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A novel bifunctional chelating agent based on *bis*(hydroxamamide) for ^{99m}Tc labeling of polypeptides

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This paper describes the synthesis and biological evaluation of a novel bifunctional chelating agent (BCA) based on *bis*(hydroxamamide) for ^{99m}Tc labeling of polypeptides. We successfully designed and synthesized $C_3(BHam)_2$-COOH as a new BCA. $C_3(BHam)_2$-COOH formed a stable ^{99m}Tc complex and enabled us to prepare ^{99m}Tc-labeled polypeptides by using a 2,3,5,6-tetrafluorophenol (TFP) active ester of $C_3(BHam)_2$-COOH. ^{99m}Tc-$C_3(BHam)_2$-HSA prepared with $C_3(BHam)_2$-TFP was stable in both murine plasma and an excess of L-cysteine without any dissociation of ^{99m}Tc from polypeptides. Furthermore, the blood clearance of ^{99m}Tc-$C_3(BHam)_2$-HSA in mice was similar to that of ^{125}I-HSA, suggesting that $C_3(BHam)_2$-COOH retained stable binding between ^{99m}Tc and the polypeptides *in vivo*. When ^{99m}Tc-$C_3(BHam)_2$-NGA was injected into mice, the radioactivity showed high hepatic uptake early on and a rapid clearance from the liver, indicating that $C_3(BHam)_2$-COOH did not affect the pharmacokinetics of polypeptides *in vivo* and gave radiometabolites, which displayed a rapid elimination from the liver. Such characteristics would render $C_3(BHam)_2$-COOH attractive as a new BCA for ^{99m}Tc labeling of polypeptides.

Keywords: ^{99m}Tc; bifunctional chelating agent; polypeptide; hydroxamamide

Introduction

Many polypeptides such as antibodies, single-chain Fv fragments, diabodies, affibodies, minibodies, and bioactive peptides have been used as scaffolds of radiolabeled probes for targeted imaging of sites of tumors, infection, and thrombosis.[1–10] Among radionuclides for radiolabeling of these polypeptides, ^{99m}Tc is ideal for scintigraphic imaging because of its excellent physical properties, low cost, and ready availability.[11,12] Generally, polypeptides do not possess binding sites to form ^{99m}Tc chelates of high stability *in vivo*. To prepare ^{99m}Tc-labeled peptides for application *in vivo*, therefore, one must incorporate appropriate chelating agents into polypeptide molecules.

^{99m}Tc labeling using bifunctional chelating agents (BCAs), which possess both a binding site for polypeptides and a site for complexation with ^{99m}Tc, is required. It has been reported that tetradentate ligands with N_3S or N_2S_2 (containing one or two thiol groups)[13–15] and hydrazino nicotinamide (HYNIC) (thiol-free chelating agent)[16–19] serve as a BCA for ^{99m}Tc labeling. However, some N_3S or N_2S_2 ligands require harsh ^{99m}Tc complexation (elevated temperatures or high pH) to prepare ^{99m}Tc chelates with high radiochemical yields.[13–15] When ^{99m}Tc-HYNIC-labeled polypeptides were administered *in vivo*, they showed not only localized radioactivity in target tissues but also strong, persistent radioactivity in non-target tissues.[17]

To improve BCAs, we have developed 4′-aminomethyl-*N*,*N*′-trimethylene bisbenzohydroxamamide [$C_3(BHam)_2$-NH_2], which has $C_3(BHam)_2$ for chelating with ^{99m}Tc and a primary amino group for binding with polypeptides (Figure 1).[20–24] A recent report showed that the ^{99m}Tc complexes of benzohydroxamamide (BHam) possessed square base pyramid coordination geometry, and the equatorial plane was formed by two-amine nitrogen and two-oxime oxygen atoms in a trans-orientation, whereas the oxo core of the Tc(V) occupied the apical position.[25] Indeed, $C_3(BHam)_2$-NH_2 provided stable ^{99m}Tc-labeled antibodies in mild conditions to image tumor sites in tumor-bearing mice.[23] However, as $C_3(BHam)_2$-NH_2 cannot directly conjugate to polypeptides, *N*-(6-maleimidocaproyloxy)succinimide (EMCS) and 2-iminothiolane (2-IT) were essential for the preparation of ^{99m}Tc-labeled polypeptides by using $C_3(BHam)_2$-NH_2 as a BCA. When $C_3(BHam)_2$-NH_2 is applied to ^{99m}Tc labeling for lower molecular weight polypeptides, the incorporation of spacers (EMCS and 2-IT) between ^{99m}Tc-$C_3(BHam)_2$ and polypeptides may affect the pharmacokinetics and bioactivity of the polypeptides.[26,27]

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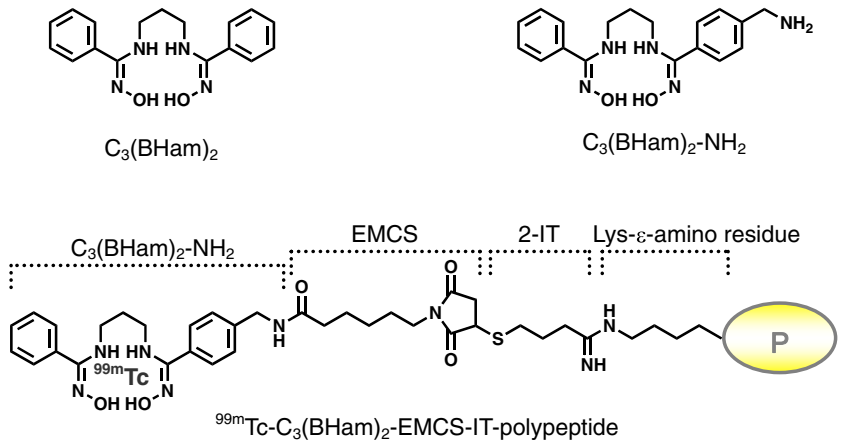


Figure 1. Chemical structure of $C_3(BHam)_2$, $C_3(BHam)_2$-NH_2, and ^{99m}Tc-$C_3(BHam)_2$-EMCS-IT-polypeptide.

