New ^{64}Cu PET imaging agents for personalised medicine and drug development using the hexa-aza cage, SarAr

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The success of positron emission tomography (PET) in personalised medicine and drug development requires radioisotopes that provide high quality images and flexible chemistry for a broad application. ^{64}Cu is arguably one of the most suitable PET isotopes for imaging with the evolving target agents, but there are not many appropriate chelating agents for ^{64}Cu and this has limited its wider application. The bi-functional chelator, SarAr is known to bind $^{64}Cu^{2+}$ quantitatively (*i.e.* one metal per ligand present) and rapidly (<2 min) at 10^{-6} M over a range of pH (4–9). In this paper the conjugation of SarAr to the whole and fragmented antibody is described. Conjugation of the SarAr to the protein does not impair its coordination of the ^{64}Cu. It complexes the $^{64}Cu^{2+}$ rapidly, quantitatively and essentially irreversibly at pH 5. Animal studies show that the ^{64}Cu–SarAr–immunoconjugates maintain their specificity for the target and are stable *in vivo*. Also, SarAr is a platform technology, is easy to use in a kit formulation and is readily adaptable for the wider application in ^{64}Cu PET imaging.

Introduction

There is a strong drive from both government and industry for the development of imaging tools that have application for personalised medicine and drug risk assessment.[1,2] Positron emission tomography (PET) offers an unparalleled sensitivity (10^{-9} to 10^{-12} M) in the clinical setting for these purposes. However, it is important that the half-lives and the chemistry of the PET isotopes are compatible with the localisation and clearance of the molecular target agents *in vivo*. In this respect the half-lives of commercially available PET isotopes such as ^{11}C and ^{18}F are far too short for applications with many evolving target agents (*e.g.* proteins, oligonucleotides and peptides) and the chemical techniques employed for radiolabelling with radio-halogens can also damage these agents.

A number of PET isotopes (^{45}Ti, ^{61}Cu, ^{64}Cu, ^{68}Ga, ^{76}Br, ^{77}Br, ^{86}Y, ^{94m}Tc and ^{124}I) have been used for radiolabelling proteins and peptides. All except ^{64}Cu have significantly higher mean positron energies (>300 keV) than ^{18}F which result in comparatively poor image resolution and/or high energy gamma emissions that can interfere with imaging processing.[3] ^{64}Cu (β^+; E_{av} 278 keV, 17.9%) has a mean positron energy similar to that of ^{18}F and a half-life of 12.7 h. Advances in the cyclotron production routes for ^{64}Cu using low and high energy cyclotrons have also led to its recognition as a commercially viable PET isotope for molecular imaging.[4–7]

Meares and co-workers pioneered the use of polyazacarboxylate macrocycles for radiolabelling antibodies with ^{67}Cu and ^{64}Cu for imaging.[8–10] Since that time various tetraazamacrocycles[11] and polyazacarboxylate macrocycles[12] have been synthesised for radiolabelling antibodies and peptides with $^{64}Cu^{2+}$ or $^{67}Cu^{2+}$.[13] Their choice has been largely based on these ligands forming thermodynamically stable complexes with Cu^{2+} (log K = 20–28).[14,15] Unfortunately, the formation of their respective $^{64}Cu^{2+}$ complexes is not necessarily fast or very selective for Cu^{2+} ion in the presence of high electrolyte concentrations.[16] Often, ^{64}Cu radiolabelling of the immunoconjugates incorporating polyazacarboxylates requires an excess of the ligand and/or immunoconjugate, heating, and lengthy incubation times (⩾30 min) as well as extensive purification procedures. In contrast, the simple cyclam derivatives have shown greater thermodynamic stability with Cu^{2+} than their analogous polyaminocarboxylate derivatives, but they also suffer from kinetic instability when exposed to high concentrations of acid or competing metal ions.[17]

The hexa-aza cages **1** and **2** (illustrated in Fig. 1)[18] designed with the additional linking strand incorporating two nitrogen atoms, form a three-dimensional 'cage' around the Cu^{2+} ion which leads to an increased thermodynamic and kinetic stability.[19–21] Once formed, the Cu^{2+} complex of the hexa-aza cage, Diamsar

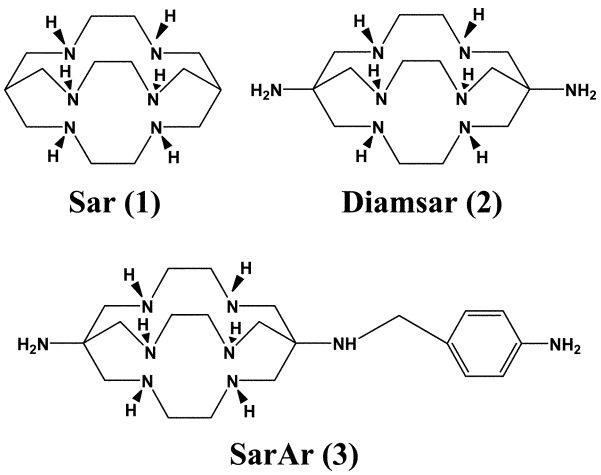

Fig. 1 Hexa-aza cages Sar (**1**) and Diamsar (**2**) and SarAr (**3**).

