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Stoichiometry-focused ^{18}F-labeling of alkyne-substituted oligodeoxynucleotides using azido([^{18}F]fluoromethyl)benzenes by Cu-catalyzed Huisgen reaction

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ABSTRACT

A novel method for ^{18}F-radiolabeling of oligodeoxynucleotides (ODNs) by a Cu-catalyzed Huisgen reaction has been developed by using the lowest possible amount of the precursor biomolecule for the realization of stoichiometry-oriented PET (positron emission tomography) chemistry. Under the optimized cyclization conditions of *p*- or *m*-azido([^{18}F]fluoromethyl)benzene and alkyne-substituted ODN (20 nmol) at 40 °C for 15 min in the presence of $CuSO_4$, TBTA [tris((1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl)amine], and sodium ascorbate (2:1:2), the synthesis of ^{18}F-labeled ODNs with sufficiently high radioactivities of 2.1–2.5 GBq and specific radioactivities of 1800–2400 GBq/μmol have been accomplished for use in animal and human PET studies.

1. Introduction

Positron emission tomography (PET) is a powerful noninvasive molecular imaging technique for the in vivo investigation of the distribution and dynamics of bioactive molecules labeled with positron-emitting radionuclides in the brain, heart, and other organs. PET has been extensively used for clinical diagnoses and drug development.[1] Positron-emitting radionuclides such as ^{18}F, ^{68}Ga, and ^{64}Cu are often utilized for radiolabeling of biomolecules such as peptides, oligonucleotides, and antibodies. Recently, ^{68}Ga and ^{64}Cu have gained importance because of their ability to form chelate complexes with 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) which is conjugated to target biomolecules.[2a] On the other hand, many practical ^{18}F-labeling methods that involve the use of sophisticated ^{18}F-labeling agents, the so-called ^{18}F-prosthetic groups, have been reported.[2b] ^{18}F radionuclides have a moderate half-life of 109.8 min, which make them suitable for use in pharmacokinetic studies, and relatively high specific radioactivity; further, they form chemically stable covalent bonds at the labeling position. For these reasons, ^{18}F radionuclide is widely used in most PET institutes and clinics.

However, in the research field of ^{18}F-labeling, the excessive use of a precursor biomolecule (ca. 30–5000 nmol)[3] for enhancing the labeling reaction rate remains an outstanding problem, compared with ^{68}Ga or ^{64}Cu-chelation labeling accomplished by the use of several nmol of a biomolecule, because the available quantities of biomolecules are often very tiny due to the expensive cost and time-consuming process for the synthesis. This problem has limited the usefulness and expansion of biomolecular ^{18}F-labeling. Hence, we focused on the realization of more efficient ^{18}F-labeling with the use of the lowest possible amount, that is, ca. 20 nmol, of our original oligodeoxynucleotide (ODN). This figure is related to the level of ca. 25 nmol of an ^{18}F-prosthetic group, which can be quantitatively converted from an [^{18}F]fluoride ion whose radioactivity and specific radioactivity are 50 GBq and 2000 GBq/μmol, respectively.[4] In this paper, we describe a novel and effective method for ^{18}F-labeling of our original alkyne-substituted ODNs[5] with the aim of achieving stoichiometric PET chemistry.

Abbreviations: PET, positron emission tomography; ODN, oligodeoxynucleotide; SPE, solid phase extraction; TBTA, tris((1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl)-amine; DCY, decay-corrected radiochemical yield; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; TEAA, triethylammonium acetate.

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2. Results and discussion

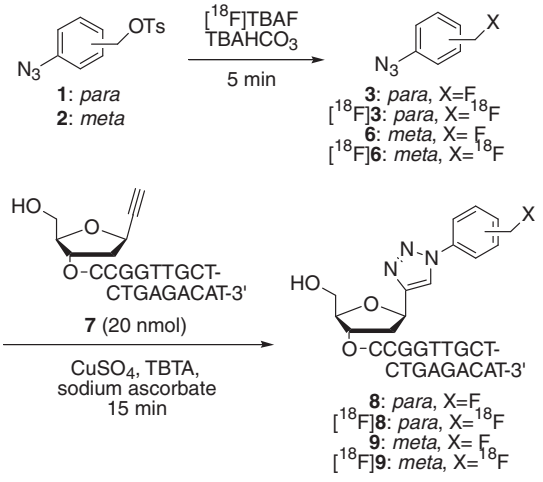
A Cu-catalyzed Huisgen reaction, so-called 'click chemistry',[6] which connects two chemically stable moieties of azide and alkyne with high chemoselectivity in mild biocompatible conditions, is frequently used in bioconjugate chemistry. Actually, many groups have applied this reaction to the ^{18}F-labeling of biomolecules such as ODNs,[7] oligopeptides, azidothymidine, and sugar analogs, etc.[8] According to the previous reports, more than 300 nmol of precursor biomolecules have generally been used. In order to decrease the substrate amount toward stoichiometric PET chemistry, two main problems must be solved. One is that of obtaining a highly pure ^{18}F-prosthetic group because the purity is a crucial factor for achieving 'PET stoichiometric reaction'. Solid phase extraction (SPE) and distillation are usually preferred as the methods of purification in a PET radiolabeling system because they are fast and convenient. However, we could not completely remove large excesses of unreacted precursor, remaining reagents, and byproducts using these methods. Consequently, we opted to use HPLC purification to overcome this problem. The second is that a Cu-catalyzed Huisgen reaction must be further accelerated because previous conditions cannot satisfy our demands for two substrates with amounts as low as ~20 nmol being coupled in a short time. These problems prompted us to explore more effective conditions that would be applicable for PET tracer synthesis.

2.1. For discussion of the synthetic efficiency of ^{18}F-labeling

Before a study, we here briefly mention the synthetic efficiency of our ^{18}F-labeling method by two parameters. One is HPLC analytical yield showing the reaction efficiency of ^{18}F-labeling, which is calculated by the peak area ratio of the desired [^{18}F]fluorinated product by radio-HPLC analysis of the reaction mixture. Another decay-corrected radiochemical yield (DCY) shows the production efficiency of the ^{18}F-labeled compound based on decay-corrected radioactivity of isolated [^{18}F]fluorinated product. DCY is usually lower than HPLC analytical yield mainly because DCY involves the radioactivity loss owing to the absorption of ^{18}F in glass vessels[9] and mechanical losses[10] in PET tracer synthesizer.