The objective of this study was to develop a novel BCA based on $C_3(BHam)_2$, which can be introduced into polypeptides without a long linker such as EMCS and 2-IT. We designed and synthesized 4′-carboxyl-*N*,*N*′-trimethylene bisbenzohydroxamamide [$C_3(BHam)_2$-COOH], which has carboxylic acid for binding with the lys-ε-amino group of polypeptides. Next, using $C_3(BHam)_2$-COOH, we investigated the labeling of ^{99m}Tc with human serum albumin (HSA) as a model polypeptide and evaluated the stability of ^{99m}Tc-$C_3(BHam)_2$-HSA *in vitro* and *in vivo*. Furthermore, we used galactosyl-neoglycoalbumin (NGA) as a model polypeptide and evaluated the pharmacokinetics of radiometabolites formed in the liver after the injection of ^{99m}Tc-$C_3(BHam)_2$-NGA into mice.

Materials and methods

Reagents and chemicals

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian Gemini 300 (300 MHz) (Varian Medical Systems, Inc., Calfornia, USA). Electron impact mass spectra (EI-MS) and fast atom bombardment mass spectra (FAB-MS) were obtained with a JEOL IMS-DX300 mass spectrometer (JEOL Ltd., Tokyo, Japan). Na[^{125}I]I (3.7 GBq/mL, 0.01 N NaOH solution) was obtained from MP Biomedicals. [^{99m}Tc]Pertechnetate ($^{99m}TcO_4^-$) (111 MBq/mL) was purchased from Nihon-Medi-Physics (Tokyo, Japan). Size-exclusion HPLC (SE-HPLC) was performed using a TSK G3000SW (7.8 × 300 mm) column (Tosoh Corporation, Tokyo, Japan), eluted with 0.1 M phosphate buffer (PB) (pH 7.0) containing 0.3 M sodium chloride at a flow rate of 0.5 mL/min. Reversed-phase HPLC (RP-HPLC) was performed with a Cosmosil 5 C_{18}-MS column (4.6 × 150 mm, Nacalai Tesque, Kyoto, Japan) at a flow rate of 1 mL/min with a gradient mobile phase from 85% A (PB, 0.01 M, pH 7.4) and 15% B (acetonitrile) to 20% A and 80% B in 10 min. Cellulose acetate electrophoresis (CAE) was run on Separax SP (Joko, Tokyo, Japan) at a constant current of 0.8 mA/cm for 30 min in 0.072 M veronal buffer (pH 8.6). The distance migrated by HSA was determined by Ponceau 3R staining.

Synthesis of benzohydroxamamide

To a solution of hydroxylammonium chloride (10.5 g, 145.2 mmol) and $NaHCO_3$ (12.3 g, 146.0 mmol) in H_2O (40 mL) was gradually added a solution of benzonitrile (15.3 g, 145.2 mmol) in EtOH (100 mL), and the reaction mixture was stirred at 80 °C for 4 h. EtOH was removed *in vacuo*, and the mixture was extracted with ethyl acetate. After drying of the organic layer on Na_2SO_4, evaporation gave 16.6 g of BHam (83.1%). ^{1}H NMR (DMSO-d_6) δ: 5.81 (s, 2H, -NH_2), 7.36–7.38 (m, 3H, aromatic), 7.66–7.69 (m, 2H, aromatic), 9.64 (s, 1H, -NOH).

Synthesis of O-carbethoxybenzohydroxamamide (**1**)

To a solution of BHam (19.0 g, 140.0 mmol) in dry acetone (90 mL) was gradually added a solution of ethyl chlorocarbonate (16.8 g, 156.0 mmol) in acetone (30 mL) in an ice bath for 1 h. After a 5% NaOH solution (126 mL) was added, the mixture was stirred at room temperature for 1 h. The precipitate formed was filtered, and the residue was recrystallized from H_2O–acetone (4:3) to give 15.9 g of **1** (54.9%). ^{1}H NMR ($CDCl_3$) δ: 1.37 (t, 3H, –CH_3), 4.33 (q, 2H, J = 7.2 Hz, –CH_2), 5.11 (s, broad, 2H, -NH_2), 7.41–7.48 (m, 3H, aromatic), 7.68–7.71 (m, 2H, aromatic).

*Synthesis of 3-phenyl-Δ^2-1,2,4-oxadiazolin-5-one (**2**)*

To a solution of **1** (15.8 g, 75.7 mmol) in 5% NaOH solution (80 mL) was added excess acetic acid (26.3 mL, 460 mmol). The precipitate formed was filtered to give 10.7 g of **2** (87.5%). ^{1}H NMR (DMSO-d_6) δ: 7.56-7.67 (m, 3H, aromatic), 7.81–7.85 (q, 2H, J = 6.3 Hz, aromatic).

*Synthesis of potassium 3-phenyl-Δ^2-1,2,4-oxadiazolin-5-one (**3**)*

To a solution of **2** (7.50 g, 46.3 mmol) in MeOH (20 mL) was added a solution of KOH (2.60 g, 46.3 mmol) in MeOH (30 mL). After the mixture was stirred at room temperature for 1 h, evaporation gave 8.97 g of **3** (96.8%). ^{1}H NMR (DMSO-d_6) δ: 7.37–7.42 (m, 3H, aromatic), 7.76–7.80 (m, 2H, aromatic).

*Synthesis of 3-phenyl-4-(3-bromopropyl)-Δ^2-1,2,4-oxadiazolin-5-one (**4**)*

To a solution of **3** (9.28 g, 32.8 mmol) in DMF (31 mL) was added 1,3-dibromopropane (19.9 g, 98.4 mmol) in DMF (25 mL). The reaction mixture was stirred at room temperature for 3 days. After the filtrating precipitate of potassium bromide was filtered and the solvent was removed, the residue was purified by silica gel chromatography (ethyl acetate/hexane = 1:5) to give 4.56 g of **4** (49.1%). ^{1}H NMR ($CDCl_3$) δ: 2.22–2.26 (m, 2H, -$NCH_2\underline{CH_2}CH_2Br$), 3.35 (t, 2H, -$NCH_2CH_2CH_2Br$), 3.86 (t, 2H, -$NCH_2CH_2\underline{CH_2}Br$), 7.58–7.63 (m, 5H, aromatic).

