Improved ^{18}F Labeling of Peptides with a Fluoride-Aluminum-Chelate Complex

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We reported previously the feasibility to radiolabel peptides with fluorine-18 (^{18}F) using a rapid one-pot method that first mixes $^{18}F^-$ with Al^{3+} and then binds the $(Al^{18}F)^{2+}$ complex to a NOTA ligand on the peptide. In this report, we examined several new NOTA ligands and determined how temperature, reaction time, and reagent concentration affected the radiolabeling yield. Four structural variations of the NOTA ligand had isolated radiolabeling yields ranging from 5.8% to 87% under similar reaction conditions. All of the $Al^{18}F$ NOTA complexes were stable in vitro in human serum, and those that were tested in vivo also were stable. The radiolabeling reactions were performed at 100 °C, and the peptides could be labeled in as little as 5 min. The IMP467 peptide could be labeled up to 115 GBq/μmol (3100 Ci/mmol), with a total reaction and purification time of 30 min without chromatographic purification.

INTRODUCTION

^{18}F is the most commonly used isotope for positron-emission tomography (PET), due to its nearly ideal imaging properties (β+ 0.635 MeV 97%, $T_{1/2}$ 110 min). Conventionally, ^{18}F is attached to peptides by binding it to a carbon atom (*1–4*), but attachments to silicon (*5, 6*) and boron (*7*) also have been reported. Binding to carbon usually involves multistep syntheses, in some instances taking several hours to complete, which can be problematic for an isotope with a 110 min half-life.

The most common use of ^{18}F-labeled peptides is to image receptor sites in vivo (*2*). The number of receptor sites is usually limited, so a high specific activity is necessary to avoid blocking target receptors with the excess unlabeled peptide. In most ^{18}F-peptide applications, the final peptide must be purified by high-pressure liquid chromatography (HPLC) to separate the unlabeled peptide from the radiolabeled peptide in order to obtain the specific activity needed for imaging. In one case (*8*), the radiolabeled peptide was generated with a high specific activity and an excellent yield (79%) without the need for HPLC purification. However, most of the ^{18}F-peptide radiolabeling processes are complicated, take several reaction steps, and require specialized equipment and highly trained personnel. For example, Table 1 lists the properties of several of the more commonly reported fluorination procedures. Peptide labeling through carbon often involves ^{18}F-binding to a prosthetic group through nucleophilic substitution, usually in 2 or 3 steps, where the $^{18}F^-$ is first boiled to dryness in the presence of $KHCO_3$ and Kryptofix 2.2.2 (K222), then mixed with acetonitrile and dried 2 more times (dry-down step). The prosthetic group is then labeled with $^{18}F^-$ in the presence of excess precursor. The ^{18}F-prosthetic group is purified, attached to the targeting peptide, and then purified again. This method has been used to attach prosthetic groups through amide bonds, aldehydes, and "click chemistry" (*8–12*).

The most common amide bond-forming reagent has been *N*-succinimidyl 4-^{18}F-fluorobenzoate (^{18}F-SFB), but a number of other groups have been tested (*1–3*). In some cases, such as when ^{18}F-labeled active ester amide-forming groups are used, it may be necessary to protect certain groups on a peptide during the coupling reaction, after which they are cleaved. The synthesis of the ^{18}F-SFB reagent and subsequent conjugation to the peptide requires many synthetic steps and takes about 1.5−3 h (*11, 12*).

A simpler, more efficient ^{18}F-peptide labeling method was developed by Poethko et al. (*10*), where a 4-^{18}F-fluorobenzaldehyde reagent was conjugated to a peptide through an oxime linkage in about 75 min, including the dry-down step. The newer "click chemistry" method attaches ^{18}F-labeled molecules onto peptides with an acetylene or azide in the presence of a copper catalyst (*8, 9*). The reaction between the azide and acetylene groups forms a triazole connection, which is quite stable and forms very efficiently on peptides without the need for protecting groups. Click chemistry produces the ^{18}F-labeled peptides in good to excellent yield (∼50−79%) in about 30−90 min, including the dry-down step.

A more recent method of binding ^{18}F to silicon uses isotopic exchange to displace ^{19}F with ^{18}F (*5*). Performed at room temperature in 10 min, this reaction produces the ^{18}F-prosthetic aldehyde group with high specific activity (225−680 GBq/μmol; 6100−18 400 Ci/mmol). The ^{18}F-labeled aldehyde is subsequently conjugated to a peptide and purified by HPLC, and the purified labeled peptide is obtained within 40 min (including dry-down) with ∼55% yield. The ^{18}F-silicon approach was modified subsequently to a single-step process by incorporating the silicon into the peptide before the labeling reaction (*6*). Biodistribution studies in mice with an ^{18}F-silicon-bombesin derivative showed increasing bone uptake over time (1.35 ± 0.47% injected dose (ID)/g at 0.5 h vs 5.14 ± 2.71% ID/g at 4.0 h), suggesting a release of ^{18}F from the peptide, since unbound ^{18}F is known to localize in bone. HPLC analysis of urine showed a substantial amount of ^{18}F activity in the void volume, which may be due to $^{18}F^-$ released from the peptide.

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Table 1. Summary of Selected ^{18}F-Peptide Labeling Methods

author/ref	Schirrmacher *5*	Höhne *6*	Marik *8*	Glaser *9*	Poethko *10*	Wester/Mäding (*11, 12*)	Becaud *13*	McBride
attachment	silicon	silicon	click	click	aldehyde/oxime	amide	direct-substitution	aluminum complex
Rx steps	2	1	2	2	2	many	1	1
process time (min)[a]	40	115−155	30	65[b]	75	60$^+$	35	30[c]
yield (%)[d]	55	13	79	50	40	10	57	51
HPLC-purification steps	1	1	distillation + Sep-Pak	1 + distillation	1	2	1	SPE
specific activity (GBq/μmol)	225−680	62	>35	high	high	high	74	115

[a] Includes dry-down step. [b] Estimated. [c] Dry-down step is not required. [d] Decay-corrected.

Substantial hepatobiliary excretion also was reported, attributed to the lipophilic nature of the ^{18}F-silicon-binding substrate, and requiring future derivatives to be more hydrophilic. Methods of attaching ^{18}F to boron also have been explored; however, the current process produces conjugates with low specific activity (*7*).

A direct ^{18}F-labeling method has also been explored (*13*). Here, the peptide contains a trimethylammonium-leaving group attached to an aromatic ring containing an electron-withdrawing group. The $^{18}F^-$ is dried down (10 min) with K222/K_2CO_3 or Cs_2CO_3 and heated (50−90 °C) with the peptide for 15 min to substitute the ^{18}F for the trimethylammonium group. The reaction mixture is then purified by HPLC (∼10 min) to produce the high-specific-activity ^{18}F-labeled peptide (74 GBq/μmol) in 20−57% isolated yield. The radiolabeled peptide was stable in vitro in mouse plasma.

