Published in final edited form as:
Bioconjug Chem. 2012 September 19; 23(9): 1902–1908. doi:10.1021/bc300273m.

An Efficient Method for Site-specific ^{18}F-Labeling of Biomolecules Using the Rapid Condensation Reaction between 2-Cyanobenzothiazole and Cysteine

Jongho Jeon[†,‡], **Bin Shen**[†,‡], **Liqin Xiong**[†], **Zheng Miao**[†], **Kyung Hyun Lee**[†], **Jianghong Rao**[†,*], and **Frederick T. Chin**[†,*]

[†]Molecular Imaging Program at Stanford, Department of Radiology, Stanford University, 1201 Welch Road, Stanford, CA, 94305–5484, United States

Abstract

An efficient method based on a rapid condensation reaction between 2–cyanobenzothiazole (CBT) and cysteine has been developed for ^{18}F–labeling of *N*–terminal cysteine–bearing peptides and proteins. An ^{18}F–labeled dimeric cRGD ([^{18}F]CBTRGD$_2$) has been synthesized with an excellent radiochemical yield (92% based on radio–HPLC conversion, 80% decay–corrected and isolated yield) and radiochemical purity (>99%) under mild conditions using ^{18}F–CBT, and shown good *in vivo* tumor targeting efficiency for PET imaging. The labeling strategy was also applied to the site–specific ^{18}F–labeling of a protein, *Renilla* lucifierase (RLuc8) with a cysteine residue at its *N*–terminus. The protein labeling was achieved with 12% of decay–corrected radiochemical yield and more than 99% radiochemical purity. This strategy should provide a general approach for an efficient and site–specific ^{18}F–labeling of various peptides and proteins for *in vivo* molecular imaging applications.

INTRODUCTION

Positron emission tomography (PET) is a non–invasive molecular imaging technique with excellent sensitivity.[1] In the past two decades, various biomolecules have been radiolabeled for PET imaging studies of receptor activity in living subjects and for disease diagnostics including tumor detection.[2–5] As fluorine–18 (^{18}F) can be easily produced in high quantities on a medical cyclotron and has an ideal half–life of 110 min for imaging subjects, it is one of commonly–used radioisotopes for producing many PET tracers. Direct ^{18}F–fluorination however, requires harsh reaction conditions such as high temperature, pH and harmful reagent (*e.g.* fluorine gas), that are not suitable for most biomolecules. Therefore, an indirect ^{18}F–labeling strategy is generally applied where the ^{18}F is first introduced into a small organic molecule (so called ^{18}F–prosthetic group) followed by subsequent coupling to a specific functional group (*i.e.,* –NH_2, –CO_2H or –SH) in the biomolecules. Well–established ^{18}F prosthetic groups include: 1) ^{18}F–labeled benzaldehyde for labeling aminooxy groups via formation of an oxime bond;[6–7] 2) ^{18}F–labeled activated ester and maleimide for labeling amino and thiol groups, respectively;[8–11] 3) [^{18}F]fluoroazide and [^{18}F]fluoroalkyne that can react with a biomolecule equipped with an alkyne and an azide

*Corresponding Authors: jrao@stanford.edu, chinf@stanford.edu.
‡**Author Contributions**
These authors contributed equally.

Supporting Information
Synthesis and characterization of compounds, stability test of 2-cyanobenzothiazole (CBT) structures, radiochemical procedures for peptide and protein labeling and *in vivo* microPET imaging. This material is available free of charge via the Internet at http://pubs.acs.org.

group respectively through copper–catalyzed click chemistry.[12–16] In many cases, originally designed ^{18}F–prosthetic groups require lengthy synthetic procedures, relatively harsh reaction conditions, or difficult remote controls, which often result in poor radiochemical yield (RCY) and difficulty in purification. Recently, to overcome these problems, fast and specific ligation methods such as copper–free click reaction[17–18] and the [4+2] inverse electron demand Diels–Alder reaction between tetrazine structure and trans–cyclooctene[19–21] have been applied to the ^{18}F–labeling of biomolecules.

We have previously reported a versatile bioorthogonal conjugation using 2–cyanobenzothiazole (CBT) that can rapidly react with a cysteine moiety.[22–23] The observed second–order rate constant for this reaction was determined to be 9.19 $M^{-1}s^{-1}$. This condensation reaction enables rapid and site–specific fluorescent labeling of target proteins *in vitro* and at the surface of live cells without the need of catalysts under ambient conditions. Its rapid reaction rate along with biocompatibility makes this CBT–cysteine condensation reaction attractive for radio–labeling of biomolecules such as peptides and proteins for PET imaging applications. Herein we describe a facile and efficient ^{18}F–labeling method using ^{18}F–fluorinated–2–cyanobenzothiazole (^{18}F–CBT) as a novel prosthetic group and its application to radiolabeling of a dimeric cRGD peptide for *in vivo* cancer targeted PET imaging. We further demonstrated the site–specific ^{18}F–labeling of a cysteine–bearing protein using ^{18}F–CBT, and evaluated its biodistribution in a living mouse with PET imaging.

EXPERIMENTAL SECTION

^{18}F–labeling of tosylated CBT (3)

[^{18}F]–Fluoride (1000 mCi) was prepared by proton bombardment of 2.5 mL [^{18}O] enriched water target via the ^{18}O(p,n)^{18}F nuclear reaction. The [^{18}F]–Fluoride was then trapped onto a Sep–Pak QMA cartridge. 18–Crown–6/K_2CO_3 solution (1 mL, 15:1 MeCN/H_2O, 16.9 mg of 18–Crown–6, 4.4 mg of K_2CO_3) was used to elute the [^{18}F]–Fluoride from QMA cartridge into a dried glass reactor. The resulting solution was azeotropically dried with sequential MeCN evaporations at 90 °C. A solution of compound **3** (2 mg in 1 mL of anhydrous MeCN) was added to the reactor and heated at 90 °C for 10 min. After cooling to 30 °C, 0.05 M HCl (2.5 mL) was added to quench the reaction mixture and prevented basic hydrolysis of the product ^{18}F–**4**. The crude mixture was then purified with a semi–preparative HPLC (Phenomenex Gemini column: 10 × 250 mm, 5μ, 3 mL/min, and eluent gradient: 0–3 min 40% (0.1% TFA containing MeCN in 0.1% TFA containing H_2O); 3–35 min 40–100% (0.1% TFA containing MeCN in 0.1% TFA containing H_2O), R_t = 21.0 min. The collected ^{18}F–**4** was diluted with H_2O (20 mL) and passed through a C18 cartridge. The trapped ^{18}F–**4** was eluted out with Et_2O (2.5 mL). The Et_2O was removed by helium stream and used for next reaction. The isolated radiochemical yield of ^{18}F–**4** was ca. 20% (140–150 mCi, decay–corrected to end of bombardment).

