Site-Specific Labeling of Annexin V with F-18 for Apoptosis Imaging

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Annexin V is useful in detecting apoptotic cells by binding to phosphatidylserine (PS) that is exposed on the outer surface of the cell membrane during apoptosis. In this study, we examined the labeling of annexin V-128, a mutated form of annexin V that has a single cysteine residue at the NH_2 terminus, with the thiol-selective reagent ^{18}F-labeling agent N-[4-[(4-[^{18}F]fluorobenzylidene)aminooxy]butyl]maleimide ([^{18}F]FBABM). We also examined the cell binding affinity of the ^{18}F-labeled annexin V-128 ([^{18}F]FAN-128). [^{18}F]FBABM was synthesized in two-step, one-pot method modified from literature procedure. (Toyokuni et al., *Bioconjugate Chem.* **2003**, *14*, 1253−1259). The average yield of [^{18}F]FBABM was 23 ± 4% (n = 4, decay-corrected) and the specific activity was ~6000 Ci/mmol. The total synthesis time was ~92 min. The critical improvement of this study was identifying and then developing a purification method to remove an impurity *N*-[4-[(4-dimethylaminobenzylidene)aminooxy]butyl]maleimide **4**, whose presence dramatically decreased the yield of protein labeling. Conjugation of [^{18}F]FBABM with the thiol-containing annexin V-128 gave [^{18}F]FAN-128 in 37 ± 9% yield (n = 4, decay corrected). Erythrocyte binding assay of [^{18}F]FAN-128 showed that this modification of annexin V-128 did not compromise its membrane binding affinity. Thus, an *in vivo* investigation of [^{18}F]FAN-128 as an apoptosis imaging agent is warranted.

INTRODUCTION

Apoptosis, or programmed cell death, is an essential component for embryogenesis and homeostasis. Dysregulation of apoptosis is associated with many diseases such as cancer, autoimmunity, and neurodegenerative disorders (*1*). Many anticancer therapies exert their therapeutic effects by inducing apoptosis in tumor cells. A suitable *in vivo* marker for noninvasive imaging of apoptosis would be useful for both clinical care and drug development.

One of the early characteristics of apoptosis is the externalization of phosphatidylserine (PS) molecules on cell membranes, which promotes recognition and phagocytic removal (*2, 3*). In culture, once apoptosis is triggered, the PS externalization begins to occur within one hour (*2*). Annexin V, a 36 kDa endogenous cytoplasmic protein, binds with high affinity (K_d ~10 nM) to membrane-bound PS in a Ca^{2+}-dependent manner (*4*). Annexin V staining has been a standard practice for the assessment of apoptosis *in vitro* for the past decade. More recently, radiolabeled annexin V has been actively investigated to image cell death (*5–8*). ^{99m}Tc-labeled annexin V showed promising results in early clinical SPECT studies (*9–11*), but its low sensitivity has limited its broader application. To take advantage of the higher resolution and more accurate quantification of PET, labeling annexin V with short half-life positron-emitters such as ^{18}F is of particular interest.

We (*12*) and others (*13, 14*) have reported the labeling of wild-type annexin V with *N*-succinimidyl-4-^{18}F-fluorobenzoate ([^{18}F]SFB). We have also demonstrated that the ^{18}F-labeled annexin V ([^{18}F]fluoroannexin or [^{18}F]FAN) showed increased uptake in rat liver proportional to apoptosis after apoptosis was chemically induced (*15*). The rapid blood clearance and urinary excretion of [^{18}F]FAN suggest its potential as a PET imaging agent. This radiotracer, however, has its own disadvantages. The predominant renal clearance increases the radiation burden to the kidneys and bladder, and a patient would have to be catheterized if the diagnostic area of interest were in the abdomen (*15*). Moreover, this labeling method for annexin V is nonspecific, as the reaction is between the prosthetic group ([^{18}F]SFB) and any available NH_2 groups in this protein. There are at least 23 potential sites for this reaction to occur in each annexin V molecule, some near the active binding site of the protein. A recent report confirms that amine-directed chemical modification of annexin V reduces its membrane-binding activity even at low stoichiometries (*16*). Multiple substitutions of annexin V with [^{18}F]SFB could mean disproportional signaling by low-affinity molecules in PET imaging. Therefore, a site-specific method to label annexin V with only one substitution per molecule should have an advantage over the [^{18}F]SFB-modified version.

