Imaging Agents

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One-Pot Synthesis of an ^{125}I-Labeled Trifunctional Reagent for Multiscale Imaging with Optical and Nuclear Techniques**

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Molecular imaging has transformed biomedical research, drug development, and clinical practice. Yet, our understanding of biological processes in vivo remains limited because no imaging modality meets the need for high resolution, high sensitivity, and deep tissue penetration. The ability to study biological processes across the cellular and macroscopic scales therefore remains a fundamental goal for molecular imaging. Tracers equipped with dual reporter groups can overcome some of the current restraints by allowing modalities with overlapping strengths to be used in combination.[1–3] Here, we report a new approach to multiscale multimodal imaging based on one-pot synthesis of dual optical and nuclear labeling reagents.

The ideal labeling method should be technically simple and versatile, and should preserve the biological properties of the parent molecule. To date, attempts to prepare dual optical and nuclear imaging agents have relied on stepwise conjugation of a fluorescent group and a radionuclide to the biological "hook", using nanoparticles or small-molecule linkers as the central building block.[4,5] This modular approach is technically challenging, and in the case of nanoparticle-based probes, often results in unfavorable pharmacokinetic properties.[6,7] Efficient assembly of multifunctional biocompatible small-molecule scaffolds is therefore key to overcome the experimental hurdles of current techniques.

We envisaged that a three-component reaction combining a fluorescent group, a radioactive element, and a group for bioconjugation could yield dual-labeling reagents in a single step, providing a more suitable platform for tracer development (Figure 1). One chemical reaction that can achieve this is the copper-catalyzed reaction of azides, alkynes, and

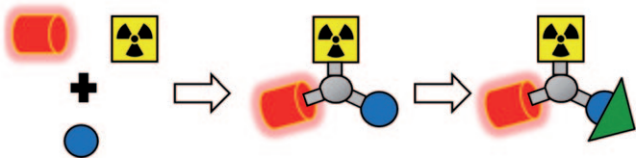
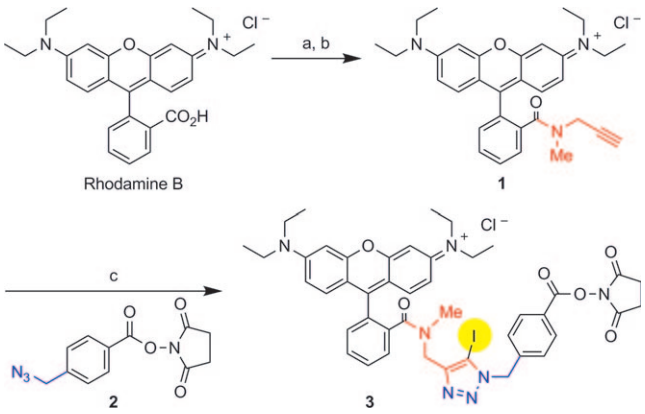


Figure 1. Reaction of a fluorescent group (red), a radioactive element (yellow), and a group for bioconjugation (blue) to yield a dual-labeling reagent. Subsequent reaction with biomolecules (green) provides dual optical and nuclear tracers.

electrophilic iodine to give 5-iodo-1,2,3-triazoles.[8,9] Iodine is attractive for multiscale imaging as its numerous radioisotopes enable a wide range of applications: the low-energy γ-emission of ^{125}I ($t_{1/2}=60$ days) for autoradiography, the more energetic γ-rays of ^{123}I ($t_{1/2}=13.2$ h) for single photon emission computed tomography (SPECT), β particles from ^{131}I ($t_{1/2}=8.0$ days) for radioimmunotherapy (RIT), and β^+ from ^{124}I ($t_{1/2}=4.18$ days) for positron emission tomography (PET).

We demonstrated our new approach to multifunctional labeling reagents by using the chemical route depicted in Scheme 1. Rhodamine B was chosen as the fluorescent group as it has excellent properties for imaging, including broad fluorescence in the red spectrum of visible light, high quantum yield, and good photostability.[10] The alkyne-



Scheme 1. Synthesis of the non-radioactive labeling reagent **3**. Reagents: a) oxalyl chloride, CH_2Cl_2; b) *N*-methylpropargylamine, Na_2CO_3, CH_2Cl_2; c) CuI, NIS, TEA, CH_3CN.

