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Labeling approaches for the GE11 peptide, an epidermal growth factor receptor biomarker

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The epidermal growth factor receptor (EGFR) is involved in the proliferation and differentiation of normal and malignant cells and is a major therapeutic target for a variety of human cancers. The peptide GE11 was reported to bind efficiently to the EGFR. Labeling GE11 with radionuclides may aid in the quantification of EGFR expression in tumors via noninvasive imaging. To this end, a GGGK linker was attached to the peptide (GE11′), which was conjugated with prosthetic-labeled groups such as [^{18}F]*N*-succinimidyl 4-fluorobenzoate ([^{18}F]SFB), [^{18}F](2-{2-[2-(2-fluoro-ethoxy)-ethoxy]-ethoxy}-ethoxy)-propyne ([^{18}F]F-PEG4-propyne), [^{124}I]*N*-succinimidyl 4-iodobenzoate, and the NOTA-Bn-NCS chelator for ^{111}In labeling. All labeled analogs were successfully prepared with radiochemical purity >95% and were identified by HPLC based on their non-labeled standards. [^{18}F]SFB and [^{18}F]F-PEG4-propyne were obtained with a decay-corrected yield (DCY) of 26% and 30% and a specific activity (SA) of 844 and 2580 Ci/mmol, respectively. [^{124}I]*N*-Succinimidyl 4-iodobenzoate was obtained with a DCY of 65% and an SA of 3600 Ci/mmol. The DCYs for [^{18}F]4-fluoro-benzoate-GE11′, [^{18}F]F-PEG4-1,3-triazole-GE11′, and [^{124}I]4-iodo-benzoate-GE11′ were 8%, 10%, and 30%, and the SAs were 108, 500, and 300 Ci/mmol, respectively. [^{111}In]-NOTA-GE11′ was prepared with 60% radiochemical yield (RCY) and a SA of 0.35 Ci/mmol. GE11 was also labeled directly with ^{124}I, using chloramine-T, yielding [^{124}I]GE11 with 47% RCY and a SA of 1030 Ci/mmol.

Keywords: EGFR; F-18; I-124; In-111; Cancer; PET

Introduction

The epidermal growth factor receptor (EGFR/HER1) belongs to the ErbB receptor family involved in the proliferation and differentiation of normal and malignant cells.[1] Overexpression of EGFR and its enhanced signaling are a frequent hallmark of human epithelial cancers and play a major role in the initiation, progression, and/or invasiveness of human cancers.[2–5] Overexpression of the EGFR is present in at least 70% of human cancers,[6] such as non-small cell lung carcinomas, breast cancers, gliomas, squamous cell carcinoma of the head and neck, and prostate cancer.[7–9] Furthermore, correlations between EGFR overexpression and metastasis formation, therapy resistance, poor prognosis, and short survival have been described previously.[2,3,5,10,11] As a result, the EGFR tyrosine kinase has become a major target for the development of pharmaceutical agents that may block the receptor and inhibit the signal transduction pathway.[12] A variety of potential drugs, either small molecules targeting the adenosine triphosphate internal binding domain or antibodies targeting the external binding domain, have been developed as anti-EGFR targeted drugs for the treatment of cancer.[13–15] In addition, major efforts have been invested in the development of labeled EGFR biomarkers as imaging agents.[16–22] Recently, a novel peptide, namely GE11 (Figure 1), was reported as a potent EGFR ligand.[23,24] This peptide was shown to bind efficiently to the receptor ($K_d \approx 22$ nM) with a limited mitogenic effect compared with the epidermal growth factor. These reports indicated that GE11-conjugated vectors could be used as efficient and selective EGFR-mediated drug delivery systems.[23,24] In addition, based on these previous reports, GE11, when labeled with appropriate radionuclides, may be used as an imaging agent for the noninvasive diagnosis of EGFR-positive tumors. In this work, we have developed and synthesized a novel GE11-GGGK peptide (GE11′), with three glycine amino acids serving as spacers and one lysine amino acid at its end for chemical transformation on its ω-amino moiety (Figure 1). This modified peptide (GE11′) was conjugated to radiolabeled prosthetic groups, such as [^{18}F]*N*-succinimidyl 4-fluorobenzoate ([^{18}F]SFB), [^{18}F](2-{2-[2-(2-fluoro-ethoxy)-ethoxy]-ethoxy}-ethoxy)-propyne ([^{18}F]F-PEG4-propyne), [^{124}I]*N*-succinimidyl 4-iodobenzoate ([^{124}I]SIB), and *S*-2-(4-isothiocyanatobenzyl)-1,4,7-triazacyclononae-1,4,7-triacetic acid (p-SCN-Bn-NOTA), for radiometal labeling with ^{111}In and ^{68}Ga. The labeled lead will be later identified by a series of microPET and biodistribution studies, and could potentially serve for the noninvasive diagnosis and quantification of EGFR expression in tumors and the selection of patients who are expected to benefit from anti-EGFR targeted therapies.

Results and discussion

Chemistry

It is well known that conjugation of labeled prosthetic groups through a linker tends to preserve the affinities and biological

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(a): YHWYGYTPQNVI (b): Acetyl-YHWYGYTPQNVI-GGGK-Amide

Figure 1. (a) Sequence of the native peptide GE11 (YHWYGYTPQNVI). (b) Sequence of the modified peptide GE11′ (acetyl-YHWYGYTPQNVI-GGGK-amide).

activities of peptides. Furthermore, in most cases, such indirect radiolabeling via a linker and conjugation to labeled prosthetic groups yield a more stable labeled peptide than the direct labeling approaches.[25] In general, the addition of the linker and prosthetic group might change the biological activity of the studied peptides. However, in the case of GE11, as reported previously,[23,24] and as supported by a more recent publication, the addition of linkers such as $(GGGGS)_3$ and PEG chain coupled to polyethylene imine did not affect binding.[26] Thus, the GGGK was attached to the GE11 sequence as a spacer and linker to the prosthetic groups SFB, SIB, F-PEG4-propyne, and NOTA-Bn-NCS to prepare four different radiolabeled probes, [^{18}F]4-fluoro-benzoate -GE11′ ([^{18}F]FB-GE11′, **[^{18}F]-1**), [^{124}I]4-iodo-benzoate-GE11′ ([^{124}I] IB-GE11′, **[^{124}I]-2**), [^{18}F]F-PEG4-1,3-triazole-GE11′ (**[^{18}F]-3**), and [^{111}In]-NOTA-GE11′ (**[^{111}In]-4**) (Figure 2). The various prosthetic groups that react with the peptide enable the use of diverse chemistry approaches (click, coupling, and metal-based chemistry) and the utilization of various radionuclides, such as ^{18}F, ^{124}I, and ^{111}In, with a wide range of half lives (109 min to 4.2 days), thus improving the evaluation and identification of the labeled lead for EGFR imaging via microPET and biodistribution studies.

