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Synthesis and evaluation of an ^{125}I-labeled azide prosthetic group for efficient and bioorthogonal radiolabeling of cyclooctyne-group containing molecules using copper-free click reaction

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ABSTRACT

Herein we report the radiosynthesis of a pyridine derived azide prosthetic group for iodine radioisotope labeling of dibenzocyclooctyne (DBCO) conjugated molecules. The radiolabeling of the stannylated precursor **2** was conducted using [^{125}I]NaI and chloramine-T to give ^{125}I-labeled azide ([^{125}I]**1**) with high radiochemical yield (72 ± 8%, n = 4) and radiochemical purity (>99%). Using ^{125}I-labeled azide ([^{125}I]**1**), cyclic RGD peptide and near infrared fluorescent molecule were efficiently labeled with modest to good radiochemical yields. The biodistribution study and SPECT/CT images showed that [^{125}I]**1** underwent rapid renal clearance. These results clearly demonstrated that [^{125}I]**1** could be used as an useful radiotracer for in vivo pre-targeted imaging as well as efficient in vitro radiolabeling of DBCO containing molecules.

Copper-free click reaction using azide and stained cyclooctyne is extensively applied to the efficient bioconjugation as well as the bioorthogonal labeling of a wide range of biomolecules, cells and living subjects.[1–7] Due to the recent advancements of biochemical methods, the azide group can be introduced into various biomolecules using several strategies including metabolic labeling, post-transitional modification and enzymatic transfer of the desired functional group.[8–11] The artificially modified biomolecules prepared by the above methods can be labeled with fluorescent dyes for molecular biology and optical imaging studies. Because of excellent bioorthogonality and rapid reaction rate of the strain-promoted copper-free click reaction, it has also been used as an useful tool for radiolabeling of positron emitter. Several ^{18}F-labeled azide or dibenzocyclooctyne (DBCO) prosthetic groups have been synthesized for in vitro labeling of cancer targeting peptides as well as in vivo pre-targeted imaging of tumors.[12–17] In addition to these results, the same conjugation reaction was applied to ^{64}Cu-labeling of a few nanomaterials for the long-term and specific PET imaging studies.[18–20]

Recently we have reported an azido group containing 4-[^{125}I] iodobenzamide prosthetic group for rapid and efficient radiolabeling of cyclooctyne group conjugated molecules via copper-free click reaction.[21] The labeling reactions using this method provided good radiochemical results. Despite usefulness and efficiency of these results, the radiolabeled tracer in our previous report showed some disadvantages for in vivo imaging study. Especially, quite large amount of radioactivity was accumulated in the intestines over the time (ca. 8%ID/g at 4 h post injection) and relatively high uptake was detected in the liver. Additionally, certain amount of radioactivity was also accumulated in the thyroid due to deiodination by enzymes in vivo. Those results would not be desirable in the pre-targeted in vivo imaging study because considerable radioactivities in the normal organs would hamper the specific imaging of the region of interest.

These observations led us to design and synthesize a new radiolabeled prosthetic group. The previous report indicated that iodine radioisotope labeled pyridine showed less thyroid uptake than that of aryl iodide analog because electron-deficient heterocycle decreases the structural similarity to iodotyrosine which undergoes facile dehalogenation in vivo.[22] Moreover, an ether linkage between azide group and iodine radioisotope labeled aromatic ring was expected to have higher in vivo stability than an amide linkage. Therefore, in the present study, pyridine group and ether structure were used for preparing the new prosthetic group which can be applied to radiolabeling of DBCO conjugated molecule.