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functionalized rhodamine derivative **1** was obtained in 72% overall yield by treatment of rhodamine B with oxalyl chloride, and reaction of the resulting acid chloride with *N*-methylpropargylamine. 4-Azidomethyl-*N*-succinimidyl benzoate (**2**) was prepared in 65% yield in two steps from 4-chloromethylbenzoic acid as previously described.[11] Coupling of the two "click partners" **1** and **2** in the presence of CuI, *N*-iodosuccinimide (NIS), and triethylamine (TEA) provided the nonradioactive labeling reagent **3** in 92% yield.

For the radiochemical reaction we carried out a series of experiments with the aim to identify a new catalytic system that would allow formation of [^{125}I]-**3** directly from aqueous non-carrier-added (n.c.a.) [^{125}I]NaI. Initially, we used *N*-bromosuccinimide (NBS) to oxidize n.c.a. [^{125}I]NaI and treated the resulting mixture with solutions of CuCl, TEA, rhodamine **1** and azide **2** in DMF (Table 1, entry 1). Under these conditions no radiochemical reaction was observed. When investigating other oxidizing reagents we discovered that the use of $CuCl_2$ led to formation of the desired product [^{125}I]-**3** in 21% analytical radiochemical yield (RCY) within 30 min at room temperature (Table 1, entry 2). Encouraged by these results we attempted to use $CuCl_2$ as the sole source of copper. In DMF the reaction proceeded to give [^{125}I]-**3** in 14% analytical RCY, whereas moderate to good yields were obtained in acetonitrile (Table 1, entries 3 and 4). The addition sequence was found to influence both the efficiency and reproducibility of the reaction, and when alkyne **1** was combined with $CuCl_2$ and TEA before addition to [^{125}I]NaI, [^{125}I]-**3** was consistently obtained in good analytical yield (Table 1, entry 5). Increasing the reaction time to 90 min increased the analytical RCY to (80 ± 3)% (Table 1, entry 6; Figure 2). Following purification of the reaction mixture with radio-HPLC,[^{125}I]-**3** was isolated in (72 ± 4)% RCY with excellent radiochemical purity (> 98%). When we started with 20–25 MBq of [^{125}I]NaI, the specific activity of [^{125}I]-**3** was in the range of 2–3 GBq μmol^{-1}. Further investigation of the reaction revealed that the ratio of TEA to $CuCl_2$ was critical. The use of more than 2 equivalents of TEA led to a sharp drop in yields, whereas 1 equivalent gave the desired product only in trace amounts (Table 1, entries 7 and 8).

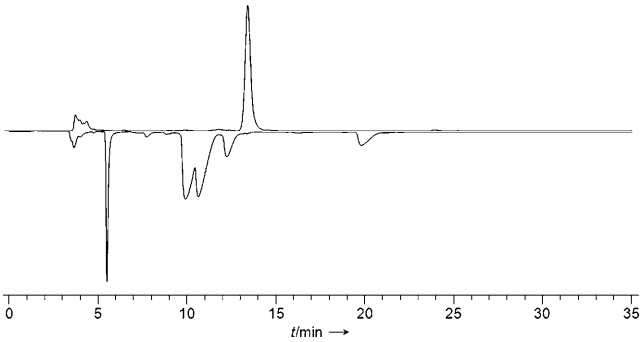

Figure 2. HPLC trace of labeling reaction mixture (Table 1, entry 6) showing radioactivity (positive trace) and UV absorption at 254 nm (negative trace) Azide **2** at 5.47 min, alkyne **1** at 9.90 min, and [^{125}I]-**3** at 13.4 min. Non-radioactive side products at 10.62, 12.24, and 19.59 min.

Table 1: Optimization of the radiochemical reaction conditions.

