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3-^{18}F-fluoropropane-1-thiol and ^{18}F-PEG$_4$-1-thiol: Versatile prosthetic groups for radiolabeling maleimide functionalized peptides

Orit Jacobson[a], Zhantong Wang[b], Guocan Yu[b], Ying Ma[a], Xiaoyuan Chen[b], Dale O. Kiesewetter[a,*]

[a] Molecular Tracer and Imaging Core Facility, National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, Bethesda, MD, USA
[b] Laboratory of Molecular Imaging and Nanomedicine, National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, Bethesda, MD, USA

A B S T R A C T

The efficient radiosynthesis of biomolecules utilizing minute quantities of maleimide substrate is important for availability of novel peptide molecular imaging agents. We evaluated both 3-^{18}F-fluoropropane-1-thiol and 2-(2-(2-(2-^{18}F-fluoroethoxy)ethoxy)ethoxy)ethane-1-thiol (^{18}F-fluoro-PEG$_4$ thiol) as prosthetic groups for radiolabeling under physiological conditions. The precursor employed a benzoate for protection of the thiol and an arylsulfonate leaving group. The radiofluorination was fully automated on an Eckert & Ziegler synthesis system using standard Kryptofix$_{222}$/K_2CO_3 conditions. In order to minimize the amount of biological molecule required for subsequent conjugation, the intermediates, S-(3-^{18}F-fluoropropyl) benzothioate and ^{18}F-fluoro-PEG$_4$ benzothioate, were purified by HPLC. The intermediates were isolated from the HPLC in yields of 37–47% and 28–35%, respectively, and retrieved from eluate using solid phase extraction. Treatment of the benzothioates with sodium methoxide followed by acetic acid provided the free thiols. The desired maleimide substrate in acetonitrile or phosphate buffer was then added and incubated at room temperature for 15 min. The final radiolabeled bioconjugate was purified on a separate HPLC or NAP-5 column. Maleimides utilized for the coupling reaction included phenyl maleimide, an Evans Blue maleimide derivative, a dimeric RGDfK maleimide (E[*c*(*RGDfK*)]$_2$), two aptamer maleimides, and PSMA maleimide derivative. Isolated radiochemical yields (non-decay corrected) of maleimide addition products based on starting ^{18}F-fluoride ranged from 6 to 22% in a synthesis time of about 90 min.

^{18}F-thiol prosthetic groups were further tested *in vivo* by conjugation to E[*c*(*RGDfK*)]$_2$ maleimide in a U87MG xenograft model. PET studies demonstrated similar tumor accumulation of both prosthetic groups. ^{18}F-fluoro-PEG$_4$-S-E[*c*(*RGDfK*)]$_2$ displayed a somewhat favorable pharmacokinetics compared to ^{18}F-fluoropropyl-S-E[*c*(*RGDfK*)]$_2$. Bone uptake was low for both indicating *in vivo* stability.

1. Introduction

Despite its favorable chemical properties and decay characteristics ($t_{1/2}$ = 109.7 min, 97% β^+, E(β^+)max = 634 keV), the use of ^{18}F for biological molecules is impeded because the incorporation reaction conditions are typically harsh and require protection of many functional groups on biomolecules. Numerous prosthetic groups have been developed, published, and reviewed for labeling biomolecules with ^{18}F in the last three decades.[1–6] The prosthetic groups are small organic molecules that can be easily radiolabeled with ^{18}F and then conjugated to the biomolecules, mainly by targeting the primary amine of lysine or the thiol of cysteine.[1–3]

In 2004, Glaser et al. reported on the labeling of ^{18}F-fluorothiols and successfully applied to the thiol selective displacement on a 2-chloroacetamide. They utilized model 2-chloroacetamide peptides (3.2 μmol) and, without intermediate purification, conducted the displacement reaction using basic conditions (pH 9–10) and heat (70–80 °C for 30 min).[7] As stated above, most of the biomolecules are unstable under such labeling conditions and attempt by the same group to label a 2-chloroacetamide-RGD peptide using these conditions failed.[8]

It is known that the reaction between a thiolated biological molecule and a high molar activity [^{18}F]fluorinated maleimide worked very effectively.[9] We believed that the reverse approach would work as well and thus developed two [^{18}F]fluorothiol prosthetic groups, similar to those described above by Glaser et al., that were easily purified by HPLC and with an easily cleaved protecting group. Both prosthetic groups were synthesized in good radiochemical yield and high molar activity. The bioconjugation of the [^{18}F]fluorothiols with maleimide functionalized biomolecules was conducted in phosphate-buffered saline (PBS, pH 7.4) at room temperature (RT) and with small amounts of precursor (Fig. 1). The whole labeling procedure was fully automated in an Eckert & Ziegler modular synthesis system. In order to assess effects of these prosthetic group on biological activity, we conducted *in vivo* evaluation of an RGD dimer peptide to which [^{18}F]fluorothiol prosthetic groups had been conjugated.

2. Material and methods

2.1. General

Solvents and reagents were purchased from Sigma Aldrich or Fisher

* Corresponding author.
E-mail address: dkiesewetter@mail.nih.gov (D.O. Kiesewetter).

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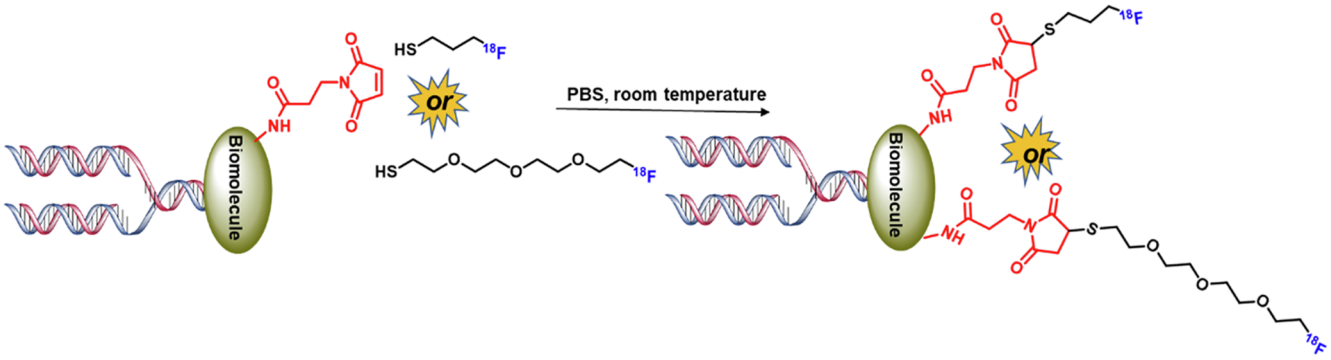


Fig. 1. Radiolabeling of various biomolecules containing maleimide *via* ^{18}F-thiol prosthetic groups in phosphate buffered saline (PBS) pH 7.4 for 15 min at room temperature.