2.2. Design and synthesis of ^{18}F-prosthetic group [^{18}F]3

Initially, we designed 1-azido-4-([^{18}F]fluoromethyl)benzene ([^{18}F]**3**) as a novel ^{18}F-prosthetic group. [^{18}F]**3** has several advantages: (1) one-step conversion from tetrabutylammonium [^{18}F]fluoride, (2) good UV absorbance providing low detection limit using conventional HPLC UV detectors, (3) low volatility and small molecular size as a labeling unit, and (4) design in consideration of the use of our Pd(0)-mediated rapid C-[^{18}F]fluoromethylation.[11] By remote control of the entire radiolabeling process, tetrabutylammonium [^{18}F]fluoride, which was converted from 50 GBq of [^{18}F]fluoride ion with tetrabutylammonium hydrogen carbonate,[12] reacted with large excesses of tosylate **1** to produce [^{18}F]**3** in 97% HPLC analytical yield (Scheme 1). Sequential HPLC isolation of [^{18}F]**3** from the reaction mixture resulted in [^{18}F]**3** with ~95% chemical purity (HPLC analysis by UV 254 nm) (Fig. 1). The operation time of the reaction and purification process was approximately 40 min. In the repeated test syntheses using a relatively small radioactivity of 3.0 GBq of [^{18}F]fluoride ion, HPLC analytical yield and the DCY of isolated [^{18}F]**3** based on [^{18}F]fluoride ion were 97% and 30 ± 13% (n = 3), respectively.



Scheme 1. Synthesis of ^{18}F-labeled ODNs, [^{18}F]**8** and [^{18}F]**9**.

2.3. Optimization of Cu-catalyzed Huisgen reaction toward stoichiometric PET chemistry

We then concentrated on the acceleration of the Cu-catalyzed Huisgen reaction in order to match time-limited ^{18}F-labeling. In our earlier study,[5] we found that the combination of $CuSO_4$, TBTA [tris((1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl)amine],[13] and sodium ascorbate as a catalyst system effectively reacted alkyne-substituted ODNs with various azides. In light of the development of this research, we pursued further optimization of this catalyst system with unlabeled **3** and *N*-propargylbenzamide **4** as a model

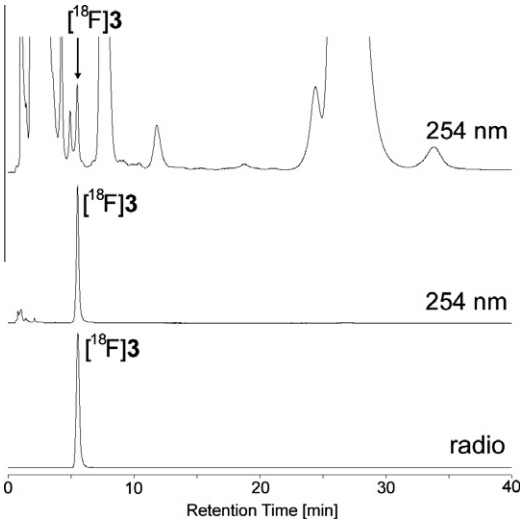
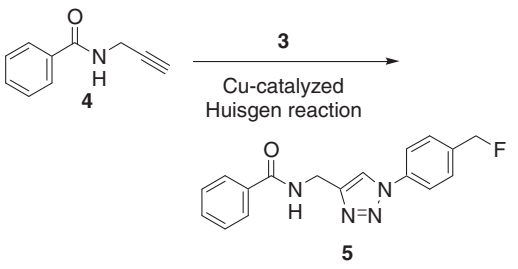


Figure 1. Top: HPLC chromatogram of the reaction mixture in [^{18}F] fluorination with **1** by UV absorbance (254 nm). Middle and bottom: HPLC chromatograms of purified [^{18}F]**3** by UV absorbance (254 nm) and γ-ray detector. Retention time of [^{18}F]**3**: 5.5 min.



Scheme 2. Cu-catalyzed Huisgen reaction using model compounds azide **3** and alkyne **4**.

Table 1
Solvent effects observed in Cu-catalyzed Huisgen reaction[a]

Entry	Organic solvent	Yield of **5**[b] (%)
1	CH_3CN	1
2	DMF	82
3	Dioxane	94
4	*t*-BuOH	95
5	DMSO	97
6[c]	DMSO	8

[a] Reaction of **3** (100 nmol) and **4** (400 nmol) was conducted in the presence of $CuSO_4$ (100 nmol), TBTA (100 nmol), and sodium ascorbate (200 nmol) in 100 μL of a 70:30 mixture of organic solvent and H_2O for 5 min at rt.
[b] Yield was determined by HPLC analysis.
[c] Reaction was conducted in the absence of TBTA.

substrate so that it can be used for PET reactions under highly diluted conditions (Scheme 2).

We first investigated the solvent effect by reacting millimolar concentrations of **3** and **4** in a mixture of an organic solvent and H_2O (70:30) for a fixed reaction time of 5 min at room temperature. As shown in Table 1, CH_3CN was not effective as an organic solvent (entry 1), whereas the desired product **5** was obtained in excellent yields when using DMSO, dioxane, and *t*-BuOH (entries 2–5). However, dioxane was known to be carcinogenic, and the solubility of TBTA in *t*-BuOH was relatively low. Therefore, we chose DMSO as the best organic solvent for our subsequent reactions. Table 2 summarizes the effect of the ratio of the additives, reaction temperature, and the mixed solvent of DMSO-buffer on the yield when low concentrations of **3** (50 μM) and **4** (100 μM) were used. The dilute reaction conditions mentioned here are similar to those adopted in PET radiolabeling experiments. The yield obtained when using a 2:1 mixture of $CuSO_4$ and TBTA in a mixture of DMSO and pH 7.0 sodium phosphate buffer (20:80) was better than that obtained when using a 1:1 mixture of $CuSO_4$ and TBTA in the same solvent system (entries 1 and 2). Decreasing the amount of Cu catalyst resulted in lower yields (entries 3–5). Interestingly, with a slight increase of the temperature to 40 °C, the reaction smoothly proceeded, giving the desired **5** in excellent yields of 87–94% (entries 6 and 7). After extensive experiments, we found that the reaction was carried out effectively when a 2:1:2 mixture of $CuSO_4$, TBTA, and sodium ascorbate was used in a 30:70 mixture of DMSO and buffer (entries 8 and 9). In particular, when the reaction temperature was 40 °C, the yield of **5** was the highest, that is, 96% (entry 9). Even with a 10-fold decrease in the amounts of **3** and **4** (the concentrations of **3** and **4**: 5 μM and 10 μM, respectively), the reaction proceeded effectively to afford **5**, although in a relatively low yield (61%; entry 10). It was encouraging that the substrates underwent click cyclization even when their concentration was lower than that in the conventional ^{18}F-labeling