Preparation of prosthetic group standards—SFB, SIB, and F-PEG4-propyne

The precursors for the radiochemistry and the standards of the prosthetic groups were prepared based on published literature. The cold standard for the coupling reaction, *N*-succinimidyl 4-fluorobenzoate (SFB), was obtained from 4-fluorobenzoic acid in the presence of *N,N*′-dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide in THF with 80% yield.[27–29] The precursor for the ^{18}F radiolabeling, ethyl 4-(trimethylammonium triflate) benzoate, was prepared from ethyl 4-(dimethylamino)benzoate and methyltriflate in THF with 80% yield.[27] The cold standard for the iodinated coupling reaction, *N*-succinimidyl 4-iodobenzoate (SIB),[25] was prepared similarly to SFB and obtained with 28% yield, while its precursor for the ^{124}I radiolabeling synthesis, *N*-succinimidyl 4-(tri-*n*-butylstannyl) benzoate (STB), was obtained in 80% yield from SIB, tetrakis(triphenylphosphine)palladium, and bis(tributyltin) in refluxed toluene for 2 h.[25] Hydroxyl-PEG4-propyne (**5**) served as a starting material for the preparation of the cold standard for the click chemistry, F-PEG4-propyne (**6**), and for the precursor **7** for the radiolabeling. Tetrapolyethylene glycol (PEG4), propargyl bromide, and sodium hydride were reacted in THF to obtain **5** with 20% yield.[30] Compound **5** was reacted with diethylaminosulfur trifluoride to give the cold standard **6** with 33% yield.[31] The synthesis of the precursor for the ^{18}F click chemistry, the tosyl derivative **7**, was performed with **5**, tosyl chloride, triethylamine (TEA), and trimethylammonium chloride in acetonitrile with 64% yield (Scheme 1).[30]

*Preparation of GE11′ derivatives (**1–4**)*

The standard FB-GE11′ (**1**) was obtained by conjugation of SFB to the GE11′ peptide through the free amine group on the lysine. The reaction was performed in dimethylsulfoxide (DMSO) in the presence of *N,N*-diisopropylethyl amine (DIPEA) at 60°C to furnish **1** with 40% yield after HPLC purification (Scheme 2).[32] The iodinated standard, IB-GE11′ (**2**), was obtained similarly to **1** by conjugation of SIB to GE11′ in dimethylformamide (DMF) in the presence of TEA at room temperature (RT) with 35% yield after HPLC purification (Scheme 2).[33] In order to obtain F-PEG4-1,3-triazole-GE11′ (**3**), which served as the standard for the click labeling, first, the azide moiety had to be introduced onto the GE11′ peptide. For this aim, 5-bromo-pentanoic acid ethyl ester was reacted with sodium azide in DMSO[34] to obtain **8** with 67% yield (Scheme 1). Basic hydrolysis[34] of **8** gave 5-azido-pentanoic acid (**9**) with 66% yield, which was conjugated to GE11′ through its lysine moiety using *O*-(*N*-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TSTU) and DIPEA to form the azido derivative of GE11′, **10**, with 41% yield.[34] The desired product, F-PEG4-1,3-triazole-GE11′ (**3**), was obtained by the click chemistry between compound **6**, dissolved in dichloromethane, and peptide **10** dissolved in a mixture of DMF/acetonitrile/H_2O and in the presence of copper (I) iodide (CuI), sodium ascorbate, and DIPEA at 0°C with 20% yield after HPLC purification (Scheme 1).[36] For radiometal labeling, the isothiocyanate group in the bifunctional chelator, NOTA-Bn-NCS, was reacted with the lysine-free amine on the GE11′ peptide in $NaHCO_3$ buffer (pH9) to furnish **11** with 55% yield after HPLC purification (Scheme 3).[37] Reacting peptide **11** with an excess of indium chloride ($InCl_3$) in 0.1 M ammonium acetate buffer (pH5.5) at