Scientific. ^{18}F-fluoride was obtained from NIH Warren Grant Magnuson Clinical Center cyclotron facility. E[*c*(*RGDfK*)]$_2$ was acquired from CS Bio. The ultraviolet absorbance for high-performance liquid chromatography (HPLC) system was monitored at 254 and 210 nm. ^{1}H, ^{13}C and ^{19}F NMR spectra were obtained on a Bruker Avance 300 MHz NMR spectrometer in $CDCl_3$. LC-MS were carried out on a Acquity Waters system equipped with an electrospray interface (ESI) similarly to the published protocol.[17]

2.2. Chemistry

2.2.1. 2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (PEG_4-monotosylate)

(PEG_4 monotosylate) were synthesized according to the published procedure.[18]

2.2.2. S-(2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl) benzothioate (PEG-4-benzothioate)

PEG_4-monotosylate (186 mg, 0.53 mmol) was dissolved in 3 mL CH_3CN. Et_3N (73 μL, 0.53 mmol) was added, followed by thiobenzoic acid (73 mg, 0.53 mmol). TLC (1:1 EtOAc: hexane) was used to follow the reaction. The reaction was stirred for 24 h, a second portion of 20 mg thiobenzoic acid added, and stirred for three days. The solvent was removed under vacuum and the residue subjected to silica gel chromatography (ISCO system, EtOAc: CH_2Cl_2) using the standard linear gradient. The product was isolated (109 mg, 65%). The product, which contained about 10 mol percent starting material, was used directly in subsequent reactions without additional purification. ^{1}H NMR δ 3.34 (t, J = 6.47 Hz, 2H), 3.60–3.67 (m, 2H), 3.67–3.81 (m, 12H), 7.44–7.53 (m, 2H), 7.57–7.65 (m, 1H), 7.96–8.04 (m, 2H).

2.2.3. S-(2-(2-(2-(2-(((4-nitrophenyl)sulfonyl)oxy)ethoxy)ethoxy)ethyl) benzothioate (nosylate PEG_4 benzothioate)

PEG_4 benzothioate (475 mg, 1.5 mmol) was dissolved in 5 mL ethyl acetate. 4-Nitrobenzenesulfonyl chloride (335 mg, 1.5 mmol) was added, followed by Et_3N (209 μL, 1.5 mmol). The mixture was stirred overnight. A precipitate was filtered off and the solvent evaporated. The residue was subjected to silica gel chromatography (CH_2Cl_2 / EtOAc gradient) on a 40-gram cartridge using an ISCO system. The purest fraction was collected for subsequent use (218 mg, 29%). ^{1}H NMR δ 3.28 (t, J = 6.2 Hz, 2H), 3.56 (brs, 4H), 3.59–3.75 (m, 8H), 4.24–4.32 (m, 2H), 7.39–7.50 (m, 2H), 7.52–7.62 (m, 1H), 7.90–8.00 (m, 2H), 8.13 (dm, J = 8.5 Hz, 2H), 8.39 (dm, J = 8.55, 2H). ^{13}C NMR 191.76, 153.45, 137.11, 133.68, 129.57, 128.85, 127.44, 124.58, 70.94, 70.83, 70.76, 70.59, 70.07, 68.76, 28.85.

2.2.4. S-(2-(2-(2-(2-fluoroethoxy)ethoxy)ethoxy)ethyl) benzothioate (Fluoro-PEG_4-benzothioate)

PEG_4 benzothioate (109 mg, 0.347 mmol) was dissolved in 2 mL CH_2Cl_2. The solution was cooled in ice prior to addition of diethylaminosulfur trifluoride (DAST) (46 μL, 0.347). After 150 min, the reaction was quenched with bicarbonate solution and extracted with CH_2Cl_2. The organic layers were washed with brine and dried. After evaporation, the products were separated by silica gel chromatography (CH_2Cl_2/hexane gradient) to yield the desired product (24 mg, 22%). Products resulting from cleavage of the PEG chain were also observed. ^{1}H NMR δ 3.3 (t, J = 6.7 Hz, 2H), 3.67–3.78 (m, 11H), 3.80 (m, 1H), 4.59 (dm, J = 48.3 Hz, 2H), 7.43–7-53 (m, 2H), 7.56–7.65 (m, 1H), 7.96–8.05 (m, 1H). ^{19}F NMR (external standard) −222.9 (tt, J = 48.4, 29.6 Hz, 1F). GC–MS (CI-CH_4) 105 (100), 149 (25), 165 (50), 257 (5), 297 (1), 315 (trace), 317 (trace).

2.2.5. S-(3-hydroxypropyl) benzothioate

Thiobenzoic acid (514 mg, 3.7 mmol), 3-bromopropanol (517 mg, 3.72 mmol), and diisopropylethylamine (480 mg, 3.72 mmol) were combined in 10 mL CH_3CN. The mixture was heated at 80 °C for 5 h. The CH_3CN was evaporated and the residue partitioned between ether and water. The aqueous layer was extracted with a second portion of ether and the combined ether layers were washed with brine, dried, and evaporated. The residue was chromatographed on silica gel (EtOAc:hexane, ISCO system, standard gradient) to provide the product (313 mg, 42%). GC–MS (EI) T_R 6.38 min, m/z 196 (0.28), 123 (22), 105 (100), 77 (45). ^{1}H NMR δ 1.92 (quin, J = 6.63 Hz, 2H), 3.21 (t, J = 6.8 Hz, 2H), 3.706 (t, J = 5.8 Hz, 2H), 7.44 (t, J = 7.4 Hz, 2H), 7.57 (tm, J = 7.3 Hz, 1H), 7.91–8.02 (m, 2H). ^{13}C NMR δ 25.40, 32.73, 60.50, 127.44, 128.79, 133.69, 137.02, 193.39.

2.2.6. S-(3-fluoropropyl) benzothioate

A flask was charged with argon; thiobenzoic acid (1 g, 7.2 mmol) and CH_3CN (20 mL) were added. Then, 3-bromo-1-fluoropropane (1.0 g, 7.2 mmol) and iPr_2NEt (1.26 mL) were added. The reaction was heated at 90 °C for 6 h, partitioned between water and CH_2Cl_2. The organic layers were dried with Na_2SO_4 and evaporated. The residue was subjected to silica gel chromatography using a gradient of EtOAc and hexane. The product was obtained (846 mg, 59%). GC–MS $C_{10}H_{11}FOS$: calculated 198.05: observed (EI) 7.03 min, m/z 198 (2), 105 (100), 77 (40). ^{1}H NMR δ 2.08 (dm, J = 26 Hz, 2H), 3.19 (t, J = 7 Hz, 2H), 4.55 (d, t, J = 47.7, 5.6 Hz, 2H), 7.92–8.01 (m, 2H), 7.52–7.62 (m, 1H), 7.4–7.51 (m, 2H). ^{13}C NMR 191.82, 137.144, 133.65, 128.83, 127.41, 82.5 (d, J = 166 Hz), 30.76 (J = 20 Hz), 25.06 (d, J + 5.2 Hz). ^{19}F NMR (external standard) −221 (tt, J = 48, 25 Hz).

