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Development of a new thiol-reactive prosthetic group for site-specific labeling of biomolecules with radioactive iodine

Ha Eun Shim[a,b,d], Sajid Mushtaq[a,c,d], Lee Song[a], Chang Heon Lee[a], Hyosun Lee[b], Jongho Jeon[a,c,*]

[a] Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, Jeongeup 56212, Republic of Korea
[b] Department of Chemistry, Kyungpook National University, Daegu 41566, Republic of Korea
[c] Radiation Biotechnology and Applied Radioisotope Science, University of Science and Technology, Daejeon 34113, Republic of Korea

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ABSTRACT

In this report, we describe the radiosynthesis of a new thiol-targeting prosthetic group for efficient radioactive iodine labeling of biomolecules. Radioiodination using the precursor **3** was performed to obtain ^{125}I-labeled tetrazole **4b** with high radiochemical yield (73%) and radiochemical purity. Using the radiolabeled **4b**, a single free cysteine containing peptide and human serum albumin were labeled with ^{125}I in modest-to-good radiochemical yields (65–99%) under mildly reactive conditions. A biodistribution study of [^{125}I]**7** in normal ICR mice exhibited lower thyroid uptake values than those of ^{125}I-labeled human serum albumin prepared via a traditional radiolabeling method. Thus, [^{125}I]**7** could be employed as an effective radiotracer for molecular imaging and biodistribution studies. The results clearly demonstrate that **4b** has the potential to be effectively implemented as a prosthetic group in the preparation of radiolabeled biomolecules.

In the last few decades, radioactive iodines have been extensively used to perform small molecule and biomolecule radiolabeling,[1,2] and a variety of radioactive iodine-labeled small molecules and biomacromolecules have been developed as specific radioligands for molecular imaging, pharmacokinetics study, and cancer therapy.[3–12] One of the most frequently used methods for the radioiodination of biomolecules generally proceeds as follows: oxidation of radioactive iodine anions, followed by an electrophilic substitution reaction of a phenol or tyrosine residue.[13] Although such direct radioiodination of biomolecules has typically provided good radiochemical results in a short time, the radiolabeled product synthesized via this labeling technique has been shown to subsequently undergo considerable deiodination in animals.[14,15] This process then resulted in the liberated radioactive iodines rapidly accumulating in the thyroid and stomach, thereby producing significant false-positive signals. Moreover, a strong oxidant, which is involved in the electrophilic radiolabeling reaction, was often found to eliminate or reduce the biological activity of the biomolecules.[16] To solve these problems, indirect methods that use several prosthetic groups comprising an activated ester and maleimide have been developed to facilitate efficient conjugation with the amino and thiol groups, respectively.[17–20] Recently, the authors have reported on the implementation of ^{125}I-labeled azide and tetrazine analogs for rapid and efficient radiolabeling of biomolecules and nanomaterials.[21–24] These prosthetic groups were found to not only yield highly efficient radiochemical results, but also provide effective radiotracers for biodistribution and *in vivo* imaging studies. However, to apply these methods, the target molecules must be modified to support other artificial functional groups that can effectively react with the radiolabeled prosthetic group. For example, a *trans*-cyclooctene analog must be conjugated with the biomolecules to react with a radiolabeled tetrazine. These modifications require additional synthetic and purification steps, which generate randomly conjugated functional groups that may reduce the bioactivity of the target molecules. Considering this, there is a need for the development of a new prosthetic group that is able to react with non-modified biomolecules in a site-specific manner. In 2017, the Furdui group reported that (4-(5-methane-sulfonyl-[1,2,3,4]tetrazole-1-yl)-phenol) (MSTP) can be used as a highly reactive thiol blocking agent.[25] The conjugation reaction resulting from the use of this heteroaromatic sulfone has some advantages over that induced by the maleimide chemistry in terms of the reaction rate and thiol group specificity in aqueous media as a maleimide structure can also be reacted with a primary amine group (e.g., lysine side chain) under a weakly basic condition (pH ~ 8). In addition, thiol-maleimide condensation adduct in a conjugated product is known to be potentially unstable under certain physiologically relevant conditions.[26] In consideration of these findings, we purposed to synthesize radiolabeled

* Corresponding author at: 29 Geumgu-gil, Jeongeup, Jeonbuk 56212, Republic of Korea.
E-mail address: jeonj@kaeri.re.kr (J. Jeon).
[d] These authors contributed equally to this work.

