Simplified one-pot $^{18}$F-labeling of biomolecules with \textit{in situ} generated fluorothiophosphate synthons in high molar activity

Hongzhang Yang$^1$, Lei Zhang$^2$, Huanhuan Liu$^1$, Yunming Zhang$^1$, Zhaobiao Mou$^1$, Xueyuan Chen$^1$, Jingchao Li$^1$, Fengming He$^3$, and Zijing Li$^{1,2}$

1. Center for Molecular Imaging and Translational Medicine, State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, Department of Laboratory Medicine, School of Public Heath, Xiamen University, Xiamen, Fujian 361102, China.
2. Tianjin Engineering Technology Center of Chemical Wastewater Source Reduction and Recycling, School of Science, Tianjin Chengjian University, Tianjin 300384, China.
3. School of Pharmaceutical Sciences, Xiamen University, Xiamen, Fujian 361102, China.

Corresponding author: Zijing Li Ph.D., Email: zijing.li@xmu.edu.cn

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Abstract

\textbf{Rationale:} Conventional $^{18}$F-labeling methods that demand substrate pre-modification or lengthy radiosynthesis procedures have impeded the visualization and translation of numerous biomolecules, as biomarkers or ligands, using modern positron emission tomography techniques \textit{in vivo}. Moreover, $^{18}$F-labeled biomolecules in high molar activity ($A_m$) that are indispensable for sensitive imaging could be only achieved under strict labeling conditions.

\textbf{Methods:} Herein, $^{18}$F-labeled fluorothiophosphate (FTP) synthons in high $A_m$ have been generated rapidly \textit{in situ} in reaction solutions with < 5% water via nucleophilic substitution by wet $[^{18}\text{F}]$F–, which required minimal processing from cyclotron target water.

\textbf{Results:} Various $^{18}$F-labeled FTP synthons have been prepared in 30 sec at room temperature with high radiochemical yields > 75% (isolated, non-decay-corrected). FTP synthons with unsaturated hydrocarbon or activated ester group can conjugate with typical small molecules, peptides, proteins, and metallic nanoparticles. 337–517 GBq $\mu$mol$^{-1}$ $A_m$ has been achieved for $^{18}$F-labeled c(RGDyK) peptide using an automatic module with 37–74 GBq initial activity.

\textbf{Conclusion:} The combination of high $^{18}$F-fluorination efficiency of FTP synthons and following mild conjugation condition provides a universal simplified one-pot $^{18}$F-labeling method for broad unmodified biomolecular substrates.

Key words: radiolabeling, radiosynthet, fluorine-18, fluorothiophosphate, positron emission tomography probe

Introduction

Positron emission tomography (PET) is a non-invasive, real-time functional imaging technique that provides abundant physiological and biochemical information. It relies on the spatiotemporal tracing of molecular probes composed of functional scaffolds and positron-emitting nuclides [1, 2]. Biomolecules, including small molecules, peptides and proteins, come from a treasury house of lead compounds with high specificity and low immunogenicity [3-5]. $^{18}$F ($t_{1/2} = 109.7$ min, 97% $\beta^+$, maximum positron energy 0.64 MeV) is the most popular positron-emitting nuclide due to its favorable chemical, nuclide properties and production feasibility [6]. Numerous target-specific biomolecules merit $^{18}$F-labeling with optimum radiochemical parameters for pre-clinical and clinical PET imaging evaluation [7-10].

To achieve the mild $^{18}$F-labeling of biomolecules,
two/multi-step/indirect approaches have been widely applied, which go through the incorporation of $^{18}$F into a prosthetic/linker synthon and then the gentle coupling of the synthon to the lead-compound biomolecules [4, 11-21] (Table 1).Since the hydration effect eliminates the reactivity/nucleophilicity of $[{^{18}}\text{F}]^{-}$ in aqueous media directly obtained from a high efficient $^{18}$F-labeling methods that do not biomolecules [4, 11-21] (22-24) energy of P-F bonds, varied P(V) compounds exhibit a separable $^{18}$F-labeled fluorothiophosphates (FTPs) are prostheses that are pre-coupled to biomolecules [10, 22-24] synthesized fluorination pathway overcoming low activation but time-consuming alterations of reaction conditions, purification of intermediates, and use of inseparable precursors for high molar activity ($A_{m}$) are still in open request [25].

Due to the low heat of formation and high bond energy of P-F bonds, varied P(V) compounds exhibit a capacity for rapid mild $^{18}$F-labeling via $^{18}$F/$^{19}$F-exchange or Al$^{18}$F chelation in aqueous solutions at a specific pH/temperature on non-carbon-centered prostheses that are pre-coupled to biomolecules [10, 22-24] (Table 1). Nevertheless, universally accessible, highly efficient $^{18}$F-labeling methods that do not require the pre-coupling of a prosthetic group, time-consuming alterations of reaction conditions, purification of intermediates, and use of inseparable precursors for high molar activity ($A_{m}$) are still in open request [25].

Table 1. Important parameters of selected $^{18}$F-labeling methods for biomolecules.

