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$^{67/68}$Ga-Labeling Agent That Liberates $^{67/68}$Ga-NOTA-Methionine by Lysosomal Proteolysis of Parental Low Molecular Weight Polypeptides to Reduce Renal Radioactivity Levels

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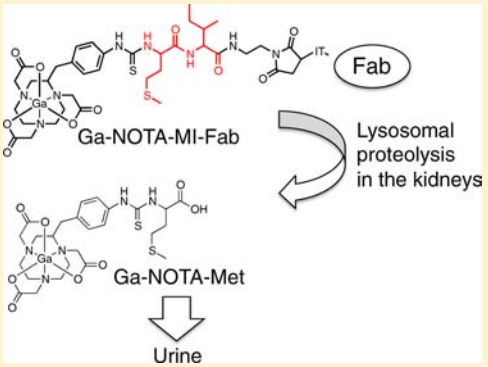
ABSTRACT: The renal localization of gallium-67 or gallium-68 ($^{67/68}$Ga)-labeled low molecular weight (LMW) probes such as peptides and antibody fragments constitutes a problem in targeted imaging. Wu et al. previously showed that ^{67}Ga-labeled *S*-2-(4-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (SCN-Bz-NOTA)-conjugated methionine (^{67}Ga-NOTA-Met) was rapidly excreted from the kidney in urine following lysosomal proteolysis of the parental ^{67}Ga-NOTA-Bz-SCN-disulfide-stabilized Fv fragment (*Bioconjugate Chem.*, (**1997**) 8, 365−369). In the present study, a new $^{67/68}$Ga-labeling reagent for LMW probes that liberates $^{67/68}$Ga-NOTA-Met was designed, synthesized, and evaluated using longer-lived ^{67}Ga in order to reduce renal radioactivity levels. We employed a methionine-isoleucine (MI) dipeptide bond as the cleavable linkage. The amine residue of MI was coupled with SCN-Bz-NOTA for ^{67}Ga-labeling, while the carboxylic acid residue of MI was derivatized to maleimide for antibody conjugation in order to synthesize NOTA-MI-Mal. A Fab fragment of the anti-Her2 antibody was thiolated with iminothiolane, and NOTA-MI-Mal was conjugated with the antibody fragment by maleimide−thiol chemistry. The Fab fragment was also conjugated with SCN-Bz-NOTA (NOTA-Fab) for comparison. ^{67}Ga-NOTA-MI-Fab was obtained at radiochemical yields of over 95% and was stable in murine serum for 24 h. In the biodistribution study using normal mice, ^{67}Ga-NOTA-MI-Fab registered significantly lower renal radioactivity levels from 1 to 6 h postinjection than those of ^{67}Ga-NOTA-Fab. An analysis of urine samples obtained 6 h after the injection of ^{67}Ga-NOTA-MI-Fab showed that the majority of radioactivity was excreted as ^{67}Ga-NOTA-Met. In the biodistribution study using tumor-bearing mice, the tumor to kidney ratios of ^{67}Ga-NOTA-MI-Fab were 4 times higher (6 h postinjection) than those of ^{67}Ga-NOTA-Fab. Although further studies including the structure of radiometabolites and/or cleavable linkages are required, the results of the present study indicate that the current chemical design is applicable to the development of ^{67}Ga-labeled Fabs for low renal radioactivity levels.



INTRODUCTION

Gallium radioisotopes are of great interest for molecular imaging. Gallium-67 (^{67}Ga) is a γ-emitter (half-life: 3.3 d) that is currently used in the diagnosis of infection and inflammatory processes as well as tumor imaging by SPECT.[1,2] Gallium-68 (^{68}Ga) is a PET radioisotope that is obtained from long-lived ^{68}Ge/^{68}Ga generator systems, allowing the potentially cost-effective production of ^{68}Ga radiotracers far away from a cyclotron facility.[3] Its physical half-life of 67.7 min is suitable for labeling low molecular weight (LMW) probes such as small peptides and antibody fragments with rapid pharmacokinetics.[3−6] To prepare $^{67/68}$Ga-labeled LMW probes, a bifunctional chelating agent is conjugated with an LMW probe, followed by a complexation reaction between $^{67/68}$Ga and the conjugate. Of the various bifunctional chelating agents reported so far, the macrocyclic chelator, 1,4,7-triazacyclononane-*N*,*N*′,*N*″-triacetic acid (NOTA) is preferably used due to the formation of a stable gallium complex under mild reaction conditions.[3−6]

Radiolabeled LMW probes allow for rapid targeting and uniform distribution in target tissues.[7,8] However, the use of LMW probes, particularly those labeled with metallic radionuclides, has been associated with high and persistent radioactivity levels in the kidneys, which compromises target visualization near the kidneys.[8,9] Previous studies demonstrated that radiolabeled antibody fragments were reabsorbed by proximal tubules via luminal endocytosis after glomerular filtration. Persistent renal radioactivity levels were attributed to the long residence time of radiometabolites generated following the lysosomal proteolysis of radiolabeled antibody fragments in renal cells.[10−13] These findings indicated that the in vivo behaviors of radiometabolites played crucial roles in renal radioactivity levels and also that the liberation of a radiometabolite by rapid excretion from the kidneys in urine following the lysosomal degradation of parental $^{67/68}$Ga-labeled LMW probes may be useful for reducing renal radioactivity levels.

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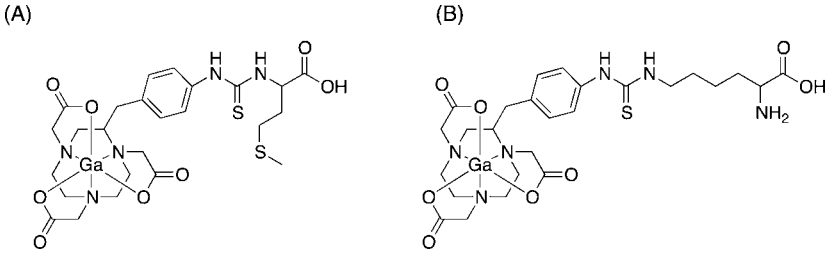

Figure 1. Structures of Ga-NOTA-Met (A) and Ga-NOTA-Lys (B).