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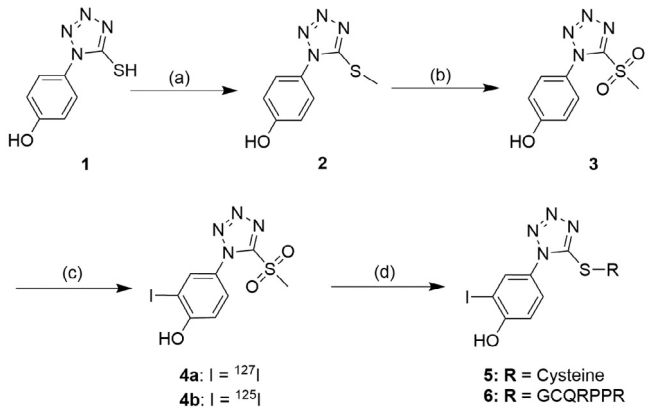

Scheme 1. Synthesis of ^{125}I-labeled MSTP **4b**, and radiolabeling procedure. *Reagent and conditions:* (a) CH_3I, TEA, THF, 0 °C to room temperature, 16 h; (b) Na_2WO_4, H_2O_2, ethanol, 0 °C to room temperature, 16 h, 55% yield from **1**; (c) For **4a**: NaI, chloramine T, DMSO/H_2O (1:1), room temperature, 15 min, 50% yield; For **4b**: [^{125}I]NaI, chloramine T, DMSO/H_2O (1:1), room temperature, 15 min, 73% isolated RCY; and (d) Reaction conditions and results detailed in Table 1.

MSTP and apply it to the rapid and site-specific labeling of cysteine-bearing biomolecules. Thus, we propose a new radioactive iodine-labeled prosthetic group and demonstrate its ability to efficiently radiolabel biomolecules. Additionally, we discuss an initial *in vivo* evaluation of radioiodine-labeled human serum albumin (HSA), as prepared by using MSTP, to investigate its stability with respect to deiodination in animals.

For the preparation of radioactive iodine-labeled MSTP, the sulfone **3** was synthesized from a commercially available tetrazole **1** in two steps, generating a 55% yield (Scheme 1). Because a phenol moiety of the precursor **3** is readily available for reaction with the iodo (I^+) species, it is implemented in the iodination reaction without further modification of the structure. For HPLC characterization of the radioiodinated product, non-radioactive analog **4a** was prepared by inducing an electrophilic aromatic substitution reaction. Radioiodination of phenol **3** was accomplished by using [^{125}I]NaI and chloramine T to a mixture of dimethyl sulfoxide (DMSO) and water (1:1) and letting it settle for 15 min at room temperature. Following HPLC purification of the crude product, we obtained the ^{125}I-labeled product **4b**, with a specific radioactivity of 74 GBq/μmol and 73 ± 5% radiochemical yield (RCY, n = 3) (Fig. 1). Furthermore, radiochemical purity was found to exceed 99%, as determined via analytical radio-HPLC.

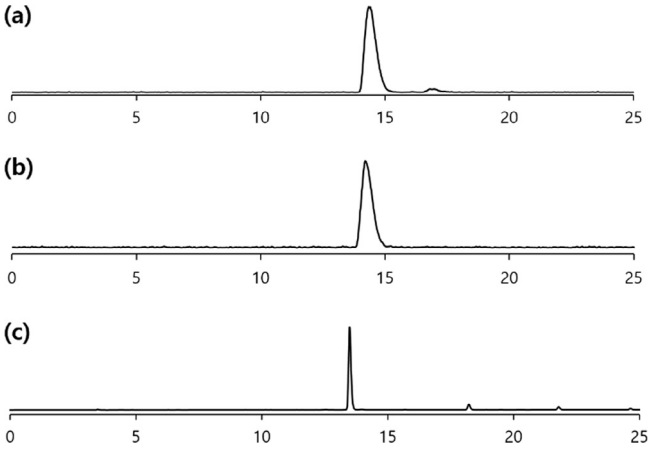

Fig. 1. Analytical-HPLC chromatograms (for 25 min). (a) Radio-HPLC-chromatogram of the crude product, (b) Radio-HPLC-chromatogram of the purified product **4b**, and (c) UV-HPLC-chromatogram of the standard compound **4a**.