*Synthesis of 4-carboxylbenzohydroxamamide methyl ester (BHam-COOMe, **5**)*

The reaction mixture of 4-cyanobenzoic acid methyl ester (10.0 g, 61.8 mmol), $NaHCO_3$ (5.35 g, 66.7 mmol), and hydroxylammonium chloride (4.30 g, 61.8 mmol) in MeOH (120 mL) was stirred at room temperature for 30 min. The mixture was then stirred at 80–90 °C for 3 h. After it had cooled to room temperature, 200 mL of water was added to produce a precipitate. **5** was obtained after washing the precipitate with ether in a yield of 68.6% (8.24 g). ^{1}H NMR ($CDCl_3$) δ: 3.94 (s, 3H, $-CH_3$), 4.89 (s, broad, 2H, $-NH_2$), 7.72 (t, 2H, aromatic), 8.89 (t, 2H, aromatic).

*Synthesis of 3-(4-carboxylphenyl)-Δ^2-1,2,4-oxadiazolin-5-one methyl ester (**6**)*

To a suspension of **5** (4.96 g, 25.5 mmol) in 1,4-dioxane (35 mL) was added 1′-carbonyldiimidazole (5.30 g, 30.6 mmol). The mixture was stirred at 110 °C for 30 min. The solvent was removed, the residue was dissolved in water, and 3 N HCl was added to produce a precipitate. The precipitate was washed with water, ether, and ethyl acetate to give 4.91 g of **6** (87.5%). ^{1}H NMR (DMSO-d_6) δ: 3.90 (s, 3H, $-CH_3$), 7.96 (d, 2H, J = 8.7 Hz, aromatic), 8.15 (t, 2H, aromatic).

*Synthesis of potassium 3-(4-carboxylphenyl)-Δ^2-1,2,4-oxadiazolin-5-one methyl ester (**7**)*

To a solution of **6** (8.62 g, 39.2 mmol) in MeOH (23 mL) was added a solution of KOH (2.25 g, 40.0 mmol) in MeOH (28 mL). After the reaction mixture was stirred at room temperature for 1 h, evaporation of the solvent gave 9.78 g of **7** (96.6%). ^{1}H NMR (DMSO-d_6) δ: 3.87 (s, 3H, $-CH_3$), 7.93 (t, 2H, aromatic), 8.00 (t, 2H, aromatic).

*Synthesis of methyl 4-(4,5-dihydro-5-oxo-4-(3-(5-oxo-3-phenyl-1,2,4-oxadiazol-4-yl)propyl)-1,2,4-oxadiazol-3-yl)benzoate (**8**)*

To a solution of **4** (1.15 g, 4.06 mmol) in DMF (20 mL) was added a solution of **7** (1.11 g, 4.30 mmol) in DMF (20 mL) at room temperature. The reaction mixture was then stirred at 40 °C for 7 days. The solvent was evaporated, water was added, and the mixture was extracted with ethyl acetate. The organic layer was dried over Na_2SO_4 and filtered. The residue was purified by silica gel chromatography (ethyl acetate/hexane = 1:2) to give 850 mg of **8** (49.6%). ^{1}H NMR (DMSO-d_6) δ: 1.77 (m, 2H, $-CH_2\underline{CH_2}CH_2-$), 3.55–3.62 (q, 4H, J = 7.2 Hz, $-\underline{CH_2}CH_2\underline{CH_2}-$), 3.93 (s, 3H, $-CH_3$), 7.56–7.60 (m, 4H, aromatic), 7.64–7.69 (m, 1H, aromatic), 7.75 (d, 2H, J = 7.8 Hz, aromatic), 8.12 (d, 2H, J = 8.1 Hz, aromatic). EI-MS calc $C_{21}H_{18}N_4O_6$ (M^+): m/z 422, found: 422.

Synthesis of C_3(BHam)$_2$-COOH

8 (850 mg, 2.01 mmol) was added to 5 mL of 1 N NaOH, and the mixture was heated with stirring at 90–100 °C for 1.5 h, then allowed to cool to room temperature. The pH was adjusted to 3 with 1 N HCl. The mixture was neutralized with 1 N NaOH, and the solvent was removed *in vacuo*. The residue was purified by silica gel chromatography (MeOH/$CHCl_3$/acetic acid = 10:50:1) to give 620 mg of C_3(BHam)$_2$-COOH (61.5%). ^{1}H NMR (DMSO-d_6) δ: 1.57 (m, 2H, $-CH_2\underline{CH_2}CH_2-$), 3.00–3.08 (m, 4H, $-\underline{CH_2}CH_2\underline{CH_2}-$), 7.53–7.62 (m, 7H, aromatic), 8.05 (d, 2H, J = 9.0 Hz, aromatic), 8.92 (s, 1H, -COOH). FAB-MS calc $C_{18}H_{21}N_4O_4$ (MH^+): m/z 357, found: 357.

Preparation of ^{99m}Tc-C_3(BHam)$_2$-COOH

A solution of C_3(BHam)$_2$-COOH in DMSO (0.1 M) was prepared. This solution (5 μL) was added to H_2O (495 μL). A solution of stannous tartrate (375 μL, 3×10^{-4} M) in H_2O and $Na^{99m}TcO_4$ (74 MBq/mL, 125 μL) was then added. After incubation for 15 min, radiochemical yields of ^{99m}Tc-C_3(BHam)$_2$-COOH were determined by RP-HPLC, CAEP, and TLC.

Preparation of ^{99m}Tc-C_3(BHam)$_2$-TFP

2,3,5,6-Tetrafluorophenol (TFP) (2 mg) was added to a solution of ^{99m}Tc-C_3(BHam)$_2$-COOH in saline (200 μL). Next, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (4 mg) was added, the reaction mixture was stirred at room temperature for 30 min, DMSO (60 μL) was added, and the mixture was purified using Sep-Pak. The Sep-Pak was replaced with water. The reaction mixture (260 μL) was applied, the column was washed with water (6 mL) and diethylether (1 mL), and ^{99m}Tc-C_3(BHam)$_2$-TFP was eluted with acetonitrile (1 mL). After evaporation of the acetonitrile, the radioactivity in the residue was analyzed by SE-HPLC and CAEP.

Preparation of ^{99m}Tc-C_3(BHam)$_2$-HSA

To 200 μL of HSA solution (10 mg/mL in 0.1 M carbonate buffer, pH 9.5) was added an equal volume of ^{99m}Tc-C_3(BHam)$_2$-TFP prepared above, and the reaction mixture was incubated for 1 h at room temperature. ^{99m}Tc-C_3(BHam)$_2$-HSA was then purified by the centrifuged column procedure using a Sephadex G-50 column equilibrated and eluted with PB (0.1 M, pH 7.4). The radiochemical yield was assessed by SE-HPLC and CAEP.