<table>
<thead>
<tr>
<th>Tracer</th>
<th>Labeling strategy</th>
<th>Mechanism</th>
<th>T (°C)</th>
<th>Solvent (v/v)</th>
<th>pH</th>
<th>Total synthesis time (min)</th>
<th>Precursor load (μmol)</th>
<th>RCC &amp; RCY</th>
<th>$A_{m}$ (GBq μmol$^{-1}$)/Starting activity (GBq)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>[R]F-FTP-c(RGDyK)</td>
<td>Instant FTP</td>
<td>$S_{0}$</td>
<td>RT</td>
<td>Borate buffer/DMSO (91/9)</td>
<td>8.0</td>
<td>15(§)</td>
<td>0.2</td>
<td>&gt; 90% &amp;</td>
<td>34 ± 5%</td>
<td>49-107/3,7-5.6</td>
</tr>
<tr>
<td>[R]F-FTP-HSA</td>
<td>Instant FTP</td>
<td>$S_{0}$</td>
<td>RT</td>
<td>Borate buffer</td>
<td>8.0</td>
<td>15(§)</td>
<td>0.2</td>
<td>&gt; 90% &amp;</td>
<td>34 ± 5%</td>
<td>49-107/3,7-5.6</td>
</tr>
<tr>
<td>[R]F-FTP-SF7</td>
<td>Instant FTP</td>
<td>$S_{0}$</td>
<td>RT</td>
<td>Borate buffer</td>
<td>8.0</td>
<td>15(§)</td>
<td>0.2</td>
<td>&gt; 90% &amp;</td>
<td>34 ± 5%</td>
<td>49-107/3,7-5.6</td>
</tr>
<tr>
<td>[R]F-FTP-RGD</td>
<td>Multi-step</td>
<td>$S_{0}$</td>
<td>RT</td>
<td>Borate buffer/DMSO</td>
<td>7.4</td>
<td>130(§)</td>
<td>13</td>
<td>&gt; 90% &amp;</td>
<td>34 ± 5%</td>
<td>49-107/3,7-5.6</td>
</tr>
<tr>
<td>[R]F-FTP-GSH</td>
<td>Multi-step</td>
<td>$S_{0}$</td>
<td>RT</td>
<td>PBS</td>
<td>7.4</td>
<td>&gt; 60(§)</td>
<td>30</td>
<td>&gt; 90% &amp;</td>
<td>34 ± 5%</td>
<td>49-107/3,7-5.6</td>
</tr>
<tr>
<td>(a)-[R]F-FTP-RGD</td>
<td>One-step</td>
<td>Catechol reaction</td>
<td>RT</td>
<td>Ammonium acetate buffer</td>
<td>4</td>
<td>&lt; 35(§)</td>
<td>5.6</td>
<td>&gt; 90% &amp;</td>
<td>34 ± 5%</td>
<td>49-107/3,7-5.6</td>
</tr>
<tr>
<td>(a)F]-SFA-peptide</td>
<td>One-step</td>
<td>IEX(§)</td>
<td>RT</td>
<td>HEO/CHClCN (10/90)</td>
<td>4</td>
<td>25(§)</td>
<td>0.07</td>
<td>55-65%</td>
<td>2.96 ± 0.18/0.248</td>
<td>-</td>
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<tr>
<td>(a)F]-AMB-M-TrisRGD</td>
<td>One-step</td>
<td>IEX</td>
<td>80</td>
<td>Pyridazine-HCl buffer</td>
<td>2.5</td>
<td>12(§)</td>
<td>0.05-0.08</td>
<td>23 ± 5%</td>
<td>111-148/29.6-37</td>
<td>-</td>
</tr>
<tr>
<td>(a)F]-BPDFO-c(RGDyK)</td>
<td>One-step</td>
<td>IEX</td>
<td>RT</td>
<td>HEO/DMSO (95/5)</td>
<td>7</td>
<td>25(§)</td>
<td>1.3</td>
<td>15-25%</td>
<td>2.22-4.81/0.821-1.64</td>
<td>-</td>
</tr>
</tbody>
</table>

16 Nucleophilic substitution. 17 Isotope exchange. 18 The probe was manually synthesized. 19 The probe was synthesized by automated radiosynthesis module. 20 The probe was synthesized manually. 21 min was reported for the $^{18}$F-labeling step alone. 22 RCCs were detected by radio-HPLC. 23 RCYs were isolated yields. 24 Not found.
Figure 1. Analysis of rapid kinetics and condition scopes of the $^{18}$F-labeling of FTPs. (A) Reaction scheme showing the fluorination of FTPs. (B) Reaction rate constant ($k$) values that were predicted by density functional calculations at B3LYP/6-311+G* level of theory (at 298 K) and $k$ values that were measured at 298 K. The pseudo-first order initial rate constant ($k'$) of $[^{18}$F]$^1$ (C) and $[^{18}$F]$^2$ (E) were determinated from the kinetic curves reflecting the change in ln [1/(1 – RCC)] in DMF at different temperatures. The $E_a$ of $[^{18}$F]$^1$ (D) and $[^{18}$F]$^2$ (F) were determined from Arrhenius plots of ln $k$ against 1/T. (G) RCCs of $[^{18}$F]$^1$ and $[^{18}$F]$^2$ at different time periods from 10 to 300 sec in acetonitrile at RT. (H) RCCs of $[^{18}$F]$^1$ and $[^{18}$F]$^2$ at different temperatures and water contents (The solvents are mixtures of acetonitrile and water, v/v. “0%” means no additional water is added.) post 30 sec. (J) RCCs of $[^{18}$F]$^1$ and $[^{18}$F]$^2$ with different precursor loads in acetonitrile post 30 sec at RT. (L) The time-bone uptake curves of $[^{18}$F]$^1$, $[^{18}$F]$^2$, $[^{18}$F]$^3$, and $[^{18}$F]$^4$ during 0–60 min post-injection. Bone uptake was measured with microPET in healthy mice (n = 3).