The radiolabeling efficiency of **4b** was evaluated by using L-cysteine and cysteine-bearing model peptide, which has previously been implemented in an apoptosis imaging study.[27] The labeling reaction was carried out in H_2O/DMSO (9:1) solution, and the pH of the reaction mixture was adjusted to approximately 8 by adding aqueous $NaHCO_3$ solution (Scheme 1). The RCY was determined by performing radio-HPLC integration, and the radiolabeled products (i.e., [^{125}I]**5** and [^{125}I]**6**) were confirmed by comparing the HPLC to that of the corresponding non-radioactive ^{127}I-analogs. To examine the thiol group chemoselectivity, **4b** was respectively incubated with 1 mM of cysteine and glycine. As is shown in Table 1, the radiolabeled sulfone was able to efficiently react with cysteine after a period of 30 min at 37 °C (Entry 1); conversely, **4b** remained intact in the presence of an excess amount of glycine (Entry 2), suggesting that MSTP exclusively reacted with a thiol group. Although **4b** was found to be consumed in less than 30 min at a relatively high temperature of 60 °C, an impurity peak, which reduced the RCY, was also detected in the HPLC chromatogram (Fig. S3). Therefore, the peptide labeling reaction was performed at 37 °C. We added tris(2-carboxyethyl)phosphine (TCEP) in the reaction mixture to minimize the formation of a disulfide bond in the peptide. Subsequent radio-HPLC analysis showed that the conversion yields were concentration-dependent, and notably, that full conversion of **4b** to [^{125}I]**6** was achieved in 30 min under the condition of 2.0 nmol (0.1 mM) of peptide substrate (Entries 3–5). Because a cysteine residue can be easily incorporated into the process of solid-phase peptide synthesis, it is believed that MSTP will further optimize site-specific radiolabeling of the biologically active peptides.

Next, we investigated the site-specific ^{125}I-labeling of protein by using **4b** in the presence of HSA (Scheme S1). HSA is composed of 585 amino acids (66.5 kDa) that only contain a single free cysteine (Cys34), which can react with the MSTP structure. Thus, HSA is considered to be a suitable model protein to evaluate the labeling efficiency and thiol selectivity of the proposed labeling method. Moreover, HSA has been extensively investigated in various biomedical research studies, such as studies on the development of a molecular imaging probe, therapeutic agent, and drug delivery system.[28,29] For example, several types of radiolabeled HSA, which were respectively radiolabeled by using ^{68}Ga, ^{18}F, and ^{99m}Tc, were utilized in the blood vessel or sentinel lymph node imaging agent. Additionally, ^{125}I- or ^{131}I-labeled HSA have come to be known as effective radiotracers to employ in the measurement of the total blood and plasma volume of patients.[30,31] In a radiolabeling reaction, **4b** (0.37 MBq) was mixed with 100 nmol of protein at 37 °C to produce ^{125}I-labeled HSA; the conversion yield, which is listed in Table 2, was determined by radio-TLC integration. After a period of 1 h, the observed RCY was 13%; however, by 18 h the RCY was observed to have increased to 65% (Entries 2 and 3). The purification was accomplished by performing size exclusion chromatography (PD-10 column) on the saline-eluted column prior to centrifugal filtration to obtain ^{125}I-labeled HSA with a 55% RCY. However, it should be noted that, because a portion of the radiolabeled protein remained in the column during the purification step, the isolated RCY was lower than the conversion yield as determined by radio-TLC. In comparison, the same radiolabeling reaction was performed in the presence of an excess amount of **4a**; radio-TLC analysis revealed that there was no radiolabeled product under this condition, as a free thiol group in HSA predominantly reacted with non-radioactive MSTP (Entry 4). Thus, these results indicate that site-specific ^{125}I-labeling of HSA was accomplished by using **4b**.

A biodistribution study was then carried out using the [^{125}I]**7** prepared by labeling HSA with **4b**. Each mouse was injected with 0.37 MBq of radiolabeled HSA, and the biological uptake in the blood and nine different organs was quantified at five time points (5 mice per each time point). As is detailed in Table S1, there was large initial uptake in the blood (34.7% ID/g tissue); moreover, although there was significant reduction, a high level of radiolabeled HSA was retained up to 24 h after injection (6.45% ID/g tissue), indicating that **7** circulated through