Entry	Solvent/water 10:1	Catalyst system	Radiochemical yield[e]
1[a]	DMF	CuCl/NBS/TEA (1:1:1.5)	0%
2[a]	DMF	CuCl/$CuCl_2$/TEA(1:1:2.2)	21%
3[a]	DMF	$CuCl_2$/TEA (1:1.5)	14%
4[a]	MeCN	$CuCl_2$/TEA (1:1.5)	(33±15)% (n=3)
5[b]	MeCN	$CuCl_2$/TEA (1:1.5)	(56±7)% (n=3)
6[b]	MeCN	$CuCl_2$/TEA (1:1.5)	(80±3)% (n=8)[c] (72±4)% (n=5)[c,d]
7[b]	MeCN	$CuCl_2$/TEA(1:2.5)	13%
8[b]	MeCN	$CuCl_2$/TEA(1:1.0)	< 5%

[a] A solution of rhodamine **1** (1 μmol) and azide **2** (1 μmol) was added to a mixture of the copper catalyst, TEA, and [^{125}I]NaI and left to react for 30 min. [b] A solution of rhodamine **1** (1 μmol), $CuCl_2$ (1 μmol), and TEA was added to a mixture of azide **2** (1 μmol) and [^{125}I]NaI and left to react for 30 min. [c] Reaction time 90 min. [d] RCY of isolated product. [e] RCY is a mean value of n experiments ± standard deviation; when not specified n = 1.

For proof-of-concept imaging studies we coupled our dual-labeling reagent [^{125}I]-**3** to the carcinoembryonic antigen (CEA) specific antibody A5B7 (150 kDa). CEA is expressed by most gastrointestinal tumors, and A5B7 and its fragments are in regular preclinical and clinical use.[12–14] Antibody labeling was achieved by incubating a solution of A5B7 with a mixture of the non-radioactive labeling reagent **3** (20 equiv) and [^{125}I]-**3** (20 MBq) at room temperature for 1 hour. The resulting solution was purified with a size-exclusion column to give the dual-labeled imaging agent [^{125}I]-**3**/A5B7 in 22% RCY with an average of six to eight fluorescent groups per antibody.[^{125}I]-**3**/A5B7 was subsequently evaluated in mice bearing human colorectal xenografts.[13] The isotype-matched dual-labeled antibody [^{125}I]-**3**/MOPC was included as a negative control. The overall organ distribution of [^{125}I]-**3**/A5B7 (see Figure S7 in the Supporting Information) was similar to that of the antibody when it was labeled with ^{125}I alone using the Chloramine-T method.[13,15] The tumor uptake of [^{125}I]-**3**/A5B7 24 h and 48 h after injection was (13.9 ± 4.5)% ID g^{-1} (ID g^{-1}: injected dose per gram of tissue) and (12.3 ± 1.3)% ID g^{-1}, respectively. Good clearance was observed for all organs apart from the liver, which had an increased uptake, (6.99 ± 0.16)% ID g^{-1} at 48 h). Interestingly, the increased uptake by the liver was accompanied by a more rapid blood clearance, resulting in tumor-to-blood ratios of 3.5:1 and 4.8:1 at 24 h and 48 h postinjection, respectively. In contrast, the tumor uptake of [^{125}I]-**3**/MOPC was (1.10 ± 0.02)% ID g^{-1} at 48 h postinjection, with a tumor-to-blood ratio of 0.28:1. Frozen tumor sections were used to visualize antibody localization across the whole tumor 6 h after injection of 50 μg of [^{125}I]-**3**/A5B7. The distribution of radioactivity (Figure 3A) was in good agreement with the fluorescence signal (Figure 3B), and demonstrated antibody uptake across the viable areas of the tumor (Figure 3D). High-magnification fluorescence imaging (Figure 3C) showed that the antibody was associated mainly with tumor cells around perfused blood vessels, but also had started to diffuse away from vessels into the tumor mass at this early time post-injection.

