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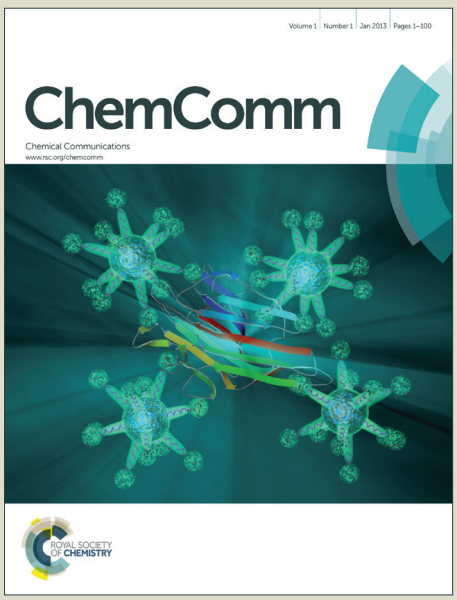

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Enabling [^{18}F]-bicyclo[6.1.0]nonyne for oligonucleotide conjugation for positron emission tomography applications: [^{18}F]-anti-microRNA-21 as an example

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A bicyclononyne-based prosthetic group has been developed for ^{18}F-labeling of anti-microRNA-21, an oligonucleotide, in a near-stoichiometric manner.

MicroRNAs are non-coding oligonucleotides that are about 22-nucleotides in length. In humans, there are about 2000 different microRNAs and they are involved in almost all cellular processes.[1] Accordingly, microRNAs are well justified targets for drug development and diagnostic applications.[2] Indeed, the first anti-microRNA drug, Miravirsen (Roche, previously Santaris Pharma), has reached clinical phase II studies and shown very promising results.[3] Anti-microRNAs are inhibitory oligonucleotides with complimentary nucleotide sequences to microRNAs. We have recently embarked on a research program on development of anti-microRNA-based tracers for positron emission tomography (PET) imaging and monitoring *in vivo* fate of anti-microRNAs. To pursue that, the first step is to set up suitable radiolabeling platforms for anti-microRNAs with different types of positron emitters,[4a] based on the previous work.[4b] This work focuses on labeling strategies with fluorine-18 (^{18}F). ^{18}F is the most often used positron emitter in clinical PET, due to its favorable physical properties (e.g. clean positron emitting process, low positron energy, etc.). Regarding ^{18}F-labeling of oligonucleotides, a number of ^{18}F-labeled prosthetic groups have been used based on different conjugation chemistry (Figure 1). Compounds 2-bromo-*N*-[3-(2-[^{18}F]fluoropyridin-3-yloxy)propyl]acetamide ([^{18}F]FPyBrA) and *N*-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide ([^{18}F]FBBA) are for alkylation of oligonucleotides with a single phosphorothioate-modification.[5,6] Compound succinimido 4-[^{18}F]-fluorobenzoate ([^{18}F]SFB) is an acylation agent for amino-functionalized oligonucleotides.[7] Azides (e.g. compound **1**) and alkynes (e.g. compound **2**) have been developed for copper-catalyzed 1,3-dipolar [3 + 2] cycloaddition (CuAAC) reactions.[8] Maleimides (e.g. compound **3**) are thiol-reactive agents.[9] In addition, amino-bearing oligonucleotides have been conjugated to **4** photochemically and isothiocyanate **5**, respectively.[10a,b] Silicon- and boron-based chemistry have also been explored.[10c,d] Because of the short physical half-life (109.8 min) of ^{18}F, the tiny amount of cyclotron-generated [^{18}F]-fluoride (in terms of molarity) and clinical setting for PET imaging, efficient and site-specific conjugation methods are ever needed. Among the aforementioned labeling strategies (Figure 1), CuAAC seems to be one of the most efficient labeling reactions. However, in typical cases CuAAC still has limited conjugation efficiency, in addition to the concerns about Cu^{I}-induced damages to biomolecules including oligonucleotides.[11]

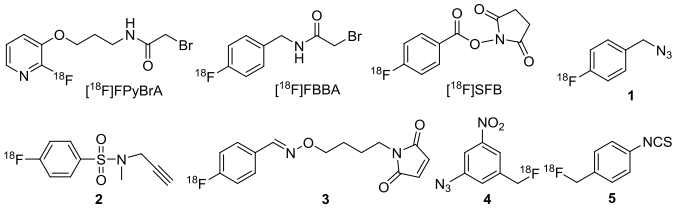

Figure 1. Prosthetic groups previously used for ^{18}F-labeling of oligonucleotides.[5-10]

