Imaging Agents

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Ortho-Stabilized ^{18}F-Azido Click Agents and their Application in PET Imaging with Single-Stranded DNA Aptamers

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Abstract: *Azido ^{18}F-arenes are important and versatile building blocks for the radiolabeling of biomolecules via Huisgen cycloaddition ("click chemistry") for positron emission tomography (PET). However, routine access to such clickable agents is challenged by inefficient and/or poorly defined multistep radiochemical approaches. A high-yielding direct radiofluorination for azido ^{18}F-arenes was achieved through the development of an ortho-oxygen-stabilized iodonium derivative (OID). This OID strategy addresses an unmet need for a reliable azido ^{18}F-arene clickable agent for bioconjugation reactions. A ssDNA aptamer was radiolabeled with this agent and visualized in a xenograft mouse model of human colon cancer by PET, which demonstrates that this OID approach is a convenient and highly efficient way of labeling and tracking biomolecules.*

Biologics labeled with fluorine-18 (^{18}F, β^+, $t_{1/2} = 109.7$ min), including nucleic acids, peptides, and proteins, are extensively used for molecular imaging in positron emission tomography (PET).[1] ^{18}F incorporation in these sensitive biomolecules requires fast, high yielding, and site-specific bioconjugation methods that can be carried out under mild conditions.[2] Huisgen reactions (chemoselective and regiospecific 1,3-dipolar cycloaddition between an azide and an alkyne) are the most commonly used bioconjugation strategy.[3] The resulting 1,2,3-triazole linker is stable in vivo[4] and is considered to be an isosteric surrogate for peptide bonds.[5] Compared to the many applications of azides in non-radioactive bioconjugation, only a small number of ^{18}F-labeled azides have been developed for PET radiochemistry,[3] among which only limited examples are attractive for routine production for PET imaging. This can be attributed to lengthy multistep syntheses with short half-life ^{18}F and/or the requirement for special handling owing to the strong ionization energy. For instance, [^{18}F]fluoroethyl azide was the first aliphatic ^{18}F-azide and was prepared in high yield (55%, decay corrected) through a nucleophilic displacement reaction with [^{18}F]fluoride.[6] A disadvantage of this type of low-molecular-weight agent is its high volatility and the need for intermediate purification by distillation, which is challenging to carry out in radiochemical laboratories.[6,7] To the best of our knowledge, 4-[^{18}F]fluorobenzyl azide is the only azido ^{18}F-arene used in PET imaging studies.[8] However, the preparation of 4-[^{18}F]fluorobenzyl azide involves laborious multistep processes[8b,d] or the utilization of a specialized flow device.[8e] We have recently reported a manual one-step synthesis of [^{18}F]fluorobenzyl azide with high radiochemical yield and specific activity,[9] but the subsequent problems associated with high volatility made this agent unsuitable for widespread use. Since ^{18}F-arenes are generally stable in vivo (high resistance to deradiofluorination)[2a,8e,10] and possess a strong chromophore (UV-detectable), there is an unmet need for a convenient and highly efficient radiofluorination method to provide azido ^{18}F-arene click agents for bioconjugation through robust click chemistry.

There is a new emerging class of targeting vector for molecular imaging, namely aptamers or single stranded oligonucleotides, which show highly specific binding to protein targets with efficient tissue penetration and rapid blood clearance.[11] Aptamers are chemically assembled and can be easily modified to improve stability and pharmacokinetic profiles without notable immunogenicity.[11b,c] There are ongoing efforts to explore the feasibility of labeled aptamers for in vivo imaging.[12] The single-stranded DNA aptamer sgc8 was identified as specifically targeting protein tyrosine kinase 7 (PTK-7),[12b] which is over-expressed in several human malignancies.[13] However, the mapping of PTK-7 in various tumors has not been realized in clinical use, mainly owing to the lack of a method to noninvasively probe this target in vivo. A reliable and efficient method to radiolabel an aptamer targeting PTK-7 would enable us to utilize PET imaging to further our understanding of this kinase in cancer biology and could lead to the development targeted therapies through precision medicine.