Chemistry

To synthesize the aliphatic (1, 5–9) and aromatic (2, 10–14) FTPs with either electron-donating (10–12) or electron-withdrawing (13, 14) substituents, 1,3,2-dithiaphospholanes (1a, 2a, 5a–14a) were first obtained through the phosphitylation of N,N-diethyl-1,3,2-dithiaphosolan-2-amine (19), in the presence of an alcohol/phenol and S-ethylthiotetrazole (Figure 1A, Scheme S1). Dithiaphospholanes were oxidized with elemental sulfur to obtain oxodithiaphospholane 2-sulfide substrates (1b, 2b, 5b–13b) at yields exceeding 50%. When these substrates were treated with an excess of tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF), 1, 2, 5–13 were obtained at yields exceeding 95% in 2 min. In addition, 1b and 2b were entirely consumed upon treatment with an equivalent amount of TBAF in THF.
in less than 2 min, as shown by $^{31}$P NMR spectroscopy results (Figure S2). Same routes were used to synthesize 2-(but-3-yn-1-yl oxy)-1,3,2-oxathioaphospholane 2-sulfide (3b) and O-(but-3-yn-1-yl) phosphorfluoridothioate (3, a monothio-derivative). The oxidation of 14a with elemental sulfur failed to obtain 14b. FTPs with typical active groups, N-hydroxysuccinimide ester (NHS) for 15, cyclooctene for 16, were fluorinated from 15b (Scheme S3) and 16b (Scheme S4) at yields exceeding 95%. 17b and 18b were treated with TBAF, and then deprotected to obtain 17-18, two bioactive phosphate analogs (Scheme S5, S6).

**Measurement of fluorination kinetics and energetics**

Two simple substrates, O-(But-3-yn-1-yl) $[^{18}$F$]$phosphorfluoridothioate ($[^{18}$F$]$1) and O-phenyl $[^{18}$F$]$phosphorfluoridothioate ($[^{18}$F$]$2), were used as model compounds to evaluate fluorination kinetics and activation energies ($E_a$). The pseudo-first order model is applied since the trace amount $[^{18}$F$]^{-}$ is negligible compared to the precursors. The pseudo-first order initial rate constant ($k_i$) under different temperatures were calculated from the exponential fit equation (Figure 1C, 1E) and then divided by the concentration of 1b or 2b to determine the second-order rate constants ($k$). For the conversion of 1b to 1, $k_{223} = 8.53$ L mol$^{-1}$ s$^{-1}$, $k_{228} = 25.66$ L mol$^{-1}$ s$^{-1}$, $k_{233} = 34.93$ L mol$^{-1}$ s$^{-1}$, $k_{243} = 61.20$ L mol$^{-1}$ s$^{-1}$, $k_{248} = 69.38$ L mol$^{-1}$ s$^{-1}$. For the conversion of 2b to 2, $k_{218} = 5.91$ L mol$^{-1}$ s$^{-1}$, $k_{223} = 8.60$ L mol$^{-1}$ s$^{-1}$, $k_{228} = 19.72$ L mol$^{-1}$ s$^{-1}$, $k_{240} = 47.85$ L mol$^{-1}$ s$^{-1}$, and $k_{248} = 66.20$ L mol$^{-1}$ s$^{-1}$. The values of $E_a$ were measured to be 9.0 ± 0.1 kcal mol$^{-1}$ for the conversion of 1b to 1, and 9.5 ± 0.6 kcal mol$^{-1}$ for the conversion of 2b to 2 from Arrhenius plots (ln $k$ versus $T^{-1}$) (Figure 1D-F).

**Radiochemistry**

$^{18}$F-Labeling conditions were optimized in aprotic solvents containing graded proportions of water for different reaction durations (10–300 sec) under specific temperatures (RT–80 °C). Non-decay-corrected radiochemical conversions (RCCs) were detected using both radio-TLC and radio-HPLC (F–adsorption might occur to radio-HPLC column) at continuous time points ($n = 3$). The activity adsorption by the vial/glass was measured to be ~10% of the total initial activity. RCC values were 98 ± 5% for $[^{18}$F$]$1 and 99 ± 4% for $[^{18}$F$]$2 after incubation for 30 sec at RT in acetonitrile and water, with > 90% of RCCs being achieved in just 10 sec (Figure 1G). Although the RCC values decreased with the increasing of solvent water contents, satisfactory RCC values of 48 ± 5% for $[^{18}$F$]$1 and 10 ± 3% for $[^{18}$F$]$2 could be achieved in a mixture of acetonitrile and water ($\nu/\nu = 9/1$) at RT. RCC values of 98 ± 2% for $[^{18}$F$]$1 and 40 ± 5% for $[^{18}$F$]$2 was achieved using a mixture of acetonitrile and water ($\nu/\nu = 9/1$) at 80 °C (Figure 1H-I). Substrate 2b showed higher sensitivity to water than 1b, in consistence with the calculated and experimental fluorination kinetics and energetics. After assessing precursor loads of 0.04–4.5 μmol/100 μL, the optimal precursor load was determined to be 0.20–2.00 μmol/100 μL (Figure 1J). This $^{18}$F-labeling method exhibited high efficiency in aprotic solvents (Figure 1K).

