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Synthesis of a new cage ligand, SarAr, and its complexation with selected transition metal ions for potential use in radioimaging †

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A new hexaazamacrobicyclic cage ligand, 1-*N*-(4-aminobenzyl)-3,6,10,13,16,19-hexaazabicyclo[6.6.6]eicosane-1,8-diamine (SarAr) has been designed for conjugation to proteins. SarAr was synthesised and characterised by microanalyses, ^{1}H NMR and electrospray mass spectrometry. The complexation of selected transition metal ions (Cu(II), Ni(II) and Co(II) at 10^{-6} M) by SarAr was complete within 30 min over pH 6 to 8. The $[^{64}Cu(SarAr)]^{2+}$ complex was investigated with a view to applications in radioimaging. The $[^{64}Cu(sar)]^{2+}$ complex was found to be stable in human plasma for at least 174 h and biodistribution studies in mice, showed that the $[^{64}Cu(SarAr)]^{2+}$complex was rapidly excreted through the renal system unlike the free $^{64}Cu^{2+}$. Overall, the simple synthesis, ready complexation behaviour of SarAr, the kinetic inertness of the $[Cu(SarAr)]^{2+}$ complex to dissociation of ^{64}Cu and its facile elimination from mice make it an attractive prospect for use in nuclear medicine.

Introduction

Numerous hexaazamacrobicyclic cage ligands (Fig. 1) have now been synthesised and some prospects for biological and medical applications have been investigated.[1–4] The periphery of the sar ligand has also been derivatised significantly.[1,4] The substitutions are well documented and include amine, hydroxyl, halide, carboxylate, amide and aromatic groups attached to the apical carbon of the bridgehead. These groups and others such as glycine, allow for final conjugation to peptides, sugars, steroids, intercalating agents, paraffins and biologically compatible polymers, in order to target various types of biological sites.[1,2]

The syntheses of the sarcophagine (sar) type cages are simply and readily effected through a template method as outlined previously.[5] They involve the reaction of the kinetically inert tris(ethylenediamine)cobalt(III) complex (as the template), formaldehyde and a nucleophile such as nitromethane in basic conditions at ~ 25 °C. The Co(III) complexes of these ligands are extremely stable both kinetically and thermodynamically. However, if reduced to Co(II), the metal ion may be effectively removed with either cyanide or concentrated HBr.[3] The free cage so obtained may then be complexed with various metal ions, including Cu(II), Ni(II), Co(II), Fe(II), Mn(II), Mg(II), Zn(II), Cd(II), Hg(II), Cr(III), Ga(III), In(III), V(IV), Ag(II), Re(V) and Ru(II). Rh(III), Ir(III) and Pt(IV) cages have also been synthesised directly by the template process and the X-ray structures of many of these products have been established. [5–9]

The metal ion is encapsulated in the ligand tightly to give extraordinarily stable complex cations which are mostly kinetically inert to dissociation of the metal. For example, the transition metal complexes of sar can be expected to be more stable than those of commonly known tetraazamacrocycles (*e.g.* cyclam, 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)). Stability constants (log *K* at 25 °C, 0.1 M) reported for Hg^{2+}, Cu^{2+}, Ni^{2+} and Co^{2+} complexes of cyclam are 23.0, 27.2, 22.2 and 12.71, for DOTA they are 26.4, 22.2, 20.0 and 20.2 in 0.1 M $[(CH_3)_4N(NO_3)]$ and for TETA, they are 25.7, 21.6, 19.9 and 16.6 at 25 °C 0.1 M ionic strength, respectively.[10–12] The Hg^{2+} complex with sar is the only molecule to date where the stability constant could be measured and it has a log *K* value of 28.1 ($[OH^-] = 0.1$ M, $I = 0.5$ M $NaClO_4$, at 25 °C),[8] which is significantly larger than those of Hg^{2+} cyclam, DOTA or TETA complexes. The $[Cu(sar)]^{2+}$ complex is expected to be even more stable but the very slow dissociation of the Cu^{2+} cation precludes the determination of its log *K* value. The exchange of radiolabelled Cu^{2+} ion with the copper complex (< 6% in 18 h at ~ 25 °C)[13] is consistent with this analysis.

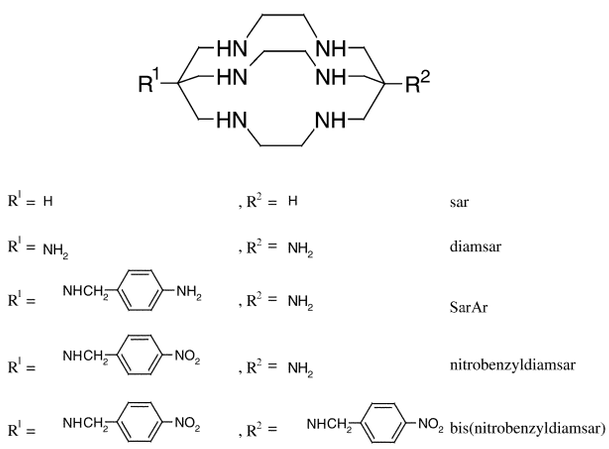

Fig. 1 Abbreviation of cage ligands described in this study.

Administration of the $[^{57}Co(sar)]^{3+}$ and the $^3H[Co(sar)]^{3+}$ type complexes both orally and intra-peritoneally in rats and mice showed similar biodistribution patterns for both complexes, indicating that the Co(III) complexes of these ligands

† Abbreviations. Because the IUPAC names for the cage ligands are long and complicated, those ligands described in this paper have been abbreviated as follows: 3,6,10,13,16,19-hexaazabicyclo[6.6.6]eicosane-1,8-diamine (diamsar); 1-*N*-(4-nitrobenzyl)-3,6,10,13,16,19-hexaazabicyclo[6.6.6]eicosane-1,8-diamine (nitrobenzyldiamsar), 1-*N*-(4-aminobenzyl)-3,6,10,13,16,19-hexaazabicyclo[6.6.6]eicosane-1,8-diamine (SarAr) and 3,6,10,13,16,19-hexaazabicyclo[6.6.6]eicosane (sar). Complexes of these ligands will be denoted as M[(ligand)]$^{n+}$, *e.g.* $[Cu(sar)]^{2+}$.