With the goal to develop a highly efficient radiolabeling method for azido ^{18}F-arene click agents and a robust platform for aptamer radiolabeling with application to PTK-7, we designed an unusual precursor scaffold based on iodine(III) chemistry (Scheme 1). On the basis of early discoveries of an

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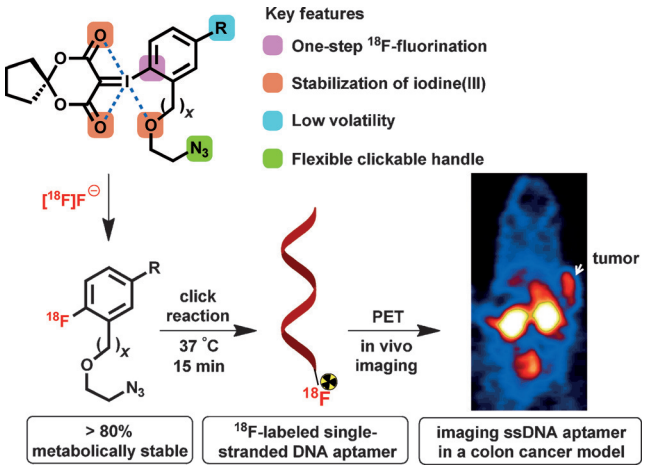
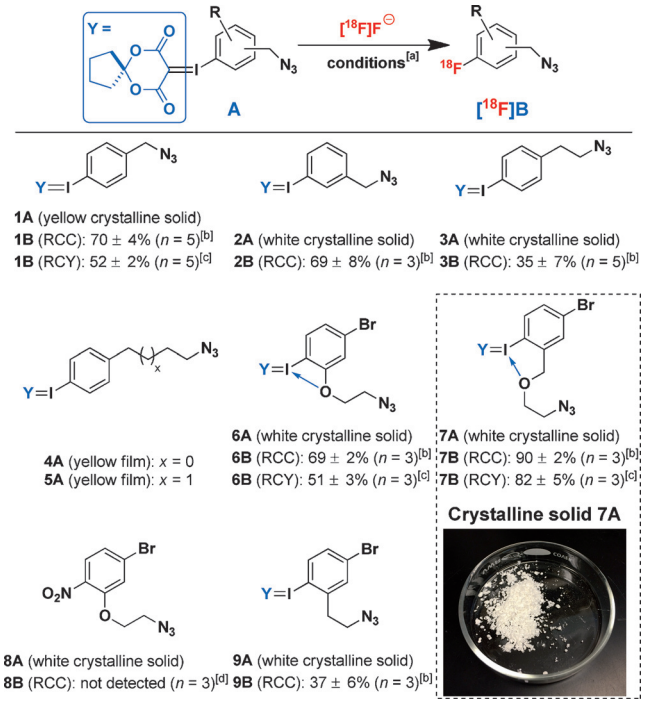


Scheme 1. A new ^{18}F-azido agent for PET imaging of a ssDNA aptamer in a xenograft tumor model.



Scheme 2. Synthesis and radiolabeling of different iodonium(III) precursors. a) Conditions: precursor (4 mg), Et_4NHCO_3 (2 mg), DMF (0.4 mL), [^{18}F]F$^-$ (1–3 mCi), 120 °C. b) RCC = radiochemical conversion. c) RCY = radiochemical yield. d) No desired product was found.

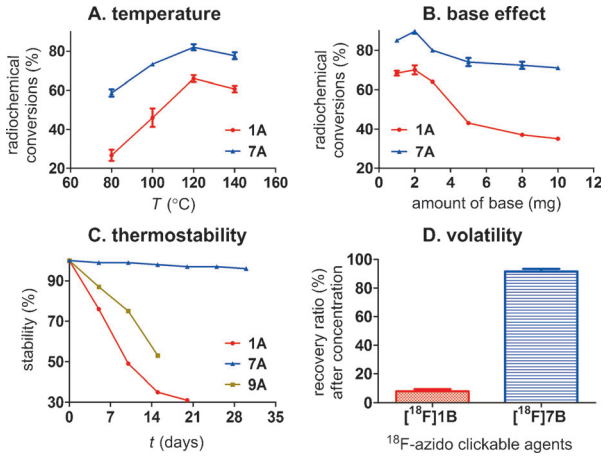


Figure 1. Characterization and comparison of labeling precursors and ^{18}F-azido clickable agents. A) Precursor (2 mg), Et_4NHCO_3 (1 mg), DMF (0.4 mL), [^{18}F]fluoride (1–3 mCi). B) Precursor (4 mg), DMF (0.4 mL),[^{18}F]fluoride (1–3 mCi), 120 °C. C) Precursors (2 mg) were stored at ambient temperature. D) [^{18}F]1B or [^{18}F]7B (3–5 mCi) in CH_3CN (1 mL) was concentrated under N_2. DMF = *N*,*N*-dimethylformamide.