An alternative strategy to label proteins involves targeting the free thiol groups that are present only in cysteine residues. Thiol reactive agents such as N-substituted maleimides and iodoacetamide can be used to modify proteins at cysteines at specific sites (*17–20*). Annexin V-128 is a mutated form of annexin V containing only one cysteine. The initiator Met at position one is deleted and a six amino acid extension containing one cysteine is added at the N-terminus. The N-terminus does not play a role in the binding activity of annexin V and so this modification does not change the binding affinity of annexin V-128 for PS. Site-specific labeled (with ^{99m}Tc) annexin V-128 derivatives showed twice as much *in vivo* apoptosis-specific uptake in rat liver models as did amine-derivatized forms of annexin V (*16*). A previous approach to label thiol-containing molecules with *N*-[4-[(4-[^{18}F]fluorobenzylidene)aminooxy]butyl]maleimide ([^{18}F]FBABM) used a two-step/two-pot synthesis that was labor intensive and gave low yield with larger biomolecules such as 5′-S-ODN (*21*). In this paper, we report an improved synthesis and efficient purification of [^{18}F]FBABM,

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and the production of [^{18}F]annexin V-128 ([^{18}F]FAN-128) with this thiol-reactive reagent. The PS binding affinity of [^{18}F]FAN-128 and its potential as a PET imaging agent for cell death are also discussed.

MATERIALS AND METHODS

General. All reagents and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI) as reagent grade and were used without purification unless otherwise noted. The labeling precursor, 4-trimethylammoniumbenzaldehyde triflate **1**, was prepared as described by Haka et al. (*24*) and stored in a sealed vial over desiccant in the refrigerator. The coupling precursor, *N*-[4-(aminooxy)butyl]maleimide **3**, as a HCl salt was synthesized according to Toyokuni et al. (*21*) with modifications and stored over desiccant in the refrigerator. Enriched (97%) [^{18}O]water was obtained from Medical Isotopes, Inc. (Pelham, NH). Chromafix 30-PS-HCO_3 ^{18}F separation cartridges were obtained from GE Medical Systems and C-18 SepPak (light) cartridges were obtained from Waters Corp. (Milford, MA). The first part of the radiosynthesis (the production of the radioprecursor [^{18}F]FBABM) was performed using a GE Tracerlab FX_{FN} automatic synthesis module. The second part of the radiosynthesis (the radio-labeling of annexin V-128) was done manually outside the GE box. ^{1}H NMR data were obtained with a Varian 300 MHz NMR instrument. Quality control of the purified radioactive product was performed on a Waters 2690 HPLC module coupled with an in-line Micromass ZMD mass spectrometer.

Preparation of Annexin V-128. Annexin V-128 (~2 mg/mL in 20 mM HEPES sodium, 100 mM NaCl, pH 7.4) was prepared and purified as described (*26*). The protein was reduced with 1 mM dithiothreitol (DTT) for 15 min at 37 °C and then applied to a Sephadex G-25 column equilibrated with deoxygenated buffer (20 mM HEPES-sodium, 100 mM NaCl, pH 7.4) to remove the reductant. Protein concentration was measured by absorbance at 280 nm and the reduced protein was used for labeling within 6 h.

Synthesis of *N*-[4-[(4-Dimethylaminobenzylidene)aminooxy]butyl]maleimide 4. A solution of 4-dimethylaminobenzaldehyde (17 mg, 0.1 mmol) and **3**·HCl (20 mg, 0.09 mmol) in anhydrous DMF (1 mL) was stirred at r.t. for 16 h. The reaction mixture was then poured into water and extracted with diethyl ether. After drying over anhydrous Na_2SO_4, the ether solution was decanted, concentrated under reduced pressure, and then loaded onto a silica TLC plate (Alltech Silica Gel 60 F_{254} 5 × 20 cm). The TLC plate was developed with 40% diethyl ether in hexanes (v:v) and the product (R_f ~ 0.34) was washed off with ether as a yellowish solid (14 mg, 0.05 mmol, 50%). mp 51 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.65−1.75 (m, 4H), 2.99 (s, 6H), 3.57 (t, J = 6.0 Hz, 2H), 4.12 (t, J = 6.0 Hz, 2H), 6.66 (d, J = 9.0 Hz, 2H), 6.67 (s, 2H), 7.43 (d, J = 9.0 Hz), 7.97 (s, 1H). MS (ESI+): 316 $[M+H]^+$ HRMS (ESI+): $[M+Na]^+$ Calc. 338.1475; Found: 338.1481.