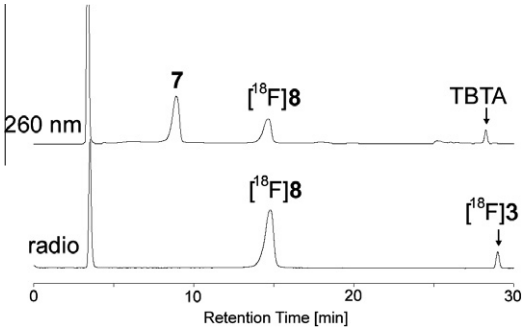


Figure 2. Preparative HPLC profile of the reaction mixture in Huisgen type ^{18}F-labeling with [^{18}F]**3** and **7**.[14] Top: UV chromatogram analyzed at 260 nm. Bottom: radioactivity chromatogram obtained with a γ-ray detector. Retention time: **7**, 8.9 min; [^{18}F]**8**, 14.8 min; [^{18}F]**3**, 29.0 min. A portion of [^{18}F]**8** was contained in the first peak of DMSO with the retention time of 3.5 min without affinity for HPLC column.

reactions. It was also found in our experiments that the product yield decreased slightly when the pH of sodium phosphate buffer was 6.5 or 7.5. As optimized in Table 2, when using the pH 7.0 buffer, the reaction efficiency was well satisfying, and the ODNs in the solution were stabilized.

2.4. ^{18}F-labeling of ODN under optimized conditions

Thus, we concluded that such optimized conditions (entry 9 in Table 2) would have high potential for conducting the stoichiometry-focused Huisgen reaction for the ^{18}F-labeling of our target ODN (Scheme 1). In the actual PET radiolabeling, 20 nmol of ethynyl ODN **7**[14] (0.50 mM aqueous solution, 40 μL) was mixed with a solution of [^{18}F]**3** in DMSO–H_2O (180–290 μL). Then, a mixture of TBTA (50 mM in DMSO, 6 μL, 300 nmol), $CuSO_4$ (50 mM in H_2O, 12 μL, 600 nmol), sodium ascorbate (50 mM, 12 μL, 600 nmol), and sodium phosphate buffer (pH 7.0, 100 mM, 60 μL) was added. The mixture with a total volume of 600 μL was heated for 15 min at 40 °C. The reaction mixture was directly analyzed by radio-HPLC and the analytical yield of [^{18}F]**8** was calculated to be up to 92% (typically ~60%).[15] Subsequent HPLC purification of the reaction mixture (Fig. 2) gave the desired ^{18}F-labeled ODN [^{18}F]**8** with satisfactory radioactivity (ca. 2.5 GBq) and fairly high specific radioactivity (2400 GBq/μmol). The entire synthesizing time, including the preparation of [^{18}F]**3** with [^{18}F]fluoride ion, was 84 min and the DCY was up to 8.6%,[12,15] calculated on the basis of 50 GBq [^{18}F]fluoride ion in a single cyclotron bombardment. The DCY of the desired [^{18}F]**8** based on the ^{18}F-prosthetic group [^{18}F]**3** was ca. 30%. The chemical and radiochemical purities of [^{18}F]**8** were 95% and 87%, respectively. [^{18}F]**8** was identified by HPLC analysis using the unlabeled authentic compound **8** as the reference.[16]

Table 2
Optimizations of Cu-catalyzed Huisgen reaction[a]

Entry	$CuSO_4$, *M* (μM)	TBTA, *M* (μM)	Sodium ascorbate, *M* (μM)	*T*	Solvent DMSO:buffer	Yield of **5**[b] (%)
1	1000	1000	1000	rt	20:80	71
2	1000	500	1000	rt	20:80	79
3	500	250	500	rt	20:80	81
4	200	100	200	rt	20:80	67
5	100	50	100	rt	20:80	41
6	1000	500	1000	40 °C	20:80	94
7	500	250	500	40 °C	20:80	87
8	1000	500	1000	rt	30:70	92
9	1000	500	1000	40 °C	30:70	96
10[c]	1000	500	1000	40 °C	30:70	61

[a] Reaction of **3** (5 nmol) and **4** (10 nmol) was conducted in 100 μL of a mixture of DMSO and sodium phosphate buffer (10 mM, pH 7.0) for 15 min.
[b] Yield was determined by HPLC analysis.
[c] Reaction of **3** (0.5 nmol) and **4** (1 nmol) was conducted in 100 μL of a mixture of DMSO and buffer.

2.5. Synthesis of ^{18}F-labeled meta-isomer

By following the procedure for synthesizing [^{18}F]**8**, we succeeded in synthesizing [^{18}F]**9**, a *meta* structural isomer of [^{18}F]**8** in the labeling position,[17] under the same reaction conditions using **7** and a *meta* prosthetic group 1-azido-3-([^{18}F]fluoromethyl)benzene ([^{18}F]**6**) which was synthesized from tosylate **2**.[9] The radioactivity and specific radioactivity of this compound were 2.1 GBq and ca. 1800 GBq/μmol, respectively. The chemical and radiochemical purities of [^{18}F]**9** were 99% and 93%, respectively. The DCY was approximately 7.2%, calculated on the basis of 50 GBq of [^{18}F]fluoride ion in a single cyclotron bombardment (see Section 4).

