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Rapid, one-step, high yielding ^{18}F-labeling of an aryltrifluoroborate bioconjugate by isotope exchange at very high specific activity†

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A rapid, single step, aqueous ^{18}F-labeling method that proceeds under mild conditions to provide radiotracers in high radiochemical yield and at high specific activity represents a long-standing challenge. Arylboronates capture aqueous ^{18}F-fluoride ion in buffered pH 2–3 at moderate temperature to provide a highly polar ^{18}F-ArBF$_3^-$ anion. Similarly, ^{19}F-^{18}F isotope exchange on a ^{19}F-ArBF$_3^-$ should create an ^{18}F-ArBF$_3^-$. We hypothesized that this reaction would proceed in volumes that would be amenable to the high levels of ^{18}F-activity used in clinical hospitals. In order to measure both radiochemical and chemical yields, along with specific activity, we linked an alkyne-^{19}F-ArBF$_3^-$ to rhodamine azide by standard click chemistry to afford a precursor Rh-^{19}F-ArBF$_3^-$. This precursor was aliquoted in portions of 50 nmol and lyophilized for on-demand use. Using robotic manipulators in a hot cell, we combined >29.6 GBq (800 mCi) and 50 nmol of the Rh-^{19}F-ArBF$_3^-$ in aqueous dimethylformamide at buffered pH 2–3. Following mild heating (40 °C) for 10-15 min, the reaction was quenched and analyzed. We observed radiochemical yields of 50% and specific activities of nearly 555 GBq/μmol (15 Ci/μmol). Similar radiochemical yields and slightly lower specific activities were also obtained with ~400 mCi ($n = 2$). With radiochemical yields in the hundreds of millicuries and specific activities that are 3–10-fold higher than most radiotracers, this method is very attractive method for preparing clinically useful radiotracers. Moreover, the ability to produce tracers at extraordinarily high specific activities expands the distribution time window from production labs to distant positron emission tomography scanners.

Keywords: one-step ^{18}F-labeling; fluorescent radiotracers; high specific activity; bioconjugates

Introduction

Positron emission tomography imaging provides some of the highest *spatio*-temporal *in vivo* resolution of deep-tissue localization of targets for validation, preclinical evaluation, and patient diagnosis.[1] Although isotope choice is based on various considerations, the main deciding factors are ease of labeling, isotope availability, specific activity, and half-life. Of various β^+-radionuclides, fluorine-18, with its high isotopic purity, single decay process, low β^+ emission energy, moderate half-life, and facile on-demand production by hospital cyclotrons accounts for its widespread demand.

^{18}F-fluoride ion is produced by bombardment ^{18}O(p,n)^{18}F from ^{18}OH$_2$. Whereas carrier-free fluoride ion has a specific activity of 63.6 TBq/μmol (1720 Ci/μmol), in practice, the specific activity of no carrier added (NCA) ^{18}F-fluoride ion falls to 925–1320 GBq/μmol (25–40 Ci/μmol).[2,3] Drying and anion exchange trapping leads to further reduction of specific activity and hence small molecule tracers[4,5] have been labeled at 185–296 GBq/μmol (5–8 Ci/μmol), whereas most are labeled at even lower specific activities.[6–9] Similarly, the specific activities of most ^{18}F-labeled peptides fall below 74 GBq/μmol (2 Ci/μmol).[8,10–15] Nevertheless, a value of 37–74 GBq/μmol (1–2 Ci/μmol), is commonly described as 'high' and therefore is considered very useful for imaging.[16,17] For instance, Ritter and coworkers used 37 GBq (1 Ci) of NCA ^{18}F-fluoride ion to produce radiotracers in radiochemical yields of 6–30% at a specific activity of 37 GBq/μmol (1 Ci/μmol) that was also described as 'high'.[9] These reports underscore the need for radiosynthetic methods whereby labeling occurs rapidly, under aqueous conditions, and in a single step.[15,18–25] Toward these ends, several new captors of aqueous fluoride ion based on aluminum, silicon, and boron may expedite a one-step aqueous labeling, as recently reviewed.[22–25]

Previously, we proposed that arylboronates would capture aqueous ^{18}F-fluoride ion to provide an ^{18}F-ArBF$_3^-$.[26] ArBF$_3^-$ salts, which are considered to be non-toxic, are synthesized from potassium ^{18}F-fluoride under mildly acidic conditions according to Figure 1.[27–29] Labeling requires only microgram quantities of arylboronate precursor and proceeds rapidly with mild heating under mildly acidic conditions, that is, pH 2–3. Only the

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†Supporting information may be found in the online version of this article.