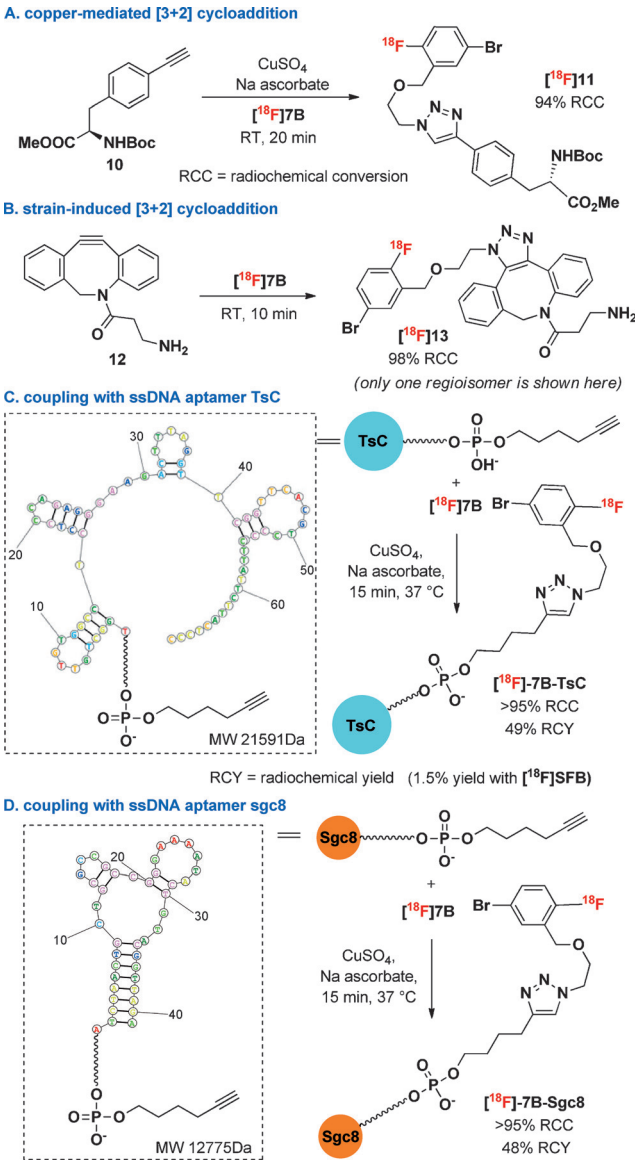
ortho effect on iodonium species[9,14] and a secondary bonding interaction observed in hypervalent iodine compounds,[15] we speculated that *ortho*-oxygen-coordinated iodonium derivative (OID) would provide stabilization for iodine(III) to yield thermally stable and highly reactive labeling precursors. We synthesized a series of labeling precursors featuring different lengths and positions of the aliphatic linkers (**1**–**5**) and *ortho* coordinating substituents (**6** and **7**; Scheme 2). The synthesis was highly efficient from commercially available starting materials (Scheme S1–S4 in the Supporting Information). While most of the precursor molecules were crystalline, precursors with long alkyl chains (**4** and **5**) led to the formation of semisolids or oils (Figure S1 in Supporting Information), which are not ideal for handling in PET radiochemistry and were thus not further pursued. With the aim to decrease ^{18}F-product volatility (see below) and generate a readily reactive crystalline precursor, we studied the combined effect of a *para* bromine atom and an *ortho* oxygen-containing group in precursors **6** and **7**, and we discovered that this combination increases not only the radiochemical yield but also the thermostability of the precursors. In the ^{18}F-labeling experiments, benzyl azides **1A**–**3A** and **9A** provided 24–70% ^{18}F-incorporation yields, while OIDs **6A** and **7A** afforded 69% and 90% conversions with excellent product yields of 51% and 82%, respectively. This is the first time that the *ortho* effect in hypervalent iodine(III) chemistry has been utilized in the design of ^{18}F-labeled agents. The OIDs **6A** and **7A** also proved to be highly efficient in ^{18}F-labeling compared to the traditional nucleophilic aromatic substitution (S_NAr) approach. A control experiment was carried out to test labeling efficiency from the nitro precursor (Scheme 2, compound **8**) via the S_NAr pathway, but this approach did not afford any desired product and gave 35% conversion to other unidentified byproducts. This was also supported by a literature report of limited conversion (< 0.5%) from 1-bromo-4-nitrobenzene.[16]