2.6. PET study of ^{18}F-labeled ODNs using rats

PET imaging of [^{18}F]**8** was conducted on male rats under isoflurane anesthesia (n = 4). After an intravenous injection of [^{18}F]**8**, the radioactivity was first detected in the inferior vena cava for a few seconds. Subsequently, the radioactivity was detected in the kidneys, liver, urinary bladder, and intestines. Afterwards, the radioactivity accumulated in the bones, such as the vertebrae, costae, femur, etc. (Fig. 3). It is likely that [^{18}F]**8** was gradually metabolized into [^{18}F]fluoride ion, which accumulated in the bones.[18] Meanwhile, the in vivo behavior of the *meta*-labeled ODN [^{18}F]**9** was substantially different from that of [^{18}F]**8**. These PET studies are currently still in progress.

3. Conclusion

In summary, we succeeded in developing a method for the site-specific ^{18}F-labeling of ODNs using novel ^{18}F-prosthetic groups, azido([^{18}F]fluoromethyl)benzenes ([^{18}F]**3** and [^{18}F]**6**), via a Cu-catalyzed Huisgen reaction. The labeling was conducted using a mere 20 nmol of alkyne-substituted ODN, an amount similar to that of ^{18}F-prosthetic groups, under mild conditions at 40 °C for 15 min to provide the desired ^{18}F-labeled ODNs with an adequate radioactivity of ~2.5 GBq applicable for animal and human PET studies. This ^{18}F-labeling procedure provides the firm chemical basis for stoichiometry-oriented PET labeling reactions, potentially useful for the PET tracer syntheses of middle molecular weight biomolecules, such as ODNs, and would be especially valuable as requisites for the promotion of recently attracted ^{18}F-labelings with microscale reactors.[19] That is, the advantage of our optimized conditions for stoichiometry-focused ^{18}F-labeling will be preferably insured by use of the integrated microfluid devices for multistep ^{18}F-radiosynthesis,[19c] which are making remarkable progress in PET chemistry. In addition, it is worth mentioning that since a compact aryltriazole-substituted deoxyribose unit is structurally similar to a nucleoside, it can be easily incorporated into an ODN as a dummy nucleoside. Thus, with the proposed method, site-specific ^{18}F-labeling can be carried out at any position in the ODN without causing any drastic change in the intrinsic biological activities of the resulting oligonucleotides.[16] The applications of the proposed labeling method and further biological studies will be reported in due course.[20]

4. Experimental section

4.1. General

Melting points were measured with a Yanagimoto micro melting point apparatus. IR spectra were recorded on a Shimadzu IR Prestige-21 Fourier Transform Infrared Spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM-AL400. EI and FAB mass spectra were recorded on a JEOL JMS-D300 or JMS-600 mass spectrometer. MALDI-TOF mass spectra were recorded on an Applied Biosystems Voyager®-DE. HPLC analysis was performed on two JASCO PU-980 Intelligent HPLC pumps, an MD-910 multiwavelength detector, and a CU-965 column oven. Radio-HPLC analysis was performed on a Shimadzu Prominence CBM-20A communication bus module, an LC-20AB liquid chromatograph, and a CTO-20AC column oven; an SPD-20A UV/VIS detector and an RLC-700 Aloka radio analyzer. The amount of a radioacitve compound was determined by UV_{254} or UV_{260} calibration curve provided by radioinactive authentic samples. The specific activity of a radioactive compound was calculated as the ratio of the dose measured on a Biodex Mecical Systems ATOMLAB 300 by the amount of the compound. ODNs were synthesized utilizing an Applied Biosystems 394 DNA/RNA synthesizer. An anion exchange resin cartridge SAIKA-SPE SAX-30 (AiSTI SCIENCE), Sep-Pak Plus C18 (Waters), and other chemicals (Sigma–Aldrich or Nacalai Tesque) were used for ^{18}F-labeling reactions and for synthesizing the ODN precursors, model compounds, and authentic compounds. ^{18}O-enriched water (Taiyo-Nissan) was irradiated by 12 MeV protons in a Sumitomo CYPRIS HM-12S cyclotron (Sumitomo Heavy Industries) to generate [^{18}F]fluoride ion. Literature procedures were used for the syntheses of 4-azidobenzyl alcohol[21a] and 3-azidobenzyl alcohol.[21b] ^{18}F-labeling reaction was conducted using an automated radiolabeling system at RIKEN CMIS.

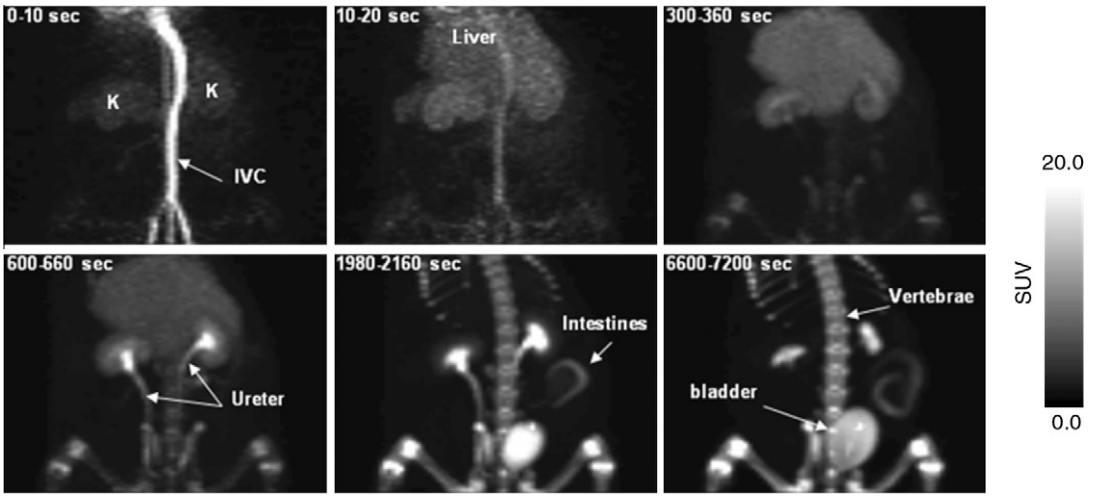


Figure 3. Maximum-intensity-projection PET image of [^{18}F]**8** in the rat abdomen.

