Synthesis of a novel bifunctional chelator AmBaSar based on sarcophagine for peptide conjugation and ^{64}Cu radiolabelling†

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Copper-64 shows promise as both a suitable PET imaging and therapeutic radionuclide due to its nuclear characteristics. Stable attachment of radioactive $^{64}Cu^{2+}$ to targeted imaging probes requires the use of a bifunctional chelator. Sarcophagine (Sar) ligands coordinate the metal ion $^{64}Cu^{2+}$ within the multiple macrocyclic rings comprising the cage structure, yielding extraordinarily stable complexes that are inert to dissociation of the metal ion *in vivo*. Several ^{64}Cu labelled RGD derivatives have been applied in imaging of the $\alpha_v\beta_3$ integrin receptor expression during tumour angiogenesis. In order to design and develop novel molecular imaging probes containing RGD and Sar ligands, we designed a novel versatile Sar cage-like bifunctional chelator named AmBaSar, synthesized using a conventional synthetic strategy. Conjugation with the cyclic peptide RGD, and subsequent labelling with ^{64}Cu, provided a new PET probe ^{64}Cu-AmBaSar-RGD for imaging the $\alpha_v\beta_3$ integrin receptor.

Introduction

Molecular imaging is an emerging technology that allows for visualization of interactions between molecular probes and biological targets. Various modalities, which include nuclear medicine imaging, such as positron emission tomography (PET) and single photon emission tomography (SPECT), functional magnetic resonance imaging (fMRI), magnetic resonance spectroscopy (MRS), optical imaging including bioluminescence and fluorescence, targeted ultrasound, and the others have been applied.[1–3] The advancement of molecular imaging is driven mainly by the development of smart imaging probes targeted to specific receptors and enzymes.[1,4]

PET, micro-PET and PET/CT, are state-of-the-art nuclear medicine imaging modalities, which use nano- to picomolar concentrations of the corresponding probes (radiotracers) to achieve images of biological processes within the living system. The selection of the proper radionuclide and synthetic approach for radiotracer design are critical.[1] Positron-emitting isotopes frequently used include ^{11}C and ^{18}F. Recently, non-traditional PET radionuclides have gained considerable interest because of increased production and availability, such as ^{124}I, ^{68}Ga, ^{86}Y and $^{62/64}$Cu. One of them, ^{64}Cu, shows promise as both a suitable PET imaging and therapeutic radionuclide due to its nuclear characteristics ($t_{1/2} = 12.7$ h, β^+: 17.4%, $E_{\beta+\max} = 656$ keV; β^-: 39%, $E_{\beta-\max} = 573$ keV), and the availability of its large-scale production with high specific activity.[3] The stable attachment of radioactive $^{64}Cu^{2+}$ to targeted imaging probes requires the use of a bifunctional chelator (BFC), which is used to connect a radionuclide and bioactive molecule to form the ^{64}Cu-radiopharmaceutical. Optimization of the binding between the copper-64 and the BFC is crucial for achieving stable and high uptake of targeted ^{64}Cu-radiopharmaceuticals.[5]

The stability of ^{64}Cu–radiopharmaceuticals *in vivo* is a critical factor for optimal ligand design. A significant effort has been devoted to the development of ligands that can stably chelate $^{64}Cu^{2+}$ *in vivo*. Two of the most common chelators studied have been the macrocyclic ligands DOTA (1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid) and TETA (1,4,8,11-tetraazacyclotetradecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid). Recent studies have shown that these commonly used BFCs are unstable *in vivo* due to increased dissociation relative to more recently developing BFCs, such as the cross-bridged tetraamine ligands, the sarcophagine ligands and the triaminocyclohexane ligands.[6–9] Among these new BFCs, the Sar ligands containing hexaazamacrobicyclic cages have been developed by Sargeson and co-workers, and have recently been used for the preparation of ^{64}Cu–radiopharmaceuticals by Smith *et al.*[10–13] These ligands coordinate the metal ion Cu^{2+} within the multiple macrocyclic rings comprising the Sar cage structure, yielding extraordinarily stable complexes that are inert to dissociation of the metal ion.

A cage-like hexaazamacrobicyclic sarcophagine (Sar, Fig. 1A), $(NH_2)_2$-Sar (Diamsar, Fig. 1B), and their derivatives, have shown the potential for biology applications, including developing imaging and therapeutic agents.[14] Recently, a derivative of the DiamSar ligand, named SarAr (Fig. 1C), has been conjugated to antibodies and radiolabelled with ^{64}Cu. The resulting ^{64}Cu-SarAr immunoconjugates have shown high specific activity, antigen binding affinity, and *in vivo* target specificity to neuroblastoma and melanoma, with minimal uptake in normal tissues.[12,13] The cage-like BFC Sar ligands are unique in their ability to selectively label $^{64}Cu^{2+}$ rapidly over a wider range of pH values.[11] The cage-like Sar ligand is not only flexible for the incorporation of a range of functional linker groups and quantitatively complexing $^{64}Cu^{2+}$ at low concentrations, but also is adaptable for kit

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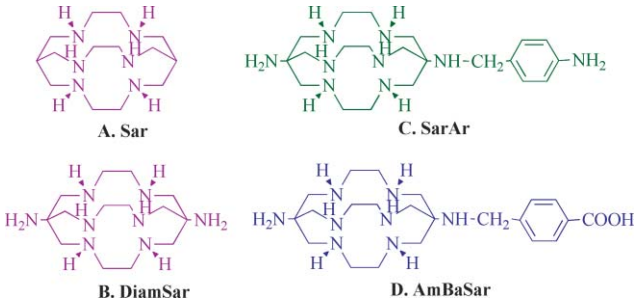