2.2.7. S-(3-(tosyloxy)propyl) benzothioate

S-(3-hydroxypropyl) benzothioate (1.3 g, 6.6 mmol) was dissolved in 15 mL of pyridine and cooled in ice. Tosyl chloride (1.26 g, 6.6 mmol) was added. The reaction sat in refrigerator for three days. The solution was poured into water and extracted with 2 × 50 mL ether. The ether layer was washed with portions of 2 N H_2SO_4 until the

aqueous layer remained acidic. The ether layer was dried with Na_2SO_4 and evaporated. TLC indicated unreacted starting materials and the chlorinated side product. The residue was subjected to flash chromatography on silica gel (20% EtOAc in hexane) to provide the product (624 mg, 27%). GC–MS 10 min, *m/z* 105 (100), 77 (18), 178 (4), 277 (trace), 350 (trace). ^{1}H NMR δ 2.07 (p, J = 6.4 Hz, 2H), 2.46 (s, 3H), 3.11 (t, J = 7.0 Hz, 2H), 4.18 (t, J = 5.8 Hz, 2H), 7.37 (d, J = 8.2 Hz, 2H), 7.48 (tm, J = 7.1 Hz, 2H), 7.61 (tm, J + 7.5 Hz, 1H), 7.84 (dm, J + 8.4 Hz, 2H), 7.94 (dm, J = 7.1 Hz, 2H).

2.2.8. 3-((3-fluoropropyl)thio)-1-phenylpyrrolidine-2,5-dione

N-phenyl maleimide (1 g, 5.78 mmol) was treated with 3-mercaptopropanol (532 mg, 5.78 mmol) in 10 mL benzene with a catalytic amount of Et_3N (10 μL). The reaction was stirred overnight, and the solvent evaporated. The residue was subjected to chromatography on silica gel (EtOAc:hexane, ISCO system standard gradient) to provide the product (589 mg, 76%). GC–MS (EI), T_R 8.51 min, *m/z* 265 (trace), 175 (100), 119 (95). ^{1}H NMR δ 1.74–2.05 (m, 3H), 2.67 (dd, J = 18.59, 3.65 Hz, 1H), 2.86–3.14 (m, 2H), 3.30 (dd, J = 18.5, 9.17 Hz, 1H), 3.75 (brt, J = 5.4 Hz, 2H), 3.89 (dd, J = 9.17, 3.60 Hz, 1H), 7.22–7.33 (m, 2H), 7.36–7.53 (m, 3H). ^{13}C NMR 28.69, 31.87, 36.44, 61.17, 126.59, 129.03, 129.42, 131.723, 173.89, 175.95.

3-((3-hydroxypropyl)thio)-1-phenylpyrrolidine-2,5-dione (218 mg, 0.82 mmol) was dissolved in 10 mL CH_2Cl_2, cooled in ice and treated with DAST (108 μL, 0.82 mmol). After 3 h the reaction was quenched with saturated $NaHCO_3$, partitioned with CH_2Cl_2, dried and evaporated. The residue was subjected to silica gel chromatography (ISCO system, EtOAc/hexane) using a 12 g silica cartridge. The desired product was obtained (45 mg, 16%). ^{1}H NMR δ 1.94–2.22 (m, 2H, alkyl CH2), 2.68 (dd, J = 19.4, 3.5, 1H, ring CH2), 2.88–3.02 (m, 1H, CH2-S), 3.04–3.18 (m, 1H, CH2-S), 3.20 (dd, J = 18.8, 9.2, 1H, ring CH), 3.87 (dd, J = 9.2, 3.6, 1H, ring CH2), 4.56 (dt, J + 46.9, 5.7, 2H, CH2F), 7.24–7.34 (m, 2H), 7.34–7.54 (m, 3H). ^{19}F NMR (external standard) −220.78 (tt, J = 47.3, 25.6 Hz). ^{13}C NMR 28.1 (d, J = 4.9 Hz), 30.17 (d, J = 20.4 Hz), 36.20, 39.38, 82.2 (d, J = 166.5 Hz), 125.49, 126.58, 128.42, 129.03, 129.23, 129.44, 173.74, 175.60.

2.2.9. General procedure for conjugation of primary amine to maleimide substrates

Substrate containing free amine group (1 eq, 40 mg/mL in DMSO was dissolved in 0.5 mL dimethyl sulfoxide (DMSO). Then 1.2 eq of 3-maleimidopropionic acid NHS ester (80 mg/mL in DMSO) were added followed by addition of neat triethylamine (4 eq). The mixture was stirred at RT for 1–2 h. The reaction was diluted with water and purified by HPLC (Higgins C-18 column (5 μm, 250 × 20 mm), gradient 95% solvent A [0.1% trifluoroacetic acid (TFA)/$H_2$0] and 5% solvent B [0.1%TFA/CH_3CN] and changing to 65% solvent B at 35 min, flow rate of 12 mL/min.

For aptamers, a concentration of 10 mg/mL aptamer-5AmMC6-modification (free NH_2) in H_2O was used and purification was done on NAP-5 column using phosphate-buffered saline (PBS) as an eluent.

2.2.10. Synthesis of E[c(RGDfK)]$_2$-maleimide for biological studies

E[*c*(*RGDfK*)]$_2$-maleimide was prepared similarly to the above procedure, using 1.2 eq of NHS-maleimidopropionate and 4 eq of triethylamine, to give 34% isolated product with chemical purity > 95%. LC-MS analysis confirmed mass of 1467 $[M-H]^+$.