Preparation of ^{99m}Tc-C_3(BHam)$_2$-NGA

Galactosyl-neoglycoalbumin was synthesized by conjugation of cyanomethyl-2,3,4,6-tetra-*O*-acetyl-1-thio-β-*D*-galactopyranoside, synthesized according to the procedure of Lee *et al.*,[28] with HSA. The phenol–sulfuric acid reaction indicated that 25 galactose units were attached to each HSA molecule.[29] ^{99m}Tc-C_3(BHam)$_2$-NGA was prepared similar to ^{99m}Tc-C_3(BHam)$_2$-HSA, and radiochemical yield was assessed by SE-HPLC and CAEP.

Preparation of ^{125}I-HSA

To 200 μL of HSA solution (4 mg/mL in 0.1 M PB, pH 7.4) was added 1 μL of $Na^{125}I$ (3.7 GBq/mL) solution and 10 μL of chloramine-T solution (2 mg/mL in 0.1 M PB, pH 7.4). The reaction mixture was incubated for 10 min. A solution of $NaHSO_3$ (1 mg/mL, 6 μL) was added, and the mixture was purified by the centrifuged column procedure using a Sephadex G-50 column equilibrated and eluted with PB (0.1 M, pH 7.4). The radiochemical yield of ^{125}I-HSA was assessed by CAEP and TLC.

Stability of ^{99m}Tc-C_3(BHam)$_2$-HSA *in vitro*

^{99m}Tc-C_3(BHam)$_2$-HSA (50 μL, 8.3 kBq) was diluted 20-fold with 0.1 M PB (pH 7.4, 200 μL) or freshly prepared murine plasma, and the solution was incubated at 37 °C. After 1, 3, 6, and 24 h of incubation, the radioactivity of ^{99m}Tc-C_3(BHam)$_2$-HSA was analyzed by CAEP. To a solution of ^{99m}Tc-C_3(BHam)$_2$-HSA (12.2 kBq) in 0.1 M PB (pH 7.4, 135 μL) was added L-cysteine (15 μL, 5×10^{-6} to 5×10^{-2} M). After incubation for 1 h at 37 °C, the radioactivity of the reaction mixture was analyzed by SE-HPLC. ^{99m}Tc-cysteine was prepared by incubating for 1 h at room temperature after mixing a solution of 0.1 M L-cysteine (100 μL) with an aqueous solution of stannous tartrate (75 μL, 3×10^{-4} M) and $Na^{99m}TcO_4$ (25 μL, 3.7 MBq).

Experiments in vivo

Animal experiments were conducted in accordance with our institutional guidelines and approved by the Nagasaki University Animal Care Committee. Biodistribution experiments were performed by intravenously administering ^{99m}Tc-C_3(BHam)$_2$-HSA (37 MBq/mL in 66 mM PB, pH 7.4) or ^{125}I-HSA (37 kBq/mL in 66 mM PB, pH 7.4) to 5-week-old male ddY mice (20–25 g). Groups of four to five mice each were administered 100 μL (3.7 kBq) of ^{99m}Tc-C_3(BHam)$_2$-HSA prior to sacrifice at 5, 10, 30, and 60 min postinjection by decapitation. Tissues of interest were removed and weighed, and radioactivity was measured with an auto well gamma counter. Data in the biodistribution experiments were analyzed using the unpaired *t*-test. Differences were considered statistically significant when the *p* value was less than 0.05.

The concentration of ^{99m}Tc-C_3(BHam)$_2$-NGA was adjusted to 90 μg/mL with PB (66 mM, pH 7.4). Biodistribution experiments were performed by intravenously administering ^{99m}Tc-C_3(BHam)$_2$-NGA to 6-week-old male ddY mice (25–30 g). Groups of five to eight mice each were administered 9 μg of ^{99m}Tc-C_3(BHam)$_2$-NGA prior to sacrifice at 5, 10, 30, 60, 180, and 360 min postinjection by decapitation. Tissues of interest were removed and weighed, and radioactivity was measured with an auto well gamma counter.

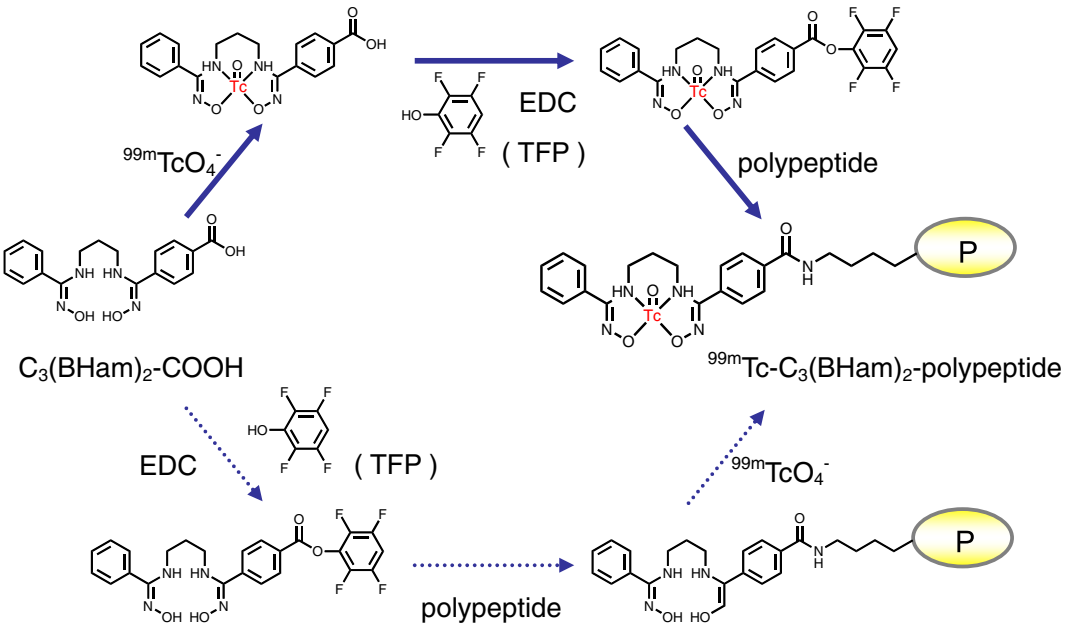
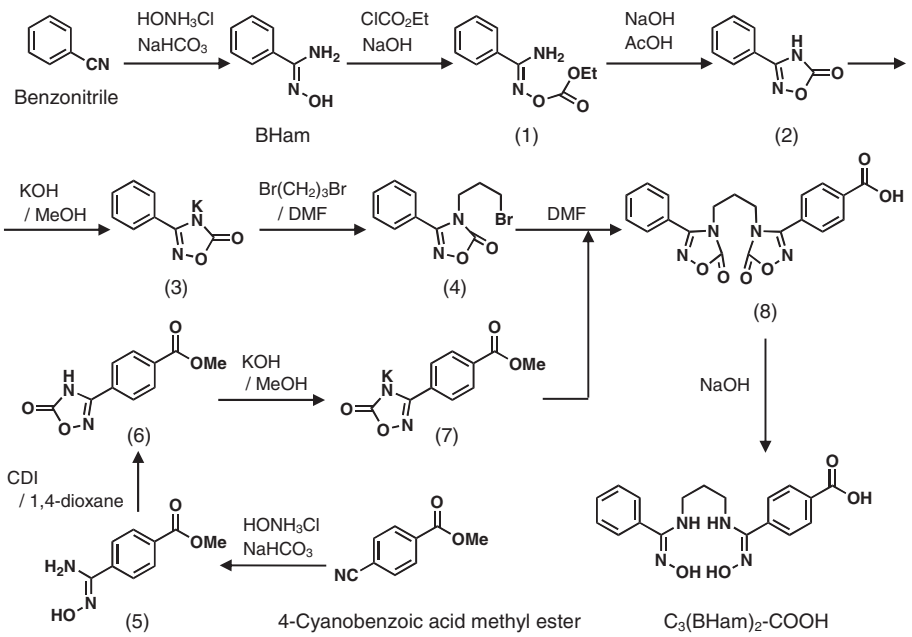


