Video Article

Microwave-assisted One-pot Synthesis of *N*-succinimidyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB)

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Abstract

Biomolecules, including peptides,[1-9] proteins,[10,11] and antibodies and their engineered fragments,[12-14] are gaining importance as both potential therapeutics and molecular imaging agents. Notably, when labeled with positron-emitting radioisotopes (e.g., Cu-64, Ga-68, or F-18), they can be used as probes for targeted imaging of many physiological and pathological processes.[15-18] Therefore, significant effort has devoted to the synthesis and exploration of ^{18}F-labeled biomolecules. Although there are elegant examples of the direct ^{18}F-labeling of peptides,[19-22] the harsh reaction conditions (i.e., organic solvent, extreme pH, high temperature) associated with direct radiofluorination are usually incompatible with fragile protein samples. To date, therefore, the incorporation of radiolabeled prosthetic groups into biomolecules remains the method of choice.[23,24]

N-Succinimidyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB),[25-37] a Bolton-Hunter type reagent that reacts with the primary amino groups of biomolecules, is a very versatile prosthetic group for the ^{18}F-labeling of a wide spectrum of biological entities, in terms of its evident *in vivo* stability and high radiolabeling yield. After labeling with [^{18}F]SFB, the resulting [^{18}F]fluorobenzoylated biomolecules could be explored as potential PET tracers for *in vivo* imaging studies.[1] Most [^{18}F]SFB radiosyntheses described in the current literatures require two or even three reactors and multiple purifications by using either solid phase extraction (SPE) or high-performance liquid chromatography (HPLC). Such lengthy processes hamper its routine production and widespread applications in the radiolabeling of biomolecules. Although several module-assisted [^{18}F]SFB syntheses have been reported,[29-32, 41-42] they are mainly based on complicated and lengthy procedures using costly commercially-available radiochemistry boxes (**Table 1**). Therefore, further simplification of the radiosynthesis of [^{18}F]SFB using a low-cost setup would be very beneficial for its adaption to an automated process.

Herein, we report a concise preparation of [^{18}F]SFB, based on a simplified one-pot microwave-assisted synthesis (**Figure 1**). Our approach does not require purification between steps or any aqueous reagents. In addition, microwave irradiation, which has been used in the syntheses of several PET tracers,[38-41] can gives higher RCYs and better selectivity than the corresponding thermal reactions or they provide similar yields in shorter reaction times.[38] Most importantly, when labeling biomolecules, the time saved could be diverted to subsequent bioconjugation or PET imaging step.[28,43] The novelty of our improved [^{18}F]SFB synthesis is two-fold: (1) the anhydrous deprotection strategy requires no purification of intermediate(s) between each step and (2) the microwave-assisted radiochemical transformations enable the rapid, reliable production of [^{18}F]SFB.

Video Link

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Protocol

1. Initial preparations

1. A V-vial (5-mL) RV1 (with stirring bar) is used as the main reaction vessel for performing microwave synthesis. It is connected to a PEEK adaptor with seven inlet/outlet ports connects and placed inside the microwave cavity (see Figure 2). RV2 is connected to SPE cartridge (I) to collect the crude [^{18}F]SFB. RV3 is connected to SPE cartridge (II) for collecting the final [^{18}F]SFB solution. It can be placed in a warm

water bath (40°C) to concentrate the corresponding solution before reconstituting in PBS buffer, especially for the downstream radiolabeling of biomolecules.
2. **Setup for collecting crude [^{18}F]SFB:** Fill up MeCN/H_2O [6 mL; 1:4 (v:v)] solution, 5% aqueous AcOH (8 mL), MeCN (2 mL) for reservoir A,B and C, respectively. Then activate a SPE cartridge (I) (polystyrene, Merck LiCholut EN) with ethanol (10 mL), followed by 5% aqueous AcOH (10 mL) washing.
3. **Setup for collecting purified [^{18}F]SFB:** Prepare reservoir D and E filled up with 10 ml of H_2O and 3 ml of diethyl ether respectively. The second SPE cartridge (II) (polystyrene, Merck LiCholut EN) is activated by the same procedure mentioned above.
4. Start the HPLC (elution buffer: MeCN/ H_2O, 1:1 (v/v) containing 0.2% TFA; flow rate: 3 ml/min) for pre-conditioning the HPLC column [a reverse-phase semi-prep column (Luna, 5 µm C18(2) 100 Å, 250 x 10 mm), Phenomenex, Torrance, CA, USA].