2.3. Radiolabeling

2.3.1. Radiosynthesis of ^{18}F-Fluoropropyl benzothioate and ^{18}F-Fluoro-PEG$_4$ benzothioate

Radiosynthesis of both, ^{18}F-Fluoropropyl benzothioate and ^{18}F-Fluoro-PEG_4 benzothioate, was done on a modular system (Eckert & Ziegler Eurotope GmbH). 130–270 mCi of ^{18}F-fluoride were added to a reactor vial containing 12 μmol of K_2CO_3 in 60 μL H_2O and 24 μL $K_{2.2.2}$ in 500 μL CH_3CN. Azeotropic drying was done using CH_3CN and heating to 120 °C and under a stream of argon and vacuum. Then, 20–24 μmol of the precursors (either tosyloxypropyl benzothioate or nosyl-PEG_4 benzothioate) were added in 500 μL CH_3CN. The reactor was sealed and heated to 95 °C for 10 min. Then after, the reactor was cooled to 40 °C and 1 mL of H_2O was added. The crude mixture was injected into built-in HPLC system using Agilent column (Zorbax 300SB-C18, 5 μm, 250 × 20 mm) isocratic conditions of 40% CH_3CN, 60% H_2O, flow 6 mL/min. ^{18}F-fluoropropyl benzothioate and ^{18}F-fluoro-PEG_4 benzothioate were eluted with retention times of 13.8 and 8.2 min, respectively, trapped on Waters light C18 cartridge, and eluted with 500 μL CH_3CN. Quality control and specific activity calculations for radiolabeled compounds were done on a Phenomenex Luna C18 column (5 μm, 4.6 × 150 mm) using 0.1% trifluoracetic acid (TFA) in water (solvent A) and CH_3CN (solvent B) with a gradient system starting from 80% of solvent A and 20% of solvent B for 2 min, changing to 10% solvent A and 90% solvent B at 22 min, with a flow rate of 1 mL/min.

2.3.2. Radiosynthesis of ^{18}F-Fluoropropyl thiol and ^{18}F- Fluoro-PEG$_4$ thiol

Deprotection of benzothioate group was done as follows; 200 μL of sodium methoxide solution (0.5 M in methanol) were added to a solution of either, ^{18}F-Fluoropropyl benzothioate and ^{18}F-Fluoro-PEG_4 benzothioate in 500 μL CH_3CN for 10 min incubation at RT, followed by treatment with 400 μL of 25% acetic acid in H_2O for additional 2 min. The thiolate compounds were used as is for the next step, with no further purification.

2.3.3. Conjugation with maleimide substrates

To the thiol solutions described above, were added 0.008–3 μmol of maleimide substrate in 400 μL PBS pH 7.4. The reaction mixture was incubated at RT for 15 min, followed by either NAP-5 purification (for ^{18}F-labeled aptamers) or in all other cases, HPLC purification on Phenomenex Luna C18 column (C-18, 5 μm, 250 × 10 mm) using a gradient starting from 95% solvent A and 5% solvent B and changing to 65% solvent B at 35 min at flow rate of 5 mL/min. The labeled compounds were collected, diluted with excess of water, trapped on Waters light C-18 cartridge and eluted with 0.2 mL of 0.5% acetic acid in ethanol solution.

2.4. Biological evaluation of ^{18}F-Fluoro-propyl-thio-E[c(RGDfK)]$_2$ and ^{18}F-Fluoro-PEG$_4$-thio- E[c(RGDfK)]$_2$

2.4.1. PBS and serum stability

20 μL of either ^{18}F-E[*c*(*RGDfK*)]$_2$ derivative were incubated in 200 μL PBS at RT or mouse serum at 37 °C for 2 h. The stability was tested by analytical HPLC using the system described above. Extraction of radioactivity from mouse serum was done using 2-fold volume excess of CH_3CN, followed by short vortex and centrifugation at 10,600*g* for 5 min.

2.4.2. Cell lines and mice models

In vivo studies were conducted in accordance to animal protocol NIBIB 16-01, which had been approved by the Animal Care and Use Committee of the NIH Clinical Center. Athymic nude mice (Envigo) were kept in an animal facility under pathogen free conditions. U87MG cells were purchased from ATCC and cultured in Eagle's Minimum Essential Medium fortified by addition of 10% fetal bovine serum. Cells were kept in a humidified incubator containing 5% CO_2 in air at 37 °C.

2.4.3. PET imaging studies

150–170 μCi radiolabeled compounds were diluted with PBS and injected intravenously to U87MG-tumor bearing mice (n = 5). PET scans were acquired on Inveon scanner (Siemens Preclinical Solutions) at 0.5, 1 and 2 h post-injection using acquisition time of 5–10 min. The images were reconstructed using ASIPRO (Siemens Preclinical

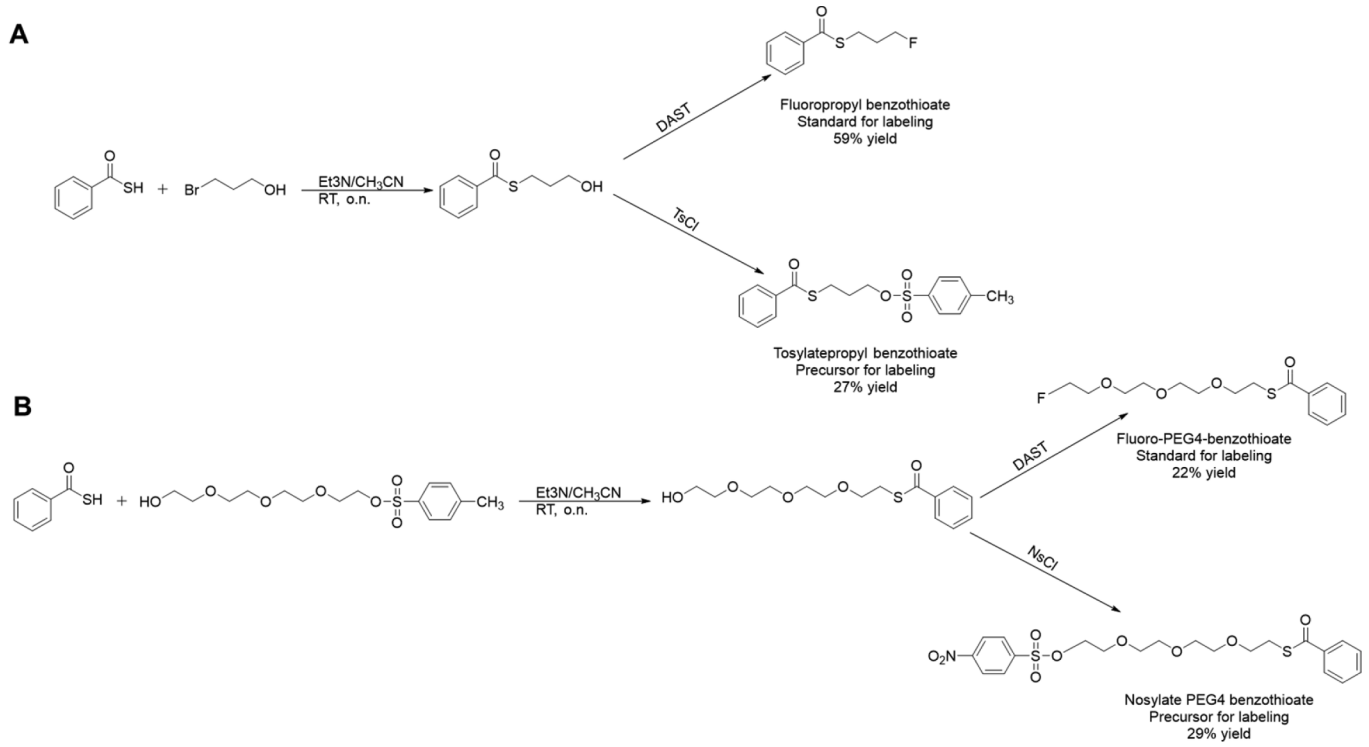


Fig. 2. Synthetic routes for precursors and/or standards. Reagents and yields are indicated in the scheme. The thiol is introduced by nucleophilic reaction of thiobenzoic acid with a mono activated diol. The remaining alcohol is converted to the activated sulfonate ester or to the fluoride using the appropriate reagent.