Approaches using metabolizable linkers cleaved by lysosomal enzymes, ester bonds, or diester bonds have been employed as metabolizable linkers.[10−14] However, the stability of ester bonds as metabolizable linkers was shown to be dependent on the molecular size of probes and the development of plasma stable metabolizable linkers was also needed for LMW probes.[15] Moreover, few studies have examined radiolabeling reagents using macrocyclic chelates that release radiometabolites designed for rapid excretion from the kidneys in urine following the lysosomal degradation of parental radiolabeled LMW probes.

Wu et al. previously reported that when a ^{67}Ga-labeled disulfide-linked Fv fragment of an antibody (dsFv) using 2-(*p*-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (SCN-Bz-NOTA) as a bifunctional chelating agent was administered, the ^{67}Ga-NOTA-Bz-SCN-adduct of methionine (^{67}Ga-NOTA-Met, Figure 1) and ^{67}Ga-NOTA-Bz-SCN-adduct of lysine (^{67}Ga-NOTA-Lys, Figure 1) were generated as radiometabolites in the kidneys because the *N*-terminal amino acid of the dsFv was methionine.[12] This study also indicated that the former had a significantly shorter residence time in the murine kidney than the latter. These findings suggested that $^{67/68}$Ga labeling reagents for LMW probes that liberate $^{67/68}$Ga-NOTA-Met following proteolysis of the parental compound in lysosomes may be useful for reducing renal radioactivity levels after Ga-labeled LMW probes are injected. Since the second amino acid from the *N*-terminal of the light chain of the dsFv used by Wu et al. was isoleucine (Ile),[16] NOTA-Met-Ile-Maleimide (NOTA-MI-Mal, Figure 2) was designed, synthesized, and conjugated to the Fab fragment of a mAb against Her2 after the treatment of the antibody fragment with 2-iminothiolane (NOTA-MI-Fab). In this molecular design, Ga-NOTA-Met was released from the conjugate when the peptide bond between methionine and isoleucine in Ga-labeled-NOTA-MI-Fab (Ga-NOTA-MI-Fab) was cleaved. The tissue distribution of radioactivity and radiometabolites in the kidneys and urine after an injection of Ga-NOTA-MI-Fab were compared to those of a Ga-NOTA-SCN-conjugated Fab fragment (Ga-NOTA-Fab). The validity of the new Ga-labeling reagent of LMW probes, NOTA-MI-Mal, in reducing the renal radioactivity levels was estimated.

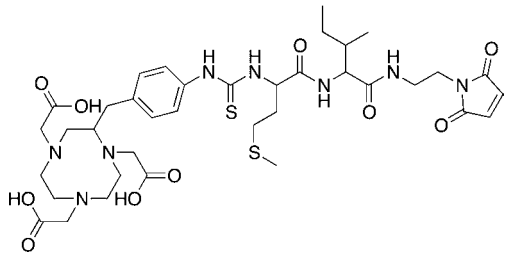

Figure 2. Structure of NOTA-MI-Mal.

RESULTS

Preparation of NOTA-MI-Fab and NOTA-Fab (Scheme 1). NOTA-MI-Mal was synthesized by reacting SCN-Bz-NOTA with Met-Ile-Mal. The conjugation of Fab with NOTA-MI-Mal was performed by maleimide−thiol chemistry using thiol groups generated by the addition of 2-IT. The number of NOTA-MI introduced per molecule of Fab was estimated to be 0.75−0.9 by measuring the thiol groups of the Fab fragment before and after the conjugation reaction. The conjugation of SCN-Bz-NOTA with the Fab fragment was achieved by reacting isothiocyanate with primary amine residues of the Fab fragment. Unpurified NOTA-Fab conjugates labeled by ^{67}Ga and subsequent analyses by thin layer chromatography estimated the number of SCN-Bz-NOTA attached per molecule of Fab to be 1.0.

Preparation of ^{67}Ga-NOTA-MI-Fab and ^{67}Ga-NOTA-Fab. Both NOTA-MI-Fab and NOTA-Fab were labeled by ^{67}Ga in the presence of acetate and the resulting compounds were analyzed by TLC, CAE, and SE-HPLC. The radiochemical yields of both ^{67}Ga-labeled-Fabs were over 95%. The radiochemical purities of both ^{67}Ga-labeled-Fabs were over 99% with centrifugation and column purification after the addition of EDTA.

Stability Estimation of ^{67}Ga-Labeled Fabs. After ^{67}Ga-labeled Fabs had been incubated for 24 h in murine plasma at 37 °C, more than 90% of radioactivity remained in intact fractions in both cases.

Biodistribution Study Using Normal Mice. The biodistribution of radioactivity after the injection of ^{67}Ga-NOTA-MI-Fab and ^{67}Ga-NOTA-Fab to normal mice is summarized in Table 1. The radioactivity levels of ^{67}Ga-NOTA-MI-Fab in blood were similar to those of ^{67}Ga-NOTA-Fab. Although ^{67}Ga-NOTA-Fab showed high radioactivity levels in the kidney from 1 to 6 h postinjection, ^{67}Ga-NOTA-MI-Fab showed significantly lower renal radioactivity levels during the same time period. More ^{67}Ga-NOTA-MI-Fab was excreted in urine (55.63%ID) than ^{67}Ga-NOTA-Fab (20.81% ID).

Radiolabeled Species in Urine and the Kidney. Figure 3 shows radiochromatograms of urine samples obtained 6 h after the injection of ^{67}Ga-NOTA-IT-Fab. SE-HPLC analyses revealed that 95% of the radioactivity excreted in urine was observed in the low molecular weight fraction with a retention time of 24 min. RP-HPLC analyses showed that most of the radioactivity in urine had a retention time that was identical to that of Ga-NOTA-Met (14.5 min).

