Fast Indirect Fluorine-18 Labeling of Protein/Peptide using the useful 6-Fluoronicotinic acid-2,3,5,6-Tetrafluorophenyl prosthetic group: A Method Comparable to direct Fluorination

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Abstract

Fluorine-18 labeling of biomolecules are mostly performed by an indirect labeling method using a prosthetic group. Fluorine-18 labeled 6-fluoronicotinic acid-2,3,5,6-tetrafluorophenyl ester is a useful prosthetic group to radiolabel a protein. Recently we reported an improved preparation of this prosthetic group. To test the conjugation efficiency of the labeled ester prepared by this method we have performed conjugation reactions with two different peptides and a protein. Prostate-specific membrane antigen (PSMA) targeting peptide [^{18}F]DCFPyL, αvβ3 integrin receptors targeting peptide [^{18}F]c(RGDfK) and [^{18}F]albumin were prepared in good radiochemical yields. The conjugation reactions were completed at 40 - 50 °C in 10 min. The overall radiochemical yield was 25-43 % in 30-45 min.

1. Introduction

An increasing number of peptide and protein based drugs have been developed for clinical use due to their therapeutic efficacy *in vivo*.[1-4] This has prompted researchers to develop corresponding radiolabeled peptides and proteins for diagnosis and therapy.[5-8] Among the clinically established non-invasive imaging techniques, positron emission tomography (PET) is the most sensitive imaging modality which provides a specific view of normal or abnormal biological processes or conditions.[9-11] The functional information provided by PET imaging allows an earlier diagnosis and clear visualization of the disease state, which is crucial to provide reliable prognosis and therapeutic intervention. Among the many available PET radionuclides, fluorine-18 is the most commonly used positron emitter for routine imaging due to ease of production and appropriate half-life ($t_{1/2}$ = 110 min) to allow syntheses, transportation, and imaging procedures.[12-18]

Fluorine-18 incorporation into the large biomolecules can be achieved by either direct or an indirect labeling approach. However, labeling of biomolecules such as peptides or proteins is difficult due to the number and diversity of the functional groups present. Conventional nucleophilic fluorine-18 labeling to form C-^{18}F bond often requires base and high temperature. Although there are a few examples of direct fluorine-18 labeling of peptide, [19-22] protein labeling is typically performed using an indirect labeling method. In this approach a small molecule containing a suitable functional group is radiolabeled by conventional nucleophilic substitution chemistry using [^{18}F]fluoride and conjugated with proteins of interest. The most widely used prosthetic group is N-succinimydyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB) [23-27] for conjugation with amine functionalities. Other commonly used prosthetic groups include 4-[^{18}F]fluorobenzaldehyde ([^{18}F]FBA) [28-33] for reaction with amine functionalities and ^{18}F-N-[2-(4-Fluorobenzamido)ethyl]maleimide [^{18}F]FBEM [34-36] for conjugation with thiols on cysteine residues. Click chemistry, widely applied in many fields of chemical sciences, has also been applied to radiolabel peptides/proteins.[37, 38] However, while each of these synthesis methods has certain advantages, other complications have limited their applications for clinical purposes. Therefore, there is a continuing effort to develop a rapid, clean and mild synthesis technique for the radiolabeling of peptides/proteins.

Several very useful aqueous based fluorine-18 labeling methods such as [^{18}F]AlF,[39, 40] B-^{18}F [41, 42] and Si-^{18}F [43, 44] have been developed to radiolabel biomolecules. Fluorine-18 labeling was usually achieved at elevated temperature, therefore direct fluorination was restricted to only thermally stable peptides. Recently room temperature fluorination using [^{18}F]AlF was achieved by modifying the chelate.[40] In all approaches, the precursor cannot be separated from the labeled product. Hence there is a fine balance between the amount of precursor used

and specific activity (SA) of the tracers. Large amounts of starting fluorine-18 are often required to produce tracers with high SA.

In 2010, Olberg et al. reported synthesis of fluorine-18 labeled 6-fluoronicotinic acid-2,3,5,6-tetrafluorophenyl ester for radiolabeling peptides.[45] The radiolabeling method is straight forward (one-step) in comparison with multi-step synthesis of [^{18}F]SFB.[23-27] The synthesis time is significantly shorter as there is no need of time consuming HPLC purification. Moreover, stability of tetrafluorophenyl ester is better at higher pH. This prosthetic group has been used by us and others to radiolabel peptides/proteins.[46, 47] Recently, we reported a simple preparation of 6-[^{18}F]fluoronicotinic acid-2,3,5,6-tetrafluorophenyl ester on an anion-exchange cartridge (Chromafix 30-PS-HCO_3) without azeotropic drying of fluorine-18.[48] Purification of the labeled product was done by Oasis MCX Plus cartridge. The overall synthesis time is only 5 min, similar to the catch and release of the fluorine-18 on the anion exchange cartridge. Herein we report the rapid conjugation of this 6-[^{18}F]fluoronicotinic acid-2,3,5,6-tetrafluorophenyl ester prepared by our new technique with peptide (RGD), protein (albumin) and a small molecule (PSMA targeting) in short synthesis time.