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{[Cu(II)Diamsar]$^{2+}$}, is resistant to Cu^{2+} dissociation in 4 M HCl at 20 °C for more than 7 months.[15]

Understanding the rates of metal ion exchange with the complex at physiological pH is also important for defining the role and safe use of radiometal complexes in humans. The concentration of exchangeable Cu^{2+} and Zn^{2+} in plasma is *ca.* 1 and *ca.* 10 μM, respectively, while the concentrations of radiolabelled target agents injected into the body are $<10^{-8}$ M.[22] Many products (*e.g.* whole and fragmented antibodies and peptides) can circulate in the body from hours up to days and they can be exposed to concentrations of Cu^{2+} and Zn^{2+} that are up to 1000-fold higher for relatively long periods of time.

The free metal ions along with the radioisotope will compete for the bi-functional ligands of the immunoconjugate. This may limit the radioisotope from being effectively localised at the target site or clearing from the body. Such a loss of the radioisotope can result in its uptake in non-target organs and be responsible for poor image quality and radiation toxicity.

Interestingly, studies with the hexa-aza **2** have shown that when they are incubated with hepatocytes, up to 80% of the Cu^{2+} within the hepatocytes is released and coordinated with **2**, thereby preventing Cu^{2+} incorporation into ceruloplasmin.[23] Further testimony to the kinetic inertness of the hexa-aza cage complexes are the reported exchange studies with [Zn(II)Diamsar]$^{2+}$ and [Cu(II)Diamsar]$^{2+}$ complexes that show neither Cu^{2+} nor Zn^{2+} interchange, respectively at 20 °C for several days near neutral pH.[15] Perhaps the most outstanding and unique feature of the hexa-aza cages is their ability to bind micromolar concentrations of Cu^{2+} ions, quantitatively (*i.e.* one metal to one ligand) within minutes at ambient temperatures over a pH range of 4–9 and in the presence of relatively high concentrations of alkali metal ions or electrolytes. This characteristic is highly desirable for a radiopharmaceutical and creates a potential for kit formulation of an emerging range of molecular targeting agents. Furthermore, the ability to produce the desired radiopharmaceutical without the use of expensive automation systems makes it a compelling technology for radiopharmaceutical applications.[24]

The synthesis of the new bi-functional hexa-aza cage ligand, 1-*N*-(4-aminobenzyl)-3,6,10,13,16,19-hexa-aza-bicyclo-[6.6.6]eicosane-1,8-diamine or SarAr (Fig. 1), has been reported elsewhere.[24] The present study explores conditions for conjugating SarAr to the well-characterised B72.3 antibody and its fragments. B72.3 is an IgG_1 that has been shown to react with the high-molecular-weight tumour-associated glycoprotein, TAG-72, expressed in a variety of adenocarcinomas.[25] Radiolabelling of the resultant immunoconjugates with $^{64}Cu/^{67}Cu$ and their biodistribution in colon carcinoma tumour-bearing nude mice is evaluated. The potential of SarAr technology for use in a kit formulation is also tested and the extension of this technology for radiolabelling other molecular targets and the production of new ^{64}Cu PET radiopharmaceuticals is discussed.

Results

Conjugation chemistry

The conjugation of SarAr *via* its amine functional group to the whole antibody (Mab) B72.3, using the carbodiimide 1-ethyl-3-(3-dimethyaminopropyl)carbodiimide hydrochloride (EDC) was investigated under various reaction conditions, such as pH, reaction time, concentration of antibody and molar ratios of EDC and SarAr to Mab at 37 °C. The immunoconjugate from each reaction mixture was then purified using size-exclusion high pressure liquid chromatography (SE-HPLC) and radiolabelled with ^{64}Cu to determine the amount of radioactivity associated with the protein. The optimum pH for the conjugation of the antibody to the SarAr using EDC was found to be 5 (see Fig. 2).

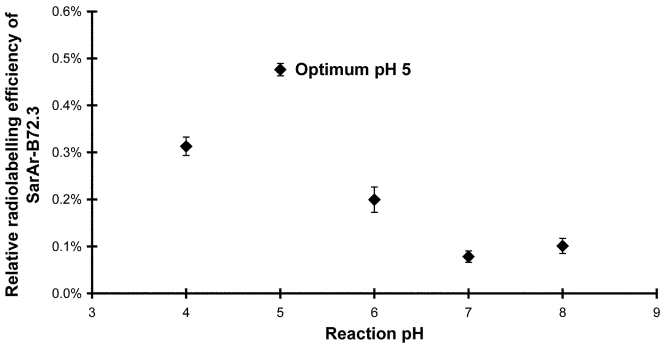

Fig. 2 Effect of pH on SarAr conjugation to B72.3. [B72.3] = 5 mg mL^{-1}, 30 min, 37 °C, B72.3 : EDC : SarAr = 1 : 100 : 20.

The optimum time for conjugation of SarAr to the protein was found to be at approximately 30 min when the reaction was studied over a 15 min to 2 h time period. The effect of Mab concentrations on the conjugation reaction was also investigated and the most efficient radiolabelling occurred at 5 mg mL^{-1}. Fig. 3 summarises the effect that EDC and SarAr concentrations have on the conjugation reaction with Mab. SE-HPLC showed that for all conditions investigated, <10% cross-linking of the Mab occurred. In general, an increase in the molar ratio of SarAr and EDC to Mab resulted in an increase in specific activity. However, when the EDC : Mab molar ratio was >500 there was a negligible increase in specific activity. For SarAr : Mab molar ratios >500 the SE-HPLC showed evidence of lower molecular weight impurities