4.2. Product analyses

HPLC analysis of [^{18}F]**3** and [^{18}F]**6**; column: COSMOSIL 5C_{18}-MS-II 4.6 × 150 mm (Nacalai Tesque, Inc.), eluent: 50:50 (v/v) mixture of CH_3CN and phosphate buffer (10 mM NaH_2PO_4 + 10 mM Na_2HPO_4), flow rate: 1.5 mL min^{-1}, UV wavelength: 240 and 254 nm, column temperature: 35 °C, retention time of [^{18}F]**3**: 5.5 min (see Fig. 1), retention time of [^{18}F]**6**: 5.8 min.

HPLC analysis of [^{18}F]**8** and [^{18}F]**9**; column: COSMOSIL 5C_{18}-MS-II 4.6 × 100 mm (Nacalai Tesque, Inc.), linear gradient elution: 10–24% CH_3CN in 0.1 M TEAA buffer (tetraethylammonium acetate) from 0 min to 20 min, and 24–80% CH_3CN in 0.1 M TEAA buffer from 20 min to 30 min, flow rate: 1.0 mL min^{-1}, UV wavelength: 260 nm, column temperature: 50 °C, retention time of [^{18}F]**8**: 12.1 min, retention time of [^{18}F]**9**: 12.1 min.

HPLC analysis of **5** in the model reaction; column: COSMOSIL 5C_{18}-MS-II 4.6 × 150 mm (Nacalai Tesque, Inc.), eluent: 40:60 (v/v) mixture of CH_3CN and phosphate buffer (10 mM NaH_2PO_4 + 10 mM Na_2HPO_4), flow rate: 1.5 mL min^{-1}, UV wavelength: 240 nm, column temperature: 35 °C, retention time of **5**: 4.0 min. Yield of **5** was determined by using *n*-butyl 4-hydorxybenzoate as an internal standard.

4.3. ^{18}F-Labeling of ODNs and preparation of the corresponding precursors and unlabeled authentic compounds

4.3.1. Syntheses of prosthetic group [^{18}F]3 and ODN PET tracer [^{18}F]8

[^{18}F]fluoride ion with a radioactivity of approximately 50 GBq in 2 mL of [^{18}O]water was transferred to an automated radiolabeling system, and [^{18}F]fluoride ion was trapped by a short anion-exchange resin cartridge SAIKA-SPE SAX-30 which had been preconditioned with aqueous sodium hydrogen carbonate (1.0 M, 5 mL) and water (10 mL). The trapped [^{18}F]fluoride ion was eluted into the first reaction vessel with tetrabutylammonium hydrogen carbonate (0.6 mL of 0.025 M solution in 80% CH_3CN–water mixture) and rinsed with CH_3CN (0.6 mL). CH_3CN and water were evaporated under reduced pressure at 110 °C under a He gas stream, and the residue was azeotropically dried using an additional 1 mL of CH_3CN.

A solution of **1** (6.0 mg) in CH_3CN (1 mL) was added to the residue. The resulting mixture was heated at 85 °C for 5 min. A sampling solution (~5 μL) withdrawn from the reaction mixture was directly analyzed by HPLC (HPLC analytical yield of [^{18}F]**3**: 97%). Then, the reaction mixture was diluted with 0.3 mL of H_2O and injected into preparative HPLC. The purification conditions are as follows: COSMOSIL 5C_{18}-MS-II 10 × 250 mm (Nacalai Tesque, Inc.), eluent: 40:60 (v/v) CH_3CN/H_2O mixture from 0 min to 6 min, and then 50:50 CH_3CN/H_2O mixture from 6 min to 14 min, flow rate: 4.0 mL min^{-1}, UV wavelength: 254 nm, column temperature: rt, retention time of [^{18}F]**3**: 15–16 min.

The fraction containing [^{18}F]**3** was collected and transferred to the second reaction vessel that was charged with 0.18 mL of DMSO. CH_3CN was gently evaporated under reduced pressure at 40 °C with a He gas stream. To the resulting DMSO–H_2O (0.18 mL—ca. 0.29 mL) solution of [^{18}F]**3**, sodium phosphate buffer (60 μL, 100 mM, pH 7.0), the ethynyl precursor of our ODN **7** (0.50 mM in H_2O, 40 μL, 20 nmol), $CuSO_4$ (50 mM in H_2O, 12 μL, 600 nmol), TBTA (50 mM in DMSO, 6 μL, 300 nmol), and sodium ascorbate (50 mM in H_2O, 12 μL, 600 nmol) were added. The mixture was heated at 40 °C for 15 min. A sampling solution (~5 μL) withdrawn from the reaction mixture was diluted with 0.1 M TEAA buffer and analyzed by HPLC (HPLC analytical yield of [^{18}F]**8**: 92%). Then, the reaction mixture was diluted with 0.3 mL of TEAA buffer and injected into HPLC. The purification conditions are as follows: COSMOSIL 5C_{18}-AR-II 10 × 250 mm (Nacalai Tesque, Inc.), linear gradient elution: 10–20% of CH_3CN in 0.1 M TEAA buffer from 0 min to 20 min, flow rate: 4.0 mL min^{-1}, UV wavelength: 260 nm, column temperature: 50 °C, retention time of [^{18}F]**8**: 14–15 min. The fraction containing the desired ^{18}F-labeled compound was collected, and CH_3CN was evaporated under reduced pressure to afford a solution of [^{18}F]**8** in TEAA buffer (~5 mL).

[^{18}F]**8** was identified by HPLC analysis using the unlabeled authentic compound **8** as the reference. The total time taken for the synthesis, from the generation of [^{18}F]fluoride ion in the cyclotron to the preparation for the TEAA buffer solution of [^{18}F]**8**, was 84 min. The isolated radioactivity and specific radioactivity at the end of synthesis: 2.53 GBq and 2366 GBq/μmol, respectively. The chemical purity determined at 260 nm and radiochemical purity: 95% and 87%, respectively. The isolated yield of the product was calculated to be 5.1% on the basis of 50 GBq of the [^{18}F]fluoride ion in a single cyclotron bombardment, and the DCY was 8.6%.