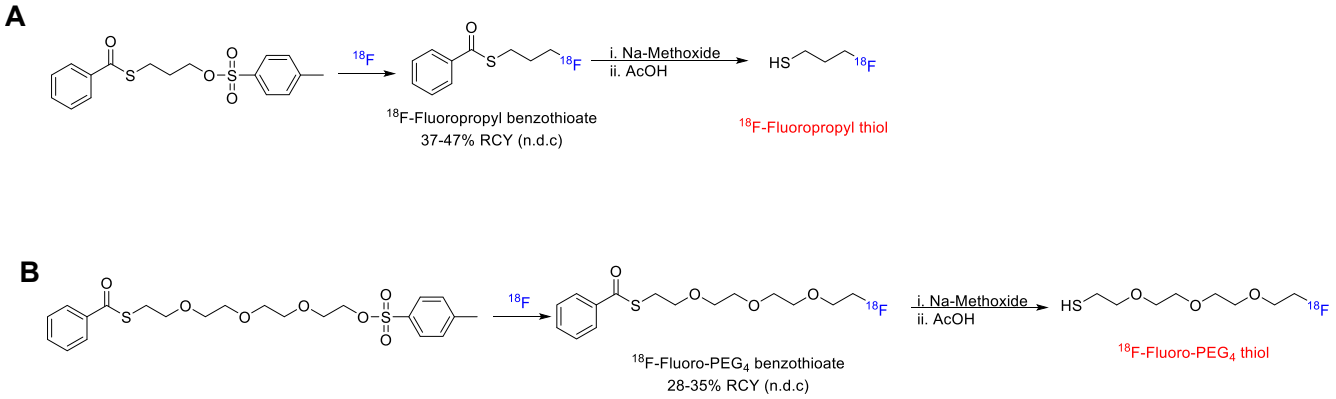


Fig. 3. Radiosynthesis of ^{18}F-thiol prosthetic groups. [^{18}F]Fluoride displacement is conducted using standard K222/K_2CO_3 conditions in CH_3CN. The intermediate is purified by HPLC. Subsequently, the protective group is cleaved with $NaOCH_3$.

Solutions) and/or VivoQuant (Invicro).

2.4.4. Statistical analysis

Results are reported as mean ± SD. Two-tailed paired and unpaired Student's t tests were used to determine significance differences.

3. Results

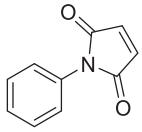
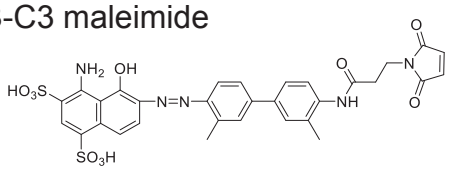
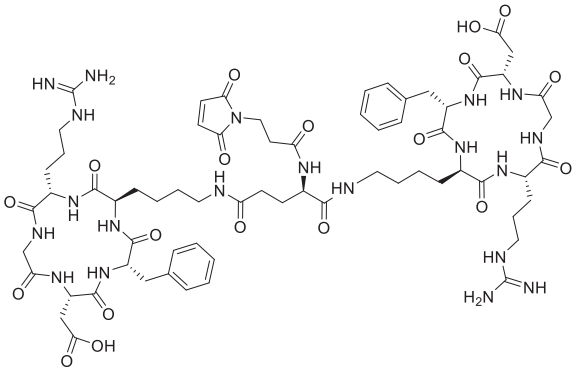
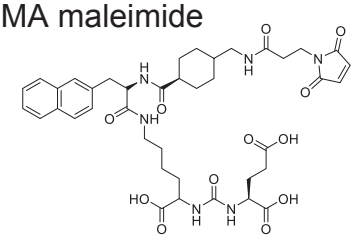
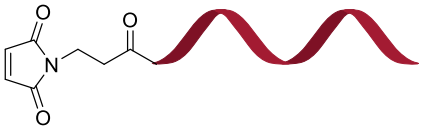
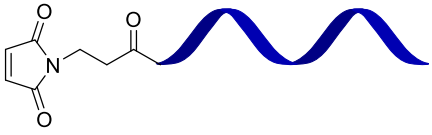
3.1. Chemistry

Synthesis of precursors, tosylate-propyl benzothioate and nosylate-PEG_4 benzothioate was done in two steps and resulted in reasonable yields and high chemical purity (> 98%) (Fig. 2). These two examples provide prosthetic groups with differing hydrophilicity that may be of importance for a particular application. ^{19}F standards for the first labeling step, were synthesized *via* the hydroxyl group using (Diethylamino)sulfur trifluoride (DAST) as a fluorination reagent. This reaction was more efficient for the propyl benzothioate derivative (59% isolated yield compares to 22% for the PEG_4-benzothioate, Fig. 2). Excluding the commercially available phenyl maleimide, the other maleimide substrates were synthesized by conjugation of free amine to 3-maleimidopropionic acid. ^{19}F standards reported in this study, were synthesized following the radiolabeling procedure, using ^{19}F-fluoropropyl benzothioate or ^{19}F-fluoro-PEG_4 benzothioate. The chemical purities of all fluorinated standards were greater than 95%, as determined by analytical HPLC and LC-MS analysis (Supp. Figs. S1–S7).

3.2. Radiolabeling

Synthesis and purification of ^{18}F-fluoropropyl benzothioate and ^{18}F-fluoro-PEG_4 benzothioate was done on a modular system using the built-in HPLC module. ^{18}F-Fluoropropyl benzothioate yields were superior to ^{18}F-fluoro-PEG_4 benzothioate (37–47% *vs.* 28–35% non-decay corrected isolated yields, n = 5). Overall synthesis time for this step was 50 min. Both tracers were obtained with high radiochemical purity (> 98%) and molar activity of 3.4 ± 1.2 Ci/μmol (n = 5).

