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Facile synthesis of *N*-succinimidyl 4-[^{18}F]fluorobenzoate ([^{18}F]SFB) for protein labeling

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An efficient preparation of *N*-succinimidyl 4-[^{18}F]fluorobenzoate ([^{18}F]SFB) based on a convenient three-step, one-pot procedure is described. [^{18}F]Fluorination of the precursor ethyl 4-(trimethylammonium triflate)benzoate gave ethyl 4-[^{18}F]fluorobenzoate. Saponification of the ethyl 4-[^{18}F]fluorobenzoate with aqueous tetrapropylammonium hydroxide yielded the corresponding 4-[^{18}F]fluorobenzoate salt ([^{18}F]FBA), which was then treated with *N,N,N,N′*-tetramethyl-*O*-(*N*-succinimidyl)uronium hexafluorophosphate. The purified [^{18}F]SFB was used for the labeling of Avastin™ (Bevacizumab) through [^{18}F]fluorobenzoylation of the Avastin's α-amino groups. The decay-corrected radiochemical yields of [^{18}F]SFB were as high as 44% (based on [^{18}F]fluoride ($n = 10$) with a synthesis time of less than 60 min. [^{18}F]Avastin was produced in decay-corrected radiochemical yields of up to 42% ($n = 5$) within 30 min (based on [^{18}F]SFB). The radiochemical purities of [^{18}F]SFB and [^{18}F]Avastin were greater than 95%.

Keywords: protein labeling; ^{18}F; VEGF; SFB; Avastin; one-pot; radiosynthesis

Introduction

The application of biomolecules such as proteins, peptides, and antibodies labeled with positron-emitting nuclides has emerged as an important field in targeted molecular imaging for *in vivo* studies of many physiological and pathological processes.[1,2] The incorporation of fluorine-18 into proteins, peptides, and antibodies usually requires the use of prosthetic groups, also referred to as bifunctional labeling agents. A large number of ^{18}F-labeled prosthetic groups have been developed that can be attached to biomolecules via acylation, amidation, imidation, alkylation reactions, photochemical conjugation, and solid-phase syntheses.[1,3] The acylation approach using *N*-succinimidyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB) is one of the most versatile ^{18}F-labeling methods with respect to the *in vivo* stability and radiochemical yield.[2,3]

The published radiosyntheses of [^{18}F]SFB involve a three-step multi-vessel procedure: (1) [^{18}F]fluorination of an appropriate aromatic precursor, (2) formation of the 4-[^{18}F]fluorobenzoate salt ([^{18}F]FBA), and (3) conversion of the salt to [^{18}F]SFB.[1] In the past few years, a number of modifications have been made in an effort to improve the synthesis of [^{18}F]SFB for routine application.[1] Recently, two different fully automated preparations of [^{18}F]SFB on modified commercial modules were described,[2,4] but further improvements in the [^{18}F]SFB synthesis would be beneficial.

Angiogenesis, the process of new blood vessel growth, plays a critical role in several physiological and pathological states, particularly in tumor growth, invasion, and metastasis. Several growth factors have been implicated in tumor angiogenesis and one of the most potent positive regulators of angiogenesis is vascular endothelial growth factor (VEGF). VEGF binds to two receptor tyrosine kinases: VEGF receptor VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR). VEGFR-1 and VEGFR-2 are found predominantly on the surface of vascular endothelial cells and bind to VEGF with high affinity.[5,6] There is very low or undetectable expression of VEGFRs in most normal tissues (with the exception of renal glomeruli), whereas VEGF is upregulated in most human tumor types. VEGF expression is associated with tumor progression and patient survival in a variety of human cancers. Therefore, VEGF is considered an attractive target for anticancer diagnosis and therapy. Avastin™ (Bevacizumab) is a recombinant humanized monoclonal IgG1 antibody (93% human, 7% murine sequences—molecular weight 149 kDa), which selectively binds with high affinity to all isoforms of human VEGF and inhibits the VEGF's biologic activity through a steric blocking of the binding of VEGF to its receptors VEGFR-1 and VEGFR-2 on the surface of endothelial cells.[6] Bevacizumab and its analogues are potentially valuable for therapeutic agents due to their specific inhibition of tumor angiogenesis (microvascular growth) and, thereby, inhibition of tumor growth and metastatic disease progression.[5] [^{18}F] Avastin ([^{18}F] Bevacizumab) holds promise as a Nuclear Medicine diagnostic agent using positron emission tomography.

