B) "Blocking" images with a coinjection of nonradioactive di-cRGD-PEG$_5$-ADIBOT-F (10 mg/kg). T = tumor, K = kidney.

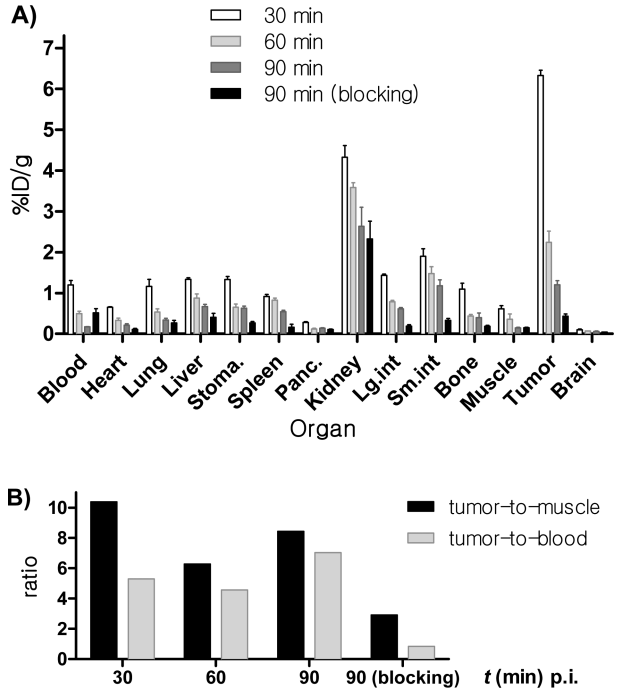

Figure 4. (A) Biodistribution of di-cRGD-PEG$_5$-ADIBOT-^{18}F in U87MG tumor bearing mice at 30, 60, and 90 min time points after microPET scans with or without nonradioactive di-cRGD-PEG$_5$-ADIBOT-F as a blocking agent. The data are expressed as normalized accumulation of activity in %ID/g ± SD (n = 4). (B) Tumor-to-muscle and tumor-to-blood ratio.

dimeric RGD radiotracers. It is supposed that the efficient removal of nonlabeled peptide precursor by chemo-orthogonal scavenger-assisted separation using the azide-resin enabled enhancement of the tumor uptake of the tracer.[17,22,35,36] However, this tracer may be rapidly washed out considering the highest tumor-to-muscle ratio occurs at 30 min p.i. (Figure 4B). In terms of the tumor-to-blood ratio, the highest tumor-to-muscle ratio was observed at 90 min p.i. because the tracer showed a relatively high blood uptake initially and was rapidly cleared of blood. In addition to tumor uptake, this radiotracer exhibited relatively high kidney uptake, indicating its predominant renal clearance. A blocking experiment where the tracer was coinjected with the nonradiolabeled compound also confirmed the specific tumor uptake of di-cRGD-PEG$_5$-ADIBOT-^{18}F. At 90 min postinjection, the uptake of a tumor coinjected with the nonradioactive RGD peptide was 0.4 ± 0.05%ID/g, which is significantly lower than that in the unblocked mouse model. Consequently, these biodistribution results were well-matched with the PET imaging study.

In summary, four fluorine-substituted monomeric and dimeric cRGD peptide derivatives were synthesized based on the SPAAC conjugation reaction. In an *in vitro* evaluation of these cRGD derivatives via competition binding assay, the di-cRGD-PEG$_5$-ADIBOT-F derivative, which has both dimeric cRGD and a mini-PEG linker, exhibited the highest binding affinity compared to the other derivatives and even original cRGDyk. On the basis of these *in vitro* results, di-cRGD-PEG$_5$-ADIBOT-^{18}F tracer was prepared via the SPAAC radiolabeling reaction of the ADIBO precursor with a ^{18}F-labeled azide in 95% dcRCY for use in *in vivo* PET imaging studies. Despite the same retention time as its precursor on HPLC, it could be separated with high specific activity by subsequent chemo-orthogonal scavenger-assisted separation using an azide-resin without the need for HPLC purification. This radiotracer provided successful visualization of a tumor with good tumor-to-background contrast as well as significantly higher tumor uptake compared to other major organs in PET imaging and biodistribution studies. These *in vivo* study results suggest that the di-cRGD-PEG$_5$-ADIBOT-^{18}F produced by this straightforward ^{18}F-labeling protocol platform based on the SPAAC reaction, can act as a promising PET imaging probe for cancer diagnosis. This ^{18}F-labeling method is also expected to be useful for radiosynthesis of other peptide probes for PET molecular imaging.

■ ASSOCIATED CONTENT

Supporting Information

All experimental procedure and characterization data of all RGD derivatives. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

PET, positron emission tomography; CT, computed tomography; HPLC, high performance liquid chromatography; SPAAC, strain-promoted alkyne azide cycloaddition; ADIBO, aza-dibenzocyclooctyne; ADIBOT, azadibenzocyclooctatriazole; dcRCY, decay-corrected radiochemical yield

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