Table 1
^{18}F-Fluoropropyl benzothioate radiolabeling with various maleimides. RCY calculated for isolated product starting from ^{18}F-Fluoropropyl thiol (n = 3).

Substrate	MW	μmol	% RCY
Phenyl maleimide 	173.2	3	78 ± 6
EB-C3 maleimide 	693.7	0.29	49 ± 8
Cyclo(RGDfk) dimer maleimide 	1468.7	0.13	72 ± 3
PSMA maleimide 	806.3	0.21	61 ± 2
Tenascin-C aptamer 	21,764	0.008	9 ± 3
Sgc8 aptamer 	12,827	0.0024	14*

* n = 1.

Deprotection of the benzothioate group was efficient using excess of sodium methoxide solution, resulted in high conversion to the desired thiol intermediate, followed by addition of acetic acid to quench the sodium methoxide and neutralize the crude mixture before addition of the maleimide biomolecule (Fig. 3). Optimization of the conditions for maleimide conjugation were first done using the commercial phenyl maleimide. Fifteen minutes at RT in the presence of PBS was sufficient to get high conversion to the desired ^{18}F-phenyl maleimide, therefore, those conditions were used for the other maleimide substrates. We applied this new labeling approach on various maleimide substrates such as small molecule, peptide and aptamers, using small amount of maleimide. Radiochemical yields, based on ^{18}F-fluoropropyl benzothioate, with various substrates (0.0024–3 μmol) are presented in Table 1. The radiochemical yields based on ^{18}F-fluoro-PEG4-benzothioate with phenyl maleimide, E[*c*(*RGDfK*)]$_2$, and PSMA maleimide were similar to those obtained with ^{18}F-fluoropropyl benzothioate. In all cases, the reaction was relatively clean and resulted in high conversion to the desired labeled product (Fig. 4).

3.3. Biology

In order to evaluate these two ^{18}F-thiol synthons for applications in

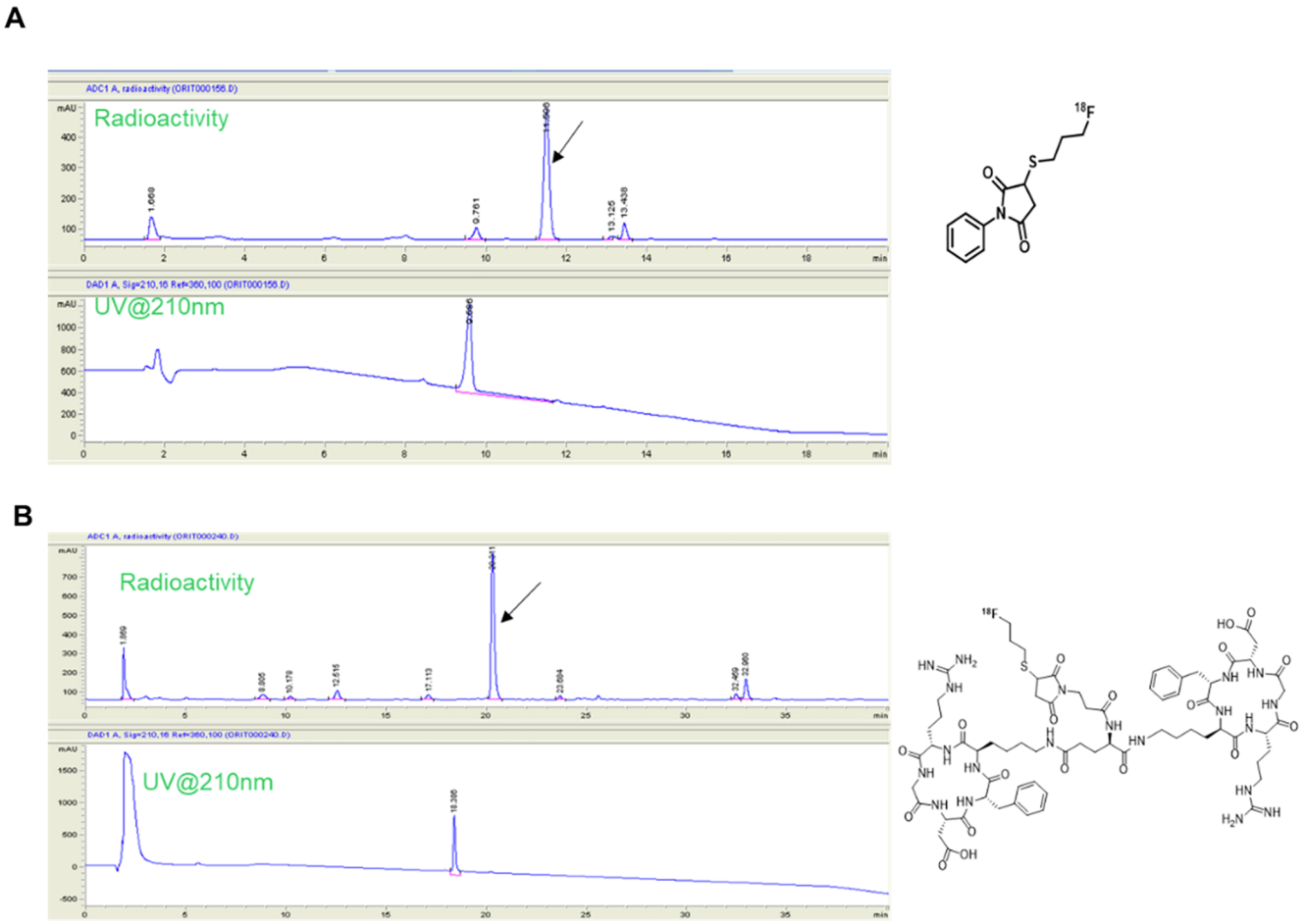


Fig. 4. Representative analytical HPLC of crude mixture from conjugation of ^{18}F-fluoro-propyl-thiol to phenyl maleimide (A) or E[*c*(*RGDfk*)]$_2$ (B) Arrows point to the peak of the desired product.