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remain intact and are stable *in vivo*.[4] Incubation of diamsar as the free ligand, with rat hepatocytes after exposure to $^{67}Cu^{2+}$ shows this type of ligand has the ability to remove 80% of the bound copper.[13,14] Also, it prevents the incorporation of $^{67}Cu^{2+}$ into ceruloplasmin, the protein responsible for the recirculation of copper into the blood after hepatobiliary clearance.[14] It does seem therefore that the copper cage complexes of this type are very unlikely to release their copper to proteins or other chelating agents in biological systems simply because they are too inert to dissociation.

Of the various radionuclides suitable for clinical application in nuclear medicine, ^{64}Cu is expected to exhibit potential for both the diagnosis and therapy of cancer for the following reasons.[15] It displays both electron capture decay (61%) with associated Auger electron emission ($E = 0.84$ keV, 59.1% and 6.5 keV, 23.3%) and β^- ($E_{av} = 190$ keV, 37.2%) and β^+ ($E_{av} = 278$ keV, 17.87%) nuclear decay paths, the positron emission allowing imaging on the high resolution PET camera for determination of dosimetry and ultimately accurate dosing of the radioimmunoconjugate for therapy. The half-life of ^{64}Cu (12.7 h) is appropriate for radiolabelling both antibodies and peptides. The ^{64}Cu decays to ^{64}Zn (a stable isotope). Exchange studies with $[Zn(sar)]^{2+}$ and $[Cu(sar)]^{2+}$ complexes have shown neither Cu^{2+} nor Zn^{2+} interchange, respectively, at 20 °C.

In the development of radioimmunoconjugates researchers have generally used derivatised tetraazamacrocycles for radiolabelling carrier molecules and the conjugation of these ligands is readily achieved *via* an aromatic amine linker. However recent work reported that the skeleton and the formal charge of the complexes can affect the biodistribution of the final product and that in some instances decomposition of the complex occurs.[16] The macrobicyclic structures of sar and diamsar complexes with Cu^{2+} appear to show greater stability than any other saturated amine chelator reported that we are aware of. Also, the comparative ease by which the diamsar can be functionalised and its relatively rapid complexation rate make it a potentially exciting addition to the list of bifunctional ligands that can be used for radiolabelling proteins and peptides. This paper describes the synthesis, properties and metal complexation behaviour of a modified sarcophagine, SarAr, incorporating an aromatic amine. It also critically evaluates aspects of the ligand's potential for application in nuclear medicine.

Experimental

Materials and equipment

All reagents and solvents used were of analytical grade (used without further purification) and obtained from commercial sources. All water used for experimental purposes was Milli-Q grade. ^{64}Cu was obtained from the National Medical Cyclotron, Australia (specific activity typically 2000 Ci g^{-1}). ^{67}Cu was obtained from Los Alamos or Brookhaven Laboratories (specific activity typically 15000 Ci g^{-1}). ^{57}Ni and ^{57}Co were isolated from the waste of the ^{67}Ga production at the National Medical Cyclotron. The specific activity of the ^{57}Ni and ^{57}Co was > 0.5 Ci g^{-1}.

The following equipment was used for measurement: electrospray mass spectrometry – Quatro II; UV-Vis – Varian S100 or Pharmacia detector UV-1; ^1H NMR – Bruker Avance DPX 400; radioactivity – Wallac Wizard 1480 gamma counter. All evaporations were carried out in a Buchi rotatory evaporator at ~ 20 Torr and a temperature of < 50 °C.

Animal studies were carried out with the approval of the Animal Care and Ethics Committee (ACEC) at Australian Nuclear Science and Technology Organisation (ANSTO) under protocol numbers 96/124, 97/124 and 98/124. Nu/nu mice were purchased from Combined Universities Laboratory Animal Supplies (CULAS, Australia).

Preparations

$[Cu(diamsar)]^{2+}$. Diamsar was prepared and characterised by ^1H NMR as described previously.[3] A copper complex of diamsar was formed quantitatively by stoichiometric addition of copper(II) acetate (2.0 g) to a solution of diamsar in dry ethanol (3.7 g in 150 mL). The bright blue solution was evaporated to dryness. The residue was then dissolved in dry ethanol and evaporated (twice) to remove excess water. [Cu(diamsar)]$(OAc)_2$ (5.6 g) was used without further purification.

$[Cu(nitrobenzyldiamsar)]^{2+}$. [Cu(diamsar)]$(OAc)_2$ (5.6 g) was dissolved in dry ethanol (100 mL) and treated with 4-nitrobenzaldehyde (1.51 g), dried/activated 4 Å molecular sieves and glacial acetic acid (0.6 mL). The mixture was stirred under N_2 (g) for 45 min. Sodium cyanoborohydride (0.63 g) was added to this solution and the reaction mixture was stirred under N_2 (g) for 4 days at ~ 25 °C. After filtration and evaporation to dryness the residue was extracted into chloroform (2 × 100 mL). The combined chloroform fractions were filtered and the filtrate evaporated to dryness. The glassy blue residue obtained, was then diluted in water (2 L) and sorbed onto a Sephadex SP C-25 column (55 × 7.5 cm swelled in water). The column was eluted with sodium citrate (2 L, 0.1 M) and a wide blue band formed. Increasing the sodium citrate concentration (5 L, 0.3 M) resulted in three blue bands eluting in order as $[Cu(diamsar)]^{2+}$, $[Cu(nitrobenzyldiamsar)]^{2+}$ and [Cu(bis-(nitrobenzyl)diamsar)]$^{2+}$.