The supernatant of each kidney homogenate was extracted with efficiencies of over 70% for all experiments. When the

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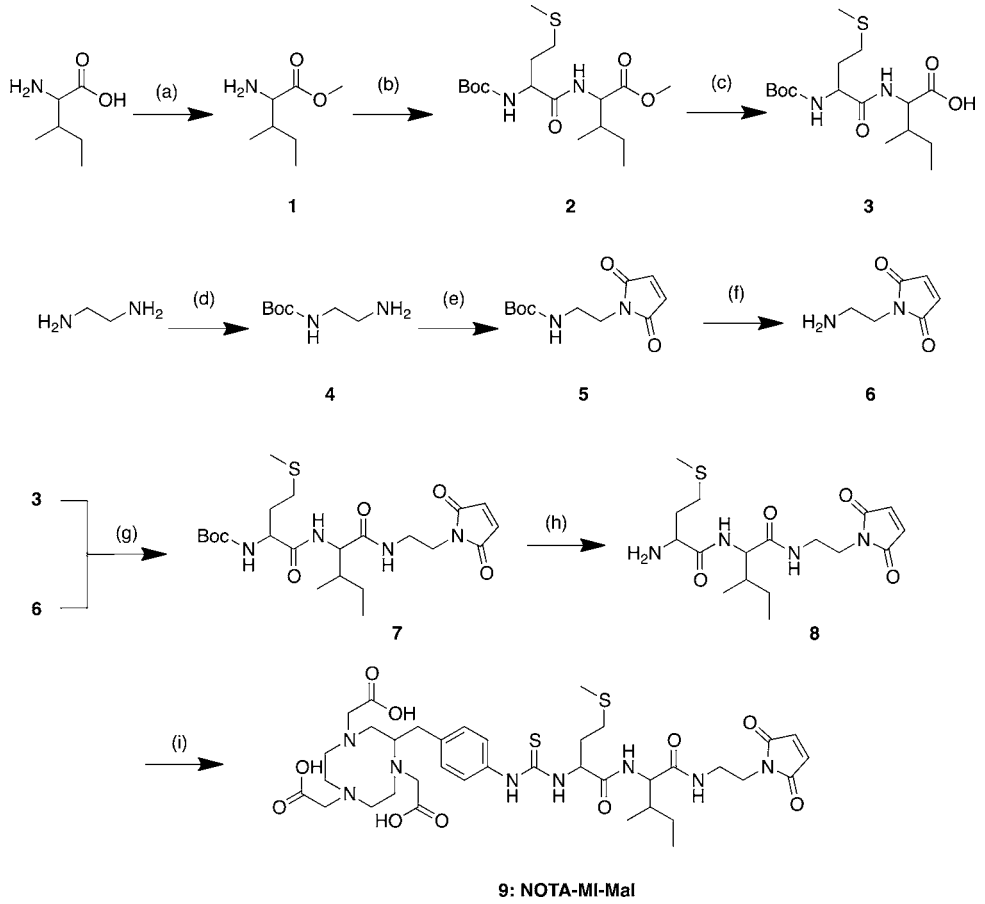
Scheme 1. Synthetic Procedure to Prepare NOTA-MI-Mal[a]



[a](a) $SOCl_2$, MeOH; (b) Boc-Met, HOBt, DCC; (c) 4 N NaOH/EtOH; (d) Boc_2O; (e) NMCM, $NaHCO_3$; (f) 4 N HCl/ethyl acetate; (g) HOBt, DCC; (h) 4 N HCl/ethyl acetate; (i) NOTA-Bz-SCN, triethylamine.

Table 1. Biodistribution of Radioactivity in Mice after an Injection of ^{67}Ga-NOTA-MI-Fab and ^{67}Ga-NOTA-Fab[a]

	time after the injection			
	10 min	1 h	3 h	6 h
		^{67}Ga-NOTA-MI-Fab		
blood	16.56 (3.90)	2.94 (0.13)*	1.02 (0.11)*	0.59 (0.07)
liver	3.38 (0.28)*	1.49 (0.11)	0.99 (0.15)	0.61 (0.11)*
kidney	62.74 (7.29)	85.34 (11.68)*	28.34 (5.22)*	12.41 (0.39)*
intestine	0.69 (0.19)	1.02 (0.21)	1.53 (0.27)*	2.41 (0.43)*
urine[b]				55.63 (9.35)*
feces[b]				1.26 (1.41)
kidney/blood	3.94 (0.89)	28.99 (3.82)	28.18 (6.86)*	20.96 (2.55)*
		^{67}Ga-NOTA-Fab		
blood	14.81 (1.17)	3.68 (0.23)	1.27 (0.16)	0.65 (0.08)
liver	4.61 (1.23)	1.30 (0.12)	2.86 (0.28)	2.75 (0.49)
kidney	61.59 (7.00)	114.98 (6.25)	117.41 (10.6)	101.02 (6.46)
intestine	2.09 (0.59)	3.44 (0.21)	2.20 (0.20)	3.28 (0.59)
urine[b]				20.81 (2.72)
feces[b]				0.69 (1.10)
kidney/blood	4.19 (0.68)	31.39 (2.74)	92.70 (1.19)	157.59 (22.8)

[a]Tissue radioactivity is expressed as % ID/g for each group (n = 3–4); results are expressed as the mean (SD). Significance was determined by an unpaired Student's t-test; (*) $p < 0.05$ significantly different from ^{67}Ga-NOTA-Fab. [b]Expressed as %ID.

radiolabeled species in the kidney at 1 and 6 h after the injection of ^{67}Ga-labeled Fabs were analyzed by SE-HPLC in each case, over 95% of radioactivity was eluted in the low molecular weight fractions (data not shown). Figure 4 shows the RP-HPLC chromatograms of kidney samples after filtration through a 10 000 cutoff membrane. The majority of radioactivity in the low molecular weight fractions after the administration of ^{67}Ga-NOTA-MI-Fab was detected at a

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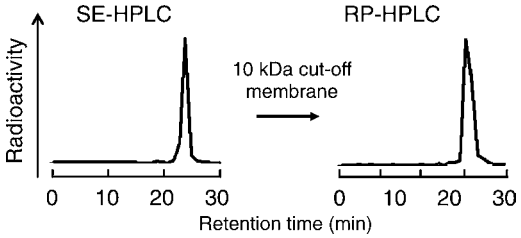

Figure 3. Radioactivity profiles of urine collected 6 h after the injection of ^{67}Ga-NOTA-MI-Fab by SE-HPLC and RP-HPLC. Urine samples were analyzed after filtration through a polycarbonate membrane with a pore diameter of 0.45 μm (SE-HPLC) and after filtration through a 10 kDa cutoff membrane (RP-HPLC).