**Stabilities of $^{18}$F-labeled FTPs and precursors**

Each oxydithiaphospholane 2-sulfide substrate and representative FTP was incubated for 2 h in a mixture of acetonitrile and water ($\nu/\nu = 1/9$) with pH values of 1 to 13. The HPLC analysis results showed that the substrates could tolerate acids and weak bases but were unstable in strong alkaline solutions (Figure S33, S34). High stabilities of the FTP motif were observed in both acidic and alkaline solutions (pH values of 1 to 13, Figure S35, S36), which is critical for deprotection reaction in some occasion. The extent of defluorination (bone uptake, resistant to enzymatic hydrolysis in vivo) of $[^{18}$F$]$1 and $[^{18}$F$]$2 was insignificant in microPET imaging evaluation ($n = 3$) (Figure 1L, S48). While $[^{18}$F$]$1 was detected only in a small percentage of the parent compound in the urine, 5 min after administering an i.v. injection, $[^{18}$F$]$2 existed in the urine mainly in the form of the parent compound, as shown in Figure S45, S46. All procedures and animal use and care procedures have been approved by the Animal Care and Use Committee of Xiamen University.

A subsequent study showed that equally high stabilities were observed both in vitro and in vivo, if at least one O atom of phosphates was substituted by S atom, such as O-(but-3-yn-1-yl) $[^{18}$F$]$phosphorfluoridothioate ($[^{18}$F$]$3) and $[^{18}$F$]$1. However, $[^{18}$F$]$3 was cleared rapidly from the kidney probably due to its higher hydrophilicity, which might lead to insufficient internalization (Figure S47, S48). Defluorination occurred slowly in S-benzyl O-(but-3-yn-1-yl) $[^{18}$F$]$phosphorfluoridothioate ($[^{18}$F$]$4) in vitro and in vivo (bone uptake reached ~6 %ID g$^{-1}$ at 60 min), where the $\text{-SH}$ moiety was substituted intentionally with an alkylthio group (Figure 1L, S48). This suggests that the coulombic force contributes to the high stability of FTPs. The biodistribution study confirmed the high in vivo stabilities of $^{18}$F-labeled FTPs, where $[^{18}$F$]$1, $[^{18}$F$]$2, $[^{18}$F$]$3, and $[^{18}$F$]$15 ($[^{18}$F$]$FTP-NHS, a typical N-hydroxysuccinimide ester synthon) all exhibited only background bone uptakes (0.9–1.1 %ID g$^{-1}$) at 2 h post injection (Figure S49).
Structure scope of 18F-labeled FTPs

As illustrated in Figure 2, high RCC values of 88–98% were observed in anhydrous acetonitrile with a wide substrate scope of 18F-labeled FTPs, including alkanes, alkynes, heterocycles, halides, amino acid derivatives and nucleotide derivatives (condition i, ii). Notably, despite the absence of a phase transfer reagent, RCCs of 87–99% were observed with the use of wet [18F]- in solvents with 1–3% water (condition iii, iv). The RCCs decreased gradually with an increase in the water content; when the water content was 5% (condition vi) and 10% (condition vii), RCCs were 63–96% and 7–53%, respectively. 18F-Labeled FTPs with functional groups, such as [18F]1, [18F]3, [18F]15 and [18F]16, could be coupled to biomolecules and acted as ready-to-use radiosynthons. High RCC values > 95% and high radiochemical yield (RCY, isolated yields, non-decay-corrected, n = 3) values > 75% were observed for these FTP synths that were in situ generated with wet [18F]- at RT (condition v, in solvents with 3–5% water). The Am of [18F]15 ([18F]FTP-NHS) reached 128.2 GBq μmol⁻¹ by manual labeling with 3.7–5.6 GBq initial activity. Biologically active phosphate analogs, such as the 18F-labeled L-Tyr phosphate (p-Tyr) mimic ([18F]FTP-Tyr, [18F]17) and the 18F-labeled adenosine monophosphate mimic ([18F]FTP-AMP, [18F]18), were labeled directly with RCYs > 55%.

