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Ortho-stabilized ^{18}F -azido click agents and application in PET imaging of single-stranded DNA aptamer

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Abstract

Azido ^{18}F -arenes are important and versatile building blocks for radiolabeling of biomolecules via Huisgen cycloaddition ('click chemistry') in positron emission tomography (PET). However, routine access of such clickable agents is challenged by inefficient multi-step and esoteric radiochemical approaches. Herein we describe a high-yielding direct radiofluorination for azido ^{18}F -arenes by developing an oxygen ortho-stabilized iodonium derivative (OID). This OID strategy addresses an unmet need for a reliable azido ^{18}F -arene clickable agent in bioconjugation reactions. A ssDNA aptamer is radiolabeled and visualized in a xenograft mouse model of human colon cancer by PET, which demonstrates a convenient and highly efficient way of labeling biomolecules and tracking them by OID approach.

Keywords

Bioconjugation; fluorine-18; PET; protein tyrosine kinase 7; aptamer

Fluorine-18 (^{18}F , β^+ , $t_{1/2} = 109.7$ min) labeled biologics, 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 necessitates the need of fast, high yielding and site-specific bioconjugation methods under mild conditions.^[2] Huisgen reactions (1,3-dipolar cycloaddition between azide and alkyne in a chemoselective and regiospecific manner) are the most routinely-used bioconjugation strategy.^[3] The resulting 1,2,3-triazole linker is stable in vivo^[4], and considered to be an isosteric surrogate for

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peptide bonds.^[5] Compared to many applications of azides in non-radioactive bioconjugation, only a small number of ¹⁸F-labeled azides have been developed for PET radiochemistry,^[3] among which only limited examples are attractive for routine production for PET imaging, and is likely attributed to lengthy multi-step syntheses with short half-life ¹⁸F and/or requirement of special handling due to strong ionization energy. For instance, [¹⁸F]fluoroethyl azide was the first aliphatic ¹⁸F-azide, prepared in high yield (55%, decay corrected) *via* a nucleophilic displacement reaction with [¹⁸F]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-[¹⁸F]fluorobenzyl azide is the only azido ¹⁸F-arene used in PET imaging studies.^[8] However, the preparation of 4-[¹⁸F]fluorobenzyl azide involves laborious multi-step processes^[8b, 8d] or utilization of a specialized flow device.^[8e] We have recently reported a manual one-step synthesis of [¹⁸F]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. As ¹⁸F-arenes are generally stable *in vivo* (high resistance to de-radiofluorination),^[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 ¹⁸F-arene click agent for bioconjugation via robust click chemistry.

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

With the goal to develop a highly efficient radiolabeling method for azido ¹⁸F-arene click agent and a robust platform for aptamer radiolabeling with application in PTK-7, we designed an unusual scaffold of precursors based on iodine(III) chemistry (Scheme 1). On the basis of early discoveries of ortho effect on iodonium species^[9, 14] and a secondary bonding interaction observed in hypervalent iodine chemistry,^[15] we speculated that oxygen *ortho*-coordinating iodonium derivative (OID) would provide stabilization of iodine(III) to yield thermally stable and highly reactive labeling precursors. We synthesized a series of labeling precursors which feature different lengths and positions of aliphatic linkers **1–5** and *ortho*-coordinating substituents **6** and **7** (Scheme 2). The synthesis was highly efficient from commercially available starting materials (Schemes S1–S4 in supporting information). While most of the precursor molecules were crystalline, precursors with long alkyl chains **4** and **5** led to the formation of semi-solids or oils (Figure S1 in supporting information),

which are not ideal for handling in PET radiochemistry and thus not further pursued. With the aim to decrease ^{18}F -product volatility (vide infra) and generate a readily reactive crystalline precursor, we studied the combined effect of a *para*-bromine atom and oxygenated *ortho* substitution in precursors **6** and **7**, and discovered that this combination increased not only radiochemical yield but also 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 isolated yields of 51% and 82%, respectively. This is the first time that *ortho*-effect in hypervalent iodine (III) chemistry was utilized to design ^{18}F -labeled agents. The OIDs **6A** and **7A** also proved to be highly efficient in ^{18}F -labeling compared with traditional nucleophilic aromatic substitution ($\text{S}_{\text{N}}\text{Ar}$) approach. A control experiment was carried out to test labeling efficiency from nitro precursor (Scheme 2, compound **8**) via $\text{S}_{\text{N}}\text{Ar}$ pathway, but did not afford any desired product albeit to 35% conversions to other unidentified by-products. This was also supported by a literature report of limited conversion ($< 0.5\%$) from 1-bromo-4-nitrobenzene.^[16]