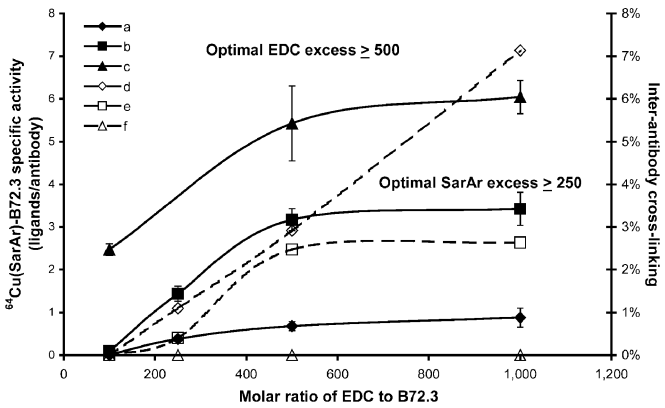

Fig. 3 Effect of EDC and SarAr concentrations on specific activity and inter-antibody cross-linking.†

† The conjugation reaction was effected in acetate buffer, pH 5 at 37 °C for 30 min, [B72.3] = 5 mg mL^{-1}. Filled symbols show specific activity at molar ratios of SarAr : B72.3 = 100 (a), 250 (b) and 500 (c). Open symbols show inter-antibody cross-linking at molar ratios of SarAr : B72.3 = 100 (d), 250 (e) and 500 (f).

that resulted in a lower yield of the desired product. Hence, the optimum conditions for the conjugation of SarAr to Mab were at a molar ratio of SarAr : Mab > 100 but at <250 and with a 500-fold molar excess of EDC : Mab at pH 5 for 30 min at 37 °C. Each immunoconjugate was then radiolabelled with ^{64}Cu, and SE-HPLC and instant thin layer chromatography (ITLC-SG) used to determine the average number of SarAr attached to the antibody was 3.3 with ⩽3% inter-antibody cross-linking (B72.3 = 5 mg mL^{-1}). The immunoreactivity assay showed that the ^{64}Cu–SarAr–B72.3 prepared under optimum conditions was ⩾80%. Preliminary studies to investigate the conjugation of SarAr to other whole murine and chimeric antibodies, showed that similar specific activities were achievable.[26] The SarAr immunoconjuguate of the B72.3-F(ab′)$_2$ antibody fragment was prepared using the same optimum conditions [*i.e.* pH 5.0; F(ab′)$_2$ B72.3 = 5.0 mg mL^{-1}; molar ratio of SarAr : EDC : F(ab′)$_2$ = 250 : 500 : 1; 30 min]. Analysis by SE-HPLC showed no evidence of inter-antibody cross-linking and an average of 1.3 SarAr ligands conjugated to each F(ab′)$_2$ molecule. The sandwich immunoreactivity assay of the resultant ^{64}Cu–SarAr–B72.3-F(ab′)$_2$ showed that there was a reduction in the immunoreactivity to 46% compared with the unconjugated B72.3-F(ab′)$_2$.

^{64}Cu radiolabelling of SarAr-immunconjugates

The SarAr–immunoconjugates could be rapidly radiolabelled by incubation with high specific activity $^{64}Cu^{2+}$ or $^{67}Cu^{2+}$ in acetate buffer at pH 5.0 for 5 min at *ca.* 20° C. The $^{64/67}$Cu–SarAr–immunconjugates were washed with 0.1 M EDTA on centrifugation filters and resuspended in the appropriate buffer. Characterisation by SE-HPLC and ITLC-SG showed that the final $^{64/67}$Cu radiolabelled immunoconjugates were >95% radiochemically pure.

Stability of SarAr–immunoconjugate

Each SarAr–B72.3 immunoconjugate prepared as previously described was divided into several aliquots of at least 50 μg but more typically 100 μg. An aliquot of the prepared immunoconjugate solution was radiolabelled with ^{64}Cu immediately, and the remaining unlabelled aliquots were stored in buffer (0.1 M sodium acetate buffer pH 5.0) or lyophilised and stored at 4 °C for at least 35 d and up to 48 d. Stored samples were removed at set intervals and radiolabelled with ^{64}Cu. The specific activities of resultant ^{64}Cu–SarAr–immunoconjugate samples were determined and compared with the specific activity of the original batch. In all instances the specific activity of the resultant ^{64}Cu–SarAr–B72.3 was equivalent to the original batch indicating that the SarAr–immunoconjugate was stable whether stored in buffered solution or lyophilised at 4 °C. Immunoreactivity assays of each batch also showed that the immunoreactivities of both SarAr–B72.3 and SarAr–B72.3-F(ab′)$_2$ were maintained under these storage conditions.

Integrity of ^{64}Cu–SarAr–immunoconjugates

The ^{64}Cu–SarAr–B72.3 and ^{64}Cu–SarAr–B72.3-F(ab′)$_2$ radiolabelled immunoconjugates were prepared as previously described and stored in sodium acetate (0.1 M, pH 5.0) at 4 °C for up to 5 d. ITLC-SG was used to monitor for dissociated ^{64}Cu and/or ^{64}Cu–SarAr from the immunoconjugate. In all instances the radioactivity associated with the SarAr–immunoconjugate was >98%, indicating negligible loss of SarAr from the antibody and/or loss of ^{64}Cu from SarAr occurred during the time period investigated.

Animal biodistribution studies

The tumour localisation and biodistribution of ^{64}Cu–SarAr–B72.3 and ^{64}Cu–SarAr–B72.3-F(ab′)$_2$ were determined in nude mice bearing LS174t tumours. The ^{64}Cu–SarAr–B72.3 and ^{64}Cu–SarAr–B72.3-F(ab′)$_2$ were injected intravenously and the animals were sacrificed and dissected at various time intervals. Injected doses per gram of organ are plotted for ^{64}Cu–SarAr–B72.3 and ^{64}Cu–SarAr–B72.3-F(ab′)$_2$ in Figs. 4 and 5, respectively.