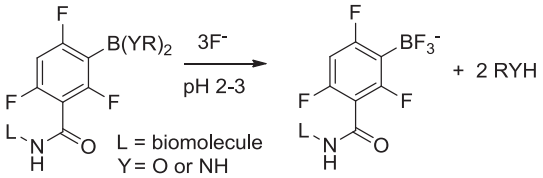

Figure 1. General labeling of an arylboronic acid (Y=O) or arylborimidine (Y=NH) where L is a biomolecule or clickable handle such as an alkyne.

^{18}F-ArBF$_3^-$ is stable at physiological pH; all other monofluorinated and difluorinated species solvolyze within seconds once the reaction is quenched at pH 7.5.[30]

Synthesis, fluorescent screening, ^{19}F-NMR kinetics, HRMS, pulse-chase-TLC, and serum stability assays verified physiological stability of certain aryltrifluoroborates,[30] whereas ^{18}F-ArBF$_3^-$ labeled conjugates of marimastat,[20,31] biotin, and[32] Lympho-seek™[33] demonstrated *in vivo* stability with little if any signal in bone, the appearance of which would have suggested solvolytic/metabolic defluorination. Recently, we labeled peptidic RGD conjugates either in a single step or in a one-pot-two-step click labeling of an RGD azide with an alkyne-modified ^{18}F-ArBF$_3^-$ as shown in Figure 2 (Li *et al.*, manuscript in press). In both cases, good tumor images were obtained despite the rather low specific activity of the RGD-conjugates (2.2–5.9 GBq/μmol or 0.06–0.16 Ci/μmol).

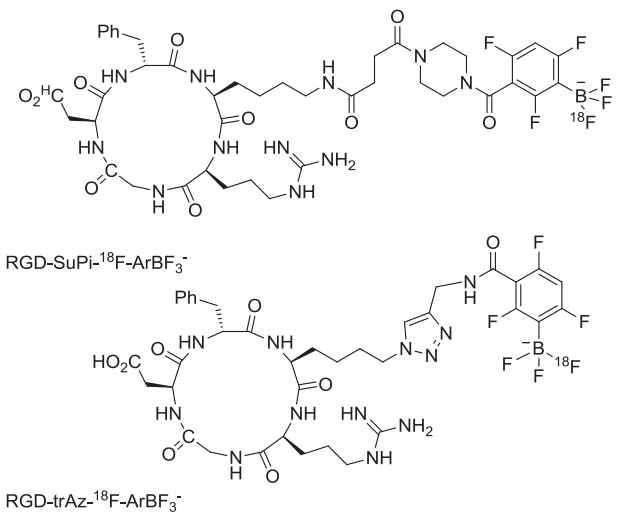

Figure 2. Two ^{18}F-labeled RGD species that were imaged; top—RGD-SuPi-^{18}F-ArBF$_3^-$ was directly labeled from the arylboronates precursor by treatment with K^{18}F in acidic conditions; bottom—an alkyne-^{18}F-ArBF$_3^-$ was first synthesized from the alkyne-arylborimidine and then conjugated to an RGD azide by standard Cu$^+$-catalyzed click conjugation.

