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One-step ^{18}F labeling of biomolecules using organotrifluoroborates

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Abstract

Herein we present a general protocol for the functionalization of biomolecules with an organotrifluoroborate moiety so that they can be radiolabeled with aqueous ^{18}F fluoride ($^{18}\text{F}^-$) and used for positron emission tomography (PET) imaging. Among the β^+ -emitting radionuclides, fluorine-18 (^{18}F) is the isotope of choice for PET, and it is produced, on-demand, in many hospitals worldwide. Organotrifluoroborates can be ^{18}F -labeled in one step in aqueous conditions via ^{18}F – ^{19}F isotope exchange. This protocol features a recently designed ammoniomethyltrifluoroborate, and it describes the following: (i) a synthetic strategy that affords modular synthesis of radiolabeling precursors via a copper-catalyzed ‘click’ reaction; and (ii) a one-step ^{18}F -labeling method that obviates the need for HPLC purification. Within 30 min, ^{18}F -labeled PET imaging probes, such as peptides, can be synthesized in good chemical and radiochemical purity (>98%), satisfactory radiochemical yield of 20–35% ($n > 20$, non-decay corrected) and high specific activity of 40–111 GBq/ μmol (1.1–3.0 Ci/ μmol). The entire procedure, including the precursor preparation and ^{18}F radiolabeling, takes 7–10 d.

INTRODUCTION

PET is a fast-expanding, noninvasive imaging technology for visualizing and quantifying the dynamic distribution of target-specific ligands^{1–4}. Peptides, which are a class of relatively large water-soluble molecules with good target selectivity, often demonstrate high-contrast tumor-specific PET images, and thus they are promising imaging agents. A one-step ^{18}F -labeling method, which is sufficiently versatile to afford broad applications for most peptides, has been a longstanding challenge in radiochemistry^{5,6}. To achieve this goal,

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several approaches have been reported over the past few years that represent substantial advances in the radiolabeling of peptides and small molecules^{5,7–15}. However, to date, most of the reported strategies require anhydrous conditions, which involve an azeotropic ‘drying’ procedure that enhances the reactivity of $^{18}\text{F}^-$, thereby ensuring good radiochemical yields¹⁶. Furthermore, to achieve high purity and high specific activity, which defines as GBq/ μmol (Ci/ μmol) and represents an impartial standard of radiotracer quality, time-consuming HPLC purification is often required¹⁷. These laborious procedures, such as azeotropic drying and HPLC purification, often reduce the radiochemical yield, while at the same time they increase the risk of synthetic failure. To solve these problems, a one-step and easy-to-follow protocol is described here for peptide radiolabeling that is based on the use of a recently developed organotrifluoroborate¹⁸.

In 2005, the Perrin group published a proof-of-concept study that described the labeling of an aryltrifluoroborate as a radiosynthon that could be ^{18}F labeled in aqueous conditions¹⁹. In the following years, this research group obtained promising biological data using this approach to develop PET imaging agents^{20,21}. Despite this initial success, early reports on PET imaging agents obtained via boron-mediated ^{18}F capture suffered from several apparent drawbacks¹⁰, namely, (i) insufficient *in vivo* stability, (ii) limited specific activity and (iii) stepwise and laborious operational procedure.

Over the past few years, most of these disadvantages have been addressed. The first problem was solved by designing and synthesizing a new generation of organotrifluoroborates that are stable *in vivo*^{22,23}. The second problem was largely solved by exploring ^{18}F radiolabeling at high levels of activity to increase the specific activity of ^{18}F radiotracers up to ~555 GBq/ μmol (refs. 7,24), which is orders of magnitude higher than in our previous reports^{25–27}. Nevertheless, to advance this approach to a general and clinician-friendly method, we developed a novel B- ^{18}F radiosynthon by screening a series of organotrifluoroborates²². This compound combines high *in vivo* stability, synthetic and bioconjugation simplicity, and convenient ^{18}F -labeling technology, which is based on ^{18}F - ^{19}F isotope exchange (IEX) of the ^{19}F -containing modified biomolecule²⁸, and it represents the core of this protocol and has smoothly solved the third problem²². On the basis of this method, a number of bioligands have been radiolabeled and evaluated, and some of them have demonstrated excellent *in vivo* performance^{18,29–31}. Particularly, ammoniomethyltrifluoroborate (AMBF₃)-TATE (Fig. 1), which is an octreotate derivative, showed excellent somatostatin receptor imaging properties, and the relevant bench-to-bedside translation is now underway²⁹.

In contrast to mainstream ^{18}F -labeling methods, which either involve multiple steps or are water sensitive³², this approach has a one-step radiolabeling reaction, and the intermediates are stable in water. For large doses of $^{18}\text{F}^-$, a small quaternary methyl amine (μ -QMA) cartridge can be used (instead of performing azeotropic distillation) to concentrate the radioactivity before radiolabeling. In this approach, Curie levels of $^{18}\text{F}^-$ can be directly eluted with <60 μl of saline (recovery rate: >95%, $n > 50$), and no further concentration is necessary (Step 17A; concentration by azeotropic distillation is still advisable for lower $^{18}\text{F}^-$ doses—e.g., 50 mCi doses).