Synthesis of FBABM. A solution of 4-fluorobenzaldehyde (31 μL, 0.29 mmol) and **3**•HCl (41 mg, 0.18 mmol) in DMF (2 mL) was stirred at r.t. for 30 min. The reaction mixture was then poured into water and extracted with diethyl ether. After drying over anhydrous Na_2SO_4, the ether solution was decanted and concentrated under reduced pressure, and then loaded onto a silica flash chromatography column and eluted with 40% diethyl ether in hexanes (v:v) to give FBABM (50 mg, 0.17 mmol, 91%) as a white solid. mp 81 °C.(Lit. (*21*) 79−81 °C) ^{1}H NMR (CD_3OD, 300 MHz) δ 1.55−1.70 (m, 4H), 3.49 (t, J = 6.0 Hz, 2H), 4.09 (t, J = 6.0 Hz, 2H), 6.74 (s, 2H), 7.07 (dd, J = 9.0 Hz, 9.0 Hz, 2H), 7.57 (dd, J = 9.0 Hz, 6.0 Hz, 2H), 8.03 (s, 1H). MS (ESI+): 291 $[M+H]^+$.

Radiosynthesis of [^{18}F]FBABM. Cyclotron-produced [^{18}F]fluoride in $H_2{}^{18}O$ was forced through a Chromafix ^{18}F separation cartridge with helium pressure using a GE Tracerlab FX_{FN} automated synthesis unit. The concentrated $^{18}F^-$ on the cartridge was then eluted with 1.4 mg K_2CO_3 in 0.5 mL 1:1 (v:v) acetonitrile/water solution into the reaction vessel of the FX_{FN} box. A solution of 7.5 mg Kryptofix[2,2,2] in 2 mL anhydrous acetonitrile was then added to the same reaction vessel and the whole mixture was azeotropically evaporated to dryness under heat and reduced pressure. A solution of 4−5 mg 4-trimethylammoniumbenzaldehyde triflate **1** in 1 mL anhydrous acetonitrile was then pushed into the reactor, pressurized (~200 mbar), and heated at 100 °C. After 15 min, the reaction vessel was cooled to r.t. before 8−9 mg **3** in 1.5 mL methanol was added. After stirring at r.t. for 15 min, the mixture was slowly evaporated to <0.5 mL with heat (50 °C) under vacuum. After cooling to r.t., 1.5 mL of 45% ethanol in water (v:v) was added to the reaction vessel and the whole mixture was transferred to the HPLC loop. Semipreparative HPLC was performed with a C18 column (Phenomenex Prodigy ODS(3), 5 μm, 250 × 10 mm) eluted with 45% ethanol/water (v:v) at 3 mL/min and 50 °C. The chemically and radiochemically (>99%) pure fraction containing [^{18}F]FBABM was collected (~12 mL; t_R ~ 38−40 min). The chemical and radiochemical purity was assessed with a C18 analytical HPLC column (Phenomenex Inertisil ODS-3, 100 × 2.1 mm, 5 μm, eluted with 55% methanol/water (v:v) at 0.3 mL/min flow rate and 40 °C with in-line mass detection.

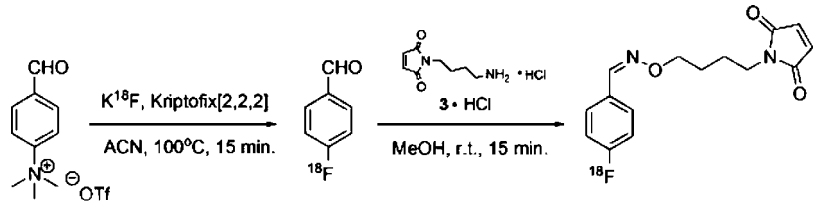
Labeling of Annexin V-128 with [^{18}F]FBABM. Purified [^{18}F]FBABM from the previous step (~12 mL) was diluted with water (~50 mL) and passed through a Waters C18 SepPak that had been preconditioned with 5 mL ethanol and 5 mL water. The SepPak was washed with additional 10 mL water and the excess water was expelled with a push of air. 2 mL of ether was then pushed through the SepPak to elute the product into a clean 5 mL glass test tube. The ether phase of the biphase mixture was drawn out and transferred into a clean 5 mL BD Vacutainer test tube. The ether was then removed by evaporation under a gentle flow of argon at 50 °C. After cooling to r.t., approximately 0.7 mg freshly prepared annexin V-128 in 0.5 mL buffer (20 mM HEPES, 100 mM NaCl, pH 7.4) was carefully added to the residual film and incubated for 15 min with periodic gentle agitation. Labeled [^{18}F]fluoroannexin V-128 ([^{18}F]FAN-128) was isolated by a semipreparative size-exclusion column (BioSep SEC-S 2000, 300 × 7.8 mm) eluted at 1 mL/min with 10 mM phosphate/150 mM NaCl (pH = 7). [^{18}F]FAN-128 was the first radioactive peak eluted from the column with a retention time of ~7−9 min.