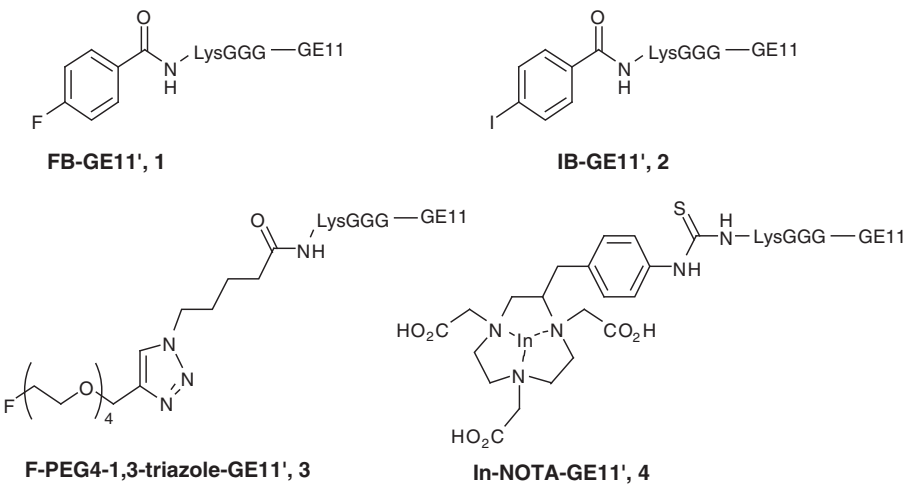
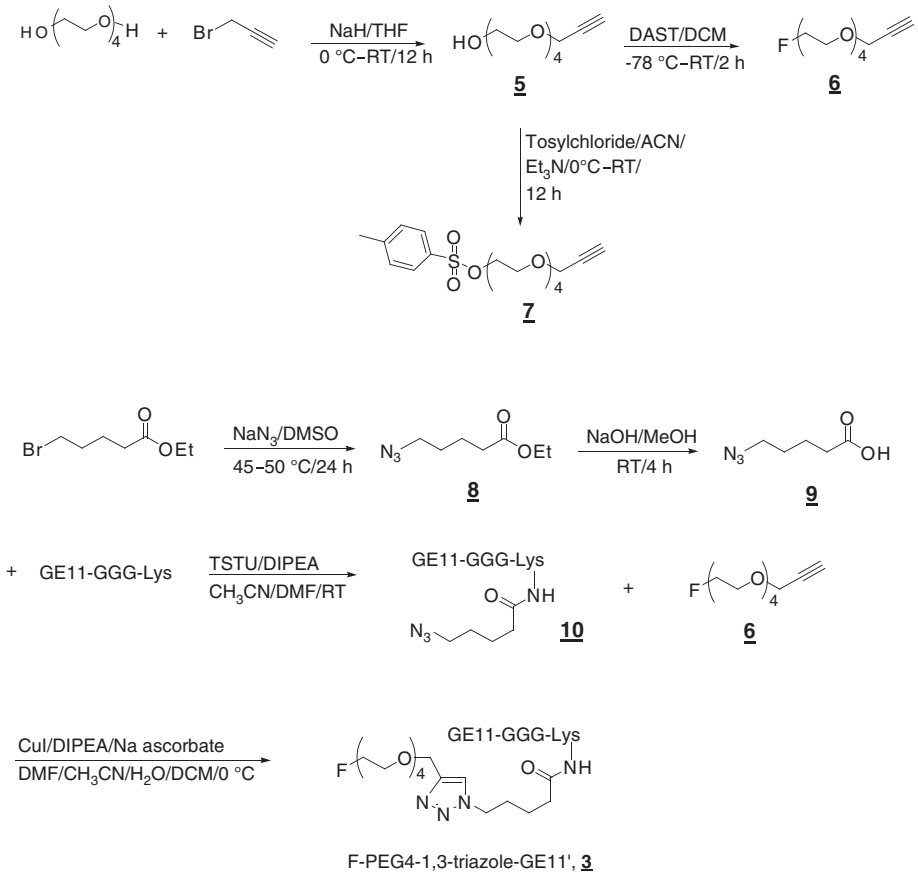
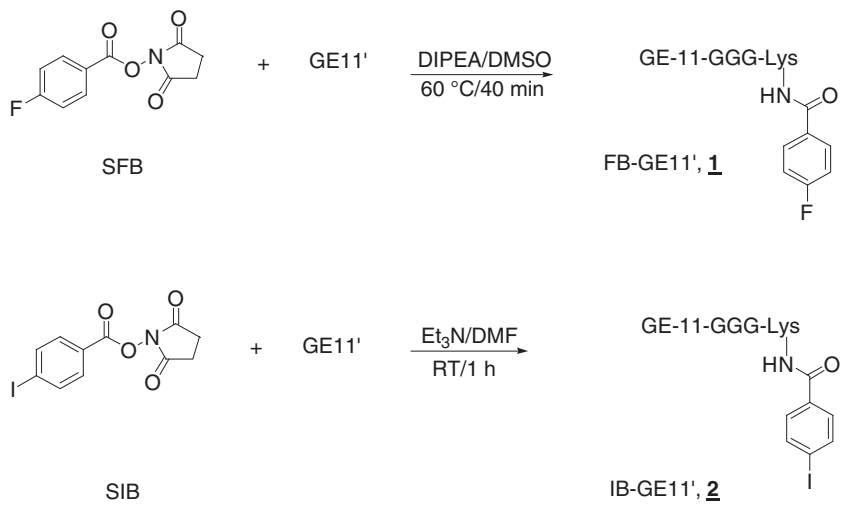


Figure 2. Chemical structures of novel conjugated GE11′ derivatives **1** to **4**.



Scheme 1. Conjugation of tetraethylene glycol (PEG4) residue to the GE11′ peptide by click chemistry.



Scheme 2. Conjugation of SFB and SIB to the GE11-GGGK (GE11′) peptide.

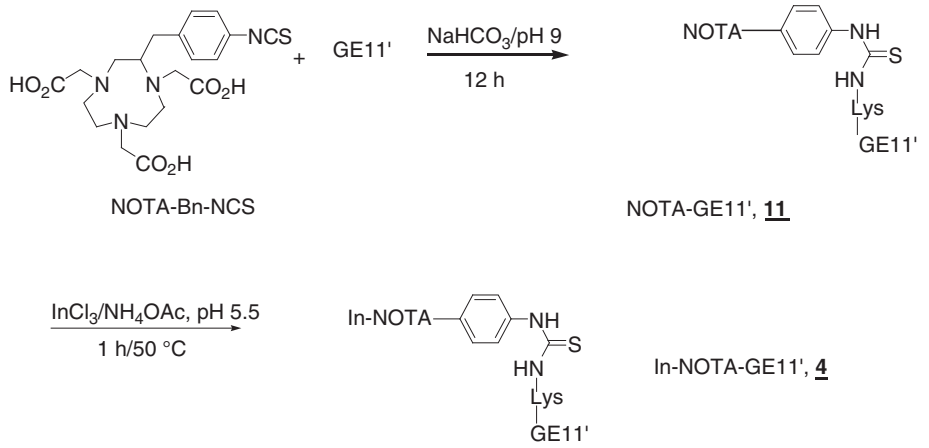
50 °C[38] furnished the desired standard In-NOTA-GE11′ (**4**) with 35% yield after HPLC purification (Scheme 3).

Radiochemistry

Labeling of the GE11 peptide was performed directly on tyrosine residues with (1) free [^{124}I]iodine; (2) through coupling of labeled prosthetic groups, [^{124}I]SIB and [^{18}F]SFB, to the lysine moiety in GE11′; (3) by the click reaction between [^{18}F]F-PEG4-propyne and the azido derivative of GE11′, **10**; or (4) with ^{111}In through the NOTA chelator conjugated to GE11′. It should be noted that the availability of iodine-124 is limited. However, the growing interest in this isotope for PET application may make it more readily accessible in the near future.

Direct radiolabeling of GE11 with ^{124}I

The direct labeling of GE11 with ^{125}I and its biodistribution in mice were reported in the original publication on GE11.[23,24]



Scheme 3. Conjugation of NOTA-Bn-NCS chelator to the GE11′ peptide.

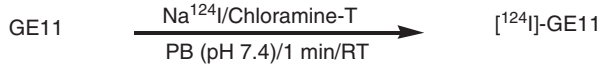
Thus, in order to have a reference point in future comparative biodistribution studies with the newly developed labeled derivative, GE11 was labeled directly with ^{124}I, using Na^{124}I solution (0.02 M NaOH) and chloramines-T in phosphate buffer (pH 7.4).[39] The labeled peptide, [^{124}I]GE11, was obtained with 47% radiochemical yield (RCY), a specific activity (SA) of 1030 Ci/mmol, and 98% radiochemical purity (RCP) (n=2) (Scheme 4).