In ^{18}F – ^{19}F IEX, the chemical structure of the radiolabeled product is identical to that of the precursor. In this case, a simple Sep-Pak purification affords the tracer in high radiochemical purity, and no HPLC purification is required. Furthermore, because of its operational simplicity, the radiolabeling can be simply performed with remote telemanipulators in a fully shielded hot cell. Thus, Curie levels of radioactivity are routinely used and a >7.4 GBq (>200 mCi) product can be reproducibly obtained at high specific activity. A limitation of this protocol is that when starting with a small amount of $^{18}\text{F}^-$ (e.g., 50 mCi) for radiolabeling, as described in Step 17B, an azeotropic-concentrating procedure will be recommended in order to obtain the radiotracer of high specific activity. Overall, this protocol provides researchers a user-friendly tool that can overcome the challenges of preparing ^{18}F -labeled PET tracers.

In summary, this protocol simplifies the radiosynthesis of ^{18}F -labeled biomolecules, an improvement that will potentially promote the development of new PET tracers and accelerate their use from the bench to bedside. Herein, we describe the detailed protocols of (i) μ -QMA cartridge assemblage, (ii) copper-catalyzed radiosynthon conjugation and (iii) ^{18}F – ^{19}F IEX.

Experimental design

This protocol describes the synthesis of $[^{18}\text{F}]\text{BF}_3$ -conjugated PET imaging probes. As a representative, $[^{18}\text{F}]\text{AMBF}_3\text{-TATE}$ is synthesized and used as an actual example to implement this approach. The procedure is outlined in Figure 1. This protocol is generally suitable for ^{18}F labeling at the N termini of peptides; however, the authors would recommend the readers to implement the PROCEDURE exactly as detailed, so as to acquaint themselves with it, before moving on to using a different fluorinated compound. For very acid-sensitive or fragile peptides, one-pot two-step approaches can be considered³³. In addition to radiolabeling peptides, this method can also be used to radiolabel other molecules after minor modification. The precursors will be prepared in similar manners by conjugating alkynyl AMBF_3 on biomolecule azide via copper-catalyzed ‘click’ reaction.

As a general applicable radiosynthon, the preparation of alkynyl AMBF_3 (Fig. 2) has been described in detail in this protocol. The peptide azide can be prepared either using solid-phase synthesis (as described in this PROCEDURE) or in liquid phase by directly conjugating azidoacetic acid on an amine-terminated peptide. In addition, there are many publications that describe how to prepare other types of azide compounds, and there are also commercially available azides that could be used.

The crucial step of this protocol is ^{18}F labeling, and the key factor for this step is the reaction pH. On the basis of our previous studies, a pH of 2–2.5 generally gives better radiochemical yield and lower by-product (boronic acid) levels than other conditions³⁴. To maintain the reaction pH within the desired window, a series of buffers were screened and eventually a pyridazine-HCl buffer was chosen for BF_3 -mediated ^{18}F labeling. A pyridazine-HCl buffer was chosen not only for its ideal pK_a but also because of its high solubility in water and low toxicity (LD_{50} in mice is >2.6 g/kg). The experimental details for the preparation and use of this buffer are described in the Reagent Setup and Step 17 of the PROCEDURE, respectively.

Before radiolabeling, the $^{18}\text{F}^-$ starting solution needs to be concentrated. By using an amount of radioactive precursor that affords 1 Curie level of radioactivity (~ 37 GBq), which can be easily obtained in many clinical PET centers, the azeotropic evaporation step can be obviated, as the $^{18}\text{F}^-$ can be concentrated by using a μ -QMA cartridge. The details and tips related to the preparation of this cartridge are reported in Step 17A(i–vi) of the PROCEDURE. In this case, noncarrier added $^{18}\text{F}^-$ is directly eluted into the reaction vial with ~ 60 μl of saline solution, and the total reaction volume is ~ 150 μl . By starting with 100 nmol of precursor, generally 200 mCi (7.4 GBq) of product can be obtained by the end of synthesis (EOS) to give the specific activity of 2.0 Ci/ μmol (74 GBq/ μmol). However, if you are starting with ~ 50 mCi of $^{18}\text{F}^-$ (~ 1.85 GBq), which is more suitable for research purpose or preclinical studies, azeotropic evaporation is highly recommended in order to achieve a good radiochemical yield, as well as high specific activity (Step 17B(i–v)). In such cases, <10 nmol of precursor is used for radiolabeling, and consequently the reaction volume is reduced to 15 μl . Therefore, the $^{18}\text{F}^-$ solution needs to be concentrated before radiolabeling. The composition of the vial in which the $^{18}\text{F}^-$ is dried is crucial, and glass vials should be avoided because of the presence of fluorophilic silicates on the glass surface. After drying (or concentration), the $^{18}\text{F}^-$ is resuspended with buffered stock solution to initiate the ^{18}F – ^{19}F IEX.

One notable limitation of this protocol might stem from the acidic radiolabeling conditions. Nevertheless, to date, in our hands, these conditions have not been an issue for most bioligands, as they are generally stable in acidic conditions for a short period of time and many of them are even prepared in acidic conditions^{35,36}. For instance, peptides are typically cleaved from the solid base support and purified under the conditions that are substantially more acidic than those used herein (e.g., deprotection in 80% (vol/vol) trifluoroacetic acid (TFA), pH = -2 , purification in 0.1–0.5% (vol/vol) TFA, pH = $1-2$; refs. 37,38).