Fig. 1 Chemical structures of Sar, DiamSar, SarAr and AmBaSar.

formulations. However, there are only a few reports that describe the complexation, stability and biodistribution of the ^{64}Cu Sar complexes. Unfortunately, only one compound, a SarAr BFC for $^{64}Cu^{2+}$ conjugated with a monoclonal antibody (mAb), has been applied in PET imaging.[13]

The development of other linkage strategies for Sar is of interest to our laboratory. Primary amines and carboxylic acids are the most common examples of pendent arms that have been introduced to the DiamSar periphery. Direct introduction the carboxymethyl entities to DiamSar may be complicated and bring many by-products due to the side reactions on the secondary amines.[15] Likewise, using benzoic acid to functionalize DiamSar is an option. Based on this, we have designed a new Sar cage-like BFC named AmBaSar (Fig. 1D) by introducing benzoic acid to DiamSar. Tagged small peptides have attracted much attention recently as molecular imaging probes due to their relatively low immunogenicity, good pharmacokinetic properties, and binding affinities.[16] For example, conjugates containing the cyclic peptide Arg-Gly-Asp (RGD), bind to cancer cells and/or neoplastic vascular endothelial cells *via* the $\alpha_v\beta_3$ integrin receptor, and therefore have potential as angiogenesis-related diagnostic and therapeutic agents.[17,18] Several ^{64}Cu labelled RGD derivatives have been applied in imaging of the $\alpha_v\beta_3$ integrin receptor expression during tumour angiogenesis.[17–19] However, work remains to be done to optimize the pharmacokinetics and dynamics of these agents for use in animal and human studies. A reasonable next step is to select a new sarcophagine chelator for ^{64}Cu labelling RGD. In this study, we have designed a novel molecular imaging probe containing bioactive cyclic peptide RGD with a versatile Sar cage-like BFC AmBaSar. We have synthesized AmBaSar using a conventional synthetic strategy, and applied it conjugating the small cyclic peptide RGD for ^{64}Cu radiolabelling.

Abbreviations

The IUPAC names for the cage ligands are long and complicated. In this study, the names have been abbreviated as follows: 3,6,10,13,16,19-hexaazabicyclo[6.6.6]icosane = "sarcophagine" or "Sar"; 1, 8-diamine-3,6,10,13,16,19-hexaazabicyclo[6.6.6]icosane = "DiamSar"; 1-*N*-(4-aminobenzyl)-3,6,10,13,16,19-hexaazabicyclo[6.6.6]icosane-1,8-diamine = "SarAr"; methyl-4-((8-amino-3,6,10,13,16,19-hexaazabicyclo[6.6.6]icosane-1-ylamino)methyl)benzoate = "AmMBSar"; 4-((8-amino-3,6,10,13,16,19-hexaazabicyclo[6.6.6]icosane-1-ylamino)methyl)benzoic acid = "AmBaSar".

For complexes of Sar ligands, we have used nomenclature which represents these M-hexaamine cage complexes by "M-(X,Ysar)", where M represents the metal ion, and X and Y are the substituents in the 1- and 8-positions of the Sar cage. (1,8-Dinitro-Sar)cobalt(III) trichloride = $[Co(DiNosar)]Cl_3$; (1,8-diamine-Sar)cobalt(III) pentachloride = $[Co(DiAmSar)]Cl_5$; (1,8-diamine-Sar)copper(II) tetrachloride = $[Cu(DiAmSar)]Cl_4$; (1-amine,8-(aminomethyl)-4′-methylbenzoate-Sar)copper(II) = $[Cu(AmMBSar)]^{2+}$; (1-amine,8-(aminomethyl)-benzoic acid-Sar)copper(II) = $[Cu(AmBaSar)]^{2+}$; (1,8-(aminomethyl)-4′-methylbenzoate-Sar)copper(II) = $[Cu(DiMBSar)]^{2+}$.

Experimental

Methods and materials

^{1}H NMR spectra were obtained using a Varian Mercury 400 MHz instrument (USC NMR Instrumentation Facility), and the chemical shifts were reported in ppm on the δ scale relative to an internal TMS standard. Microanalyses for carbon, hydrogen, nitrogen and chlorine, cobalt, copper were carried out by the Columbia Analytical Services, Inc (Tucson, AZ). Mass spectra using LC-MS were operated by the Proteomics Core Facility of the USC School of Pharmacy. Thin-layer chromatography (TLC) was performed on silica gel 60 F-254 plates (Sigma-Aldrich) using a mixture solution of 70% MeOH and 30% aqueous NH_4OAc (NH_4OAc solution is 20% by weight) as the mobile phase. Ion-exchange chromatography was performed under gravity flow using Dowex 50WX2 (H^+ form, 200–400 mesh) or SP Sephadex C25 (Na^+ form, 200–400 mesh) cation exchange resins. All evaporations were performed at reduced pressure (*ca.* 20 Torr) using a Büchi rotary evaporator.

HPLC was accomplished on two Waters 515 HPLC pumps, a Waters 2487 absorbance UV detector and a Ludlum Model 2200 radioactivity detector, operated by Waters Empower 2 software. Purification of the conjugate AmBaSar-RGD was performed on a Phenomenex Luna C18 reversed phase column (5 μm, 250 × 10 mm); the flow was 3.2 mL min^{-1}, with the mobile phase solvent A (12% acetonitrile in water), and the absorbance monitored at 254 nm. The analytical HPLC was done on a Phenomenex Luna C18 reversed phase column (5 μm, 250 × 4.6 mm) and monitored using a radiodetector and UV at 218 nm. The flow was 1.0 mL min^{-1}, with the mobile phase starting from 99% solvent B (0.1% TFA in water) and 1% solvent C (0.1% TFA in acetonitrile) (0–1 min) to 70% solvent B (0.1% TFA in water) and 30% solvent C (0.1% TFA in acetonitrile) (1–30 min).