Cell-Binding Assay of [^{18}F]FAN-128. The membrane binding affinity of [^{18}F]FAN-128 was determined following the procedure described by Tait et al. (*22, 16*) Briefly, mixtures of red blood cells (RBCs) with exposed PS on the outer surface of the cell membrane (Coulter "4C Plus" Cell Control, Fullerton, CA), [^{18}F]FAN-128 (diluted to a final concentration of 1 nM) in buffer (100 mM NaCl, 50 mM Hepes-Na, pH 7.4, 0.02% NaN_3, 1 mg/ml BSA) and $CaCl_2$ at precise concentrations (between 0 and 3 mM) were prepared. The mixtures were incubated for 10 min. at r.t. After centrifugation, the supernatant was discarded. Then the RBCs with bound [^{18}F]FAN-128 were washed with the same buffer containing $CaCl_2$ at the same concentrations and recentrifuged and the supernatant was discarded. Membrane-bound [^{18}F]FAN-128 was then released with assay buffer containing 5 mM EDTA and counted in the gamma counter.

RESULT AND DISCUSSION

Chemistry. Our synthesis of [^{18}F]FBABM builds on the approach reported by Toyokuni et al. (*21*) (Scheme 1). Briefly, triflate **1** was heated with dried [^{18}F]KF in anhydrous acetonitrile in the presence of Kryptofix[2,2,2] to generate [^{18}F]FBA **2**,

Scheme 1. Synthesis of [^{18}F]FBABM



1 **2** **[^{18}F]FBABM**

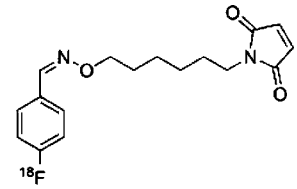
Table 1. Yield of FBA (2) under Different Reaction Conditions[a]

solvent	amt. of precursor **1** (mg)	temperature (°C)	yield (%)[c]
DMSO	8	100	90
THF	4	80[b]	0
CH_3CN	2	100[b]	10
CH_3CN	3	100[b]	20
CH_3CN	4	100[b]	75
CH_3CN	8	100[b]	90

[a] All reactions were carried out by heating the precursor in 1 mL solvent for 15 min in a closed reaction vessel in a GE Tracerlab FX_{FN} automated synthesis module. [b] Heated under helium pressure (200 mbar). [c] Determined from radio-HPLC chromatograms of the crude product. Data represent an average of 3 runs.

followed by the addition of maleimide **3** in methanol and purification by HPLC. Their two-step/two-pot synthesis of [^{18}F]FBABM was used to label the thiol-containing tripeptide glutathione (GSH) and a 5′-thiol-functionalized oligodeoxynucleotide (5′-S-ODN). This synthesis started with the generation of **2** followed by a C18 SepPak purification. After coupling to the heterobifunctional linker *N*-[4-(aminoxy)butyl]maleimide **3** using the oxime O-ether formation reaction and HPLC purification, [^{18}F]FBABM was formulated in acetonitrile and successfully labeled GSH or 5′-S-ODN. This method, however, has disadvantages for routine protein labeling. First, the synthesis of [^{18}F]FBABM was labor-intensive, with a two-pot setup and a C18 SepPak purification step in between. Second, although the labeling of GSH gave good yield (70% decay corrected), the yield in labeling larger biomolecules such as 5′-S-ODN (5% decay corrected) was low. The concentrations of the GSH or the 5′-S-ODN in these reactions were 6 mM and 0.1 mM, respectively. The radiochemical yield of radiolabeled peptides/proteins or other biomolecules via prosthetic groups typically decreases as the concentration of the biomolecule decreases. For an even larger molecule such as annexin V-128, which is usually obtained in ~30 μM concentration, the radiochemical yield might be even lower than 5%. Indeed, our initial attempts to label annexin V-128 with [^{18}F]FBABM synthesized by closely following the literature procedure (*12*) gave no yield. In addition to the slow rate of reaction associated with dilute conditions, the presence of nonradioactive maleimide-containing impurities, which would compete with [^{18}F]FBABM for the available thiol groups, could be an important factor that results in the dramatic decrease in yield. In the Toyokuni report, there was little information on the chemical purity of the [^{18}F]FBABM produced. We focused our investigation on simplification of the radiosynthesis and purification of the [^{18}F]FBABM.