Despite these *in vivo* images, a significant limitation is that ^{18}F-ArBF$_3^-$ conjugates have been labeled at low specific activity ~7.4 GBq/μmol (~0.2 Ci/μmol). We and others have argued that low specific activities reflect the use of low levels of starting ^{18}F-activity, (370–1850 MBq or 10–50 mCi) and that use of high levels of ^{18}F-activity routinely produced by hospital cyclotrons would provide much higher specific activities.[9,34,35] Yet, the use of high levels of ^{18}F-activity requires robotic manipulation in shielded hot cells, which might not accommodate the low reaction volumes needed for good radiochemical yields. Encouraged by *in vivo* tumor images gleaned from two differently linked RGD-^{18}F-ArBF$_3^-$ conjugates, we hypothesized that a ^{19}F-ArBF$_3^-$ bioconjugate, prepared in advance, would undergo facile ^{19}F-^{18}F isotope exchange (either directly or through re-equilibration *in situ* with the arylboronate) to provide an ^{18}F-ArBF$_3^-$ conjugate in high radiochemical and chemical yield and at high specific activity thereby greatly simplifying radiosynthesis. Such would be analogous to ^{19}F-di-tertbutylphenylfluorosilane-bioconjugates designed to undergo isotope exchange.[36] During the preparation of this manuscript, a recent study showed demonstrated *in vivo* stability of a phosphonium-stabilized ^{19}F-ArBF$_3^-$ would undergo ^{19}F-^{18}F isotope exchange although the specific activities reported were 18.5 GBq/μmol (0.5 Ci/μmol) or lower.[37]

Here, we sought very *high* specific activity with good radiochemical yields in conjunction with production levels of ^{18}F-activity in fully shielded hot cells. In order to measure the very high specific activity anticipated from using high levels of ^{18}F-activity, we labeled a conjugate containing a suitable chromophore that would enable accurate mass measurement from the HPLC trace, from which specific activity would be determined. Hence, we conjugated an ^{19}F-ArBF$_3^-$ to rhodamine, ($\lambda_{max} = 569$ nm, $\varepsilon = 100{,}000\ M^{-1}\ cm^{-1}$). In keeping with prior conjugation chemistries where we linked the arylboron moiety to the RGD peptide for tumor visualization, rhodamine was first conjugated to the nonradioactive alkyne-^{19}F-ArBF$_3^-$ via a copper mediated 2 + 3 cycloaddition 'click' reaction to give the radiosynthetic precursor shown in Figure 3.

This conjugate was then subjected to a ^{19}F-^{18}F isotope exchange reaction, either directly or through re-equilibration, for the rapid production of a fluorescent ^{18}F-ArBF$_3^-$-bioconjugate at very high specific activity. The salient advantages embodied in this labeling method are the following:(i) rapid synthesis time: <15 min; (ii) excellent radiochemical yields ~50%; (iii) the use of aqueous conditions; (iv) the use of high levels of radiation common to production labs; (v) a kit-like approach that uses only 50 nmol of lyophilized precursor; and (vi) the production of a dual-modal ^{18}F-labeled fluorophore at very high specific activity.[38–40]

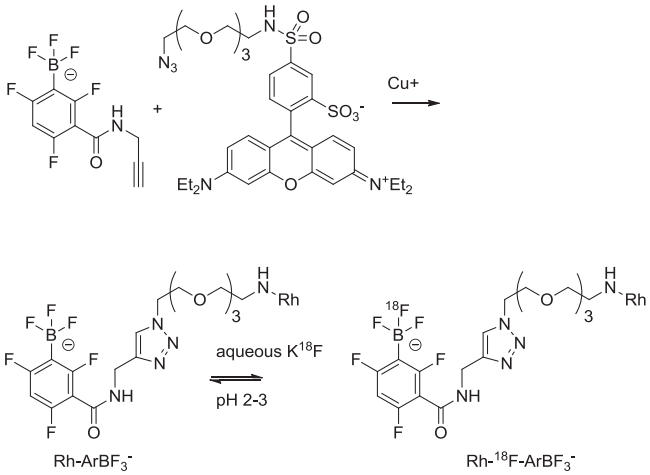

Figure 3. Synthetic scheme for click conjugation of the unlabeled alkyne-^{19}F-ArBF$_3^-$ to an azide-rhodamine to give the Rh-ArBF$_3^-$ precursor that undergoes isotope exchange.

Experimental

Materials

Chemicals were purchased from Sigma-Aldrich unless stated otherwise. The azidotriethyleneglycol-bis-sulfonyl rhodamine was purchased from Click Chemistry Tools Inc. as predominantly one of two regio-isomers.