In contrast to these multistep, time-consuming procedures, antibodies and peptides are radiolabeled routinely with radiometals typically in 15 min and in quantitative yields (*14, 15*). For PET imaging, copper-64 and, more recently, gallium-68 have been bound to peptides via a chelate, and have shown reasonably good PET-imaging properties (*16*). Since fluoride binds to most metals (*17*), we sought to determine if an ^{18}F-metal complex could be bound to a chelate on a targeting molecule. We focused on the binding of an $(Al^{18}F)^{2+}$ complex, since the aluminum−fluoride bond is one of the strongest fluoride−metal bonds and the $(AlF)^{2+}$ complex was known to bind to ligands (*18*). The AlF_n complex is stable in vivo, since this is part of the mechanism that the body uses to incorporate fluoride into tooth enamel, and thus, low doses of AlF_n should be compatible for human use (*19, 20*). We reported previously initial studies that showed the feasibility of this approach, using an ^{18}F-labeled peptide for in vivo targeting of cancer with a bispecific antibody (bsMAb) pretargeting system (*21*), a procedure that was shown to be a highly sensitive and specific technique for localizing cancer, in some cases better than ^{18}F-FDG (fluorodeoxyglucose) (*22–30*). In this initial report, we found that an $(Al^{18}F)^{2+}$ complex could bind stably to a 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) ligand, but the yields were low and the labeled peptide had to be purified by HPLC to obtain the specific activity needed for the imaging study.

As shown in this report, we have improved the radiolabeling yields and specific activity of this NOTA ligand, but have also prepared several new NOTA ligands to determine if structural changes would yield further improvements. Our goal is to develop a one-pot radiolabeling method that provides the radiolabeled peptide with a specific activity greater than 37 GBq/μmol with no more than a simple filtration-type solid-phase extraction (SPE) purification needed before formulation for injection. Three new NOTA ligands were chosen: the NODA-GA ligand (IMP460), because it was commercially available in a form suitable for peptide synthesis; a simple NOT2A derivative (IMP461) because our experience with IMP449 suggested that only two carboxyl groups of the NOTA were needed for the Al^{18}F-NOTA complex (*21*); and the *C*-NETA-containing peptide, IMP467, because the literature had indicated that this NOTA ligand could have increased binding kinetics for metals (*31*). As shown herein, IMP467 proved to be the best of the 4 NOTA ligands we have evaluated to date, and thus, additional studies were performed to assess optimal labeling conditions for IMP467.

EXPERIMENTAL PROCEDURES

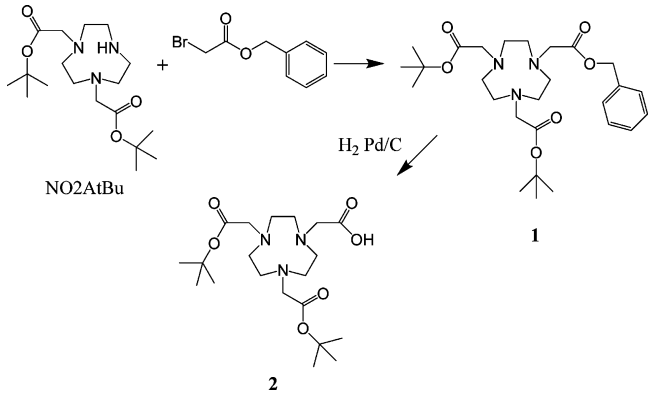
Materials. The *p*-SCN-Bn-NOTA and TACN were purchased from Macrocyclics, Inc. (Dallas, TX). The DiBocTACN, NODA-GA$(tBu)_3$, and NO2AtBu were obtained from CheMatech (Dijon, France). Protected amino acids, other peptide synthesis reagents, and resins were procured from Creosalus (Louisville, KY), Chem Impex (Wood Dale, IL), Bachem (Torrance, CA), and EMD Biosciences (San Diego, CA). The peptides were synthesized by the Fmoc method either manually or on a Protein Technologies, Inc. (Tucson, AZ) peptide synthesizer. The aluminum chloride hexahydrate was acquired from Sigma-Aldrich (Milwaukee, WI). The remaining solvents and reagents were obtained from Fisher Scientific (Pittsburgh, PA) or Sigma-Aldrich. The analytical and preparative reverse-phase HPLC (RP-HPLC) columns were bought from Phenomenex (Torrance, CA) and Waters Corp. (Milford, MA). The Sep-Pak Light Waters Accell Plus QMA and CM cartridges used to purify the $^{18}F^-$ and Waters Oasis HLB 1 cm^3 flangeless cartridges, which were used to purify the radiolabeled peptides, were purchased from Waters Corp. The size exclusion HPLC (SE-HPLC) column and the AG 1-X8 resin were from Bio-Rad (Hercules, CA). The Tricorn 5/20 Column was acquired from GE Healthcare (Piscataway, NJ). $^{18}F^-$ was supplied by IBA Molecular (Somerset, NJ). Female nude mice (NCr num), 23.1 ± 2.3 g, were obtained from Taconic Farms, Germantown, NY.

The recombinant, humanized, tri-Fab bsMAb, TF2, was provided by IBC Pharmaceuticals, Inc. (Morris Plains, NJ). TF2 binds divalently to carcinoembryonic antigen (CEACAM5 or CD66e) and monovalently to the synthetic hapten, HSG (histamine-succinyl-glycine) (*30*). The bsMAb was >95% immunoreactive against CEACAM5 and the divalent-HSG NOTA-peptide, IMP449, using a SE-HPLC method described previously (*30*).

^{1}H and ^{13}C NMR spectra were obtained using a Varian Inova NMR Spectrometer (Varian Inc., Palo Alto, CA) at 500 MHz for ^{1}H and 125.7 MHz for ^{13}C at Rutgers University Chemistry Department (Newark, NJ). Chemical shifts were referenced to tetramethyl silane. High-resolution mass spectra (HRMS) were obtained on an Agilent ESI-TOF instrument at the Scripps Center for Mass Spectrometry (La Jolla, CA) or at Immunomedics, Inc. (Morris Plains, NJ).

Methods. *Synthesis of IMP460: NODA-GA-D-Ala-D-Lys(HSG)-D-Tyr-D-Lys(HSG)-NH$_2$.* The peptide was synthesized on Sieber

Scheme 1. Synthesis of Bis-t-butyl NOTA



amide resin with the amino acids and other agents added in the following order: Aloc-D-Lys(Fmoc)-OH, Trt-HSG-OH, Aloc removal, Fmoc-D-Tyr(But)-OH, Aloc-D-Lys(Fmoc)-OH, Trt-HSG-OH, Aloc removal, Fmoc-D-Ala-OH, and NODA-GA(t-Bu)$_3$. The peptide was then cleaved and purified by HPLC. HRMS $C_{61}H_{92}N_{18}O_{18}$ MH$^+$ calcd 1365.6909, found 1365.6912.