2. Preparation of dried [i.e. non-carrier-added, (n.c.a)] [^{18}F]fluoride

1. [^{18}F]fluoride solution in [^{18}O]H_2O (100 µL) was added to a mixture of Kryptofix 222 (20 mg), 1M aqueous K_2CO_3 (26 µL) and MeCN (0.8 mL) in an Eppendorf tube. The entire solution is then mixed well before transferring to the RV1 via inlet line 1. The [^{18}F]fluoride solution can be also passed through an anionic exchange cartridge (e.g. QMA-light Sep-Pak from Waters) to trap the fluoride-18 and then eluted out with a mixture of K_2CO_3 and Kryptofix in MeCN.
2. Execute the Drying sequence (20W, 3 min) under the Microwave control program to remove residual water in RV1 [under vacuum]. After the cooling down as system temperature is below 50°C, additional MeCN (1.0 mL) was introduced into the reactor and the sequence is repeated once.

3. Synthesis of ethyl 4-[^{18}F]fluorobenzoate

1. To a DMSO solution (0.4 mL) containing ethyl 4-(*N,N,N*-trimethylammonium)benzoate triflate (1.5 mg) was added into RV1 via inlet line 2.
2. Execute the Labeling sequence (50W, 1 min) under the Microwave control program with stirring, vessel cooling and all valves closed to afford ethyl 4-[^{18}F]fluorobenzoate ([^{18}F]2).

4. Synthesis of potassium 4-[^{18}F]fluorobenzoate

1. To a DMSO solution (0.5 mL) containing KOtBu (13 mg) was added into RV1 via inlet line 3.
2. Execute Deprotect program (40 W, 1 min) under the Microwave control program with stirring, vessel cooling and all valves closed to afford the 4-[^{18}F]fluorobenzoate salt ([^{18}F]3).

5. Synthesis of crude [^{18}F]SFB

1. To an acetonitrile solution (2.5 mL) containing TSTU (30 mg) was added to RV1 via inlet line 6. TSTU is moisture- and light-sensitive. It should be aliquoted into small vial and stored at 4°C in a closed container covered by aluminum foil.
2. Execute Coupling sequence (30W, 2 min) under the Microwave control program with stirring, vessel cooling and all valves closed to afford the crude [^{18}F]SFB.

6. The Preparation of SPE-purified [^{18}F]SFB

1. 5% aqueous AcOH (1.0 mL) was added to RV1 via inlet line 7 to neutralize the reaction mixture. The solution was then transferred into vial B containing 8 ml of 5% aqueous AcOH (Figure 2).
2. Pass the diluted reaction mixture through SPE cartridge (I) to trap crude [^{18}F]SFB using nitrogen (10 psi).
3. WASH SPE cartridge (I) with a mixture of MeCN and H_2O [10 mL, 1:4 (v:v)] from reservoir A.
4. [^{18}F]SFB was eluted out into RV2 using MeCN (2 mL) from reservoir C.

7. Purification of Crude [^{18}F]SFB with Radio-HPLC

1. Dilute either crude [^{18}F]SFB or SPE-purified [^{18}F]SFB with H_2O (2 mL) in RV2 and transfer the mixture into the HPLC loop (5 mL). The the solution was injected into radio-HPLC [MeCN/ H_2O, 1:1 (v/v) containing 0.2% TFA; flow rate: 3 mL/min].
2. Collect the fraction containing purified [^{18}F]SFB (retention time: 8-10 minutes) into the vial D (pre-filled with 10 ml of H_2O) (Figure 2). Critical step: If performed correctly, the fraction volume collected here should be 4-5 ml.
3. Pass the diluted reaction mixture through SPE cartridge (II) to trap purified [^{18}F]SFB using nitrogen (10 psi). Dry the cartridge with a stream of nitrogen for 2-3 minutes.
4. [^{18}F]SFB was eluted out into RV3 using diethylether (3 mL) from reservoir E..
5. Evaporate the solvent in RV3 to dryness by a gentle stream of nitrogen gas (10 psi) using a water bath (40° C). The final dried [^{18}F]SFB can be reconstituted into PBS buffer for the downstream application.

8. Representative Results:

We developed a simplified, rapid, one-pot method for synthesizing [^{18}F]SFB using a deprotection strategy under anhydrous conditions and microwave heating during each radiochemical/chemical transformation. Figure 1 presents the details of our radiosynthesis. The identity of final product was confirmed by comparison of HPLC retention time with a non-radioactive SFB reference. The purified [^{18}F]SFB was also analyzed through radio-TLC and -HPLC to determine its radiochemical and chemical purity. The RCY of [^{18}F]SFB was 35 ± 5% within 60 min after HPLC

purification (n > 30), with high radiochemical purity (>99%) and good chemical purity (see the UV trace in the HPLC profile, Figure 3). The specific activity was ca. 67-330 GBq/μmol (1.8-9.0 Ci/μmol), depending on the starting radioactivity.