Figure 2. Two schemes for ^{99m}Tc labeling of polypeptides using $C_3(BHam)_2$-COOH.



Scheme 1. Synthesis of $C_3(BHam)_2$-COOH.

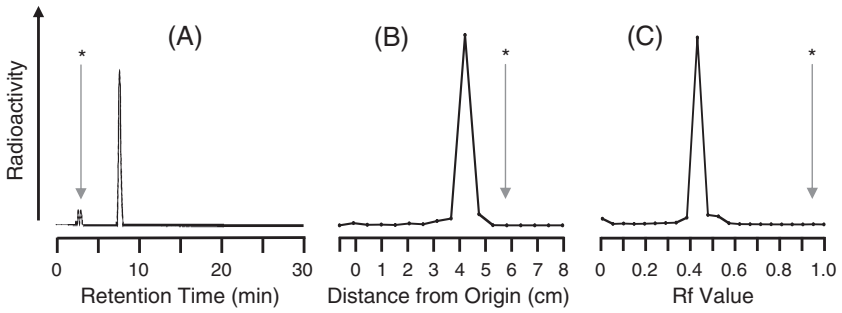


Figure 3. Radiochromatograms of ^{99m}Tc-C_3(BHam)$_2$-COOH obtained by RP-HPLC (A), CAEP (B), and TLC (C). Arrows with single asterisks show $^{99m}TcO_4^-$.

Results and discussion

In the present study, in order to introduce ^{99m}Tc-C_3(BHam)$_2$ into polypeptides, we designed and synthesized C_3(BHam)$_2$ which has a carboxylic acid for binding to the lys-ε-amino group of polypeptides. As shown in Figure 1, we tried two methods of preparing ^{99m}Tc-labeled polypeptides by using C_3(BHam)$_2$-COOH as a BCA. Considering the half-life of ^{99m}Tc, and convenience of labeling experiments or radiation exposure, a method of labeling polypeptides with ^{99m}Tc after conjugation with a BCA is preferred. Therefore, first of all, we tried to synthesize a TFP active ester of C_3(BHam)$_2$-COOH in order to conjugate polypeptides with a BCA before ^{99m}Tc labeling (Figure 2, the scheme shown with a dotted line). We performed the reaction of (BHam)$_2$-COOH with TFP under several different conditions. However, all the reactions gave multiple products, and we could not obtain C_3(BHam)$_2$-TFP. This may be one of the reasons why C_3(BHam)$_2$-TFP reacts with an active secondary amino group or hydroxy group in the hydroxamamide scaffold. As we had difficulty producing C_3(BHam)$_2$-TFP, we used an alternative method to prepare ^{99m}Tc-labeled polypeptides, through the conjugation of polypeptides with ^{99m}Tc-C_3(BHam)$_2$-TFP after active esterification of ^{99m}Tc-C_3(BHam)$_2$-COOH prepared by labeling C_3(BHam)$_2$-COOH with ^{99m}Tc (Figure 2). Previous papers have reported that ^{99m}Tc-labeled polypeptides were successfully prepared using N_2S_2 and N_3S type ligands as BCAs by such a pre-chelating method without a loss of bioactivity.[13,14,30] Furthermore, as the chemical form of ^{99m}Tc-labeled polypeptides is the same even using different methods, in the present study, we synthesized ^{99m}Tc-labeled polypeptides through the latter approach, and evaluated the utility of C_3(BHam)$_2$-COOH as a BCA for ^{99m}Tc labeling.

C_3(BHam)$_2$-COOH was synthesized by using benzonitrile and 4-cyanobenzoic acid methyl ester as the starting materials according to the route shown in Scheme 1. Compounds **4** and **6** were synthesized as reported previously. **6** was converted to its potassium salt (**7**). **7** was reacted with **4** to give **8**. **8** was hydrolyzed in a 5% NaOH solution to give C_3(BHam)$_2$-COOH in a total yield of 5.7%.

The radioactivity of the reaction mixture of C_3(BHam)$_2$-COOH and ^{99m}Tc was analyzed by RP-HPLC. The radioactivity of $^{99m}TcO_4^-$ at 3 min disappeared, and a new peak derived from ^{99m}Tc-C_3(BHam)$_2$-COOH was detected at a retention time of 7 min (Figure 3(A)). A new peak was detected 4 cm (anode) from the origin in the analysis by CAEP (Figure 3(B)) and at Rf = 0.4 in the analysis by TLC (Figure 3(C)). The results suggested that ^{99m}Tc-C_3(BHam)$_2$-COOH was produced, because radioactivity of $^{99m}TcO_4^-$ was detected at 6 cm (anode) by CAEP and at Rf = 1.0 by TLC. The radiochemical yield of ^{99m}Tc-C_3(BHam)$_2$-COOH was over 93% in the RP-HPLC, TLC, and CAEP analyses. These results indicated that C_3(BHam)$_2$-COOH can function as the coordinating site of ^{99m}Tc in mild labeling conditions.