Tumour localisation of the ^{64}Cu–SarAr–B72.3 to the target site reached 38 ± 5% ID/g within 48 h. Blood clearance was bi-phasic (α = 7.3 ± 0.4 h, β = 100 ± 30 h) and the tumour : blood ratio increased from 0.1 to 2.1 over a two day period (see Fig. 4). Clearance of the radiolabelled antibody from other organs was typical of a whole IgG antibody.

The blood clearance of ^{64}Cu–SarAr–B72.3-F(ab′)$_2$ was also bi-phasic (α phase = 3.9 ± 0.2 h and β phase = 7 ± 1 h). At 5 h, the tumour contained up to 10 ± 1% ID/g and this uptake persisted for up to 24 h. Tumour to blood ratios of 2.2 ± 0.6 were achieved within 9 h (see Fig. 5). Kidney uptake was high (>200% ID/g) and persisted over the 24 h.

Fig. 6 shows a comparison of tumour : blood ratios for the ^{64}Cu–SarAr–B72.3 and ^{64}Cu–SarAr–B72.3-F(ab′)$_2$. The data clearly illustrate comparatively faster clearance of the lower molecular weight F(ab′)$_2$ species [^{64}Cu–SarAr–F(ab′)$_2$] relative to the whole antibody (^{64}Cu–SarAr–B72.3) and confirms that the stability of the ^{64}Cu–SarAr complex is maintained *in vivo*.

Discussion

This study clearly demonstrates that SarAr can be used to effectively capture and retain the $^{64}Cu^{2+}$ radiolabel on an array of target agents and therefore its allows a wide application in PET imaging for drug risk assessment and personalised medicine.

As the carbodiimide EDC is perhaps one of the most popular reagents to conjugate biological substances, it was used to mediate the conjugation of SarAr to the proteins.[27] EDC is classified as a zero-length cross-linker that effects the formation of an amide bond between a carboxylate and an amine. Its water solubility allows it to be used with aqueous biological systems without predissolution in organic solvents. Furthermore, excess reagent and the isourea formed as the by-product of the cross-linking reactions are both water-soluble and are easily removed either by dialysis or gel filteration. The conditions for the conjugation of SarAr to the B72.3 antibody using EDC were optimised to achieve the highest specific activity (*i.e.* the number of ligands bound per molecule of target agent) by minimising the effect on the antibody function or immunoreactivity. Reaction conditions, such as the time of the conjugation reaction, the pH, the concentration of the antibody and the excess of EDC and SarAr were therefore investigated.

To activate the carboxylic acid groups of the antibody (*e.g.* aspartate and glutamate residues) using EDC, pH values approximately one unit higher than their pK_a value (3.65 and 4.26, respectively) are required. Of the six secondary amines in the SarAr

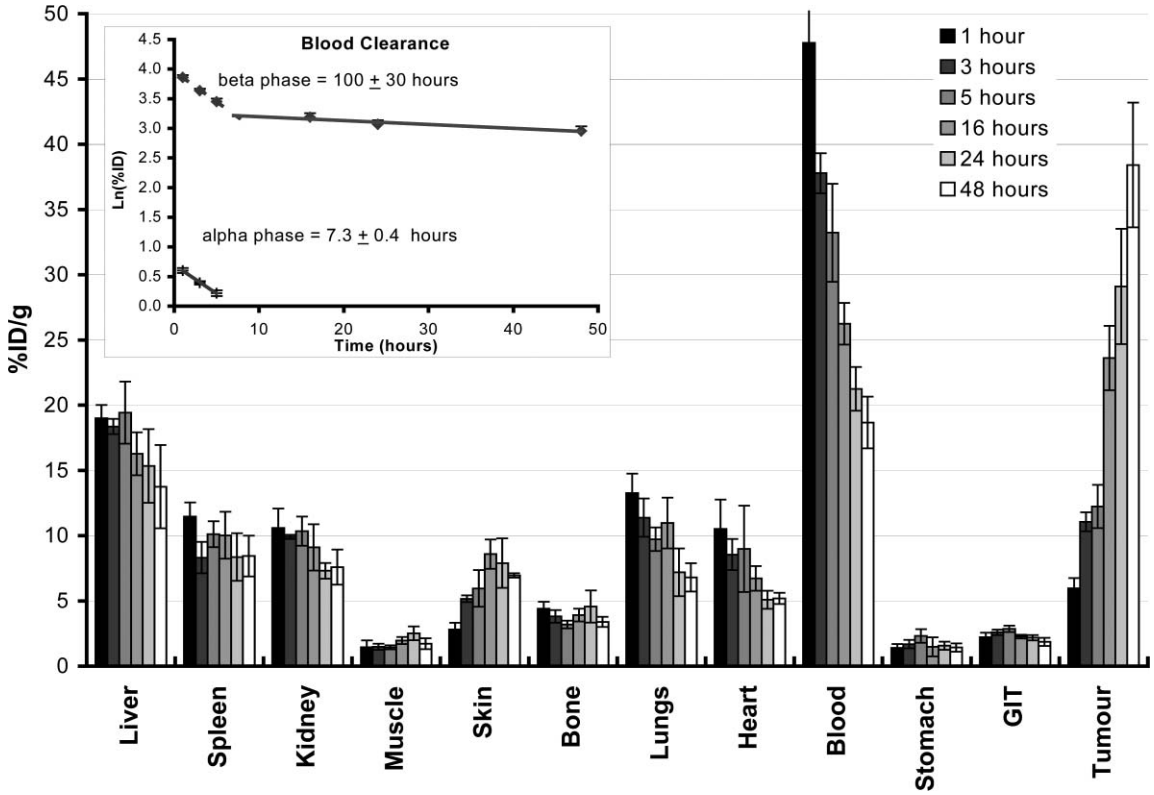

Fig. 4 Biodistribution and biphasic blood clearance (inset) of ^{64}Cu–SarAr–B72.3 in LS174t tumour-bearing nude mice.