Generally, inverse electron demand Diels-Alder (IEDDA) reaction is a fastest chemistry scheme in bioconjugation and a powerful alternative to existing ligation chemistries.[12] Biocyclo[6.1.0]nonyne (BCN, Scheme 1, a) is one of the dienophiles in IEDDA chemistry. BCN is readily accessible, relatively hydrophilic and have balanced stability and reactivity.[13] Among others, BCN has been used for fluorescence labeling in living cells, for preparing nanoparticles and for development of carbon monoxide (CO)-based prodrugs.[14] Very

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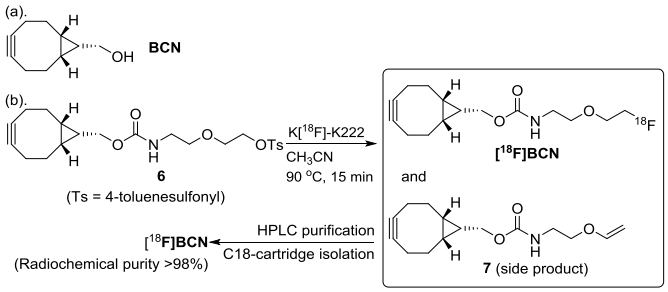
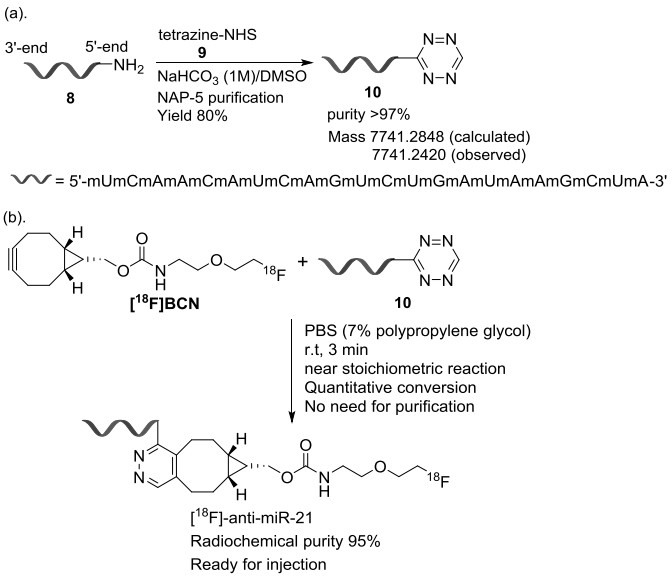
† Electronic Supplementary Information (ESI) available: Experimental details for [^{18}F]BCN, [^{18}F]-anti-miR-21 and other compounds and See DOI: 10.1039/b000000x/

importantly, the conjugation products of BCN have simpler isomeric profile compared with some other strained alkynes and alkenes.[15] In development of radiopharmaceuticals for PET applications, simple isomeric profile may facilitate the interpretation of imaging results and drug approval process. Thus, we have set out to develop the prosthetic group [^{18}F]-biocyclo[6.1.0]nonyne ([^{18}F]BCN, Scheme 1, b) for labeling of oligonucleotides such as anti-microRNAs. Accordingly, activated sulfonate ester **6** was prepared (Electronical supplementary information ESI) and subjected to ^{18}F-fluorination reactions with the protocol routinely used in our laboratory.[16] [^{18}F]BCN was formed in the presence of K[^{18}F]-Kryptofix 2.2.2 (K222) in acetonitrile at 90 ºC for 15 min, and compound **7** was a non-radioactive side product. After HPLC purification, [^{18}F]BCN was isolated with C18-cartridges and formulated in ethanol (clinical grade) for subsequent conjugation reactions. To confirm the identity of [^{18}F]BCN, the "cold" counterpart [^{19}F]BCN was prepared and used as a reference in the radiochemical quality control analyses (ESI). The total preparation time of [^{18}F]BCN was about 85 min and the decay-corrected radiochemical yield (RCY) starting from end of bombardment (EOB) was 20 ± 3% (n = 6). The RCYs didn't change significantly when the fluorination reactions were carried out at temperatures ranging from 85 to 100 ºC. The radiochemical purity of [^{18}F]BCN was higher than 98% according to both HPLC and radio-TLC analyses. The radiosynthesis was scaled up to obtain 1.4 GBq/mL of [^{18}F]BCN and radiolysis was not observed.