2. Materials and Methods

PSMA precursor, di-tert-butyl (((S)-6-amino-1-(tert-butoxy)-1-oxohexan-2-yl)carbamoyl)-L-glutamate formate salt, and cold standard were prepared according to the literature method.[49] Cyclic peptide c(RGDfK) was obtained from Peptides International Inc. (Louisville, KY, USA). PBS 1X buffer (Gibco) was obtained from Life Technologies (Carlsbad, CA, USA). Normal saline was obtained from Quality Biological (Gaithersburg, MD, USA). PD10 MiniTrap™ columns were obtained from GE Healthcare Bioscience (Pittsburg, PA, USA). All other chemicals and solvents were received from Sigma-Aldrich (St. Louis, MO, USA)

and used without further purification. The precursor N,N,N-trimethyl-5-((2,3,5,6-tetrafluorophenoxy)carbonyl)pyridin-2-aminium fluoromethanesulfonate (**1**) and cold standard 6-fluoronicotinic acid-2,3,5,6-tetrafluorophenyl ester were prepared as previously described.[45, 46] Fluorine-18 was obtained from National Institutes of Health cyclotron facility (Bethesda, MD, USA). Chromafix 30-PS-HCO_3 anion-exchange cartridge was purchased from Macherey-Nagel (Düren, Germany). Columns and all other Sep-Pak cartridges used in this synthesis were obtained from Agilent Technologies (Santa Clara, CA, USA) and Waters (Milford, MA, USA), respectively. tC18 environmental cartridge was activated by passing 5 mL ethanol followed by 10 mL water. Oasis MCX Plus cartridge was conditioned with 5 mL anhydrous acetonitrile. Semi-prep HPLC purification and analytical HPLC analyses for radiochemical work were performed on an Agilent 1200 Series instrument equipped with multi-wavelength detectors. Mass spectrometry (MS) was performed on a 6130 Quadrupole LC/MS Agilent Technologies instrument equipped with a diode array detector.

2.1. Preparation of ^{19}F standard of c(RGDfK)

To a solution of c(RGDfK) (10 mg, 0.016 mmol) in acetonitrile (1 mL) and water (1 mL) was added 6-fluoronicotinic acid-2,3,5,6-tetrafluorophenyl ester (4.67 mg, 0.016 mmol) and N,N-diisopropylethylamine (6.2 mg, 0.048 mmol). The reaction mixture was stirred at 50 °C for 1 h. The product was purified by semi-preparative HPLC (conditions: Agilent Eclipse plus C18 column (9.4 × 250 mm, 5 µm), mobile phase: 5 - 50% acetonitrile in water (0.1% TFA), with a flow rate of 4.0 mL/min.) The product peak was collected and freeze-dried to obtain the ^{19}F cold standard of c(RGDfK). (3 mg, 27% yield). LC/MS calculated for $C_{33}H_{43}FN_{10}O_8$, 726.32, found 727.20 $(M + H)^+$.

2.2. Radiosynthesis of 6-[^{18}F]fluoronicotinic acid-2,3,5,6-tetrafluorophenyl ester (3)[48]

Fluorine-18 labeled target water (10-25 mCi) was diluted with 2 mL water and passed through an anion-exchange cartridge (Chromafix 30-PS-HCO_3). The cartridge was washed with anhydrous acetonitrile (6 mL) and dried for 1 min under vacuum. The [^{18}F]fluoride from the Sep-Pak was eluted with quaternary ammonium triflate precursor (5-7 mg, **1**) in 0.5 mL 1:4, acetonitrile : *t*-butanol through a conditioned Oasis MCX Plus cartridge. The cartridge was flushed with 1 mL acetonitrile and collected in the same vial for small molecule and peptide labeling. The cartridges were flushed with 2 mL diethyl ether for protein labeling.

2.3. Radiosynthesis and stability test of [^{18}F]c(RGDfK)

To the solution of **3** (1.5 mL) was added a mixture of c(RGDfK) (3-5 mg) and sodium bicarbonate (10-15 mg) in 1 mL water. The solution was stirred for 10 min at 50 °C. The product was purified by either Sep-Pak or semi-prep HPLC. For Sep-Pak purification, the mixture was diluted with 30 mL of water and passed through tC18 environmental cartridge. The cartridge was washed with water (10 mL) followed by 10% ethanol in water (10 mL). The product was eluted with 3 mL 30% ethanol in water. For semi-prep HPLC purification, the crude reaction mixture was diluted with 2 mL HPLC buffer and injected to the HPLC (conditions: Phenomenex Luna (2) C18 column (10 × 250 mm, 5 µm), mobile phase: 25% ethanol in 50 mM o-phosphoric acid, with a flow rate of 4 mL/min). The identity and purity of the product was confirmed by analytical HPLC.