Deuterated solvents were purchased from Cambridge Isotope Laboratories. TLC analysis and preparation were performed by Silica Gel 60 F_{254} glass TLC plates from EMD Chemicals.

HPLC methods

Unless otherwise stated, all samples were loaded onto a Phenomenex Jupiter 10 μ C18 300Å 4.6 × 250 mm column and material was eluted using a step gradient (solvent A: 0.04 M ammonium formate pH 6.8; solvent B: MeCN); 0–5 min: 0–5% B, 5–10 min: 5–35% B, 10–20 min, 35–45% B, 20–22 min: 45–100% B, 22–28 min: 100–100% B, 28–30 min: 100–20% B, 30–33 min: 20–5% B; flow rate: 1 mL/min, column temperature: 19–21 °C.

Synthesis of alkyne-^{19}F-ArBF$_3^-$

Briefly, 1,3,5-trifluoro-2-carboxyphenyl-4-boronic acid was prepared according to previous reports.[20,41] Instead of converting the boronate to the tretaphenylpinacolate, a diaminonaphthalene (dan) group was installed according to previous reports.[42–44] The carboxylate was activated in the presence of EDC according to previous reports and condensed with propargyl amine and purified by flash chromatography. Following previous reports for the production of the 1,3,5-trifluoro-4-ArBF$_3^-$,[32] (50 nmol) was dissolved in 5 μL DMF to which was added 2 μL 1 M pyridazine-HCl buffer in 50% DMF/H_2O, and 1 μL 100 mM KHF_2 aqueous solution were added to make a cocktail. The solvent was removed under vacuum in speed-vac under 45 °C. Generally, this procedure took 15 min. And the alkyne-ArBF$_3^-$ has been previously reported in which we have disclosed the ^{1}H- and ^{19}F-NMR spectra (Li *et al.* AJNMMI in press), here, the residue was directly used without further purification.

Synthesis of rhodamine-^{19}F-ArBF$_3^-$

Approximately 250 nmol of alkyne-^{19}F-ArBF$_3^-$ was resuspended in 8 μL 5% NH_4OH in 50% MeCN/H_2O. A 5 μL MeCN solution that contained 500 nmol rhodamine azide, 250 nmol $CuSO_4$, and 600 nmol sodium ascorbate was added and the reaction was placed in a speed-vac at room temperature. After ~10 min, a dark reddish solid remained. The residue was resuspended with 20 μL 50% MeCN/H_2O then applied to a TLC plate, which was developed with 15% methanol in dichloromethane. The TLC band, corresponding to the desired product was excised and extracted into methanol. NOTE: If the plate was not completely dry prior to resolution, R_f values were quite variable. An aliquot of the TLC purified solution was injected into HPLC to verify purity and elution time. Because of the small amount of material in this case and the commercial availability of the azido-rhodamine for use in copper catalyzed 2 + 3 cycloaddition, the ^{1}H-NMR and ^{19}F-NMR spectra were not acquired following click conjugation. Instead, TLC purification followed by HPLC purification along with low-res MS and UV-vis spectra were considered sufficient to confirm the purity of this conjugate: ESI-MS (M-H) = 1038; λ_{max} = 568–569 nm.

NCA ^{18}F-fluoride ion preparation

About >29.6 GBq (>800 mCi) of ^{18}F-fluoride ion was prepared by ^{18}O-water bombardment with 12–14 MeV protons. A QMA cartridge was pretreated with (i) deionized (DI) water (6 mL); (ii) saturated brine (6 mL); and (iii) DI water (6 mL) whereupon the irradiated water containing the NCA ^{18}F-fluoride ion was passed from the target over the cartridge. The NCA ^{18}F-fluoride ion was eluted with 0.25 mL, 8 mg/mL $NaClO_4$ in water was used to elute the radioactivity into a well-sealed plastic polypropylene vial. Approximately 0.5 mL of MeCN was added and the fluoride ion was azeotropically concentrated at 100 °C for 10 min. This step served to simply concentrate rather than dry the ^{18}F-fluoride ion, which appeared to be a pasty, damp, translucent residue in the tube.