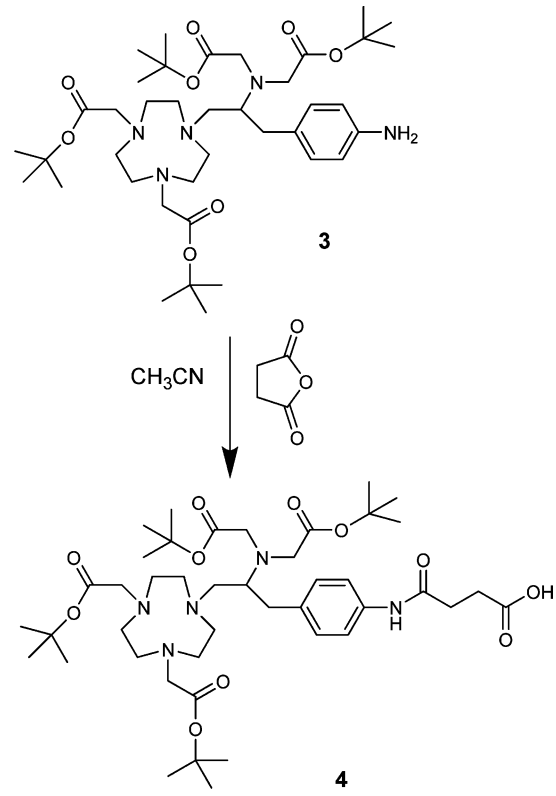
Synthesis of IMP461: NOT2A-D-Ala-D-Lys(HSG)-D-Tyr-D-Lys-(HSG)-NH$_2$ 2-{4,7-bis-tert-butoxycarbonylmethyl-[1,4,7] triazocyclononan-1-yl }-acetic acid (Bis-t-butyl-NOTA). The NO2AtBu (Scheme 1) (0.501 g 1.4×10^{-3} mol) was dissolved in 5 mL anhydrous acetonitrile. The benzyl-2-bromoacetate (0.222 mL, 1.4×10^{-3} mol) was added to the solution followed by 0.387 g of anhydrous K_2CO_3. The reaction was stirred at room temperature overnight, then filtered and concentrated to obtain 0.605 g (86% yield) of the benzyl ester conjugate (*1*). The crude product was dissolved in 50 mL of isopropanol, mixed with 0.2 g of 10% Pd/C (under Ar), and placed under 50 psi H_2 for 3 days. The product was then filtered and concentrated under vacuum to obtain 0.462 g of the desired product (*2*). HRMS $C_{20}H_{37}N_3O_6$ MH$^+$ calcd 416.2755, found 416.2759. ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C, TMS) δ 1.45 (s, 18 H), 2.8−3.3 (m, 12 H), 3.44 (s, 4 H), 3.67 (s, 2 H); ^{13}C (125.7 MHz, $CDCl_3$) δ 28.15, 49.75, 52.43, 53.54, 57.25, 58.99, 81.56, 168.77, 170.68.

Synthesis of IMP461. The peptide was synthesized as described above with Bis-t-butyl-NOTA-OH added last. The peptide was then cleaved and purified by HPLC to obtain the product. HRMS $C_{58}H_{88}N_{18}O_{16}$ MH$^+$ calcd 1293.6698, found 1293.6707.

*Synthesis of IMP467: tert-Butyl {4-[2-(Bis-tert-butyoxycarbonylmethylamino-3-(4-succinylamidophenyl)propyl]-7-tert-butyoxycarbonylmethyl-[1,4,7]triazacyclononan-1-yl}acetate (Succinyl-C-NETA) (***4***).* To a solution of **3** (*31*) (Scheme 2) (148.1 mg, 0.202 mmol) in anhydrous CH_3CN (3 mL) was added (21.1 mg, 0.210 mmol) succinic anhydride. After 3 h, the solvent was evaporated and the reaction was purified by preparative RP-HPLC to yield pure **4** (137.7 mg, 82%) as dark brown oil. HRMS $C_{43}H_{71}N_5O_{11}$ MH$^+$ calcd 834.5223, found 834.5221. ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C, TMS) δ 1.43 (s, 18 H), 1.44 (s, 18 H), 2.65−2.66 (m, 4 H), 2.85−2.88 (m, 5 H), 3.18−3.32 (m, 10 H), 3.43−3.46 (m, 10 H), 6.99 (d, 2H), 7.44 (d, 2H); ^{13}C (125.7 MHz, $CDCl_3$) δ 27.95, 28.05, 29.96,31.58, 33.36, 58.10, 58.74, 58.77, 60.69, 81.93, 82.40, 120.76, 129.20, 132.21, 137.26, 170.19, 170.21, 171.61, 171.75, 175.10.

Synthesis of IMP467: C-NETA-succinyl-D-Lys(HSG)-D-Tyr-D-Lys(HSG)-NH$_2$. IMP467 was made on a Sieber amide resin, as described above, except when the last Aloc was cleaved, the *tert*-butyl{4-[bis-(*tert*-butoxycarbonylmethyl)amino)-3-(4-succinylamidophenyl)propyl]-7-*tert*-butoxycarbonylmethyl[1,4,7]-triazanonan-1-yl}acetate (**4**) was added. The peptide was then cleaved from the resin and purified by RP-HPLC to yield 6.3 mg of IMP 467. HRMS $C_{70}H_{101}N_{19}O_{20}$ MH$^+$ calcd 1528.7543, found 1528.7565.

Scheme 2. Synthesis of Succinyl C-NETA Ligand (4)



Preparation of Aluminum Acetate Stock Solution. An aluminum acetate buffer solution was prepared by dissolving $AlCl_3 \cdot 6H_2O$ in a 0.1 M, pH 4, sodium acetate solution to provide a 2 mM Al-stock solution.

Concentration and Purification of $^{18}F^-$. Radiochemical-grade $^{18}F^-$ needs to be purified and concentrated before use. We examined four different SPE purification procedures to process the $^{18}F^-$ prior to its use.

Most of the radiolabeling procedures were performed using $^{18}F^-$ prepared by a conventional process (*32*). The $^{18}F^-$ in 2 mL of water was loaded onto a Sep-Pak Light, Waters Accell QMA Plus Cartridge that was prewashed with 10 mL of 0.4 M $KHCO_3$, followed by 10 mL water. After loading the $^{18}F^-$ onto the cartridge, it was washed with 5 mL water to remove any dissolved metal and radiometal impurities. The isotope was then eluted with ~1 mL of 0.4 M $KHCO_3$ in several fractions to isolate the fraction with the highest concentration of activity. The eluted fractions were neutralized with 5 μL of glacial acetic acid per 100 μL of solution to adjust the eluent to pH 4−5.

In the second process, the QMA cartridge was washed with 10 mL pH 8.4, 0.5 M NaOAc followed by 10 mL DI H_2O. $^{18}F^-$ was loaded onto the column as described above and eluted with 1 mL, pH 6, 0.05 M KNO_3 in 200 μL fractions with 60−70% of the activity in one of the fractions. No pH adjustment of this solution was needed.

In the third process, the QMA cartridge was washed with 10 mL pH 8.4, 0.5 M NaOAc followed by 10 mL DI H_2O. The $^{18}F^-$ was loaded onto the column as described above and eluted with 1 mL, pH 5−7, 0.154 M commercial normal saline in 200 μL fractions with 80% of the activity in one of the fractions. No pH adjustment of this solution was needed.

Finally, we devised a method to prepare a more concentrated and high-activity $^{18}F^-$ solution, using tandem ion exchange. Briefly, Tygon tubing (1.27 cm long, 0.64 cm OD) was inserted into a Tricorn 5/20 column and filled with ~200 μL of AG 1-X8 resin, 100−200 mesh. The resin was washed with 6 mL 0.4 M K_2CO_3 followed by 6 mL H_2O. A Sep-Pak light Waters

Accell Plus CM cartridge was washed with DI H_2O. Using a syringe pump, the crude $^{18}F^-$ that was received in a 5 mL syringe in 2 mL DI H_2O flowed slowly through the CM cartridge and the Tricorn column over ~5 min followed by a 6 mL wash with DI H_2O through both ion-binding columns. Finally, 0.4 M K_2CO_3 was pushed through only the Tricorn column in 50 μL fractions. Typically, 40−60% of the eluted activity was in one 50 μL fraction. The fractions were collected in 2.0 mL free-standing screw-cap microcentrifuge tubes containing 5 μL glacial acetic acid to neutralize the carbonate solution. The elution vial with the most activity was then used as the reaction vial.