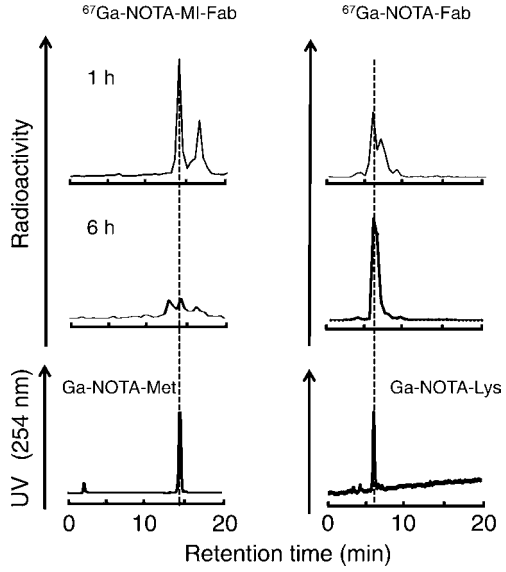

Figure 4. RP-HPLC radioactivity and UV profiles of kidney homogenates 1 and 6 h after the injection of ^{67}Ga-labeled Fab fragments. Samples were analyzed after filtration through a 10 kDa cutoff membrane.

retention time that was similar to that of Ga-NOTA-Met at 1 h postinjection. However, this peak decreased at 6 h postinjection. On the other hand, the majority of radioactivity in the low molecular weight fractions after the administration of ^{67}Ga-NOTA-Fab was detected at a retention time that was similar to that of Ga-NOTA-Lys at both 1 and 6 h postinjection.

Biodistribution in Tumor-Bearing Mice. The biodistribution of radioactivity after the administration of ^{67}Ga-NOTA-MI-Fab and ^{67}Ga-NOTA-Fab in nude mice bearing NCI-H2170 xenografts is summarized in Figure 5. No significant differences were observed in tumor uptake, blood levels, or tumor/blood ratios between the two ^{67}Ga-labeled Fabs. However, the tumor/kidney ratio of ^{67}Ga-NOTA-MI-Fab registered 4 times higher than that of ^{67}Ga-NOTA-Fab at 6 h postinjection.

DISCUSSION

Of the various bifunctional chelating agents reported for $^{67/68}$Ga to date, the macrocyclic chelator, NOTA, is used preferably due to the formation of a stable gallium complex under mild reaction conditions.[3−6] Wu et al. also demonstrated that ^{67}Ga-NOTA-Met was rapidly excreted from the kidneys in urine following lysosomal proteolysis of the parental ^{67}Ga-NOTA-Bz-SCN-disulfide-stabilized Fv fragment.[12] In the present study,

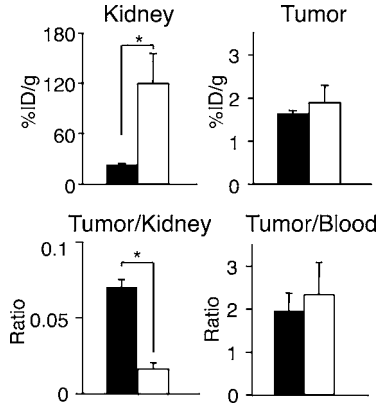

Figure 5. Bar graphs (top) showing %ID/g of kidney and tumor and (bottom) showing tumor/kidney and tumor/blood ratios of radioactivity 6 h after the injection of ^{67}Ga-NOTA-MI-Fab (black) and ^{67}Ga-NOTA-Fab (white) into nude mice (n = 3) bearing NCI-H2170 lung squamous cell carcinoma. Significance was determined using an unpaired Student's t-test (*: $p < 0.05$ significantly different from ^{67}Ga-NOTA-Fab).

the molecular design of Ga-labeling reagents for LMW probes that liberate Ga-NOTA-Met after proteolysis of the parental probe in lysosomes was evaluated for their ability to reduce renal radioactivity levels after an injection of Ga-labeled LMW probes.

A metabolizable linker in Ga-labeling reagents was designed in order to release Ga-NOTA-Met after proteolysis of the parental probe. Ester or diester bonds have been used as metabolizable linkers in the approach using metabolizable linkers cleaved by lysosomal enzymes.[10−14] However, the stability of ester bonds as metabolizable linkers was shown to depend on the molecular sizes of probes, and when Fab fragments were used as parental probes, ester bonds in the radiolabeling reagent were cleaved in the blood.[15] Thus, peptide bonds were selected as metabolizable linkers. Since the second amino acid from the N-terminal of the light chain of the dsFv used by Wu et al. was isoleucine (Ile),[16] the Met-Ile sequence (MI) was selected as the metabolizable linker. Moreover, maleimide−thiol chemistry was selected as the conjugation method of NOTA-MI to antibody fragments. Because thiourea bonds between SCN-Bz-NOTA and methionine were previously reported to cause Edmann degradation under strong acid conditions,[17] acid-labile protecting groups could not be used for the carboxylic groups of NOTA after the formation of thiourea bonds. Therefore, difficulties have been associated with synthesizing the NOTA-MI-adducts of active esters. Thus, NOTA-MI-Mal was synthesized by reacting SCN-Bz-NOTA with Met-Ile-Mal and the results obtained with ^{67}Ga-NOTA-MI-Fab were then compared with those of ^{67}Ga-NOTA-Fab.

