Comparison of Two Site-Specifically 18F-Labeled Affibodies for PET Imaging of EGFR Positive Tumors

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ABSTRACT: The epidermal growth factor receptor (EGFR) serves as an attractive target for cancer molecular imaging and therapy. Our previous positron emission tomography (PET) studies showed that the EGFR-targeting affibody molecules 64Cu-DOTA-ZEGFR:1907 and 18F-FBEM-ZEGFR:1907 can discriminate between high and low EGFR-expression tumors and have the potential for patient selection for EGFR-targeted therapy. Compared with 64Cu, 18F may improve imaging of EGFR-expression and is more suitable for clinical application, but the labeling reaction of 18F-FBEM-ZEGFR:1907 requires a long synthesis time. The aim of the present study is to develop a new generation of 18F labeled affibody probes (Al18F-NOTA-ZEGFR:1907 and 18F-CBT-ZEGFR:1907) and to determine whether they are suitable agents for imaging of EGFR expression. The first approach consisted of conjugating ZEGFR:1907 with NOTA and radiolabeling with Al18F to produce Al18F-NOTA-ZEGFR:1907. In a second approach the prosthetic group 18F-labeled 2-cyanobenzothiazole (18F-CBT) was conjugated to Cys-ZEGFR:1907 to produce 18F-CBT-ZEGFR:1907. Binding affinity and specificity of Al18F-NOTA-ZEGFR:1907 and 18F-CBT-ZEGFR:1907 to EGFR were evaluated using A431 cells. Biodistribution and PET studies were conducted on mice bearing A431 xenografts after injection of Al18F-NOTA-ZEGFR:1907 or 18F-CBT-ZEGFR:1907 with or without coinjection of unlabeled affibody proteins. The radiosyntheses of Al18F-NOTA-ZEGFR:1907 and 18F-CBT-ZEGFR:1907 were completed successfully within 40 and 120 min with a decay-corrected yield of 15% and 41% using a 2-step, 1-pot reaction and 2-step, 2-pot reaction, respectively. Both probes bound to EGFR with low nanomolar affinity in A431 cells. Although 18F-CBT-ZEGFR:1907 showed instability in vitro, biodistribution studies revealed rapid and high tumor accumulation and quick clearance from normal tissues except the bones. In contrast, Al18F-NOTA-ZEGFR:1907 demonstrated high in vitro and in vivo stability, high tumor uptake, and relatively low uptake in most of the normal organs except the liver and kidneys at 3 h after injection. The specificity of both probes for A431 tumors was confirmed by their lower uptake on coinjection of unlabeled affibody. PET studies showed that Al18F-NOTA-ZEGFR:1907 and 18F-CBT-ZEGFR:1907 could clearly identify EGFR positive tumors with good contrast. Two strategies for 18F-labeling of affibody molecules were successfully developed as two model platforms using NOTA or CBT coupling to affibody molecules that contain an N-terminal cysteine. Al18F-NOTA-ZEGFR:1907 and 18F-CBT-ZEGFR:1907 can be reliably obtained in a relatively short time. Biodistribution and PET studies demonstrated that Al18F-NOTA-ZEGFR:1907 is a promising PET probe for imaging EGFR expression in living mice.

KEYWORDS: affibody, EGFR, PET, 18F, NOTA, CBT

INTRODUCTION

The epidermal growth factor receptor (EGFR) plays an important role in neoplastic processes of cell proliferation, inhibition of apoptosis, angiogenesis, and metastatic spread. Overexpression of EGFR in tumors has been associated with resistance against conventional drug treatment and radiation and may predict poor prognosis. Detection of EGFR expression by molecular imaging could be a useful tool for evaluation of antitumor drug
effect, stratification of cancer patients for molecularly targeted therapy, and prognosis of cancer patients, as it could provide real time data with fewer false-negative results.5

Affibody molecules are based on a 58 amino acid residue protein domain, derived from one of the IgG-binding domains of staphylococcal protein A, and has been engineered to be chemically stable and to bind target proteins with high affinity.6,7 Because of their small size (~7 kDa) and high affinity, affibody molecules generally show fast and good tumor tissue penetration and accumulation, and rapid clearance from the blood, resulting in high imaging contrast within a short period (for example, 0.5–1 h) after injection. Antihuman epidermal growth factor receptor 2 (HER2) affibody molecules (ZHER2) and their derivatives have been radiolabeled with various radionuclides for imaging of tumors overexpressing HER2 in animal models.7–10 Subsequently, 111In- or 68Ga-labeled ZHER2 have been successfully and safely used to visualize HER2-expressing tumors in patients with metastatic breast cancer. These clinical studies clearly demonstrate that affibody molecules have great potential to become a promising new class of cancer-targeting ligands for clinical translation.11 Overall the previous preclinical and clinical studies encourage us to further develop clinical translatable affibody probes to image other tumor targets such as EGFR.12,13

We have previously reported the site-specific coupling of an anti-EGFR affibody molecule (ZEGFR:1907) with maleimido-monoamide-DOTA (MMA-DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7-trisacetic acid-10-maleimidoethylmonoamide) to produce the bioconjugate, DOTA-ZEGFR:1907, that was radiolabeled with 64Cu.13 This conjugate allowed high-contrast imaging of EGFR-expressing xenografts. However, imaging of EGFR expression with affibody molecules and further clinical translation of them can be further improved by 18F-labeling. Not only are 18F probes more clinically relevant than 64Cu but also they have good imaging characteristics and a suitable half-life for relatively low molecular weight proteins and peptides. Therefore, we recently radiolabeled ZEGFR:1907 with N-2-(4-18F-fluorobenzamido)-ethyl maleimide (18F-FBEM) to produce the positron emission tomography (PET) probe, 18F-FBEM-ZEGFR:1907, for imaging EGFR expression in a variety of tumor models.12 Although 18F-FBEM-ZEGFR:1907 PET allowed us to visualize EGFR-expressing tumors, the labeling procedure to obtain the probe is complex and tedious, and requires a long radiosynthesis time (4-step radiosynthesis, 3 h, 10% decay corrected yield), which severely limits further applications of 18F-FBEM-ZEGFR:1907.