MATERIALS

REAGENTS

Synthesis of AMBF₃-conjugated biomolecules

- *N,N*-dimethylformamide (DMF; Sigma-Aldrich, cat. no. 270547)
- Anhydrous diethyl ether (Sigma-Aldrich, cat. no. 346136)
- Iodomethyl-pinacol boronate (Combi-Blocks, cat. no. FM-6291)
- Potassium hydrogen fluoride (KHF₂; Sigma-Aldrich, cat. no. 239283)
- NH₄OH (aqueous (aq.), concentrated (conc.), Sigma-Aldrich, cat. no. 320145)
- CuSO₄·5H₂O (Sigma-Aldrich, cat. no. C1297)
- Sodium ascorbate (Sigma-Aldrich, cat. no. A7631)
- Dichloromethane (Sigma-Aldrich, cat. no. 650463)
- Trifluoroacetic acid (TFA; for spectroscopy; Merck Chemicals, cat. no. 108262)

- Triisopropylsilane (99%; Sigma-Aldrich, cat. no. 233781)
- *N,N*-Diisopropylethylamine (98.0%; Fluka, cat. no. 03440)
- Acetonitrile anhydrous (ACN; Sigma-Aldrich, cat. no. 271004)
- Methanol (Sigma-Aldrich, cat. no. 34860)
- DMSO (Sigma-Aldrich, cat. no. D8148)
- 1 N hydrochloric acid (HCl; Sigma-Aldrich, cat. no. 71763)
- Ethanol (Sigma-Aldrich, cat. no. 459844)

Solid-phase peptide synthesis (optional)

- Bromoacetyl *N*-hydroxysuccinimide (NHS) ester (Sigma-Aldrich, cat. no. B8271)
- *N*-methyl-2-pyrrolidone (Sigma-Aldrich, cat. no. 328634)
- Sodium azide (Sigma-Aldrich, cat. no. S2002)
- Phenol (Sigma-Aldrich, cat. no. P1037)
- Piperidine (99%, for peptide synthesis; Sigma-Aldrich, cat. no. 104094)
- O-(Benzotriazol-1-yl)-*N,N,N,N*-tetramethyluronium hexafluorophosphate (98.0%, Sigma-Aldrich, cat. no. 12804)
- Diisopropylcarbodiimide (Sigma-Aldrich, cat. no. 38370)
- Fmoc-Thr(tBu)-Wang resin (100–200 mesh; Novabiochem, cat. no. 856017)
- Fmoc-Cys(Acm)-OH (Novabiochem, cat. no. 852006)
- Fmoc-Thr(tBu)-OH (Novabiochem, cat. no. 852000)
- Fmoc-Lys(Boc)-OH (Novabiochem, cat. no. 852012)
- Fmoc-D-Trp(Boc)-OH (Novabiochem, cat. no. 852164)
- Fmoc-Tyr(tBu)-OH (Novabiochem, cat. no. 852020)
- Fmoc-D-Phe-OH (Novabiochem, cat. no. 852148)
- Thallium(III) trifluoroacetate (Technical grade; Sigma-Aldrich, cat. no. 150533)

Radiolabeling

- Pyridazine (Sigma-Aldrich, cat. no. P57204)
- Deionized water (obtained from Milli-Q integral water purification system)
- HCl (aq. 37%; Sigma-Aldrich, cat. no. 258148)
- Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S9888)

- Saline (Thermo Fisher, cat. no. 721016)
- Phosphate buffer (Thermo Fisher, cat. no. BP399-500)
- Fluorine-18 (1.5 ml, purchased from a supplier or generated by on-site cyclotron from bombardment via the $^{18}\text{O}(\text{p},\text{n})^{18}\text{F}$ reaction)³⁹ ! **CAUTION** Working with radioactive materials needs extra caution. If possible, try to minimize the exposure time to radiation. In addition, to work with radioactivity of more than 200 mCi, all operations should be performed using a telemanipulator in a lead-shielded hot cell.

EQUIPMENT

Synthesis of AMBF₃-conjugated biomolecules

- Round-bottom flask (Sigma-Aldrich, cat. no. Z723134)
- Sand-core funnel (National Institutes of Health (NIH) self-service store or institutional glassware shop)
- High-vacuum pump (direct-drive high-vacuum pumps, Welch, cat. no. 8917)
- Sep-Pak alumina N light cartridge (Waters, cat. no. WAT023561)
- Argon (Robert Oxygen)

Preparation and concentration of $^{18}\text{F}^-$

- BD PrecisionGlide needle, 16 gauge $\times$ 1 1/2-inch (Med Plus Physical Supplies, cat. no. BD 305198)
- μ -QMA cartridge (This product was previously available from ORTG with the item name as $^{18}\text{F}^-$ Trap & Release Columns. However, ORTG has been out of business since 2014, and this type of QMA cartridge is currently available from MedChem Imaging)
- pH meter (Thermo Fisher, cat. no. S90528)
- Wire cutters (Thermo Fisher, cat. no. 17-467-435)