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**Figure 2. Substrate structure scope of 18F-labeled FTPs.** Labelling condition i: 0.1 mg precursor, [18F]KF/K222 (1–5 mCi), 100 μL anhydrous acetonitrile, 80 °C, 30 sec. Labeling condition ii: 0.1 mg precursor, [18F]KF/K222 (1–5 mCi), 100 μL anhydrous acetonitrile, RT, 30 sec. Labeling condition iii: 0.1 mg precursor, [18F]F– in cyclotron target water (1 μL, 0.3–0.5 mCi), 100 μL acetonitrile, RT, 30 sec. Labeling condition iv: 0.1 mg precursor, [18F]F– in cyclotron target water (3 μL, 1–3 mCi), 100 μL acetonitrile, RT, 30 sec. Labeling condition v: 0.1 mg precursor, [18F]F– in cyclotron target water (3 μL, 1–3 mCi), tetrabutylammonium hydroxide (0.52 mg), 100 μL acetonitrile, RT, 30 sec. Labeling condition vi: 0.1 mg precursor, [18F]F– in cyclotron target water (5 μL, 1–5 mCi) and acetonitrile 90 μL, RT, 30 sec. Each 18F-labeling reaction was performed thrice with all reported RCCs determined by radio-HPLC or radio-TLC. RCCs mean ± standard deviation values. **(A)** Aliphatic substrates with different levels of steric hindrance. **(B)** Aromatic substrates with different electron density distributions. **(C)** 18F-Labeled FTPs as radiosynthons. **(D)** Small molecular FTPs as phosphate analogs. **(E)** 18F-Labeled peptide/protein biomolecules generated using FTP radiosynthons. **(F)** 18F-Labeled metallic nanoparticle generated using an FTP radiosynth. * Cis-cyclooctene was used as a substitute.
Figure 3. Selected 18F-labeled biomolecules via FTPs and their microPET imaging. (A) MicroPET images of [18F]FTP-c(RGDyK) showing specific accumulation in 4T1 xenografts at 15 min post-injection (The white dotted line indicates the tumor margin). (B) Quantitative PET imaging results. Data represent the mean ± standard deviation values (n = 3), ***P < 0.001. (C) Dynamic microPET images of healthy female mice reconstructed at 15, 30, and 60 min after administering an [18F]FTP-HSA injection. LV: left ventricle; RV: right ventricle; K: kidneys. (D) Time-activity curves of [18F]FTP-HSA in indicated organs. Uptake values are presented in terms of mean ± standard deviation values (n = 3). (E) MicroPET images of [18F]FTP-5F7 showing specific accumulation in MDA-MB-453 xenografts at 60 min post-injection (The white dotted line indicates the tumor margin). (F) Quantitative PET imaging results. Data represent the mean ± standard deviation values (n = 3), ***P < 0.001. (G) MicroPET images showing the specific accumulation of [18F]FTP-Tyr in B16 xenografts at 15 min post-injection (the white dotted line indicates the tumor margin). (H) Quantitative PET imaging results. Data represent the mean ± standard deviation values (n = 3), ***P < 0.001.

[18F]FTP-c(RGDyK)

The Arg-Gly-Asp (RGD) sequence exhibits high affinity and selectivity for alpha(v)beta3 integrin, which is a significant peptide-based ligand [27]. Under mild coupling conditions, i.e., 10 min, 37 °C, borate buffer (pH = 8.0), [18F]FTP-NHS was efficiently conjugated with c(RGDyK) to obtain [18F]FTP-c(RGDyK). The total RCC values were ~90% determined by radio-HPLC, and the average RCC value was 34 ± 5% and Am value was 107 GBq μmol⁻¹ after manually labeling with 5.6 GBq initial activity (Figure S25, S31). The purification of [18F]FTP-c(RGDyK) using a C18 cartridge followed by HPLC resulted in > 98% radiochemical purity (RCP) (Figure S20). An automated procedure involving a commercial multifunctional radiosynthesis module was established (Figure S24). The total auto-synthesis time was ~60 min, and the RCC was 39 ± 8%, with an Am value of 337–517 GBq μmol⁻¹ (initial activity 37–74 GBq). The microPET/CT imaging data indicated that [18F]FTP-c(RGDyK) was accumulated specifically within 4T1 xenografts. The average tumor uptake of this monomeric RGD tracer, based on the whole tumor region of interest (ROI), was 1.30 ± 0.28 %ID g⁻¹, with a tumor-to-muscle ratio of 2.38 at 15 min post-injection (Figure 3A–B), in comparison to a value of roughly 1.8 %ID g⁻¹ for [18F]AMBF3 labeled trimeric RGD [19].

[18F]FTP-HSA

HSA, a heat-sensitive protein, has routinely been radiolabeled and used to evaluate plasma distribution volumes in specific organs, quantify cardiac function-related parameters, and assess vascular “leakage” in pathological tissues [28]. [18F]FTP-HSA was obtained via [18F]FTP-NHS after 10 min conjugation in borate buffer (pH = 8.0) at 40 °C, with an overall RCC of 83 ± 5% (n = 3). The average RCC value was 46 ± 9% (RCP > 99%) after purification by a PD-10 column (GE Healthcare Bio-Science AB) (Figure S21). As shown by the dynamic microPET/CT images in Figure 3C, the central vasculature, including the cardiac ventricular chambers, were clearly visualized. There was no apparent radioactivity in the skeleton, which suggested the absence of significant amounts of free [18F]F⁻ and other metabolites (Figure 3D).

[18F]FTP-5F7

Nanobodies (VHH, 12–15 kDa), the antigen-binding fragments of heavy-chain-only antibodies derived from Camelidae, have biological half-lives of 1–2 h that are comparable with 18F [29, 30]. Herein, we explored the feasibility of utilizing the 18F-labeled 5F7 anti-HER2 nanobody as a probe for evaluating the HER2 expression. [18F]FTP-5F7 was obtained via [18F]FTP-NHS after 10 min conjugation in borate
buffer (pH = 8.0) at RT. Overall RCCs values were determined via radio-HPLC to be ~90%, and the average RCY value was 29 ± 11% (RCP > 95%) after purification by a size exclusion chromatography column (Figure S22). MicroPET/CT images of mice with HER2-overexpressing MDA-MB-453 xenografts demonstrated the rapid tumor accumulation (1.40 ± 0.22%) and clearance of [18F]FTP-5F7 from the background. Pre-treatment with excessive amounts of 5F7 substantially reduced the tumor uptake in the background (Figure 3E–F).