Comparison of Al^{18}F Radiolabeling Yields of IMP449, IMP460, IMP461, and IMP467. Three microliters of 2 mM Al^{3+} stock solution was added to 60 μL of $^{18}F^-$ (44 MBq) followed by the addition of 10 μL of 0.05 M peptide solution in pH 4.1, 0.5 M NaOAc. The four reaction mixtures were formulated and placed in a 103 °C heating block for 19 min. The reaction mixtures were purified by an HLB column, as described above, to determine the radiochemical reaction yield. All of the reaction yields noted in this work are isolated radiochemical yields.

Kinetics of ^{18}F-IMP467 Radiolabeling. Three microliters of 2 mM Al^{3+} stock solution was mixed with 40 μL of $^{18}F^-$ followed by the addition of 20 μL of 2 mM IMP467 in 0.1 M, pH 4, acetate buffer. Four reaction mixtures were formulated and placed in a 107 °C heating block for 5, 10, 15, and 30 min. The crude products were each purified on a Waters Oasis HLB 1 cm^3 (30 mg) Flangeless Cartridge. Briefly, 200 μL of water was added to the reaction solution, which was then removed via pipet and drawn into the HLB column. The reaction solution was drawn into the column. The reaction vessel was rinsed with 1 mL of H_2O, which was then transferred and drawn into the column. The column was eluted with 2 × 200 μL portions of 1:1 EtOH/H_2O, with the product being isolated in a 3 mL vial that contained 15 mg of ascorbic acid that was adjusted to pH 6 and previously lyophilized. The yield was determined by measuring the activity left in the reaction vessel, remaining on the HLB column, in the water wash, and in the 1:1 EtOH/H_2O. The amount of activity in the 1:1 EtOH/H_2O fraction divided by the total activity from the other fractions gave the isolated radiochemical yield. The radiolabeled peptides were then analyzed by RP-HPLC, which showed that the unbound $^{18}F^-$ was removed in all cases.

Comparison of ^{18}F-IMP467 Reaction Yield vs Moles of Peptide Used. Three microliters of 2 mM Al^{3+} stock solution was added to 40 μL of $^{18}F^-$ (24 MBq) followed by the addition of 5, 10, 15, or 20 μL of 2 mM IMP467 in 0.1 M, pH 4.1 acetate buffer and 15, 10, 5, or 0 μL of water, respectively. The reaction solutions were heated to 99 °C for 15 min, then purified by HLB column as described above to determine the isolated radiochemical yield.

Comparison of ^{18}F-IMP467 Reaction Yield Vs Reaction pH. A CM cartridge (to remove metals) was washed with H_2O and placed upstream from the QMA cartridge. The $^{18}F^-$ was purified by the nitrate method described above. The peptide and $AlCl_3$ were both dissolved in pH 4, 0.1 M NaOAc. The peptide was radiolabeled with the purified $^{18}F^-$ in duplicate over the pH range 4−7.2. Each sample was labeled by mixing 20 μL of $^{18}F^-$, 2 μL of 0.01 M $AlCl_3$ in 0.05 M NaOAc pH 4.9, 20 μL 2 mM IMP467 in 0.1 M NaOAc pH 6, and 158 μL of 0.1 M reaction buffer. One sample was purified and the duplicate was allowed to decay while sealed. The pH of the decayed sample was measured with a calibrated pH meter equipped with a Calomel glass microcombination pH electrode.

Radiolabeling of IMP467 with Carbonate-Eluted ^{18}F-Fluoride. ^{18}F-fluoride (5.39 GBq) in 50 μL 0.4 M K_2CO_3 was neutralized with 5 μL glacial acetic acid and then mixed with 1 μL 0.01 M ($AlCl_3 \cdot 6H_2O$) in 0.1 M NaOAc (pH 4), followed by 2 μL 0.01 M IMP467 (20 nmol) in 0.1 M NaOAc (pH 4), and incubated in a 105 °C heating block for 17 min. The solution was cooled briefly and diluted with 1 mL H_2O, and then placed in an HLB 1-cc (30 mg) cartridge. The solution was eluted under vacuum into an empty crimp-sealed vial as follows. The reaction vessel and the column were rinsed with 2 × 1 mL portions of H_2O. The column was moved to a vial containing lyophilized ascorbic acid (15 mg, buffered at pH 5.5) and eluted with 2 × 200 μL of 1:1 ethanol/water.

Radiolabeling of IMP467 Using Saline-Eluted ^{18}F-Fluoride Ion. ^{18}F-fluoride ion (70.3 MBq) in 50 μL saline was mixed with 2 μL 0.01 M ($AlCl_3 \cdot 6H_2O$) in 0.1 M NaOAc (pH 4) followed by 20 μL IMP467 (2 mM, 40 nmol) in 0.1 M NaOAc (pH 4), and incubated in a 105 °C heating block for 15 min. The solution was cooled briefly, diluted with 1 mL PBS (pH 7.4), and then placed in a HLB cartridge. The solution was purified as described above to obtain 48.3 MBq of the radiolabeled product in 85% isolated yield. The total reaction and purification time was 30 min.

Synthesis and Characterization of ^{19}F-IMP467. The ^{19}F-IMP467 was prepared by mixing 20 μL of 2 mM IMP467 in 2 mM NaOAc buffer, pH 4.18, with 50 μL of 2 mM $AlCl_3$ and 100 μL of 2 mM NaF in the same acetate buffer in a 2 mL screwcap microcentrifuge vial and heated (sealed) for 5 min at 101 °C. The sample was then examined by HPLC on a Waters 2695 HPLC system equipped with a Phenomenex Gemini C_{18} reverse-phase column (250 × 4.6 mm, 5 μm, 110 Å), using a linear gradient of 90% A (0.1% TFA) to 20% B (90% acetonitrile, 10% water, 0.1% TFA) over 20 min at a flow rate of 1 mL/min, absorbance was detected at 254 nm using Waters PDA 2996 detector. The ^{19}F-IMP467 forms two complexes (12.92 and 14.84 min), which were isolated by HPLC. The isolates were stored in the eluent buffer and reinjected 0.5, 1, 1.5, and 2 h after isolation. The complexes were examined by HPLC on the Agilent ESI-TOF.

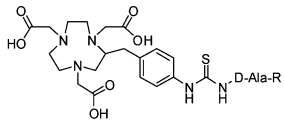
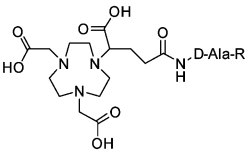
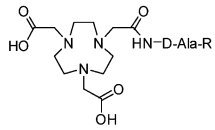
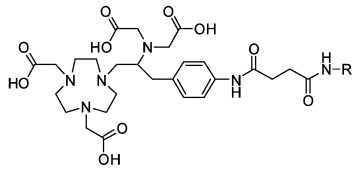
HPLC Separation of ^{19}F-IMP467 from Unreacted Peptide. The unreacted peptide could be separated from the ^{19}F or ^{18}F-peptide by using a Phenomenex Kinetex C-18 column, 50 × 4.60 mm, 2.6 μ, 100A and eluting at 0.6 mL/min using 0.01% formic acid in 5% acetonitrile/water as Buffer A and 0.01% formic acid in 90% acetonitrile as Buffer B. The gradient ran for 1 min with 100% Buffer A, then to 80:20 A/B over 6 min. Under these conditions, the Al-peptide and the unreacted peptide were eluted before the fluoride-bound peptides. These were the HPLC conditions used to examine the peptide reaction mixture on the Agilent ESI-TOF.