To test the serum stability, 2 mCi of [^{18}F]c(RGDfK) was added to whole human serum (2 mL) and kept at room temperature. At different time interval (0, 1, 2 and 4h), 20 µL of the incubated sample was directly injected to the analytical HPLC (supporting information, **Figure S3**) without further processing.

2.4. Radiosynthesis of [^{18}F]DCFPyL

To the solution of **3** (1.5 mL) was added an acetonitrile solution (300 µL) of di-tert-butyl (((S)-6-amino-1-(tert-butoxy)-1-oxohexan-2-yl)carbamoyl)-L-glutamate formate salt (3-5 mg)[49] with triethylamine (5 µL). The solution was stirred for 10 min at 50 °C. Solvent was evaporated under N_2 and vacuum, and TFA (400 µL) was added. The mixture was stirred for 10 min at 50 °C. TFA was removed under N_2 and vacuum. Ethanol in 50 mM phosphoric acid (10%, 3 mL) was added to the crude mixture, which was purified by semi-prep HPLC (conditions: Agilent Eclipse plus C18 column (9.4 × 250 mm, 5 µm), mobile phase: 12% ethanol in 50 mM phosphoric acid, with a flow rate of 4 mL/min). The identity and purity of the product was confirmed by analytical HPLC.

2.5. Radiosynthesis of [^{18}F]albumin

The solvent from **3** was removed under nitrogen at 40 °C. The conjugation reaction with albumin was performed according to the literature method.[46] Briefly, to the vial containing **3** was added albumin (20 mg in 450 µL of phosphate buffer of pH 9 + 50 µL dimethylsulfoxide) and incubated for 15 min at 40 °C. The product was purified by PD10 Minitrap size exclusion column using phosphate buffer (pH 7.4) as an eluent. The product fraction was collected in 0.8 mL. Formation of the product was confirmed by analytical HPLC.

3. Results and Discussion

Fluorine-18 labeled 6-fluoronicotinic acid-2,3,5,6-tetrafluorophenyl ester (**3**), first reported by Olberg et al., is a useful prosthetic group for radiolabeling of biomolecules.[45-47] Compound **3** for this study was prepared according to the literature method [48] recently reported by us and purified by passing through an activated Oasis MCX Plus cartridge. In this

method, **3** is formed directly by passing the precursor solution (**1**) through the anion-exchange cartridge (Chromafix 30-PS-HCO_3). The use of anhydrous acetonitrile, DMSO or mixture of acetonitrile/ *t*-butanol is necessary for nucleophilic displacement to form the product (**3**). Aqueous acetonitrile, methanol or ethanol solution of **1** only elutes the fluorine-18 from the anion-exchange cartridge as a fluoride salt (supporting information, **Figure S1**). Compound **3** was purified by passing through the activated Oasis MCX Plus cartridge. The activated ester (**3**) was eluted from the cartridge by flushing with either acetonitrile for peptide labeling (**Figure 1**) or diethyl ether for protein labeling. In this work, our main focus is to develop a simple and straightforward labeling method to synthesize fluorine-18 labeled peptides and proteins containing a C-^{18}F bond using fluorine-18 labeling prosthetic group **3**. We chose to radiolabel cyclic RGD peptide c(RGDfK), PSMA inhibitor (DCFPyL) and albumin (**Figure 1**) due to their current interests in nuclear imaging research.

3.1. [^{18}F]c(RGDfK)

Integrin $\alpha_v\beta_3$ is a potential molecular marker for angiogenesis during imaging and therapy due to its significant up-regulation on activated endothelial cells.[50-52] The tripeptide Arg-Gly-Asp (RGD) has been extensively used as imaging tracer for integrin $\alpha_v\beta_3$ because of its high affinity and specificity. In a recent review Chen et al. compared the radiosynthesis, dosimetry, pharmacokinetics and clinical efficacy of clinically available RGD-based PET tracers.[53] [^{18}F]Glacto-RGD, radiolabeled by an indirect approach using 4-nitrophenyl-2-[^{18}F]fluoropropionate, was the first fluorine-18 labeled PET tracer of this class tested clinically.[54, 55] There are other conventional C-^{18}F bond containing RGD-based tracers.[28, 56-60] These tracers are prepared in multi-step syntheses with several HPLC purifications, thus requiring a long synthesis time.