All reagents and solvents were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA) and used without further purification unless otherwise stated. *N*-hydroxysulfosuccinimide sodium salts (SNHS) and *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl) were obtained from the Sigma-Aldrich Chemical Co. The cyclic RGDyK peptide was purchased from Peptides International, Inc (Louisville, KY, USA). $^{64}CuCl_2$ was purchased from MDS Nordion (Vancouver, BC, Canada). Water was purified using a Milli-Q ultra-pure water system from Millipore Corp. (Milford, MA, USA).

The bifunctional chelator AmBaSar synthesis and characterization

Synthesis of $[Co(DiNoSar)]Cl_3$ (1). To a solution of tris(ethylenediamine)cobalt(III) chloride dehydrate (1.2 g,

3.0 mmol) in water (4.0 mL) was added nitromethane (0.7 g, 12 mmol) and aqueous formaldehyde (37%, 2.4 g, 30 mmol). The resulting solution was cooled to 4 °C on an ice–water bath. Aqueous NaOH (4.0 M, 3.0 mL) was cooled to 4 °C and mixed with the resulting solution above. The combined solution was stirred on ice-water bath where it rapidly turned deep violet-brown from the initially orange colour, and the reaction temperature was raised to room temperature 23–25 °C. After 90 min, the reaction was quenched by the addition of HCl (6.0 M, 2.0 mL). The orange precipitate formed was collected by filtration after cooling on ice for 2 h, washed with methanol, and dried to provide **1** (1.46 g, 90.2%). ^{1}H-NMR (D_2O): δ 3.55–3.65 (d, 6 H, $NCCH_2N$); 3.15–3.35 (d, 6 H, $NCCH_2N$); 3.00–3.10 (d, 6 H, NCH_2CH_2N); 2.55–2.65 (d, 6 H, NCH_2CH_2N). MS: calcd for $C_{14}H_{31}CoN_8O_4$ [M + 1 – 3HCl] $^+$ m/z 431.2, found 431.5. Elemental analysis calculated for $C_{14}H_{30}Cl_3CoN_8O_4$, requires C 31.15, H 5.60, N 20.76, Cl 19.71, Co 10.92. Found: C 30.90, H 5.46, N 20.13, Cl 20.80, Co 10.70.

Synthesis of [Co(DiamSar)]Cl_5·H_2O (2). [Co(DiNoSar)]Cl_3 (2.0 g, 3.7 mmol) was dissolved in deoxygenated water (100 mL) under N_2 atmosphere. Zinc dust (2.3 g, 35 mmol) was added into the solution with stirring for 5 min, followed by addition of HCl (6 M, 15 mL) dropwise. The resulting solution continued to stir for an additional 60 min under a N_2 atmosphere. The N_2 flow was stopped and 30% H_2O_2 (1.0 mL) was added. The resulting solution was warmed for 15 min on 75 °C water bath, then cooled and placed on a Dowex 50WX2 cation exchange column and washed with water (120 mL), followed by HCl (1.0 M, 120 mL). The complex was then eluted with HCl (3.0 M, 400 mL) and the yellow elute was collected and dried under vacuum to yield **2** (1.78 g. 87%). ^{1}H-NMR (D_2O): δ 3.30–3.10 (m, 12 H, $NCCH_2N$); 2.50–2.65 (m, 12 H, NCH_2CH_2N). MS calcd for $C_{14}H_{35}CoN_8$ [M + 1 – 5HCl – H_2O] $^+$ m/z 371.2, found 371.7. Elemental analysis calculated for $C_{14}H_{38}Cl_5CoN_8O$, requires C 29.46, H 6.71, N 19.63, Cl 31.06, Co 10.33. Found: C 29.06, H 6.73, N 19.04, Cl 25.30, Co 9.50.

Synthesis of DiamSar·$5H_2O$ (3). Co(DiamSar)]Cl_5·H_2O (3.58 g, 6.3 mmol), NaOH (0.58 g, 14.5 mmol, sufficient to neutralize the protonated primary amino groups) and cobalt(II) chloride hexahydrate (1.6 g, 6.4 mmol) were dissolved in deoxygenated water (50 mL) under nitrogen. Sodium cyanide (5.60 g, 114 mmol) was added to the resulting solution. The reaction mixture was heated to 70 °C being stirred under nitrogen until the solution had become almost colourless (overnight). This final solution was taken to dryness under vacuum, with the residue extracted with boiling acetonitrile (3 × 25 mL). The total extract was filtered, reduced under vacuum to a white solid, and cooled to −10 °C to precipitate white crystals of the product. Drying *in vacuo* provided **3** (1.72 g, 58.5%). Mp 91–94.0 °C. ^{1}H-NMR (D_2O): δ 2.51 (s, 12 H, $NCCH_2N$); 2.42 (s, 12 H, NCH_2CH_2N). MS calcd for $C_{14}H_{35}N_8$ [M + 1 – $5H_2O$] $^+$ m/z 315.3, found 315.8. Elemental analysis calculated for $C_{14}H_{44}N_8O_5$, requires C 41.56, H 10.96, N 27.70. Found: C 41.53, H 10.28, N 27.12.