Stability of ^{18}F-IMP467 in Phosphate-Buffered Saline (PBS) and Human Serum. The purified radiolabeled peptide in 50 μL 1:1 EtOH/H_2O was mixed with 150 μL of human serum and placed in the HPLC autosampler heated to 37 °C. Another sample of ^{18}F-IMP467 was diluted in the same manner with PBS and heated to 37 °C. The samples were analyzed by RP-HPLC.

Biodistribution and in Vivo Stability of ^{18}F-IMP467. All animal studies were approved in advance by the Institutional Animal Care and Use Committee of the Center for Molecular Medicine and Immunology.

The stability of ^{18}F-IMP467 was examined by comparing HPLC elution profiles of the peptide prior to injection to that found in the urine at 0.5 and 1.5 h after the intravenous injection ^{18}F-IMP467 (18.5 MBq (500 μCi), 2.67 × 10^{-10} mol in 1% human serum albumin) in nude mice. The materials were analyzed by RP-HPLC and by SE-HPLC alone and in the presence of TF2 anti-CEACAM5 × anti-HSG tri-Fab bsMAb,

Table 2. NOTA Ligands and Maximum Isolated Yields after Radiolabeling with 500 nmol Peptide (R = D-Lys(HSG)-D-Tyr-D-Lys(HSG)-NH_2)

PEPTIDE	Structure	Maximum ^{18}F-Labeling Yield
IMP449		44%
IMP460		5.8%
IMP461		31%
IMP467		87%

which was used to illustrate the continued association of the HSG-hapten with the ^{18}F-IMP467 as an indication of product stability.

Biodistribution studies were performed in nude mice bearing subcutaneous LS174T human colon cancer xenografts. Mice in the pretargeting group received 163 μg (1 nmol) of TF2 intravenously, followed with ^{18}F-IMP467 (3.91 MBq (105 μCi), 0.05 nmol in 1% human serum albumin) 16 h later. Mice were necropsied 1 and 3 h post-peptide injection, and the tissues were weighed and co-counted in a gamma scintillation counter with standards prepared from the injected product. The data are expressed as the percent-injected dose per gram (% ID/g).

RESULTS

IMP449, a benzyl-NOTA derivative (Table 2) of a peptide used for pretargeting, was the first chelate that formed a stable complex with $(Al^{18}F)^{2+}$ suitable for in vivo targeting (*21*). IMP449 was labeled previously by mixing 6 nmol of Al^{3+} with $^{18}F^-$ in a pH 4 sodium acetate buffer to form $(Al^{18}F)^{2+}$, then mixed with 521 nmol of IMP449 (total volume 213 μL) and heated for 15 min at ~100 °C. Labeling yields were between 5% and 20% after HPLC purification. We found subsequently that radiolabeling yields with this derivative could be improved to as much as 44% by reducing the reaction volume (73 μL) by one-third. Several products also were observed by RP-HPLC after radiolabeling the IMP449 peptide. Adding ascorbic acid to the reaction mixture markedly reduced these side products (not shown).

Three new NOTA and NOT2A derivatives were synthesized as part of a pretargeting peptide in an attempt to find a ligand that would improve labeling yields and maintain stability of the $Al^{18}F$-NOTA complex. Table 2 shows the maximum radiolabeling yields obtained with the three new ligands using the same conditions that enabled 44% yields with IMP449. One of the derivatives, IMP460, had only 5.8%, while another, IMP461, had a 31% yield. However, radiolabeling yields nearly doubled to 87% with IMP467. The reported improved binding properties of the ligand may be responsible for the increased labeling yield (*33*). While all of these products were tested and found to be stable in human serum, further studies focused on IMP467 to assess the impact that other aspects of the radiolabeling procedure might have on the yield and specific activity.

Table 3. ^{18}F-IMP467 Yield as a Function of pH

reaction buffer pH	final reaction pH	isolated yield (%)
2.88	3.96	54
3.99	4.27	70
	4.25	77
5.00	5.05	70
	4.25	69
6.00	6.04	41
7.30	7.23	3.0

Decreasing the amount IMP467 in the reaction mixture from 500 nmol to 40 nmol reduced the labeling yield to 65−75%, which was still better than the 44% yield achieved with 500 nmol of IMP449. When the amount of peptide added to the reaction was varied from 40 to 20 nmol (at concentrations from 0.63 to 0.32 mM), the yields remained fairly constant at around 75−82%, but decreased to 49% when only 10 nmol was used at a concentration of 0.16 mM. Other studies found that binding was nearly complete within 5 min at 107 °C (5 min, 68%; 10 min, 61%; 15 min, 71%; and 30 min, 75%), with only moderate increases in isolated yield with reaction times as long as 30 min, but no binding was achieved with labeling at 50 °C.

In an effort to enhance the specific activity further, we devised a procedure for purifying and concentrating $^{18}F^-$, starting with substantially higher levels of $^{18}F^-$ than were employed in all previous studies. When using only 20 nmol of IMP467 with 5.39 GBq (145.6 mCi) of $^{18}F^-$, the labeling yield was 52% (isolated radiochemical yield, without correcting for decay, 51% decay-corrected). Thus, this process further improved the specific activity of the preparation [i.e., the isolated peptide contained 2.29 GBq (61.9 mCi) of the purified peptide, with an effective specific activity of 115 GBq/μmol (3100 Ci/mmol)], albeit at the expense of somewhat reduced, but acceptable, yields. This radiolabeled product was prepared in 28 min ($^{18}F^-$ post purification delay = 6 min; reaction time = 17 min; purification time = 5 min). A second experiment using 7 min heating afforded a similar yield and specific activity in only 14 min. Overall, these studies confirmed that increasing the concentration of reagents improves the reaction kinetics and the specific activity of the labeled product, with some reduction in labeling efficiency.

We next assessed how the concentration of Al^{3+} might impact the labeling yields. When IMP467 (40 nmol) was labeled in the presence of increasing amounts of Al^{3+} (0, 5, 10, 15, 20 μL of 2 mM Al in pH 4 acetate buffer and keeping the total volume constant), yields of 3.5%, 80%, 77%, 78%, and 74%, respectively, were achieved. These results indicated that (a) nonspecific binding of $^{18}F^-$ to this peptide in the absence of Al^{3+} was low, (b) 10 nmol of Al^{3+} was sufficient to allow for maximum ^{18}F-binding, and (c) higher amounts of Al^{3+} did not reduce binding substantially, indicating that there was sufficient chelation capacity at this peptide concentration.

The optimal pH for labeling was between 4.3 and 5.5 (Table 3). The process could be expedited by eluting the $^{18}F^-$ from the anion exchange column with nitrate or chloride ion instead of carbonate ion, which eliminates the need to adjust the eluent to pH 4 with glacial acetic acid before mixing with the $AlCl_3$.