Radiosynthesis of [^{18}F]CBTRGD$_2$ (9)

cRGD dimer **8** (1.2 mg) was dissolved in DMF (200 μL) containing 2 equiv. of TCEP·HCl and 15 equiv. of DIPEA. The resulting solution was added to ^{18}F–**4** (40 mCi) in DMF (200 μL) at room temperature. At different time points (1, 5, 10 and 20 min), the sample was taken from the crude mixture and the reaction was quenched with 10% AcOH aqueous solution. After 20 min, the conversion yield was 92% determined by analytical HPLC. The reaction was quenched by adding 10% AcOH aqueous solution and then the crude product was purified by a semi–preparative HPLC to give [^{18}F]CBTRGD$_2$ **9** with 80% RCY (decay–corrected to the end of synthesis). [Phenomenex Gemini column: 10 × 250 mm, 5μ, 5 mL/

min, and eluent gradient: 0–50 min 10–50% (0.1% TFA containing MeCN in 0.1% TFA containing H_2O), R_t = 34.4 min]. The specific radioactivity was 1.3 ± 0.15 Ci/μmol.

Radio–labeling of Cys–RLuc8 (11)

^{18}F–**4** (10.7 mCi, 7.5 μL) in DMSO solution was added to a solution of Cys–RLuc **11** (5 nmol,) in PBS buffer (150 μL, pH = 7.5 with 2 mM TECP), and stirred at 37 °C for 30 min. After the reaction, the crude mixture was diluted with PBS buffer until total volume was up to 1 mL. The crude mixture was directly loaded a NAP–10 column which was pre–conditioned with elution buffer (PBS, pH = 7.4). The crude solution (1 mL) was allowed to enter into the column completely and then 1.5 mL of elution buffer (PBS, pH = 7.4) was added into the column to collect the product [^{18}F]CBT–RLuc **12** (12 ± 0.7%, n = 3, decay–corrected to end of synthesis, isolated yield). Overall reaction and purification steps were completed within 40 min. The specific radioactivity was 262 mCi/μmol.

microPET imaging of U87MG tumor xenografts in mice

Image analyses were carried out using a microPET R4 rodent model scanner (Siemens Medical Solutions). By tail–vein injection, each mouse was administered approximately 100 μCi of [^{18}F]CBTRGD$_2$ under isoflurane anesthesia. At 0.5 h, 1 h and 2 h post–injection (n = 3 group), five-minute static PET scans were acquired. For the blocking experiments, the tumor bearing mice were co–injected with 20 mg/kg mouse body weight of cRGD$_2$ **7** and [^{18}F]CBTRGD$_2$. PET images were acquired at 0.5 h, 1 h and 2 h post–injection (n = 3 each group). For each scan, region of interests (ROIs) were drawn over the tumor, liver and kidney by using vendor software (ASI Pro 5.2.4.0; Simens Medical Solutions) on decay–corrected whole–body coronal images. The maximum radiochemistry concentration (accumulation) within a tumor or an organ was obtained from mean pixel values within the multiple ROI volume, which were converted to percent injected dose per gram (%ID/g).

RESULTS AND DISCUSSION

Synthesis of CBT prosthetic group and its ^{18}F–labeling procedure

To carry out ^{18}F–labeling of the CBT structure, tosylated CBT **3** was prepared from commercially available 6–methoxy–CBT **1** (Scheme 1). The methyl group in compound **1** was removed using pyridine hydrochloride at 200 °C. The resulting hydroxyl–CBT derivative was converted to tosylate **3** by reacting with excess amount of ethylene glycol ditosylate. A ^{19}F–analog, ^{19}F–**4**, was synthesized using 2–fluoroethyl 4–methylbenzenesulfonate as a reference for HPLC characterization of ^{18}F–**4**.

^{18}F–labeling of tosylate **3** was first performed with traditional phase transfer catalyst (PTC) Kryptofix 222 (K_{222}) (Fig. 1), however, an ^{18}F–byproduct was mainly observed on radio–HPLC chromatograph instead of the desired ^{18}F–**4** (Fig. 1b), which might be due to the hydrolysis of the cyano group on CBT under this condition. Indeed, stability tests with tosylated CBT **3** and fluorinated CBT (^{19}F–CBT) showed that the CBT compounds were hydrolyzed under K_{222}/K_2CO_3 condition within 1 min and also unstable in the presence of Cs_2CO_3 or tetrabutylammonium bicarbonate (Fig. S1). K_{222} contains nucleophilic nitrogen atoms that could attack the cyano group of **3** and cause facile hydrolysis under this common radiofluorination condition. To discover alternate ^{18}F–labeling conditions, we tested another PTC and to our delight, the CBT precursor was stable for 10 min in the presence of 18–Crown–6/K^+ complex at 90 °C. Tosylated CBT **3** (2 mg) in MeCN was then added to the anhydrous 18–Crown–6/K^+/[^{18}F]F^- complex and the labeling reaction was carried out at 90 °C for 10 min. Radio–HPLC chromatograph indicated by–product ^{18}F–**5** was suppressed under these conditions (Fig. 1c). Automated syntheses have been accomplished with up to 1000 mCi of radioactivity and the expected ^{18}F–**4** was obtained at a 20% RCY (decay–

corrected to end of bombardment, isolated yield). Analytical HPLC revealed that the radiochemical/chemical purity of ^{18}F–**4** was more than 99%.

A condensation reaction between ^{18}F–4 and free L–cysteine

The efficiency of condensation reaction between the prepared ^{18}F–**4** and free cysteine was subsequently investigated (Scheme 1). The reaction was carried out in MeOH/H_2O (1:1, 1 mL) at room temperature. TCEP·HCl (tris(2–carboxyethyl)phosphine hydrochloride, 2 equiv.) was added to the reaction mixture for preventing undesired oxidation of the thiol group and pH was adjusted to 7.0–7.5 using aqueous $NaHCO_3$. The condensation reaction was quenched by adding 0.05 M aqueous HCl and the conversion yield in the reaction was estimated by analytical HPLC. As shown in Fig. S2, ^{18}F–**4** was converted to ^{18}F–**6** in more than 95% yield within 1 min. Given that ^{18}F–**4** was fully converted to ^{18}F–**6** in a few minutes at room temperature, the condensation reaction should be appropriate for ^{18}F–labeling of biomolecules.