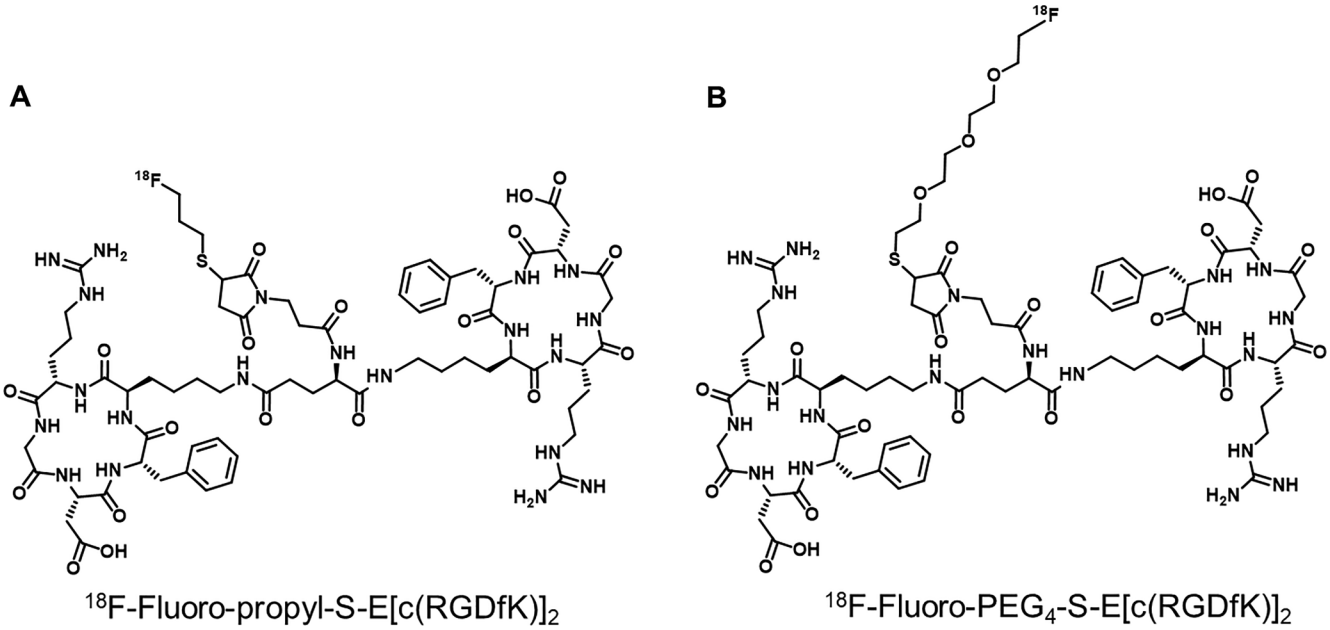


Fig. 5. Chemical structures of ^{18}F-fluoro-propyl-S-E[*c*(*RGDfK*)]$_2$ and ^{18}F-fluoro-PEG$_4$-S-E[*c*(*RGDfK*)]$_2$ which were used for biological evaluation.

PET imaging, we need to evaluate their *in vitro* and *in vivo* stabilities. We chose E[*c*(*RGDfK*)]$_2$ as a model molecule and prepared ^{18}F-fluoro-propyl-S-E[*c*(*RGDfK*)]$_2$ and ^{18}F-fluoro-PEG$_4$-S-E[*c*(*RGDfK*)]$_2$ (Fig. 5). Both ^{18}F-E[*c*(*RGDfK*)]$_2$ tracers showed high stability in PBS and mouse serum (Fig. 6), with extraction efficiency of 87–92% using CH_3CN. ^{18}F-fluoro-PEG$_4$-S-E[*c*(*RGDfK*)]$_2$ displayed a small amount of a new polar

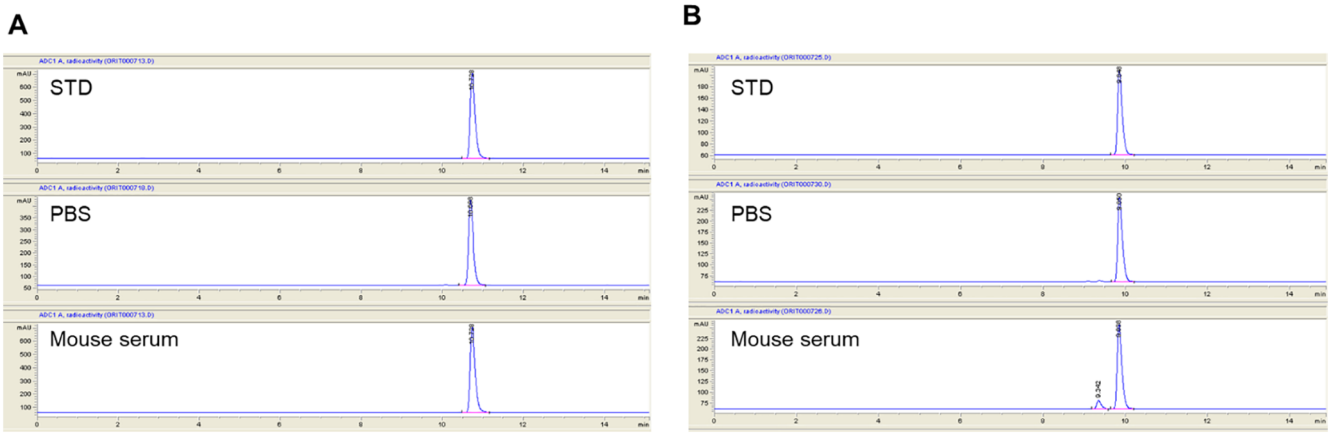


Fig. 6. Stabilities of ^{18}F-Fluoro-propyl-S-E[*c*(*RGDfK*)]$_2$ (A) and ^{18}F-Fluoro-PEG$_4$-S-E[*c*(*RGDfK*)]$_2$ (B) in ethanol (STD), PBS and mouse serum at 2 h time point.