Radiolabeling of GE11′ with the labeled prosthetic` groups

The modified GGGK-GE11 (GE11′) peptide was conjugated to the radiolabeled prosthetic groups: [^{18}F]SFB, [^{18}F]FPEG4-propyne, [^{124}I]SIB, and NOTA for ^{111}In labeling. The indirect radiolabeling reactions to obtain [^{18}F]FB-GE11′ (**[^{18}F]-1**) [^{124}I]IB-GE11′ (**[^{124}I]-2**), [^{18}F]F-PEG4-propyne (**[^{18}F]-3**), and [^{111}In]NOTA- GE11′ (**[^{111}In]-4**) are outlined in Scheme 5.

*Radiosynthesis of [^{18}F]FB-GE11 (**[^{18}F]-1**)*

The automated synthesis of [^{18}F]SFB was performed in a two-step reaction using two connected GE Tracerlab® modules. The first module was used for the [^{18}F]-fluorination of ethyl 4–(trimethylammonium triflate)benzoate in acetonitrile at 95°C, using kryptofix®$_{222}$/K_2CO_3 followed by basic hydrolysis with 0.5 M NaOH at 90°C. After Sep-Pak C18 (Oasis) purification, the desired labeled compound, [^{18}F]4-fluoro-benzoic acid ([^{18}F]FBA), was obtained in acetonitrile with 50% decay-corrected yield (DCY) and 95% RCP (n=5).[40,41] The [^{18}F]FBA was automatically transferred to the reactor of the second module (from the product vial in module 1 via peek tubing directly to the reactor of module 2); the acetronitrile was evaporated; and 0.5 M tetramethylammonium hydroxide was added. TSTU dissolved in acetonitrile was added, and the reaction proceeded at 95°C for 2 min.[40,41] After HPLC purification, [^{18}F]SFB was obtained with 26% DCY, a SA of 844 Ci/mmol, and 100% RCP (n=5). The conjugation of [^{18}F]SFB to GE11′ was first performed in borate buffer (pH 8.4), yielding the labeled peptide **[^{18}F]-1** with a poor DCY of 7%. However, when the solvent was replaced with DMSO, DIPEA was added, and the reaction temperature was decreased to 60°C for 40 min,[32] the DCY was significantly increased to 22%. The total radiosynthesis time, including purification and formulation, for the labeled peptide [^{18}F]FB-GE11′ (**[^{18}F]-1**) was 3.5 h, with a RCP of 95% and a SA of 108 Ci/mmol (n=2).



Scheme 4. Direct labeling of the GE11 peptide with iodine-124.

*Radiosynthesis of [^{124}]IB-GE11′ (**[^{124}I]-2**)*

The stanyl precursor STB dissolved in methanol was added to dried [^{124}I]NaI followed by the addition of *N*-chlorosuccinimide and acetic acid (5% in methanol) as oxidant. The reaction mixture was stirred at RT for 20 min.[24] After purification on a Sep-Pak C18 (Oasis), the desired [^{124}I]SIB was obtained with 95% RCP, a SA of 3600 Ci/mmol, and a RCY of 65%. The conjugation between GE11′ and dried [^{124}I]SIB performed in DMF in the presence of TEA[33] at RT yielded [^{124}I]IB-GE11′ (**[^{124}I]-2**) with 98% RCP, 30% RCY, and a SA of 300 Ci/mmol (n=2). The total radiosynthesis time was 2 h.

*Radiosynthesis of [^{18}F]FPEG4-1,3-triazole-GE11′ (**[^{18}F]-3**)*

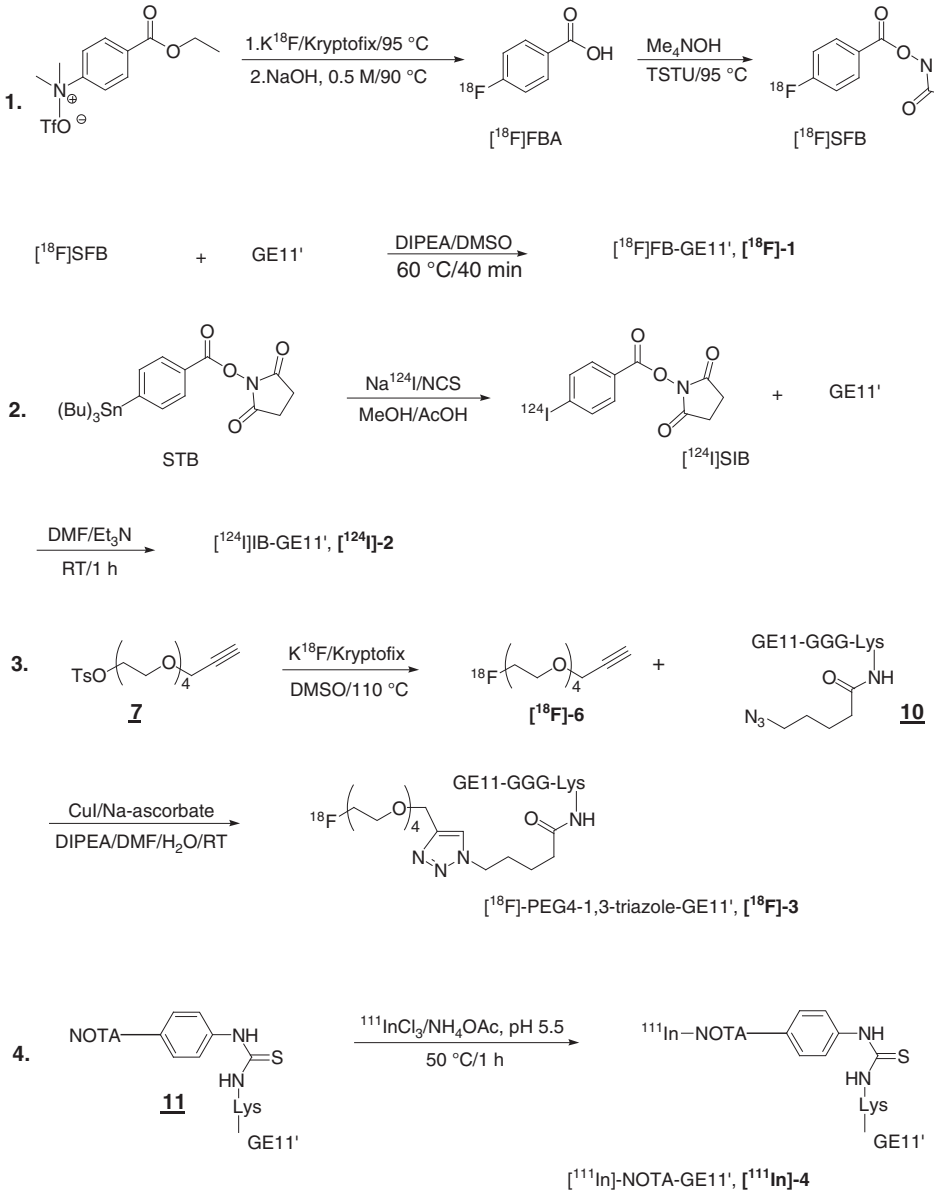
The tosyl precursor **7** was reacted with dried ^{18}F-fluoride in the presence of K_2CO_3/kryptofix®$_{222}$.[35] The reaction mixture was purified by HPLC to yield **[^{18}F]-6** with 30% DCY, 100% RCP, and a SA of 2850 Ci/mmol. The click reaction between **[^{18}F]-6** and the azido derivative of GE11′, **10**, to obtain the triazole ring was performed in the presence of CuI, sodium ascorbate, and DIPEA for 10 min at RT.[36] The labeled click product **[^{18}F]-3** was obtained with 10% DCY, a SA of 500 Ci/mmol, and 98% RCP (n=2) after a total radiosynthesis time of 2 h.