^{18}F–labeling of a dimeric cRGD peptide

We selected a dimeric cRGD peptide for our labeling because cRGD peptides have been shown to target $\alpha_v\beta_3$ integrin expression in tumors.[24–25] A number of ^{18}F prosthetic groups have been employed for labeling cRGD, some of which are currently in clinical trials such as [^{18}F]Galacto–RGD[26] and [^{18}F]FPPRGD$_2$.[27–28] However, most of these ^{18}F labeling chemistries require multi–step syntheses that resulted in low radiochemical yield and could be challenging to routinely produce for clinic use[26–30] Since ^{18}F–CBT could be easily obtained by direct ^{18}F/tosylate substitution and the condensation reaction between ^{18}F–CBT and cRGD proceeds readily at room temperature, this novel ^{18}F–prosthetic group could provide more benefits over those previously reported. In detail, *N*–terminal cysteine was introduced to a cRGD dimer **7** using *N*–Boc–Cys(Trt) succinimidyl ester in 2 steps (Scheme 2). The cRGD derivative **8** was then reacted with ^{18}F–**4** in anhydrous DMF at room temperature. The conversion yield reached 92% after incubating 20 min with the ^{18}F–labeling agent at room temperature as determined by analytical radio–HPLC (Fig. S3). After the labeling reaction was finished, the crude product was purified by semi–preparative HPLC. The observed RCY of the ^{18}F–labeled product **9** was 80% (decay–corrected to end of synthesis) and the radiochemical purity was more than 99%. The final product [^{18}F]CBTRGD$_2$ **9** was obtained in an isolated overall yield of 7.5 ± 2.7% (decay–uncorrected) with a total synthesis time of 100 min (Table 1). Specific radioactivity of the final product was 1.3 ± 0.15 Ci/μmol. It is important to note that, during the HPLC purification of crude [^{18}F]CBTRGD$_2$, all the impurity, reagent and starting material have distinct retention time from the product (> 10 min) and no other peaks appeared around the product peak on the HPLC chromatograph at UV 254 nm (Fig. S4). In comparison to [^{18}F]FPPRGD$_2$ as previously reported in clinical studies, two impurities were identified to have retention times close to the final product (< 1 min) and are difficult to remove.[27] Therefore, CBT–based ^{18}F labeling chemistry could offer the following advantages: 1) higher RCY; 2) fewer synthetic steps and shorter synthesis time; and 3) easier HPLC purification.

In vivo evaluation of [^{18}F]CBTRGD$_2$ with U87MG tumor xenograft model

Tumor targeting efficiency of [^{18}F]CBTRGD$_2$ **9** was evaluated in U87MG tumor bearing nude mice by static small animal imaging PET scans (Fig. 2). PET imaging results showed that the tumor site was clearly visualized with good tumor–to–background contrast within 30 min and the clearance of tracer uptake was observed at 120 min (Fig. 2a). PET quantification results demonstrated that tumor-to-background (or muscle) ratios were 4.5±0.83 (30 min), 5.4±0.72 (60 min) and 6.3±1.1 (120 min). The tumor uptake of

[^{18}F]CBTRGD$_2$ was 5.62 ± 1.15, 4.25 ± 0.82, 3.35 ± 0.71% at 30, 60, 120 min, respectively (Table 2). A blocking experiment where the tracer was co–injected with cRGD dimer (20 mg/kg) showed significantly reduced tumor uptake of the tracer (1.62 ± 0.10, 1.26 ± 0.23, 0.81 ± 0.04% at 30, 60, 120 min, respectively), suggesting that [^{18}F]CBTRGD$_2$ specifically bound integrin $\alpha_v\beta_3$ receptor (Fig. 2b). We also carried out a comparison experiment with tracer **9** and [^{18}F]FPPRGD$_2$ in the same mice. PET imaging results showed that the specific tumor uptake of [^{18}F]CBTRGD$_2$ was 50% higher than that of [^{18}F]FPPRGD$_2$ at 30 min (Fig S5, Table S1). [^{18}F]CBTRGD$_2$ also showed higher uptakes in organs such as liver and kidney than [^{18}F]FPPRGD$_2$, likely due to the increased lipophilicity from the aromatic structure in ^{18}F–CBT.

Recently the [4+2] inverse electron demand Diels–Alder reaction was applied to ^{18}F–labeling of a cRGD peptide.[31] The rate of this conjugation method was faster than the other reactions described, but this ligation unfortunately created a mixture of isomers. Moreover, the ligation product from the reaction between tetrazine and *trans*–cyclooctene was large and hydrophobic, and thus large amounts of radioactivity could be detected in normal organs. Considering that hydrophobic derivatization of isotope labeled tracers often spoils their desired *in vivo* pharmacokinetics properties, a relatively small hydrophobic part used in the CBT–cysteine reaction may have less adverse effect on the biodistribution of the final tracer.

Site–specific ^{18}F–labeling of *N*–terminal cysteine bearing RLuc8

We next investigated site–specific ^{18}F–labeling of a protein using ^{18}F–**4**. Several ^{18}F–prosthetic groups targeting lysine[8–9, 32–34] and cysteine[35–37] residues have been applied to protein labeling by means of amidation, conjugate addition and so on. But these methods normally result in a mixture of randomly labeled proteins. Moreover, non site–specific labeling of proteins often resulted in decreased biological activity. For example, ^{18}F–labeled anti–carcinoembryonic agent diabody using *N*–succinimidyl–4–[^{18}F]fluorobenzote ([^{18}F]SFB) showed lower immunoreactivity compared with non–labeled protein.[33] Therefore, site–specific labeling of an amino acid residue away from the active site of the protein is highly desirable. Additionally, an efficient reaction under mild conditions is preferred for ^{18}F–labeling of protein.

For this study, the bioluminescent protein *Renilla* luciferase (RLuc8) was used as a model protein. The peptide substrate of tobacco etch virus (TEV) protease was fused at the *N*–terminus region of RLuc8 **10** to generate *N*–terminal cysteine (Scheme 3). TEV protease was added to the purified fusion protein to cleave the peptide substrate. After the reaction, it was purified with Ni–NTA agarose by using 6xHis tag in front of the TEV protease sequence to provide the *N*–terminal cysteine RLuc (Cys–RLuc) **11**. During this procedure, uncleaved RLuc8 **10** and TEV protease with 6xHis tag could be separated from the product. Cys–RLuc **11** was used in next step without furthur purification. In the ^{18}F–labeling reaction, ^{18}F–**4** was added to Cys–RLuc **11** (5 nmol) in phosphate buffer saline (PBS, pH = 7.5 with 2 mM TECP), and then the labeling reaction proceeded at 37 °C for 30 min. The purification was accomplished by size exclusion chromatography (NAP–10 column) eluted with PBS (pH = 7.4) to afford [^{18}F]CBT–RLuc **12** with 262 mCi/μmol of specific activity. The observed RCY was 12 ± 0.7% (n = 3, decay–corrected to end of synthesis, isolated yield) and radiochemical purity was more than 99% as determined by radio–TLC analysis (Fig. S6). In comparison, the same labeling reaction with RLuc8 **10**, which did not contain the required *N*–terminal cysteine group for coupling, provided no ^{18}F–labeled product. Therefore we can conclude that ^{18}F–labeling of **11** with ^{18}F–**4** is site–specific. After the labeling reaction, the bioluminescent property of RLuc8 remained unchanged. The observed bioluminescent intensities of Cys–RLuc **11** and the purified product from the ^{18}F–labeling reaction were nearly the same (Fig. S7).

