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Radiochemistry

Stoichiometric Leverage: Rapid ^{18}F-Aryltrifluoroborate Radiosynthesis at High Specific Activity for Click Conjugation**

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Positron emission tomography (PET) imaging is on the forefront of cancer diagnosis, clinical drug evaluation, and patient management.[1] Hence, radiotracers have been labeled with myriad β^+-nuclides.[2] Whereas isotope choice involves several considerations, the specific activity (SA), which is defined as Ci μmol^{-1} of radiotracer, represents an impartial measure of radiotracer quality.[3] A high SA is crucial for early cancer detection where target concentration is low and for which regulatory agencies will usually require that tracer concentration be low enough ($< 0.1 \times K_d$) that $< 10\,\%$ of the target is bound to avoid pharmacological effects.[2b]

As ^{18}F ($t_{1/2} = 109.8$ min) is a mainstay isotope for PET imaging, labeling methods that ensure high SA are of paramount importance. Whereas the SA of carrier-free ^{18}F-fluoride ion is 1712 Ci μmol^{-1}, in practice, the SA of no-carrier-added (NCA) ^{18}F-fluoride ion is usually ≤ 25 Ci μmol^{-1} owing to adventitious ^{19}F-fluoride ion, irrespective of where in the world it is prepared.[4] Ion exchange trapping and drying usually lowers SAs to ≤ 10 Ci μmol^{-1}. Hence, most small molecule tracers[5] and some radiosynthons are labeled at SAs of ca. 8 Ci μmol^{-1}[6] whereas most peptides are labeled at ≤ 2 Ci μmol^{-1}.[6c,7] Nevertheless, SA values of ca. 1 Ci μmol^{-1} are still described as high, and therefore deemed useful for imaging.[2b,3,6d,8] In view of the maximum SA of NCA ^{18}F-fluoride ion generally obtainable in practice, many reports have underscored the need to ensure the highest possible SAs through rapid one-step and one-pot two-step labeling.[6d,7e,9]

Herein, we report on a method that increases SAs to 15 Ci μmol^{-1}, which is circa three times the normal maximum, and which can be generalized to many ligands because of the mild conditions used. This is achieved by 1) a very rapid (ca. 30 min) one-pot two-step click labeling that minimizes decay, and 2) the formation of an ^{18}F-aryltrifluoroborate anion ([^{18}F]-ArBF$_3^-$) that leverages specific activity threefold compared to that of the ^{18}F-fluoride ion. The [^{18}F]-ArBF$_3^-$ anions are produced in one step from arylboronic acids, esters, and imidines in KF (buffered at pH 2) according to Equation (1).

$$\mathrm{ArB(YR)_2 + KF + 2\,HF \rightarrow ArBF_3K + 2\,RYH\ (Y = O\ or\ N)} \qquad (1)$$

Upon quenching to pH 7, any intermediate mono- or difluoroborates solvolyze to leave the [^{18}F]-ArBF$_3^-$.[10] Recently, we labeled an RGD-boronate (RGD = L-arginyl-glycyl-L-aspartic acid cyclopentapeptide) in one step, and azides of both RGD and bombesin in one pot by (2 + 3) cycloaddition to an alkyne–[^{18}F]-ArBF$_3^-$.[20] In all cases, tumor-specific in vivo PET images showed the utility of this method for peptide labeling and functional imaging. Tumor-specific images could be obtained from two RGDs labeled at SA values of ca. 0.1 Ci μmol^{-1} and from bombesin labeled at ca. 1 Ci μmol^{-1}. Because higher SA values are desirable, we investigated whether an [^{18}F]-ArBF$_3^-$ could be labeled at NCA or near-NCA conditions to provide for very high SA.

A unique advantage of this labeling method is that the SA of the [^{18}F]-ArBF$_3^-$ (defined as Ci μmole^{-1} of [^{18}F]-ArBF$_3^-$) is triple that of the ^{18}F-fluoride ion owing to the stoichiometry of Equation (1), even though there is only one ^{18}F-fluoride atom per [^{18}F]-ArBF$_3^-$ (see the Supporting Information).[9c,11] Yet, this hypothetical advantage is important only if the ArBF$_3^-$ species can be labeled with NCA or near-NCA ^{18}F-fluoride ion to provide an [^{18}F]-ArBF$_3^-$ with SAs of 7.5–15 Ci μmol^{-1}, values which are higher than those of most other radiotracers. Herein, we show the feasibility of this method in terms of one-step labeling and click conjugation, along with experimental proof of such high SAs.