Synthesis of [Cu(DiamSar)]Cl_4·$5H_2O$ (4). $CuCl_2$·$2H_2O$ (0.17 g, 1.0 mmol), was dissolved in 10 mL water, followed by adding **3** (0.41 g, 1.0 mmol). The solution was acidified to pH = 4.0 with HCl (0.1 M), and stirred overnight. Evaporation on heating yielded a blue precipitate. The blue solid was cooled, filtered, and washed with ethanol (3 × 5 mL), and dried, yielding a blue crystalline product **4** (0.53 g, 97%). ^{1}H-NMR (D_2O): δ 3.20 (s, 12 H, NCH_2CH_2N); 2.90 (s, 12 H, $NCCH_2N$). MS calcd for $C_{14}H_{33}CuN_8$ [M + 1 – 4HCl – $5H_2O$] $^+$ m/z 376.2, found 376.0. Elemental analysis calculated for $C_{14}H_{46}Cl_4CuN_8O_5$, requires C 27.48, H 7.58, N 18.31, Cl 23.17, Cu 10.38. Found: C 27.72, H 7.17, N 17.87, Cl 22.40, Cu 9.71.

Synthesis of [Cu(AmMBSar)]$(CH_3COO)_2$·$5H_2O$ (5). [Cu(DiAmSar)]Cl_4·$5H_2O$ (0.73 g, 1.2 mmol) was dissolved in dry ethanol (30 mL), followed by addition of methyl 4-formylbenzoate (0.28 g, 1.7 mmol), dried/activated 4 Å molecular sieves (1.0 g) and glacial acetic acid (60 μL). The resulting solution was stirred for 3 h under argon gas, followed by addition of sodium cyanoborohydride (0.82 g, 14 mmol). The reaction mixture continued to stir under argon gas for 4 d at room temperature 20–25 °C. The mixture was filtered, and filtrate was evaporated to dryness and extracted with ethyl acetate (3 × 15 mL), dried and then diluted to 300 mL. It was placed onto a SP Sephadex C25 column and eluted with sodium citrate (0.1 M, 400 mL) and a wide blue band formed. Increasing the sodium citrate concentration (0.3 M, 500 mL) resulted in three blue bands eluting in order as $[Cu(DiamSar)]^{2+}$, $[Cu(AmMBSar)]^{2+}$ (compound **5**), and $[Cu(DiMBSar)]^{2+}$ by TLC monitoring. The second band (compound **5** solution, 100 mL) was isolated and diluted with water (10 fold, 1.0 L), and placed onto another SP Sephadex C25 column. A single blue band eluted with sodium acetate (1.0 M, 200 mL), was evaporated to dryness and the residue extracted with 2-propanol (3 × 50 mL). Fine white crystals of sodium acetate were separated, filtered and the process of evaporation and extraction repeated 3 times. The final residue was dried *in vacuo* to a dark blue solid **5** [Cu(AmMBSar)]$(Ac)_2$·$5H_2O$ (246.5 mg, 27.9%). ^{1}H-NMR (D_2O): δ 8.0–7.9 (d, 2 H, aromatic); 7.48–7.42 (d, 2 H, aromatic); 4.70 (s, 3 H, OCH_3); 4.02 (s, 2 H, CH_2-Ar); 3.54–3.30 (m, 12 H, NCH_2CH_2N); 2.93–2.78 (m, 12 H, $NCCH_2N$). MS calcd for $C_{23}H_{43}CuN_8O_2$ [M + 1 – 2HAc – $5H_2O$] $^+$ m/z 526.3, found 526.8.

Synthesis of AmMBSar (6). Sodium borohydride (150 mg) was dissolved in 0.4 mL water and stirred under a nitrogen atmosphere, following addition of Pd/C (60 mg) in 1.0 mL water. Compound **5** (164 mg) was dissolved in sodium hydroxide (3 mL; 1% NaOH) and added dropwise to the above mixture solution. Stirring was continued under nitrogen at 25 °C until the colour turned from blue to clear. The resulting solution was filtered (0.22 μm) and the filtrate was collected in an ice-cooled glass vial. Concentrated hydrochloric acid was added dropwise (50 μL) to the cooled solution until gas evolution ceased (~ 450 μL, HCl). The solution was acidified to pH 4–6, and then dried under vacuum to provide AmMBSar, **6** (56 mg, 47.6%). ^{1}H-NMR (D_2O): δ 7.97–7.92 (d, 2 H, aromatic); 7.50–7.45 (d, 2 H, aromatic); 4.65 (s, 3 H, OCH_3); 4.08 (s, 2 H, CH_2-Ar); 3.45–3.20 (m, 12 H, NCH_2CH_2N); 3.12–2.98 (m, 12 H, $NCCH_2N$); 1.98–1.85 (m, 9 H, NH). MS calcd for $C_{23}H_{42}N_8O_2$ [M + 1] $^+$ m/z 463.0, found 462.3.

Synthesis of AmBaSar (7). Compound **6** (46.2 mg, 0.1 mmol) was dissolved in methanol (3 mL) and water (1.0 mL), followed by addition of NaOH (1.5 mL, 0.1 M). The resulting solution was refluxed and stirred for 5 h, then neutralized with HCl (1.0 M) to pH 6–7. Drying the solution under reduced pressure formed

solids, which were dissolved with hot MeOH (3 × 2 mL). The total extract was filtered and dried under vacuum to yield AmBaSar, **7** (36 mg, 80.2%). ^{1}H-NMR (D_2O): δ 7.76–7.67 (m, 2 H, aromatic); 7.45–7.37 (m, 2 H, aromatic; 3.66 (s, 2 H, NCH_2C) 3.26–3.04 (m, 12 H, $NCCH_2N$); 2.99–2.84 (m, 12 H, NCH_2CH_2N); 1.84–1.77 (m, 9 H, NH). MS calcd for $C_{23}H_{41}N_8O_2$ $[M + 1]^+$ m/z 449.3, found 449.7.