After the active esterification of ^{99m}Tc-C_3(BHam)$_2$-COOH with TFP using EDC, we analyzed the radioactivity eluted from the Sep-Pak column by RP-HPLC. The peak for ^{99m}Tc-C_3(BHam)$_2$-TFP was observed at a retention time of 14 min, which was later than that

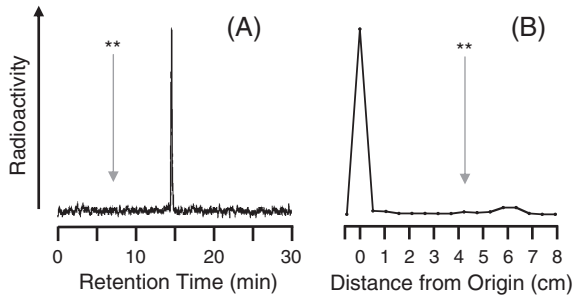


Figure 4. Radiochromatograms of ^{99m}Tc-C_3(BHam)$_2$-TFP obtained by RP-HPLC (A) and CAEP (B). Arrows with double asterisks show ^{99m}Tc-C_3(BHam)$_2$-COOH.

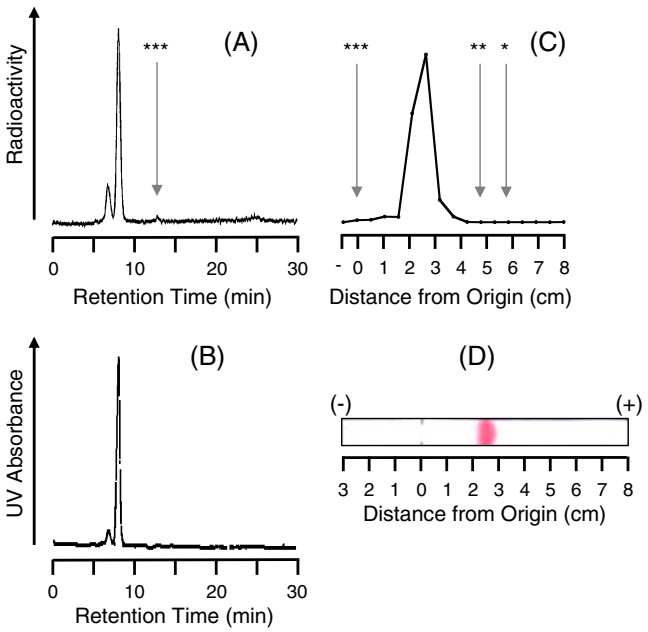


Figure 5. SE-HPLC profiles of ^{99m}Tc-C_3(BHam)$_2$-HSA (radioactivity, A) and unmodified HSA (UV absorbance 254 nm, B). A typical radioactivity profile of ^{99m}Tc-C_3(BHam)$_2$-HSA (C) and Ponceau 3R staining of unmodified HSA analyzed by CAEP (D). Arrows with single, double, and triple asterisks show $^{99m}TcO_4^-$, ^{99m}Tc-C_3(BHam)$_2$-COOH, and ^{99m}Tc-C_3(BHam)$_2$-TFP, respectively.

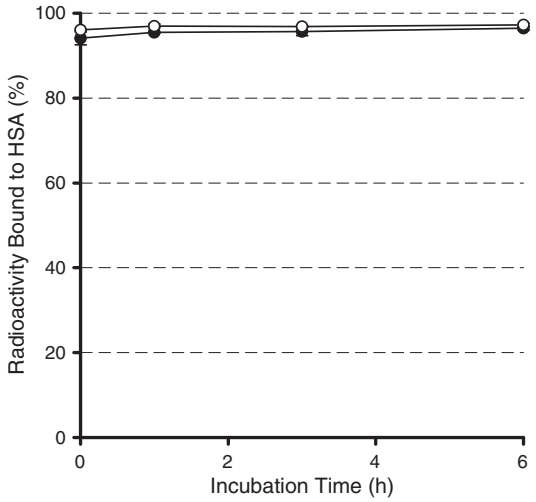


Figure 6. Percent radioactivity in HSA fractions following incubation of ^{99m}Tc-C_3(BHam)$_2$-HSA in phosphate buffer (●) and murine plasma (○) at 37 °C. Each value was determined by CAEP.

of ^{99m}Tc-C_3(BHam)$_2$-COOH (Rt = 7 min) (Figure 4(A)). In the analysis by CAEP, almost all radioactivity existed at the origin different from the site where the radioactivity of $^{99m}TcO_4^-$ and C_3(BHam)$_2$-COOH was detected, suggesting the production of ^{99m}Tc-C_3(BHam)$_2$-TFP (Figure 4(B)). The recovery rate of radioactivity was ca. 50% after purification by Sep-Pak, and the rest remained in the Sep-Pak column.

We performed by gel filtration chromatography after the conjugation of ^{99m}Tc-C_3(BHam)$_2$-TFP with HSA and analyzed the radioactivity eluted from the gel filtration column by SE-HPLC and CAEP (Figure 5). In the SE-HPLC analysis, the peaks of radioactivity at retention times of 7 (dimer) and 8 min (monomer) corresponded with the peaks of UV (254 nm) for unmodified HSA (Figure 7(A, B)). In the CAEP analysis, over 95% of radioactivity was detected at the same position as HSA stained with Ponceau 3R (Figure 5(C, D)). The radiochemical yield and purity were

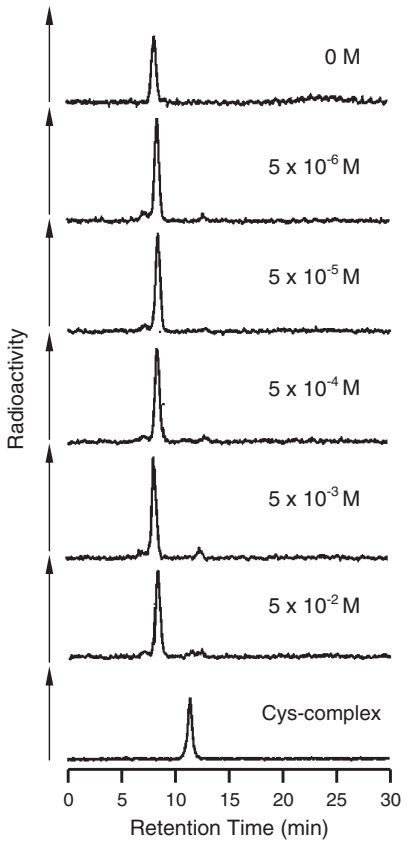


Figure 7. SE-HPLC profiles of ^{99m}Tc-C_3(BHam)$_2$-HSA after incubation with L-cysteine for 1 h at 37 °C.

ca. 75% and >95%, respectively. The results suggested that C_3(BHam)$_2$-COOH may serve as a BCA for the labeling of polypeptides with ^{99m}Tc.