Recently, two new and simple methods for labeling of biomolecules with 18F have been developed. In the first one, peptides conjugated to MMA-NOTA (1,4,7-triazacyclononane-N,N,N′,N″-tetraacetic acid maleimidoethylmonoamide) and its analogues have been labeled with 18F via the formation of aluminum 18F-fluoride (Al18F) and its complexation by NOTA directly (one step radiosynthesis).14,15 The second method involves 18F-labeling of N-terminal cysteine-bearing peptides and proteins and is based on a rapid condensation reaction between 18F-fluorinated-2-cyanobenzothiazole (18F-CBT) and cysteine (2-step reaction).16 Both methods allow rapid and efficient labeling of peptides and proteins with 18F. Al18F-NOTA in particular has been applied to label many peptides including RGD and anti-HER2 affibody molecules.17,18 The Al18F-NOTA labeled RGD peptides have also been successfully used for PET imaging of a lung cancer patient recently.19

Our ultimate goal is to translate an 18F-labeled ZEGFR into clinical applications. Therefore, in the current study, we aimed to use the above radiofluorination strategies (Al18F-NOTA and 18F-CBT) to site-specifically label ZEGFR:1907 and further determine whether the resulting PET probes, Al18F-NOTA-ZEGFR:1907 and 18F-CBT-ZEGFR:1907, are suitable agents for imaging mice bearing EGFR expressing A431 tumors. For this purpose, NOTA-conjugated ZEGFR:1907 was prepared and radiolabeled with 18F to produce Al18F-NOTA-ZEGFR:1907, and the prosthetic group (18F-CBT) was conjugated to Cys-ZEGFR:1907 to produce 18F-CBT-ZEGFR:1907 (Figure 1). The in vitro properties and in vivo...
performance of $^{18}$F-NOTA-ZEGFR$_{1907}$ were then compared with those of $^{18}$F-CBT-ZEGFR$_{1907}$ in A431 cells and tumor xenografts.

**MATERIALS AND METHODS**

**General.** MMA-NOTA was purchased from CheMatech Inc. (Dijon, France). Phosphate-buffered saline (PBS), high-glucose Dulbecco’s modified eagle medium (DMEM), 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 0.1% trypsin, trypsin-EDTA, and TrypLE-Express were purchased from Invitrogen Life Technologies (Carlsbad, California). Dimethyl sulfoxide (DMSO) and acetonitrile (MeCN) were purchased from Fisher Scientific (Pittsburgh, Pennsylvania). Dimethylformamide (DMF), trifluoroacetic acid (TFA), thioanisole (TIS), ethanedithiol (EDT), ethylene-diamine-tetra-acetic acid (EDTA), tris(2-carboxyethyl)-phosphate hydrochloride (TCEP HCl), N,N-diisopropyl-ethylamine (DIPEA), ethyl acetate, dithiothreitol (DTT), mouse serum, and all other standard synthesis reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Missouri). All chemicals were used without further purification.

The affibody molecules Ac-Cys-ZEGFR$_{1907}$ (Ac-CVDKNFNKEMWAAWEIRNLPLNLGWMQMTAFIALSVDDPSQSANLLAEKKALDAPQK-NH$_2$) and Cys-ZEGFR$_{1907}$ (CVDKKNFKEMWAAWEIRNLPLNLGWMQMTAFIALSDLVDDPSQSANLLAEKKALDAPQK-NH$_2$) were synthesized on a CS Bio CS336 instrument (CS Bio Company, Menlo Park, California) in our laboratory as previously described.$^{13}$ The purified peptide was dissolved in water, and the concentration was determined by amino acid analysis (Molecular Structure Technologies, Park, California) in our laboratory as previously described.$^{13}$ The human epidermoid carcinoma cancer cell line A431 was obtained from the American Type Tissue Culture Collection (Manassas, Virginia). Female nude mice were purchased from Charles River Laboratories (Boston, Massachusetts).

**Radioisotopes of $^{18}$F-CBT**. $^{18}$F-NOTA-ZEGFR$_{1907}$ was radioiodinated with $^{18}$F according to a previously reported method.$^{14,15,18}$ (Figure 1A). First, Ac-Cys-ZEGFR$_{1907}$ was conjugated with the bifunctional chelator MMA-NOTA using the method described below: Ac-Cys-ZEGFR$_{1907}$ was dissolved in freshly degassed phosphate buffer (0.1 M, pH 7.4) at a concentration of 1 mg/mL. Twenty equivalents of MMA-NOTA dissolved in DMSO (10 mM) were added. After mixing by vortexing for 2 h, the product was purified by RP-HPLC with a protein-and-peptide C4 column (Grace Vydac 214TP54, Columbia, Maryland) using a gradient system of solvent A (0.1% TFA/H$_2$O) and solvent B (0.1%TFA/MeCN) at a flow rate of 3 mL/min. The flow rate was 4 mL/min, with the mobile phase starting from 90% solvent A and 10% solvent B (0–3 min) to 35% solvent A and 65% solvent B at 33 min. Fractions containing the product were collected and lyophilized.

The identity of the products was confirmed by MALDI-TOF-MS.

Second, nonradioactive $^{19}$F-NOTA-ZEGFR$_{1907}$ (90% purity) was dissolved in 20 μL of sodium acetate buffer (0.1 M, pH 4) and added to AlCl$_3$ (2 mM, 5 μL). Then, NOTA-ZEGFR$_{1907}$ (50 μg) dissolved in 50 μL of sodium acetate buffer (0.1 M, pH 4) was added, and the reaction mixture was incubated for 15 min at 100 °C. The resulting conjugate, $^{19}$F-NOTA-ZEGFR$_{1907}$ was purified by HPLC.

Lastly, $^{18}$F radioiodination of NOTA-ZEGFR$_{1907}$ was performed. $^{18}$F-fluoride (37 × 10$^3$ MBq) was prepared by proton bombardment of 2.5 mL of $[^3]$H$_2$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 (Waters, Milford, Massachusetts), washed with 3 mL of metal-free water, and eluted from the cartridge with 100 μL of 0.9% NaCl. $^{18}$F was prepared by adding AlCl$_3$ (2 mM, 2 μL) in sodium acetate buffer (0.1 M, pH 4). NOTA-ZEGFR$_{1907}$ (150 μg) was dissolved in 25 μL of sodium acetate buffer (0.5 M, pH 4). To the dissolved affibody molecule, acetonitrile (25 μL) and Al$^{18}$F (50 μL, 1.3–1.6 × 10$^3$ MBq) were added, then the reaction mixture was incubated for 15 min at 100 °C. An Oasis HLB cartridge (30 μg; Waters) was used to remove unincorporated $^{18}$F and the desired product was purified with HPLC using the same elution gradient described for NOTA-ZEGFR$_{1907}$ purification. The HPLC fractions containing $^{18}$F-NOTA-ZEGFR$_{1907}$ were collected, combined, and evaporated.