To evaluate our new imaging probe in a living subject, we then carried out a PET imaging experiment by injecting [^{18}F]CBT–RLuc **12** (ca. 100 μCi) in non–tumor bearing mice. PET images of different time points (20, 60 and 100 min) showed that primary radioactivity uptake was observed in the renal system within 20 min of injection and then subsequently cleared into bladder by 100 min (Fig. 3). Based on PET quantification of the collected images, non–specific liver accumulation was not observed (Table 3). These results were correlated well with a previous biodistribution study using ^{124}I–labeled RLuc8.[38]

Among various ^{18}F–precursors, ^{18}F–fluorobenzaldehyde ([^{18}F]FBA)[39–40] targeting an aminooxy group and ^{18}F–maleimide containing prosthetic groups[41–42] reacting with a thiol have been developed as site–specific labeling agents for proteins. They normally provided good ^{18}F–labeling results in terms of RCY and purity for each target protein. But the labeling reaction using [^{18}F]FBA has to be done under aqueous acidic condition (pH < 4.5) that would potentially abolish the bioactivity of pH sensitive enzymes or proteins. In case of maleimide–containing prosthetic groups, their synthesis always require an additional coupling step because maleimide structure is quite labile under the required basic ^{18}F–labeling conditions that often results in lengthy synthesis times and decreased RCYs. Moreover, these site–specific methods involved relatively complicated chemical ligation reactions or protein engineering procedures along with a couple of purification steps to generate a single reactive functional group on the protein. Compared with these methods, the *N*–terminal cysteine residue can be easily produced by a simple enzymatic cleavage with TEV protease and additional modification and purification steps are unnecessary for the ^{18}F–labeling reaction. Using this procedure, an *N*–terminal cysteine group can be readily prepared in a fusion protein. Therefore, our method has just three steps in total for protein labeling: 1) protein modification with TEV protease, 2) radio–synthesis of ^{18}F–**4**, and 3) a condensation reaction between ^{18}F–**4** and *N*–terminal cysteine protein under neutral condition, and it provides a useful and general protocol for efficient ^{18}F–labeling of various biomolecules.

CONCLUSIONS

This study demonstrated a highly efficient ^{18}F–labeling strategy of biomolecules such as peptide ligands and proteins based on a rapid condensation reaction of ^{18}F–**4**. The tosylated CBT derivative used for preparing ^{18}F–**4** can be easily synthesized from a commercially available precursor in two simple steps. The fast and specific reactivity of ^{18}F–**4** toward a free cysteine residue provides excellent RCYs under ambient temperature. The fast and specific reactivity of ^{18}F–**4** toward a free cysteine residue provides excellent RCYs under ambient temperature. Depending on the cysteine-containing molecules, the labeling can proceed efficiently in a number of different solvent systems, as demonstrated in DMF, MeOH/water and PBS buffer solutions. An *N*–terminal cysteine could be introduced into the target molecules by chemical synthesis or enzymatic processing of specific peptide sequence fused to the target protein. Using this convenient labeling method, [^{18}F]CBTRGD$_2$ has been prepared in much shorter synthesis time with a higher RCY compared to the PET tracer currently under clinical trial, [^{18}F]FPPRGD$_2$, and has shown better tumor targeting efficiency in mice than [^{18}F]FPPRGD$_2$ prepared by a different prosthetic group, 4–nitrophenyl 2–^{18}F–fluoropropionate (^{18}F–NFP). The increased accumulation of [^{18}F]CBTRGD$_2$ in livers may be due to the lipophilic structure of the CBT group, which should be ameliorated with modifications of the CBT structure that increase its hydrophilicity. On the other hand, when ^{18}F–**4** was used for site–specific ^{18}F–labeling of RLuc8, the ^{18}F–labeled protein showed little alteration in its biodistribution as revealed by PET imaging in healthy mice. We anticipate that this method could be generally applied to afford efficient and site–specific ^{18}F–labeling of peptides, proteins or antibodies that contain an *N*–terminal cysteine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by an IDEA award from Department of Defense Breast Cancer Research Program (W81XWH–09–1–0057) and the NCI ICMIC@Stanford (1P50CA114747–06).