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In this report we describe an efficient preparation of [^{18}F]SFB based on a convenient three-step, one-pot procedure, consisting of [^{18}F]fluorination of the precursor ethyl 4-(trimethylammonium triflate)benzoate, saponification to generate the [^{18}F]FBA salt with tetrapropylammonium hydroxide, and conversion of the [^{18}F]FBA salt to [^{18}F]SFB. The resultant [^{18}F]SFB was used for labeling the Avastin through [^{18}F]fluorobenzoylation of the Avastin's α-amino groups.

Experimental

General

Ethyl 4-(trimethylammonium triflate)benzoate was synthesized in our laboratory according to published procedures.[7] Sephadex G-50 was purchased from Sigma-Aldrich. All other reagents used in the synthesis were commercial products and were used without further purification unless otherwise indicated. Sep-Pak QMA, Sep-Pak C18, and Sep-Pak alumina cartridges were purchased from Waters (Milford, MA) and Lichrolut SCX cartridge was obtained from Waters (Eschborn, Germany). Thin-layer chromatography (TLC) was carried out using pre-coated aluminum-backed silica gel 60 F254 TLC plates (E. Merck Company, Darmstadt, Germany) to verify product purities. All analytical high-performance liquid chromatography (HPLC) were performed using a Perkin-Elmer system and a Nova-Pak® reversed-phase Symmetry analytical C18 column (3.9 mm × 300 mm, 5 μm, Waters) or a PRP-3 reversed-phase column (4.1 mm × 150 mm, Hamilton Company), consisting of a pump, a variable wavelength UV detector, and a radioflow detector.

K_{222}/K[^{18}F]F complex

No-carrier-added [^{18}F]F^- was obtained through the nuclear reaction ^{18}O(p, n)^{18}F by irradiation of >95% ^{18}O-enriched water with an 11 MeV proton beam using a Siemens RDS-112 negative ion cyclotron. After the delivery of [^{18}F]F^- from the cyclotron, the radioactivity was passed through a Sep-Pak light QMA cartridge to trap [^{18}F]F^- ([^{18}O]water was collected for recycling). [The Sep-Pak light QMA cartridge was pre-conditioned sequentially with 5 mL of 0.5 M potassium bicarbonate, 10 mL of deionized water, and 10 mL of acetonitrile before use.] The [^{18}F]F^- was eluted with 1.5 mL of a solution prepared by mixing aqueous K_2CO_3 (0.11 g, 0.8 mmol in 1.0 mL of water) with Kryptofix 222 (K_{222}) (0.60 g, 1.6 mmol in 19 mL of acetonitrile). The solvent was evaporated under a stream of nitrogen at 120°C. Azeotropic drying was repeated twice with 1 mL portions of MeCN to generate the anhydrous K_{222}/K[^{18}F]F complex.

Radiosynthesis of [^{18}F]SFB

Ethyl 4-(trimethylammonium triflate)benzoate (**1**) (5.0 mg, 20 μmol) in anhydrous MeCN (1 mL) was added to the dried K_{222}/K[^{18}F]F and the mixture heated at 90°C for 10 min to produce ethyl 4-[^{18}F]fluorobenzoate (**2**). The ethyl ester was subsequently hydrolyzed to form **3** using 20 μL of tetrapropylammonium hydroxide (1.0 M in water) at 120°C for 3 min, and then the mixture azeotropically dried using MeCN (1 mL). Subsequently, a solution of *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium hexafluorophosphate (HSTU) (12 mg, 33 μmol) in MeCN (1 mL) was added and the solution heated at 90°C for 5 min. After cooling, 5% aqueous acetic acid (9 mL) and water (15 mL) were added. The reaction mixture was passed through a C18 Sep-Pak cartridge, a Sep-Pak alumina cartridge and a Lichrolut SCX cartridge (200 mg, Merck) in series. The Sep-Pak C18 cartridge trapped the [^{18}F]SFB, the Sep-Pak alumina cartridge removed the free $^{18}F^-$, and the SCX cartridge removed the impurities. The cartridges were washed with 10% aqueous MeCN (15 mL) and then the product [^{18}F]SFB eluted with MeCN (2 mL). The solvent was then removed under a stream of nitrogen at 60°C to provide the dry [^{18}F]SFB.