Isotope exchange reaction

Just prior to labeling, 50 nmol ^{19}F-Rhodamine-ArBF$_3^-$ was resuspended in 20 μL DMF and 20 μL water and 10 μL containing 2 M pyridazine-HCl (pH 2). This solution was transferred via syringe to the tube containing the NCA ^{18}F-fluoride ion. The tube was then placed in a heating block set at 40 °C. After 10–15 min, the reaction was removed from the heating block and quenched by the addition of 2 mL 5% NH_4OH in 50% MeCN/H_2O via syringe to resuspend the contents of the labeling reaction. From this resuspension, a small volume 50 μL was removed from the hot cell, counted, and injected onto an RPC18 analytical HPLC column for analysis.

Results and discussion

The alkyne-^{19}F-ArBF$_3^-$ was prepared as previously described (Li *et al.*, AJNMMI in press) and reacted with commercially available azide-triethyleneglycolyl-rhodamine bis-sulfonate in the presence of Cu^{2+} and ascorbate to provide the Rh-^{19}F-ArBF$_3^-$ bioconjugate shown in Figure 3. Following TLC and HPLC purification, the conjugate was lyophilized in aliquots of 50 nmol. For labeling, 816.5 mCi NCA ^{18}F-fluoride ion was concentrated at 100 °C for 10 min in a polypropylene tube. An aliquot of Rh-^{19}F-ArBF$_3^-$ (50 nmol) was resuspended in a DMF-water solution (50 μL) containing a pyridazine-HCl buffer (pH = 2). This solution was transferred by syringe to the plastic tube containing the NCA ^{18}F-fluoride ion. After 10–15 min at 40 °C, the reaction was quenched by the addition of 2 mL 5% NH_4OH in 50% MeCN/H_2O that served to resuspend the entire reaction contents. About 2 h following quench, a small portion of the crude reaction (4.1 mCi) was removed and injected onto the HPLC.

Figure 4 shows the radioactive and corresponding UV-vis HPLC traces of the crude labeling reaction and leads to several important observations as follows: (i) there are essentially only two radiopeaks, one corresponding to free ^{18}F-fluoride ion and one corresponding to the desired Rh-^{18}F-ArBF$_3^-$; (ii) the radiochemical yield, decay corrected to the end of synthesis, appears to be 75% (63% not decay corrected), which implies the production of >500 mCi of radiotracer starting with 816 mCi although isolated radiochemical yields are slightly lower, that is, 40–50%; (iii) there is a ~50% chemical radiochemical yield as the UV trace shows a second, well-separated fluorescent product that elutes at 28.5 min and which is the arylboronic acid whose formation either competes with isotope exchange or occurs through an *in situ* re-equilibration between the arylboronic acid, the ArBF$_3^-$, and free fluoride.

Encouraged by these high radiochemical yields, we next sought to measure specific activity. To do this, we relied on the excellent absorbance properties of the rhodamine chromophore. Although peptides generally do not have chromophores that afford reliable measurement of the low concentrations characteristic of high specific activity, this rhodamine bioconjugate has a reliable UV-vis signature and a very high molar ε. In measuring and reporting the specific activity, we took into account two important considerations, (i) there was an unnecessary lag between the time the reaction was quenched and when it was injected on the HPLC that was due to instrument availability in a shared hospital facility and (ii) there was a 25-min lag between the time the sample was loaded on the HPLC and when the radiotracer eluted. To report specific activity values in a meaningful way, we