References

1. Gambhir SS. Molecular imaging of cancer with positron emission tomography. Nat Rev Cancer. 2002; 2:683–693. [PubMed: 12209157]
2. Wester HJ, Schottelius M, Scheidhauer K, Meisetschläger G, Herz M, Rau FC, Reubi JC, Schwaiger M. PET imaging of somatostatin receptors: design, synthesis and preclinical evaluation of a novel ^{18}F-labelled, carbohydrated analogue of octreotide. Eur J Nucl Med Mol Imaging. 2003; 30:117–122. [PubMed: 12483418]
3. Stoeklin, G.; Wester, HJ. Positron emission tomography: A critical assessment of recent trends. Kluwer Academic Publishers; the Netherlands: 1998. p. 57-90.
4. Liu S. Radiolabeled multimeric cyclic RGD peptides as integrin $\alpha_v\beta_3$ targeted radiotracers for tumor imaging. Mol Pharm. 2006; 3:472–487. [PubMed: 17009846]
5. Miller PW, Long NJ, Vilar R, Gee AD. Synthesis of ^{11}C, ^{18}F, ^{15}O, and ^{13}N radiolabels for positron emission tomography. Angew Chem Int Ed. 2008; 47:8998–9033.
6. Thumshirn G, Hersel U, Goodman SL, Kessler H. Multimeric cyclic RGD peptides as potential tools for tumor targeting: solid-phase peptide synthesis and chemoselective oxime ligation. Chem Eur J. 2003; 9:2717–2725. [PubMed: 12772286]
7. Poethko T, Schottelius M, Thumshirn G, Hersel U, Herz M, Henriksen G, Kessler H, Schwaiger M, Wester HJ. Two-step methodology for high-yield routine radiohalogenation of peptides: ^{18}F-labeled RGD and octreotide analogs. J Nucl Med. 2004; 45:892–902. [PubMed: 15136641]
8. Wüst F, Hultsch C, Bergmann R, Johannsen B, Henle T. Radiolabelling of isopeptide N^{ε}-(γ-glutamyl)-L-lysine by conjugation with *N*-succinimidyl-4-[^{18}F]fluorobenzoate. Appl Radiat Isot. 2003; 59:43–48. [PubMed: 12878121]
9. Wester HJ, Hamacher K, Stöcklin G. A comparative study of N.C.A. Fluorine-18 labeling of proteins via acylation and photochemical conjugation. Nucl Med Biol. 1996; 23:365–372. [PubMed: 8782249]
10. Cai W, Zhang X, Wu Y, Chen X. A thiol-reactive ^{18}F-labeling agent, *N*-[2-(4-^{18}F-fluorobenzamido) ethyl]maleimide, and synthesis of RGD peptide-based tracer for PET imaging of $\alpha_v\beta_3$ integrin expression. J Nucl Med. 2006; 47:1172–1180. [PubMed: 16818952]
11. de Bruin B, Kuhnast B, Hinnen F, Yaouancq L, Amessou M, Johannes L, Samson A, Boisgard R, Tavitian B, Dollé F. 1-[3-(2-[^{18}F]Fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione: design, synthesis, and radiosynthesis of a new [^{18}F]fluoropyridine-based maleimide reagent for the labeling of peptides and proteins. Bioconjugate Chem. 2005; 16:406–420.
12. Gill HS, Marik J. Preparation of ^{18}F-labeled peptides using the copper(I)-catalyzed azide-alkyne 1,3-dipolar cycloaddition. Nat Protoc. 2011; 6:1718–1725. [PubMed: 22011654]
13. Maschauer S, Einsiedel J, Haubner R, Hocke C, Ocker M, Hübner H, Kuwert T, Gmeiner P, Prante O. Labeling and glycosylation of peptides using Click chemistry: a general approach to 18F-glycopeptides as effective imaging probes for positron emission tomography. Angew Chem Int Ed. 2010; 49:976–979.
14. Glaser M, Årstad E. "Click labeling" with 2-[^{18}F]fluoroethylazide for positron emission tomography. Bioconjugate Chem. 2007; 18:989–993.
15. Li ZB, Wu Z, Chen K, Chin FT, Chen X. Click chemistry for 18F-labeling of RGD peptides and microPET imaging of tumor integrin $\alpha_v\beta_3$ expression. Bioconjugate Chem. 2007; 18:1987–1994.
16. Marik J, Sutcliffe JL. Click for PET: rapid preparation of [^{18}F]fluoropeptides using Cu^{I} catalyzed 1,3-dipolar cycloaddition. Tetrahedron Lett. 2006; 47:6681–6684.

17. Campbell–Verduyn LS, Mirfeizi L, Schoonen AK, Dierckx RA, Elsinga PH, Feringa BL. Strain-promoted copper-Free "Click" chemistry for ^{18}F radiolabeling of bombesin. Angew Chem Int Ed. 2011; 50:11117–11120.
18. Evans HL, Slade RL, Carroll L, Smith G, Nguyen Q, Iddon L, Kamaly N, Stöckmann H, Leeper FJ, Aboagye EO, Spivey AC. Copper-free click–a promising tool for pre-targeted PET imaging. Chem Commun. 2012; 48:991–993.
19. Li ZB, Cai H, Hassink M, Blackman ML, Brown RCD, Conti PS, Fox JM. Tetrazine-*trans*-cyclooctene ligation for the rapid construction of ^{18}F labeled probes. Chem Commun. 2010; 46:8043–8045.
20. Keliher EJ, Reiner T, Turetsky A, Hilderbrand SA, Weissleder R. High-yielding, two-step ^{18}F labeling strategy for ^{18}F-PARP1 inhibitors. ChemMedChem. 2011; 6:424–427. [PubMed: 21360818]
21. Reiner T, Keliher EJ, Earley S, Marinelli B, Weissleder R. Synthesis and in vivo imaging of a ^{18}F-labeled PARP1 inhibitor using a chemically orthogonal scavenger-assisted high-performance method. Angew Chem Int Ed. 2011; 50:1922–1925.
22. Ren H, Xiao F, Zhan K, Kim Y–P, Xie H, Xia Z, Rao J. A biocompatible condensation reaction for the labeling of terminal cysteine residues on proteins. Angew Chem Int Ed. 2009; 48:9658–9662.
23. Liang G, Ren H, Rao J. A biocompatible condensation reaction for controlled assembly of nanostructures in living cells. Nature Chem. 2010; 2:54–60. [PubMed: 21124381]
24. Chen X, Tohme M, Park R, Hou Y, Bading JR, Conti PS. Micro-PET imaging of $\alpha_v\beta_3$-integrin expression with 18F-labeled dimeric RGD peptide. Mol Imaging. 2004; 3:96–104. [PubMed: 15296674]
25. Xiong JP, Stehle T, Zhang R, Joachimiak A, Frech M, Goodman SL, Arnaout MA. Crystal structure of the extracellular segment of integrin $\alpha_v\beta_3$ in complex with an Arg-Gly-Asp ligand. Science. 2002; 296:151–155. [PubMed: 11884718]
26. Haubner R, Kuhnast B, Mang C, Weber WA, Kessler H, Wester HJ, Schwaiger M. [^{18}F]Galacto-RGD: synthesis, radiolabeling, metabolic stability, and radiation dose estimates. Bioconjugate Chem. 2004; 15:61–69.
27. Chin FT, Shen B, Liu S, Berganos RA, Chang E, Mittra E, Chen X, Gambhir SS. First experience with clinical-grade [^{18}F]FPP(RGD)$_2$: an automated multi-step radiosynthesis for clinical PET studies. Mol Imaging Biol. 2012; 14:88–95. [PubMed: 21400112]
28. Mittra ES, Golis ML, Iagaru AH, Kardan A, Burton L, Berganos R, Chang E, Liu S, Shen B, Chin FT, Chen X, Gambihr SS. Pilot pharmacokinetic and dosimetric studies of ^{18}F-FPPRGD$_2$: a PET radiopharmaceutical agent for imaging $\alpha_v\beta_3$ integrin levels. Radiology. 2011; 260:182–191. [PubMed: 21502381]
29. Poethko T, Schottelius M, Thumshirn G, Herz M, Haubner R, Henriksen G, Kessler H, Schwaiger M, Wester HJ. Chemoselective pre-conjugate radiohalogenation of unprotected mono- and multimeric peptides *via* oxime formation. Radiochim Acta. 2004; 92:317–328.
30. Liu S, Liu Z, Chen K, Yan Y, Watzlowik P, Wester HJ, Chin FT, Chen X. ^{18}F-labeled galacto and PEGylated RGD dimers for PET Imaging of $\alpha_v\beta_3$ integrin expression. Mol Imaging Biol. 2010; 12:530–538. [PubMed: 19949981]
31. Selvaraj R, Liu S, Hassink M, Huang C, Yap L, Park R, Fox JM, Li ZB, Conti PS. Tetrazine-trans-cyclooctene ligation for the rapid construction of integrin $\alpha_v\beta_3$ targeted PET tracer based on a cyclic RGD peptide. Bioorg Med Chem Lett. 2011; 21:5011–5014. [PubMed: 21601452]
32. Kostikov AP, Chin J, Orchowski K, Niedermoser S, Kovacevic MM, Aliaga A, Jurkschat K, Wängler B, Wängler C, Wester HJ, Schirrmacher R. Oxalic acid supported Si–^{18}F-radiofluorination: one-step radiosynthesis of *N*-succinimidyl 3-(Di-tert-butyl[^{18}F]fluorosilyl)benzoate ([^{18}F]SiFB) for protein labeling. Bioconjugate Chem. 2012; 23:106–114.
33. Cai W, Olafsen T, Zhang X, Cao Q, Gambhir SS, Williams LE, Wu AM, Chen X. PET imaging of colorectal cancer in xenograft-bearing mice by use of an ^{18}F-labeled T84.66 anti–carcinoembryonic antigen diabody. J Nucl Med. 2007; 48:304–310. [PubMed: 17268029]
34. Grierson JR, Yagle KJ, Eary JF, Tait JF, Gibson DF, Lewellen B, Link JM, Krohn KA. Production of [F-18]fluoroannexin for imaging apoptosis with PET. Bioconjugate Chem. 2004; 15:373–379.