$^{18}$F-NOTA-ZEGFR$_{1907}$ was reconstituted in PBS (0.1 M, pH 7.4) and passed through a 0.22 μm Millipore filter into a sterile vial for in vitro and animal experiments.

**Radioisotopes of $^{18}$F-CBT**. Nonradioactive $^{18}$F-CBT-ZEGFR$_{1907}$ was used as a reference for characterization of $^{18}$F-CBT-ZEGFR$_{1907}$ and prepared by reaction of Cys-ZEGFR$_{1907}$ with $^{18}$F-CBT. Briefly, TCEP HCl solution (2.4 μL, 10 mM) and DIPEA (360 nmol) were added to Cys-ZEGFR$_{1907}$ solution (30 μL, 200 μM in DMF) and then the resulting solution was mixed with $^{18}$F-CBT solution (1.8 μL, 10 mM, 3 equiv). The resulting mixture was heated to 60°C for 1 h. The crude product was purified with semipreparative HPLC using Phenomenex Gemini column (10 mm × 250 mm, 5 μm) using a linear gradient from deionized water with 0.1% TFA to MeCN with 0.1% TFA: 0–3 min 0–40% (MeCN); 3–35 min 40–100% (MeCN); and the flow rate was 3 mL/min.

Cys-ZEGFR$_{1907}$ was labeled with $^{18}$F-CBT according to the procedure we recently described.$^{16}$ (Figure 1B). First, $^{18}$F labeling of tosylated CBT was performed. 18-Crown-6/K$_2$CO$_3$ solution (1 mL, 15:1 MeCN/H$_2$O, 16.9 mg of 18-Crown-6 and 4.4 mg of K$_2$CO$_3$) was used to elute the activity of $^{18}$F-fluoride from QMA cartridge into a dried glass reactor. The resulting solution was azeotropically dried with sequential MeCN evaporation at 90°C. A solution of [2-((2-cyanobenzothiazol-6-yl)-oxy)ethyl-4-methylbenzenesulfonate] $^2$H$_2$O (2 mg in 1 mL of anhydrous MeCN) was added to the reactor, and the mixture heated at 90°C for 10 min. After cooling to 30°C, HCl (0.05 M, 2.5 mL) was added to quench the reaction and prevent basic hydrolysis of the product $^{18}$F-CBT. The crude mixture was then purified with a semipreparative HPLC using the same elution gradient described for $^{18}$F-CBT purification. The collected $^{18}$F-CBT solution was diluted with H$_2$O (20 mL) and passed through a C18 cartridge. The trapped $^{18}$F-CBT was eluted out from the cartridge with Et$_2$O (2.5 mL), and the Et$_2$O was removed by a helium stream. The isolated radiochemical yield of $^{18}$F-CBT was ca. 20% (5.18–5.55 × 10$^3$ MBq, decay-corrected to end of bombardment). For the radiosynthesis of $^{18}$F-CBT-ZEGFR$_{1907}$, Cys-ZEGFR$_{1907}$ (150 μg, 7.5 nmol) was dissolved in PBS buffer (0.1 M, pH 7.4) containing 5 equiv of TCEP HCl and 50 equiv of NaHCO$_3$. The resulting solution was added to $^{18}$F-CBT (1.85 × 10$^5$ MBq) in DMF (200 μL) at 60°C. After 20 min, the reaction was quenched with 5% AcOH aqueous solution. The crude product was purified with a semipreparative HPLC using Phenomenex Gemini column.
(10 mm × 250 mm, 5 μm) using a linear gradient from deionized water with 0.1% TFA to MeCN with 0.1% TFA: 0–5 min 0–5% (MeCN); 5–42 min 5–65% (MeCN); and the flow rate was 5 mL/min.

**Cell Assays.** Cell uptake and receptor saturation assays were performed as previously described with minor modifications. Briefly, the EGFR positive A431 cell line was cultured in high glucose DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. The cells were maintained in a humidified atmosphere of 5% CO₂ at 37 °C, with the medium changed every 2 days. A 70–80% confluent monolayer was detached by 0.1% trypsin and dissociated into a single cell suspension for further cell culture.

**Cell Uptake Assays.** The A431 cells were washed three times with PBS and dissociated with 0.25% trypsin–EDTA. DMEM medium was then added to neutralize trypsin–EDTA. Cells were spun down and resuspended with serum-free DMEM. Cells (0.5 × 10⁶) were incubated at 37 °C for 0.25 to 2 h with 7.4 × 10⁻³ MBq Al¹⁸F-NOTA-ZEGFR:1907 or ¹⁸F-CBT-ZEGFR:1907 in 0.5 mL of serum-free DMEM medium. The nonspecific binding of Al¹⁸F-NOTA-ZEGFR:1907 or ¹⁸F-CBT-ZEGFR:1907 with A431 cells was determined by coincubation with 0.6 μM nonradioabeled NOTA-ZEGFR:1907 or Cys-ZEGFR:1907. The cells were washed three times with 0.01 M PBS (pH 7.4) at room temperature. Cell were then washed three times with chilled PBS and spun down at a speed of 7000–8000 rpm. The cell pellets at the bottom of the tube were spliced, and the radioactivity of the pellets was measured using a γ-counter (PerkinElmer 1470, Waltham, Massachusetts). The uptake (counts/min) was normalized to the percentage of binding for analysis using Excel (Microsoft Software Inc., Redmond, Washington).

**Receptor Saturation Assays.** A431 cells (0.3 × 10⁶) were plated on 6-well plates 1 day before the experiment. Cells were washed with PBS three times. Serum-free DMEM (1 mL) was added to each well, followed by the addition of either Al¹⁸F-NOTA-ZEGFR:1907 (8.9–532.8 × 10⁻³ MBq, 2–120 nM final concentration) or ¹⁸F-CBT-ZEGFR:1907 (8.9–532.8 × 10⁻³ MBq, 2–120 nM final concentration). The nonspecific binding of Al¹⁸F-NOTA-ZEGFR:1907 or ¹⁸F-CBT-ZEGFR:1907 with A431 cells was determined by coincubation with 100 times excess of nonradioabeled Ac-Cys-ZEGFR:1907 or Cys-ZEGFR:1907, respectively, through a tail vein. At 3 h after injection, the mice were sacrificed, and tumors and normal tissues of interest were removed and weighed, and their radioactivity was measured in a γ-counter. The radioactivity uptake in the normal tissues was expressed as a percentage of the injected radioactivity per gram of tissue (%ID/g). In order to study the in vivo EGFR targeting specificity of Al¹⁸F-NOTA-ZEGFR:1907 and ¹⁸F-CBT-ZEGFR:1907, unlabeled Ac-Cys-ZEGFR:1907 or Cys-ZEGFR:1907 protein (300 μg) was coinjected with the corresponding ¹⁸F-labeled ZEGFR:1907 in nude mice bearing A431 tumors (n = 4) via a tail vein, and biodistribution studies were conducted at 3 h after injection.