The second band eluate was diluted with water (10 fold) sorbed onto another Sephadex SP-C25 column. The single blue band which eluted with sodium acetate (0.5 L, 1 M), was evaporated to dryness and the residue extracted with 2-propanol (0.2 L). Fine white crystals of sodium acetate separated, were filtered off and the process of evaporation and extraction was repeated until only a gel-like product remained. The final residue was then redissolved in water (50 mL) and lithium nitrate (~ 0.3 g) added to precipitate [Cu(nitrobenzyldiamsar)]$(NO_3)_2$ as fine dark blue crystals (1.1 g, 15%). Electrospray mass spectrometry of an aqueous solution (10^{-4} M) at a cone voltage of 25 V, gave a major signal at *m*/*z* 256.0 consistent with the expected 2+ cation and a minor signal at *m*/*z* 511.1 consistent with the same molecular weight less H^+ (*i.e.* a 1+ cation).

Since nitrate ion interfered with the reduction of $[Cu(nitrobenzyldiamsar)]^{2+}$ to SarAr, the counter ion was changed to chloride by dissolving the complex nitrate salt in water (20 mL) and then absorbing the complex on a Dowex 50W × 2 column (10 × 1.2 cm swelled in water). After washing the column with 1 M HCl (20 mL), the Cu complex eluted as a single blue band with 6 M HCl. Then the solution was evaporated to dryness, redissolved in water and re-evaporated to give blue-green crystals (Found: C, 33.7; H, 6.9; N, 16.9. $CuC_{21}H_{41}N_9Cl_4O_2 \cdot 5H_2O$ requires C, 34.1; H, 7.2; N, 16.9%).

SarAr. Sodium borohydride (22 mg dissolved in 0.2 mL water) was added slowly to the catalyst [10% palladium on activated charcoal (9.5 mg) suspended in water (0.2 mL)] and stirred under a nitrogen atmosphere. After addition of sodium borohydride was complete, the reaction mixture was stirred for a further 5 min. Then $[Cu(nitrobenzyldiamsar)]Cl_2 \cdot 5H_2O$ (15 mg) dissolved in sodium hydroxide (0.3 mL; 1% NaOH) was added dropwise to the reaction mixture. The reduction was allowed to proceed under nitrogen until the colour turned from blue to clear (~ 30 min at ~ 25 °C).

The Pd/C catalyst was filtered off (0.22 μm) and the eluent collected in an ice-cooled glass vial. Hydrochloric acid (11 M) was added dropwise (5 μL) to the cooled solution until gas evolution ceased (~ 45 μL, HCl). The final pH was < 1. This procedure was also carried out in D_2O with deuterated reagents (NaOD and DCl) and the final product (DCl, pD < 1) was

characterised by ^{1}H NMR: 3.34 (s, 6H, NCH_2CH$_2$N); 3.45 (s, 6H, NCH$_2$CH_2N); 3.62 (s, 6H, NCCH_2N); 3.79 (s, 6H, NCCH_2NDCH$_2$); 4.50 (s, 2H, ArCH_2); 7.53 (d, 2H, Ar-*H*); 7.74 (d, 2H, Ar-*H*). Electrospray mass spectrometry of an aqueous solution of SarArH$^+$ (10^{-4} M) at a cone voltage of 40 V, gave a signal at *m*/*z* 420.1 consistent with the expected ligand molecular weight plus H$^+$. CHN analyses of the neutral ligand were not obtained because the sample was hygroscopic; however, the ligand was crystallised as the hexahydrochloride salt (Found: C, 38.0; H, 8.1; N, 19.0; Cl, 32.8. $C_{21}H_{47}N_9Cl_6\cdot H_2O$ requires C, 38.4; H, 7.5; N, 19.2; Cl, 32.4%).

Complexation of Cu, Ni and Co by SarAr

As rates of complexation can vary with pH and concentration levels, it was important to assess SarAr's ability to complex a series of transition metal ions such as Co, Cu and Ni at the microscopic level (≈ 10^{-6} M) and compare this behaviour to previous published and unpublished work at macroscopic levels (10^{-3} M). The pH investigated ranged from 3 to 9 using various buffer conditions: 0.1 M glycine in 0.1 M sodium chloride for pH 3.0; 0.1 M sodium acetate for pH 4.0 and 5.0; 0.1 M potassium dihydrogen phosphate in 0.1 M disodium phosphate for pH 6.0, 7.0 and 8.0; 0.1 M glycine in 0.1 M sodium chloride for pH 9.0. A typical procedure involved dissolving the ligand (~ 10^{-6} M) in the appropriate buffer. Then, the relevant metal ion in the corresponding buffer (spiked with a tracer amount of $^{64}Cu^{2+}$, $^{57}Ni^{2+}$ or $^{57}Co^{2+}$) was added to achieve a 1 : 1 ratio of metal to ligand in the final reaction mixture. The complexation reaction was monitored at ~ 25 °C by instant thin layer chromatography (ITLC-SG) [mobile phase aqueous sodium acetate (0.1 M, pH 4.5)] and ethanol (9 : 1, v/v); R_f: $M^{2+} > 0.9$; $[M(SarAr)]^{2+} < 0.1$.

Stability of [Cu(sar)]$^{2+}$ in human plasma

The stability of the [Cu(sar)]$^{2+}$ complex in human plasma was investigated by forming [^{67}Cu(sar)]$^{2+}$ at ~ 25 °C in a similar manner to that described above. ITLC-SG showed complexation was > 99% [mobile phase aqueous 0.1 M sodium acetate (pH 4.5)–ethanol (9 : 1, v/v)]. The complex was diluted 1/10 in human plasma (0.22 μm filtered) and incubated at 37 °C. At various time intervals, 0.10 mL of solution was removed and analysed for breakdown on a Sephadex G-25 size exclusion column [0.8 × 46 cm, equilibrated with 0.1 M sodium acetate (pH 5.0), pre-blocked with 10% bovine serum albumin (0.4 mL)]. The elution of the plasma proteins was monitored by UV-Vis at 280 nm and the radioactivity monitored in collected fractions (1 mL/fraction) by the gamma counter. The two profiles were overlaid and the percentage of radioactivity associated with the protein peak determined.