Reaction of **3** with c(RGDfK) in the presence of base (sodium carbonate) proceeded with over 80% radiochemical conversion to the product (**Figure S2b**) by analytical HPLC. The compound was purified by Sep-Pak to produce > 98% radio-chemically pure (**Figure 1a**) product with a SA of 1000-2200 Ci/mmol (end of synthesis, n = 12). The overall radiochemical yield was 32-43% (uncorrected, n = 6) in a 30 min synthesis time. A minor UV impurity peak at ~5 min was observed in analytical HPLC chromatogram (**Figure 1a**). In a typical radiosynthesis starting from 103 mCi of [^{18}F]F$^-$, the amount of impurity was <7 ug/mL in 39 mCi (3 mL) of product. The crude product was also purified by semi-prep HPLC to remove the impurity peak (**Figure 1b**). The identity of the product [^{18}F]c(RGDfK) was confirmed by comparing its HPLC retention time with co-injected, authentic nonradioactive standard (**Figure 1c**). [^{18}F]c(RGDfK) showed excellent serum stability up to 4 h post synthesis (**Figure S3**).

3.2. [^{18}F]DCFPyL

Prostate cancer (PC) is the most common cancer in men in the United States. It is the second leading cause of death from cancer in men.[61] Therefore over the decades, there has been an increasing interest in synthesis and evaluation of PET tracer for PC. [^{18}F]FDG, the most widely used metabolic radiotracer for PET imaging of tumors, gave mixed results in PC.[62] Although carbon-11 or fluorine-18 choline PET/CT showed promising results for the detection of bone metastases, this approach has limitations in terms of sensitivity and specificity. Therefore routine clinical use of carbon-11 or fluorine-18 choline PET/CT is debatable.[63] This unmet clinical need led to the development of another class of prostate-specific membrane antigen (PSMA) target specific tracers. Over expression of PSMA has been linked to PC and is an important target in patients with negative bone scan who are at high risk of metastatic disease. A recent review by Maurer et al. summarized the current use

of PET tracers such as [^{11}C]choline, [^{18}F]fluorocholine, gallium-68, and fluorine-18 labeled low-molecular weight PSMA inhibitors including DCFBC and DCFPyL in PC management.[64] The second generation PSMA inhibitor reported by Chen et al. showed high tumor: background ratio and favorable pharmacokinetics compared to other small molecules.[47, 65, 66] Therefore development of reproducible radiochemical synthesis with high radiochemical yield for this tracer is of interest. Synthesis of [^{18}F]DCFPyL was firstly reported by an indirect method using **3**.[47] Recently synthesis of [^{18}F]DCFPyL has been reported by a direct fluorination method using a single precursor.[49, 67] In this report we prepared [^{18}F]DCFPyL by an indirect labeling method. Compound **3** was prepared on a Sep-Pak following the method recently published by our group [48] and purified by passing through an Oasis MCX plus cartridge. The cartridge efficiently removed unreacted precursor (**1**) from the product **3**. Hence this method of preparation of **3** is comparable to initial anion exchange catch and release of fluorine-18 containing target water (**Table 1**). Final conjugation, de-protection and purification were done according to the literature method.[47] The overall radiochemical yield was 25-32% (uncorrected, n = 6) in a 45 min synthesis time. Both radiochemical and chemical purity were >98% determined by analytical HPLC (**Figure 2a)** with a SA of 1200-2600 Ci/mmol (end of synthesis, n = 15). The identity of the product was confirmed by comparing its HPLC retention time with co-injected, authentic non-radioactive standard (**Figure 2b**). The total labeling method is comparable with the direct method of radiolabeling (**Table 1**).

3.3. [^{18}F]albumin

Recently fluorine-18 labeling of albumin by conjugation with **3** has been reported by our group.[46] The labeled albumin showed excellent blood pool imaging property. We therefore set out to further simplify the radiolabeling using the current method. By conjugating **3** to target pendant amine groups, albumin can be radiolabeled in 30 min with moderate

radiochemical yield (**Table 2**). The radiochemical purity (>98%) and chemical purity (>98%) of the labeled albumin were determine by size exclusion chromatography (**Figure 3**).

In summary, the yield and synthesis time of this current method has been compared with the literature reported methods for two known PET tracers ([^{18}F]DCFPyL and [^{18}F]Albumin) in **Table 2**. The RGD peptide (cRGDfK) has not been radiolabeled using **3**, therefore the yield and synthesis time of this tracer is compared with the known C-^{18}F bonded RGD tracers (**Table 2**). The current method requires much less time with comparable or higher radiochemical yield.