**Small-Animal PET Imaging.** PET imaging of tumor-bearing mice was performed on a microPET R4 rodent model scanner (Siemens Medical Solutions USA, Inc., Malvern, Pennsylvania). The mice bearing A431 tumors (for each group n = 4) were injected with Al¹⁸F-NOTA-ZEGFR:1907 (1.9–2.6 MBq) or ¹⁸F-CBT-ZEGFR:1907 (1.48–2.22 MBq) with 30 μg of nonradioactive Ac-Cys-ZEGFR:1907 or Cys-ZEGFR:1907, respectively, through a tail vein. At 3 h after injection, the mice were anesthetized with 2% isoflurane and placed near the center of the field of view of the microPET scanner in prone position. Three-minute static scans were obtained, and the images were reconstructed by a two-dimensional ordered subsets expectation maximization (OSEM) algorithm. No background correction was performed. Regions of interest (ROIs; 5 pixels for coronal and transaxial slices) were drawn over the tumors on decay-corrected whole-body coronal images. The maximum counts per pixel per minute were obtained from the ROIs and converted to counts per milliliter per minute using a calibration constant. Tissue density was assumed to be 1 g/mL, and the ROIs were converted to counts per gram per minute. Image ROI-derived %ID/g values were determined by dividing counts per gram per minute by the injected dose. No attenuation correction was performed.

In Vivo Stability Assay. Two groups of A431 mice (for each group n = 3) were injected with Al¹⁸F-NOTA-ZEGFR:1907 (5.8 MBq) or ¹⁸F-CBT-ZEGFR:1907 (7.4 MBq) via a tail vein and euthanized at 1 h after injection. The tumors were removed and homogenized with DMF (0.5 mL) with 1% Triton X-100 (Sigma-Aldrich). Blood samples were centrifuged immediately after collection to remove the blood cells. The plasma portions were added to DMF (0.5 mL) with 1% Triton X-100. After centrifugation, the supernatant portions were diluted with solution A (99.9% H₂O with 0.1% TFA) and centrifuged again at 16,000 g for 2 min with a nylon filter. The filtrates were analyzed by radio-HPLC under conditions identical to those used for analyzing the original radiolabeled peptide.

**Biodistribution Studies.** The animal procedures were performed according to a protocol approved by the Stanford University Institutional Animal Care and Use Committee. Approximately 5 × 10⁶ cultured A431 cells suspended in PBS were implanted subcutaneously in the right upper or lower shoulders of nude mice. Tumors were allowed to grow to around 0.5–1.0 cm in diameter (10–15 days) and then the tumor-bearing mice underwent in vivo biodistribution and imaging studies.

For biodistribution studies, A431 tumor-bearing mice (for each group n = 4) were injected with ¹⁸F-NOTA-ZEGFR:1907 (1.9–2.6 MBq) or ¹⁸F-CBT-ZEGFR:1907 (1.48–2.22 MBq) with 30 μg of nonradioactive Ac-Cys-ZEGFR:1907 or Cys-ZEGFR:1907, respectively, via a tail vein. At 3 h after injection, the mice were sacrificed, and tumors and normal tissues of interest were removed and weighed, and their radioactivity was measured in a γ-counter. The radioactivity uptake in the tumor and normal tissues was expressed as a percentage of the injected radioactivity per gram of tissue (%ID/g). In order to study the in vivo EGFR targeting specificity of Al¹⁸F-NOTA-ZEGFR:1907 and ¹⁸F-CBT-ZEGFR:1907, unlabeled Ac-Cys-ZEGFR:1907 or Cys-ZEGFR:1907 protein (300 μg) was coinjected with the corresponding ¹⁸F-labeled ZEGFR:1907 in nude mice bearing A431 tumors (n = 4) via a tail vein, and biodistribution studies were conducted at 3 h after injection.
Statistical Methods. Statistical analysis was performed using Student’s two-tailed t-test for unpaired data. A 95% confidence level was chosen to determine the significance between groups, with a P value less than 0.05 being indicated as a significant difference.

RESULTS

Chemistry and Radiochemistry. The affibody molecules Ac-Cys-ZEGFR:1907 and Cys-ZEGFR:1907 with a cysteine at the N-terminal were successfully synthesized using conventional solid phase peptide synthesis and purified by semipreparative HPLC. The peptides were generally obtained in 10% yield. The retention time for both on analytical HPLC was 26 min. The purified Ac-Cys-ZEGFR:1907 and Cys-ZEGFR:1907 were characterized by MALDI-TOF-MS. The measured molecular weights (MWs) for both constructs were consistent with the expected MWs (for Ac-Cys-ZEGFR:1907, calculated MW = 6690.0 and found MW = 6690.7; for Cys-ZEGFR:1907, calculated MW = 6646.0 and found MW = 6645.7). Ac-Cys-ZEGFR:1907 was then conjugated with MMA-NOTA and purified by HPLC. The measured MW of the final product (NOTA-ZEGFR:1907) was m/z = 7112.0 for [M + H]+ (calculated MW[M+H]+ = 7112.6), and the purity of NOTA-ZEGFR:1907 was over 95% (retention time = 29 min). Lastly, purified 19F-NOTA-ZEGFR:1907 and 19F-CBT-ZEGFR:1907 were also characterized by MALDI-TOF-MS. The measured MWs for both constructs were consistent with the expected MWs (for 19F-NOTA-ZEGFR:1907, calculated MW = 7256.0 and measured MW = 7256.6; for 19F-CBT-ZEGFR:1907, calculated MW = 6852.0 and measured MW = 6852.9). The recovery yields of 19F-NOTA-ZEGFR:1907 and 19F-CBT-ZEGFR:1907 were 70% and 85%, respectively, after purification (retention time, 29 and 26.4 min).