AmBaSar conjugating peptide RGD and radiolabelling

Synthesis of AmBaSar-RGD (8). AmBaSar was activated by EDC at pH 5.5 for 30 min (4 °C), with a molar ratio of AmBaSar : EDC : SNHS = 5 : 5 : 4. Typically, 15.0 mg of AmBaSar (30 μmol) was dissolved in 500 μL of water. Separately, 5.76 mg of EDC (30 μmol) was dissolved in 500 μL of water. The two solutions were mixed, and 0.1 M NaOH (250 μL) was added to adjust the pH to 4.5. SNHS (5.2 mg, 24 μmol) was then added to the stirring mixture on an ice-bath, followed by 0.1 M NaOH (50 μL) to finalize the pH to 5.4. The reaction was allowed to stir for 30 min at 4 °C. The theoretical concentration of active ester AmBaSar-OSSu was calculated to be 24 μmol. Cyclic RGD peptide (2.5 mg, 4.0 μmol) dissolved in 500 μL (5.0 mg mL^{-1}) of water was cooled to 4 °C and added to the AmBaSar-OSSu reaction mixture. The pH was adjusted to 8.6 with 0.1 M NaOH (280 μL). The reaction was allowed to proceed overnight at room temperature (20–25 °C). The AmBaSar-RGD conjugate was purified by semi-preparative HPLC. The peak containing the RGD conjugate was collected, lyophilized, and dissolved in water at a concentration of 1.0 mg mL^{-1} for use in radiolabelling reactions.

Synthesis of reference compound [Cu-AmBaSar-RGD](Ac)$_2$·2HAc. The AmBaSar-RGD (1.0 mg) was dissolved in 0.5 mL of a 0.1 M ammonium acetate–0.80 mM copper(II) acetate solution. The mixture was stirred at 37 °C for 40 min and allowed to cool to room temperature. The crude Cu-AmBaSar-RGD solution was purified and quantified by HPLC. MS calcd for $C_{58}H_{94}N_{16}O_{17}Cu$ $[M + 1]^+$ m/z 1352.0, found 1351.9.

Radiolabelling of ^{64}Cu-AmBaSar-RGD. [^{64}Cu]acetate (^{64}Cu-$(OAc)_2$) was prepared by adding 111 MBq (3 mCi) of $^{64}CuCl_2$ in 0.1 M HCl into an 1.5 mL microfuge tube containing 300 μL 0.1 M ammonium acetate (pH 5.0), followed by mixing using a mini vortex and incubating for 15 min at room temperature. The AmBaSar-RGD (1–2 μg in 100 μL 0.1 M ammonium acetate) was labelled with $^{64}Cu(OAc)_2$ by addition of 1–3 mCi of ^{64}Cu. The chelation reaction was performed in 0.1 M sodium acetate buffer, pH 5.0, for 60 min at room temperature (23–25 °C). Labelling efficiency was determined by HPLC. ^{64}Cu-AmBaSar-RGD was purified by radio-HPLC. The eluant was evaporated and the activity reconstituted in saline, followed by passage through a 0.22 μm Millipore filter into a sterile dose vial for use in animal experiments.