Biodistribution of ^{64}Cu and ^{64}Cu complexes of sar, diamsar and SarAr in balb/c mice

Complexes of [^{64}Cu(sar)]$^{2+}$, [^{64}Cu(diamsar)]$^{2+}$, [^{64}Cu(SarAr)]$^{2+}$ (typically at 2×10^{-4} M) were prepared in a similar manner to that described above. ITLC-SG showed complexation was > 99% [mobile phase aqueous 0.1 M sodium acetate (pH 4.5)–ethanol (9 : 1, v/v)]. Complex solutions were diluted to ~ 4 MBq mL^{-1} (typically 5×10^{-6} M) in 0.9% saline and filtered (0.22 μm) prior to injection into Balb/C mice.

Mice between the ages of 7–29 weeks (males and females) were injected intravenously *via* the tail vein with 0.1 mL of [^{64}Cu(sar)]$^{2+}$, [^{64}Cu(diamsar)]$^{2+}$, [^{64}Cu(SarAr)]$^{2+}$ or $^{64}Cu_{aq}^{2+}$ (control) in 0.9% saline. At time intervals of 3, 5, 10, 15, 20 and 30 min, a group of five animals was exposed to CO_2 prior to cardiac puncture and then sacrificed by cervical dislocation. Each animal was dissected and selected tissues removed and weighed and the associated radioactivity in each organ was determined. Percent injected dose for each organ, as well as percent injected dose per gram was calculated (Figs. 5–8). Biodistribution of each complex was repeated.

Results and discussion

Considerable research has been devoted to establishing the best type of linkers to attach to ligands for conjugation to carrier molecules. Generally, it is considered that C_6 linkers that are rigid and contain a reactive amino group are most readily conjugated to protein and peptide alike. Typical conjugation strategies between the amino group of the linker of the bifunctional ligand have employed such reagents as; carbodiimides to activate the carboxylic acid group of the protein, reaction of imidoesters to form an amidine product containing a free SH, conversion of the amino linker group to a diazonium ion, or alternatively cyanuric chloride for bridging the alcohol of a carbohydrate group to an amine of the bifunctional ligands. However, typically water soluble carbodiimides are preferred for conjugation of bifunctional ligands to proteins. The reaction can be conducted in water at neutral pH, with an excess of bifunctional ligand to reduce polymerisation, and yield a relatively stable peptide bond.[17] Preliminary work attempting to attach the diamsar directly to protein using EDC was unsuccessful, hence the selection of an aromatic amine as the linker of choice to form SarAr was a natural progression given these results, prior art and literature knowledge. Once a linker chosen and the modification of the chelator effected it is important to ensure that the modified ligand meets the following criteria:

1. Complexation of the radionuclide is discrete (*i.e.* a 1 : 1 M : L species) and fast (*i.e.* min) at concentrations < 10^{-6} M.
2. Complexation is complete (> 95%) over a range in pH, generally 4.0–9.0.
3. Once formed the complex is stable in serum.
4. Clearance of the complex from the blood and excretory systems is relatively rapid.
5. The complexes are preferably excreted quantitatively and the radionuclide is not released by metabolic pathways.
6. Functionalising the ligand for attachment to carrier molecules does not adversely affect the above qualities.

Synthesis

The synthesis of the SarAr ligand involved the condensation of [Cu(diamsar)]$^{2+}$ complex with one mole of 4-nitrobenzaldehyde which took a surprisingly long time (45 min) at ~ 25 °C. The resulting nitrobenzylimine and bis(nitrobenzylimine) adducts were reduced with cyanoborohydride ion to give the saturated [Cu(nitrobenzyldiamsar)]$^{2+}$ complex and the corresponding bis derivative. These products were then separated from each other and unreacted [Cu(diamsar)]$^{2+}$ by ion exchange chromatography. The mono nitrobenzyl derivative was characterised by electrospray mass spectrometry.

Various methods including Zn/HCl, $SnCl_2$ and electrochemistry were investigated for reducing the nitro group of the [Cu(nitrobenzyldiamsar)]$^{2+}$ complex. However, they all resulted in a mixture of products and were low yielding. Finally, the effective method used provided not only a conversion of the nitro species to the amine in high yield (> 95%) and purity but also the ligand, free of metal ion. A typical reduction reaction involved converting the nitrate salt of the [Cu(nitrobenzyldiamsar)]$^{2+}$ ion to its chloride salt and then dissolving the resultant complex in a basic solution. This solution was then added to an aqueous solution of sodium borohydride in the presence of palladium on activated charcoal under nitrogen at room temperature. The nitro group was reduced cleanly to the amine and the Cu^{2+} ion was reduced to Cu^+ and immediately extruded from the cage (Fig. 1). Completion of the reaction was indicated when the blue solution became clear. The Cu^+ released from the cage is likely to be reduced further to the

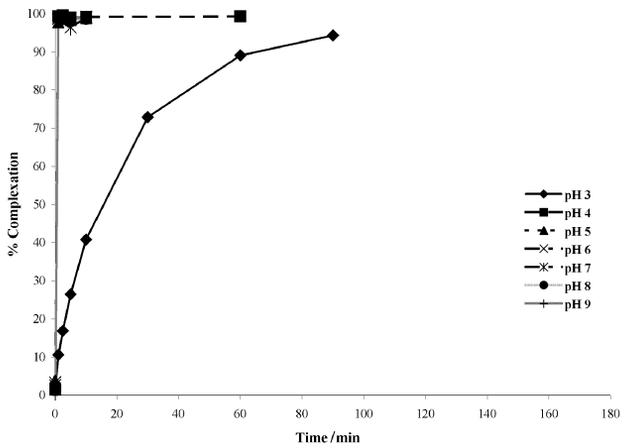

Fig. 2 Complexation of Cu(II) with SarAr.

metal and is probably bound to the palladium on the activated charcoal. The solution containing the free ligand, SarAr, was filtered to remove the catalyst. This reaction was repeated using the ^{64}Cu complex of nitrobenzyldiamsar under similar conditions and both the filtrate and charcoal were monitored for associated radioactivity. The results showed that all the radioactivity was associated with the charcoal, confirming that the SarAr ligand so isolated was metal free. An additional reduction reaction was conducted in D_2O and the resultant filtrate monitored by ^{1}H NMR spectrometry showed complete conversion of the nitro derivative to a single product, SarAr. There was no evidence of any other organic impurity.