The whole radiosynthesis of 18F-NOTA-ZEGFR:1907 was accomplished within 40 min. For 18F-CBT-ZEGFR:1907, the total radiosynthesis was completed within 120 min. 18F-NOTA-ZEGFR:1907 and 18F-CBT-ZEGFR:1907 showed a retention time of 29 and 26.4 min on HPLC, respectively. Both products were found to be more than 95% radiochemically pure, as determined by analytic HPLC. The overall radiochemical yields with decay correction at the end of synthesis for 18F-NOTA-ZEGFR:1907 and 18F-CBT-ZEGFR:1907 were 15% and 41%, respectively. The specific activity of 18F-NOTA-ZEGFR:1907 and 18F-CBT-ZEGFR:1907 were approximately 1.5 × 10⁷ and 22.2 × 10⁵ MBq/μmol, respectively.

In Vitro Stability and Metabolite Analysis. In vitro stability studies allowed us to observe that more than 90% of 18F-NOTA-ZEGFR:1907 remained intact during 1 to 2 h of incubation in mouse serum (Figure 2A,B). More than 90% of 18F-CBT-ZEGFR:1907 remained intact after 1 h incubation in mouse serum, while there was about 75% intact 18F-CBT-ZEGFR:1907 after 2 h of incubation (Figure 2C,D). Next, the in vivo stability studies are shown in Figure 3. In plasma and tumor, 90% and 85%, respectively, of 18F-NOTA-ZEGFR:1907 remained intact (Figure 3A,B) at 1 h after injection, indicating excellent stability in vivo. However, 18F-CBT-ZEGFR:1907 showed much faster degradation in vivo, with only 40% and 24% of intact tracer product in plasma and tumor, respectively (Figure 3C,D).

In Vitro Cell Binding Assays. Cell uptake levels for 18F-NOTA-ZEGFR:1907 and 18F-CBT-ZEGFR:1907 are shown in Figure 4A,C, respectively. 18F-NOTA-ZEGFR:1907 quickly accumulated in A431 cells and reached a highest value of 12% of applied activity at 1 h. A similar cell uptake pattern was observed for 18F-CBT-ZEGFR:1907, but the uptake level was much lower than that observed for 18F-NOTA-ZEGFR:1907 at 1 h (7% of applied activity). When both probes were incubated with large excesses of nonradioactive affibody molecules (Ac-Cys-ZEGFR:1907 or Cys-ZEGFR:1907), their uptake levels in A431 cells were significantly inhibited (P < 0.05) at all incubation time points (Figure 4A,C).

The binding affinity of 18F-NOTA-ZEGFR:1907 and 18F-CBT-ZEGFR:1907 to EGFR was determined through the receptor saturation assay. As shown in Figure 4B,D, the mean ± SD of Kᵦ values of 18F-NOTA-ZEGFR:1907 and 18F-CBT-ZEGFR:1907 were 12.72 ± 1.25 and 25.82 ± 3.62 nM, respectively. Al18F-NOTA-ZEGFR:1907 showed a lower Kᵦ value compared to 18F-CBT-ZEGFR:1907. Overall, these results strongly suggested that PET probes Al18F-NOTA-ZEGFR:1907 and 18F-CBT-ZEGFR:1907 had high...
EGFR-binding specificity and affinity, which warranted their further evaluation in vivo.

**In Vivo Biodistribution Studies.** At 3 h after injection, the biodistribution profiles of $^{18}$F-NOTA-Z$_{EGFR,1907}$ and $^{18}$F-CBT-Z$_{EGFR,1907}$ are presented in Table 1. Both $^{18}$F-labeled affibody molecules displayed relatively high levels of radioactivity accumulation in A431 tumors (4.77 ± 0.36 and 4.08 ± 0.54 %ID/g for $^{18}$F-NOTA-Z$_{EGFR,1907}$ and $^{18}$F-CBT-Z$_{EGFR,1907}$, respectively). The value of tumor uptake for $^{18}$F-NOTA-Z$_{EGFR,1907}$ was higher than that of $^{18}$F-CBT-Z$_{EGFR,1907}$. $^{18}$F-NOTA-Z$_{EGFR,1907}$ also exhibited significantly higher kidney and liver uptake than $^{18}$F-CBT-Z$_{EGFR,1907}$ (112.26 ± 12.57, 13.31 ± 0.80 and 8.12 ± 1.0, 3.08 ± 0.15 %ID/g, respectively, $P < 0.05$). Conversely, bone uptake of $^{18}$F-NOTA-Z$_{EGFR,1907}$ was significantly lower than that of $^{18}$F-CBT-Z$_{EGFR,1907}$ (1.75 ± 0.35 and 12.99 ± 2.37 %ID/g, respectively, $P < 0.05$). Interestingly, most other organ uptakes of $^{18}$F-CBT-Z$_{EGFR,1907}$, such as blood, heart, lungs, spleen, pancreas, stomach, brain, intestine, skin, and muscle, were higher.
Table 1. Biodistribution Results for $^{18}$F-NOTA-ZEGFR:1907 and $^{18}$F-CBT-ZEGFR:1907 in A431 Xenografts