4. Conclusions

We have successfully prepared [^{18}F]c(RGDfK), [^{18}F]DCFPyL and [^{18}F]albumin in short synthesis time (30-50 min) with moderate to high radiochemical yield. For the first time RGD-peptide c(RGDfK) has been radiolabeled with **3**. This method is comparable with direct fluorine-18 labeling approaches. Due to the simplicity of the method, it could easily be automated for routine clinical production.

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Table 1: Key steps of direct labeling method and current indirect labeling method to prepare [^{18}F]DCFPyL

Direct labeling method (Literature)[49, 67]	Indirect radiolabeling method (Current)
F-18 catch on the anion exchange resin Wash with water Elution with base Azeotropic drying Reaction with precursor De-protection, purification	F-18 catch on the anion exchange resin Wash with anhydrous acetonitrile Drying under vacuum Elution with precursor (**1**) through Oasis MCX Reaction with second precursor (peptide) De-protection, purification

Table 2: Comparison of yield, time and SA for literature and current method

Compound	Yield (%)		SA (Ci/mmol)		Time (min)		References
	Literature	Current[c]	Literature	Current	Literature	Current	
[^{18}F]RGD[a]	10-35[b]	39-43	2-2700	1000-2200	90-218	30	28, 54-60
[^{18}F]DCFPyL	5-53[b]	25-32	340-120000	1200-2600	55-128	45	47, 49, 66
[^{18}F]Albumin	18-35[c]	26-35	n/a	n/a	90	30	46

[a]Only C-^{18}F bonded tracers are included in this table; [b]decay corrected; [c]decay uncorrected

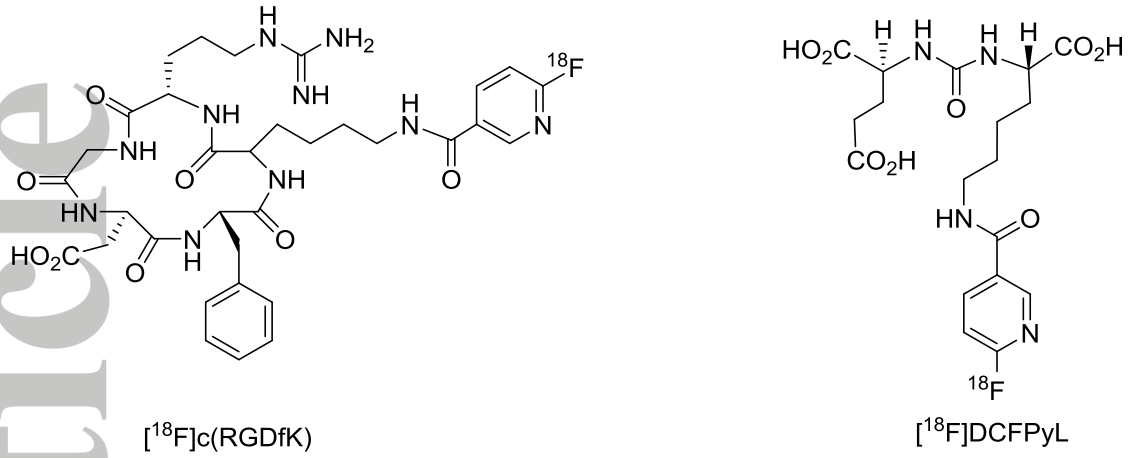
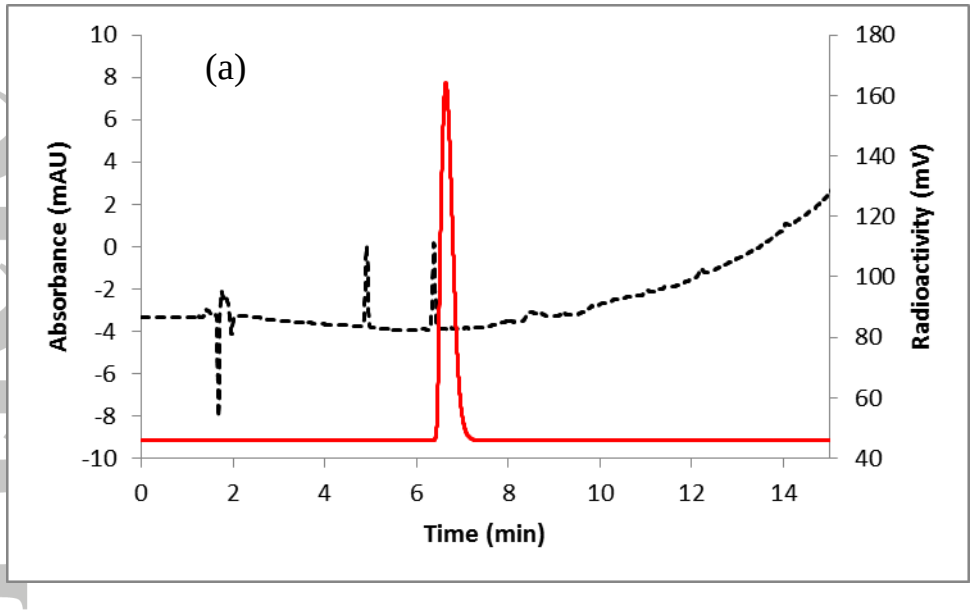
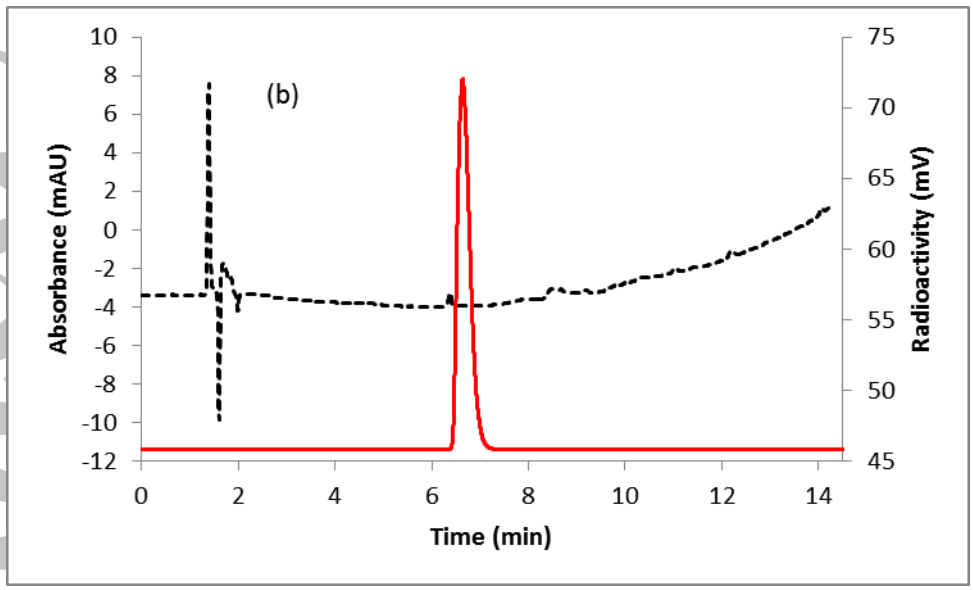