Propargylated arylborimidine **1** was synthesized according to previous reports as a shelf-stable B(dan)-protected[12] radiosynthon precursor that is rapidly converted in buffer to the corresponding in vivo stable [^{18}F]-ArBF$_3^-$ **2**[11] (Scheme 1) with high SA. However, measuring high SAs on subnanomole quantities is inherently challenging because the molar extinction coefficients (ε) of peptides are either unreliable at 220 nm or too low at 280 nm. Likewise, the molar ε for ArBF$_3^-$ **2** ($\lambda_{max} = 325$ nm), is low (ca. 3750 M^{-1} cm^{-1}) and not absolutely ascertainable. Therefore, we repeated the one-pot two-step labeling method used for both RGD and bombesin, but with **2** conjugated to rhodamine ($\lambda_{max} = 568$ nm), a chromophore with a high ε value (100000 M^{-1} cm^{-1}) and a high fluorescence emission yield at 584 nm. The triazole-linked [^{18}F]-ArBF$_3^-$-rhodamine bioconjugate **3** was prepared according to Scheme 1.

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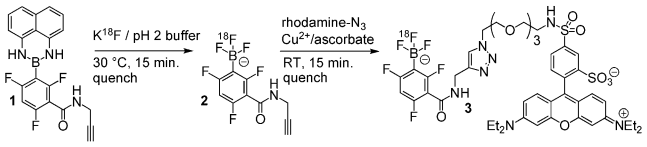
Supporting information, including detailed experimental procedures for radiolabeling, specific activity calculation, additional HPLC traces, and ^{19}F-fluoride ion concentration measurements, for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201208551.



Scheme 1. Preparation of an ^{18}F-labeled fluorescent-ArBF$_3$ **3** by Cu-catalyzed [2+3] cycloaddition of ^{18}F-labeled **2** with azido-rhodamine

The UV/Vis spectra of rhodamine and of **3** are identical at >400 nm, because **2** does not absorb at >400 nm. Thus, the optical properties imparted by rhodamine permitted unambiguous concentration determination, which in turn provided a direct measurement of the SA of **3**.

To begin, 98 mCi NCA ^{18}F-fluoride ion at end of bombardment (EOB) was obtained by typical ion exchange trapping to remove trace radiometals. The NCA ^{18}F-fluoride was then concentrated in a polypropylene tube within ca. 15 min, and resuspended in water to provide an NCA fluoride ion mixture that contained 89 mCi at beginning of synthesis (BOS).[10b] Two labeling reactions (15–20 μL total volume each) were set up to ensure high SA (Table 1). For reaction 1 (entry 1), NCA fluoride ion (19.3 mCi) was used; for reaction 2 (entry 2) NCA fluoride ion, (18.7 mCi) was spiked with 4 nmol [^{19}F]-KF to give a SA that is defined to be 2.7 Ci μmol^{-1}. To each reaction was added a buffered mixture containing precursor **1**. Based on many cited reports, we assumed that the SA of the NCA fluoride would be ca. 6 Ci μmol^{-1} at BOS, an assumption that we experimentally validated by analyzing for total ^{19}F-fluoride ion following decay (see below).[14] Both labeling reactions were set up to produce labeled **3** at high SAs: >7 Ci μmol^{-1} at end of synthesis (EOS), and which were deliberately measured in the validation of this hypothesis.

Table 1: Summary of measured specific activity data.

Entry	^{19}F added [nmol]	^{18}F at BOS [mCi]	SA of ^{18}F at BOS[a] [Ci μmol^{-1}]	SA of ^{18}F at EOS[b] [Ci μmol^{-1}]	SA of **3** at EOS[c] [Ci μmol^{-1}]	SA of **3** at EOS[d] [Ci μmol^{-1}]
1	0	19.3	6.5	5.3	14.6	15.5
2	4	18.7	2.7	2.2	8.0	7.4

[a] The specific activity of the (NCA) ^{18}F-fluoride ion was measured at 6.5 Ci μmol^{-1} using the fluoride sensor in Scheme 2, such that the 19.3 mCi used in entry 1 contained 3 nmol total fluoride ion and the 18.7 mCi used in entry 2 contained 2.9 nmol fluoride ion. Entry 2 was supplemented with fluoride ion (4 nmol, 6.9 nmol total) to give a final SA of 2.7 Ci μmol^{-1} at BOS. [b] Calculated by correcting for 30 min of decay. [c] Measured by dividing the injected activity by the concentration determined directly by integrating the absorbance value of the HPLC trace. [d] Measured by dividing the activity of radiochemically pure **3** by the concentration measured by fluorescence emission spectroscopy. BOS = beginning of synthesis, EOS = end of synthesis, SA = specific activity.