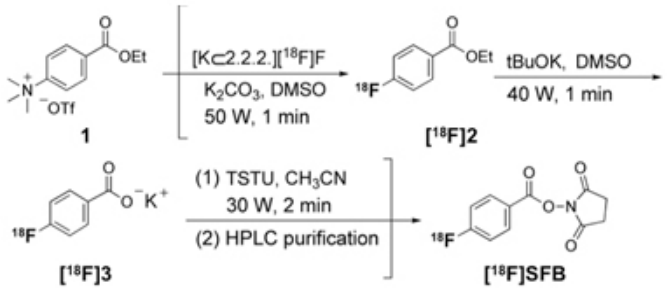

Figure 1. Microwave-assisted one-pot radiosynthesis of [^{18}F]SFB. First, the radiofluorination of ethyl 4-(*N,N,N*-trimethylammonium)benzoate triflate (1) was performed under microwave heating (50 W, 1 min) in the presence of [K#2.2.2][^{18}F]F- complex in dimethylsulfoxide (DMSO) to afford ethyl 4-[^{18}F]fluorobenzoate ([^{18}F]2). Without purification, a DMSO solution of potassium tert-butoxide (*t*BuOK) was added and the reaction vessel was microwave irradiated (40 W, 1 min) to complete the anhydrous deprotection. The final conversion of [^{18}F]3 into [^{18}F]SFB was achieved using O-(*N*-succinimidyl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TSTU) activation. TSTU in acetonitrile was added to the reaction mixture containing the 4-[^{18}F]fluorobenzoate ([^{18}F]3) salt; this last synthetic step yielded crude [^{18}F]SFB after heating (30 W, 2 min).

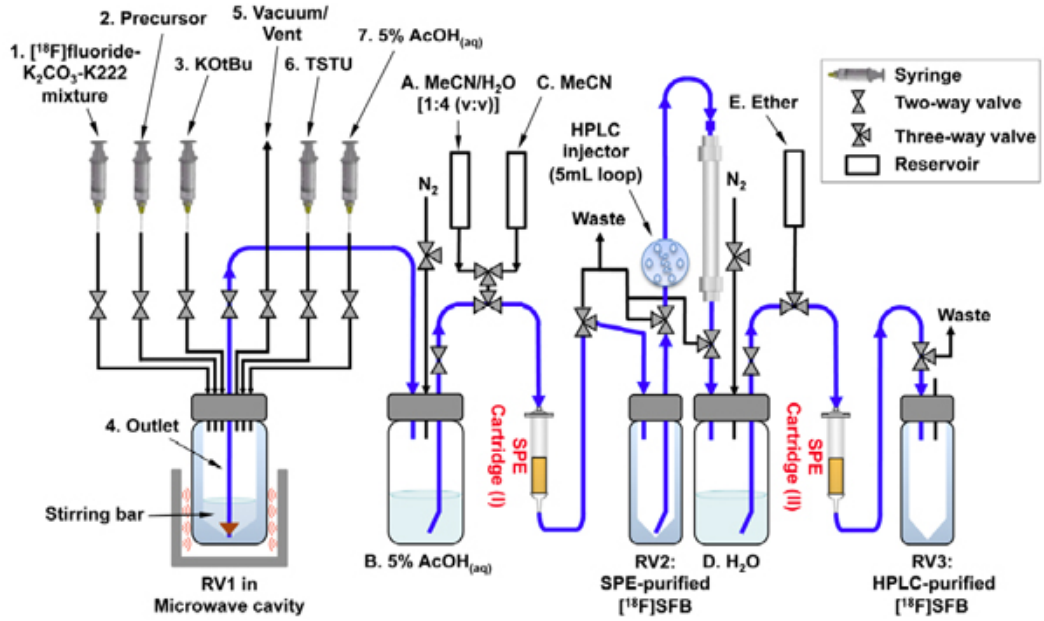

Figure 2. The schematic diagram of setup for microwave-assisted one-pot [^{18}F]SFB synthesis.