We found that the C18 SepPak purification was not required for [^{18}F]FBABM synthesis; the two-step reactions proceed smoothly in one pot. Starting with ~850 mCi [^{18}F]fluoride, 110 ± 14 mCi (n = 4) of purified [^{18}F]FBABM was obtained in ~92 min (23 ± 4% decay corrected radiochemical yield) with >99% radiochemical purity and a specific activity of ~6000 Ci/mmol. This yield is comparable to that from the two-pot procedure (*21*), but with a process that can be automated. Although theoretically, [^{18}F]FBABM could be formed as both *E*- and *Z*-isomers, we observed only one isomer (tentatively assigned to possess the *E*-configuration) (*21*) under our reaction condition. This was confirmed by coeluting with the cold standard of FBABM, whose NMR spectrum revealed only one isomer, on HPLC. During the preparation of this manuscript, Berndt et al. reported a one-pot method to prepare a similar thiol-reactive labeling agent *N*-[6-(4-[^{18}F]fluorobenzylidene)-aminooxyhexyl]maleimide ([^{18}F]FBAM) with similar yield to ours (*23*). Their method, however, includes an extra prepurification step using solid-phase extraction on LiChrolut RP18 cartridge before semipreparative HPLC purification.

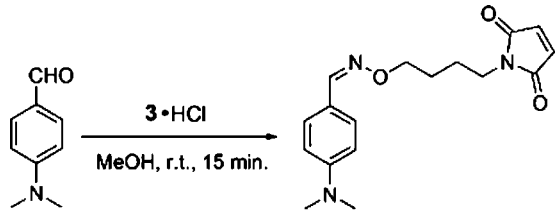


[^{18}F]FBAM

The synthesis of [^{18}F]FBA **2** from the triflate precursor **1** was traditionally carried out by heating the dried [^{18}F]KF/Kryptofix[2,2,2] in a high-boiling polar aprotic solvent such as DMSO (*24, 25*). We chose the lower boiling acetonitrile because it could be easily removed by evaporation. Compared with DMSO, the use of acetonitrile did not compromise the yield of [^{18}F]FBA (Table 1). Similar yields were obtained while the same amounts of precursors were used. A smaller amount of precursors resulted in decreased yields of **2**. Using 4 mg triflate precursor struck a balance between reasonably high yield and acceptably lower quantity of material introduced into the system. Compared to the Toyokuni report (*21*), the yield of the coupling reaction dropped from ~70% to ~40% (calculated from the yield of the first step and the overall yield), but the advantage of easier production of this radiotracer outweighed this reduction of yield. It is important to note that, because FBA is a volatile compound at elevated temperature, it is crucial to cool the reaction mixture to r.t. before opening the reaction vessel to add the coupling precursor.

The critical factor for optimal protein labeling with [^{18}F]FBABM is the chemical purity of the product. We suspected that a maleimide side product was present that would compete with [^{18}F]FBABM for reaction with thiol groups. HPLC-MS was used to identify this major contaminant as *N*-[4-[(4-dimethylaminobenzylidene)aminooxy]butyl]maleimide (**4**) (estimated >100 μg from a typical run described above) derived from the 4-dimethylaminobenzaldehyde formed inevitably during the initial nucleophilic fluorination step (Scheme 2). In a typical run, about 300 μg 4-dimethylaminobenzaldehyde was detected after this first step. The identity and quantity was determined by HPLC-MS analysis, in comparison to an authentic sample. Our initial attempt to label annexin V-128 with [^{18}F]FBABM containing **4** gave no yield, presumably due to a competition for the single thiol group by the vast molar excess of the alternative maleimide contaminant **4**. In a typical labeling reaction, the molar ratio of FBABM to annexin V-128 (generally 0.6 mg was used)