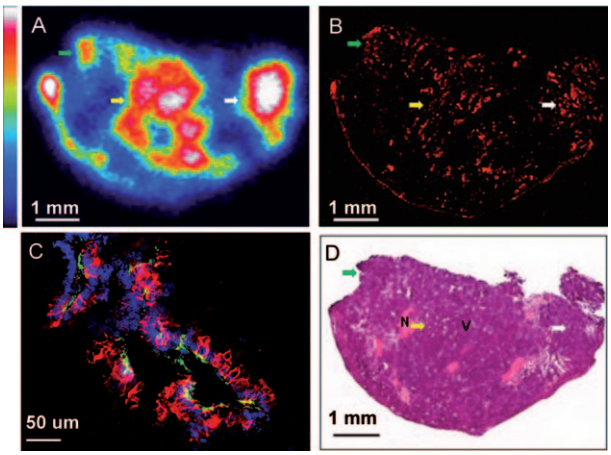

Figure 3. A) Radioluminograph (color bar shows counts per pixel from 0 (blue) to 6000 (white)) and B) fluorescence image of adjacent tumor sections demonstrating uptake of [^{125}I]-**3**/A5B7 6 h postinjection of 50 μg antibody; C) high-power image; red: distribution of [^{125}I]-**3**/A5B7 antibody; blue: Hoechst staining showing perfused blood vessels; green: CD31 staining showing blood vessel distribution. D) H&E (haematoxylin and eosin) staining showing tumor histology: viable tumor (V); necrosis (N). Colored arrows (color definitions in (A)) indicate that the distribution of radioactivity colocalizes with the fluorescence signal (B) and demonstrates antibody uptake in viable tumor (D).

In conclusion, we have developed a new approach to multiscale imaging based on the one-pot formation of dual optical and nuclear labeling reagents. The concept has been demonstrated using a highly efficient and rapid Cu^{II}-mediated three-component radiochemical reaction that yields 5-[^{125}I]iodo-1,4,5-trisubstituted-1,2,3-triazoles directly from aqueous [^{125}I]NaI. The method reported is technically simple, inexpensive, and robust, and can therefore make multiscale imaging accessible as a routine tool for biomedical research. The added flexibility of trisubstituted triazoles brings a new dimension to the field of molecular imaging with the potential to transform tracer design and harvest the synergies between individual modalities.

Experimental Section

Synthesis of [^{125}I]-**3**: A solution of copper(II) chloride (134 μg, 1.0 μmol) and triethylamine (151 μg, 1.5 μmol) in acetonitrile (40 μL) was added to rhodamine **1** (530 μg, 1.0 μmol). After 5 min the resulting solution was added to a mixture of the azide **2** (275 μg, 1.0 μmol) in acetonitrile (20 μL) and [^{125}I]NaI (20–40 MBq) in water (6.0 μL). After 90 min the reaction mixture was diluted with water and acetonitrile (10:1, 1.0 mL) and the resulting solution was purified by HPLC using a ZORBAX column (300SB-C18, 9.4 × 250 mm, 5 μm) with the following eluent: water (0.1 % formic acid) as solvent A and methanol (0.1 % formic acid) as solvent B, going from 60 % of B to 70 % of B in 30 min and going back to 60 % of B in 2 min and remaining at 60 % of B for an additional 3 min with a flow rate of 3 mL min^{-1}. The retention time of the compound [^{125}I]-**3** was 13.40 min. The labeled compound co-eluted with the non-radioactive reference compound. For antibody labeling the fraction that contained [^{125}I]-**3** was diluted with water (12 mL), the solution was passed though a Sep-Pak C18 light cartridge (Waters), the cartridge was washed with water (5 mL), and the radioactive product was eluted with acetonitrile (0.5 mL). The solvents were removed by a stream of nitrogen prior to labeling.

Antibody labeling: A solution of non-radioactive labeling reagent **3** (62 μg, 20 equiv) in DMF (13 μL) was added to [^{125}I]-**3** (20 MBq) followed by a solution of A5B7 (507 μg) in phosphate buffer (130 μL, pH 7) and sodium carbonate buffer (130 μL, 1.0 M, pH 9.0). The reaction mixture was incubated at room temperature for one hour and purified using a PD MiniTrap G-25 column (GE Healthcare) following the manufacturer's instructions. The dual-labeled [^{125}I]-**3**/A5B7 (4.74 MBq, six to eight fluorescent groups per antibody) was collected in a total volume of 0.8 mL. The recovery efficiency of the purification step was determined to be (80 ± 7) % (n = 3) in separate experiments. Under the conditions used the dual-labeling reagent [^{125}I]-**3** was completely retained on the size-exclusion column. The number of fluorescent groups incorporated was calculated by taking into account the specific activity of the dual-labeling reagent [^{125}I]-**3**, and correcting the RCY for residual activity in the reaction vial and loss of the antibody on the size-exclusion column.

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