4.3.2. Syntheses of the prosthetic group [^{18}F]6 and the ODN PET tracer [^{18}F]9

[^{18}F]**6** and [^{18}F]**9** were synthesized from **2** by following the same procedure described above. The HPLC analytical yields of [^{18}F]**6** and [^{18}F]**9** were 96% and 90%, respectively. [^{18}F]**9** was identified by HPLC analysis using the unlabeled authentic compound **9** as the reference. The total time taken for the synthesis, starting from the generation of [^{18}F]fluoride ions by the cyclotron, 83 min. The isolated radioactivity and specific radioactivity of the product at the end of synthesis: 2.12 GBq and 1809 GBq/μmol, respectively. Chemical purity (based on UV_{260}) and radiochemical purity: 99% and 93%, respectively. The isolated yield was calculated to be 4.2% on the basis of 50 GBq of the [^{18}F]fluoride ion in single cyclotron bombardment, and the DCY was 7.2%.

4.3.3. Desalting of the solution [^{18}F]8 or [^{18}F]9 in TEAA buffer

If necessary, the solution of [^{18}F]**8** or [^{18}F]**9** in the TEAA buffer can be desalted by passing it through a Sep-Pak Plus C18 (preconditioned with 40 mL of EtOH and 40 mL of H_2O), washed twice with 5 mL H_2O, dried under N_2 flow for 1 min, and eluted using 1 mL EtOH. Then, EtOH was evaporated under a N_2 gas stream and the residue was dissolved in saline to be used as the injection solution in the animal PET studies. The total process time was 30 min and 90% (DCY) of the radioactivity was recovered.

4.3.4. Synthesis of 4-azidobenzyl 4-methylbenzenesulfonate (1)

Pyridine (0.162 mL, 2.00 mmol) and *p*-toluenesulfonic anhydride (343 mg, 1.05 mmol) were added to a stirred solution of 4-azidobenzyl alcohol (149 mg, 1.00 mmol) in dichloromethane (5.0 mL) at 0 °C. After 30 min, the resulting mixture was partitioned between H_2O and dichloromethane. The organic phase was washed with 1.0 M hydrochloric acid, saturated aqueous sodium bicarbonate, and brine, then, it was dried over anhydrous magnesium sulfate, filtered, and concentrated under reduced pressure. The residue was suspended with diethyl ether (3 mL) and filtered through a cotton plug. The filtrate was diluted with hexane (5 mL) and cooled in an ice bath. After 30 min, the supernatant fluid was pipetted off and the crystals were washed twice with hexane and dried under reduced pressure to afford **1** (218 mg, 0.719 mmol, 72% yield; colorless crystals). Since **1** was found to decompose rapidly at room temperature, it was stored in a freezer and kept away from moisture.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.45 (3H, s), 5.02 (2H, s), 6.97 (2H, d, J = 8.3 Hz), 7.24 (2H, d, J = 8.3 Hz), 7.34 (2H, d, J = 8.3 Hz), 7.79 (2H, d, J = 8.3 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 21.7, 71.3, 119.2, 127.9, 129.9, 129.9, 130.3, 133.3, 141.0, 144.9. HRMS (EI) calcd for $C_{14}H_{13}N_3O_3S$ (M^+) 303.0677, found 303.0650.

4.3.5. Synthesis of 3-azidobenzyl 4-methylbenzenesulfonate (2)

We synthesized **2** from 3-azidobenzyl alcohol by following the same procedure reported for **1** (yield of **2**: 52%). Compound **2** decomposes gradually at room temperature and is stored in a freezer and kept away from moisture.

IR (film, KBr): 2114, 1593, 1489, 1452, 1360, 1292, 1177, 945, 835, 814, 781, 665 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ: 2.45 (3H, s), 5.03 (2H, s), 6.85 (1H, s), 6.97 (1H, d, J = 7.8 Hz), 7.03 (1H, d, J = 7.8 Hz), 7.30 (1H, t, J = 7.8 Hz), 7.33 (2H, d, J = 8.5 Hz), 7.79 (2H, d, J = 8.5 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 21.6, 71.0, 118.8, 119.5, 124.7, 128.0, 129.9, 130.1, 133.1, 135.3, 140.5, 145.0. MS (EI): m/z 303 (M^+). Anal. Calcd for $C_{14}H_{13}N_3O_3S$: H, 4.32; C, 55.43; N, 13.85. Found: H, 4.32; C, 55.59; N, 13.89.

4.3.6. Synthesis of 1-azido-4-(fluoromethyl)benzene (3)

A solution of **1** (348 mg, 1.15 mmol) in CH_3CN (5 mL) was added to a stirred suspension of potassium fluoride (267 mg, 4.59 mmol) and 18-crown-6 (303 mg, 1.15 mmol) in CH_3CN (5 mL). The mixture was heated to 50 °C and stirred for 30 h. Then the mixture was cooled to room temperature and partitioned between H_2O and AcOEt. The aqueous layer was extracted once with AcOEt. The combined organic extracts were dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (eluent: 5% AcOEt in hexane) to give **3** (123 mg, 0.814 mmol, yield: 71%) as a colorless oil.

IR (film on KBr): 2920, 2112, 1284, 1016, 912, 742 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ: 5.34 (2H, d, J = 47.8 Hz), 7.05 (2H, d, J = 8.4 Hz), 7.38 (2H, dd, J = 8.4, 2.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 84.0 (d, J = 165.5 Hz), 119.2 (d, J = 1.7 Hz), 129.4 (d, J = 5.8 Hz), 132.8 (d, J = 17.3 Hz), 140.7 (d, J = 3.3 Hz). HRMS (EI) calcd for $C_7H_6FN_3$ $(M+H)^+$ 151.0546, found 151.0555.

4.3.7. Synthesis of 1-azido-3-(fluoromethyl)benzene (6)

We synthesized **6** from **2** by the following same procedure reported for the synthesis of **3** (yield of **6**: 66%).

IR (film on KBr): 2114, 1589, 1485, 1450, 1295, 990, 786 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ: 5.37 (2H, d, J = 47.6 Hz), 7.02 (1H, d, J = 7.8 Hz), 7.04 (1H, s), 7.13 (1H, d, J = 7.8 Hz), 7.37 (1H, d, J = 7.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 83.8 (d, J = 168.0 Hz), 117.6 (d, J = 6.6 Hz), 119.2 (d, J = 3.3 Hz), 123.5 (d, J = 6.6 Hz), 130.0, 138.2 (d, J = 17.4 Hz), 140.5. MS (EI): m/z 151 $(M+H)^+$. Anal. Calcd for $C_7H_6FN_3$: H, 4.00; C, 55.63; N, 27.80. Found: H, 4.19; C, 55.74; N, 27.95.