Both ^{67}Ga-NOTA-MI-Fab and ^{67}Ga-NOTA-Fab exhibited the stable attachment of radiolabels to the respective Fab in mice serum. This was reflected in the similar radioactivity levels of the two radiolabeled Fab fragments in the blood (Table 1). Thus, similar amounts of the radiolabeled Fab fragments could be filtered through the glomerulus and transported to the proximal tubules of the kidneys after their administration. Radioactivity levels in the kidneys 10 min after the injection of ^{67}Ga-NOTA-MI-Fab were similar to those of ^{67}Ga-NOTA-Fab (Table 1). However, although ^{67}Ga-NOTA-Fab exhibited high renal radioactivity levels from 1 to 6 h postinjection, ^{67}Ga-

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NOTA-MI-Fab demonstrated markedly lower renal radioactivity levels from 1 h postinjection onward (Table 1). Moreover, the amount of radioactivity excreted in urine after the injection of ^{67}Ga-NOTA-MI-Fab (55.63%ID) was higher than that of ^{67}Ga-NOTA-Fab (20.81%ID). SE-HPLC analyses of urine revealed that 95% of the radioactivity excreted in urine after the administration of ^{67}Ga-NOTA-IT-Fab was observed in the low molecular weight fraction with a retention time of 24 min (Figure 3). RP-HPLC analyses of the low molecular weight fraction showed that the majority of radioactivity was observed at a retention time identical to that of Ga-NOTA-Met (14.5 min, Figure 3). These results suggested that the significantly lower renal radioactivity levels achieved by ^{67}Ga-NOTA-MI-Fab were attributed to the rapid release of ^{67}Ga-NOTA-Met from the kidneys.

To further investigate the low renal radioactivity levels of ^{67}Ga-NOTA-MI-Fab, the fates of radiometabolites in the kidney were compared with those of ^{67}Ga-NOTA-Fab (Figure 4). While both ^{67}Ga-NOTA-Lys and intermediate radiometabolites were observed in the kidneys 1 h after the injection of ^{67}Ga-NOTA-Fab, the majority of radioactivity that remained in the kidneys 6 h after the injection of ^{67}Ga-NOTA-Fab was ^{67}Ga-NOTA-Lys. On the other hand, both ^{67}Ga-NOTA-Met and the intermediate radiometabolites were observed in the kidneys 1 h after the injection of ^{67}Ga-NOTA-MI-Fab. However, small amounts of radioactivity remained in the kidneys in the form of Ga-NOTA-Met or other intermediate radiometabolites. This result suggested that ^{67}Ga-NOTA-Met may have had a shorter residence time than ^{67}Ga-NOTA-Lys in the kidneys, which was consistent with the findings of Wu et al.[12] On the other hand, previous findings indicated that the residence time of ^{111}In-labeled diethylenetriamine pentaacetic acid (DTPA)-conjugated methionine (^{111}In-DTPA-Met) in the kidneys was similar to that of the other ^{111}In-DTPA-adducts of amino acids so far reported.[18] These findings suggested that the short residence time of ^{67}Ga-NOTA-Met in the lysosome compartment may represent one of its specific characteristics.

The ability of NOTA-MI-Mal to reduce renal radioactivity levels without impairing the target radioactivity levels delivered by the antibody fragment was demonstrated in the biodistribution study using nude mice. Although ^{67}Ga-NOTA-MI-Fab showed similar radioactivity levels in the tumor as ^{67}Ga-NOTA-Fab, the tumor/kidney ratios of radioactivity after the injection of ^{67}Ga-NOTA-MI-Fab were 4 times higher than those of ^{67}Ga-NOTA-Fab 6 h postinjection (Figure 5). These results suggested that the metabolizable linker in ^{67}Ga-NOTA-MI-Fab at tumor tissues may be stable. Since the Fab fragment of herceptin was previously shown to not be internalized in Her2-expressing cells,[19,20] the metabolizable linker, MI, may not have been cleaved at tumor tissues.

In conclusion, ^{67}Ga-NOTA-MI-Fab registered significantly lower renal radioactivity levels from 1 to 6 h postinjection than those of ^{67}Ga-NOTA-Fab in normal mice. Moreover, the tumor/kidney ratios of radioactivity after the injection of ^{67}Ga-NOTA-MI-Fab were 4 times higher than those of ^{67}Ga-NOTA-Fab at 6 h postinjection. Although further studies including the structure of radiometabolites and/or cleavable linkages are required to reduce renal radioactivity levels shortly after the injection of ^{67}Ga-labeled Fabs, the results of the present study demonstrated that the present chemical design is applicable to the development of ^{67}Ga-labeled Fabs for low renal radioactivity levels.