Figure 1: Structures of fluorine-18 labeled cyclic RGD and [^{18}F]DCFPyL





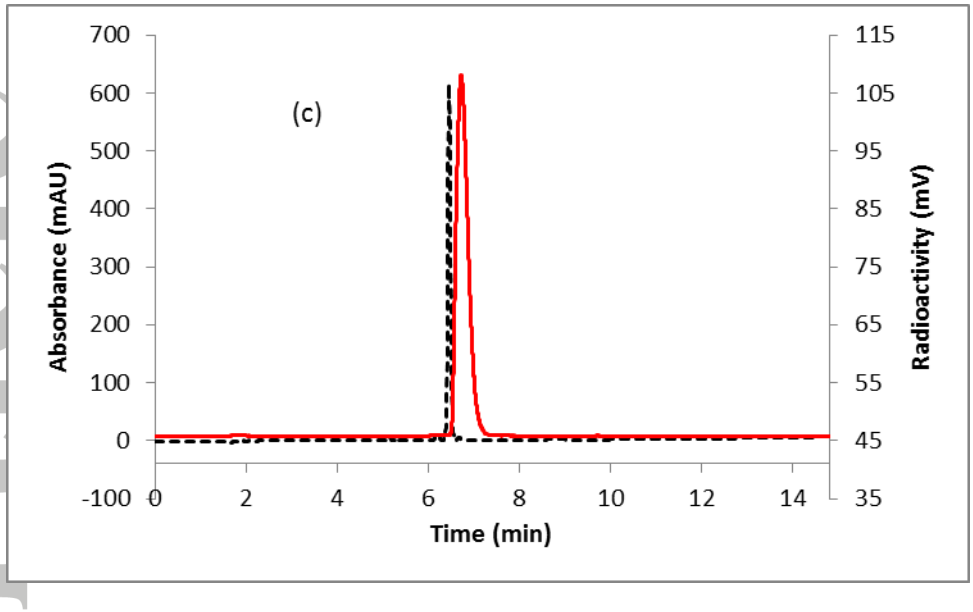


Figure 2. HPLC analysis of a) Sep-Pak purified [^{18}F]c(RGDfK); b) HPLC purified [^{18}F]c(RGDfK); c) [^{18}F]c(RGDfK), co-injected with the non-radioactive standard. HPLC condition: Agilent Eclipse plus C18 column (4.6 × 150 mm, 3.5 µm), mobile phase: 10 - 50% A in 8 min, 50 – 90 % A in 15 min. A = acetonitrile (0.1% TFA), B = water (0.1% TFA), with a flow rate of 1.0 mL/min. Solid line, in-line radiodetector; dotted line, UV detector at 254 nm.