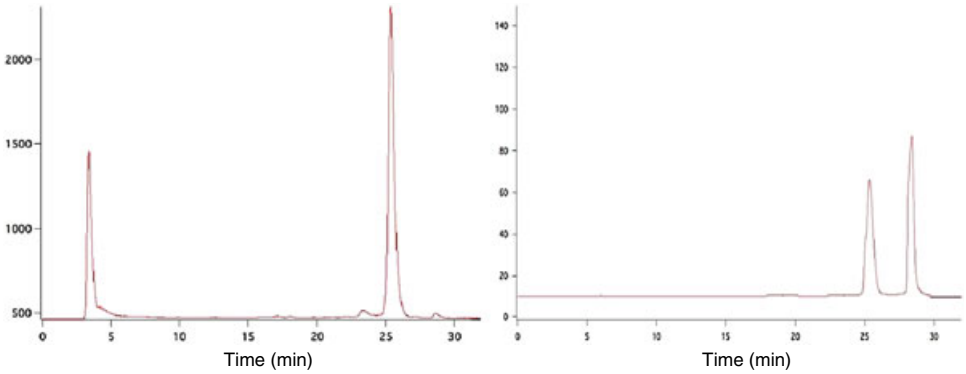

Figure 4. HPLC trace of the crude reaction; left—radiotrace where the y-axis is in mV; right—a UV-vis trace at 569 nm where the y-axis is in mAu.

corrected for the unnecessary decay between quench and HPLC injection but did not correct for the HPLC purification time that necessarily erodes specific activity. Hence, specific activities are reported at time of collection (TOC) and not back-corrected to EOS, which would otherwise reflect even higher specific activities.

For the radiosynthesis featured in Figure 4, a small portion of the crude reaction that contained 4.1 mCi was loaded on the HPLC, from which was eluted 81.4 MBq (~2.2 mCi) (53% isolated), 25–26 min later. Integration of the visible peak at 569 nm that eluted at 25.5 min provided a quantitative mass value of 330 pmol and therefore gave a specific activity of 248 GBq/μmol (6.7 Ci/μmol) at TOC. Correcting for the 2 h lag time between quenching and loading, the real specific activity at TOC was an extraordinary 529 GBq/μmol (14.3 Ci/μmol). On the basis of the assumption that only one atom of fluoride is exchanged, the calculated specific activity of 592 GBq/μmol (16 Ci/μmol, that is, 0.8 Ci/50 nmol $ArBF_3^-$), corrected to beginning of synthesis is consistent with the specific activity that was measured. Gratifyingly, this reaction was repeated using 17.9 (485 mCi) and 15.2 GBq (412 mCi) in radiochemical yields of 50% and 45%, respectively, and specific activities in the range of 111–259 GBq/μmol (3–7 Ci/μmol), corrected to TOC (see supporting information). These slightly lower specific activities are consistent with the use of commensurately lower levels of ^{18}F-activity.

Whereas separation herein gave radiochemically pure material at very high specific activity, the separation of labeled bioconjugates from other unlabeled products may not be required for imaging. For instance, although bisRGD-^{18}F-Al-NOTA could be prepared at ~6 Ci/μmol, it could not be separated from unlabeled bisRGD. Consequently, the effective specific activity was reduced to 18.5 GBq/μmol (0.5 Ci/μmol) although no time was "wasted" on HPLC separation.[15] Had we not separated labeled Rh-^{18}F-$ArBF_3^-$, the effective specific activity (defined as GBq/μmol of all tracer components), corrected to TOC, would have been 259 GBq/μmol (7 Ci/μmol), a value that is still on par with some of the highest values obtained for any radiotracer. Thus, this work shows that high effective specific activities will prevail even *sans* separation. Finally, cartridge-based separation methods may eventually obviate the need for HPLC separation; for instance, a sandwich of (i) silica to remove free fluoride ion; (ii) a diol resin to remove the boronic acid side product; and (iii) a reverse phase resin to remove hydrophobic organics, may provide rapid purification.

In order to measure specific activity, we conjugated an alkyne-^{19}F-$ArBF_3^-$ to rhodamine azide in order to provide a surrogate bioconjugate whose chromophore unequivocally afforded a measure of low concentrations. These results now suggest that this method will find use in labeling a fluorescent peptide to create bimodal imaging agents in which there is considerable interest.[33,41,45] Furthermore, because we previously labeled RGD, both by direct conversion of an RGD-boronate to an RGD-^{18}F-$ArBF_3^-$ and by preparing an alkyne-^{18}F-$ArBF_3^-$ followed by click conjugation onto an RGD azide, chemical logic suggests this method of isotope exchange is applicable to many other peptides.