Complexation of Cu, Ni and Co by SarAr

Discrete, complete and relatively rapid (*i.e.* minutes) complexation of the radioactive metal ion is an essential feature for bifunctional ligands which are to be used for radiolabelling proteins and peptides. While macroscopic studies often indicate that complexation is rapid, tracer studies ($< 10^{-6}$ M) using radionuclides often require excess ligand (> 10 fold) in order to achieve complete > 95% complexation.[18] Also, while stability constants for metal complexes are an indication of the degree of ligand binding of the metal ion, they are usually determined in media at constant ionic strength containing non-coordinating anions. Conditions employed for radiolabelling are considerably different and the process is often conducted in the presence of competing coordinating agents in excess (*e.g.* buffers). As a result the rates of complexation can be diminished by the competition.

While ^{64}Cu displays greatest potential for use in the development of radiopharmaceuticals, ^{57}Co and ^{57}Ni, with their long half-lives provide an opportunity to assess the biological stability of the radiobioconjugates *in vivo*. The longer half-lives of ^{57}Ni and ^{57}Co (35.7 h and 270 d, respectively) and respective positron and gamma emissions also allow analysis of radiolabelled products over extended periods of time (*e.g.* loss of free radionuclide and/or loss of radionuclide complex from the carrier molecule). This is often required for product development and stability studies in biological systems in order to monitor any breakdown products. Complexation studies of SarAr with the $^{57}Ni^{2+}$ and $^{57}Co^{2+}$ also provided an opportunity to compare their behaviour with previously reported work, and to observe if trends in behaviour at the macroscopic level were maintained at tracer level. Complexation at tracer levels (10^{-6} M) of Cu(II), Ni(II) and Co(II) with SarAr (10% excess) in various buffer systems are illustrated in Figs. 2, 3 and 4, respectively. Complexation of Cu(II) was fast (< 2 min, 10^{-6} M Cu^{2+} and ligand) for pH > 3. However at pH 3, complexation was comparatively slow only nearing completion at ~ 100 min. Overall, this behaviour is not inconsistent with previously reported complexation rates for sar and diamsar ($t_{1/2}$ = 5 s for

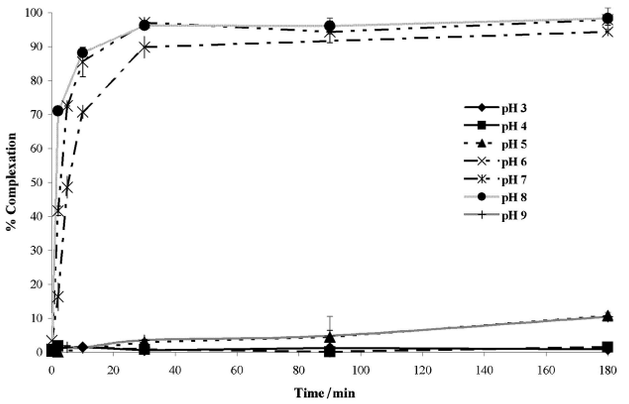

Fig. 3 Complexation of Ni(II) with SarAr.

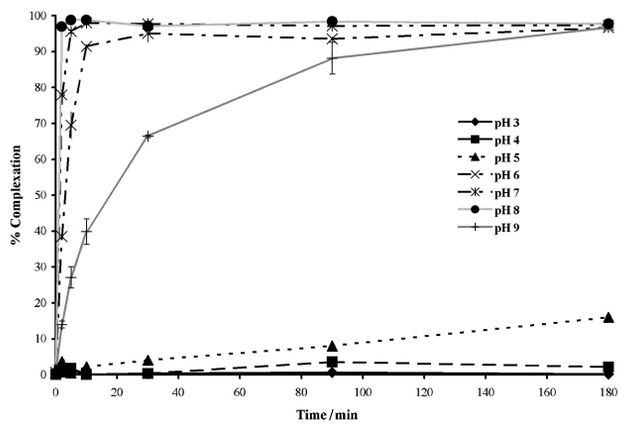

Fig. 4 Complexation of Co(II) with SarAr.

4×10^{-3} M solutions of Cu^{2+} ion at 25 °C and neutral pH)[13] given the differences in concentration and conditions.

The reactions of Ni(II) and Co(II) with the SarAr ligand show that complexation is comparatively slower for both compared to Cu(II) over the pH range investigated. However reasonable complexation (80%) for both Ni(II) and Co(II) in buffers at pH 6, 7 and 8 was achieved within 30 min. For pH ≤ 5 the rate of complexation was significantly slower for Ni(II) and Co(II) (approximately 10% complexation in 3 h). These conditions would therefore be considered inappropriate for radiolabelling molecules with short lived radioactive Ni and Co isotopes. At pH 9, the complexation of Ni(II) and Co(II) ions is even slower and such behavior would be considered a significant limitation in the development of a radiopharmaceutical.