Next, we tested the stability of ^{99m}Tc-C_3(BHam)$_2$-HSA in PB or murine plasma at 37 °C by CAEP (Figure 6). The amount of radioactivity bound to HSA was over 95% until 6 h. We also

Table 1. Biodistribution of radioactivity after injection of ^{99m}Tc-C_3(BHam)$_2$-HSA in mice

	Percentage of injected dose per tissue				
Tissue	5 min	10 min	30 min	1 h	3 h
Blood[a]	35.60	32.92	31.42	27.94	19.54
	(2.69)	(2.01)	(1.47)	(3.17)	(2.62)
Liver	7.39	8.72	8.07	7.28	5.68
	(1.60)	(1.09)	(0.70)	(1.07)	(0.62)
Kidney	1.68	1.47	1.88	1.76	1.31
	(0.41)	(0.29)	(0.21)	(0.29)	(0.08)
Intestine	2.04	2.62	4.81	7.27	11.56
	(0.45)	(0.35)	(0.39)	(1.18)	(0.78)
Spleen	0.33	0.35	0.32	0.30	0.21
	(0.01)	(0.05)	(0.06)	(0.04)	(0.03)
Stomach	0.51	0.63	1.00	1.02	0.95
	(0.10)	(0.02)	(0.24)	(0.10)	(0.11)
Lung	3.50	3.65	2.62	3.13	1.80
	(0.47)	(0.72)	(0.38)	(0.44)	(0.52)

Each value represents the mean (SD) for four to five animals.
[a]Expressed as% injected dose per gram.

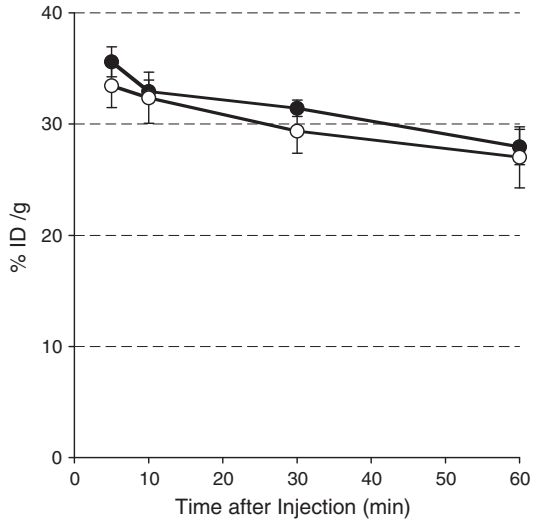


Figure 8. Blood clearance of radioactivity after injection of ^{99m}Tc-C_3(BHam)$_2$-HSA (●) and ^{125}I-HSA (○) in mice.

analyzed the radioactivity of ^{99m}Tc-C_3(BHam)$_2$-HSA by SE-HPLC after incubation with excess L-cysteine (Figure 7). No marked change in the peak of radioactivity was observed at 5×10^{-5} M, 5×10^{-4} M, 5×10^{-3} M, or 5×10^{-2} M of L-cysteine. These results suggested that ^{99m}Tc stably binds to HSA via C_3(BHam)$_2$-COOH.

Furthermore, we determined the biodistribution of radioactivity after the injection of ^{99m}Tc-C_3(BHam)$_2$-HSA into mice (Table 1). At 5-min postinjection, 36% ID/g was observed in the blood, and at 180 min, 20%ID/g. The profile of radioactivity was similar to that for ^{125}I-HSA (Figure 8). The results suggested that ^{99m}Tc-C_3(BHam)$_2$-HSA may reflect the pharmacokinetics of HSA without the dissociation of ^{99m}Tc from HSA, and in addition, C_3(BHam)$_2$-COOH serves as a BCA, which can give stable ^{99m}Tc-labeled polypeptides. As no marked increase in radioactivity was detected in the stomach and lungs, the reoxidation to $^{99m}TcO_4^-$ or production of ^{99m}Tc-colloid did not seem to occur, supporting the notion that C_3(BHam)$_2$-COOH serves as a BCA that produces stable ^{99m}Tc-labeled polypeptides *in vivo*.

When radiolabeled polypeptides were administered *in vivo*, persistently high levels of radioactivity were observed in the liver and kidney where catabolism of the parent proteins and peptides occurs, which compromises the diagnostic accuracy of these radiopharmaceuticals. Previous investigations regarding the radiometabolites produced in the liver and kidney indicated that slow elimination rates are responsible for the persistent localization of radioactivity in the lysosomal compartment of hepatic and renal cells.[31–34] Several recent studies suggested that an experimental system using NGA, which is incorporated by hepatic parenchymal cells via receptor-mediated endocytosis immediately after its administration, is suitable for investigating radiometabolites formed in the liver.[31–34] To investigate the pharmacokinetics of the radiometabolites produced in the metabolic tissues after the injection of ^{99m}Tc-labeled polypeptides by using C_3(BHam)$_2$-COOH as a BCA, NGA was selected as a model polypeptide, ^{99m}Tc-C_3(BHam)$_2$-NGA was prepared, and the biodistribution in mice was evaluated. The biodistribution of radioactivity after the injection of ^{99m}Tc-C_3(BHam)$_2$-NGA is summarized in Table 2. At 5-min postinjection, more than 89% of the radioactivity was accumulated in the liver. The radioactivity was rapidly eliminated from the liver by hepatobiliary excretion. At 6-h postinjection, the radioactivity retained in the liver was just 7% of the injected dose. No marked accumulation in the blood or the other tissues was observed.