RP-HPLC showed unlabeled IMP467 elutes as a single peak at 13.454 min (Figure 1A), but when radiolabeled, ^{18}F-IMP467 elutes as two peaks 13.399 and 15.388 min (Figure 1B), suggesting that the aluminum fluoride forms two complexes with the peptide. To examine this further, we prepared and characterized cold ^{19}F-IMP467 by RP-HPLC (UV) and HPLC-mass spectroscopy (ESI-TOF). The HPLC of the ^{19}F-IMP467 (Figure 1C) confirmed that the ^{19}F-IMP467 peaks 12.927 and 14.887

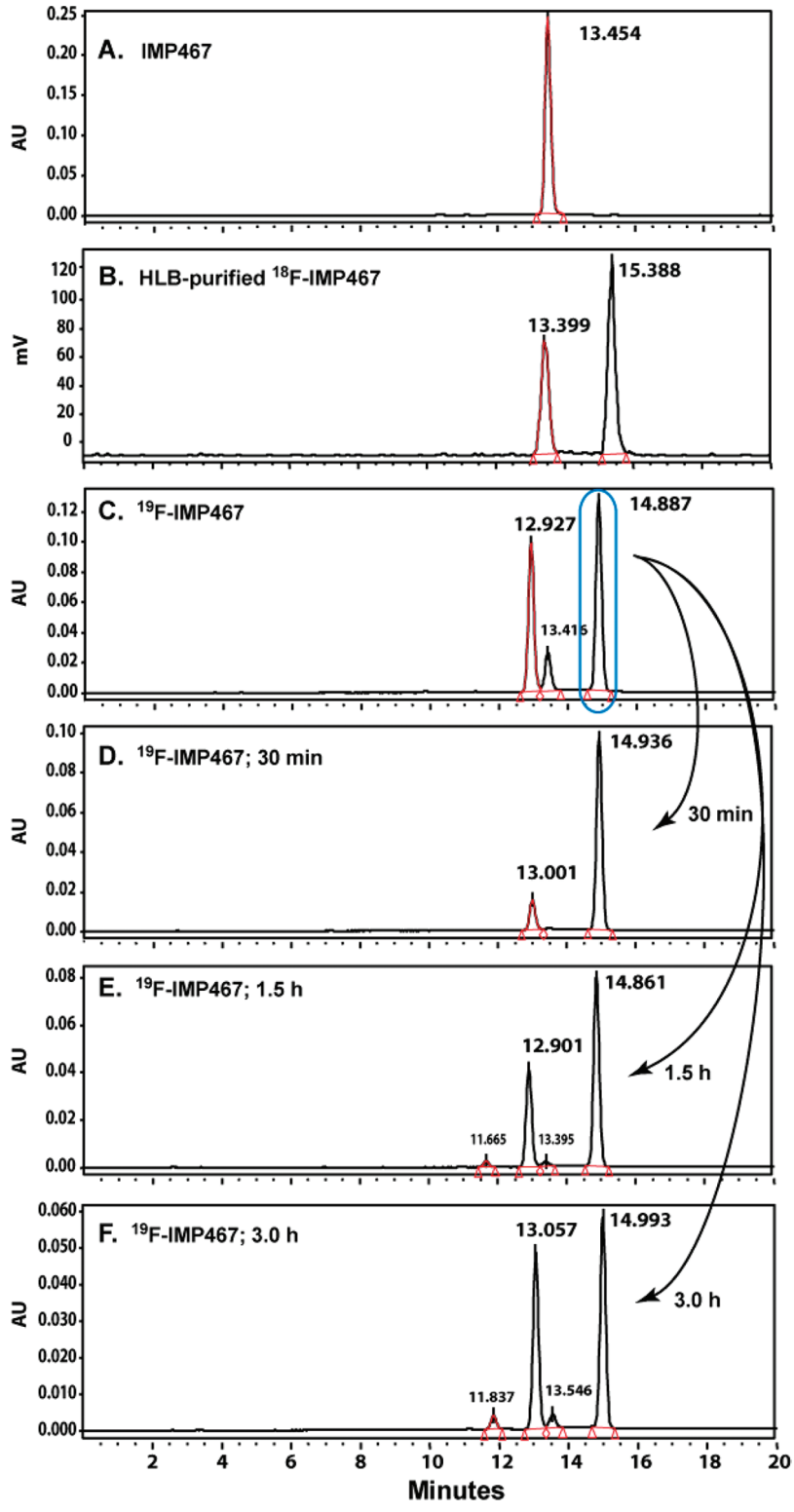


Figure 1. RP-HPLC analysis of IMP467, ^{18}F-IMP467, and ^{19}F-IMP467. The unlabeled peptide elutes as a single peak (A), but when radiolabeled with Al^{18}F (^{18}F-IMP467), two peaks are observed (B). ^{19}F-IMP467 also showed two main peaks (C) [13.4 min peak is unlabeled peptide as seen in (A)]. The 14.9 min peak was isolated and evaluated over time (D−F), showing the equilibration of the isomeric counterpart (∼13.0 min).

min corresponded to the same peaks seen with the ^{18}F-IMP467 with the retention times of the radiometric detector being slightly later since it is downstream from the UV detector. When this reaction mixture was analyzed by HPLC-mass spectrometry (high resolution), the results confirmed that each of the two peaks contained the peptide, one aluminum, and one fluorine (ESI-TOF $C_{70}H_{99}FN_{19}O_{20}Al$ calcd 1571.7113 found 786.8635 $[M+2H]^{2+}$).

The individual ^{18}F and ^{19}F peaks could be isolated by HPLC. Over time, the isolated isomers interconvert, as shown in Figure 1C−F, where the ∼14.9 min ^{19}F-IMP467 product was isolated and then re-evaluated over 3 h. This interconversion also was seen previously with the simple NOT2A ligand (*34*). Under the HPLC conditions shown in Figure 1, the unlabeled peptide is eluted between the two ^{19}F-IMP467 complexes (∼13.4 min). This peak also was seen to develop over the 3 h evaluation of

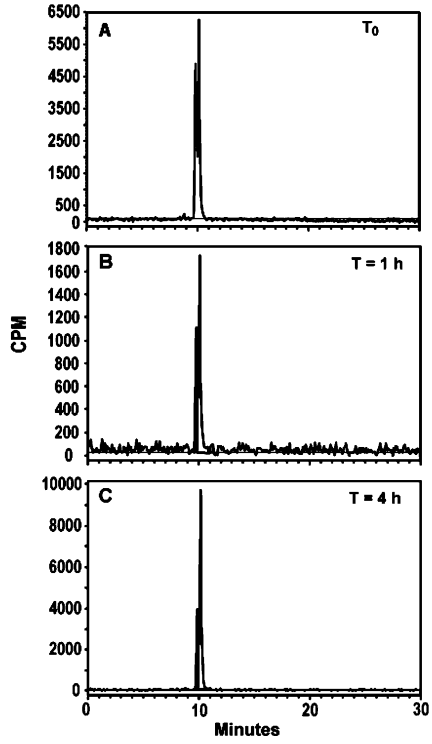


Figure 2. Serum stability of IMP467 as analyzed by RP-HPLC: (A) ^{18}F-IMP467 in serum at time-zero, (B) ^{18}F-IMP467 in serum after 1 h, (C) ^{18}F-IMP467 in serum after 4 h.

the isolated 14.9 min ^{19}F-IMP467 peak, suggesting some loss of Al^{19}F from the product over time when held in 0.1% TFA buffer. Al-peptide complexes are eluted before these peaks (i.e., between 9.8 and 12.2 min). If a different C-18 column is used (Phenomenex Kinetex C-18), the Al peptide complexes and the unreacted peptide are eluted first followed by the ^{18}F- or ^{19}F-complexed peptide. The purification on the Kinetex column would permit isolation of the labeled peptide if further increases in effective specific activity were desired (data not shown).

Stability studies with ^{18}F-IMP467 were performed in PBS and initially, in previously frozen human serum. At time zero, both samples showed no detectable $^{18}F^-$ above background at the void volume of the column. At 5.5 h, the PBS sample had 2.3% of the $^{18}F^-$ activity in the void volume. At 1 and 4 h, there was no detectable activity in the void volume, but the ratio of the two radiolabeled peaks changed over time, with a higher portion found in the second peak (Figure 2). The serum stability study was later repeated in fresh human serum at 37 °C for 5 h and the RP-HPLC indicated that 0.5% of the injected activity was present at the void volume of the column (not shown). ^{18}F-IMP467 analyzed by SE-HPLC showed that nearly all of the activity shifted to a shorter retention time on addition of TF2, indicating that the binding of the Al^{18}F complex did not compromise the binding of the HSG hapten (Figure 3).