**[18F]FTP-Pds-PEG**

The use of radiolabeled nanoparticles for molecular imaging has attracted broad attention due to their large functional surface area and easy-to-control surface chemistry, which allows them to be tailored for the purpose of personalized cancer management [31]. The sulfhydryl groups on FTPs can form stable coordinate covalent bonds, which can be used to attach various metal nanoparticles efficiently. PEGylated Pd nanosheets (Pd-PEGs) with an average size of 40 nm were conjugated with [18F]I upon stirring the reaction mixture for 15 min at RT. The overall RCYs of [18F]FTP-Pds-PEG were > 90% after ultrafiltration (same as measured by radio-TLC, RCP > 99%, Figure S23). 2 h after administering an i.v. injection, a significant tumor uptake of 4.87 ± 0.31 %ID g⁻¹ based on the whole tumor ROI was illustrated by microPET/CT imaging, as well as a high tumor-to-muscle ratio of 3.4 in subcutaneous 4T1 tumors (Figure S56).

**[18F]FTP-Tyr**

Evidence has shown that greater uptake rate, solubility, and oxidative stability were observed during the uptake of L-DOPA phosphate and p-Tyr by melanomas than those observed with L-DOPA and L-Tyr [32, 33]. Thus, they were recruited as melanogenesis markers for the early diagnosis of melanomas. In this study, [18F]FTP-Tyr was synthesized as a non-hydrolytic (overcoming the in vivo enzymatic-instabilities of phosphates) p-Tyr analog with an RCC of 80 ± 9% and A_m of > 3.91 GBq μmol⁻¹ (manual labeling; initial activity 1.48–1.85 GBq; FTP-Tyr exhibited weak UV absorption to determine the A_m; Figure S29). The results of the in vitro cell uptake study revealed that the significant specific uptake of [18F]FTP-Tyr by a B16 tumor within 120 min, with maximum uptake occurring at 30 min. This uptake could be either entirely inhibited by p-Tyr or partially inhibited by L-Tyr (Figure S53). More evidence for the similarity between FTP-Tyr and p-Tyr was provided in the supplementary material (Figure S50–S52, S54, S55). MicroPET/CT imaging of [18F]FTP-Tyr was performed in B16 xenograft mice, and specific accumulation in tumors and a tumor-to-muscle ratio of 6.4 were observed at 30 min after administering an i.v. injection. This specific accumulation was blocked by p-Tyr and only partially blocked by Tyr as well, indicating the specific uptake of [18F]FTP-Tyr by melanomas (Figure 3G–H).

**Discussion**

The easily oxidized trivalent phosphorus intermediates (19, 20, 1a–3a, 5a–18a, monitored by 31P NMR) were not isolated and directly treated with sulfur to obtain the separable pentavalent precursors liable to oxidation (1b–3b, 5b–13b, 15b–18b) with total yields ranged from 10–70%. Functional groups exhibiting higher steric repulsion and stronger electron withdrawal are unfavorable for precursor synthesis, e.g., the unformed 14b and 14. Although the predicted three-step pathway mechanism requires further experimental evidence, the extremely rapid fluorination kinetics were in accordance with the experimental rate constants and pseudo-first order analysis. Ultimately, the high [18F]-fluorination efficiency at RT was attributed to the low free-energy barriers and trace amount [18F]F⁻. Favorable energy barriers also resulted in a higher selectivity for F⁻ over other nucleophiles, such as, OH⁻, which is many-fold more in quantity in wet solvents. Thus, the routine azeotropic drying processing of [18F]F⁻ can be simplified to evaporating processing to obtain reaction solutions that contain less than 5% water in automatic production.

Non-structure-biased [18F]-labeled FTPs were generated at high RCCs within seconds in solvents with a water content of < 5%, using a very low molar quantity/concentration of storage-stable precursors (0.1 mg/0.22–0.45 μmol per 100 μL). Varied [18F]-labeled FTP synths with respective functional/ linker groups, e.g., unsaturated hydrocarbon or activated ester group, are accessible. Insignificant levels of defluorination but multiple degradation products were observed in vivo for [18F]I and [18F]2. The in vivo metabolic stabilities of [18F]-labeled FTPs are attributed not only to the coulombic repulsion between the FTP group and anions, which protects the P-F bond from hydrolysis, but also the substitution of P=O with P=S, which enhances its resistance to enzymatic hydrolysis (Figure 1L, S48, S51).

A simplified [18F]-labeling strategy has been consequently developed via the rapid in situ generation of instant FTP synths that could couple with delicate biomolecules. This strategy does not require the pre-modification of biomolecules, preparation of dry [18F]F⁻, exposure of biomolecules to...
harsh conditions, and purification of radioactive intermediates. The negligible time-related costs associated with FTP synthon formation and application of the same mild conditions used for the conjugation step during multi-step labeling results in the high $^{18}$F-labeling efficiency. Typical medically conjugation step during multi-step labeling results in pharmacokinetic profile of the radiolabeled peptides the development of PET tracers from unmodified chemical motif or precursor ($^{18}$F)F– and separable precursors that allows site-specific labeling methods with certain chemical motif or precursor ($^{18}$F)F– was azeotropically dried as previously described [14]. Briefly, $^{18}$F)F– was produced via the $^{18}$O(p, n)$^{18}$F reaction and delivered as $^{18}$F)F– in $^{18}$O-enriched-water using QMA and subsequently released with by a solution of 8.0 mg kryptofix 222 ($K_{222}$) and 1.0 mg K$_2$CO$_3$ in 1.0 mL of acetonitrile/H$_2$O (4/1, v/v). The solution was