EXPERIMENTAL SECTION

Materials. $^{67}GaCl_3$ was supplied by FUJIFILM RI Pharma Co., Ltd. (Tokyo, Japan). Analytical reversed phase HPLC (RP-HPLC) was performed with a Cosmosil $5C_{18}$-AR-300 column (4.6 mm × 150 mm, Nacalai Tesque Inc., Kyoto, Japan) at a flow rate of 1 mL/min with a gradient mobile phase starting from 95% A (0.1% aqueous trifluoroacetic acid (TFA) and 5% B (acetonitrile with 0.1% TFA) to 70% A and 30% B at 20 min. Preparative RP-HPLC was performed with a Cosmosil $5C_{18}$-AR-300 column (20 mm × 150 mm, Nacalai Tesque Inc.) at a flow rate of 5 mL/min with a gradient mobile phase starting from 95% C (0.05% TFA) and 5% D (acetonitrile with 0.05% TFA) to 55% C and 45% D at 25 min, and then to 55% C and 45% D at 35 min. Size-exclusion HPLC (SE-HPLC) was performed with a Cosmosil Diol-300-II column (7.5 × 600 mm, Nacalai Tesque Inc.) eluted with 0.1 M phosphate buffer (pH 6.8) at a flow rate of 1 mL/min. The eluent was monitored online with an UV/Visible single beam spectroscopy detector (L-7405, Hitachi Co. Ltd., Tokyo) coupled to a NaI(Tl) radioactivity detector (Gabi star, Raytest, Strubenhardt, Germany). Each eluent was collected with a fraction collector (RediFrac, GE Healthcare Bio-Science, Tokyo) at 0.5 min intervals, and the radioactivity counts in each fraction were determined with an auto well gamma counter (ARC-380M, Aloka, Tokyo). TLC analyses were performed with silica plates (Silica gel 60 F_{254}, Merck Ltd., Tokyo) developed with saline. Cellulose acetate electrophoresis (CAE) strips were run in a veronal buffer (pH 8.5, I = 0.06) at a constant current of 1 mA/cm for 30 min. Radioactivity was measured using a MiniGita Star Gamma TLC Scanner (Raytest) and an auto well gamma counter. Mass spectrometry was carried out using an Agilent 6130 Series Quadrupole LC/MS electrospray system (Agilent Technologies, Tokyo). ^{1}H NMR spectra were recorded on a JEOL JNM-ECP-400 (400 MHz) spectrometer (JEOL Ltd., Tokyo). High resolution mass spectrometry was carried out using JMS-T100LP (JEOL Ltd.). Apo-transferrin (apo-Tf) Iron Free was purchased from Nacalai Tesque Inc. (Kyoto, Japan). *N*-(Methoxycarbonyl)maleimide (NMCM) was synthesized according to the procedure of Keller et al.[21]

Synthesis of the Isoleucine Methyl Ester (1, Ile-OMe). Thionyl chloride (2.0 mL) was added dropwise to dry methanol (20 mL) at −15 °C. After standing for 10 min at the same temperature, L-isoleucine (1.0 g, 7.6 mmol) was added to the solution. The temperature of the solution was gradually increased to room temperature and the solution was mixed for 6 h. After the solvent was evaporated in vacuo, the residue was suspended in saturated aqueous $NaHCO_3$ solution (30 mL). Compound **1** was extracted using chloroform (10 mL × 3) and then dried over anhydrous $MgSO_4$. After removing the solvent, compound **1** was obtained as a brown oil (0.96 g, 87%). ^{1}H NMR ($CDCl_3$) δ: 0.89 (6H, m, CH_3), 1.30 (2H, dm, CH_2), 1.42 (1H, m, CH), 3.36 (1H, d, CH), 3.77 (3H, s, CH_3). ESI-MS m/z $[M + H]^+$ 146, Found 146.

Synthesis of the *tert*-Butoxycarbonyl-Methionyl-Isoleucine Methyl Ester (2, Boc-Met-Ile-OMe). Compound **1** (742 mg, 5.1 mmol), *tert*-butoxycarbonyl-methionine (Boc-Met-OH, 1.27 g, 5.11 mmol), and 1-hydroxybenzotriazole (HOBt, 759 mg, 5.61 mmol) were dissolved in dimethylformamide (DMF, 15 mL) and the mixture was then cooled on ice before the addition of *N*,*N*′-dicyclohexylcarbodiimide (DCC, 1.16 g, 5.61 mmol). The reaction mixture was stirred for 10 min on ice and for an additional 18 h at room temperature. After

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removing the precipitate by filtration, the solvent was evaporated in vacuo. The residue dissolved in ethyl acetate (10 mL) was washed with saturated aqueous $NaHCO_3$ solution (10 mL × 3) and 5% citric acid (10 mL × 2). The organic layer was dried over anhydrous $MgSO_4$. After removing the solvent, compound **2** was obtained as a white solid (1.42 g, 73.8%). ^{1}H NMR ($CDCl_3$) δ: 0.89 (6H, m, CH_3), 1.30 (11H, m, s, CH_2, Boc), 1.97 (3H, m, CH, CH_2), 2.10 (3H, s, CH_3), 2.57 (2H, t, CH_2), 3.70 (3H, s, OCH_3), 4.28 (1H, d, CH), 4.53 (1H, q, CH); ESI-MS m/z $[M + Na]^+$ 399, Found 399.

Synthesis of *tert*-Butoxycarbonyl-Methionyl-Isoleucine (3, Boc-Met-Ile-OH). Compound **2** (1.55 g, 3.9 mmol) was dissolved in ethanol (40 mL) and 4 N NaOH solution (1.8 mL) was then added to the solution. After mixing for 8 h at room temperature, ethanol was evaporated in vacuo. The pH of the solution was adjusted to **3** with 10% citric acid and the compound was then extracted using ethyl acetate (15 mL × 3). The organic layer was dried over anhydrous $MgSO_4$. After removing the solvent, compound **3** was obtained as a white solid (1.3 g, 92.3%). ^{1}H NMR ($CDCl_3$) δ: 0.85 [6H, m, CH_3], 1.29 [11H, m, s, CH_2, Boc], 1.97 [3H, m, CH, CH_2], 2.10 [3H, s, SCH_3], 2.58 [2H, t, CH_2S], 4.28 [1H, d, CH], 4.53 [1H, q, CH]; ESI-MS m/z $[M + Na]^+$ 385, Found 385.

Synthesis of *N-tert*-Butoxycarbonyl-Ethylenediamine (4, Boc-ED). Ethylenediamine (1.0 g, 16.6 mmol) was dissolved in chloroform (25 mL) and Boc_2O (1.82 g, 8.32 mmol) dissolved in chloroform (25 mL) was then added to the solution dropwise on ice. After mixing for 1 h on ice, the precipitate was removed by filtration. After removing the solvent in vacuo, the residue was purified with open column chromatography using silica gel and subsequently eluted with $CHCl_3$/MeOH/25% aqueous NH_3 (80/16/3) to obtain compound **4** as a colorless oil (1.21 g, 45.5%). ^{1}H NMR ($CDCl_3$) δ: 1.41 [9H, s, Boc], 2.76 [2H, t, CH_2], 3.13 [2H, q, CH_2], 4.88 [1H, s, NH]; ESI-MS m/z $[M + H]^+$ 161, Found 161.