4.3.8. Synthesis of ODN 7, a precursor for ^{18}F-labeling

ODN **7** was synthesized according to our previously reported procedure (see Refs. 5,14).

MALDI-TOF MS m/z **7** $[M+H]^+$ calcd 5693.52, found 5692.07.

4.3.9. Synthesis of unlabeled ODNs 8 and 9

We synthesized **8** from **3** and **7** according to our previously reported procedure (see Refs. 5,14). MALDI-TOF MS m/z **8** $[M+H]^+$ calcd 5844.66, found 5844.39.

We also prepared **9** from **6** and **7**. MALDI-TOF MS m/z **9** $[M+H]^+$ calcd 5844.66, found 5844.39.

4.4. DNA melting experiments

The thermal stabilities of the duplexes were determined using a spectrophotometer equipped with a thermoregulator (SHIMADZU UV-1650PC). Hybridization mixtures of 130 μL were prepared using a medium salt buffer (10 mM sodium phosphate buffer [pH 7.0] and 100 mM NaCl) and an equimolar amount (2.0 μmol/L) of ODNs and complementary RNA (5′-AUGUCUCAGAGCAACCGG-3′). The mixtures were incubated at 85 °C, then cooled back slowly to 4 °C before T_m measurements. The samples were heated at a rate of 0.5 °C/min. The melting temperatures (T_m calves) were determined by plotting the first derivative of the absorbance (260 nm) versus the temperature curve (n = 3). T_m values of 5′-CCGGTTGCT CTGAGACAT-3′, **8**, and **9** were 65 °C, 66 °C, and 66 °C, respectively.

4.4.1. Synthesis of *N*-((1-(4-(fluoromethyl)phenyl)-1*H*-1,2,3-triazol-4-yl)methyl)benzamide (5) for model Cu-catalyzed Huisgen reaction

To a stirred solution of 2.5 mg (0.010 mmol) of copper (II) sulfate pentahydrate and 7.9 mg (0.040 mmol) of sodium ascorbate in 0.25 mL of H_2O were added a DMF solution (1.0 mL) of *N*-propargylbenzamide **4** (31.8 mg, 0.200 mmol), and a DMF solution (0.5 mL) of **3** (30.2 mg, 0.200 mmol). The mixture was heated to 80 °C and stirred for 15 min. Then the mixture was cooled to room temperature and partitioned between 3% aqueous ammonia and AcOEt. The organic layer was washed with H_2O and brine, dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The residue was crystallized with AcOEt, filtered, washed with cooled AcOEt, and then dried under reduced pressure to give **5** (49.4 mg, 0.159 mmol, yield: 80%) as a white powder.

Mp: 190–192 °C. IR (film on KBr): 3319, 1634, 1553, 698 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ: 4.61 (2H, d, J = 5.6 Hz), 5.49 (2H, d, J = 47.6 Hz), 7.44–7.55 (3H, m), 7.62 (2H, dd, J = 8.2, 1.6 Hz), 7.88–7.91 (2H, m), 7.96 (2H, d, J = 8.2 Hz), 8.72 (1H, s), 9.08 (1H, t, J = 5.6 Hz). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 34.8, 83.5 (d, J = 166.4 Hz), 120.0, 121.2, 127.3, 128.3, 129.2 (d, J = 5.8 Hz), 131.3, 134.1, 136.4 (d, J = 16.6 Hz), 136.7 (d, J = 3.3 Hz), 146.4, 166.2. HRMS (FAB) calcd for $C_{17}H_{16}FN_4O$ $(M+H)^+$ 311.1308, found 311.1313. Anal. Calcd for $C_{17}H_{15}FN_4O$: H, 4.87; C, 65.80; N, 18.05. Found: H, 4.87; C, 65.70; N, 18.02.

4.5. PET studies using rats

Male Sprague–Dawley rats (SLC, Hamamatsu, Shizuoka, Japan) weighing approximately 250 g were used. The rats (n = 4) were anesthetized and maintained with a mixture of 1.5% isoflurane and nitrous oxide/oxygen (7:3) and positioned in the gantry of a PET scanner (microPET Focus 220, Siemens Co., Ltd, Knoxville, TN, USA). After positioning and fixation, a 30-min transmission scan with a rotating ^{68}Ge–^{68}Ga pin source was performed. Then a 120-min emission scan of the abdomen was performed with 400–650 keV as the energy window and 6 ns as the coincidence time window. Emission data were acquired in the list mode, after an intravenous bolus injection of [^{18}F]**8** (approximately 50 MBq per animal) via a tail vein. The acquired data were sorted into dynamic sinograms (6 × 10 s, 6 × 30 s, 11 × 60 s, 15 × 180 s, 6 × 600 s; a total of 44 frames). The data were reconstructed by a statistical maximum a posteriori probability algorithm (MAP) of 12 iterations with point spread function (PSF) effect. During the experiment, body temperature was maintained at 37 °C with a heating blanket. The radioactivity concentrations were normalized with cylinder phantom data, and were expressed as standardized uptake values (SUV). All experimental protocols were approved by the RIKEN's Ethics Committee on Animal Care and Use and were performed in accordance with the *Principles of Laboratory Animal Care* (NIH publication No. 85–23, revised 1985).

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Supplementary data

Supplementary data (^{13}C NMR spectra of **1** and **3**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.11.033.