We compared the clearance of ^{99m}Tc-C_3(BHam)$_2$-NGA from the liver with that of ^{99m}Tc-(HYNIC-NGA)(tricine)$_2$ (Figure 9).[17] Radioactivity in the liver cleared faster after the injection of ^{99m}Tc-C_3(BHam)$_2$-NGA than that of ^{99m}Tc-(HYNIC-NGA)(tricine)$_2$, indicating that ^{99m}Tc-labeled polypeptides prepared with C_3(BHam)$_2$-COOH produce radiometabolites, which show rapid elimination from the liver. We have not determined the radiometabolites produced in the liver after the injection of ^{99m}Tc-labeled polypeptides by using C_3(BHam)$_2$-COOH as a BCA. Previous studies using ^{111}In-labeled NGAs with cDTPA or SCN-Bz-EDTA or ^{99m}Tc-HYNIC-NGA showed that radiolabeled NGAs generated lysine-adducts ([^{111}In]

Table 2. Biodistribution of radioactivity after injection of ^{99m}Tc-C_3(BHam)$_2$-NGA in mice

	Percentage of injected dose per tissue					
Tissue	5 min	10 min	30 min	1 h	3 h	6 h
Blood[a]	1.35	0.89	1.84	1.49	0.98	0.75
	(0.28)	(0.39)	(0.39)	(0.48)	(0.42)	(0.31)
Liver	89.43	80.52	59.96	32.66	14.64	7.12
	(4.15)	(7.71)	(2.79)	(2.87)	(3.05)	(0.62)
Kidney	0.36	0.33	0.45	0.53	0.50	0.36
	(0.11)	(0.04)	(0.09)	(0.13)	(0.13)	(0.01)
Intestine	0.93	2.28	22.33	43.66	62.44	27.74
	(0.03)	(0.34)	(1.87)	(6.16)	(4.05)	(11.02)
Spleen	0.19	0.16	0.18	0.14	0.13	0.11
	(0.07)	(0.03)	(0.03)	(0.01)	(0.06)	(0.03)
Stomach	0.41	0.51	0.58	0.74	0.51	0.27
	(0.13)	(0.11)	(0.16)	(0.28)	(0.22)	(0.09)
Lung	0.24	0.17	0.19	0.21	0.21	0.12
	(0.13)	(0.03)	(0.05)	(0.03)	(0.01)	(0.03)

Each value represents the mean (SD) for five to eight animals.
[a]Expressed as% injected dose per gram.

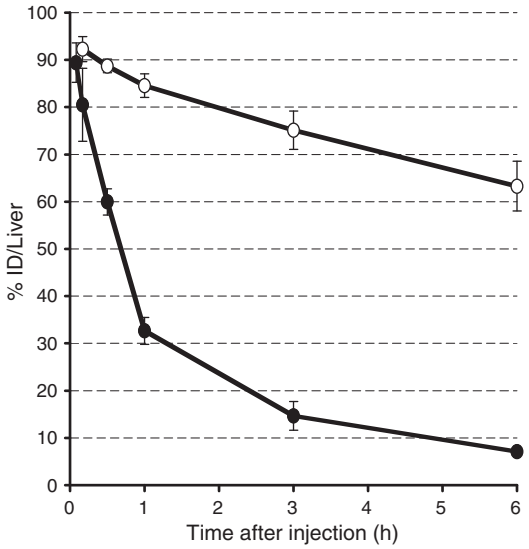


Figure 9. Elimination of radioactivity from the liver after injection of ^{99m}Tc-C_3(BHam)$_2$-NGA (●) and ^{99m}Tc-(HYNIC-NGA)(tricine)$_2$ (○) (data from Abrams *et al.*[16]) in normal mice.

DTPA-lysine, [^{111}In]SCN-Bz-EDTA-lysine, and ^{99m}Tc-HYNIC-lysine) as the major final radiometabolites in murine hepatocytes.[31,32,35,36] As ^{99m}Tc-C_3(BHam)$_2$-COOH was also conjugated to the ε-amine residues of NGA, it was speculated that a lysine adduct of ^{99m}Tc-C_3(BHam)$_2$ may be produced in the liver as the final radiometabolite. The rapid elimination of radioactivity after the injection of ^{99m}Tc-C_3(BHam)$_2$-NGA may reflect the rapid clearance of the final radiometabolites from the lysosomal compartment of hepatocytes. Although ^{99m}Tc-labeled polypeptides showed high and persistent levels of radioactivity in the liver and kidney, the present results observed for ^{99m}Tc-C_3(BHam)$_2$-NGA suggested that C_3(BHam)$_2$-COOH is a useful BCA for labeling with ^{99m}Tc to reduce the non-specific accumulation in the liver observed for ^{99m}Tc-HYNIC-polypeptides. Low molecular weight polypeptides including single-chain Fv fragments, diabodies, affibodies, minibodies, and bioactive small peptides are attractive scaffolds of ^{99m}Tc-labeled probes for targeted imaging because of the pharmacokinetic properties of these molecules. As ^{99m}Tc has an appropriate half-life to label such polypeptides, the application of C_3(BHam)$_2$-COOH as a BCA to low molecular weight polypeptides is expected in the future.

In conclusion, we successfully designed and synthesized C_3(BHam)$_2$-COOH as a new BCA for ^{99m}Tc labeling of polypeptides. C_3(BHam)$_2$-COOH formed a stable ^{99m}Tc complex and enabled us to prepare ^{99m}Tc-labeled polypeptides by using a TFP active ester of C_3(BHam)$_2$-COOH. ^{99m}Tc-C_3(BHam)$_2$-HSA existed stably in murine plasma and an excess of L-cysteine without any dissociation of ^{99m}Tc from polypeptides. Furthermore, as the blood clearance of ^{99m}Tc-C_3(BHam)$_2$-HSA in mice was similar to that of ^{125}I-HSA, C_3(BHam)$_2$-COOH retained stable binding between ^{99m}Tc and polypeptide *in vivo*. When we determined radioactivity after the injection of ^{99m}Tc-C_3(BHam)$_2$-NGA into mice, we found high liver uptake early on and rapid clearance from the liver, indicating that C_3(BHam)$_2$-COOH did not affect the pharmacokinetics of polypeptides *in vivo* and gave radiometabolites which showed rapid elimination from the liver. Such characteristics would render C_3(BHam)$_2$-COOH attractive as a new BCA for ^{99m}Tc labeling of polypeptides.

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Conflict of Interest

The authors did not report any conflict of interest.

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