Synthesis of *N-tert*-Butoxycarbonyl, *N*′-Maleoyl-Ethylenediamine (5, Boc-ED-Mal). Compound **4** (630 mg, 3.93 mmol) was dissolved in saturated $NaHCO_3$ solution (10 mL) and NMCM (610 mg, 3.93 mmol) was then added to the solution on ice. The solution was mixed for 40 min on ice and for an additional 50 min at room temperature. The pH of the solution was adjusted to 3 with concentrated H_2SO_4 on ice. The compound was extracted by ethyl acetate (5 mL × 3) and then dried over anhydrous $MgSO_4$. After removing the solvent in vacuo, compound **5** was obtained as a white solid (870 mg, 92.1%). ^{1}H NMR ($CDCl_3$) δ: 1.38 [9H, s, Boc], 3.31 [2H, q, CH_2], 3.64 [2H, t, CH_2], 4.71 [1H, s, NH], 6.69 [2H, s, maleimide]; ESI-MS m/z $[M + H]^+$ 241, Found 241.

Synthesis of *N*-Maleoyl-Ethylenediamine (6, Mal-ED). Compound **5** (1.0 g, 4.16 mmol) was dissolved in 4 N HCl/ethyl acetate and then mixed for 30 min at room temperature. After removing the solvent in vacuo, compound **6** was obtained as a white solid (711 mg, 96.7%). ^{1}H NMR (D_2O) δ: 3.09−3.12 [2H, t, CH_2], 3.70−3.73 [2H, t, CH_2], 6.78 [2H, s, maleimide]; ESI-MS m/z $[M + H]^+$ 141, Found 141.

Synthesis of Boc-Met-Ile-ED-Mal (7). Compound **3** (234 mg, 1.66 mmol), compound **6** (600 mg, 1.66 mmol), and HOBt (224 mg, 1.66 mmol) were suspended in acetonitrile (5 mL) and diisopropylethylamine (DIPEA, 289 μL, 1.66 mmol) was then added to the solution on ice. After 10 min, DCC (343 mg, 1.66 mmol) was added to the solution and mixed for 1 h on ice and for an additional 18 h at room temperature. After the precipitate was removed by filtration, the solvent was evaporated in vacuo. The residue was dissolved in chloroform (20 mL) and then washed with 5% $NaHCO_3$ (20 mL × 2) and 5% citric acid (20 mL × 2). The organic layer was dried over anhydrous $MgSO_4$. After removing the solvent in vacuo, the residue was purified by open column chromatography using silica gel and subsequently eluted with chloroform to obtain compound **7** as a white solid (402 mg, 49.9%). ^{1}H NMR ($CDCl_3$) δ: 0.87 [6H, m, CH_3], 1.21 [11H, m, CH_2, Boc], 1.94 [2H, m, CH_2], 2.13 [4H, m, CH_2, SCH_3], 2.57 [2H, t, CH_2S], 3.44 [2H, t, CH_2], 3.67 [2H, t, CH_2], 4.22 [2H, m, CH], 6.70 [2H, s, maleimide]; ESI-MS m/z $[M + Na]^+$ 507, Found 507. mp 168−169 °C.

Synthesis of Met-Ile-Mal (8). Compound **7** (85 mg, 0.175 mmol) was dissolved in 4 N HCl/ethyl acetate (5 mL) and then mixed for 30 min at room temperature. After removing the solvent, compound **8** was obtained as a white solid (50 mg, 74.4%). ^{1}H NMR (CD_3OD) δ: 0.89 [6H, s, CH_3], 1.17 [2H, m, CH_2], 1.60 [2H, m, CH_2], 1.79 [2H, m, CH_2], 2.11 [4H, m, CH, CH_3], 3.59 [4H, m, CH_2], 4.04 [1H, t, CH], 6.83 [2H, s, maleimide]; ESI-MS m/z $[M + H]^+$ 385, Found 385.

Synthesis of NOTA-MI-Mal (9). NOTA-Bz-SCN (5.1 mg, 8.99 μmol) and compound **8** (5.2 mg, 13.5 mmol) were dissolved in dry DMF (300 μL). After triethylamine (TEA, 6.3 μL) was added to the solution, it was mixed for 2 h at room temperature. After the solution was diluted with H_2O/acetonitrile (4/1, 3 mL), it was purified by preparative RP-HPLC to obtain compound **9** as a white solid (2.2 mg, 30%). ^{1}H NMR ($CDCl_3$) δ: 0.87 [6H, m, CH_3], 1.94 [2H, m, CH_2], 2.13 [4H, m, CH_2, SCH_3], 2.57 [2H, t, CH_2S], 3.44 [2H, t, CH_2], 3.67 [2H, t, CH_2], 4.22 [2H, m, CH], 6.70 [2H, s, maleimide]; ESI-MS m/z $[M + H]^+$ 835, Found 835. HRMS m/z $[M + H]^+$ calculated for $C_{37}H_{55}N_8O_{10}S_2$ 835.34825, observed 835.33769.