In comparison to labeling precursor **1A** for [^{18}F]fluorobenzyl azide, OID **7A** showed superior radiochemical conversions in terms of reaction temperature (Figure 1A), base loading (Figure 1B) as well as reaction time and amount of precursor loading (Figure S2 in supporting information). For bioorthogonal reactions that are routinely carried out in aqueous media under pH = 4 or 10, the resulting product [^{18}F]**7B** showed superior stability in alkaline or acidic solutions (Figure S3 in 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 PET routine production, while compound **1A** only showed shelf half-life of 10 day (Figure 1C; and Figure S4 in supporting information), which was partially attributed to secondary bonding at iodine(III) center.^[15] We also synthesized an *ortho*-substituted carbon analog **9A** (c.f. Scheme 2) and found this compound showed inferior radiochemical yield (37%) and thermostability (shelf $t_{1/2}$ = 15 day) to its oxygenated counterpart **7A**, suggesting there may be a stabilizing interaction between *ortho* oxygen and iodine. Since the biological agents to be labeled (e.g., aptamers or nucleic acids etc) 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 and thus 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 using a commercial radiosynthesis module and produced ca. 400 mCi of [^{18}F]**7B** in greater than 50% non-decay corrected radiochemical yield with $>95\%$ (radio)chemical purity, and greater than 2 $\text{Ci}\cdot\mu\text{mol}^{-1}$ specific activities (Figure S5 in supporting information). Among all the parameters evaluated, non-volatile clickable agent [^{18}F]**7B**, prepared from thermally stable OID **7A** in high yield, with outstanding stability in either basic or acidic conditions, revealed **7** as a promising scaffold for bioorthogonal reactions and 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 chemistry with 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) via 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% isolated yield) was obtained between [^{18}F]7B and alkyne-linked TsC aptamer, and demonstrated up to 30-fold improvement in coupling efficiency in a site-specific manner. Encouraged by these results, we also radiolabeled [^{18}F]-7B-sgc8 with 49% isolated radiochemical yield for further in vivo evaluation.

PTK-7 represents an excellent template protein to illustrate the use of the novel ^{18}F -click agent, in addition to the first in vivo PET imaging study of this important target in disease model. Affinity of aptamer **18F-7B-sgc8** towards PTK-7 was determined by receptor saturation binding assay, showing a single digital nanomolar K_d value of 1.1 nM. The uptake of **18F-7B-sgc8** was rapid and blocked by addition of excess of unlabeled aptamer, demonstrating high specificity to PTK-7 (Figure 2A). **18F-7B-sgc8** binding to PTK-7 was not due to internalization and washed away over time, suggesting that PET imaging should be completed within few hours post injection of the radiotracer. To map tumoral PTK-7 expression, we selected a colon cell line because this protein was expressed in colon carcinomas but not in normal colon tissue and thus may be utilized as a biomarker for tumor progression.^[13a, 13b] 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 administration of **18F-7B-sgc8** to female nude mice with HCT116 tumors followed by PET imaging (Figure 2C). **18F-7B-sgc8** clearly visualized PTK-7 positive tumors at all time points, with tumor uptake of 0.71 ± 0.07 %ID/g and reasonable tumor-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 relative modest tumoral uptake of aptamers relates to their rapid clearance from the body,^[12g] as **18F-7B-sgc8** had a rapid urinary elimination and to some extent an hepatic clearance with negligible defluorination in vivo (Table S1 in supporting information).

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Characterization and comparison of labeling precursors and ¹⁸F-azido clickable agents. (A) Precursor (2 mg), Et₄NHCO₃ (1 mg), DMF (0.4 mL), [¹⁸F]fluoride (1–3 mCi); (B) Precursor (4 mg), DMF (0.4 mL), [¹⁸F]fluoride (1–3 mCi), 120 °C; (C) Precursors (2 mg) were stored at ambient temperature; (D) [¹⁸F]1B or [¹⁸F]7B (3–5 mCi) in CH₃CN (1 mL) was concentrated under N₂.

Figure 2.

(A) Cell uptake, internalization, and efflux assays of ^{18}F -7B-sgc8 in HCT116 cells; (B) PTK-7 expression evaluated by flow cytometry: control antibody staining (red), and specific antibody PTK-7 staining (blue); (C) Representative coronal PET images of mouse bearing HCT116 xenograft (white arrow) over time; (D) Tumor-to-muscle (T/M) ratio of ^{18}F -7B-sgc8.

Scheme 1.

New ^{18}F -azido agent and PET imaging of ssDNA aptamer in a xenograft tumor model.

Scheme 2.

Synthesis and radiolabeling of different iodonium(III) precursors. *a.* conditions: Precursor (4 mg), Et₄NHCO₃ (2 mg), DMF (0.4 mL), [¹⁸F]F⁻ (1–3 mCi), 120 °C; *b.* RCC = radiochemical conversion; *c.* RCY = radiochemical yield; *d.* No desired product was found.

A. copper mediated [3+2] cycloaddition **B. strain induced [3+2] cycloaddition** **C. coupling with ssDNA aptamer TsC** **D. coupling with ssDNA aptamer sgc8** **Scheme 3.**

Application of ^{18}F -azide agent $[^{18}\text{F}]\text{7B}$ in copper-mediated and strain-induced click reactions (A, B) and copper-mediated bioconjugation reactions with ssDNA aptamer TsC and sgc8 (C, D).