**Experimental Section**

**Materials**

All the reagents we used in the synthesis and biology experiment were purchased from Energy Chemical Co., Ltd. (China) or J&K Co., Ltd. (China) and were used without further purification. Column chromatography purification was performed on silica gel (54–74 μm, Qingdao Haiyang Chemical Co., Ltd., China). Anhydrous dichloromethane, anhydrous tetrahydrofuran (THF), anhydrous dimethyl sulfoxide (DMSO), anhydrous acetonitrile and anhydrous dimethylformamide (DMF) were purchased from Energy Chemical Co., Ltd. (China) and used without further drying.

**Fluorination kinetics**

In order to determine the fluorination kinetics and $E_a$ for $^{18}$F-fluorination process, we decided to carry out a series of experiments at different temperatures. We found experimentally that the optimal temperature range to monitor the labeling reaction rate of $^{18}$F]1 and $^{18}$F]2 is between -55 °C and -25 °C, since the reaction is too fast at higher temperatures and too slow at lower temperatures to measure. Concentrations of precursors and in DMF solutions were kept constant at 3.82 × 10$^{-3}$ M and 4.01 × 10$^{-3}$ M, respectively. The RCCs under each individual temperature was monitored by radio-TLC. Dynamic RCCs were able to be monitored by TLC because we took 10 μL of the reaction mixture at the indicated time points, quenched it in 1.0 mL of water before TLC developing. Labeling efficiency graphs could be converted to line graphs of reaction time versus ln [1/(1-RCC)], whose slopes represent specific rate constants at specific temperatures. Pseudo-first order initial rate constants at different temperatures were calculated from the exponential fit equation, and then $k$ was divided by the concentration of 1b or 2b to determine the actual second-order rate constants. These rate constants were used to create an Arrhenius plot (ln $k$ versus T$^{-1}$) to calculate the $E_a$, which was found to be 8.41 kcal mol$^{-1}$ for $^{18}$F-labeling reaction of $^{18}$F]1 and 8.98 kcal mol$^{-1}$ for $^{18}$F-labeling reaction of $^{18}$F]2.

**General manual $^{18}$F-labeling procedures**

Labeling procedure I: $^{18}$F)F– was azeotropically dried as previously described [14]. Briefly, $^{18}$F)F– was produced via the $^{18}$O(p, n)$^{18}$F reaction and delivered as $^{18}$F)F– in $^{18}$O-enriched-water using QMA and subsequently released with by a solution of 8.0 mg kryptofix 222 ($K_{222}$) and 1.0 mg K$_2$CO$_3$ in 1.0 mL of acetonitrile/H$_2$O (4/1, v/v). The solution was
The collected product was then put on the rotary evaporator before being analyzed and purified on radio-HPLC. NaOH (1.0 M) and diluted with PBS and acetonitrile solution. \([^{18}\text{F}]\text{FTP-AMP}\) was dissolved in saline for evaporator to remove excess acetonitrile from the shaken at RT for 10 min. The solution was then neutralized to pH 7 by adding NaOH (1.0 M) and lyzed and purified on HPLC. The collected product was then put on the rotary evaporator to remove excess methanol from the solution. \([^{18}\text{F}]\text{FTP-Tyr}\) was dissolved in saline for PET imaging studies.

Radiosynthesis of \([^{18}\text{F}]\text{FTP-Tyr}\)

\([^{18}\text{F}]\text{FTP-Tyr}\) was synthesized from 17b following labeling procedure I. \([^{18}\text{F}]\text{FTP-Tyr}\) was then dissolved in 100 μL MeOH followed by adding 100 μL NaOH (2.0 M) for 15 min. The reaction mixture was acidified by 1.0 M HCl and was dried under a stream of nitrogen. The resulting residue was dissolved in acetonitrile (100 μL). 5.0 M HCl or TFA (100 μL) was added and shaken at RT for 10 min. The solution was then neutralized to pH 7 by adding NaOH (1.0 M) and diluted with PBS and acetonitrile before being analyzed and purified on HPLC. The collected product was then put on the rotary evaporator to remove excess methanol from the solution. \([^{18}\text{F}]\text{FTP-Tyr}\) was dissolved in saline for PET imaging studies.

Radiosynthesis of \([^{18}\text{F}]\text{FTP-AMP}\)

\([^{18}\text{F}]\text{FTP-AMP}\) was synthesized from 18b following labeling procedure I. Then 100 μL TFA was added to \([^{18}\text{F}]\text{FTP-AMP}\) and stirred at RT for 5 min to deprotection. The solution was then neutralized to pH 7 by adding NaOH (1.0 M) and diluted with PBS and acetonitrile before being analyzed and purified on radio-HPLC. The collected product was then put on the rotary evaporator to remove excess acetonitrile from the solution. \([^{18}\text{F}]\text{FTP-AMP}\) was dissolved in saline for PET imaging studies.