Scheme 2. Formation of 4



was approximately 1:1, whereas that of **4** was greater than 20:1. Attempts to remove the undesired 4-dimethylaminobenzaldehyde after the first step with a C18 SepPak were unsuccessful. The removal of **4** from the final product [^{18}F]FBABM proved to be challenging. The calculated log *P* values for FBABM and **4** are 1.91 and 1.85, respectively (Molinspiration program: http://www.molinspiration.com/cgi-bin/properties). The two compounds coeluted using the HPLC method from the literature (*21*). We developed a useful separation by using Phenomenex Prodigy ODS(3) 5 μm 250 × 10 mm column. At 50 °C and 45% ethanol/water as the eluent at 3 mL/min, **4** was completely removed from [^{18}F]FBABM (impurity t_R(**4**) ∼ 26−28 min, t_R(FBABM) ∼ 34−38 min). A typical semipreparative HPLC chromatogram is shown in Figure 1. No detectable amount of **4** was present in the purified [^{18}F]FBABM product. We realize that, in this isocratic HPLC method, although a complete separation of impurity **4** was achieved, the long retention time of the [^{18}F]FBABM is disadvantageous for routine production. We are currently investigating alternative purification method with gradient HPLC.

Annexin V-128 (*26*) contains one cysteine in its N-terminus. The single naturally occurring cysteine residue in its interior domain has been mutated to a serine in order to eliminate the possibility of modification by thiol-reactive prosthetic conjugating agents. Annexin V-128 is freshly reduced immediately prior to each labeling study with DTT to ensure that the free thiol is fully accessible. The protein is purified of excess DTT by eluting through a Sephadex column.

The purified [^{18}F]FBABM was concentrated onto a C18 SepPak, washed off with diethyl ether, and taken to dryness. After evaporation, the [^{18}F]FBABM was incubated with annexin V-128 in HEPES buffer (pH = 7.4, 0.5 mL). It is noted that the number of liquid transfers and the contact surface area with the reaction vessel should be minimized to reduce unnecessary loss of annexin V-128 due to surface adsorption of the protein. Even using a careful transfer technique, approximately 1/3 of the total radioactivity is typically lost in transfers for injection onto the size-exclusion column (SEC). After HPLC purification, radiochemically pure

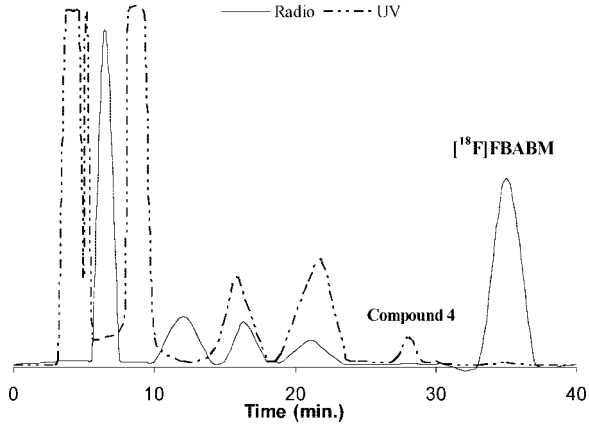


Figure 1. A typical semipreparative HPLC chromatogram for [^{18}F]FBABM purification. (Phenomenex Prodigy ODS-3, 250 × 10 mm, 5 μm, eluted with 45% ethanol/water v:v at 3 mL/min and 50 °C).