Labeling Avastin with [^{18}F]SFB

Avastin (0.20–0.50 mg, 3.3×10^{-6} mmol in 20–50 μL of phosphate-buffered saline (0.05 M, pH 7.4)) and aqueous 0.1 M Na_2HPO_4 (0.2 mL) were added to the dried [^{18}F]SFB residue. [The [^{18}F]SFB can also be dissolved in a minimal quantity of acetonitrile or dimethyl sulfoxide (DMSO) prior to the addition of the protein solution]. The mixture was allowed to react at room temperature for 15 min and then purified by passing it through two self-made Sep-Pak Gel Filtration cartridges [prepared by opening Sep Pak silica cartridges from Waters and filling them with the Sephadex G-50. The cartridges were washed with 10 mL of sterile water before use].

Purity Determination of [^{18}F]SFB and [^{18}F]Avastin

Analytical HPLC analysis on a Nova-Pak® reversed-phase analytical C18 column (Waters) was used to determine radiochemical purity of the [^{18}F]SFB at a flow rate of 1 mL/min using the following gradient (the eluent components were A: 0.01 M aqueous H_3PO_4; B: MeCN): 0.5 min—95%A/5%B; 8 min—90%A/10%B; 16 min—10%A/90%B; 1 min—95%A/5%B. The analytical HPLC on a PRP-3 reversed-phase column was used for determining radiochemical purity of [^{18}F]Avastin at a flow rate of 2 mL/min eluted with the following gradient (A: 0.01 M aqueous H_3PO_4; B: MeCN): 0.5 min—95%A/5%B; 28 min—10%A/90%B; 1 min—95%A/5%B. Radio TLC analysis on a silica gel 60 plate (MeCN/H_2O: 95/5, v/v) was also used to confirm radiochemical purity. Trichloroacetic acid (TCA) precipitation[8] was used for determination of [^{18}F]Avastin radiochemical purity. A K_{222} detection test was performed on the silica gel 60 coated plate developed with methanol/15% aqueous ammonium hydroxide (9/1, v/v) using iodine vapor for staining.[9] Radiochemical stability was monitored using radio TLC and an analytical HPLC for periods up to 6 h.

Results And discussion

Several procedures have been reported for the synthesis of [^{18}F]SFB. All utilize a three-step, two-pot procedure involving aromatic nucleophilic substitution of various ammonium triflate moieties (including ethyl, *tert*-butyl, and pentamethylbenzyl 4-(trimethylammonium triflate)), benzoates, and no-carrier-added [^{18}F]fluoride. The substitution reaction is followed by hydrolysis of the ester protecting group using aqueous NaOH,[10,11] aqueous HCl,[4] or trifluoroacetic acid.[3,12,13] The final synthetic step employs activation by *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU). When ethyl and *tert*-butyl esters were used as precursors, two purification steps were necessary: purification of the intermediate [^{18}F]FBA and purification of the final product using solid-phase extraction (cartridges). When the pentamethylbenzyl ester was used as a precursor, three purification steps were required.[12,13]

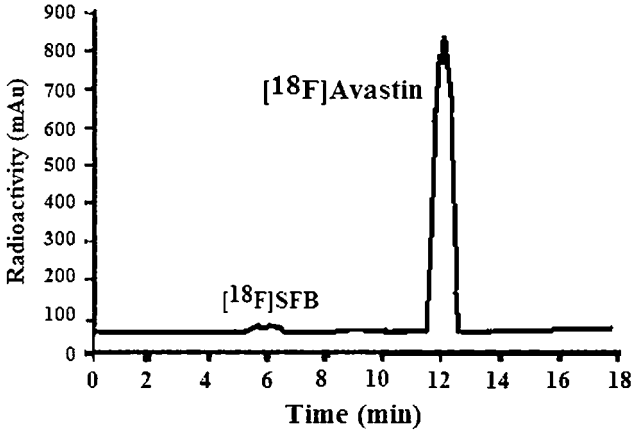


Figure 1. HPLC chromatogram of the purified [^{18}F]Avastin.

We manually synthesized [^{18}F]SFB (Scheme 1) using ethyl 4-(trimethylammonium triflate)benzoate (**1**) as a precursor via a three-step, one-pot procedure. [^{18}F]Fluorination of the ammonium triflate precursor **1** in the solvent MeCN gave **2**, which was directly used for the hydrolysis reaction. Hydrolysis of **2** with aqueous tetrapropylammonium hydroxide yielded the [^{18}F]FBA salt **3**, which was azeotropically dried using MeCN. Reaction of **3** with HSTU, followed by purification using a C18 cartridge, a Sep-Pak alumina cartridge, and a cation exchange cartridge in series yielded the purified [^{18}F]SFB. [^{18}F]SFB was produced in a decay-corrected radiochemical yield of up to 43.8 ± 4.6% (based on [^{18}F]fluoride; $n = 10$) in less than 60 min ($n = 10$). This simplified one-pot procedure should be readily adaptable to automated radiosynthesis techniques.