*Radiosynthesis of [^{111}In]NOTA- GE11′ (**[^{111}In]-4**)*

The radiometal labeling of the NOTA-GE11′ (**11**) was performed in 0.1 M ammonium acetate buffer (pH 5.5) and [^{111}In]$InCl_3$ at 50°C.[38] After HPLC purification, **[^{111}In]-4** was obtained with 60% DCY, a SA of 0.35 Ci/mmol, and 100% RCP (n=2) after a total radiosynthesis time of 2 h.

Conclusion

The modified GE11 peptide was successfully labeled with different radionuclides using the prosthetic groups [^{18}F]SFB, [^{124}I]SIB, [^{18}F]F-PEG4-PROPYNE, and the NOTA chelator for ^{111}In labeling. The impact of each labeling approach on imaging potential would be investigated via a series of microPET and biodistribution studies in order to identify the leading labeled compound that could be used for the noninvasive diagnosis and quantification of EGFR expression.



Scheme 5. Radiolabeling of the GE11′ peptide by using various prosthetic groups.

Experimental

The sequences of peptides GE11 and GE11′ were custom-synthesized by GL Biochem Ltd (Shanghai). The structures and purity of both peptides were confirmed by HPLC and mass spectrometry (MS). All chemicals were purchased from Sigma-Aldrich, Fisher Scientific, Merck, and J.T. Baker. THF was dried over sodium/benzophenone; dichloromethane was dried over phosphorous pentoxide; and toluene was dried over sodium. These solvents were freshly distilled prior to their use. All other solvents were purchased as anhydrous. ^{1}H-NMR spectra were recorded on a 300-MHz spectrometer in $CDCl_3$ or DMSO-d_6. ^{1}H-NMR signals were referenced to the residual proton (2.50 ppm for DMSO-d_6; 7.26 ppm for $CDCl_3$) of a deuterated solvent. Mass spectra were obtained on a spectrometer equipped with CI, EI, and FAB probes and on a spectrometer equipped with an ESI probe. HRMS were obtained on MALDI-TOF and ESI-MS. Thin layer chromatography was developed on silica gel 60 F_{254} plates (Merck). Solid-phase extraction (SPE) was performed on a Waters C-18 cartridge preactivated with ethanol and water. For the reversed-phase HPLC, an analytical Waters μBondapak C-18 column (10 μm, 125A, 300 × 3.9 mm) and a preparative Macherey Nagel Nucleosil C-18 column (100, 250 × 16 mm) were used with a mixture of water/acetonitrile/0.2% trifluoroacetic acid (TFA). A Varian 9012Q pump, a Varian 9050 variable wavelength detector operating at 254 or 220 nm, and a Bioscan Flow-Count radioactivity detector with a NaI crystal (GE) were used. Specific activities were determined by analytical column HPLC, using cold mass calibration curves. Radiosyntheses using [^{18}F]fluoride were carried out on a GE Tracerlab® module. [^{18}F]Fluoride was produced on an IBA 18/9 cyclotron by irradiation of 3.2 mL of target water (97%-enriched [^{18}O]water) by the [^{18}O(p,n)^{18}F] nuclear reaction and was transferred to the appropriate module. [^{124}I]NaI was purchased in a 0.02 M NaOH solution from Ritverc GmbH, Germany, and [^{111}In]$InCl_3$ was purchased in a 0.02 M HCl solution from Mallinckrodt, the Netherlands. A dose calibrator (BioDex Medical System, ATOMLAB

300) was used for the measurement of the radioactivity values. A radio-TLC scanner (Bioscan system 200 imaging scanner) was used for the measurement of the radiochemical purities of the radiolabeled compounds.

N-Succinimidyl 4-fluorobenzoate

N-Hydroxysuccinimide (0.5 g, 4.3 mmol) was added to a solution of 4-fluorobenzoic acid (0.5 g, 3.66 mmol) dissolved in dry THF (5 mL) followed by the addition of DCC (0.62 g, 3 mmol) under a stream of nitrogen.[27–29] The reaction mixture was stirred for 12 h at RT. The white precipitates were filtered, and the filtrate was evaporated. The crude product was purified by silica gel chromatography using 1% methanol in dichloromethane as eluent to obtain SFB (0.692 g, 80%). ^{1}H-NMR ($CDCl_3$): δ 8.18 (d, J=5.5 Hz, 2H), 7.2 (t, J=9 Hz, 2H), 2.9 (s, 4H).

Ethyl 4-(trimethylammonium triflate)benzoate

Methyltriflate (0.2 mL, 1.76 mmol) was added to a solution of 4-dimethylaminobenzoic acid ethyl ester (0.5 g, 2.55 mmol) in dry ether (5 mL) and stirred for 12 h at RT.[27] The produced crystals were washed with ether and dried to afford (4-ethoxy-carbonyl-phenyl)-trimethylammonium triflate (0.6 g, 90%). ^{1}H-NMR ($CDCl_3$): δ 8.19 (d, J=4.7 Hz, 2H), 7.86 (d, J=7 Hz, 2H), 4.38 (q, J=7 Hz, 2H), 3.55 (s, 9H), 1.36 (t, J=7 Hz, 3H). MS (*m/z*): 208 $[MH]^+$.

4-Fluoro-benzoate-GE11′ (**1**)

SFB (1.5 mg, 6.19 μmol) was dissolved in DMSO (50 μL) and added to the GE11′ peptide solution (DMSO, 1 mL), followed by the addition of DIPEA (10 μL).[32] The reaction mixture was heated to 60°C for 40 min, diluted with water, filtered, and purified on an HPLC preparative column. The desired product was collected, evaporated, and lyophilized to obtain **1** (5 mg, 40%). MALDI-TOF-MS (*m/z*): 2004 $[MH]^+$. HRMS (EI): calcd. for $C_{96}H_{126}N_{23}O_{24}FNa$: 2026.9232, found: 2026.9228.