Unlike previously reported $ArBF_3^-$ syntheses, which require relatively high concentrations of total fluoride ion, necessitating low volumes and/or the addition of excess ^{19}F-fluoride ion, the isotope exchange reaction herein uses no added carrier ^{19}F-fluoride ion and only very low concentrations of carrier ^{19}F-$ArBF_3^-$ (1 mM). Because isotope exchange is likely to be pseudo-first order in ^{19}F-$ArBF_3^-$ concentration, radiochemical yields are independent of the amount of activity used. This labeling has been repeated numerous times at low specific activity, and herein, three times with more than 14.8 GBq (400 mCi) of ^{18}F-activity. In each case, reaction in as little as 10–15 min provided radiochemical yields of 50% or higher.

Notably, the specific activities herein are higher than those of most other radiotracers, including clickable radiosynthons on the basis of ^{18}F-SiFA conjugates and ^{18}F-Al-NOTA chelates that have been labeled at specific activities of ≤259 GBq/μmol (≤7 Ci/μmol) prior to click.[46,47] Although we have argued that specific activities as low as 3.7 GBq/μmol (0.1 Ci/μmol) are sufficient for imaging,[48–51] it is likely that high specific activity will be crucial for *early* cancer detection where target concentration is necessarily low and for which regulatory agencies require that injected radiotracer concentration also be low ($<0.1 \times K_d$) such that <10% target is bound in order to avoid pharmacological effects.[17] Although a recent review[2] suggests that to date, there is no consensus as to the minimum specific activity needed for imaging, a value of 18.5 GBq/μmol (0.5 Ci/μmol) is a generally accepted minimum value required for imaging.[15]

As such, an interesting conclusion then arises. If this minimum threshold is not raised in the future, the ability henceforth to produce tracers at exceptionally high specific activity opens the possibility of intercontinental distribution whereby radiotracers produced at 529 GBq/μmol (14.3 Ci/μmol) can be transported 8.85 h before decaying to 0.5 Ci/μmol. We believe that this feature may prove especially significant for the industrial production, distribution, and sale of radiotracers.

Conclusion

Here, we have used click chemistry to prepare a fluorescent bioconjugate ^{19}F-ArBF$_3^-$ precursor that can be aliquoted and lyophilized for future use in kit-like fashion. For labeling, 50 nmol (52 μg) is resuspended in 50 μL aqueous DMF (pH 2 buffer) and combined with >29.6 GBq (>800 mCi) of NCA ^{18}F-fluoride ion via robotic manipulation in a shielded hot cell. After 10–15 min at 40 °C, the reaction was quenched and analyzed for radiochemical yield and specific activity. Radiochemical yields are ~50% and specific activities are as high as 529 GBq/μmol (14.3 Ci/μmol), corrected to the TOC following HPLC purification. Two other radiosyntheses each with ~14.8 GBq (~400 mCi) provided similar radiochemical yields at specific activities of approximately 111–222 GBq/μmol (3–7 Ci/μmol). In contrast to many other methods, labeling herein has several advantages as follows: (i) speed; (ii) use of aqueous conditions at mild temperatures in buffer; (iii) high radiochemical yields; (iv) high radiochemical purity; (v) extraordinarily high specific activity; (vi) the creation of a high polar anionic ^{18}F-labeled prosthetic that is readily separated by HPLC; and (vii) compatibility with both hot cell robotic manipulation and high levels of ^{18}F-activity. On the basis of successful tumor imaging with ^{18}F-ArBF$_3^-$-bioconjugates that were labeled at *much* lower specific activities, we believe this work should be applicable to imaging the same bioconjugates at specific activities ~100-fold higher than we previously reported and 3–10-fold higher than what is *normally* achieved for most other ^{18}F-labeled radiotracers reported by others. This work should empower others to label many other bioconjugate-^{19}F-ArBF$_3^-$ precursors by isotope exchange.

Acknowledgements

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Conflict of Interest

UBC has sought patent protection of various aryltrifluoroborates for use in ^{18}F-imaging.

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