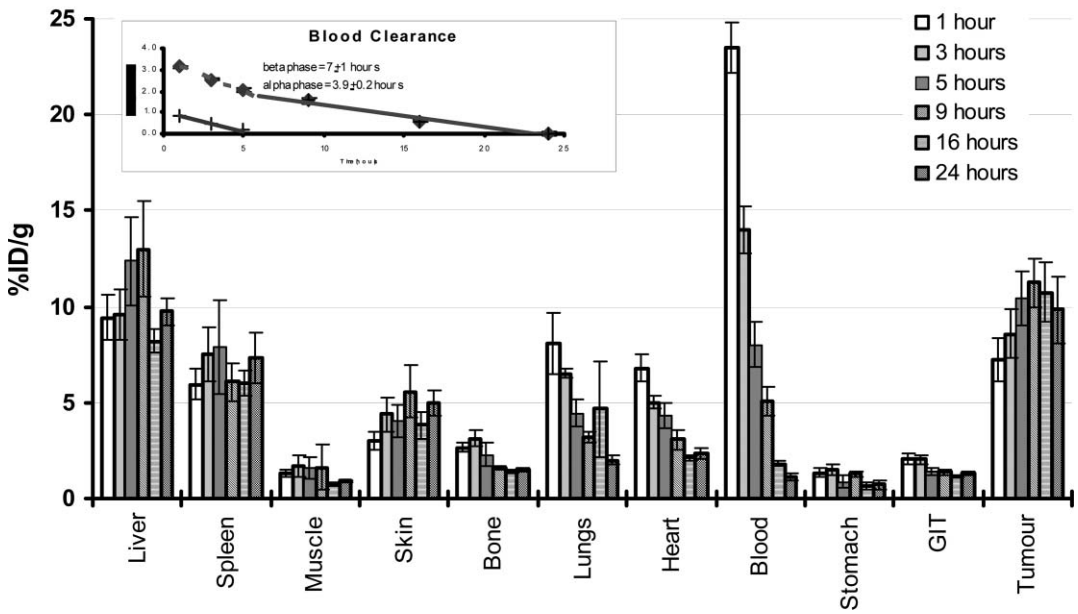

Fig. 5 Biodistribution and blood clearance (inset) of ^{64}Cu–SarAr–B72.3-F(ab′)$_2$ in LS174t tumour-bearing nude mice.†

cage at least three would be protonated at *ca.* pH 5. However, it is difficult for steric reasons, for the secondary amines in the hexa-aza cage to act as nucleophiles; therefore, the two primary amines of SarAr, *i.e.* that on the aromatic linker and that adjacent to the cage, are the most favoured. Unsuccessful attempts to conjugate the parent species, Diamsar, directly to the antibody with EDC also supports the assumption that the preferred nucleophile is the aromatic NH_2. Our studies also showed that there was no significant increase in the number of ligands bound per antibody for reaction times greater than 30 min using EDC. This is most likely due to the decomposition of EDC over time, a known competing reaction.[28] Not surprisingly, the concentration

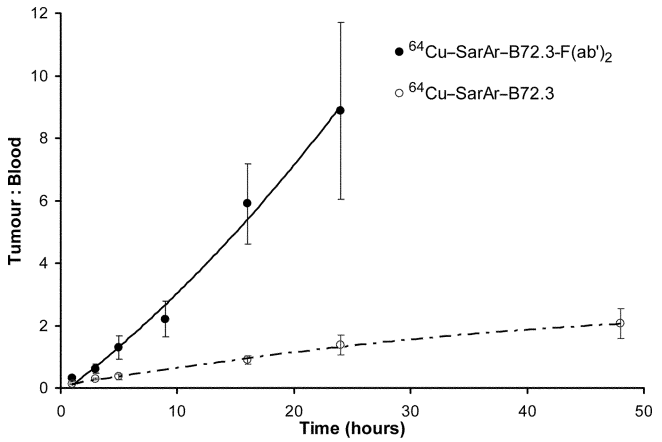

Fig. 6 Comparison of tumour : blood ratios of ^{64}Cu–SarAr–B72.3 and ^{64}Cu–SarAr–B72.3-F(ab′)$_2$ in LS174t tumour-bearing nude mice.