Radiolabeling

- Heating block (Thermo Fisher, cat. no. 11-720-10BQ)
- Eppendorf tube (Thermo Fisher, cat. no. 05-402-25)
- Capintec well counter (CRC-55TW dose calibrator/well counter)
- Falcon 15-ml conical centrifuge tubes (polypropylene; Fisher Scientific, cat. no. 14-959-70C)
- Precision Seal rubber septa (White, 14/20 joints, Sigma-Aldrich, cat. no. Z553964)
- Syringe PP/PE without needle (5 ml; Sigma-Aldrich, cat. no. Z116866)

- Syringe PP/PE without needle (3 ml; Sigma-Aldrich, cat. no. Z116858)
- Syringe PP/PE without needle (1 ml; Sigma-Aldrich; cat. no. Z230723)
- 4 × BD PrecisionGlide needles 21 gauge × 2 inch (Med Plus Physical Supplies, cat. no. BD 305129)
- Sep-Pak C18 light cartridge (Waters, cat. no. WAT022301)
- Analytical HPLC System (Agilent, 1200 series)
- Radio-HPLC detector system (Eckert & Ziegler, FC-3200 NaI detector)

REAGENT SETUP

Pyridazine-HCl buffer (1.0 M, pH 2.0–2.5)—Dissolve 720 µl of pyridazine (10 mmol) in 5 ml of DMF and 2.5 ml of deionized water in a 15-ml conical tube. Adjust the pH by using a pH meter; continue adding diluted HCl (aq., 4 M) until the pH is regulated to 2.0. Adjust the final volume to 10 ml with deionized water to obtain a final concentration of the buffer of 1.0 M. This buffer can be stored at 4 °C for up to 1 month. ▲ **CRITICAL** The pH of this pyridazine buffer may increase after being stored for more than 1 month. If the buffer has been stored for more than 1 week, please make sure to check the buffer pH before radiolabeling. In addition, the buffer should be stored at 4 °C. The color of the buffer may turn light yellow after prolonged storage, but this color change should not affect the yield of radiolabeling.

Aqueous solution of CuSO₄·5H₂O (1.0 M)—Dissolve CuSO₄·5H₂O (2.5 g, 10 mmol) with 5 ml of deionized water. Dilute this solution to 10 ml using deionized water to obtain the desired concentration. This solution is stable at room temperature (~20 °C) for at least 3 months.

Aqueous solution of sodium ascorbate (1.0 M)—Dissolve sodium ascorbate (1.98 g, 10 mmol) with 2 ml of deionized water. Dilute this solution to 10 ml using deionized water to obtain the desired concentration. This solution is stable at room temperature (~20 °C) for at least 3 months.

KHF₂ aqueous solution (7.5 mM)—Dissolve KHF₂ (5.85 mg, 75 µmol) with 1 ml of deionized water. Dilute this solution to 10 ml using deionized water to obtain the desired concentration. This solution is stable at room temperature (~20 °C) for at least 3 months.

NaCl saturated solution—Dissolve NaCl (10 g) with 10 ml of deionized water to obtain a saturated solution. This solution is stable at room temperature (~20 °C) for at least 3 months.

HCl solution (4 M)—Dilute HCl (aq., 37%) with twofold (vol/vol) deionized water to obtain a 4-M HCl aqueous solution. This solution is stable at room temperature (~20 °C) for at least 3 months.

EQUIPMENT SETUP

Self-assembled μ -QMA cartridge manufacture and precondition—Cut a 16-gauge $\times$ 1 1/2-inch needle with wire cutters and precisely assemble the μ -QMA cartridge between the two parts of the needle. Seal the joints with Parafilm to ensure no possible leaking. Flush the cartridge with 3 ml of deionized water, 3 ml of brine (NaCl-saturated aqueous solution) and 3 ml of deionized water to convert the carbonate-formed QMA into its chloride form. To better understand how the μ -QMA cartridge is assembled, a descriptive picture is available as referenced¹⁸. **▲ CRITICAL** A suitable reaction pH (2–2.5) is of paramount importance to give good radiochemical yield for ^{18}F -labeled organotrifluoroborates. To ensure that the final reaction pH is within the correct range, here we highly recommend using a chloride form instead of the regular carbonate form, which gives strongly basic solution (pH = 11) after elution⁴⁰. The presence of residual carbonate in the QMA may occasionally result in failure of radiosynthesis, and thus it might jeopardize the reproducibility of this radiolabeling protocol. Of note, this self-assembled μ -QMA cartridge can be reused for up to ten cycles of trapping-releasing, and no reconditioning is needed; however, we always recommend flushing the cartridge with 3 ml of deionized water before using it as the $^{18}\text{F}^-$ trap.

Sep-Pak C18 light cartridge preconditioning—Precondition this cartridge with 6 ml of ethanol, followed by 6 ml of deionized water.

HPLC system and radioactivity detector—Equip the Agilent 1200 series analytical HPLC system with an Eckert and Ziegler B-FC-3200 NaI detector according to the manufacturer's instructions.

Analytical HPLC for quality control (method A)—A Phenomenex Jupiter 10- μm C18, 300 Å, 4.6×250 mm analytical column is used. The HPLC setup is as follows: solvent A, 0.1% (vol/vol) TFA in water; solvent B, ACN; flow rate, 2 ml/min; and column temperature, 19–21 °C.