The order of the rates of complexation of Cu(II), Ni(II) and Co(II) at neutral pH (6–8) crudely correlates with that of H_2O exchange for the respective metal aqua species. For example, the rate constant for substitution of inner sphere H_2O for Cu^{2+} ($\sim 5 \times 10^8$ s^{-1}) is considerably larger than those for Ni^{2+} ($\sim 2 \times 10^4$ s^{-1}) and Co^{2+} ($\sim 4 \times 10^5$ s^{-1}) [*i.e.* rapid for Cu(II) and slower for Co(II) and Ni(II)].[19]

The slow complexation rates at low pH are not surprising as the metal ion has to compete with H^+ for the donor N atoms on the ligand which is a strong base. For basic conditions, the complexation behaviour of the SarAr is more complicated. Studies reporting Ni ion speciation at various concentrations and pH, indicate that at concentrations of 10^{-6} M and pH < 9 the predominant species in solution is Ni^{2+}.[19] However, at pH 9, insoluble nickel hydroxide species form. The nickel ion in these polymeric olated products is not so readily available for complexation by SarAr and is likely to result in a significant decrease in complexation rate. Similar behaviour for Co^{2+} ions is also reported under these concentration (10^{-6} M) and pH (> 9) conditions.[19] This rationale accommodates the behaviour observed here.

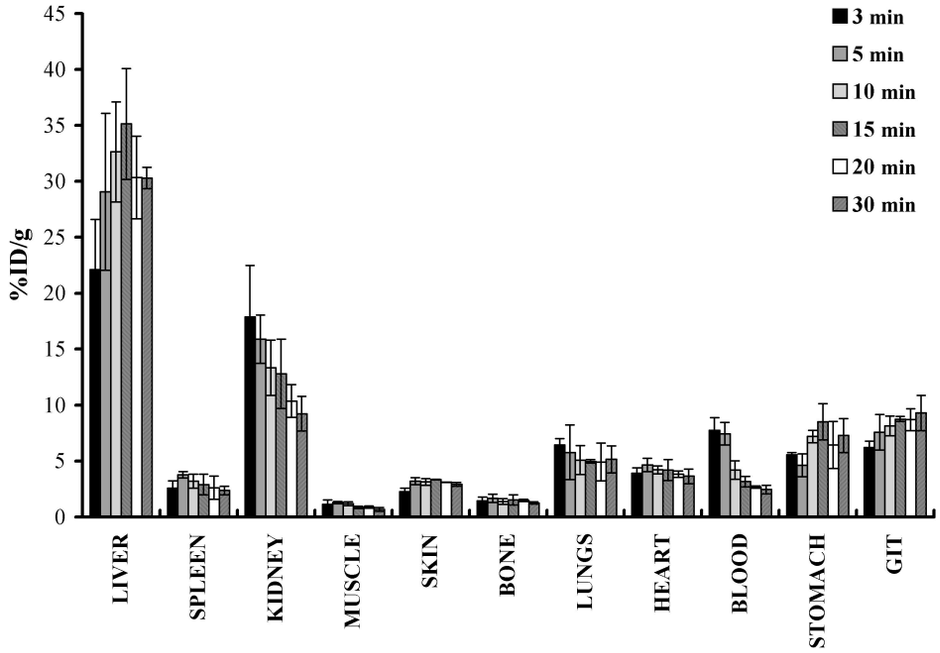

Fig. 5 Biodistribution of $^{64}Cu^{2+}$ in Balb/C mice. GIT = gastrointestinal tract.

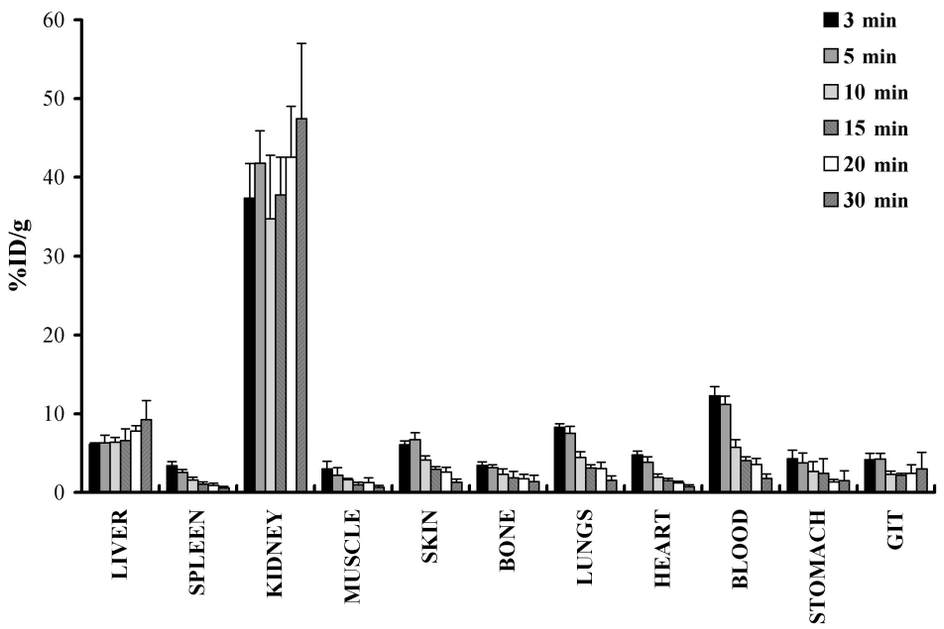

Fig. 6 Biodistribution of $[^{64}Cu(sar)]^{2+}$ in Balb/C mice.

Overall, the data indicate that the complexation rates of Cu(II), Ni(II) and Co(II) by the free SarAr ligand at pH 6–8 are satisfactory for their potential use in the radiolabelling of proteins and peptides.

Stability of $[Cu(sar)]^{2+}$ in human plasma

Copper is responsible for many enzymatic processes and hence can be bound by many inter- and intra-cellular proteins, such as metallothionein, copper transporting ATPases, cytochrome oxidase and superoxide dismutase. Its natural excretory path is *via* the hepatocytes in the liver. It may be recirculated by binding to ceruloplasmin in the liver and re-released into the plasma. Studies investigating the binding of free Cu^{2+} by sar and diamsar prior to and after exposure to hepatocytes confirm that it is unlikely that the Cu^{2+} would be released from the SarAr in the liver.