35. Berndt M, Pietzsch J, Wuest F. Labeling of low-density lipoproteins using the ^{18}F-labeled thiol-reactive reagent *N*-[6-(4-[^{18}F]fluorobenzylidene)aminooxyhexyl]maleimide. Nucl Med Biol. 2007; 34:5–15. [PubMed: 17210457]

36. Toyokuni T, Walsh JC, Dominguez A, Phelps ME, Barrio JR, Gambhir SS, Satyamurthy N. Synthesis of a new heterobifunctional linker, *N*-[4-(aminooxy)butyl]maleimide, for facile access to a thiol-reactive 18F-labeling agent. Bioconjugate Chem. 2003; 14:1253–1259.

37. Wuest F, Berndt M, Bergmann R, van den Hoff J, Pietzsch J. Synthesis and application of [^{18}F]FDG-maleimidehexyloxime ([^{18}F]FDG-MHO): a [^{18}F]FDG-based prosthetic group for the chemoselective ^{18}F-labeling of peptides and proteins. Bioconjugate Chem. 2008; 19:1202–1210.

38. Venisnik KM, Olafsen T, Loening AM, Iyer M, Gambhir SS, Wu AM. Bifunctional antibody-Renilla luciferase fusion protein for in vivo optical detection of tumors. Protein Eng Des Sel. 2006; 19:453–460. [PubMed: 16882674]

39. Flavell RR, Kothari P, Bar–Dagan M, Synan M, Vallabhajosula S, Friedman JM, Muir TW, Ceccarini G. Site-specific ^{18}F-labeling of the protein hormone leptin using a general two-Step ligation procedure. J Am Chem Soc. 2008; 130:9106–9112. [PubMed: 18570424]

40. Namavari M, De Jesus OP, Cheng Z, De A, Kovacs E, Levi J, Zhang R, Hoerner JK, Grade H, Syud FA, Gambhir SS. Direct site-specific radiolabeling of an affibody protein with 4-[^{18}F]fluorobenzaldehyde via oxime chemistry. Mol Imaging Biol. 2008; 10:177–181. [PubMed: 18481153]

41. Li X, Link JM, Stekhova S, Yagle KJ, Smith C, Krohn KA, Tait JF. Site-specific labeling of Annexin V with F-18 for apoptosis imaging. Bioconjugate Chem. 2008; 19:1684–1688.

42. Gill HS, Tinianow JN, Ogasawara A, Flores JE, Vanderbilt AN, Raab H, Scheer JM, Vandlen R, Williams S, Marik J. A modular platform for the rapid site-specific radiolabeling of proteins with ^{18}F exemplified by quantitative positron emission tomography of human epidermal growth factor receptor 2. J Med Chem. 2009; 52:5816–5825. [PubMed: 19736996]

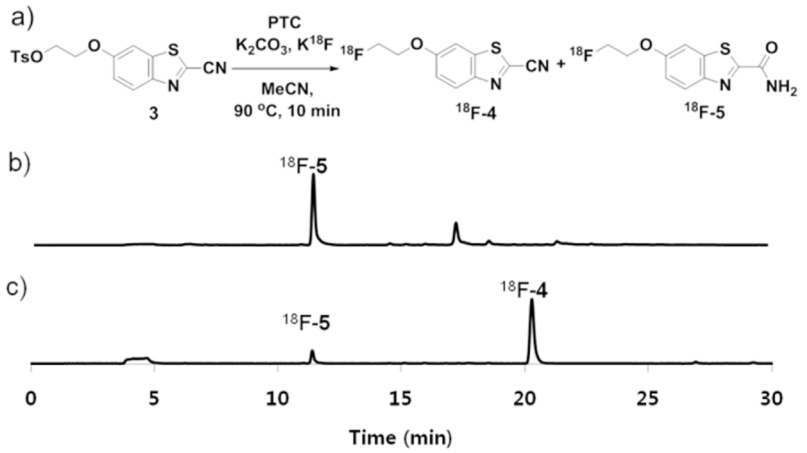


Figure 1.
(a) ^{18}F–fluorination reaction of tosylated CBT **3**. (b) Radiochromatography of the crude product using K_{222}. (c) Radiochromatography of the crude product using 18–crown–6.