N-Succinimidyl 4-iodobenzoate

DCC (0.59 g, 2.85 mmol) was added to a solution of 4-iodobenzoic acid (0.5 g, 2.01 mmol) and *N*-hydroxysuccinimide (0.255 g, 2.21 mmol) in dichloromethane (15 mL).[25] After 12 h of stirring, the white precipitates were filtered, and the filtrate was evaporated. The resulting solid was suspended in a mixture of dichloromethane/hexane 50:50 (v/v) and filtered. The insoluble material was recrystallized from methanol to yield white crystals of SIB (0.197 g, 28%). ^{1}H-NMR ($CDCl_3$): δ 7.9 (d, J=8.4 Hz, 2H), 7.83 (d, J=8.4 Hz, 2H), 2.9 (s, 4H).

N-Succinimidyl 4-(tri-*n*-butylstannyl)-benzoate

Hexabutylditin (0.535 mL, 0.924 mmol) and tetrakis(triphenylphosphine)palladium (0.013 g, 0.011 mmol) were added to a solution of SIB (0.1 g, 0.29 mmol) in anhydrous toluene (3 mL) under a stream of nitrogen.[17] The mixture was refluxed for 2 h, cooled, and evaporated to give an oily crude mixture, which was purified by silica gel chromatography using dichloromethane as eluent to yield STB (0.049 g, 33%). ^{1}H-NMR ($CDCl_3$): δ 8.039 (d, J=8 Hz, 2H), 7.62 (d, J=8 Hz, 2H), 2.91 (s, 4H), 0.89–1.55 (m, 27H).

4-Iodo-benzoate-GE11′ (**2**)

SIB (0.004 g, 0.012 mmol) dissolved in DMF (0.2 mL) was added to a solution of GE11′ peptide (0.02 g, 0.01 mmol) in DMF (1.6 mL), followed by the addition of TEA (20 μL).[33] The mixture was stirred at RT for 1 h, diluted with water, filtered, and purified on an HPLC preparative column. The desired product was collected, evaporated, and lyophilized to obtain **2** (7 mg, 35%). MALDI-TOF-MS (*m/z*): 2112.26 $[MH]^+$. HRMS (EI): calcd. for $C_{96}H_{126}N_{23}O_{24}INa$: 2134.8269, found: 2134.8289.

2-{2-[2-(2-Proponyloxy-ethoxy)-ethoxy]-ethoxy}-ethanol (**5**)

To a solution of tetraethylene glycol (6 g, 30 mmol) in dry THF (30 mL) at 0°C, sodium hydride (0.468 g, 19.5 mmol) was added. The mixture was stirred at 0°C for 30 min under nitrogen followed by the addition of propargyl bromide (1.33 mL, 15 mmol) to the reaction flask.[30] The mixture was stirred at 0°C for an additional 2 h, warmed up to RT, and left for 24 h with stirring. The solution was poured into water, extracted with dichloromethane, washed with brine, dried over sodium sulfate, and evaporated. The crude product was purified by silica gel chromatography, eluting with 3% methanol in dichloromethane to afford **5** (1.4 g, 20%). ^{1}H-NMR ($CDCl_3$): δ 4.2 (d, J=2.4 Hz, 2H), 3.59–3.73 (m, 16H), 2.72 (br, 1H), 2.43 (t, J=2.1 Hz, 1H). MS (*m/z*): 233.1 $[MH]^+$. HRMS (EI) calcd. for $C_{11}H_{21}O_5$: 233.1384, found: 233.1389.

(2-{2-[2-(2-Fluoro-ethoxy)-ethoxy]-ethoxy}-ethoxy)-propyne (**6**)

Diethylaminosulfur trifluoride (0.553 mL, 0.422 mmol) was added dropwise to a solution of **5** (0.163 g, 0.7 mmol) dissolved in dry dichloromethane (3 mL) at −78°C, and the mixture was allowed to stir for 2 h as it warmed to RT.[31] The mixture was cooled to −20°C, quenched by the addition of 1 mL of methanol, and evaporated. The crude product was purified by silica gel chromatography, eluting with 2% methanol in dichloromethane to obtain **6** (0.055 g, 33%). ^{1}H-NMR ($CDCl_3$): δ 4.64 (m, 1H), 4.48 (m, 1H), 4.2 (m, 4H), 3.66–3.81 (m, 12H), 2.42 (t, J=2.1 Hz, 1H). ^{19}F-NMR ($CDCl_3$): δ -223.32. MS (*m/z*): 235.2$[MH]^+$. HRMS (EI): calcd. for $C_{11}H_{19}O_4FNa$: 257.1160, found: 257.1165.

Toluene-4-sulfonic acid 2-{2-[2-(2-ethynyloxy-ethoxy)-ethoxy]-ethoxy}-ethyl ester (**7**)

To a solution of **5** (0.3 g, 1.29 mmol), TEA (0.45 mL, 3.23 mmol) and trimethylamine hydrochloride (12 g, 0.012 mmol) in dry acetonitrile (4 mL), 4-methyl-benzenesulfonyl chloride (0.492 g, 2.58 mmol) was added slowly at 0°C under nitrogen; the mixture was stirred for 1 h and the stirring was continued at RT for an additional 12 h.[30] The reaction mixture was poured into water, extracted with ethyl acetate, and dried over $MgSO_4$, and the solvent was removed under reduced pressure. The crude product was purified by silica gel chromatography, eluting with 2% methanol in dichloromethane to afford **7** (0.322 g, 64%). ^{1}H-NMR ($CDCl_3$): δ 7.8 (d, J=8.1 Hz, 2H), 7.35 (d, J=8.1 Hz, 2H), 4.2 (m, 2H), 4.17 (t, J=4.8 Hz, 2H), 3.58–3.7 (m, 14H), 2.44 (s, 3H), 2.42 (m, 1H). MS (*m/z*): 409.2 $[M+Na]^+$. HRMS (EI): calcd. for $C_{18}H_{27}O_7S$: 387.1474, found: 387.1478.