To ensure reasonable radiochemical yields, reactions were concentrated in vacuo for 15 min and then quenched. Half of each reaction was removed for analysis by both TLC and HPLC, and the remainder was combined with azido-rhodamine and $CuSO_4$/sodium ascorbate, for 15 min, and then quenched. To monitor the reactions, TLC analysis was performed before and after click conjugation. The reaction mixture from reactions 1 and 2 (1 μL; just prior to or after click reaction) was diluted into [^{19}F]-KF (10 μL, 100 mM; pH 7.5) to prevent any further formation of [^{18}F]-ArBF$_3^-$, and and aliquot (0.5 μL) was spotted onto a standard silica gel TLC plate and air-dried. Following resolution with 20% MeOH in CH_2Cl_2, an autoradiograph was acquired and compared to a fluorescent image, as shown in Figure 1.

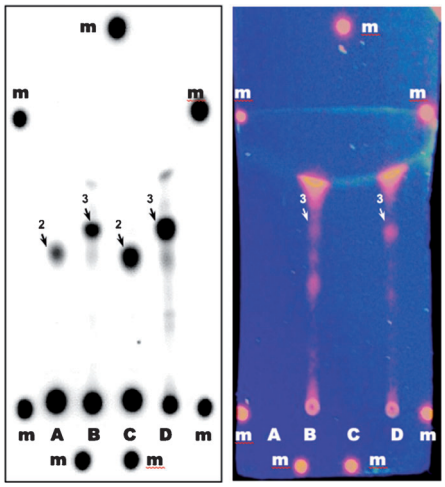


Figure 1. Left: autoradiograph, right: photograph. Lanes A and B are from crude reaction 1 (NCA), lanes C and D are from reaction 2 (near NCA); lanes A and C are before, and lanes B and D after click reaction. Spots labeled "m" are marker spots applied following resolution to correlate fluorescence with autoradiographic density. TLC plate is standard silica developed with 20% MeOH in CH_2Cl_2.

Autoradiographic density concentrates in a single spot (**3**) that coincides with fluorescence (lanes B and D), whereas in control lanes A and C (prior to click) a more polar spot (**2**) is essentially the only labeled species apart from free ^{18}F-fluoride ion. Only a single species is simultaneously fluorescent and radioactive, and this spot has an R_f identical to an authentic standard of unlabeled **3** (data not shown). TLC analysis shows near-quantitative conversion of **2** into **3**, and a high degree of radiochemical purity, as corroborated by HPLC analysis (see below). In reaction 2, both radiochemical and chemical yields are higher owing to added carrier. Because azido-rhodamine was added in a 10-fold excess and because the overall chemical yield was <100%, unlabeled fluorescent by-products likely include conjugates to **1** and the free boronic acid.

HPLC cleanly separated **3** from both **2** and the azido-rhodamine (Figure 2). The peak eluting at 21 min, which corresponds to an authentic sample of **3**, was isolated from reactions 1 and 2 in radiochemical yields of ca. 15% and 30%, respectively. As expected, the addition of small amounts of carrier in reaction 2 gave significantly higher radiochemical