In order to confirm the suitability of ^{18}F-IMP467 for in vivo use, biodistribution studies were performed in nude mice using the peptide alone or in a pretargeting setting (*21*). The peptide eliminated in the urine had an identical RP-HPLC elution profile as the ^{18}F-IMP467 that was injected, and SE-HPLC further showed the labeled peptide contained the HSG-hapten used to bind the bsMAb (Figure 4). These results indicate that the peptide was excreted intact, indicating no dissociation of the radionuclide. As shown in Table 4, the peptide cleared quickly from the blood (~0.1% ID/g at 1 h), with little residual activity in the tissues and low renal retention (~2% ID/g). Bone uptake was also low, averaging 0.4−0.5%, as compared to 6% and 9% ID/g that were reported previously for ^{18}F and Al^{18}F at 1

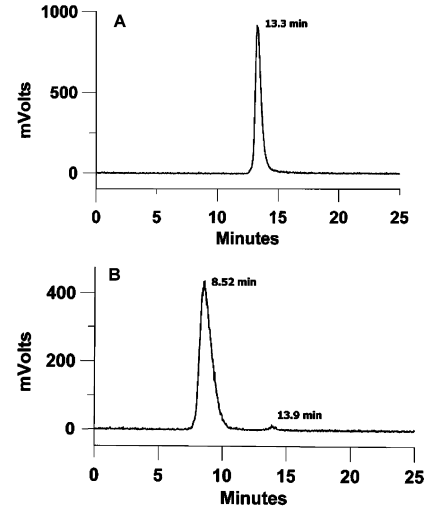


Figure 3. Binding of ^{18}F-IMP467 to TF2 by SE-HPLC: (A) ^{18}F-IMP467 after HLB purification, alone, or (B) mixed with TF2 anti-CEA × anti-HSG bsMAb.

and 3 h, respectively (*21*). Tumor uptake in the pretargeted animals 1 h after injection of the ^{18}F-IMP467 was nearly 50× higher than that observed with the ^{18}F-IMP467 alone, providing tumor/nontumor ratios of ~100:1 for the blood, liver, and other tissues, and even 5:1 for the kidney, indicating the suitability of this method to prepare functionally active and stable ^{18}F-labeled peptides for immunoPET imaging.

DISCUSSION

Molecular imaging has become an important priority, with far-reaching implications in many fields of biomedical research and clinical practice. PET imaging represents one of the more sensitive imaging modalities, with the ability to view the whole-body distribution of a radiotagged compound. A number of different positron-emitting radionuclides have been used for PET imaging, but ^{18}F is preferred because of its ideal imaging properties, and it is also widely available and relatively inexpensive because of the extensive use of ^{18}F-fluorodexoyglucose in oncology and neurobiology. However, coupling ^{18}F to compounds has been a challenge. Currently, there are a number of groups exploring ^{68}Ga, because products can be prepared at high specific activities, and also because this radionuclide can be readily coupled to compounds through simple chelation chemistry that has been widely used for radiometal labeling over the past 25 years (*14–16*). We envisioned that a similar process could be applied to metal−fluorine complex that would permit more facile radiolabeling of compounds with ^{18}F and reported previously the initial success of this approach applied to a NOTA-containing peptide used in a pretargeting procedure (*21*). However, radiolabeling yields were low (5−20%), and HPLC purification was required to isolate the ^{18}F-peptide from the unlabeled peptide.

We now report improvements to this radiolabeling procedure by changing the structure of the core NOTA compound and the reaction conditions. This method can be performed by simply mixing Al^{3+}(6−40 nmol) with $^{18}F^-$ in pH 4−5.5 acetate buffer with a 5 min incubation, followed by an HLB cartridge purification to remove unbound $^{18}F^-$ and (AlF_n^{18}F). Using these improvements, we have been able to label IMP467 with a specific activity as high as 115 GBq/μmol (3100 Ci/mmol) in ≤30 min without HPLC purification, but this required a more concentrated and high-activity $^{18}F^-$ than what is commonly supplied by the commercial vendor and resulted in somewhat lower yields. Purification of the $^{18}F^-$ to remove trace metals is important here, as well as in other methods. Importantly, the

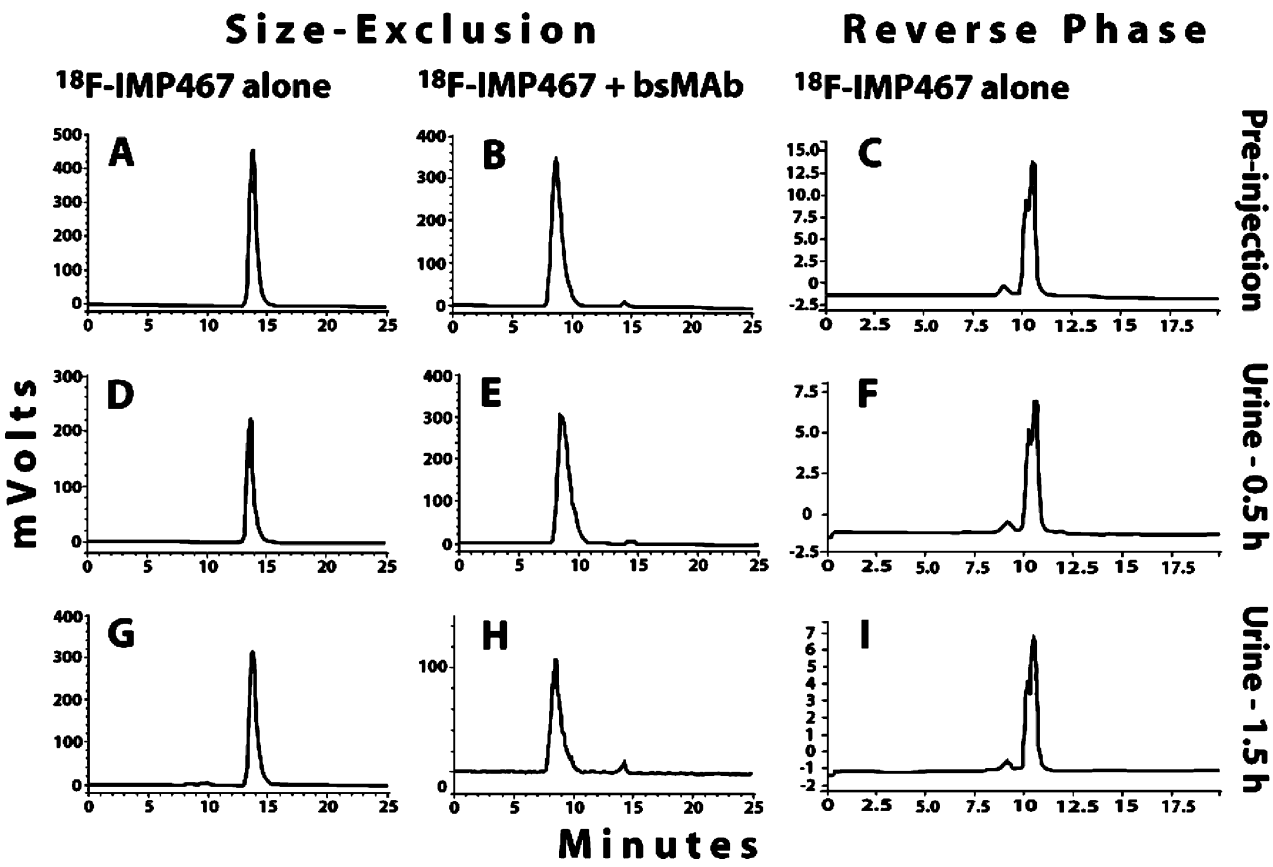


Figure 4. In vivo stability of ^{18}F-IMP467. SE- and RP-HPLC analysis of ^{18}F-IMP467 before injection and in urine samples taken from mice 0.5 and 1.5 h postinjection. RP-HPLC shows the same elution profile in the original product and in the urine, while SE-HPLC was performed to show ^{18}F-IMP467 eliminated in urine continued to retain binding to the TF2 anti-CEACAM5 × anti-HSG bsMAb.