Time (min)	%B
0–2	5
2–7	5–20
7–15	20–100
15–20	100–5

Semi-prep HPLC method for azide- AMBF_3 purification (method B)—An Agilent Eclipse XDB-C18 5 μm 9.2×250 mm semi-prep column is used. The setup is as follows: solvent A, 0.1% (vol/vol) TFA in water; solvent B, ACN; flow rate, 4.5 ml/min; and column temperature, 19–21 °C.

Time (min)	%B
0–2	20
2–20	20–35
20–22	35–20

Semi-prep HPLC method for ^{19}F -AMBF₃-TATE purification (method C)—An

Agilent Eclipse XDB-C18 5 μm 9.2 $\times$ 250 mm semi-prep column is used. The setup is as follows: solvent A: 0.1% (vol/vol) TFA in water; solvent B: ACN; flow rate: 4.5 ml/min; and column temperature, 19–21 °C.

Time (min)	%B
0–2	5–20
2–10	20–35
10–17	35–40
17–18	40–100
18–19	100–20
19–22	20–5

PROCEDURE

Preparation of *N*-propargyl-*N,N*-dimethyl-ammoniomethyl-trifluoroborate (1) ● TIMING ~1 d

- 1| Dissolve 98 μl of *N,N*-dimethylpropargylamine (1.0 mmol) into 2 ml of anhydrous diethyl ether in a flame-dried round-bottom flask. Next, add 165 μl of iodomethyl-pinacol boronate (0.9 mmol) dropwise into the solution that was previously prepared at room temperature.
- 2| Continue stirring for 5 min, during which time the solution will become cloudy followed by the formation of a white precipitate, which is the desired *N*-propargyl-*N,N*-dimethylammonio-methylboronylpinacolate intermediate product (Fig. 2).
- 3| Filter the resulting precipitate with a sand-core funnel and wash the solid with diethyl ether; retain the solid.
- 4| Dry the residue under high vacuum to give a fluffy white powder in 95% yield.
- 5| Convert 50.0 mg of the *N*-propargyl-*N,N*-dimethylammonio-methylboronylpinacolate thus synthesized (142.4 μmol) into the corresponding trifluoroborate (alkynyl-AMBF₃, 1) by adding a cocktail of KHF₂ (3 M, 300 μl in water), HCl (4 M, 300 μl in water), deionized water (200 μl) and DMF (600 μl).
- 6| Allow the reaction to proceed at 45 °C for 2 h, and then quench the reaction by adding 10 μl of NH₄OH (conc.).

- 7| Remove the free fluoride by passing the reaction mixture through a silica gel column. Next, further purify the residue by diethyl ether extraction to obtain a white powder as the chemically pure product in 90% yield (21.2 mg).

■ **PAUSE POINT** *N*-propargyl-AMBF₃ can be stored at –20 °C for up to 2 years.

Synthesis of biomolecule-azide ● TIMING ~1 week

- 8| Synthesize a biomolecule-azide adduct according to option A, if the biomolecule is a peptide, or option B, if the biomolecule is nonpeptidic.

A. Synthesis of peptide-azide ● TIMING 4–5 d

- i. Synthesize the peptide on a solid bead via a standard solid-phase peptide synthesis protocol^{41,42}.
- ii. Couple bromoacetyl NHS ester to the N terminus of the peptide sequence by using an excess amount of bromoacetyl NHS ester (20.0 eq.) in *N*-methyl-2-pyrrolidone at room temperature for 2 h.
- iii. Displace the terminal bromide of the tethered peptide resulting from implementing Step 8A(ii) with azide by incubating the peptide with an excess amount of sodium azide (27.5 eq.) in DMSO at room temperature overnight.
! CAUTION Sodium azide is severely poisonous. Even a minute amount can cause symptoms of toxicity Please wear double gloves and use extra caution in this step.
- iv. Deprotect and simultaneously cleave the peptide from the resin by adding a cocktail of TFA:H₂O:triisopropylsilane:phenol at a ratio of 90:2.5:2.5:5 to the peptide-decorated resin and by incubating the mixture for 4 h at room temperature.
- v. Purify the peptide-azide adduct by HPLC with method B (Equipment Setup) to afford chemically pure peptide-azide in quantities of ~10 mg.

B. Preparation of nonpeptide-azide ● TIMING up to ~1 week

- i. (Optional) Synthesize the azide-conjugated small molecule of choice via a classical S_N2

reaction by following published protocols^{43,44}. Alternatively, please note that, because of the rapid development and broad application of copper-catalyzed click reaction (CuAAC), many azide-conjugated functional molecules are readily available from commercial sources.

Synthesis of AMBF₃-conjugated biomolecule ● TIMING 2 d

- 9| Mix 5.0 µl of a 1.0-M aqueous solution of CuSO₄ with 12.5 µl of a 1.0-M aqueous solution of sodium ascorbate to obtain a dark solution.
- 10| Immediately quench the solution that was prepared in Step 9 with 50 µl of a 5% (vol/vol) NH₄OH in ACN/H₂O = 1:1. The resulting mixture should be a greenish, clear solution.