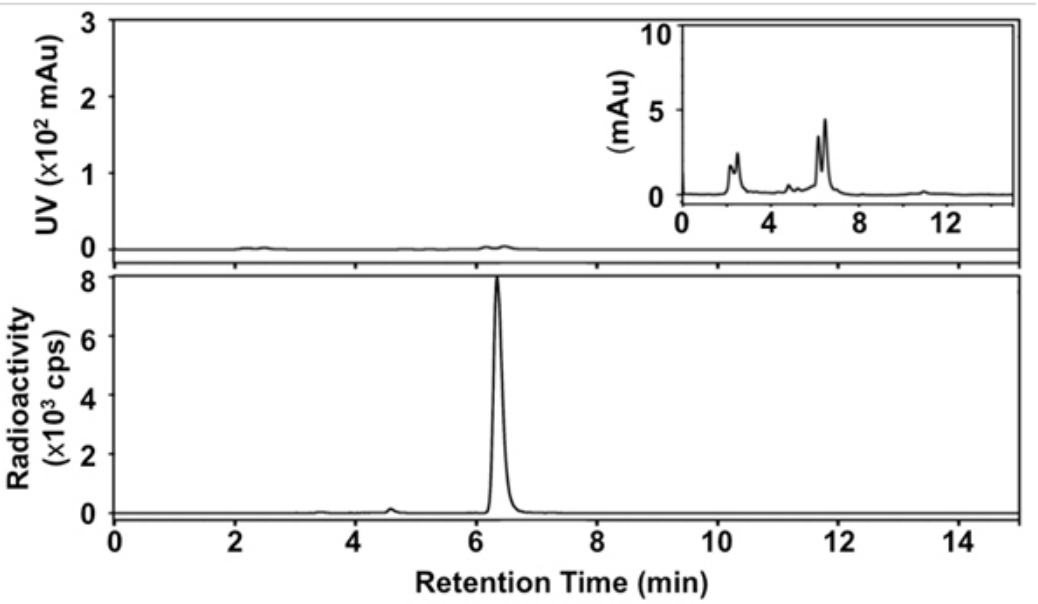

Figure 3. Radio-HPL chromatograms of final [^{18}F]SFB. Top: UV signal at 254 nm; bottom: radioactive signal; inset: UV signal at 254 nm (x 33.3).

		R=									
					-Et				-*t*Bu		-Penta-methylbenzyl
		In this method	Wester et al. 1996	Marik et al. 2007	Johnström et al. 2008	Tang et al. 2008	Tang et al. 2010	Scott et al. 2010	Wüst et al. 2003	Mäding et al. 2005	Azarian et al. 2006
Time(min)	[^{18}F] Fluorination	1	10	18	10	10	10	10	2	10	15
	Deprotection	1[a]	8[b]	9[b]	5[b]	3[b]	3[b]	3[b]	8[c]	5[d]	5[c]
	Activation	2[e]	2[e]	4.5[e]	5[e]	5[f]	5[e]	5[e]	2[e]	2[e]	5[e]
Intermediates	Cartridge purification[g]	N	Y	Y	Y	N	Y	Y	Y	Y	Y
	Solvent evaporation	N	Y	Y	Y	Y	Y	Y	Y	Y	Y
	Number of reactors	1	2	2	1[h]	1	1	1	2	2	2
	Radiochemical yield	30-40 % (n>30)	50-60% (n= N.A.)	41-51 % (n=4)	42-47% (n=25)	44% (n=10)	32-45% (n=10)	50% (n=20)	44-53 % (n=20)	34-38% (n=12)	44% (n= N.A.)
	Total synthesis time (min)	30[k] <60[l]	35[k]	128[k]	98[l]	<60[k]	40[k]	45[k]	<40[k]	68[k]	120[l]

Table 1. Summary of [^{18}F]SFB radiosyntheses reported in the literature using alkyl 4-(trimethylammonium)benzoate triflate as precursors.

Discussion

This simplified three-step, one-pot radiosynthesis of the ^{18}F-acylation reagent [^{18}F]SFB is developed based on non-aqueous chemistry. This process has excellent reproducibility and could be used reliably for the production of [^{18}F]SFB in automated radiochemistry modules, owing to two key modifications described as followings: 1. We employ a deprotection/saponification step in anhydrous KOtBu/DMSO system to replace the common aqueous basic or acidic solution. Our non-aqueous deprotection strategy enables sequential addition of reagents without intermediate SPE purification or solvent evaporation/exchange. This modification eliminates the need for a significant number of extra components and control units associated with a second reactor and SPE modules that have been required in other synthetic routes. Therefore, it reduces system complexity while increasing its reliability. Our process can be performed in a simple manual setup or in an automated radiochemistry module possessing a basic single-reactor configuration. Furthermore, elimination of SPE purification(s) between steps shortens the total synthesis time. 2. The use of microwave heating also enables the rapid, reliable production of [^{18}F]SFB (see **Table 1** for comparison with other methods). We apply microwaves in every step during this one-pot [^{18}F]SFB synthesis: F-18 drying, radiofluorination, deprotection,

and activation. As indicated in **Figure 1**, each transformation was complete within 1-2 min under microwave heating; in comparison, it generally requires 5 - 10 minutes when using conventional conduction heating (e.g., oil bath or heater block).[29-32, 41-42]

With a significant reduction in its overall complexity, we believe that this improved and concise synthesis will make the routine production of [^{18}F]SFB more practical and attractive for adoption in automated modules, enabling the use of ^{18}F-labeled biomolecules[43] as research tools to accelerate biomedical discovery and enhance clinical studies.

Disclosures

This method has been submitted for US patent application.

Acknowledgements

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