In biological systems, the release of Cu^{2+} from its complex in the presence of blood plasma is also an important factor to consider. To this extent, it is important to assess the stability of the $[Cu(SarAr)]^{2+}$ complex in the presence of human plasma. However, this complex could not be resolved from the blood proteins using size exclusion chromatography but the $[Cu(sar)]^{2+}$ ion could be resolved. Hence the stability of this parent complex, viewed as a representative of this class of molecules was investigated. The study showed that $[^{67}Cu(sar)]^{2+}$ ion does not significantly dissociate in blood plasma. Less than 2% is associated with the protein peak after 174 h and more than 98% of the ^{67}Cu was still complexed to the ligand. A comparison with serum stability reported for other copper complexes of tetraazamacrocycles and open-chain chelators such as Cu–DTPA and Cu–benzyl-TETA shows a copper loss of 77% within 24 h and 2% within 3 days, respectively.[16]

Biodistribution of $^{64}Cu^{2+}$ and $^{64}Cu^{2+}$ complexes of sar, diamsar and SarAr in balb/c mice

Biodistribution of ^{64}Cu complexes of sar, diamsar and SarAr were assessed in Balb/c mice. Each animal was sacrificed at

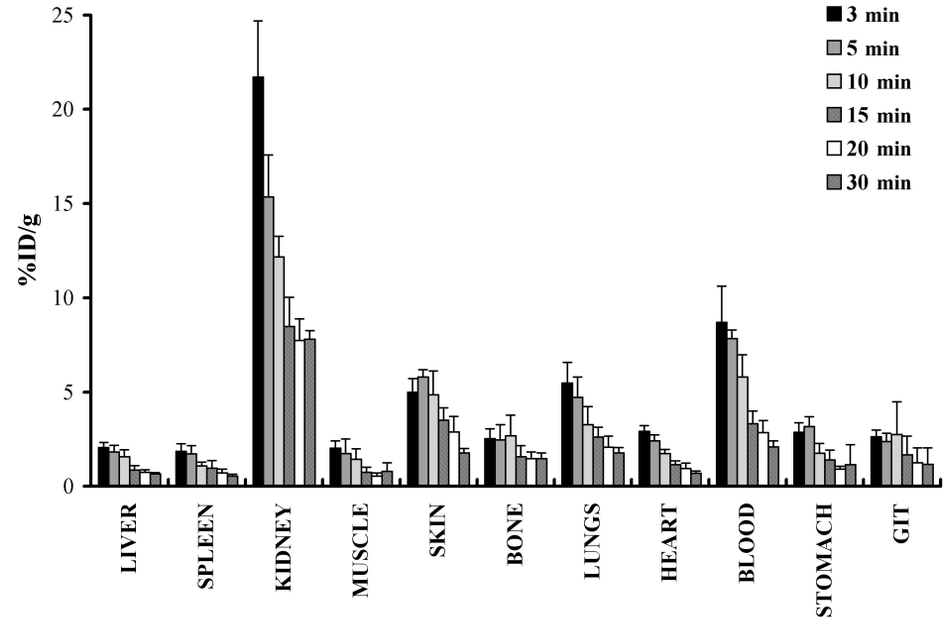

Fig. 7 Biodistribution of $[^{64}Cu(diamsar)]^{2+}$ in Balb/C mice.

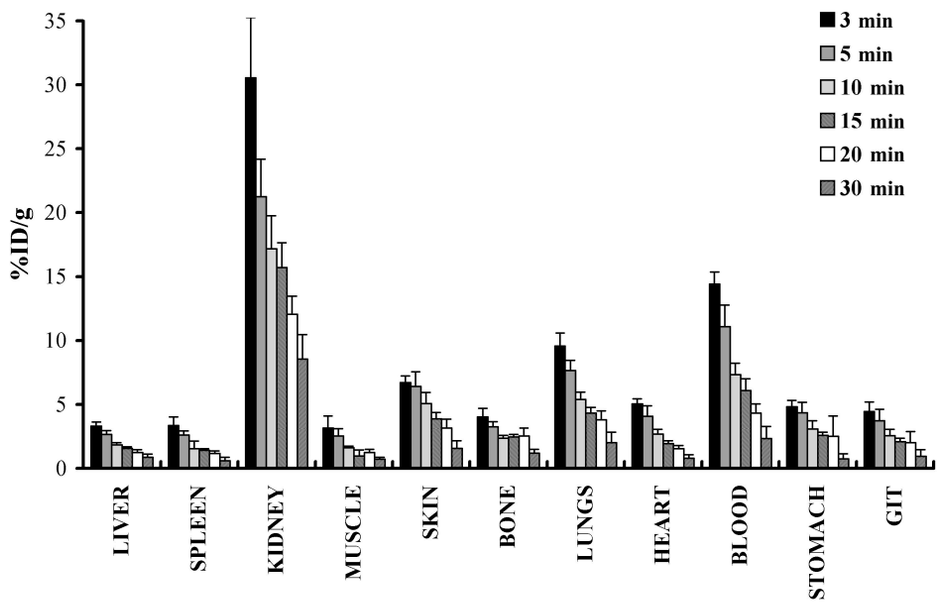

Fig. 8 Biodistribution of $[^{64}Cu(SarAr)]^{2+}$ in Balb/C mice.

various time intervals (up to 30 min) and the organs dissected. The percent injected dose/gram was plotted against time and is illustrated in Figs. 5, 6 and 7, respectively. As there was no adequate biodistribution data set for free Cu^{2+} in Balb/C mice which allows valid comparison, mice were injected with a solution of $^{64}CuCl_2$ in 0.9% saline (Fig. 8). After 30 min, about 60–70% of the ^{64}Cu had cleared from the blood for all complexes compared to free $^{64}Cu^{2+}$ where, more than 74% remained in the body.