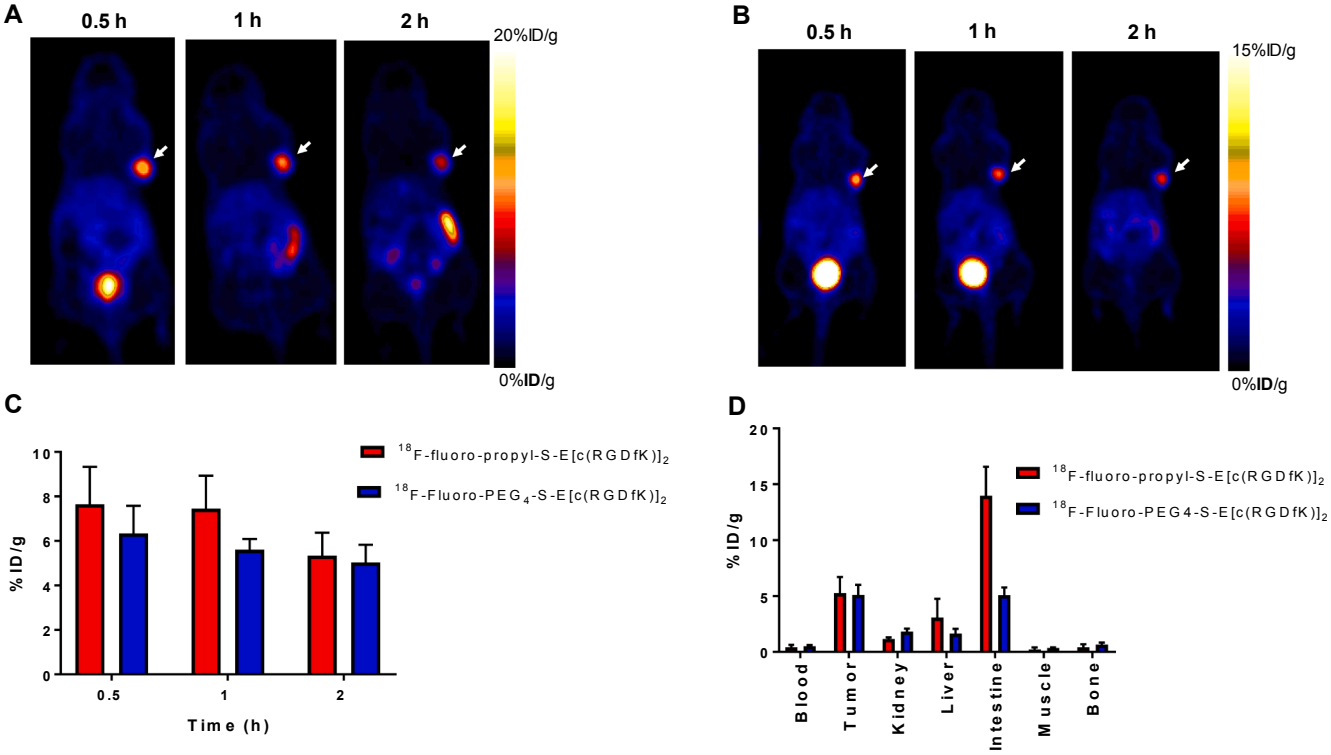


Fig. 7. Representative coronal PET images of U87MG tumor mice injected with either ^{18}F-fluoro-propyl-S-E[*c*(*RGDfK*)]$_2$ (A) or ^{18}F-fluoro-PEG$_4$-S-E[*c*(*RGDfK*)]$_2$ (B) over time. White arrows indicate tumor location. (C) Mean tumor uptake calculated from PET images of ^{18}F-fluoro-propyl-S-E[*c*(*RGDfK*)]$_2$ (red bars) and ^{18}F-fluoro-PEG$_4$-S-E[*c*(*RGDfK*)]$_2$ (blue bars). (D) Biodistribution of ^{18}F-fluoro-propyl-S-E[*c*(*RGDfK*)]$_2$ (red bars) and ^{18}F-Fluoro-PEG$_4$-S-E[*c*(*RGDfK*)]$_2$ (blue bars) at 2 h post-injection by direct tissue sampling.

peak at 2 h (Fig. 6B). PET studies showed more favorable pharmacokinetics of ^{18}F-fluoro-PEG$_4$-S-E[*c*(*RGDfK*)]$_2$ over ^{18}F-fluoro-propyl-S-E[*c*(*RGDfK*)]$_2$, with less accumulation in the intestine over time (Fig. 7). Although ^{18}F-fluoro-propyl-S-E[*c*(*RGDfK*)]$_2$ displayed slightly higher tumor accumulation at 0.5- and 1-hpost-injection, those differences were not significant (Fig. 7C). Both compounds seem to be stable *in vivo* and only low uptake in the bone was observed (0.4 ± 0.3 %ID/g and 0.6 ± 0.1 %ID/g at 2 h post-injection for ^{18}F-fluoro-propyl-S-E[*c*(*RGDfK*)]$_2$ and ^{18}F-fluoro-PEG$_4$-S-E[*c*(*RGDfK*)]$_2$, respectively).

4. Discussion

Numerous prosthetic groups have been labeled over the past two decades, mainly for conjugation to biomolecules for PET. Fluorination of the prosthetic groups is typically a multi-step process and conjugation to the sensitive biomolecule and is typically done under harsh to moderate conditions. Most of the prosthetic groups take advantage of the abundant amines and thiols that are available in peptides or proteins and are used to conjugate ^{18}F-prosthetic groups.[9–17]

In this paper, we synthesized two ^{18}F-thiol prosthetic groups which can be easily conjugated to biomolecules, such as aptamers, peptide, *etc*. under conditions that do not affect biomolecules, namely PBS, pH 7.4 at RT. The prosthetic groups described in this paper, ^{18}F-fluoropropyl thiol and ^{18}F-fluoro-PEG4-thiol, were reported by Glaser et al., in 2004 using a different precursor for labeling and different chemistry for conjugation with short peptide.[7] In 2009, the same group reported that the ^{18}F-fluoropropyl thiol prosthetic group failed to label a chloroacetylated RGD peptide.[8] Instability of the peptide precursor at high temperature and alkaline conditions were identified as the major challenge.

Our approach was to optimize the radiosynthesis and conjugation reaction of ^{18}F-fluorothiols with maleimide substrates, which are generally more reactive than chloroacetamides. In order to use small quantities of biomolecule, both ^{18}F-fluoropropyl benzothioate and ^{18}F-fluoro-PEG$_4$ benzothioate, were purified by HPLC to give high specific activity ^{18}F-intermediates. Our choice of the benzothioate protecting group allowed for rapid deprotection using NaOMe and subsequent bioconjugations could be conducted in the same reaction vessel.

The three-step radiolabeling route we developed provides good yields. The purified F-18 labeled benzothioate precursor is rapidly deprotected and then reacted with the maleimide-tagged target in the same vessel under very mild conditions. We expect that any maleimide-containing biomolecule stable at room temperature and soluble in ethanol PBS mixture would be suitable for this radiolabeling method. We did not evaluate molecules that contain disulfide bonds such as antibodies, therefore we cannot testify for the suitability of the method for such molecules.

A head-to-head comparison of the two prosthetic groups described herein in term of kinetics and yield was done using the small model molecule, *N*-phenylmaleimide, and gave marginal difference. This multi-step process was converted to fully automated system using two reactors; one for the nucleophilic fluorination and the other for deprotection of the benzothioate and reaction with maleimide substrate.

In vivo ^{18}F-fluoro-PEG$_4$-S-E[*c*(*RGDfK*)]$_2$ had favorable pharmacokinetics and less accumulation in metabolic organs than ^{18}F-fluoropropyl-S-E[*c*(*RGDfK*)]$_2$. This might be explained by its reduced hydrophobicity.

5. Conclusions

In summary, our benzthioate precursors for preparation of ^{18}F-mercaptans provided a robust method for radiolabeling of maleimide-containing biomolecules. Both prosthetic groups can be reliably synthesized from the corresponding precursor in high isolated radiochemical yields and can be reacted with maleimide-biomolecules using low molar concentration of the desired biomolecules.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2019.08.002.

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