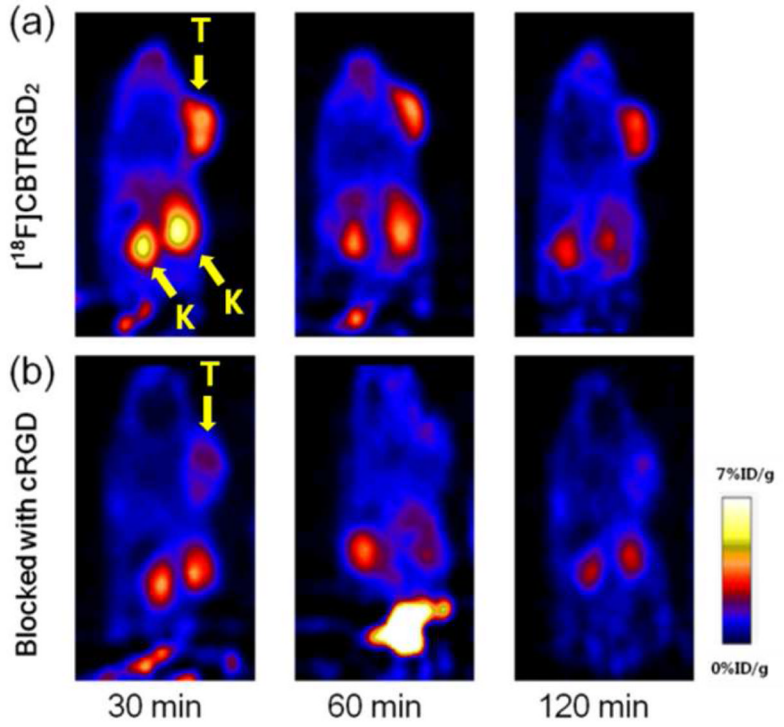


Figure 2.

Representative small–animal PET images of U87MG tumor–bearing (right shoulder) mice. (a) Whole body coronal images at 30 min, 60 min and 120 min after *i.v.* injection of [^{18}F]CBTRGD$_2$. (b) Whole body coronal images at 30 min, 60 min and 120 min after co–injection of [^{18}F]CBTRGD$_2$ with cRGD$_2$ peptide (blocking). (T: U87MG tumor, K: kidney)

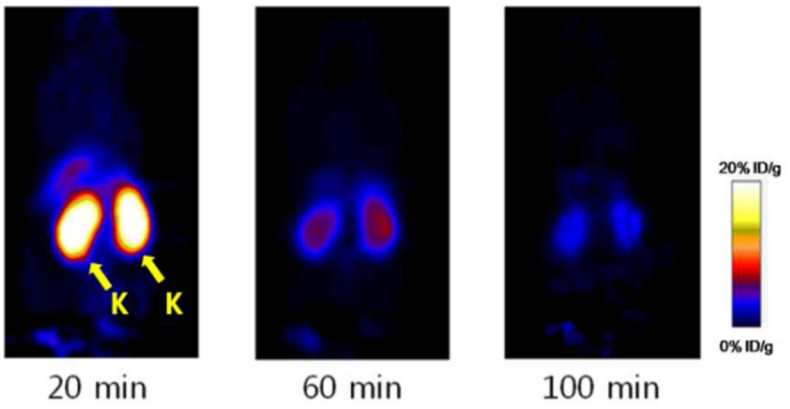
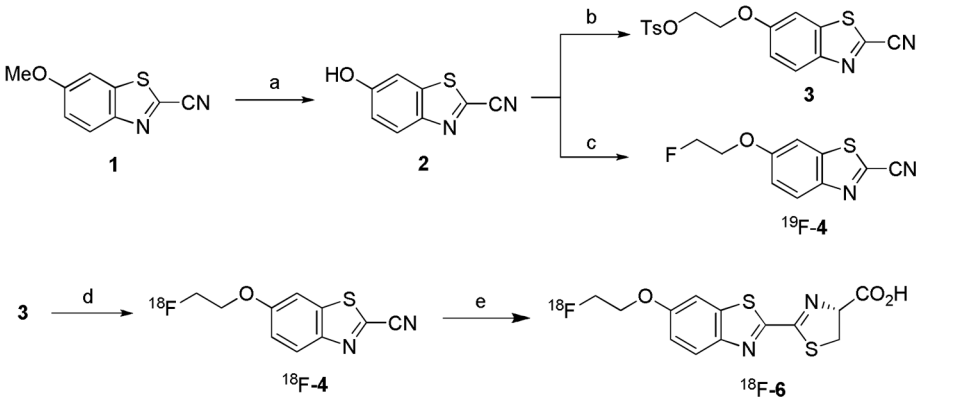


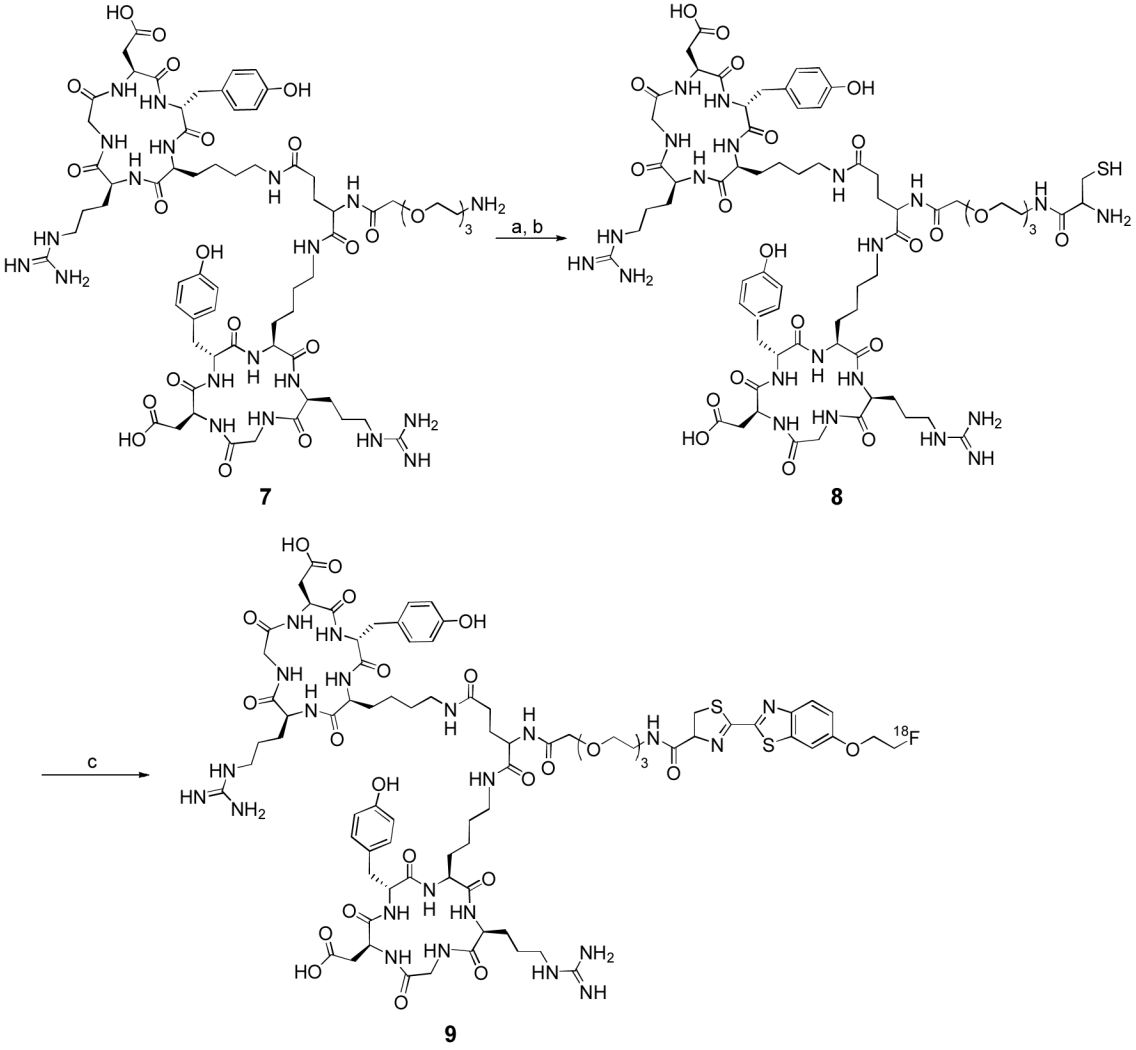
Figure 3.
Whole body coronal images at 20 min, 60 min and 100 min after *i.v.* injection of [^{18}F]CBT–RLuc8. (K: kidney)