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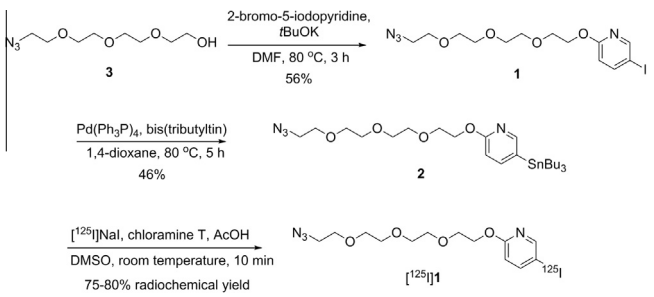
Among several iodine radioisotopes, ^{125}I was utilized in this study because large amount of ^{125}I is easily commercially available and it is also suitable for repetitive experiments to optimize radiochemical procedure due to its long half-life (59.4 days). Herein we report the synthesis and evaluation of pyridine derived ^{125}I-labeled azide tracer for efficient radiolabeling of DBCO containing molecule and in vivo imaging to show its biodistribution. Moreover, ^{125}I-labeled tracers can be applied to SPECT imaging study. We further present the radiolabeling results of DBCO conjugated peptide and near infrared fluorescent dye to determine labeling efficiency of ^{125}I-labeled prosthetic group.

Synthetic steps for the preparation of radiolabeling precursor **2** and the radiochemical procedure are outlined in Scheme 1. The alcohol **3** was reacted with 2-bromo-5-iodopyridine under basic condition gave the standard compound **1**. Tetrakis(triphenylphosphine)palladium catalyzed stannylation reaction of **1** using hexabutyldistannane provided the tin compound **2**. Radiolabeling of the precursor **2** was performed using [^{125}I]NaI (in 0.1 M aqueous NaOH), chloramine-T as an oxidant and acetic acid for adjusting the pH of the reaction condition neutral. The radiolabeling reaction has been accomplished with up to 407 MBq of [^{125}I]NaI and the desired product [^{125}I]**1** was obtained with a good radiochemical yield (72 ± 8%, $n = 4$) after preparative HPLC purification of the crude product.[23] Radiochromatogram of the radiolabeling reaction was provided in Supporting information (Fig. S1). Analytical HPLC result showed that the radiochemical purity of the purified [^{125}I]**1** was more than 99%. In vitro stability test of [^{125}I]**1** was carried out in mouse serum and analytical radio-HPLC result showed that more than 91% of [^{125}I]**1** was remained intact for 72 h (Fig. S3).

Since the level of organ distribution of ^{125}I-labeled azide tracer will be necessary for pre-targeted in vivo imaging, radiopharmacological analysis of [^{125}I]**1** was carried out in normal ICR mice through ex vivo biodistribution and single photon emission computed tomography (SPECT) imaging study. As shown in Figure 1a, most of intravenously injected [^{125}I]**1** was rapidly excreted via renal clearance because high radioactivities were shown in the kidney and bladder at 1 h post administration. The data from biodistribution study in Figure 2 was well matched with the result of SPECT/CT imaging. A large amount of [^{125}I]**1** was detected in the kidneys at the first time point (10.9%ID/g at 0.25 h) and a significant amount of radioactivity (3.22%ID/g) was still observed at 4 h post injection, suggesting that a large amount of the ^{125}I-labeled azide tracer was excreted from the kidneys. On the other hand, uptake values of liver and intestines were relatively low compared to that of kidneys. These observations were quite different in comparison to the results of our previous prosthetic group, azido 4-[^{125}I]iodobenzamide analog, which showed that large amount of radiotracer underwent hepatobiliary excretion and significant uptake values were accumulated in the intestines.[21] The measured log$P$ value of [^{125}I]**1** was 2.34 and its hydrophobicity was quite similar to the value of azido 4-[^{125}I]iodobenzamide (log$P$ = 2.49). But structural modifications of the tracer would largely affect



Scheme 1. Synthesis and radiolabeling of the precursor **2**.