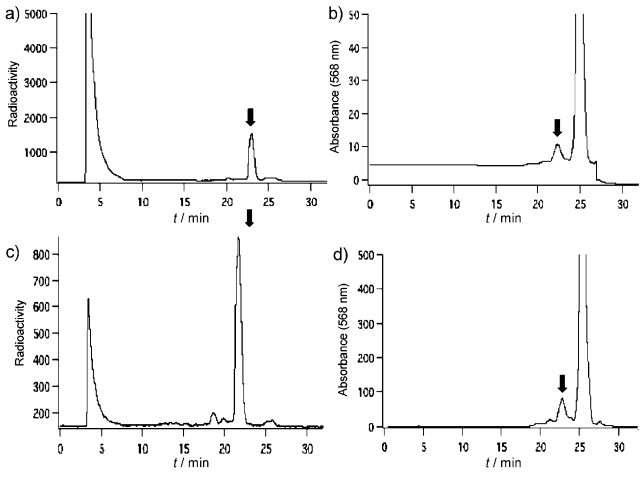


Figure 2. a) Radio-trace of click reaction 1. b) UV trace of click reaction 1. c) Radio-trace of click reaction 2. d) UV trace of click reaction 2. **3** elutes at 21 min, as indicated by the arrows. Unreacted azido-rhodamine elutes at 25 min.

yields. Fractions of pure **3** from each reaction were re-injected and radio-traces (Figure 3) demonstrate excellent radiochemical purity.

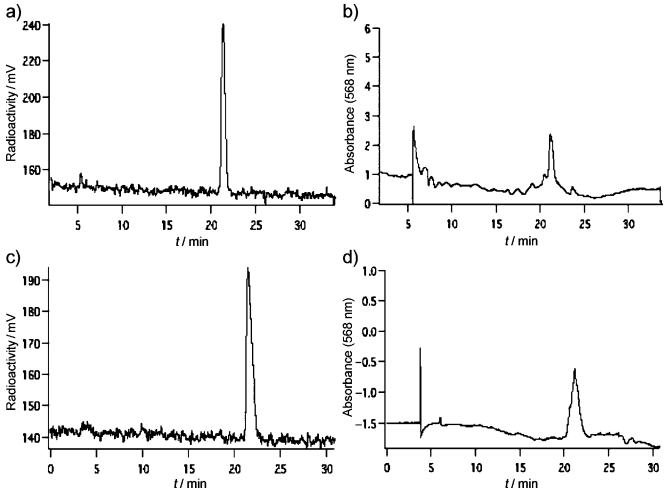
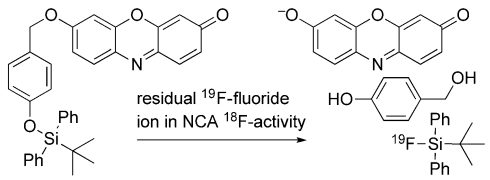


Figure 3. a) Radio-trace of re-injected sample from click reaction 1. b) UV trace of re-injected sample from click reaction 1. c) Radio-trace of re-injected sample from click reaction 2. d) UV trace of re-injected sample from click reaction 2. UV peak height is approximately 1.5 mAU.

Integration of the UV peaks gave tentative measures of concentration, from which SAs were determined for reactions 1 and 2 (Table 1). Because UV absorbance values were near the limits of accurate detection, we used fluorescent emission at 584 nm along with a standard curve to measure the concentration of purified **3**, from reactions 1 and 2 (see the Supporting Information) and thereby SA values of 15.5 Ci μmol^{-1} and 7.4 Ci μmol^{-1}, respectively, as summarized in Table 1.

The SA values determined by absorbance and fluorescence emission are consistent, both with each other, and with the reaction stoichiometry: the SA of **3** labeled in reaction 1 is three times the estimated value of NCA fluoride ion at EOS. For reaction 2, (near-NCA conditions) the SA of **3** was found to be slightly higher than thrice the SA of the fluoride ion (ca. 2.2 Ci μmol^{-1}) at EOS. This minor difference, which likely reflects experimental error, shows that, within experimental error, measured SA values necessarily equal calculated SA values. Finally, the labeling herein has been reproduced three times (see the Supporting Information).

To verify that the SA of NCA ^{18}F-fluoride ion was indeed ca. 6 Ci μmol^{-1} at BOS, we sought independent experimental evidence. The remaining 51 mCi NCA fluoride ion was decayed and analyzed for total ^{19}F-fluoride ion content with a self-immolating ratiometric fluorescent fluoride ion sensor that detects as little as 0.5 nmol fluoride ion (Scheme 2).[13] This assay showed that 51 mCi NCA ^{18}F-fluoride ion contained (7.9 ± 0.5) nmol of ^{19}F-fluoride ion, giving a SA of 6.5 Ci μmol^{-1} at BOS (5.3 Ci μmol^{-1} at EOS), which is about one third the SA of **3** labeled in reaction 1. This is noteworthy because the NCA ^{18}F-fluoride ion was prepared by standard bombardment and ion exchange trapping. A measured SA of 6 Ci μmol^{-1} is consistent with what is commonly reported for NCA ^{18}F-fluoride ion.