of the antibody was found to affect both the rate of conjugation and the species of immunoconjugate formed. At high antibody concentrations, the number of ligands per antibody increased as did the inter-antibody cross-linking (see Fig. 3). However, even at the maximum concentration (5 mg mL^{-1}) the inter-antibody cross-linking was $\leqslant$3%. The results in Fig. 3 also summarise the effect of varying the EDC and SarAr concentration on the number of ligands attached and inter-antibody cross-linking of the resulting radiolabelled B72.3. An increase in EDC generally resulted in an increase in inter-antibody cross-linking of the protein if SarAr : Mab was 100 : 1. However, an increase in SarAr concentration not only resulted in more ligands attached to the antibody but it also reduced the formation of inter-antibody cross-linking. Unfortunately, the increase in ligands attached did not necessarily result in sufficiently high immunoreactive products. Furthermore, the increase in concentration of SarAr resulted in an increase in the formation of more low molecular weight species and when characterised by SE-HPLC data low molecular weight polymers of SarAr were implicated. Although these aggregates could be easily removed by protein filtration, the optimum conditions for the conjugation of SarAr to the antibody was set at a molar excess of $\geqslant$500 for EDC and >100 to $\leqslant$250 for SarAr : Mab at a protein concentration of 5 mg mL^{-1}. Under these conditions, the amount of inter-antibody cross-linking of ^{64}Cu–SarAr–B72.3 was not significant and therefore would not inhibit its use in animal studies. For an average of 3.3 SarAr attached to each Mab molecule, the immunoreactivity of the final product, ^{64}Cu–SarAr–B72.3, was determined to be $\geqslant$80% compared with the unmodified antibody, and similar to values reported by other researchers.[29] The conditions to optimise ligand attachment to the F(ab′)$_2$ fragment were determined to be a molar ratio of $\geqslant$500 : $\geqslant$250 : 1 for EDC : SarAr : F(ab′)$_2$ with no cross-linking evident (by SE-HPLC). The resultant ^{64}Cu–SarAr–B72.3-F(ab′)$_2$ immunoconjugate was found to contain an average of 1.3 ligands per F(ab′)$_2$ molecule. It is not surprising that the number of ligands attached to F(ab′)$_2$ is considerably lower than that obtained with Mab as there are fewer carboxyl groups on the F(ab′)$_2$ (72 compared with 124). However, on modification, the immunoreactivity of ^{64}Cu–SarAr–B72.3-F(ab′)$_2$ was reduced to 46% of that of the original B72.3-F(ab′)$_2$. As the proportion of the total carboxyl groups in the antigen binding region is higher for the F(ab′)$_2$ fragment (28 of 72) than for the whole antibody (28 of 124) it is not surprising the immunoreactivity diminished. This result was found to be typical of the modified B72.2 antibody (Fab′)$_2$ and hence the resultant ^{64}Cu–SarAr–B72.3-F(ab′)$_2$ was considered appropriate for evaluation in tumour-bearing animals.

Once SarAr was conjugated to the protein it was analysed for decomposition after radiolabelling. Both the $^{64/67}$Cu–SarAr–B72.3 Mab and F(ab′)$_2$ immunoconjugates showed excellent stability in acetate buffer at 4 °C for extended periods (up to 4 d). Kit formulations of the SarAr–B72.3 immunoconjugates were prepared and stored in buffer and also in lyphophilised form at 4 °C for up to 48 d. A comparison of the specific activity of the resultant ^{64}Cu–SarAr-immunconjugates prior to storage and throughout the storage period showed that the SarAr-conjugates were stable.

PET is a highly sensitive technique that can resolve tumour to background ratios as low as 1.4.[30] Hence, an effective radioimmunoconjugate must remain intact *in vivo* while it circulates, it must target the site of interest and finally clear from the blood. Both ^{64}Cu–SarAr–B72.3 and ^{64}Cu–SarAr–B72.3-F(ab′)$_2$ showed excellent *in vivo* stability and tumour targeting. Figs. 4 and 5 display effective localisation at the target site and typical clearance profiles from other organs for the whole antibody and for the F(ab′)$_2$ fragment of a murine antibody, administered intravenously in mice. This trend is similar to those of immunoconjugates of whole B72.3 that have been radiolabelled with ^{125}I and ^{111}In, although the phases in the latter species could not be calculated from the data provided in the literature.[25] The tumour : blood ratio for ^{64}Cu–SarAr–B72.3 of 1.4 was achieved within 24 h with 29.1 + 4.42% ID/g at the tumour site. At 24 h, less than 85% of the total activity remains in the body. The faster clearance of the F(ab′)$_2$ fragment implies earlier imaging potential with a *ca.* 1.4 tumour : blood ratio achievable within 5 h (see Fig. 6). At this time the tumour contained up to 10% ID/g and this uptake persisted for at least 24 h. The results are consistent with the tumour uptake of ^{111}In–DTPA–B72.3-F(ab′)$_2$ (DTPA = diethylenetriaminepentaacetic acid) reported by Brown *et al.* and superior to that of the ^{125}I-labelled B72.3-F(ab′)$_2$ species.[25] A plot of the tumour : blood ratios of the ^{64}Cu–SarAr–B72.3-F(ab′)$_2$ compared with those of the ^{111}In– or ^{125}I–B72.3 radiolabelled species from the literature is given in Fig. 7. At 24 h, the tumour : blood ratios for the ^{64}Cu–SarAr–B72.3-F(ab′)$_2$ of 9 ± 2 were significantly higher than those achieved for the analogous ^{111}In– and ^{125}I–B72.3-F(ab′)$_2$ reagents at the same time of 3.5 ± 2.3 and 1.1 ± 1.2, respectively

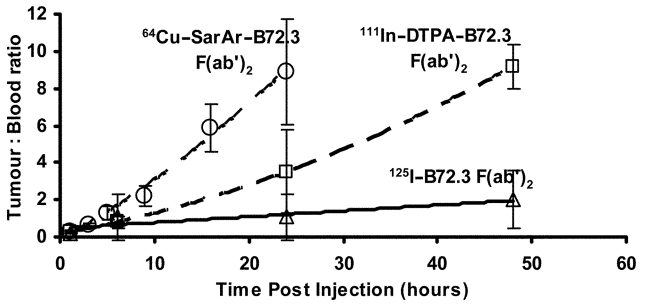

Fig. 7 Comparison of tumour : blood ratios of ^{125}I–B72.3-F(ab′)$_2$, ^{111}In–DTPA–B72.3-F(ab′)$_2$ and ^{64}Cu–SarAr–B72.3-F(ab′)$_2$ in LS174t tumour-bearing nude mice.