Table 4. Biodistribution of ^{18}F-IMP467 Alone or Pretargeted by TF2 Anti-CEACAM5 × Anti-HSG bsMAb[a]

	percent-injected dose per gram tissue (mean ± SD; $N = 5$)			
tissue	TF2-pretargeted ^{18}F-IMP467 1 h	TF2-pretargeted ^{18}F-IMP467 3 h	^{18}F-IMP467 1 h alone	^{18}F-IMP467 3 h alone
LS174T	11.8 ± 2.97	8.16 ± 4.83	0.23 ± 0.11	0.09 ± 0.05
liver	0.29 ± 0.32	0.09 ± 0.02	0.07 ± 0.01	0.06 ± 0.01
spleen	0.26 ± 0.19	0.11 ± 0.04	0.05 ± 0.01	0.03 ± 0.01
kidney	2.28 ± 0.47	1.98 ± 0.35	2.47 ± 0.56	1.96 ± 0.55
lung	0.29 ± 0.18	0.08 ± 0.02	0.12 ± 0.02	0.06 ± 0.01
blood	0.12 ± 0.06	0.02 ± 0.01	0.08 ± 0.05	0.01 ± 0.00
stomach	0.13 ± 0.06	0.05 ± 0.04	0.03 ± 0.02	0.03 ± 0.01
small intestine	0.46 ± 0.12	0.12 ± 0.03	0.21 ± 0.05	0.13 ± 0.04
large intestine	0.11 ± 0.09	0.27 ± 0.11	0.07 ± 0.02	0.20 ± 0.09
scapula	0.57 ± 0.13	0.41 ± 0.08	0.40 ± 0.11	0.44 ± 0.14
muscle	0.54 ± 0.88	0.03 ± 0.04	0.03 ± 0.01	0.02 ± 0.01
brain	0.02 ± 0.00	0.02 ± 0.02	0.01 ± 0.00	0.01 ± 0.00

[a] Nude mice bearing LS174T human colonic cancer xenografts were necropsied 1 and 3 h after ^{18}F-IMP467 injection (TF2 given 16 h earlier).

labeling process could be performed by eluting ^{18}F-fluoride with commercial sterile saline, with optimal yields occurring when mixing 71 MBq of purified $^{18}F^-$ with 20 nmol Al and 40 nmol IMP467 in a pH 4.3−5.5 acetate buffer in a total volume of 100 μL, heating to 90 to 110 °C for 15 min, and performing SPE separation to remove unbound $^{18}F^-$ from the radiolabeled peptide.

As mentioned, the specific activity and yields are contingent on the concentration of the reaction mixture, but HPLC purification also could be used to isolate the Al^{18}F-labeled peptide from the Al-peptide/peptide if higher specific activities are necessary. In the case of IMP449, Al-IMP449 had about the same HPLC retention time as the ^{18}F-IMP449, but it could be purified to a specific activity of about 1300 Ci/mmol, which provided highly favorable tumor localization with the pretargeting method (*21*). Some of the new peptides developed for this work had a much greater separation between the Al-peptide and the Al^{18}F peptide, making the HPLC purification to obtain high-specific-activity ^{18}F-peptides somewhat easier. More recently, NOT2A-octreotide (*34*) and NOT2A-bombesin (*35*) derivatives bearing the same simple NOT2A ligand as IMP461 were prepared and labeled with $(Al^{18}F)^{2+}$. As with IMP461, radiolabeling yields were in the same range, and in both of these situations, HPLC purification was able to separate radiolabeled from unlabeled peptide to improve the specific activity, but we suspect that higher specific activities may be possible by modifying the NOTA ligand. As indicated herein, radiolabeling yields vary from as low as 6% to as high as 87%, based on the NOTA ligand. The higher labeling yield (87%) observed for IMP467 may be due to the increased binding kinetics of the ligand (*33*).

In our initial studies, many known metal-binding ligands were examined for their suitability for complexing $(Al^{18}F)^{2+}$ (*21*). Diethylenetriaminepenatacetic acid-(DTPA-Al^{18}F) complexes were formed in >90% yield, but they were not stable in vitro. Other ligands known to bind Al^{3+} were tested but also were unsuitable. André et al. reported that the Al-NOTA complex was stable (*36*), and in our testing, all the NOTA-Al^{18}F compounds tested to date showed a high level of stability in serum, and both IMP449 and IMP467 were stable in vivo. A recent study also found the targeting of ^{18}F-IMP449 was similar to the same peptide radiolabeled with ^{68}Ga, attesting to the stability of the ^{18}F-product (*29*). While the HPLC analysis of the 14.9 min peak from the ^{19}F-IMP467 showed some formation of the unlabeled peptide (peak ~13.4 min) over time, this product was isolated in 0.1% TFA buffer, which is certainly not the condition in which ^{18}F-IMP467 would be held for in

vivo use. Indeed, in PBS, only ~2% loss of ^{18}F occurred, and when held in serum over 4 h, no detectable loss of ^{18}F was observed, but there was a noticeable change in the proportion of the product in the early eluting peak to the later-eluting peak. The in vivo studies also clearly support the suitability of the product's stability, with very low uptake in bone.

All of the $Al^{18}F$ peptide complexes formed two peaks (diastereomeric products), and with IMP467, the ratio of the two peaks changed over time, but this isomerization did not result in the loss of ^{18}F. Al^{3+} forms octahedral complexes with four binding sites in a plane and two axial binding sites. Many isomer possibilities with the NOTA ligands exist, but it may be that the two complexes arise from a fluoride−aluminum bond in the plane in one complex and a fluoride−aluminum bond in the axial position in the other complex. Further studies will be needed to determine the exact nature of the complexes.

CONCLUSION

In conclusion, *C*-NETA-containing peptides, as exemplified by IMP467, can be labeled with ^{18}F rapidly (15−30 min) and in high yield (up to 85%) via Al-bound ^{18}F at a specific activity of up to 115 GBq/μmol, without requiring HPLC purification. The resulting ^{18}F-IMP467 is stable in human serum and suitable for in vivo pretargeting applications. The use of the $^{18}F^-$ saline as a source of purified ^{18}F-fluoride simplifies the labeling process. We anticipate that similar results can be achieved with a broad spectrum of peptides derivatized with *C*-NETA. This labeling method provides a simple procedure for obtaining high-specific-activity ^{18}F-labeled peptides using conventional equipment and is amenable to the development of a kit formulation, requiring only the addition of $^{18}F^-$ to a peptide−aluminum mixture. Such a simple, inexpensive labeling process should expand the use of ^{18}F-radiolabeled peptides for research and clinical use.

ACKNOWLEDGMENT

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