Scheme 1. (a) Structure of BCN.[13] (b) Radiosynthesis of [^{18}F]BCN.

MicroRNA-21 (miR-21) is a highly relevant target in cardiac diseases and overexpressed in most human tumors. More and more evidences show that miR-21 inhibition may be beneficial for many diseases for which no cure is available.[17] A first therapeutic use of microRNA inhibitors in cardiovascular diseases has recently shown to block development of cardiac fibrosis.[17a] Indeed, anti-miR-21 is a microRNA antagonist with a fully complementary sequence to miR-21. To enable [^{18}F]BCN conjugation, a ligation partner needs to be attached to anti-miR-21. BCN and BCN derivatives have proved feasible to conjugate with several types of ligation partners including azides,[13] tetrazines and sydnones.[18] BCN-tetrazine ligation seems to have the fastest chemical kinetics among the tested reactions. Accordingly, 5´-amino-modified anti-miR-21 **8** (2´-*O*-methyl backbone modified) was conjugated with tetrazine **9** in a solution of DMSO in $NaHCO_3$ (1 M). The isolated yields of compound **10** were 80 ± 3% (n= 6) after purification with NAP-5 size-exclusion column and the purity was more than 97% (Scheme 2 and ESI). The identity of **10** was confirmed by high resolution mass spectroscopy (HRMS). Next, we tested the conjugation of compound **10** with [^{19}F]BCN. In the presence of one equivalent of [^{19}F]BCN (≥ 10 µM), compound **10** was completely transformed into [^{19}F]-anti-miR-21 within 3 min in phosphate-buffered saline (PBS) at r.t (ESI). Because of the stoichiometric reaction, it didn't become a need for purification of [^{19}F]-anti-miR-21. For MS analysis purposes, [^{19}F]-anti-miR-21 was desalted with a hydrophilic-lipophilic balanced (HLB) cartridge and formulated in a mixture of water (25%) in acetonitrile. With a nano-ESI-MS system, the observed average mass (7996.5154) of [^{19}F]-anti-miR-21 was quite close to the theoretical value (7996.5538). However, the initial testing results of oligonucleotide **10** conjugation with [^{18}F]BCN were surprising. In the presence of **10** (1 µM), only few percent of [^{18}F]BCN was transformed into [^{18}F]-anti-miR-21 and there was a side radioactive product formation. The use of **10** at increased concentrations (e.g. 10 µM) only increased the conversion to 20%, which did not match with the fast kinetics of tetrazine ligation as shown in the reactions with [^{19}F]BCN. This seemed to be a similar case as our previous work concerning ^{18}F-labeling of peptides with 5-deoxy-5-[^{18}F]fluororibose ([^{18}F]FDR) where ribose competed to [^{18}F]FDR for peptide conjugation.[16b,19] In ^{18}F-fluorination reaction, part of the precursor **6** was transformed into side product **7**, which could compete to [^{18}F]BCN in conjugation reactions (Scheme 2, b). Accordingly, we developed a HPLC protocol to remove **7** from [^{18}F]BCN with a Phenomenex Jupiter Proteo C18 column and the identity of **7** was confirmed with HRMS (ESI).

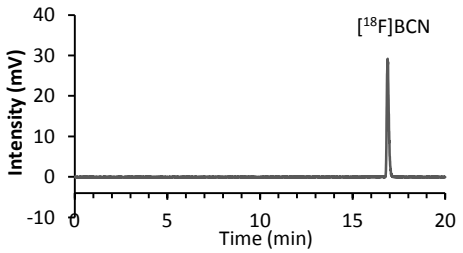


Scheme 2. (a) Synthesis of tetrazine **10**. (b) Preparation of [^{18}F]-anti-miR-21. (The chemical structures of **8**-**10** and [^{18}F]-anti-miR-21 are presented in the Electronic Supplementary Information.)