(Fig. 7). In fact, superior tumour : blood ratios for the ^{64}Cu–SarAr–B72.3-F(ab′)$_2$ were achieved within 5 h. This implies that the SarAr provides a more stable attachment for the isotope than the methods used for ^{111}In– and ^{125}I–B72.3 radiolabelled species. So while the total uptake of the immunoconjugates at the tumour site are similar over the 24 h period the lower tumour : blood ratios for the ^{111}In and ^{125}I immunoconjugates of the B72.3 indicate that the respective radioisotopes were dissociating from their respective radioimmunoconjugates.

This SarAr technology is significant for several reasons. The SarAr, even when attached to the immunoconjugate, can selectively and rapidly (<5 min) coordinate high specific activity $^{64}Cu^{2+}$ in pH 5–7 buffer at *ca.* 20 °C even in the presence of high concentrations of electrolytes. Once attached to the protein the SarAr binds copper ions irreversibly and quantitatively (*i.e.* one metal ion per ligand present) faster than any other bi-functional chelator reported in the literature under these conditions. Hence radiolabelling efficiency occurs at *ca.* 20 °C to >95% and at extremely low concentrations of copper without the need for further purification. Once the ^{64}Cu–SarAr–B72.3 is prepared even 0.1 M EDTA does not extrude the $^{64}Cu^{2+}$ which clearly demonstrates the stability of the ^{64}Cu–SarAr complex. When the SarAr immunoconjugates were stored in buffer or lyophilised for extended periods of time there was no evidence of decomposition, indicating the potential for a long shelf-life.

The conjugation chemistry described here for SarAr technology is readily adaptable to a range of target agents. Other SarAr derivatives have been developed containing linker groups that can attach to different amino acid sites of the protein.[31] Kit formulations can be readily produced and radiolabelling with $^{64}Cu^{2+}$ for these systems is simple and can be conducted without the need for expensive automation systems or a high skill base.

Perhaps the most exciting potential application of the SarAr technology is in the prognostic imaging arena. Because the ^{64}Cu–SarAr complexation is quantitative at high specific activities of copper isotopes less SarAr-conjugate needs to be administered to the patient for PET imaging. The reduction in the amount of radiolabelled immunconjugate should result in reduced pharmacological effects. More importantly, it should provide the user with an opportunity to quantify the antigen expression at the target site *in vivo*. Such information should lead to more accurate data for determining the response to treatments, rational therapeutic strategies, personalisation of dosage for patients and the drug risk assessment of new target agents both in the preclinical and clinical settings.

Experimental

Material and methods

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. The ligand SarAr was synthesised as previously described. Radiolabelled antibody conjugates were purified by either washing on ultra micro centrifuge protein filters (Centricon or Centrisart 30 or 20 kDa MWt cut-off) with 0.1 M EDTA in phosphate buffered saline (PBS 10 mM, pH 7.2) and sodium acetate buffer (NaOAc 0.1 M, pH 5.0) or using a size-exclusion column (Sephadex G-25 17 × 0.9 cm), equilibrated with NaOAc buffer (0.1 M, pH 5.0) or PBS (10 mM, pH 7.2). Eluent was monitored for protein by UV (Varian S100 or Pharmacia UV/Vis detector UV-1 spectrophotometer) at 280 nm. Radioactivity in collected fractions was measured using the Wallac Wizard 1480 gamma counter or Capintec CRC-15R dose calibrator, depending on the amount of radioactivity. Instant thin layer chromatography on silica gel-impregnated paper (Gelman Sciences, ITLC-SG mobile phase 0.1 M EDTA in 0.1 M PBS pH 7.2) was used to monitor the percentage of antibody radiolabelling (Rf radiolabelled antibody = 0, Rf of Cu^{2+} = 1). HPLC was performed on a Waters LC-626 fitted with the UV monitor (Waters 486) and a BioSep Sec 3000 column (Phenomenex, 300 × 7 mm + guard). Antibody concentration was measured using the Biorad protein assay and bovine serum albumin standard protein solutions. ^{64}Cu was obtained from the National Medical Cyclotron, Australia (specific activity up to and typically 74 × 10^6 MBq g^{-1} at calibration approximately 24 h after end of bombardment (EOB) in 0.02 M HCl. ^{67}Cu was obtained from Los Alamos or Brookhaven Laboratories (specific activity typically 555 × 10^6 MBq g^{-1}) in 0.1 M HCl. The monoclonal antibody used in this study was B72.3, a murine whole IgG_1 antibody supplied by Bioquest Pty Ltd derived from ascites of mice injected with B72.3 hybridoma cells purchased from the American Type Culture Collection (ATCC). Nu/nu mice were purchased from Combined Universities Laboratory Animal Supplies (CULAS, Australia). 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.