| organ (%ID/g)          | $^{18}$F-NOTA-ZEGFR:1907 (3 h) | $^{18}$F-CBT-ZEGFR:1907 (3 h) |
|------------------------|--------------------------------|-------------------------------|
|                        | 30 µg spike                  | 300 µg (blocking)             | 30 µg spike                  | 300 µg (blocking)             |
| blood                  | 2.36 ± 0.53 $^b$             | 1.18 ± 0.40 $^c$             | 2.90 ± 0.60 $^b$             | 1.83 ± 0.24 $^c$             |
| heart                  | 1.88 ± 0.29 $^b$             | 1.14 ± 0.27 $^b$             | 2.76 ± 0.21 $^b$             | 1.81 ± 0.26 $^c$             |
| lungs                  | 1.27 ± 0.48 $^b$             | 0.56 ± 0.25 $^b$             | 1.43 ± 0.41 $b$              | 0.65 ± 0.19 $b$              |
| liver                  | 13.31 ± 0.80 $^{a,d}$        | 3.34 ± 0.37 $^{b,c}$         | 3.08 ± 0.15 $^{b,c,d}$       | 2.01 ± 0.45 $^{b,c,d}$       |
| spleen                 | 1.65 ± 0.51 $^b$             | 0.89 ± 0.24 $^b$             | 2.52 ± 0.33 $^b$             | 1.54 ± 0.28 $^{a,b}$         |
| pancreas               | 1.54 ± 0.20 $^b$             | 0.83 ± 0.43 $^b$             | 2.45 ± 0.38 $^b$             | 1.46 ± 0.15 $^b$             |
| stomach                | 1.65 ± 0.12 $^b$             | 0.77 ± 0.12 $^b$             | 2.10 ± 0.37 $^b$             | 1.36 ± 0.34 $^b$             |
| brain                  | 0.31 ± 0.05 $^{b,d}$         | 0.16 ± 0.03 $^{b,c}$         | 2.14 ± 0.37 $^{b,d}$         | 1.06 ± 0.47 $^{b,d}$         |
| intestine              | 1.40 ± 0.38 $^b$             | 0.70 ± 0.18 $^b$             | 2.78 ± 0.71 $^b$             | 1.32 ± 0.09 $^b$             |
| kidneys                | 112.27 ± 12.57 $^{a,d}$      | 104.00 ± 15.58 $^{a}$        | 8.12 ± 1.00 $^{a,d}$         | 4.26 ± 0.96 $^{a,d}$         |
| skin                   | 1.54 ± 0.32 $^b$             | 0.73 ± 0.25 $^b$             | 1.83 ± 0.18 $^b$             | 1.05 ± 0.11 $^b$             |
| muscle                 | 1.84 ± 0.28 $^b$             | 0.75 ± 0.26 $^b$             | 2.02 ± 0.31 $^b$             | 1.10 ± 0.30 $^b$             |
| bone                   | 1.75 ± 0.35 $^{a,d}$         | 1.27 ± 0.27 $^{a,d}$         | 12.99 ± 2.37 $^{a,d}$        | 5.45 ± 0.90 $^{a,d}$         |
| tumor                  | 4.77 ± 0.36 $^b$             | 1.78 ± 0.30 $^b$             | 4.08 ± 0.54 $^b$             | 2.34 ± 0.21 $^b$             |
| uptake ratio tumor to blood | 2.08 ± 0.34 $^b$            | 1.52 ± 0.27 $^b$             | 1.44 ± 0.29 $^b$             | 1.31 ± 0.29 $^b$             |
| tumor to lung          | 4.17 ± 0.20 $^b$             | 3.77 ± 0.62 $^b$             | 3.0 ± 0.54 $^b$              | 3.85 ± 0.50 $^b$             |
| tumor to muscle        | 2.62 ± 0.33 $^b$             | 2.74 ± 0.78 $^b$             | 2.06 ± 0.41 $^b$             | 2.22 ± 0.47 $^b$             |
| tumor to liver         | 0.36 ± 0.02 $^{b,d}$         | 0.53 ± 0.06 $^{b,c}$         | 1.33 ± 0.20 $^{b,c}$         | 1.22 ± 0.32 $^{b,c}$         |
| tumor to kidney        | 0.04 ± 0.005 $^{b,d}$        | 0.02 ± 0.004 $^{b,c}$        | 0.50 ± 0.05 $^{b,d}$         | 0.56 ± 0.01 $^{b,d}$         |
| tumor to bone          | 2.81 ± 0.62 $^{b,d}$         | 1.47 ± 0.46 $^{b,d}$         | 0.32 ± 0.07 $^{b,d}$         | 0.44 ± 0.08 $^{b,d}$         |

“Data are mean ± SD, expressed as percentage administered activity (injected probe) per gram of tissue (%ID/g) after intravenous injection of probe (Al$^{18}$F-NOTA-ZEGFR:1907 or $^{18}$F-CBT-ZEGFR:1907) spiked with 30 and 300 µg of Ac-Cys-ZEGFR:1907 or ZEGFR:1907 at 3 h after injection. Significant inhibition of Al$^{18}$F-NOTA-ZEGFR:1907 or $^{18}$F-CBT-ZEGFR:1907 uptake was observed in A431 tumor of the blocked group (300 µg) ($P < 0.05$). Student’s unpaired two-tailed t-test was conducted. P < 0.05 was considered significant (for each group, n = 4).$P < 0.05$, comparing 30 µg spike and 300 µg (blocking) of dose tracer biodistribution at 3 h after injection with Al$^{18}$F-NOTA-ZEGFR:1907. $P < 0.05$, comparing 30 µg spike and 300 µg (blocking) of dose tracer biodistribution at 3 h after injection with $^{18}$F-CBT-ZEGFR:1907. $P < 0.05$, comparing 30 µg spike tracer biodistribution of Al$^{18}$F-NOTA-ZEGFR:1907 and $^{18}$F-CBT-ZEGFR:1907 at 3 h after injection. The uptake values (%ID/g) from PET image data at 3 h after injection were consistent with the findings in the biodistribution studies. Moreover, when the probes were coinjected with 300 µg unlabeled Ac-Cys-ZEGFR:1907 or ZEGFR:1907, the tumor was barely visible on PET images at 1–3 h after injection both for Al$^{18}$F-NOTA-ZEGFR:1907 and $^{18}$F-CBT-ZEGFR:1907 (Figure 5). A quantitative analysis of the PET images showed significantly ($P < 0.05$) lower tumor uptake for mice injected with 300 µg blocking dose when compared to a 30 µg spiking dose at all time points for both probes (Figure 6).”

## DISCUSSION

EGFR-targeted PET imaging is a promising tool to provide a real-time assay of EGFR expression in all tumor sites (primary and metastatic lesions) in living subjects. EGFR-targeted PET probes could not only be used for early detection of EGFR positive tumor recurrence and stratification of cancer patients but also for dose optimization of EGFR targeted therapy and monitoring the efficacy of EGFR-based tumor treatment. Preclinical literature data suggests that radiolabeled affibody molecules have superior imaging properties and higher sensitivity to detect EGFR in comparison with monoclonal antibodies and their fragments due to their small size (7 KDa), as well as excellent tumor targeting and retention, and rapid blood clearance. Furthermore, $^{18}$F, the most commonly used PET radionuclide, is widely available and presents almost ideal imaging properties, making this radionuclide highly clinically relevant. Combining the aforementioned optimal clinical characteristics to develop an imaging agent is significantly
important since our goal is to ultimately apply affibody-based PET probes for imaging patients. We have previously developed an 18F labeled affibody molecule ZEGFR:1907 (18F-FBEM-ZEGFR:1907).12 However, the 18F labeling strategies used in that work involved lengthy (3 h) and tedious multistep radiosynthetic procedures. Moreover, it is very challenging to adapt these radiosynthetic processes into a fully automated radiosynthetic platform, which creates a considerable technical barrier for using these affibody-based PET probes in the clinical setting. Very recently, we have successfully used two methods for RGD peptide radiofluorination (18F-AlF-NOTA and 18F-CBT) in no more than two radiosynthetic steps within 40 and 120 min, respectively.16,18 These recently described strategies provide straightforward, quicker, and powerful 18F labeling methods to radiofluorinate biomolecules for in vivo molecular imaging applications.