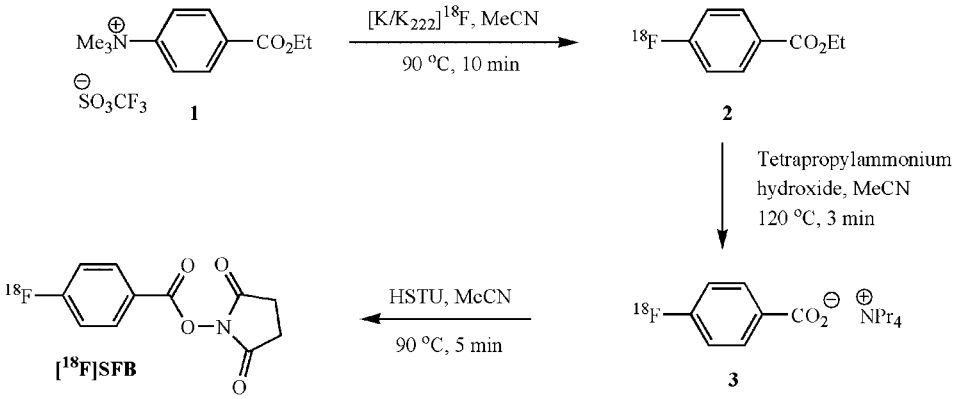
In our study, MeCN was used in place of DMSO as solvent for the [^{18}F]fluorination reaction and aqueous tetrapropylammonium hydroxide replaced aqueous NaOH in the saponification. These changes allowed us to carry out the three-step radiosynthesis in one pot because no purification of the [^{18}F]FBA was required. The [^{18}F]fluorination reaction had been carried out in MeCN in earlier studies using the *tert*-butyl benzoate precursor[4] and the pentamethylbenzyl benzoate precursor[12] HSTU can also be used in place of TSTU to achieve similar radiochemical yields. In our hands, our one-pot procedure produced the same yield as those reported earlier[1] but in a shorter time.

Using [^{18}F]SFB, we labeled Avastin in radiochemical yields up to 42.3% (decay corrected, $n = 5$). Adding a small amount of acetonitrile or DMSO to dissolve the [^{18}F]SFB prior to the addition of protein did not improve the protein labeling yield but was somewhat more convenient. Purification of [^{18}F]Avastin using Sep-Pak Gel Filtration cartridges (two Sephadex G-50 cartridges) greatly reduced the total synthesis time (~30 min).

The identities of [^{18}F]SFB and [^{18}F]Avastin were confirmed by comparison with non-radioactive reference compounds. The radiochemical purity of [^{18}F]SFB was greater than 95%, confirmed by TLC and HPLC. The retention times of [^{18}F]SFB and [^{18}F]FBA were ~17 min and ~10 min, respectively. A color spot test for K_{222} by TLC did not detect K_{222} in the final [^{18}F]SFB solution also. (The detection limit for Kryptofix using the color spot test is <50 μg/mL.) Solutions of [^{18}F]SFB in MeCN or CH_3OH were stable over a period of hours. The radiochemical purity of the [^{18}F]Avastin was greater than 95% for all preparations, confirmed by TLC, TCA, and HPLC. The purification of [^{18}F]Avastin using the Sephadex G-50 column and self-made Sep-Pak Sephadex G-50 cartridges produced excellent radiochemical purity (>95%), as shown in Figure 1. The retention times of [^{18}F]SFB and [^{18}F]Avastin were ~6 min and ~12 min, respectively, under the conditions utilized.

Conclusion

An efficient method for preparing [^{18}F]SFB, one of the most versatile ^{18}F-labeled agents used for labeling biomarkers, has been developed. [^{18}F]Avastin was successfully produced from [^{18}F]SFB. Purification of [^{18}F]Avastin was conducted using Sep-Pak Gel Filtration cartridges in place of a Gel Filtration column that further reduced the synthesis time. TLC provided a simple and practical method to routinely monitor the progress of both the [^{18}F]SFB radiosynthesis and the protein labeling reaction. The new one-pot procedure should be readily adaptable to the automated production of [^{18}F]SFB and [^{18}F]Avastin using currently available commercial synthesis modules.



Scheme 1

Acknowledgements

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