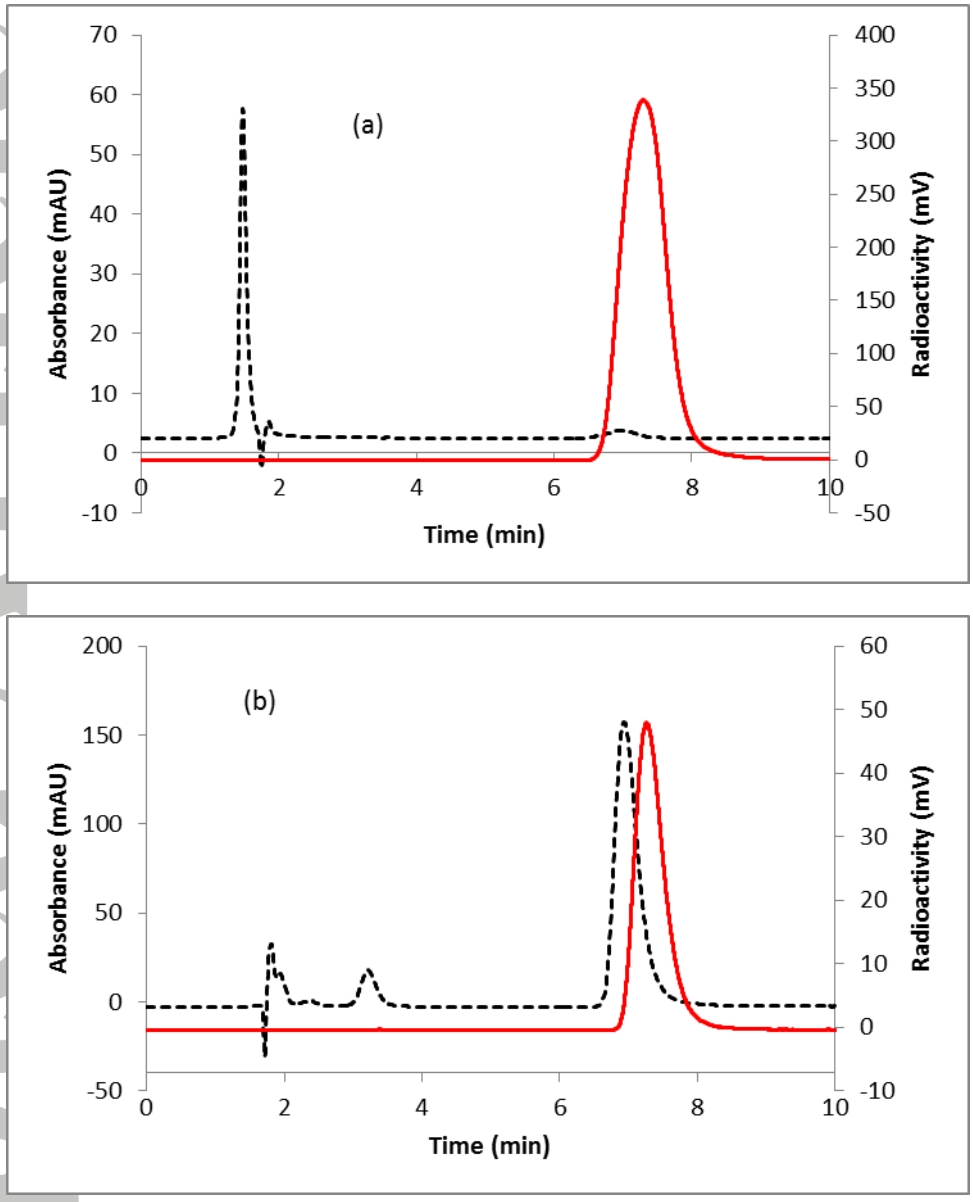


Figure 3: HPLC analysis of a) [^{18}F]DCFPyL; b) [^{18}F]DCFPyL, co-injected with the non-radioactive standard. HPLC condition: Agilent Eclipse plus C18 column (4.6 × 150 mm, 3.5 µm), mobile phase: 5% acetonitrile in 0.1 M ammonium formate (pH 3.5), with a flow rate of 1.0 mL/min. Solid line, in-line radiodetector; dotted line, UV detector at 254 nm.