In the present study, only a 1-pot reaction was involved in the procedures to obtain Al18F-NOTA-ZEGFR:1907 in a total preparation time of 40 min, with a decay-corrected yield at the end of synthesis of 15%. For 18F-CBT-ZEGFR:1907, 2-pot reactions and 2 h were required in the radiosynthesis with a yield of 41%. Compared with the radiosynthesis of 18F-FBEM-ZEGFR:1907, Al18F-NOTA-ZEGFR:1907 and 18F-CBT-ZEGFR:1907 were superior to 18F-FBEM-ZEGFR:1907 (3 h preparation time and 10% radiochemical yield). However, both radiochemical yields were less than those previously described for the 18F-labeled peptides Al18F-NOTA-RGD2 (17.9%)18 and 18F-CBT-RGD2 (80%).16 It is likely that the lower radiochemical yields observed were due to the fact that the concentration of the affibody molecules (Ac-Cys-ZEGFR:1907 or Cys-ZEGFR:1907, respectively) was much lower than that of the peptides RGD2 (36.6 or 3.55 mmol/L). Further optimization of our current labeling procedure is under investigation and may result in a higher radio-labeling yield of Al18F-NOTA-ZEGFR:1907 and 18F-CBT-ZEGFR:1907 by modifying synthetic environment (pH or temperature). Nonetheless, our study demonstrates that both Al18F-NOTA

Figure 5. Decay-corrected coronal small-animal PET images of nude mice bearing A431 tumors at 1, 2, and 3 h after tail vein injection of Al18F-NOTA-ZEGFR:1907 (A) and 18F-CBT-ZEGFR:1907 (B) spiked with 30 μg (spike) and 300 μg (blocking) of cold affibody (Ac-Cys-ZEGFR:1907 or Cys-ZEGFR:1907, respectively). Arrows indicate the location of tumors (for each group, n = 4).

Figure 6. PET quantification analysis for uptakes of tumor, liver, kidney, bone, muscle, and lungs for Al18F-NOTA-ZEGFR:1907 (A) and 18F-CBT-ZEGFR:1907 (B) in A431 xenograft mice models after coinjection of with 30 μg (spike) or 300 μg (blocking) of cold affibody (Ac-Cys-ZEGFR:1907 or Cys-ZEGFR:1907, respectively) at 1, 2, and 3 h after injection. ROI was drawn on coronal images. Uptake was calculated with the mean uptake value (for each group, n = 4).

Figure 7. Tumor and muscle time–activity curves derived from multiple-time-point small-animal PET images in A431 xenograft mice models after coinjection of Al18F-NOTA-ZEGFR:1907 or 18F-CBT-ZEGFR:1907 with 30 μg (spike) of cold affibody (Ac-Cys-ZEGFR:1907 or Cys-ZEGFR:1907, respectively) at 1, 2, and 3 h after injection. Data are shown as mean ± SD %ID/g (n = 4).
and \(^{18}\text{F}\)-CBT can be used for \(^{18}\text{F}\) labeling of small proteins and have high potential for generating PET probes for different applications. Moreover, the stability studies reveal that \(^{18}\text{F}^{-}\text{NOTA-ZEGFR:1907}\) is highly stable in vitro and in vivo, whereas the in vivo stability of \(^{18}\text{F}\)-CBT-ZEGFR:1907 is not ideal and requires further improvement.

The biologic properties of \(^{18}\text{F}^{-}\text{NOTA-ZEGFR:1907}\) and \(^{18}\text{F}\)-CBT-ZEGFR:1907 were evaluated by in vitro cell assays, biodistribution studies, and small-animal PET imaging studies. Both \(^{18}\text{F}^{-}\text{NOTA-ZEGFR:1907}\) and \(^{18}\text{F}\)-CBT-ZEGFR:1907 showed significantly high uptake in A431 cells, demonstrating their EGFR-binding specificity in vitro. \(^{18}\text{F}\)-CBT-ZEGFR:1907 showed lower cell uptake than \(^{18}\text{F}^{-}\text{NOTA-ZEGFR:1907}\). This result was likely caused by the higher EGFR-binding affinity of \(^{18}\text{F}^{-}\text{NOTA-ZEGFR:1907}\) (25.82 ± 3.62 vs 12.72 ± 1.25 nM). Compared with \(^{18}\text{F}\)-FBEM-ZEGFR:1907, \(^{18}\text{F}^{-}\text{NOTA-ZEGFR:1907}\) and \(^{18}\text{F}\)-CBT-ZEGFR:1907 both showed high binding affinity with a KD of 12.72 ± 1.25 and 25.82 ± 3.62 nM, respectively.

It is known that the high natural expression of EGFR in the liver creates a biological barrier to radiophores targeting the EGFR positive tumors by reducing tumor uptake.\(^{29}\) Saturating the EGFR in the liver can increase tumor uptake of EGFR targeted probes. Our previous study demonstrated that improved imaging contrasts of EGFR positive tumor can be achieved with optimized spiking doses (5–50 μg) along with the injection of \(^{64}\text{Cu-DOTA-ZEGFR:1907}\).\(^{13}\) Therefore, for both \(^{18}\text{F}^{-}\text{NOTA-ZEGFR:1907}\) and \(^{18}\text{F}\)-CBT-ZEGFR:1907, spiking doses of cold ZEGFR were used directly for in vivo evaluation. After evaluating two probes in mice, it was found that \(^{18}\text{F}^{-}\text{NOTA-ZEGFR:1907}\) shows some advantages over \(^{18}\text{F}\)-CBT-ZEGFR:1907 as a promising agent for EGFR imaging. \(^{18}\text{F}^{-}\text{NOTA-ZEGFR:1907}\) rapidly localizes in A431 tumors and shows good tumor uptake, retention, and tumor-to-muscle ratios, allowing clear visualization of A431 tumors by PET at even 1 h postinjection. The highest uptake observed in the kidneys and the liver is mainly attributed to the fact that they are the major organs responsible for metabolism and clearance. High kidney uptake could be associated with radiolabeled affibody molecules being reabsorbed by the organ. Additionally, liver uptake might be increased due to the fact that this organ also highly expresses EGFR.\(^{12}\) The in vivo EGFR-binding specificity of \(^{18}\text{F}^{-}\text{NOTA-ZEGFR:1907}\) was also confirmed by the reduced A431 tumor and liver uptake observed after coinjection with 300 μg of Ac-Cys-ZEGFR:1907. Moreover, low radioactivity levels were found in the lung, intestine, spleen, and stomach. The low uptake of \(^{18}\text{F}^{-}\text{NOTA-ZEGFR:1907}\) in these normal organs makes the PET probe a potential agent to detect primary or metastatic tumors expressing EGFR in the abdomen and lung region. Only low activity was observed in the brain, suggesting that \(^{18}\text{F}^{-}\text{NOTA-ZEGFR:1907}\) cannot penetrate through the blood–brain barrier. Interestingly, the kidney uptake of \(^{18}\text{F}^{-}\text{NOTA-ZEGFR:1907}\) was not reduced by the unlabeled affibody at all the time points, whereas \(^{18}\text{F}\)-CBT-ZEGFR:1907 was moderately blocked at late time point (3 h) but not at early time points (1 and 2 h). These data suggest the kidney uptake of two probes is not likely receptor mediated. The blocking effect for \(^{18}\text{F}\)-CBT-ZEGFR:1907 at 3 h p.i. may be somewhat linked to the in vivo instability of \(^{18}\text{F}\)-CBT-ZEGFR:1907. Further studies are required to reveal the observations. Overall, these results indicate that \(^{18}\text{F}^{-}\text{NOTA-ZEGFR:1907}\) is characterized by a relatively easy preparation, favorable pharmacokinetic properties, and high specificity for EGFR, which render it a useful agent for in vivo imaging of EGFR positive tumors and related applications.