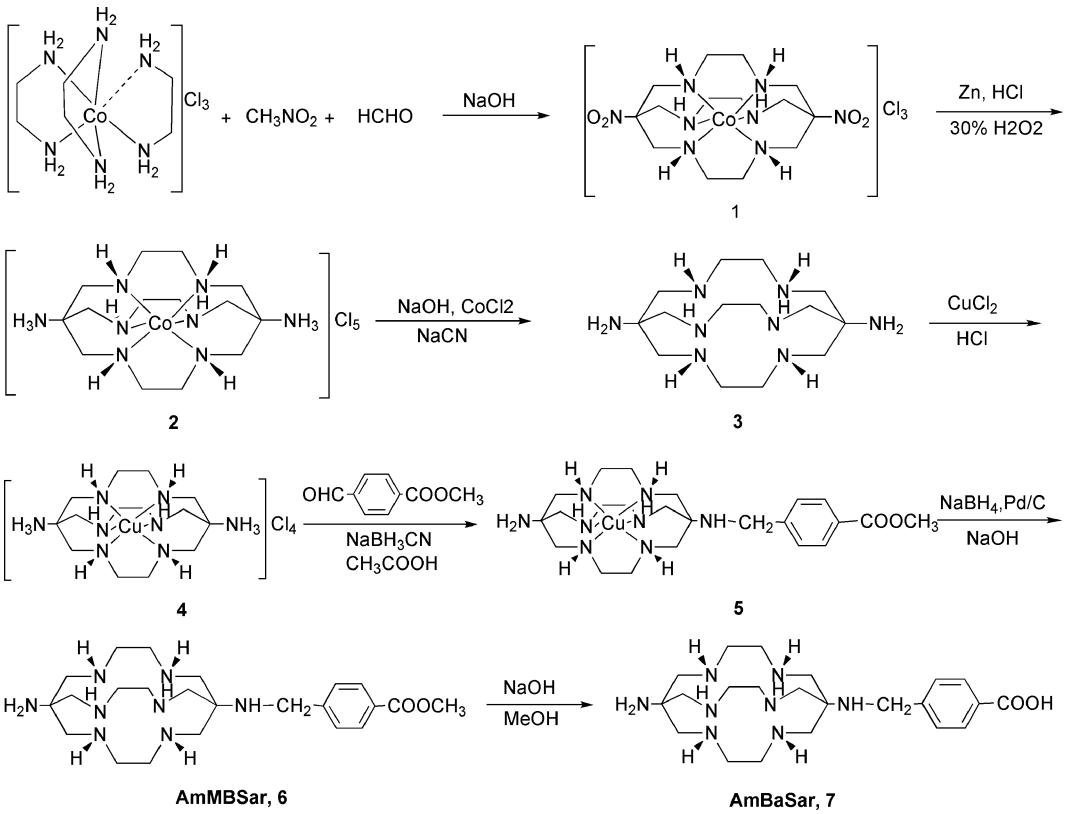
Results and discussion

Design, synthesis, and characterization of bifunctional chelator AmBaSar

In order to design a BFC such as SarAr for ^{64}Cu labelling and conjugating with RGD, it was necessary to modify the linkage of DiamSar. We replaced the aromatic amine of SarAr with an aromatic carboxyl, which can conjugate a cyclic peptide containing the RGD motif in addition to a lysine for conjugating to the chelator, to form BFC AmBaSar with minimum modification to SarAr. The novel BFC AmBaSar can efficiently label $^{64}Cu^{2+}$ due to the provision of a three-dimensional hexa-aza cage which increases thermodynamic and kinetic stability to complex $^{64}Cu^{2+}$ or other metal ions, while allowing the aromatic linker with carboxyl acid group to conjugate with the amine of lysine in the cyclic peptide containing the RGD motif. The AmBaSar-RGD can possibly be used to form new nuclear, MRI, and optical probes by complexion with other appropriate metal radioisotopes or paramagnetic metal ions using similar preparations. We anticipate that the AmBaSar can be applied to the other peptides or biomolecules, since the chemistry for conjugating AmBaSar to other peptides is similar to that described for the cyclic peptide RGD.

An efficient synthesis of the bifunctional chelator AmBaSar was shown in Scheme 1. It is worth noting that the degree of hydration of the synthesized compounds in this study is dependent on the method of purification. Although compounds **1**–**4** can be prepared following literature methods using the cobalt complexes as starting materials,[20,21] the reported syntheses of compound **1**–**4** are incomplete, with compounds not fully characterized. Here, the comprehensive and detailed synthetic procedures with full characterization of these compounds (including MS, element analysis, ^{1}H-NMR) are reported. Tris(ethylenediamine)cobalt(III) chloride initiated the encapsulation process using the reactive nucleophile nitromethane and formaldehyde in alkaline solution at room temperature to form a hexa-aza cage containing Co(III) compound **1**, which was then reduced with zinc dust in acid solution to give the very stable Co(III) DiamSar complex **2**. The Co(III) ion of compound **2** can be removed by reduction with high concentrations of hydrochloric or hydrobromic acid at high temperatures (130–150 °C) or excess cyanide ion to yield the free cage. Here, we used excess cyanide ion to remove the metal ion in compound **2** to form compound **3** in good yield (58.5%). The six nitrogen donor atoms of the hexa-aza cage allows strong binding to many metal ions, such as Cu^{2+}, so compound **3** was easy to complex with metal ion Cu^{2+}, yielding compound **4** under weak acid conditions. The compounds **1**–**4** were characterized by elemental analysis, MS and ^{1}H-NMR, the results are consistent with those reported previously.[20,21] The yields obtained using the modified procedures shown here were similar to or better than those previously reported.

It is challenging to functionalize the apical primary amines of DiamSar **3** directly because there are 2 primary and 6 secondary amines in DiamSar, which potentially could create many by-products and difficulty during purification. The initial formation of copper(II) complex of DiamSar **4** was an attractive solution as it would serve to tie up the 6 secondary amines of DiamSar, and also permit the tracking of the resultant Cu(II) complexes on the ion exchange columns.[10] Structural studies have confirmed that it is possible to exploit the relatively low nucleophilicity of the Cu(II) complex of DiamSar in acylation and alkylation reactions leading to a variety of functionalized cage amine complexes.[22,23] We utilized the hydride reducing agent sodium cyanoborohydride ($NaBH_3CN$) for reduction due to its stability in relatively strong acid solutions (about pH 3), its solubility in hydroxylic solvents such as ethanol, and its different selectivities at different pH values. At pH 3–4 it reduces the imine to an amine efficiently, while this



Scheme 1 Synthesis of bifunctional chelator AmBaSar.