In comparison to the labeling precursor **1A** for [^{18}F]fluorobenzyl azide, OID **7A** showed superior radiochemical conversions in terms of reaction temperature (Figure 1A) and base loading (Figure 1B), as well as reaction time and amount of precursor loading (Figure S2 in the Supporting Information). For bioorthogonal reactions that are routinely carried out in aqueous media at pH 4 or 10, the resulting product [^{18}F]**7B** showed superior stability in alkaline or acidic solutions (Figure S3 in the Supporting Information). The in vitro stability of [^{18}F]**7B** in mouse serum was assessed by HPLC and no degradation was observed after incubation

at 37 °C for 2 h. OID **7A** also demonstrated extraordinary thermostability, a critical and practical consideration for the routine production of radiotracers for PET, while compound **1A** only showed a shelf half-life of 10 days (Figure 1C; and Figure S4 in the Supporting Information), which was partially attributed to secondary bonding at the iodine(III) center.[15] We also synthesized an *ortho*-substituted carbon analogue **9A** (see Scheme 2) and this compound showed inferior radiochemical yield (37%) and thermostability (shelf $t_{1/2} = 15$ days) compared to its oxygenated counterpart **7A**, thus suggesting that there may be a stabilizing interaction between the *ortho* oxygen and the iodine. Since the biological agents to be labeled (e.g., aptamers and nucleic acids) are usually available in small quantities (low μg), ^{18}F-bioconjugations of these materials are usually performed in a small volume (μL scale) to provide fast reaction kinetics. One of major limitation for 4-[^{18}F]fluorobenzyl azide is associated with its high volatility, which means that it is difficult to recover in microliter level after concentration, whereas the new azide [^{18}F]**7B** exhibited up to 92% recovery and is suitable for further coupling reactions (Figure 1D). The radiosynthesis of [^{18}F]**7B** was also automated on a production scale by using a commercial radiosynthesis module, which produced approximately 400 mCi of [^{18}F]**7B** in greater than 50% non-decay-corrected radiochemical yield, with more than 95% (radio)chemical purity and greater than 2 Ci μmol^{-1} specific activity (Figure S5 in the Supporting Information). Among all the parameters evaluated, the non-volatile clickable agent [^{18}F]**7B**, which was prepared from thermally stable OID **7A** in high yield and with outstanding stability under either basic or acidic conditions, revealed **7** to be a promising scaffold for bioorthogonal reactions and this scaffold was thus transitioned for further evaluation in click chemistry and in vivo PET imaging studies.

A variety of terminal alkyne-containing small or biological molecules was evaluated in the coupling reaction with [^{18}F]**7B** under biocompatible conditions (Scheme 3). The new azide showed excellent radiochemical conversions in click reactions with a phenylalanine derivative (94%) and strained-induced DBCO cycloaddition (98%). We have previously labeled a ssDNA TsC aptamer with *N*-succinimidyl 4-[^{18}F]fluorobenzoate ([^{18}F]SFB) through peptide bond formation but obtained the corresponding ^{18}F-labeled TsC aptamer in only 1.5% yield.[12g] Significantly increased bioconjugation yield (>95%, radiochemical conversion; 48% yield of isolated product) was obtained with [^{18}F]**7B** and the alkyne-linked TsC aptamer, thereby demonstrating an up to 30-fold improvement in site-specific coupling efficiency. Encouraged by these results, we also radiolabeled sgc8 with [^{18}F]**7B**, which proceeded with 49% radiochemical yield, for further in vivo evaluation.