Preparation of the NOTA-MI-Conjugated Fab Fragment (NOTA-MI-Fab). The Fab fragment of herceptin was prepared according to the procedure of Tang et al.[22] A solution of the Fab fragment (200 μL, 4 mg/mL) in well-degassed 0.16 M borate buffer (pH 8.0) containing 2 mM EDTA was allowed to react with 7.2 μL (1.8 μL × 4) of 2-iminothiolane solution (2-IT, 2 mg/mL) prepared in the same buffer. After gentle agitation of the reaction mixture for 30 min at room temperature, excess 2-IT was removed by a centrifuged column procedure using Sephadex G-50 fine (GE Healthcare Bio-Science AB, Tokyo) equilibrated and eluted with 0.1 M phosphate buffer (pH 6.0) containing 2 mM EDTA. Aliquots of this mixture were sampled to estimate the number of thiol groups with 2,2′-dipyridyl disulfide (DPS).[23] NOTA-MI-Mal (1 μL, 100 mg/mL in DMF) was added to the filtrate (100 μL) and then mixed gently for 2 h at room temperature. Excess NOTA-MI-Mal was removed by the centrifuged column procedure using Sephadex G-50 fine equilibrated and eluted with 0.1 M phosphate buffer (pH 6.0) containing 2 mM EDTA, and aliquots of the filtrate were then sampled to estimate the number of thiol groups with DPS. After 14.8 μL of iodoacetamide (10 mg/mL) in phosphate buffer (pH 6.0) was added, the reaction mixture was further incubated for 30 min to alkylate the unreacted thiol groups. NOTA-MI-Fab was subsequently purified by the centrifuged column procedure using Sephadex G-50 fine equilibrated and eluted with 0.25 M acetate buffer (pH 5.5).

Preparation of the NOTA-SCN-Conjugated Fab Fragment (NOTA-Fab). The Fab fragment (3 mg/mL, 100 μL)

was dissolved in borate buffer (0.1 M, pH 9.0) and SCN-Bz-NOTA (7.8 μL, 8 mg/mL in DMF) was then added to the solution. After incubating for 18 h at room temperature, the conjugate was purified by Sephadex G-50 fine equilibrated and eluted with 0.25 M acetate buffer (pH 5.5).

Preparation of ^{67}Ga-NOTA-MI-Fab and ^{67}Ga-NOTA-SCN-Fab. $^{67}GaCl_3$ (0.05 N HCl, 30 μL) was added to acetate buffer (0.25 M, pH 5.5, 30 μL). After 5 min, NOTA-MI-Fab or NOTA-SCN-Fab (3 mg/mL, 0.25 M acetate buffer pH 5.5, 30 μL) was added to the solution and then incubated for 40 min at 37 °C. After the addition of EDTA (15 μM, 30 μL), the ^{67}Ga-labeled Fab fragments were purified by Sephadex G-50 fine equilibrated and eluted with 20 mM phosphate buffer (pH 7.4). Radiochemical yield and purity were determined by size-exclusion HPLC and CAE.

Plasma Stability of ^{67}Ga-NOTA-MI-Fab. ^{67}Ga-NOTA-MI-Fab (20 μL) was added to a solution of freshly prepared murine plasma (230 μL), and the solution was then incubated at 37 °C. Aliquots of samples were collected after being incubated for 1, 3, 6, and 24 h, and radioactivity was analyzed by TLC and SE-HPLC.

Cell Line. The squamous cell lung carcinoma cell line NCI-H2170 was purchased from the American Type Culture Collection (ATCC, Manassas, VA). These cells were cultured according to protocols prescribed by the ATCC.

In Vivo Study. Animal studies were conducted in accordance with institutional guidelines approved by the Chiba University Animal Care Committee. Six-week-old male ddy mice[24] (Japan SLC Inc., Shizuoka, Japan) were injected via the tail vein with either ^{67}Ga-NOTA-MI-Fab or ^{67}Ga-NOTA-Fab (100 μL, 11.1 kBq, 20 μg). Animals were then sacrificed and dissected 10 min, and 1, 3, and 6 h after this administration. Tissues of interest were removed and weighed, and radioactivity counts were determined by an auto well gamma counter. Urine and feces were collected for 6 h and radioactivity was also measured. Values were expressed as the mean (SD) for a group of 3−5 animals.

Six-week-old male BALBc nu/nu mice (Japan SLC Inc.) were xenografted by a subcutaneous injection of NCI-H2170 squamous cell carcinoma cells (5×10^6 cells/80 μL of culture medium) into their right hind legs. These mice were then subjected to biodistribution studies when tumor volumes reached 100−300 mm^3. Biodistribution studies were conducted in male BALBc nu/nu mice bearing NCI-H2170 xenografts 6 h after the administration of each radiotracer (n = 3).

Radiometabolites in Urine. Six hours after the injection of ^{67}Ga-NOTA-MI-Fab (74 kBq, 20 μg) into 6-week-old ddy-mice, urine samples were collected and filtered through a polycarbonate membrane (0.45 μm) before being analyzed by SE-HPLC. Urine samples were also filtered through a 10 000 Da cutoff ultrafiltration membrane before being analyzed by RP-HPLC.

Radiometabolites in the Kidney. To investigate the metabolites of ^{67}Ga-NOTA-MI-Fab or ^{67}Ga-NOTA-Fab in the kidney, ^{67}Ga-labeled-Fab (20 μg, 74 kBq) was intravenously injected into 6-week-old male ddy mice. At 1 or 6 h postinjection, mice were treated according to the procedure of Arano et al. with slight modifications.[25] The kidneys were excised, placed in a plastic tube, and subjected to three cycles of freezing (dry ice−methanol bath) and thawing. Two milliliters of cold 0.1 M tris-citrate buffer (pH 6.5) containing 0.15 M NaCl, 0.002% sodium azide, 1 TIU/mL aprotinin, 2 mM benzamide-HCl, 2 mM iodoacetamide, and 5 mM diisopropyl fluorophosphate (buffer A), containing an additional 35 mM of β-octyl-glucoside, was then added to each tube. The kidneys were homogenized by cell disruption with a polytron homogenizer (PT-3100 Kinematica GmbH Littau, Switzerland) at full speed for three 30 s bursts, followed by centrifugation at 48 000g for 20 min (Optima MAX Ultracentrifuge, TLA110, Beckman Instruments, Inc. CA, USA). Supernatants were separated from the pellets and radioactivity was counted. Following filtration through a polycarbonate membrane with a pore diameter of 0.45 μm, the supernatants were analyzed by SE-HPLC. The supernatants were also filtered through a 10 000 Da cutoff ultrafiltration membrane before being analyzed by RP-HPLC.

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Notes

The authors declare no competing financial interest.

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