5-Azido-pentanoic acid ethyl ester (**8**)

Sodium azide (1.85 g, 28.5 mmol) was added to a solution of 5-bromo-pentanoic acid ethyl ester (4 g, 19 mmol) in DMSO

(10 mL).[34] The suspension was heated to 50°C for 24 h, cooled, poured into water, and extracted with ether. The extracts were washed with brine and dried over sodium sulfate, and the solvent was evaporated. The crude oil **8** (2.2 g, 67%) was maintained under vacuum for 24 h and used in the next step without any further purification. ^{1}H-NMR ($CDCl_3$): δ 4.14 (q, *J*=6 Hz, 2H), 3.29 (t, *J*=6.6 Hz, 2H), 2.33 (t, *J*=5.7 Hz, 2H), 1.59–1.74 (m, 4H), 1.27 (t, *J*=6 Hz, 3H). IR: ν (2097.65, N_3), (1734.44, CO). MS (*m/z*): 172.1 $[MH]^+$.

5-Azido-pentanoic acid (9)

To a solution of **8** (0.5 g, 2.92 mmol) in 0.7 mL of methanol, 3.5 mL of 1 M NaOH was added, and the solution was stirred for 4 h at RT.[34] The organic solvent was removed, and the aqueous solution was extracted with ether and acidified with concentrated HCl. The acidic solution was extracted with ether, dried over sodium sulfate, and evaporated to yield **9** as crude oil (0.274 g, 66%), which was maintained under vacuum for 24 h and used in the next step without any further purification. ^{1}H-NMR ($CDCl_3$): δ 10.17 (br, 1H), 3.32 (t, *J*=6.6 Hz, 2H), 2.34 (t, *J*=6.6 Hz, 2H), 1.5–1.77 (m, 4H). IR: ν (2943.16, OH), (2098.54, N_3), (1708.7, CO). MS (*m/z*): 144 $[MH]^+$.

(5-Azido-pentionyl)-GE11′ (10)

To a solution of 5-azidopentanoic acid (**9**) (0.02 g, 0.14 mmol) and DIPEA (21.4 μL) in acetonitrile (0.5 mL), TSTU (0.029 g, 0.0965 mmol) was added.[35] The reaction mixture was stirred at RT for 30 min and then added to GE11′ (0.03 g, 0.016 mmol) dissolved in DMF (1 mL), and left for another 2 h at RT with stirring. The crude product was diluted with water, filtered, and purified on an HPLC preparative column. The desired product was collected, evaporated, and lyophilized to obtain **10** as a white powder (0.013 g, 41%). MALDI-TOF-MS (*m/z*): 2007 $[MH]^+$. HRMS (EI): calcd. for $C_{94}H_{130}N_{26}O_{24}Na$: 2029.9628, found: 2029.9649.

F-PEG4-1,3-triazole-GE11′ (3)

A solution of **6** (0.011 g, 0.049 mmol) in dichloromethane (0.4 mL) was added to a solution of **10** (0.01 g, 0.0049 mmol) in a mixture of DMF (0.6 mL), water (0.7 mL), and acetonitrile (0.6 mL), followed by the addition of sodium ascorbate (0.098 g, 0.49 mmol), CuI (0.0094 g, 0.049 mmol), and DIPEA (92 μL, 0.53 mmol).[36] The reaction mixture was stirred at 0°C for 1 h, diluted with water, filtered, and separated on an HPLC preparative column. The desired product was collected, evaporated, and lyophilized to obtain **3** (22 mg, 20% yield). MALDI-TOF-MS (*m/z*): 2263.7 [M+Na]. HRMS (EI): calcd. for $C_{105}H_{149}N_{26}O_{28}FNa$: 2264.0867, found: 2264.0916.

NOTA- GE11′ (11)

NOTA-Bn-NCS (8.9 mg, 15.9 μmol) was added to a solution of GE11′ (10 mg, 5.3 μmol) in 1 mL of 0.1 M $NaHCO_3$ buffer (pH9) and stirred for 12 h at RT.[37] The crude product was separated on an HPLC preparative column. The desired product was collected, evaporated, and lyophilized to obtain **11** (7.4 mg, 55%). MALDI-TOF-MS (*m/z*): 2332.48$[MH]^+$. HRMS (EI): calcd. for $C_{109}H_{148}N_{27}O_{29}S$: 2331.0652, found: 2331.0657.

In-NOTA-GE11′ (4)

$InCl_3$ (1.4 mg, 6.4 μmol) was added to a solution of **11** (3 mg, 1.28 μmol) in 0.5 mL of 0.1 M ammonium acetate buffer (pH5.5) and stirred at 50°C for 1 h, followed by the addition of 10 mM ethylenediaminetetraacetic acid (EDTA) solution.[38] The crude product was separated on an HPLC preparative column. The desired product was collected, evaporated, and lyophilized to obtain **4** (1.25 mg, 35%). MALDI-TOF-MS (*m/z*): 2448.71$[MH]^+$. HRMS (EI): calcd. for $C_{109}H_{149}N_{27}O_{29}SIn$: 2446.9773, found: 2446.9774.

Radiochemistry

[^{124}I]GE11

Two hundred micrograms of GE11′ in 0.1 M phosphate buffer (1 mg/mL, pH7.4) was added to a solution of [^{124}I]NaI (0.13 mL) in 0.02 M NaOH, followed by the addition of chloramine-T (50 μL, 1 mg/mL in H_2O). The mixture was incubated for 1 min at RT.[39] The crude product was injected into an HPLC preparative column, eluting with a mixture of water/acetonitrile 75: 25 (v/v) for 50 min (flow rate, 8 mL/min). The collected fractions containing [^{124}I]GE11′ were combined (Rt=20.17 min) and evaporated to remove acetonitrile and TFA and neutralized by the addition of 1 M NaOH. The product solution was passed through an SPE C-18 cartridge, washed with water (4 mL), and eluted with ethanol (0.3 mL). [^{124}]GE11′ was obtained after a total radiosynthesis time of 1 h with 47% DCY, a SA of 1030 Ci/mmol, and 98% RCP (*n*=2).

[^{18}F]4-Fluorobenzoic acid

[^{18}O]H_2O/$^{18}F^-$ was trapped on an ion-exchange column and eluted with 0.5 mL of K_2CO_3 (5 mg/mL).[40,41] Kryptofix®$_{222}$ (15 mg/mL in acetonitrile) was added, and solvents were removed by azeotropic distillation at 95°C under reduced pressure for 3 min. A solution of (4-ethoxycarbonyl)-trimethyl-ammonium triflate (5 mg/1 mL in acetonitrile) was added to the reactor containing the dried complex K^{18}F/kryptofix. The solution was heated to 95°C for 10 min and then cooled to 40°C; 0.5 M NaOH (0.8 mL) was added to the [^{18}F]4-fluoro-benzoic acid ethyl ester and heated to 90°C for another 10 min. The reaction mixture was cooled, neutralized with 1 M HCl (3 mL), and diluted with water. The crude product [^{18}F]FBA was passed through an SPE C-18 Sep-Pak (Oasis), washed with water (10 mL), and eluted with acetonitrile (3 mL) to afford [^{18}F]FBA with a DCY of 50% and 95% RCP (*n*=5).