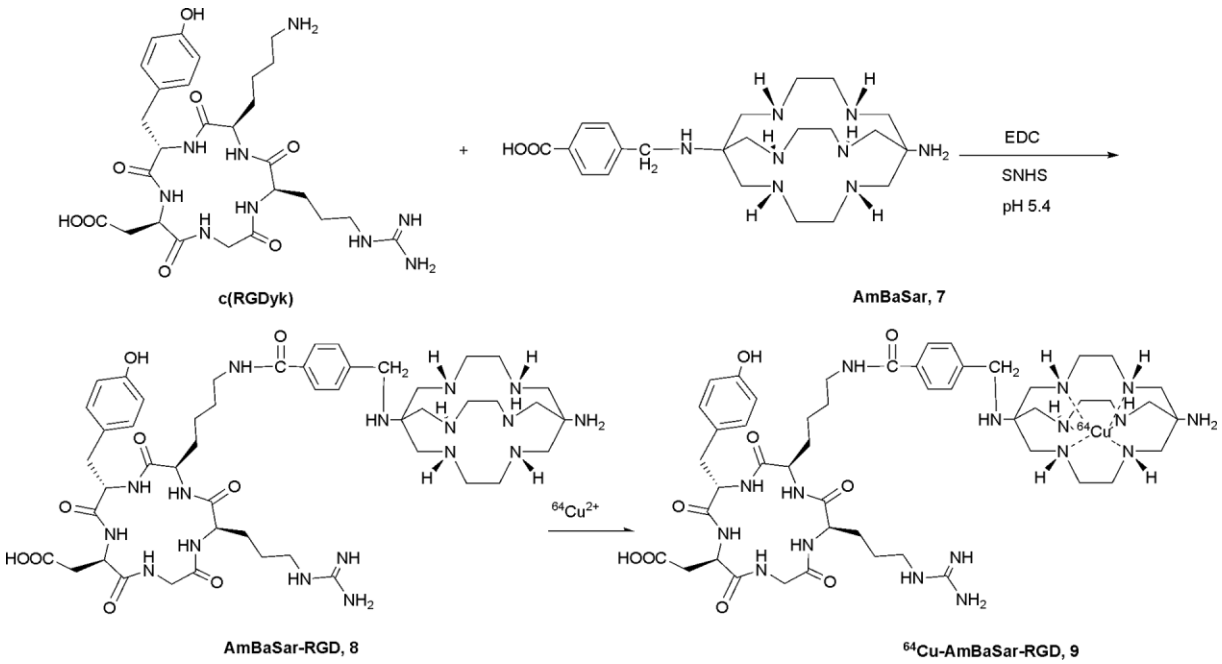
reduction becomes very slow at higher pH values.[24] Hence, the reducing reaction of the cage amine of compound **4** with aromatic methyl-4-formylbenzoate under $NaBH_3CN$ ethanol acid solution can yield aromatic functionalization of the cage amine compound **5** and the by-product bis-4-formylbenzoate diamsar complex. This reaction took a significant amount of time (4 days) and yield was 27.9% due to low nucleophilicity. Separation was carried out by ion exchange chromatography. Compound **6** was achieved by demetallation of compound **5**, followed by alkaline hydrolysis to produce the target compound **7** AmBaSar according to the reported method.[25]

Initially, we planned to use Fmoc protection of the free primary amine of AmBaSar in order to apply a solid-phase peptide synthesis (SPPS), in the event that the primary amine might perturb the conjugation with peptides. But this reaction did not happen as we expected or the yield was too low to be detected (data not shown). This likely means the primary amine of AmBaSar is inactive under these conditions. The reason for the poor reactivity of the primary amine in AmBaSar may be due to the structure of the cage hindering the reaction between Fmoc-Cl with the primary amine. This result is consistent with the inability of DiamSar to directly efficiently conjugated antibodies or proteins.[12,26] This provides additional proof that DiamSar should be functionalized and the suitable linker group should be sufficient length (~6 atom lengths) and rigidity incorporating a reactive group that could be readily attached to a range of target agents, but not interfere with DiamSar's efficient and selective complexation of metal ion like Cu^{2+}, as well as the target agent's biological activity.[10] AmBaSar appears to meet these requirements.

AmBaSar conjugating cyclic peptide RGD and ^{64}Cu radiolabelling

The bifunctional chelator AmBaSar contains one carboxyl group and an inactive primary amine, which means that it is a mono-functional molecule that can react with a cyclic peptide containing the RGD motif in addition to a lysine for conjugating to the chelator. Here, we used a simple procedure for conjugation the cyclic RGD and BFC AmBaSar and common procedures for ^{64}Cu radiolabelling conjugates.[27–29] Scheme 2 depicts AmBaSar conjugating cyclic RGD and ^{64}Cu radiolabelling. The cyclic RGD conjugate AmBaSar-RGD was synthesized in 80% yield and purified by semi-preparative HPLC. Analytical HPLC found the retention time of AmBaSar-RGD to be 3.8 min, whereas cyclic RGD peptide eluted at 25 min under the same condition. AmBaSar-RGD was analyzed by mass spectrometry, found $m/z =$ 1049.3 for $[M + H]^+$ ($M = C_{50}H_{80}N_{16}O_9$) and 1089.7 for $[M + K]^+$.

The peptide–chelator conjugate AmBaSar-RGD can be complexed with ^{64}Cu to form a new PET tracer for imaging the $\alpha_v\beta_3$ integrin receptor. AmBaSar–RGD was labelled with ^{64}Cu in 0.1 M ammonium acetate (pH 5.0) solution at room temperature (25 °C) for 1 h. The free ^{64}Cu-acetate was eluted at 3.2 min, while ^{64}Cu-AmBaSar-RGD was eluted at 15.8 min by analytical HPLC, which was confirmed by cold Cu-AmBaSar-RGD. The radiochemical yield obtained was ≥80% and the radiochemical purity was ≥95%. We did not optimize the radiolabelling here because our overall objective was to demonstrate the feasibility of preparing our novel BFC. We are currently conducting the biological evaluation of this new tracer *in vitro* and *in vivo* for PET imaging of the $\alpha_v\beta_3$ integrin receptor.



Scheme 2 Synthesis of AmBaSar-RGD and labelled with copper-64.