All three ^{64}Cu complexes, $[^{64}Cu(sar)]^{2+}$, $[^{64}Cu(diamsar)]^{2+}$ and $[^{64}Cu(SarAr)]^{2+}$, cleared rapidly from the blood. A single phase clearance pattern was observed in each instance with half-lives of 17, 21 and 21 min, respectively.

Both the ^{64}Cu complexes of diamsar and SarAr cleared predominantly through the kidneys while the $[Cu(sar)]^{2+}$ complex gradually accumulated in the kidneys over the time period investigated. Rapid clearance through the kidneys is typical of charged complexes which maintain their identity and this behaviour correlates with that found for other radiolabelled copper complexes.[20] The results are also consistent with the excretion pattern of kinetically inert $[^{57}Co(diamsar)]^{3+}$ ion following intraperitoneal injection in rats.[4] For the $[Cu(sar)]^{2+}$ complex, the behaviour in the kidneys (up to 47 (± 10)%ID/g at 15 min) was distinctly different from that of the other two complexes, implying some trapping of the Cu^{2+} complex (as opposed to free Cu^{2+}) at least for an interval of time.

Clearance through the liver, however, was rapid for all complexes while the free $^{64}Cu^{2+}$ accumulated as expected. The latter is likely to be bound to blood proteins initially and also to accumulate in the hepatocytes. Slow clearance from the blood of some of the $^{64}Cu^{2+}$ was observed in a bi-phasic mode with an α phase of 13 min and a β phase of 63 min. The contrast in behaviour between the Cu complexes and free Cu^{2+} ion supports the assumption that the copper complexes of all three ligands are stable *in vivo* for more than the time required to function as an imaging or therapeutic agent. Clearance from other organs was also relatively rapid for all the complexes investigated.

Conclusion

The synthesis of SarAr is simple and the yield could no doubt

be improved with a more detailed investigation. The conditions for rapid and complete complexation of Cu(II), Ni(II) and Co(II) by free SarAr (aqueous buffered solutions at physiological pH and at ~25 °C) are mild enough for use in radiolabelling proteins, other biological molecules and other biological investigations. The Cu complex's thermodynamic and kinetic stability in human plasma indicates that the radiometal is unlikely to be removed by other copper avid proteins or chelators present in blood. Further, if the radioactive complex of SarAr were released from the carrier molecule, would be rapidly excreted *in vivo*. Hence the simple synthesis, ready complexation and stability of SarAr therefore make it an attractive prospect for use in nuclear medicine.

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References

1 A. M. Sargeson, *Coord. Chem. Rev.*, 1996, **151**, 89.
2 G. C. Yeh, A. Beatty and M. J. K. Bashkin, *Inorg. Chem.*, 1996, **35**, 3828.
3 G. A. Bottomley, I. J. Clark, I. I. Creaser, L. M. Engelhardt, R. J. Geue, K. S. Hagen, J. M. Harowfield, 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.
4 C. A. Behm, P. F. L. Boreham, I. I. Creaser, B. Korybut-Daszkiewicz, D. J. Maddalena, A. M. Sargeson and G. M. Snowdon, *Aust. J. Chem.*, 1995, **48**, 1009.
5 A. M. Sargeson, *Pure Appl. Chem.*, 1984, **56**, 1603.
6 J. M. Harrowfield, A. J. Herlt, P. A. Lay and A. M. Sargeson, *J. Am. Chem. Soc.*, 1983, **105**, 5503.
7 P. Comba, A. M. Sargeson, L. M. Engelhardt, J. M. Harrowfield, A. H. White, E. Horn and M. R. Snow, *Inorg. Chem.*, 1985, **24**, 2325.
8 L. Grøndahl, A. Hammershøi, A. M. Sargeson and V. Thöm, *Inorg. Chem.*, 1997, **36**, 5396.
9 P. A. Anderson, I. I. Creaser, C. Dean, J. M. Harrowfield, E. Horn, L. L. Martin, A. M. Sargeson, M. R. Snow and E. R. T. Tiekink, *Aust. J. Chem.*, 1993, **46**, 449.
10 A. E. Martel and R. M. Smith, *Critical Stability Constants*, Plenum Press, New York, 1974, vol. 1, p. 204.
11 A. E. Martel and R. M. Smith, *Critical Stability Constants*, Plenum Press, New York, 1982, vol. 5, 1st Supplement.
12 A. E. Martel and R. M. Smith, *Critical Stability Constants,* Plenum Press, New York, 1989, vol. 6, 2nd Supplement.
13 H. J. McArdle, S. M. Gross, I. I. Creaser, A. M. Sargeson and D. M. Danks, *Am. J. Physiol.*, 1989, **256 (4 Pt1)**, G667.
14 M J. Bingham, A. M. Sargeson and H. J. McArdle, *Am. J. Physiol.*, 1997, G1400.
15 P. J. Blower, J. S. Lewis and J. Zweit, *Nucl. Med. Biol.*, 1996, **23**, 957.
16 W. C. Cole, S. J. DeNardo, C. F. Meares, M. J. McCall, G. L. DeNardo, A. L. Epstien, H. A. OBrien and M. K. Moi, *J. Nucl. Med.*, 1987, **28**, 83.
17 N. Nakajima and Y. Ikada, *Bioconjugate Chem.*, 1995, **6**, 123.
18 D. M. Goldenberg, in *Cancer Imaging with Radiolabelled Antibodies*, Kluwer Academic Publishers, Boston, Massachusetts, 1990.
19 M Pourbaix, in *Atlas of Electrochemical Equilibria in Aqueous Solutions*, NACE, Texes, 1974.
20 S. V. Deshpande, S. J. DeNardo, C. F. Meares, M. J. McCall, G. P. Adams, M. K. Moi and L. G. DeNardo, *J. Nucl. Med.*, 1988, **29**, 217.