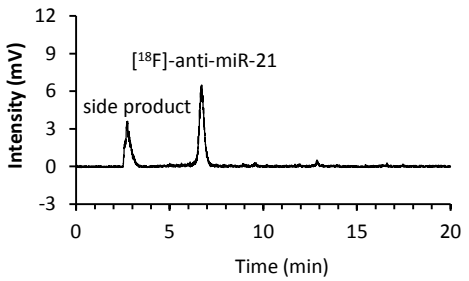
In the presence of compound **10** (10 µM, 1.1 equivalent), HPLC-purified [^{18}F]BCN (up to 1.4 GBq) was completely consumed within 3 minute and the formation of [^{18}F]-anti-miR-21 was clearly observed. However, there was a radioactive peak appearing prior to [^{18}F]-anti-miR-21 on HPLC (Figure 2 (a) and (b)) and the proportion of the side product was increasing with time. Starting with increased amount of radioactivity of [^{18}F]BCN, increased proportion of the side radioactive product was observed. However, in the syntheses of [^{19}F]-anti-miR-21 we have never encountered similar problems. [^{19}F]-Anti-miR-21 was stable in PBS at r.t for at least 24 hours (longer time was not tested). In some studies, it was evident that single-stranded oligonucleotides were prone to oxidative damage compared to double-stranded oligonucleotides.[20] As an explanation, the bases of single-stranded oligonucleotides might be easily accessible to oxidative radicals. Fluorine-18 is an ionization radiation source that could cause oxidative damages to biomolecules. This made us to assume that radioactivity-induced damage of [^{18}F]-anti-miR-21 might occur. Keeping this in mind, we decided to add polypropylene glycol (PPG) in the reactions because PPG is an approved pharmaceutical additive. In the presence of PPG (7% by volume) in PBS, the formation of the radioactive side product was

completely prevented (Figure 2 (c)). [^{18}F]-Anti-miR-21 was neatly formed and stable > 7.5 hours in PBS containing PPG (7%). We kept the amount of PPG as 7% in subsequent experiments since this amount was used for other PET radiopharmaceuticals in our hospital. However, less amount of PPG (e.g. 5%) was also applicable for [^{18}F]-anti-miR-21. Starting from 12.6 GBq of [^{18}F]-fluoride, 10–21 nmol of [^{18}F]BCN was produced in ethanol (1 ml). It was confirmed that 1.1 equivalent of oligonucleotide precursor **10** was sufficient to complete the conjugation with [^{18}F]BCN in 3 min at r.t. Because of the nature of cyclotron-produced [^{18}F]-fluoride, the molarity of [^{18}F]BCN varied in the range of 10–21 nmol per batch in our production system. In routine productions of PET tracers, a practical way is to measure the molarity of tracers post-synthesis. Likewise, the actual amount of [^{18}F]BCN in an individual batch was not known beforehand. To fit the production protocol into preclinical and clinical settings, we decided to add a fixed amount (23 nmol) of compound **10** to each batch of [^{18}F]BCN (typically 1.2–1.4 GBq per batch) in routine productions, to ensure sufficient amount of **10** for [^{18}F]BCN conjugation. Since the formation of [^{18}F]-anti-miR-21 was quantitative and the only concurrent product was nitrogen gas (Scheme 2, b), it was not necessary to perform purification for [^{18}F]-anti-miR-21. Starting from EOB, [^{18}F]-anti-miR-21 was produced with a total synthesis time of 90–95 min, and radiochemical purity was >95%. The specific radioactivity was 52–61 GBq/μmol (n = 6).

(a). Starting material [^{18}F]BCN.



(b). [^{18}F]-Anti-miR-21 synthesis in the absence of PPG.



(c). [^{18}F]-Anti-miR-21 synthesis in the presence of PPG (7% by volume).

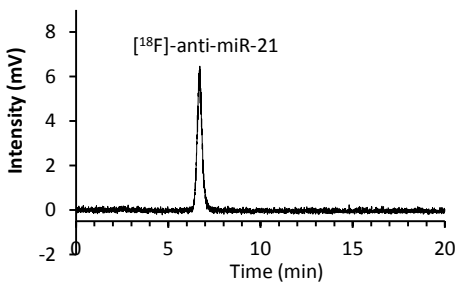


Figure 2. Monitoring [^{18}F]-anti-miR-21 radiosynthesis with HPLC analyses under radioactive detection. PPG effectively prevents radioactive side products formation for [^{18}F]-anti-miR-21.

In conclusion, an efficient ^{18}F-labeling system with [^{18}F]BCN as the prosthetic group has been developed. The labeling system has been exemplified on the radiosynthesis of [^{18}F]-anti-miR-21 in a near-stoichiometric manner under biocompatible conditions. In addition, PPG proves effective for preventing radiation-induced damages of [^{18}F]-anti-miR-21. Thus, it is feasible and practical to use [^{18}F]BCN-based ligation for radiolabeling of oligonucleotides.

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