In contrast, the performance of \(^{18}\text{F}\)-CBT-ZEGFR:1907 in vivo was not ideal: relatively high uptake in most normal tissues (such as brain, pancreas, spleen, intestine, blood, muscle, lung, and spleen), especially the remarkably high uptake in bone (130.0 ± 2.37 %ID/g), suggests in vivo release of \(^{18}\text{F}\)-fluoride (Table 1). In fact, bone uptake of \(^{18}\text{F}\)-CBT-ZEGFR:1907 was about 3-fold higher than that observed for \(^{18}\text{F}^{-}\text{NOTA-ZEGFR:1907}\). Also, the in vitro stability and metabolite analysis studies, where only about 75%, 40%, and 24% of \(^{18}\text{F}\)-CBT-ZEGFR:1907 was intact after 2 h of serum incubation or in plasma and tumor in vivo at 1 h after injection, suggest that \(^{18}\text{F}\)-CBT-ZEGFR:1907 is not stable in vivo. The kidney and the liver showed the lowest uptake (8.12 ± 1.0 and 3.08 ± 0.15 %ID/g) at 3 h after injection. These results are in agreement with our previous data\(^{17}\) indicating that polar metabolites clear more rapidly from blood.

NOTA has already been coupled to affibody molecules (\(Z_{\text{HER2:2395}}\) and \(Z_{\text{HER2:518}}\)) and the conjugates radiolabeled with \(^{111}\text{In}, ^{68}\text{Ga}, \text{and} ^{18}\text{F}\) for HER2 imaging.\(^{17,21}\) Our data are generally consistent with the findings reported in these published studies. For example, it was reported that \(^{18}\text{F}^{-}\text{NOTA-ZHER2:2395}\) displayed uptake of 4.4 ± 0.8 and 4.9 ± 0.7 %ID/g in SKOV3 tumors at 1 and 4 h after injection, respectively, whereas the corresponding levels in the kidneys were high (about 140 and 150 %ID/g, from Figure 3) and in the bone low (1 %ID/g, from Figure 3). In the present study, the uptake of \(^{18}\text{F}^{-}\text{NOTA-ZEGFR:1907}\) in A431 tumors at 3 h after injection were 4.77 ± 0.34 %ID/g, and the corresponding levels in kidney and bone were 112.27 ± 12.57 and 1.75 ± 0.35 %ID/g, respectively. Overall, \(^{18}\text{F}^{-}\text{NOTA}\) radiolabeled affibody molecules rapidly accumulated in tumors, with high uptake and good tumor-to-normal tissue ratios and low uptake in the bone indicating stable complexation in the form of \(^{18}\text{F}\) by the NOTA chelator. However, they typically showed high uptake in the kidneys as well, probably because radiolabeled affibody molecules were excreted and reabsorbed by the kidneys. In order to minimize the reabsorption of affibody molecules by the kidneys, the pharmacokinetics could be further improved with strategies such as the use of positively charged amino acids, gelofusin, or albumin fragments.\(^{22–24}\) Moreover, compared with \(^{18}\text{F}\)-FBEM-ZEGFR:1907,\(^{12}\) \(^{18}\text{F}^{-}\text{NOTA-ZEGFR:1907}\) had a lower tumor uptake (4.77 ± 0.36 vs 8.06 ± 1.44 %ID/g) at 3 h after injection, probably due to the effect of different \(^{18}\text{F}\)-radiolabeling group. In our previous study,\(^{16}\) we had also successfully developed \(^{18}\text{F}\)-CBT-RGD\(_2\) and \(^{18}\text{F}\)-CBT-RLuc. Both probes demonstrated high levels of tumor accumulation and favorable pharmacokinetic properties. However, in the present study, it has been found that \(^{18}\text{F}\)-CBT-ZEGFR:1907 was degraded in vivo. Therefore, great efforts will be focused on introducing appropriate molecular modifications, such as the use of more stable D-amino acids for L-amino acids, the use of pseudopeptide bonds,\(^{25}\) modifying synthetic environment, etc.

**CONCLUSIONS**

Two strategies for \(^{18}\text{F}\)-labeling affibody molecules have been successfully developed with either NOTA or CBT coupling to affibody molecules that contain an N-terminal cysteine as two model platforms. These two methods can potentially be translated to other applications. High activities of the probes can be reliably obtained in a relatively short radiosynthesis time. Biodistribution and small-animal PET imaging studies demonstrate that \(^{18}\text{F}^{-}\text{NOTA-ZEGFR:1907}\) is a promising PET probe for imaging EGFR expression in living mice. In contrast, \(^{18}\text{F}\)-CBT-ZEGFR:1907 may be easily degraded in vivo compared to \(^{18}\text{F}^{-}\text{NOTA-ZEGFR:1907}\). Further research is needed to improve the
stability of $^{18}$F-CBT-ZEGFR:1907 in vivo and determine whether this probe can be used for patient EGFR PET imaging.

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Notes
The authors declare no competing financial interest.

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