References and notes

1. (a) Phelps, M. E. *PET: Molecular Imaging and Its Biological Applications*; Springer: New York, Berlin, Heidelberg, 2004; (b) *Positron Emission Tomography*; Bailey, D. L., Townsend, D. W., Valk, P. E., Maisey, M. N., Eds.; Basic Science: Springer, New York, Berlin, Heidelberg, 2005; (c) Fowler, J. S.; Wolf, A. P. *Acc. Chem. Res.* **1997**, *30*, 181; (d) Ametamey, S. M.; Honer, M.; Schubiger, P. A. *Chem. Rev.* **2008**, *108*, 1501.
2. For a recent review of metal nuclides conjugates, see: (a) Tanaka, K.; Fukase, K. *Org. Biomol. Chem.* **2008**, *6*, 815–828; For non-metal nuclides, see: (b) Miller, P. W.; Long, N. J.; Vlar, R.; Gee, A. D. *Angew. Chem.* **2008**, *120*, 9136; *Angew. Chem., Int. Ed.* **2008**, *47*, 8998–9033.
3. Flavell et al. reported a coupling reaction between aldehyde [^{18}F]**FBA** and a nearly stoichiometric amount (30 nmol) of aminooxy peptide. They used the 'expressed protein ligation' technique to introduce a chemically-unstable aminooxy group into leptin. Flavell, R. R.; Kothari, P.; Bar-Dagan, M.; Synan, M.; Vallabhajosula, S.; Friedman, J. M.; Muir, T. W.; Ceccarini, G. *J. Am. Chem. Soc.* **2008**, *130*, 9106.
4. In our laboratory, the specific radioactivity of [^{18}F]fluoride was estimated to be approximately 500–2500 GBq/μmol. Therefore, [^{18}F]fluoride with 50 GBq obtained in a conventional single cyclotron bombardment would be approximately 20–100 nmol.
5. Nakahara, M.; Kuboyama, T.; Izawa, A.; Hari, Y.; Imanishi, T.; Obika, S. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3316.
6. (a) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596; Review: (b) Kolb, H. C.; Sharpless, K. B. *Drug Discovery Today* **2003**, *8*, 1128–1137; Review in oligonucleotide chemistry: (c) Amblard, F.; Cho, J. H.; Schinazi, R. F. *Chem. Rev.* **2009**, *109*, 4207.
7. Inkster, J. A. H.; Adam, M. J.; Storr, T.; Ruth, T. J. *Nucleosides Nucleotides Nucleic Acids* **2009**, *28*, 1131.
8. For review of click labeling in PET chemistry, see: Glaser, M.; Robins, E. G. *J. Labelled Compd. Radiopharm.* **2009**, *52*, 407. and see references cited therein.
9. Brodack, J. W.; Kilbourn, M. R.; Welch, M. J.; Katzenellenbogen, J. A. *Appl. Radiat. Isot.* **1986**, *37*, 217.
10. The mechanical losses of synthesized PET tracer were due to incomplete transfer of the reaction mixture from vessel to vessel, insufficient injection of the reaction mixture into preparative-HPLC, and incomplete isolation of the ^{18}F-labeled product by preparative-HPLC.
11. We have also intended the syntheses of azido[^{18}F]fluorobenzenes [^{18}F]**3** and [^{18}F]**6** by our Pd(0)-mediated rapid C-[^{18}F]fluoromethylation using arylborons and [^{18}F]fluoromethyl halide, see, (a) Doi, H.; Ban, I.; Nonoyama, A.; Sumi, K.; Kuang, C.; Hosoya, T.; Tsukada, H.; Suzuki, M. *Chem. Eur. J.* **2009**, *15*, 4165; (b) Doi, H.; Goto, M.; Suzuki, M. 'Pd^0-mediated rapid C-[^{18}F]fluoromethylation by the reaction of a [^{18}F]fluoromethyl halide and an organoboron compound', 15TH European Symposium On Radiopharmacy And Radiopharmaceuticals, Edinburgh, Scotland (UK), April 8–11, 2010, Abstract, *Q. J. Nucl. Med. Mol. Imaging*, April **2010**, *54*, Suppl. 1 to No. 2, pp 18–19.
12. Culbert, P. A.; Adam, M. J.; Hurtado, E. T.; Huser, J. M. A.; Jivan, S.; Lu, J.; Ruth, T. J.; Zeisler, S. K. *Appl. Radiat. Isot.* **1995**, *46*, 887.
13. Chan, T. R.; Hilgraf, R.; Sharpless, K. B.; Fokin, V. V. *Org. Lett.* **2004**, *6*, 2853–2855.
14. The sequence of the oligonucleotide used in this study is complementary to the 135–152 region containing AUG start codon of bcl-xL mRNA. Roongjang, S.; Takahashi, K.; Obika, S.; Imanishi, T. *Nucleic Acids Symp. Ser.* **2007**, *51*, 113.
15. In addition to the mechanical loss of radioactivity observed in the synthesis of [^{18}F]**3**, we found that when the reaction mixture containing ODN [^{18}F]**8** was purified by semipreparative HPLC, as shown in Figure 2, a portion of [^{18}F]**8** was eluted off with DMSO with the retention time of 3.5 min without affinity for HPLC column.
16. Binding of the unlabeled prosthetic group **3** to the original sequence of ODNs had no effect on T_m value (see Section 4).
17. We designed and synthesized *para* and *meta* compounds of [^{18}F]**8** and [^{18}F]**9** for the purpose of analyzing the stabilities of the labeling positions in in vivo biological process. In addition, by comparison of the chemical properties, a *para* tosylate **1** is so unstable that should be stored at −80 °C, but a *meta* structural isomer **2** is more stable and can be stored in a normal freezer (−20 °C).
18. For an example of the rapid degradation of ^{18}F-labeled DNA in baboons, see: Tavitian, B.; Terrazzino, S.; Kühnast, B.; Marzabal, S.; Stettler, O.; Dollé, F.; Deverre, J.-R.; Jobert, A.; Hinnen, F.; Bendriem, B.; Crouzel, C.; Giamberardino, L. D. *Nat. Med.* **1998**, *4*, 467.
19. (a) Lu, S. Y.; Pike, V. W. In *PET Chemistry, The Driving Force in Molecular Imaging*; Schubiger, P. A., Lehmann, L., Friebe, M., Eds.; Springer: New York, Berlin, Heidelberg, 2007; pp 271–287; (b) Cai, L.; Lu, S.; Pike, V. W. *Eur. J. Org. Chem.* **2008**, 2853; (c) Elizarov, A. M.; van Dam, R. M.; Shin, Y. S.; Kolb, H. C.; Padgett, H. C.; Stout, D.; Shu, J.; Huang, J.; Daridon, A.; Heath, J. R. *J. Nucl. Med.* **2010**, *51*, 282.
20. This ^{18}F-labeling methodology have been demonstrated by the syntheses of 20 types of ^{18}F-labeled ODNs focusing on nucleotide sequences and backbone modifications, thus far.
21. (a) Andersen, J.; Madsen, U.; Björkling, F.; Liang, X. *Synlett* **2005**, 2209; (b) Georg, G. I.; Boge, T. C.; Park, H.; Himes, R. H. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 615.