? TROUBLESHOOTING

- 11| Dissolve 2.0 mg of compound **1** (15 µmol) in 200 µl of ACN/H₂O = 1:1.
- 12| Pool together the two solutions prepared in Steps 10 and 11.
- 13| Dissolve 4.0 mg of TATE-azide (compound **2**, 3.5 µmol) in 200 µl of ACN/H₂O = 1:1.
- 14| Add the TATE-azide solution prepared in Step 13 in portions of 50 µl to the mixture prepared in Step 12. The final reaction mixture (Fig. 3) should be a clear solution without any visible precipitate.

▲ **CRITICAL STEP** The order in which the reagents are added is essential for this step to be successful.

- 15| Incubate this reaction mixture at 45 °C for 2 h and directly purify the crude reaction by using the Agilent 1200 HPLC system with method C (see Equipment Setup) to isolate chemically pure AMBF₃-TATE (compound **3**). Confirm and evaluate the identity and chemical purity of the product with LC-MS.

? TROUBLESHOOTING

- 16| For convenience and to ensure reproducibility, dilute the purified ¹⁹F-AMBF₃-TATE in ethanol and store it in aliquots of ~100 nmol in 15 µl for radiolabeling. Please note that these aliquots will be stable in ethanol solution for up to 1 month at room temperature. For longer storage times, the solvent in the aliquots needs to be removed under vacuum.

■ **PAUSE POINT** The dry AMBF₃-conjugated biomolecules should be stored at -20 °C, and it should be used within 6 months.

Preparation of the radiosynthesis reaction mixture ● TIMING 30 min

- 17| Prepare a radiolabeling mixture of AMBF₃-TATE and ¹⁸F (Fig. 4) according to option A, if you are aiming for a 1 Curie level of reactivity of the ¹⁸F reactant,

or option B, if you are aiming for a level of radioactivity of the ^{18}F reactant lower than 1.85 GBq (50 mCi); see relevant discussion in the INTRODUCTION (Experimental design).

A. Preparation of an ^{18}F -labeling mixture with 1 Curie level of radioactivity

- i.** Resuspend a 100-nmol aliquot of ^{19}F -AMBF₃-TATE in a mixture of 15 μl of pyridazine-HCl buffer (pH = 2.0–2.5, 1 M), 15 μl of DMF and 1 μl of a 7.5 mM KHF₂ aqueous solution.

▲ CRITICAL STEP Check the pH of the previously prepared precursor solution with pH strips (0–6) before proceeding with the next step.

? TROUBLESHOOTING

- ii.** Prepare the following materials, and then place them into the hot cell.

Quantity	Material
2	0.07 ml of saline in a 1-ml PP/PE syringe without needle
2	2 ml of NH ₄ OH (aq., 5% (vol/vol)) and 0.5 ml air in a 3-ml PP/PE syringe with a 21-gauge $\times$ 2-inch needle
3	4 ml of deionized water in a 5-ml PP/PE syringe without needle
2	0.5 ml of 1:1 saline/ethanol in a 1-ml PP/PE syringe without needle
2	2 ml of saline in a 3-ml PP/PE syringe with a 16-gauge $\times$ 1½-inch needle
6	1-ml PP/PE syringes equipped with 21-gauge $\times$ 2-inch needles
1	Sep-Pak C18 light cartridge

! CAUTION To minimize the radiation dose, properly shield yourself by performing most of the reactions involving radioactivity in a lead-shielded fume hood or a hot cell.

▲ CRITICAL STEP Make sure to always have a back-up sample of each reagent, as the hot cell should not be opened once large quantities of radioactivity (i.e., up to 37 GBq) have been transferred into the hot cell.

- iii.** Preheat the precursor solution prepared in Step 17A(i) in a heating block at 80 °C.

- iv. Close the door of the lead-shielded hot cell, and perform all the following operations with telemanipulators.
- v. Pass an $^{18}\text{F}^-$ solution through the self-assembled μ -QMA cartridge, followed by 5 ml of air.
- vi. Elute $^{18}\text{F}^-$ with $\sim 60\ \mu\text{l}$ of saline in a 1-ml PP/PE syringe into the reaction vial, followed by blowing the μ -QMA cartridge with 0.5 ml of air. The radiolabeling reaction initiates right after eluting $^{18}\text{F}^-$ into the reaction vial; no additional mixing procedure is required.

▲ **CRITICAL STEP** Elution must proceed gently and relatively slowly ($200\ \mu\text{l}/\text{min}$). With proper operation, $>95\%$ radioactivity can be recovered from the μ -QMA cartridge ($n = 50$).

▲ **CRITICAL STEP** The radiochemical yield of ^{18}F - ^{19}F IEX depends greatly on the reaction concentration of AMBF_3 conjugates and on the pH of the radiolabeling mixture.

B. Preparation of a ^{18}F -labeling mixture with $<1.85\ \text{GBq}$ (50 mCi) radioactivity

- i. Resuspend 20 nmol of ^{19}F - AMBF_3 -TATE in a mixture of $5\ \mu\text{l}$ of pyridazine-HCl buffer ($\text{pH} = 2.0\text{--}2.5$, 1 M) and $5\ \mu\text{l}$ of DMF.
- ii. Prepare the following list of reagents, and then put them into the hot cell or shielded fume hood.