PTK-7 is an excellent template protein to illustrate the use of the novel ^{18}F-click agent, especially since this would be the first in vivo PET imaging study of this important target in a disease model. The affinity of the labeled aptamer [^{18}F]**7B**-sgc8 towards PTK-7 was determined by receptor saturation binding assay, which showed a single digital nanomolar K_d value of 1.1 nM. The uptake of [^{18}F]**7B**-sgc8 was rapid and was blocked by the addition of excess unlabeled aptamer, thus



Scheme 3. Application of the ^{18}F-azide agent [^{18}F]**7B** in copper-mediated (A) and strain-induced (B) click reactions, as well as copper-mediated bioconjugation reactions with the ssDNA aptamers TsC (C) and sgc8 (D).

demonstrating high specificity for PTK-7 (Figure 2A). [^{18}F]**7B**-sgc8 binding to PTK-7 was not due to internalization and washed away over time, thus suggesting that PET imaging should be completed within few hours following injection of the radiotracer. To map tumoral PTK-7 expression, we selected a colon cell line because this protein is expressed in colon carcinomas but not in normal colon tissue and thus may be utilized as a biomarker for tumor progression.[13a,b] Flow cytometry analysis of the colorectal carcinoma cell line HCT116 showed positive and high PTK-7 expression (Figure 2B). In vivo studies were performed by administering [^{18}F]**7B**-sgc8 to female nude mice with HCT116 tumors followed by PET imaging (Figure 2C). [^{18}F]**7B**-sgc8 clearly visualized PTK-7-positive tumors at all time points, with tumoral uptake of 0.71 ± 0.07% ID/g and a reasonable tumor-

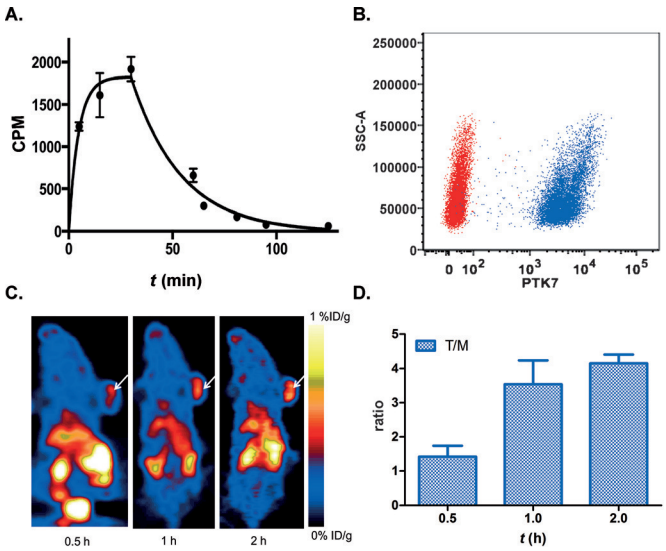


Figure 2. A) Cellular uptake, internalization, and efflux assays of [^{18}F]**7B**-sgc8 in HCT116 cells. B) PTK-7 expression evaluated by flow cytometry: red = isotype control antibody staining, blue = specific antibody staining of PTK-7. SSC-A = side scatter area. C) Representative coronal PET images of a mouse bearing a HCT116 xenograft (white arrow) over time. D) Tumor-to-muscle (T/M) ratio of [^{18}F]**7B**-sgc8.

to-muscle ratio of 3.54 ± 0.7 and 4.15 ± 0.26 at 1 h and 2 h post-injection, respectively (Figure 2D). Typically, the relatively modest tumoral uptake of aptamers relates to their rapid clearance from the body,[12g] and [^{18}F]**7B**-sgc8 was subject to rapid urinary elimination and to some extent hepatic clearance, with negligible defluorination in vivo (Table S1 in the Supporting Information).

In summary, we have developed the first-in-kind, thermally-stable and highly reactive OIDs that can be used to provide ^{18}F-azido click agents in a high-yield one-step [^{18}F]fluorination. The click agent [^{18}F]**7B** demonstrated low volatility and excellent stability, and it is highly efficient in bioconjugation via Huisgen [3+2] cycloaddition. The resulting ^{18}F-tagged ssDNA aptamer sgc8 provides the first in vivo imaging tool to visualize and track PTK-7-expressing cancers by PET. We expect this OID-based strategy to be widely utilized in the ^{18}F-labeling of biologics for tumor-targeted PET imaging.

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