Scheme 2. A self-immolating fluorescent fluoride sensor measures endogenous ^{19}F-fluoride ion present in NCA ^{18}F-fluoride ion.[13]

Encouraged by the robust and reproducible nature of this method and confident of high SAs, we labeled **2** using ^{18}F-fluoride ion at a defined SA of 4 Ci μmol^{-1}, and then reacted it with azido-RGD to yield the RGD–[^{18}F]-ArBF$_3^-$ at 12 Ci μmol^{-1} (corrected to EOS; Figure 4). As this compound provides tumor images at lower SA, the same tracer has now been labeled at very high SA.

Because of the unreliable extinction coefficients of peptides and the very high SA in this case, we could not be certain of the SA of the RGD labeled above. Instead, we measured the SA of the ^{18}F-fluoride ion by analyzing for residual ^{19}F-fluoride ion following decay to verify the SA of the ^{18}F-fluoride ion to be 4 Ci μmol^{-1} at EOS and thus that of the RGD–[^{18}F]-ArBF$_3^-$ to be ca. 12 Ci μmol^{-1}.

Previously, we[9c,11,14] and others[15] noted the potential for imaging with [^{18}F]-ArBF$_3^-$, but with added carrier that gave [^{18}F]-ArBF$_3^-$-bioconjugates at low SA (ca. 0.1 Ci μmol^{-1}). Here, we sought to leverage the stoichiometry of [^{18}F]-ArBF$_3^-$ synthesis under NCA or near-NCA conditions to give an [^{18}F]-ArBF$_3^-$ radiosynthon at a SA beyond what is normally reported. Given the great interest in click conjugations reported for both [^{18}F]-Al-NOTA (NOTA = 1,4,7-triazacy-

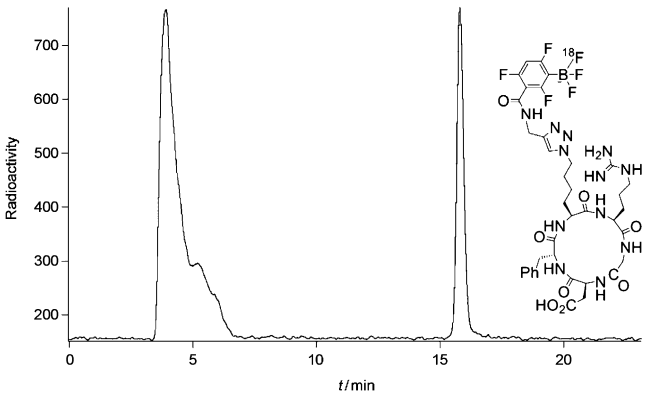


Figure 4. Crude radio-trace of RGD labeled by the same one-pot two-step method with an ^{18}F-fluoride ion specific activity set at 4 Ci μmol^{-1} to give labeled RGD at 12 Ci μmol^{-1}, corrected to EOS (elution at 16 min).

clononane-1,4,7-triacetic acid) chelates and [^{18}F]-SiFA analogues that were labeled at SAs of $\leq$7 Ci μmol^{-1} prior to conjugation,[16] we chose to show similar compatibility, but with higher SA.

To measure such high SA, we clicked **2** to rhodamine, a suitable chromophore that unequivocally allowed us to measure concentration on low (safe) amounts of ^{18}F-labeled **3**. Similar results were then obtained in a one-pot two-step labeling of azido-RGD. As we have previously labeled RGD at low SA, both directly and by one-pot two-step click labeling, and have labeled bombesin at ca. 1 Ci μmol^{-1} by one-pot two-step click labeling, this method can now be used to label the same validated ligands at much higher SA.