Conclusions

A novel versatile Sar caged-like bifunctional chelator AmBaSar containing a hexa-aza cage for ^{64}Cu radiolabelling and an aromatic linker with carboxylic acid group for conjugate cyclic RGD has been designed and synthesized using a conventional synthetic strategy, with characterizations by elemental analysis, MS and ^{1}H-NMR. AmBaSar conjugated cyclic peptide RGD was prepared in 80% yield, and then labelled with ^{64}Cu with more than 80% radiochemical yield and 95% radiochemical purity, to form a new PET probe ^{64}Cu-AmBaSar-RGD for imaging the $\alpha_v\beta_3$ integrin receptor.

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Notes and references

1 P. A. Schubiger, L. Lehmann and M. Friebe, *PET Chemistry: The Driving Force in Molecular Imaging*, Springer, New York, 2006.
2 T. F. Massoud and S. S. Gambhir, *Genes Dev.*, 2003, **17**, 545–580.
3 M. J. Welch and C. S. Redvanly, *Handbook of Radiopharmaceuticals: Radiochemistry and Applications*, Wiley, West Sussex, England, 2003.
4 C. H. Manning, A. Lander, E. McKinley and N. J. Mutic, *J. Nucl. Med.*, 2008, **49**, 1401–1404.
5 M. W. Brechbiel, *Q. J. Nucl. Med. Mol. Imaging*, 2008, **52**, 166–173.
6 T. J. Wadas, E. H. Wong, G. R. Weisman and C. J. Anderson, *Curr. Pharm. Des.*, 2007, **13**, 3–16.
7 S. Juran, M. Walther, H. Stephan, R. Bergmann, J. Steinbach, W. Kraus, F. Emmerling and P. Comba, *Bioconjugate Chem.*, 2009, **20**, 347–359.
8 C. L. Ferreira, D. T. Yapp, E. Lamsa, M. Gleave, C. Bensimon, P. Jurek and G. E. Kiefer, *Nucl. Med. Biol.*, 2008, **35**, 875–882.
9 L. Wei, Y. Ye, T. J. Wadas, J. S. Lewis, M. J. Welch, S. Achilefu and C. J. Anderson, *Nucl. Med. Biol.*, 2009, **36**, 277–285.
10 S. V. Smith, *Q. J. Nucl. Med. Mol. Imaging*, 2008, **52**, 193–202.
11 S. V. Smith, *J. Inorg. Biochem.*, 2004, **98**, 1874–1901.
12 N. DiBartolo, A. M. Sargeson and S. V. Smith, *Org. Biomol. Chem.*, 2006, **4**, 3350–3357.
13 S. D. Voss, S. V. Smith, N. DiBartolo, L. J. McIntosh, E. M. Cyr, A. A. Bonab, E. A. Carter, A. J. Fischman, S. T. Treves, S. D. Gillies, A. M. Sargeson, J. S. Huston and A. B. Packard, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 17489–17493.
14 A. M. Sargeson, *Coord. Chem. Rev.*, 1996, **151**, 89–114.
15 P. S. Donnelly, J. M. Harrowfield, B. W. Skelton and A. H. White, *Inorg. Chem.*, 2000, **39**, 5817–5830.
16 J. Fichna and A. Janecka, *Bioconjugate Chem.*, 2003, **14**, 3–17.
17 A. J. Beer and M. Schwaiger, *Cancer Metastasis Rev.*, 2008, **27**, 631–644.
18 W. Cai and X. Chen, *J. Nucl. Med.*, 2008, **49**, 113S–128S.
19 R. Haubner and H. J. Wester, *Curr. Pharm. Des.*, 2004, **10**, 1439–1455.
20 R. J. Geue, T. W. Hambley, J. M. Harrowfield and A. M. Sargeson, *J. Am. Chem. Soc.*, 1984, **106**, 5478–5488.
21 G. A. Bottomley, I. J. Clark, I. I. Creaser, L. M. Engelhardt, R. J. Geue, K. S. Hagen, J. M. Harrowfield, G. A. Lawrance, P. A. Lay, A. M. Sargeson, A. J. See, B. W. Skelton, A. H. White and F. R. Wilner, *Aust. J. Chem.*, 1994, **47**, 143–179.
22 S. Burnet, M. H. Choi, P. S. Donnelly, J. M. Harrowfield, I. Ivanova, S. J. Jeong, Y. Kim, M. Mocerino, B. W. Skelton, A. H. White, C. C. Williams and Z. L. Zeng, *Eur. J. Inorg. Chem.*, 2001, 1869–1881.
23 G. C. Yeh, A. M. Beatty and J. K. Bashkin, *Inorg. Chem.*, 1996, **35**, 3828–3835.
24 A. F. Abdel-Magid, K. G. Carson, B. D. Harris, C. A. Maryanoff and R. D. Shah, *J. Org. Chem.*, 1996, **61**, 3849–3862.
25 N. DiBartolo, A. M. Sargeson, T. M. Donlevy and S. V. Smith, *J. Chem. Soc., Dalton Trans.*, 2001, 2303–2309.
26 K. L. Bennett, S. V. Smith, R. M. Lambrecht, R. J. W. Truscott and M. M. Sheil, *Bioconjugate Chem.*, 1996, **7**, 16–22.
27 M. R. Lewis, J. Y. Kao, A. L. J. Anderson, J. E. Shively and A. Raubitschek, *Bioconjugate Chem.*, 2001, **12**, 320–324.
28 X. Chen, R. Park, M. Tohme, A. H. Shahinian, J. R. Bading and P. S. Conti, *Bioconjugate Chem.*, 2004, **15**, 41–49.
29 T. J. Wadas and C. J. Anderson, *Nature Protocols.*, 2007, **1**, 3062–3068.