Quantity	Material
2	2 ml NH_4OH (aq., 5% (vol/vol)) and 0.5 ml air in a 3-ml PP/PE syringe with a 21-gauge $\times$ 2-inch needle
2	0.5 ml of 1:1 saline/ethanol in a 1-ml PP/PE syringe without needle
2	2 ml of saline in a 3-ml PP/PE syringe with a 16-gauge $\times$ $1\frac{1}{2}$ -inch needle
1	Sep-Pak C18 light cartridge
5	1.5-ml number-labeled Eppendorf tubes

- iii. Preheat the reaction vial with the precursor solution in a heating block at $80\ ^\circ\text{C}$.

- iv. Azeotropically concentrate a mixture composed of 200 μl of ACN and 200 μl of aqueous $^{18}\text{F}^-$ using a stream of argon at 110 $^{\circ}\text{C}$ for 10 min in a Falcon tube.

▲ **CRITICAL STEP** The $^{18}\text{F}^-$ salt solution does not have to be completely dry. Indeed, overdrying this solution may lead to difficulties in suspending $^{18}\text{F}^-$.

▲ **CRITICAL STEP** The composition of the vial in which the $^{18}\text{F}^-$ is concentrated is crucial, and glass vials should be avoided, given the presence of fluorophilic silicates on the glass surface.

▲ **CRITICAL STEP** To avoid unwanted evaporation of $[^{18}\text{F}]\text{HF}$, we recommend adding 5 μl of 0.1 M NaHCO_3 (aq.) to the mixture in Step 17B(iv).

- v. Resuspend the $^{18}\text{F}^-$ salt with 20 μl of deionized water. Mix the precursor solution prepared in Step 17A(iii) with the $^{18}\text{F}^-$ salt solution just prepared in a 1.5-ml Eppendorf tube.

▲ **CRITICAL STEP** Check the pH of the precursor solution with pH strips (0–6) before mixing it with the resuspended $^{18}\text{F}^-$ salt solution; the pH of the radiolabeling reaction should be between 2 and 2.5.

^{18}F – ^{19}F IEX of ^{19}F -AMBF₃-TATE ● TIMING 20 min

- 18| Incubate the radiolabeling reaction at 80 $^{\circ}\text{C}$ for 12 min.
- 19| Quench the reaction by adding to it 2 ml of NH_4OH (5% (vol/vol) aq.) using a 3-ml PP/PE syringe with a 21-gauge $\times$ 2-inch needle. Use the same syringe to load the resulting reaction mixture onto a Sep-Pak C18 light cartridge.

▲ **CRITICAL STEP** The reaction mixture must be loaded into the Sep-Pak C18 light cartridge gently and slowly (one drop every 2 s). In addition, a 2-inch needle is required for this step, as otherwise the crude reaction would not be completely transferred to the cartridge.

? TROUBLESHOOTING

- 20| Remove the impurities (i.e., free $^{18}\text{F}^-$) from the reaction mixture by flushing the reaction mixture-loaded C18 light cartridge twice with 4 ml of deionized water each time. The flow rate of this flushing procedure should be one drop every 2 s.

? TROUBLESHOOTING

- 21|** Elute the purified product into a glass vial with 1:1 ethanol/saline (0.5 ml), and measure the radioactivity of product by using a Capintec well counter. The radiochemical yield is determined by dividing the radioactivity of finally purified product by the $^{18}\text{F}^-$ being used at the beginning of synthesis.

▲ CRITICAL STEP Do not elute too quickly (the flow rate should be <1 drop every 5 s). In regard to the radiosynthesis, the eluate should be collected in separate fractions every three drops. Measure each fraction using a dose calibrator in the hot cell. The fraction containing the highest concentration of radioactivity should be used for the following studies.

? TROUBLESHOOTING

- 22|** Dilute the solution obtained at the end of Step 21 with 2 ml of isotonic saline, and then transfer 0.1 ml of the solution thus prepared into another glass vial by using a 1-ml PP/PE syringe equipped with a 21-gauge $\times$ 2-inch needle.

! CAUTION By starting with 37 GBq of radioactivity (Step 17A(i–vi)), generally >7.4 GBq of ^{18}F -labeled product will be obtained in 2 ml of saline solution. To avoid unnecessary exposure to radiation, always take out the minimum portion of the radioactive product based on the quantities required for subsequent studies (such as imaging or biodistribution experiments).

- 23|** Take the vial with 0.1 ml of the solution out of the hot cell. Dilute the product with an additional 5–10 ml of isotonic saline to obtain a solution that can be readily injected for biological evaluation.

▲ CRITICAL STEP The final concentration of ethanol in the resulting solution should not exceed 10% (vol/vol).

- 24|** Remove a small portion from the solution obtained after implementing Step 23 to determine the radiochemical purity of the product with the analytical HPLC system (method A, see Equipment Setup).