Whereas most reports cited herein use >100 mCi, we used less than 20 mCi per labeling reaction, which requires minimal shielding. Thus, these results should find immediate use for small animal imaging and should therefore be broadly applicable to academic labs. Although both radiochemical yields (RCYs) and SAs are quite high in reaction 2, had we added 40 nmol of ^{19}F-fluoride ion, RCYs would have approached 100%, even if the SA would be reduced to a still-high value of 1.5 Ci μmol^{-1}. Two recent reports highlighted two-step click labeling of RGD in radiochemical yields of 7–12% after 100–120 min with SAs of approximately 1.3 Ci μmol^{-1}.[7d,17] Similarly, the radiochemical yields of one-step [^{18}F]-Al chelation by NOTA–bis(RGD) at 100 °C range from 5–25% after 30 min with a SA of only 0.16–0.8 Ci μmol^{-1}.[7e] Whereas RCYs of 15% in reaction 1 are competitive with these recent reports, the specific activity surpasses all reports to date.

It is not speculative to assert that RCYs will increase substantially when higher levels of ^{18}F-activity are used: whereas we achieved isolated RCYs of ca. 15% with only 4 nmol fluoride ion, 1 Ci will provide 200 nmol total fluoride ion (assuming the SA of NCA ^{18}F-fluoride ion is 5 Ci μmol^{-1}). In a similar vein, 50 nmol unlabeled **3** was isotopically exchanged in the presence of 0.8 Ci NCA ^{18}F-fluoride ion to give **3** at 15 Ci μmol^{-1} in >50% RCY in just 15 min.[21]

In terms of clinical imaging, a SA of ca. 1 Ci μmol^{-1} is generally considered to be useful, even high. Yet, if in practice the SA of NCA ^{18}F-fluoride ion is limited to ca. 6 Ci μmol^{-1}, meeting a target SA of 1 Ci μmol^{-1} will always be challenging because of the need to minimize synthesis, packaging, and transport times. Here, the 3:1 stoichiometry of [^{18}F]-ArBF$_3^-$ synthesis under NCA or near-NCA conditions along with rapid labeling and conjugation provides final SAs that are up to 10-fold higher than most tracers reported to date and ca. 100-fold higher than previously reported for [^{18}F]-ArBF$_3^-$ species.

To date, few if any tracers have been prepared at such high SAs. Hence, these results have two important ramifications for tracer production. Firstly, high SA is likely to be indispensable for early detection of primary tumors or secondary metatheses. Secondly, if current SAs of ca. 1 Ci μmol^{-1} remain sufficient for clinical use, this method expands the time window to 7 h (ca. 4 half-lives), which will enable long distance tracer distribution.

The salient advantages of our method are: 1) Extraordinarily high specific activity with low levels of ^{18}F-activity, for example, 10–20 mCi. 2) Rapid synthesis times: 15 min per step and with good radiochemical yields, for example, 15–30%. 3) The use of aqueous conditions at warm temperature. 4) The production of a highly polar ^{18}F-labeled anion. 5) The use of only 10 nmol (10 μg) of precursor. 6) Direct labeling with a Cu-catalyzed [2+3] cycloaddition that has been applied to RGD, which makes this technique applicable to any peptide, oligonucleotide, or antibody that has been click-labeled.[18] 7) The production of a dual-modal ^{18}F-labeled fluorescent tracer[15b,19] where the SA is extremely high.

In summary, we have radiosynthesized an alkyne–[^{18}F]-ArBF$_3^-$ in near-record time at very high SA using NCA ^{18}F-fluoride ion. This was then clicked to rhodamine to measure the SA at 15 Ci μmol^{-1}. These results now confirm that the SA can be reliably calculated by simply tripling the SA of the ^{18}F-fluoride ion, a value that can be measured afterwards, or reproducibly fixed by accurately adding small amounts of carrier ^{19}F-fluoride ion, as was done in reaction 2. In this manner, carrier ^{19}F-fluoride ion was added to NCA ^{18}F-fluoride ion to fix the SA at 4 Ci μmol^{-1}, which provided labeled RGD at 12 Ci μmol^{-1}. Thus, stoichiometric leverage offsets the effect of adding carrier ^{19}F-fluoride ion to ensure that SAs exceed most other NCA ^{18}F-labeling methods. Of note, NCA ^{18}F-fluoride ion at 6 Ci μmol^{-1} contains substantial amounts of ^{19}F-fluoride ion, which was quantified herein with a ratiometric fluoride sensor. This sensor should find general use for measuring SA values elsewhere. Finally, this work portends a kit-like method where lyophilized aliquots containing microgram quantities of arylborimidine radiosynthon are labeled on demand within 15 min.

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