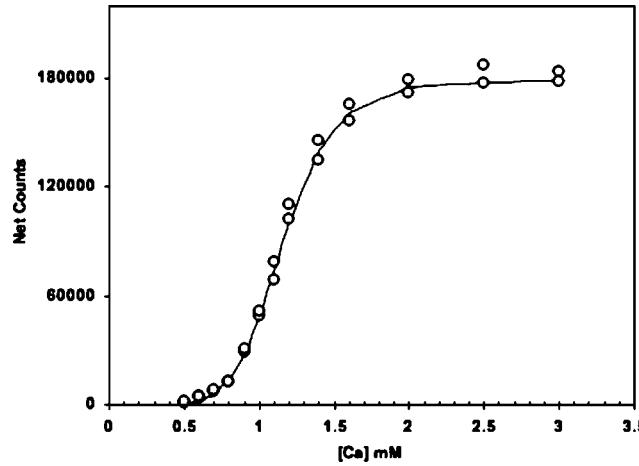


Figure 2. Typical binding curve of [^{18}F]FAN-128 to PS-enriched membranes. Typical binding curve of [^{18}F]FAN-128 as a factor of calcium concentration. [^{18}F]FAN-128 is held constant well below its K_d (at 1 nM), while calcium is titrated between 0 and 3 mM (maximal binding is observed at 2−2.5 mM Ca^{2+}). The membrane-bound radioactivity is plotted versus the concentration of Ca^{2+} present. p*K* value is calculated from slope (N) and midpoint of titration curve (EC_{50}) (*16*). Two measurements are done for each data point.

(>98%) [^{18}F]FAN-128 was obtained with 37 ± 9% yield (n = 4, decay-corrected) from [^{18}F]FBABM. The total recovery of radioactivity from the SEC was >90% with the radioactive peak at 7−9 min being [^{18}F]FAN-128. The unreacted [^{18}F]FBABM was retained for >1 h on the SEC column.

As a negative control, we repeated the reaction under the same condition using wild-type annexin V instead of mutated annexin V-128. Wild-type annexin V has one internal cysteine but it does not have a free thiol group on its N-terminus. In this reaction, the yield of ^{18}F-labeled protein was negligible (<0.2%). This result shows that, under our reaction conditions, [^{18}F]FBABM does not react with other nucleophiles such as the free NH_2 groups in annexin V, and the single cysteine residue that is present in the interior domain of wild-type annexin V is not readily accessible for maleimide binding. Therefore, we are able to conclude that the [^{18}F]FBABM labeling of annexin V-128 is site-specific. The mutation of Cys-316 to Ser in annexin V-128 may not be necessary for future annexin V based tracer design.

[^{18}F]FAN-128 PS Binding Assay. The *in vitro* membrane-binding affinity of [^{18}F]FAN-128 was determined by erythrocyte binding using the method reported by Tait et al. (*16*) The commercially available RBCs had exposed PS on their outer surfaces of the cell membranes because of the preservatives used (*27*). Annexin binding to PS is calcium dependent. By titrating calcium instead of protein, this method allows an accurate measurement of annexin binding at <1% occupancy, a value similar to that for *in vivo* imaging where the membrane is far from saturated with protein. A typical binding curve for [^{18}F]FAN-128 is presented in Figure 2. The binding affinity was measured by determining EC_{50} and slope from this experimental titration curve (*22*). The negative log of the binding constant, *p*K, is equal to $-(n \log_{10}EC_{50} + \log_{10}[\text{membrane}])$, where n is the number of Ca^{2+} ions interacting with each annexin V molecule, the EC_{50} is the molarity of Ca^{2+} at which cell binding is half of the maximal value, and [membrane] is the concentration of annexin V-binding sites in the assay (*16*). Seven batches of [^{18}F]FAN-128 gave an average p*K* of 28.5 ± 1.8, an EC_{50} of approximately 1 mM, and a slope (Hill coefficient) between 7 and 8. This compares favorably with reported values for wild-type annexin V of a p*K* around 30, EC_{50} close to 1, and a slope of 8 (*26, 16*). These data indicate that the *in vitro* binding activity

of this site-specific, monosubstituted protein was not significantly different from that of the wild-type and an *in vivo* investigation of [^{18}F]FAN-128 as an apoptosis imaging agent is warranted.

CONCLUSION

We have developed an improved, one-pot synthesis of the thiol-reactive labeling reagent [^{18}F]FBABM. It gives satisfactory radiochemical yield and can be automated for routine production. The critical improvement was identifying and then developing a purification method to remove the impurity *N*-[4-[(4-dimethylaminobenzylidene)aminooxy]butyl]maleimide (**4**). The presence of this impurity in [^{18}F]FBABM was dramatically decreasing the yield of labeled protein by competing for the available thiol groups. To our knowledge, this is the first time that this important issue has been addressed. We have also achieved an efficient synthesis of ^{18}F-labeled annexin V-128 ([^{18}F]FAN-128), a 36 kDa protein containing a single thiol group, with the [^{18}F]FBABM radioprecursor. This maleimide modification of annexin V-128 does not compromise its membrane binding affinity.

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