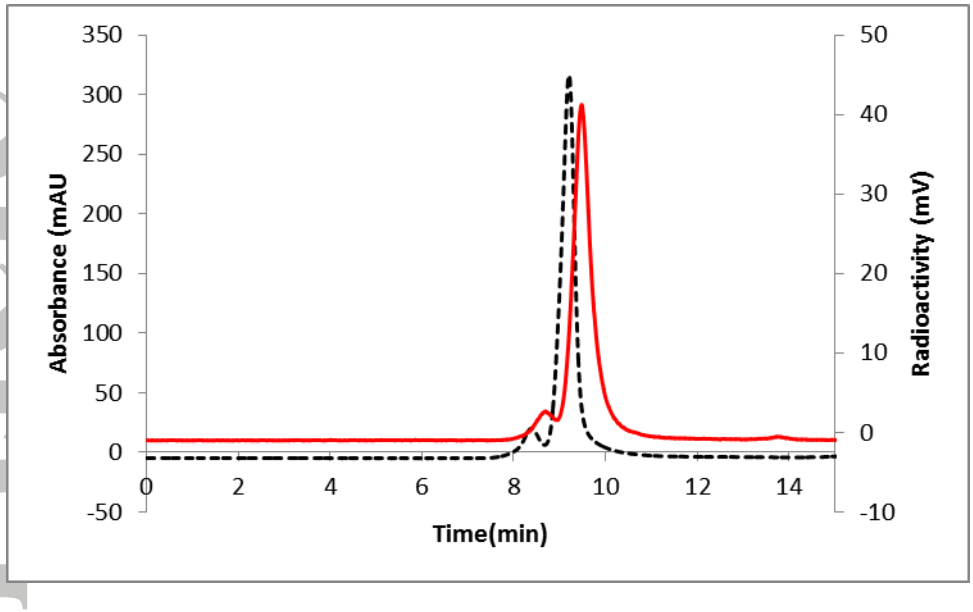
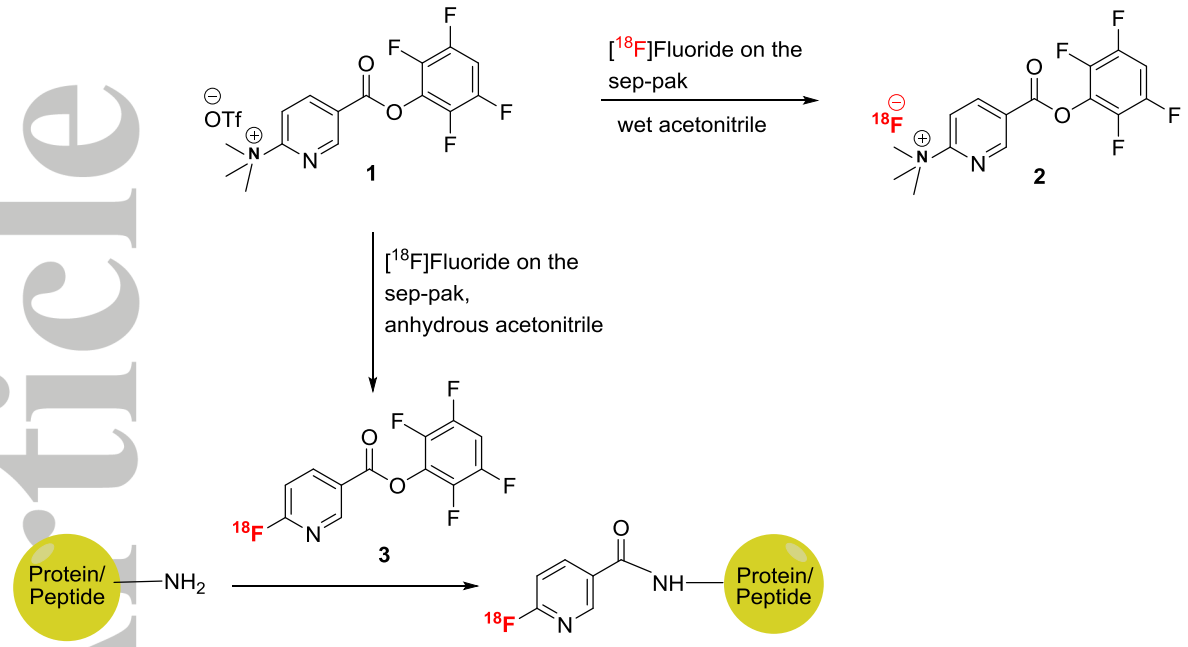


Figure 4. HPLC analysis of [^{18}F]RSA. HPLC condition: Agilent GF250 column (9.4 × 250 mm, 3.5 μm), mobile phase: PBX 1X, pH 7.4, with a flow rate of 1.0 mL/min. Solid line, in-line radiodetector; dotted line, UV detector at 254 nm.



Scheme 1: Fluorine-18 labeling of peptide and protein through nicotinic ester approach