[^{18}F]Succinimidyl 4-fluorobenzoate

[^{18}F]FBA in tetramethylammonium hydroxide (10 mg, 0.125 mL) was dried under a stream of argon at 75°C, then a solution of TSTU in acetonitrile was added at 95°C, and the mixture was stirred for 2 min.[40,41] The resulting crude product [^{18}F]SFB solution was diluted with acetonitrile/water 50:50 (v/v), injected into an HPLC preparative reversed phase column, and then eluted with water/acetonitrile/0.2% TFA (70:30) (v/v) (flow rate, 8 mL/min). The labeled product was collected (Rt=19 min) in a flask diluted with water (90 mL) and loaded onto an SPE C-18 cartridge (Oasis), washed with water (10 mL), and eluted with acetonitrile (3 mL) to obtain [^{18}F]SFB with a DCY of 26%, a SA of 844 Ci/μmol, and a RCP of 100% (*n*=5).

*[^{18}F]FB-GE11′ (**[^{18}F]-1**)*

To a solution of [^{18}F]SFB in DMSO (0.2 mL), 660 μg of GE11′ in DMSO was added, followed by the addition of DIPEA (10 μL); the mixture was stirred for 40 min at 60 °C.[32] The reaction mixture was cooled, diluted with water/acetonitrile 50:50 (v/v), and injected into an HPLC preparative column eluted with a mixture of water/acetonitrile/0.1% TFA (80:20, v/v) (flow rate, 8 mL/min). The labeled product was collected (Rt = 22 min) and evaporated to remove acetonitrile and TFA, and then neutralized by the addition of 1 M NaOH. The product solution was passed through an SPE C-18 cartridge, washed with water (10 mL), and eluted with ethanol (0.3 mL). **[^{18}F]-1** was obtained after a total radiosynthesis time of 3.5 h, with a DCY of 22%, a SA of 108 Ci/mmol, and 95% RCP (n = 2).

[^{124}I]Succinimidyl 4-iodobenzoate

Na^{124}I solution (0.02 M NaOH) was evaporated to dryness under nitrogen stream, and 0.2 mL of STB (1 mg/mL in methanol) was added followed by the addition of *N*-chlorosuccinimide (50 μL, 1 mg/mL in methanol) and 5% acetic acid (350 μL, in methanol).[25] The reaction mixture was stirred for 20 min at RT, purified, and eluted with acetonitrile (2 mL) on an SPE C-18 Sep-Pak (Oasis) to afford [^{124}I]SIB with 65% RCY, a SA of 3600 Ci/mmol, and 95% RCP (n = 2).

*[^{124}I]IB-GE11′ (**[^{124}I]-2**)*

Triethylamine (50 μL) and 2.5 mg of GE11′ (5 mg/mL in DMF) were added to dried [^{124}I]SIB. The reaction mixture was incubated for 1 h at RT,[33] diluted with water/acetonitrile 50:50 (v/v), and injected into an HPLC preparative column eluted with a mixture of water/acetonitrile 75: 25 (v/v) (flow rate, 8 mL/min). The collected fractions containing [^{124}I]IB-GE11′ were combined (Rt = 26 min) and evaporated to remove acetonitrile and TFA, and then neutralized by the addition of 1 M NaOH. The product solution was passed through an SPE C-18 cartridge, washed with water (4 mL), and eluted with ethanol (0.5 mL). **[^{124}I]-2** was obtained after a total radiosynthesis time of 2.5 h, with 30% RCY, a SA of 300 Ci/mmol, and 98% RCP (n = 2).

*[^{18}F]F-PEG4-propyne (**[^{18}F]-6**)*

A solution of **7** (5 mg/mL in DMSO) was added to the reactor containing the dried complex K^{18}F/kryptofix. The solution was heated to 110 °C for 10 min,[34] cooled to 35 °C, diluted with 1 mL of acetonitrile/water 50:50 (v/v), and injected into an HPLC preparative column eluted with water/acetonitrile/0.2% TFA (70:30) (v/v) (flow rate, 6 mL/min). The labeled product was collected (Rt = 12 min) in a flask diluted with water (50 mL) and loaded onto an SPE C-18 cartridge, washed with water (10 mL), and eluted with THF (2 mL) to obtain **[^{18}F]-6** with a DCY of 30%, a SA of 2580 Ci/mmol, and a RCP of 100% (n = 5).

*[^{18}F]F-PEG4-1,3-triazole-GE11′ (**[^{18}F]-3**)*

[^{18}F]-6 in acetonitrile (0.3 mL) was reacted with compound **10** (1 mg, 0.0005 mmol) in the presence of sodium ascorbate (32 mg, 0.16 mmol), CuI (3 mg, 0.016 mmol), DIPEA (30 μL, 0.175 mmol) in a mixture of DMF (0.2 mL) and water (0.25 mL).[36] The reaction mixture was stirred vigorously at RT for 10 min. The crude product was diluted with water, filtered, and injected into an HPLC preparative column eluted with a gradient of water/acetonitrile/0.2% TFA 85:15 (v/v) (flow rate, 8 mL/min). The product **[^{18}F]-3** was collected (Rt = 29.3 min); acetonitrile/TFA was evaporated; and the remaining mixture was neutralized by the addition of 1 M NaOH, passed through an SPE C-18 cartridge, washed with water (5 mL), and eluted with ethanol (0.5 mL). **[^{18}F]-3** was obtained after a total radiosynthesis time of 2.5 h, with a DCY of 10%, a SA of 500 Ci/mmol and 98% RCP (n = 2).

*[^{111}In]NOTA- GE11′ (**[^{111}In]-4**)*

The precursor **11** (1 mg, 0.42 μmol) dissolved in 0.1 mL of 0.1 M ammonium acetate buffer (pH 5.5) was added to a solution of [^{111}In]$InCl_3$ (0.5–2 mCi). The mixture was heated to 50 °C for 1 h,[38] and the reaction was terminated by the addition of 10 μL of 10 nM EDTA solution. The crude product was filtered and injected into an HPLC preparative column eluted with a mixture of water/acetonitrile/0.1% TFA (80:20, v/v) (flow rate, 8 mL/min). The collected fractions were combined (Rt = 13.6 min), and acetonitrile/TFA were removed. The product solution was loaded on an SPE C-18 cartridge and eluted with ethanol (0.5 mL). The final product, **[^{111}In]-4**, was obtained after a total radiosynthesis time of 2 h, with a DCY of 60%, a SA of 0.35 Ci/mmol, and 100% RCP (n = 2).

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