Scheme 1.

Synthesis of tosylated CBT **3** and a condensation reaction between ^{18}F–**4** and free cysteine[a]

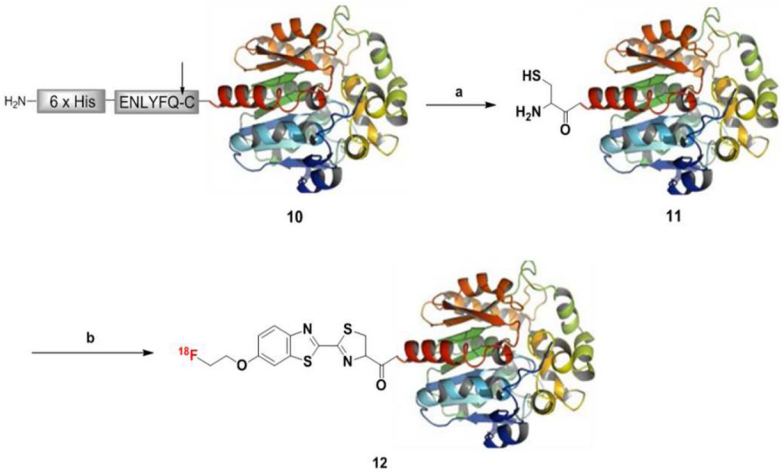
[a]Reagents and conditions: (a) pyridine–HCl, 200 °C, 2 h, 75%, (b) ethylene glycol di–tosylate, K_2CO_3, DMF, room temperature, 8 h, 53%, (c) 2–fluoroethyl 4–methylbenzenesulfonate, K_2CO_3, DMF, room temperature, overnight, 70 %, (d) K[^{18}F]F, 18–Crown–6, K_2CO_3, MeCN, 90 °C, 10 min, 20% (decay corrected to end of bombardment, isolated yield), (e) L–cysteine, TCEP·HCl, $NaHCO_3$, MeOH/H_2O, room temperature, 1 min, 95%.



Scheme 2.

^{18}F–labeling of cRGD$_2$ peptide analog using ^{18}F–**4**[a]

[a]Reagents and conditions: (a) *N*–Boc–Cys(Trt) succinimidyl ester, (b) TFA, triisopropylsilane, DCM, 79% over 2 steps, (c) ^{18}F–**4**, DIPEA, TCEP·HCl, room temperature, 20 min, 80% (decay corrected to end of synthesis, isolated yield).



Scheme 3.

^{18}F–labeling of Cys–RLuc8 **11** using ^{18}F–**4**[a]

[a] Reagents and conditions: (a) TEV protease (100 μg of protein/10 units of enzyme), 30 °C, 6 h, (b) ^{18}F–**4**, 37 °C, 30 min, 12 ± 0.7% (decay–corrected to end of synthesis, isolated yield).

Table 1

Comparison of radiochemical procedure

	$[^{18}F]FPPRGD_2$	$[^{18}F]CBTRGD_2$
Radiosynthetic steps	4	2
Total synthesis time	180 min	100 min
RCY [a] (n = 3)	3.5 ± 1.8%	7.5 ± 2.7%
Specific radioactivity	0.97 ± 0.06 Ci/μmol	1.3 ± 0.15 Ci/μmol

[a] decay–uncorrected, isolated yield

Table 2

Uptake of [^{18}F]CBTRGD$_2$ in U87MG tumor, kidney and liver derived from PET quantification (%ID/g, n = 3)

Organ	Time	[^{18}F]CBTRGD$_2$	Blocked with cRGD$_2$
Tumor	30 min	5.62 ± 1.15	1.62 ± 0.10
	60 min	4.25 ± 0.82	1.26 ± 0.13
	120 min	3.35 ± 0.71	0.49 ± 0.02
Kidney	30 min	5.46 ± 1.10	2.76 ± 0.85
	60 min	3.99 ± 1.08	1.26 ± 0.23
	120 min	2.26 ± 0.67	0.81 ± 0.04
Liver	30 min	3.64 ± 0.76	1.34 ± 0.06
	60 min	2.84 ± 0.76	0.70 ± 0.08
	120 min	2.15 ± 0.53	0.32 ± 0.01

Table 3

Uptake of [^{18}F]CBT–RLuc8 in kidney and liver derived from PET quantification (%ID/g, n = 3)

Organ	Time	[^{18}F]CBT–RLuc8
Kidney	20 min	17.3 ± 1.28
	60 min	7.94 ± 1.07
	100 min	2.98 ± 0.32
Liver	20 min	7.18 ± 0.94
	60 min	3.15 ± 0.30
	100 min	1.61 ± 0.19
Muscle	20 min	1.24 ± 0.03
	60 min	1.02 ± 0.02
	100 min	0.50 ± 0.04