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

● TIMING

Steps 1–7, preparation of AMBF_3 ($_1$): within 1 d

Step 8, preparation of ‘clickable’ bioligands (e.g., TATE-azide) including HPLC purification: ~1 week

Steps 9–16, synthesis of AMBF_3 -conjugated bioligands (e.g., ^{19}F - AMBF_3 -TATE) including HPLC purification and preparation of aliquots: within 2 d

Step 17, equipment setup and precursor preparation for radiolabeling: 30 min

Steps 18–24, ^{18}F -labeling of AMBF₃-conjugated bioligands via ^{18}F – ^{19}F IEX including Sep-Pak purification: 20 min

ANTICIPATED RESULTS

By implementing Step 17A, starting with 29.6–37.0 GBq (800–1,000 mCi) of $^{18}\text{F}^-$, no less than 7.4 GBq (200 mCi) of ^{18}F -AMBF₃-TATE should be obtained in 25 min, with a specific activity higher than 74 GBq/ μmol (2 Ci/ μmol). By implementing Step 17B, starting with 1.48–1.85 GBq (40–50 mCi) of $^{18}\text{F}^-$, 0.56–0.74 GBq (15–20 mCi) of ^{18}F -labeled product should be produced in 45 min, with a specific activity higher than 27.8 GBq/ μmol (0.75 Ci/ μmol). For both radiolabeling procedures, the radiochemical purity, which is determined by HPLC analysis, should be higher than 98% (Fig. 5), thus eliminating the need for further HPLC purification. If the radiochemical purity of the product is <95%, a second Sep-Pak purification (Steps 19–21) would be recommended to improve the purity. A representative PET imaging of ^{18}F -AMBF₃-TATE is shown in Figure 6 here to illustrate the general *in vivo* performance of AMBF₃-TATE.

Analytical data

N-propargyl-*N,N*-dimethyl-ammoniomethyl-trifluoroborate (1). ^1H NMR (300 MHz, CD₃CN) δ 4.25 (d, 2H), 3.41 (t, 1H), 3.18 (s, 6H), 2.55 (b, 2H). High-resolution electrospray ionization mass spectrum (HR-ESI-MS) (m/z): 146.10. $[\text{M}-\text{F}]^+$; calculated for C₆H₁₁BF₂N, 146.09.

TATE-azide. HR-ESI-MS (m/z): 1,132.39. $[\text{M}+\text{H}]^+$; calculated for C₅₁H₆₆N₁₃O₁₃S₂, 1132.44.

^{19}F -AMBF₃-TATE. HR-ESI-MS (m/z): 1,297.49. $[\text{M}+\text{H}]^+$; calculated for C₅₇H₇₇BF₃N₁₄O₁₃S₂, 1297.52.

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Figure 1.
Outline of the procedure to ^{18}F -label biomolecules with AMBF₃.

Figure 2.
Synthetic route of radiosynthon **1**.

Figure 3.
Synthesis of ¹⁹F-AMBF₃-TATE (**3**) via copper-catalyzed ‘click’ reaction.

Figure 4.
Radiosynthesis of ^{18}F -AMBF₃-TATE ($[^{18}\text{F}]3$) via ^{18}F - ^{19}F IEX reaction.

Figure 5. HPLC chromatograms of Sep-Pak-purified ^{18}F -AMBF₃-TATE. Top, the UV chromatogram measured at 277 nm; bottom, a radiochromatogram. UV, ultraviolet. mAU, one thousandth of an absorbance unit.

Figure 6. $[^{18}\text{F}]\text{AMBF}_3\text{-TATE}$ shows specific uptake in AR42J xenografts (dotted ovals) and low uptake in normal tissues. Maximum intensity projection $[^{18}\text{F}]\text{AMBF}_3\text{-TATE}$ PET images of AR42J tumor-bearing mice at 60 min after injection is shown here: blocked (left) and unblocked (right). The tracer specifically accumulated into the tumor, whereas the background radioactivity was rapidly cleared mainly via the kidneys to the bladder. Some

gut and gallbladder accumulation occurs because of some hepatobiliary excretion of the tracer.

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TABLE 1

Troubleshooting table.

step	problem	possible reason	solution
10	Precipitation	Cu ⁺ complex precipitates out because of its poor solubility in aqueous solution	Continue adding the organic solvent (e.g., MeCN) until the reaction is clear
15	Defluoridated product	AMBF ₃ may start losing fluoride when the reaction pH is too basic (pH >12). The defluoridated product, AMB(OH) ₂ -TATE, can be determined by mass spectrum, and it is separable from AMBF ₃ -TATE by using method B (Reagent Setup)	Adjust the final pH of the click reaction to 7–12 with phosphate buffer
17A(i)	Low efficiency of ¹⁸ F fluoride elution (<80%)	Rate of elution is too fast	Elute ¹⁸ F fluoride slower (one drop for each 10 s)
19	Low efficiency when 'trapping' radiolabeling mixture with C18 cartridge (<10%)	The product is too polar for C18 purification, or the pH of the reaction mixture is significantly different from the isoelectric point of the peptide	Adjust the pH value of the crude reaction to the pH that is similar to the isoelectric point of the peptide
20	Low efficiency when 'releasing' radiolabeled product from C18 cartridge (<50%)	The solubility of the product is not sufficient in ethanol or not enough ethanol is used	Use more ethanol for elution
21	Low isolated radiochemical yield (<10%)	The concentration of the precursor is not sufficient	The final concentration of the precursor is suggested to be no less than 1 mM. Instead of weighing the HPLC-purified AMBF ₃ conjugates with a balance, which is not accurate because of contamination (e.g., of TFA salts), we recommend the use of UV absorbance at a certain wavelength (e.g., 277 nm for AMBF ₃ -TATE) to determine the quantity of the AMBF ₃ conjugates
	Low isolated radiochemical yield (<10%)	The pH of the radiolabeling reaction is not suitable	The final concentration of the pyridazine buffer should be no less than 100 mM