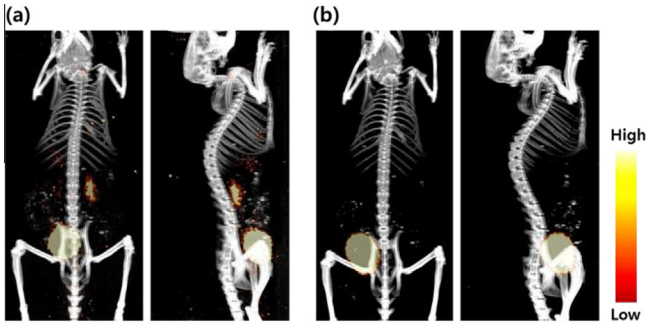


Figure 1. SPECT/CT images of [^{125}I]**1**. (a) 1 h post injection, (b) 4 h post injection.

in vivo organ distribution and major excretion pathway of [^{125}I]**1**. Certain amounts of [^{125}I]**1** were also found in some organs (i.e., heart and lung) and the blood at the first time point (0.25 h) but most of radioactivities from these organs were excreted within 4 h. We also compared the thyroid uptake values of [^{125}I]**1** with those of azido 4-[^{125}I]iodobenzamide analog (Fig. S4). At the initial time point, [^{125}I]**1** showed less accumulation in thyroid however, similar uptake values were observed at the later time points. Considering that the size of thyroid was smaller than other internal organs, the radioactivity value (5.71%ID/g at 4 h post injection) of [^{125}I]**1** in the thyroid would not be a high background. Moreover quite weak signal in the thyroid in the Figure 1b observed at 4 h post injection also supported this result. The high stability in the physiological condition, suitable biodistribution and reasonable excretion kinetics indicated that [^{125}I]**1** can be applied to the pre-targeted in vivo imaging study using DBCO group conjugated targeting molecules such as cancer targeting peptides or antibodies.

^{125}I-labeled azide ([^{125}I]**1**) was further investigated in the radiolabeling study with DBCO conjugated molecules (Fig. 3 and Table 1). The amount of DBCO substrate in the labeling reaction was 2 nmol or 20 nmol. All reactions were carried out in DMSO at 37 °C and the radiochemical yields (RCYs) were determined by integration of analytical radio-HPLC chromatogram (Fig. S2). Non-radioactive analogs **7**–**9** were prepared by using the azide **1** as a reference for HPLC characterization of ^{125}I-labeled products. [^{125}I]**1** was mixed with 2 nmol of DBCO-amine **4** to give 30% of RCY of [^{125}I]**7** after 30 min and the RCY was increased to 54% in 60 min (entries 1 and 2). When [^{125}I]**1** was reacted with 10-fold larger amount of the substrate **4**, most of the azide was converted to the product ([^{125}I]**7**) within 30 min (entry 3). [^{125}I]**1** was also used in the radiolabeling of tumor targeting peptide. DBCO group conjugated cRGD peptide **5** was used as a model substrate. This peptide was prepared by the synthetic method provided in our previous paper.[21] After reacting **5** with [^{125}I]**1** for 60 min, the obtained RCY of the labeled product [^{125}I]**8** was 52% after 60 min as determined by analytical radio-HPLC (entry 5). Next, we further applied ^{125}I-labeled azide to the radiolabeling of near infrared fluorescent (NIRF) molecule. Reaction of [^{125}I]**1** with 20 nmol of DBCO-Cy5.5 **6** under the same reaction condition provided the product [^{125}I]**9** with 82% and 95% of RCY in 30 min and 60 min, respectively (entries 6 and 7). Somewhat slower conversion rates were observed with **6** in copper-free click reaction than those of **4** due to larger molecular size of DBCO-Cy5.5 **6** than DBCO-amine **4**. Recently various nuclear and optical dual imaging probes have been synthesized for multimodality imaging studies.[24–26] Some previous reports indicated that Cy5.5 could identify some biological targets such as U87MG tumor and arthritic joints without other targeting molecule.[27,28] In the future study, the present labeling reaction can be applied to iodine radioisotope labeling of NIRF dye or NIRF dye conjugated biomolecules which contain a strained