Radiosynthesis of \([^{18}\text{F}]\text{FTP-NHS}\)

0.1 mg precursor 15b (0.2 μmol) was dissolved in dichloromethane (40 μL) and loaded onto a cotton ball (about 0.03 cm³). Then the dichloromethane was allowed to volatilize in a fume hood, and the small cotton carrying precursor 15b was fitted into a pipette tip (for manual labeling, any tube-like part for automatic modules. \([^{18}\text{F}]\text{FTP-NHS}\) was synthesized from 18b following labeling procedure II. No-carrier-added \([^{18}\text{F}]\text{F}^-\) was produced via the \(^{18}\text{O}(p, n)^{18}\text{F}\) reaction and delivered as \([^{18}\text{F}]\text{F}^-\) in \([^{18}\text{O}]\text{H}_2\text{O}\). \([^{18}\text{F}]\text{F}^-\) (3 μL, 0.30–0.37 GBq) was added to 100 μL acetonitrile with tetrabutylammonium hydroxide (TBAOH, 0.52 mg/100 μL), which was used as the eluent later. The pipette tip carrying precursor 15b was eluted by this mixed solution into a polypropylene tube and subsequently dried under a stream of nitrogen to afford \([^{18}\text{F}]\text{FTP-AMP}\) as a radiosynthon.

**Radiosynthesis of \([^{18}\text{F}]\text{FTP-c(RGDyK)}\)**

1.0 mg (1.6 μmol) c(RGDyK) was dissolved in a mixture of 10 μL DMSO and 100 μL of borate buffer (pH = 8.0). This c(RGDyK) solution was then added into the vial containing \([^{18}\text{F}]\text{I}\). After 10 min reaction at 37 °C, the reaction mixture was diluted with 10.0 mL of H₂O and loaded onto a light C18 cartridge. The cartridge was flushed twice with 10.0 mL of pure water to remove the unreacted \([^{18}\text{F}]\text{F}^-\) and 1.0 mL of ethanol to get crude product. The crude product was further purified by radio-HPLC. Purify condition: Waters XBridgeC-18 column (5 μm, 10 mm × 250 mm, USA). Phase A: PBS (0.02 mol L⁻¹ pH = 7.4); phase B: HPLC grade acetonitrile; isocratic elution at 90% phase A and 10% phase B. Flow rate: 3.0 mL min⁻¹. The HPLC fraction was dried under a stream of nitrogen at RT. \([^{18}\text{F}]\text{FTP-c(RGDyK)}\) was then dissolved in saline for injection.

**Radiosynthesis of \([^{18}\text{F}]\text{FTP-HSA}\)**

1.0 mg of HSA in 0.1 M borate buffer (pH = 8.0, 2 mg mL⁻¹, 100 μL) was added to a glass vial containing dried \([^{18}\text{F}]\text{I}\) and the mixture was incubated for 10 min at 40 °C. \([^{18}\text{F}]\text{FTP-HSA}\) was purified by a PD-10 column (GE Healthcare Bio-Science AB) using PBS (pH = 7.4) as the eluent. A size exclusion chromatography (SEC) column was applied to analyze its RCP.

**Radiosynthesis of \([^{18}\text{F}]\text{FTP-5F7}\)**

0.1 mg of 5F7 was dissolved in 100 μL of borate buffer (pH = 8.0) and added to \([^{18}\text{F}]\text{I}\). The reaction mixture was incubated for 10 min at RT. The product was purified and then analyzed by radio-HPLC equipped with an Xtimate SEC-300 column (Welch, China).

**Radiosynthesis of \([^{18}\text{F}]\text{FTP-Pds-PEG}\)**

\(^{18}\text{F}\)-Labeled FTPs were prepared following the Labeling procedure I. Pd nanosheets with an average size of 40 nm (200 μg), as an example of metal nanoparticle, dissolved in thiol-polyethylene glycol (mPEG-SH) solution (2 mg in 100 μL water) to obtain PEGylated Pd nanosheets. Then, \([^{18}\text{F}]\text{I}\) (taking \([^{18}\text{F}]\text{I}\) as an example) was added to Pd-PEG and stirred for 15 min at RT to get \([^{18}\text{F}]\text{FTP-Pds-PEG}\). RCCs was
determined by radio-TLC analysis. [\(^{18}\)F]I labeled Pd nanosheets with a high efficiency (about 93%). Then the mixture was subject to ultrafiltration (13000 rpm for 10 min, repeated 3 times) to remove unlabeled [\(^{18}\)F]I and [\(^{18}\)F]-. RCPs was determined by radio-TLC analysis.

**Abbreviations**

Activation energy (\(E_a\)); Arg-Gly-Asp (RGD); density functional theory (DFT); dimethylformamide (DMF); dimethyl sulfoxide (DMSO); fluorothiophosphate (FTP); human serum albumin (HSA); N-hydroxysuccinimide ester (NHS); cryptofix 222 (DMF); dimethyl sulfoxide (DMSO); fluorothio-

**Supplementary Material**


**Acknowledgments**

Data and materials availability

All data are available in the main text or the supplementary materials.

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**Author contributions**

Dr. Zijing Li conceived the study and coordinated all the research. Hongzhong Yang preformed the experiments. Zijing Li and Hongzhong Yang analyzed the results and prepared the manuscript. Computational chemistry was carried out by Dr. Lei Zhang. Molecular docking was carried out by Fengming He. Huanhuan Liu, Yunming Zhang, Zhaobiao Mou, Xueyuan Chen and Jingchao Li all offered help in biology and microPET imaging experiments.

**Competing Interests**

The authors have declared that no competing interest exists.

**References**