Preparation and purification of F(ab′)$_2$ fragment of B72.3

B72.3 (10.6 mg mL^{-1} in a freshly prepared buffer of 50 mM Tris/HCl pH 7.0, 2 mM EDTA and 10 mM cysteine) was reacted with 2% bromelain at 37 °C for 5 h. The reaction was terminated with 10% v/v 0.1 M *N*-ethylamide. The F(ab′)$_2$ fragment produced was isolated by fast protein liquid chromatography (FPLC) using a HR 10/10 Mono Q anion exchange column, eluted with sodium phosphate (5 mM, pH 8.0). Alternatively, the F(ab′)$_2$ fragment was generated and isolated using the commercially available ImmunoPure kit from Pierce Chemical Company.

Conjugation chemistry

Typically, B72.3 [whole antibody or F(ab′)$_2$ fragment] was reacted with a molar excess of EDC and SarAr at 37 °C for a predetermined period of time in buffer (0.1 M). The immunoconjugate that formed, SarAr–B72.3, was purified by washing the reaction mixture on a protein filter (20 000 MWt cutoff) with sodium acetate buffer (0.1 M pH 5.0). An excess of $^{64}Cu^{2+}$ (or $^{67}Cu^{2+}$) in HCl (0.02 M) was added to the purified SarAr–B72.3 (final reaction pH 5.0) at *ca.* 20 °C. Percent labelling, number of ligands per antibody (using the specific activity of the ^{64}Cu or $^{67}Cu^{2+}$) and/or specific activity (radioactivity per mass of antibody) of the radiolabelled product was calculated.

The following conditions for optimising the radiolabelling of the B72.3 whole antibody were investigated: the pH of reaction at 4.0, 5.0 (0.1 M sodium acetate buffer), 6.0, 7.0 and 8.0 (0.1 M sodium carbonate buffer); the time of immunoconjugate formation at 15,

30, 60, 90 and 120 min; the effect of antibody concentration at 1.0, 2.5 and 5.0 mg mL^{-1}; the effect of excess EDC (molar ratio to antibody of 100, 250, 500 and 1000) and excess SarAr (molar ratio to antibody excess of 100, 250 and 500). The B72.3-F(ab′)$_2$ fragment was conjugated to SarAr at pH 5.0 (0.1 M sodium acetate buffer) for 30 min at 5.0 mg mL^{-1} B72.3-F(ab′)$_2$, molar ratio of antibody : EDC : SarAr of 1 : 500 : 250.

An excess $^{64}Cu^{2+}$ (in 0.02 M HCl) was added to each conjugation reaction and allowed to react for up to 5 min at *ca.* 20 °C. Radiolabelled antibody conjugates were purified as described above and percentage labelling and/or specific activity was calculated. Inter-antibody cross-linking in the resulting radiolabelled antibody was examined by size-exclusion HPLC.

Radioimmunoassay (RIA)

The immunoreactivity of the radiolabelled antibody was determined by a modified method of Lindmo *et al*.[32] Bovine Submaxillary Mucine (BSM as antigen) or glycine (Gly as control) was serially diluted in triplicate onto Immulon 2 microtitre plates (8 μg per well serially diluted by half 10 times, blank contained only PBS). Plates were incubated overnight at 4 °C. The solution was then removed and the plates blocked with 0.05% Tween 20 in PBS for 1 h at 37 °C. The blocking solution was removed and the plates were then incubated overnight at 4 °C with an excess of ^{64}Cu–SarAr–B72.3 (in 0.05% Tween 20 in PBS). Unbound antibody was removed by washing three times with 0.05% Tween 20 in PBS. The radioactivity in each well was measured. Binding curves of percentage bound (corrected for non-specific binding) *versus* BSM concentration for both antibody solutions were constructed. Immunoreactivity was calculated by the Lindmo method, *i.e.* total radioactivity added to each plate well/radioactivity bound to the well (corrected for non-specific binding) was plotted against 1/[BSM] in the plate well and the resulting linear plot was extrapolated to the *y*-intercept to obtain 1/immunoreactive fraction. A similar method employing a second antibody (radioiodinated goat-anti-mouse antibody, ^{123}I–GAM) to detect the binding of radiolabelled B72.3 compared with unmodified B72.3 was used to confirm the immunoreactivity as calculated by the Lindmo method.

Stability of immunoconjugate

The SarAr–B72.3 immunoconjugate was prepared as described above. The batch of immunoconjugate was divided into several aliquots of at least 50 μg but more typically 100 μg samples. An aliquot of the prepared immunoconjugate solution was radiolabelled with ^{64}Cu immediately, and the remaining unlabelled aliquots of solution were stored either as a frozen solution or lyophilised at 4 °C. At a later date stored samples of immunoconjugate were radiolabelled with ^{64}Cu and the specific activity of radiolabelled immunoconjugates was monitored over time using HPLC and ITLC-SG and compared with the original batch.

Integrity of radiolabelled immunoconjugates

^{64}Cu–SarAr–B72.3 and ^{64}Cu–SarAr–B72.3-F(ab′)$_2$ radiolabelled immunoconjugates were prepared as previously described. The purified radiolabelled antibodies were stored in sodium acetate (0.1 M, pH 5.0) at 4 °C and the radioactivity dissociated from each radioimmunoconjugate was monitored by ITLC-SG.

Animal biodistribution studies

Nu/nu mice between the ages of 6 and 8 weeks (males and females) were subcutaneously injected with 0.1 mL of a 10×10^6 LS174t cells mL^{-1} in PBS. Animals were sacrificed after one week, the tumours harvested and kept in ice-chilled media under sterile conditions. Tumour sections (approx. 2 × 2 mm) were implanted subcutaneously into the hind flanks or subscapular region of another group of nu/nu mice. Tumours were allowed to grow (2 weeks) to approximately 0.7 × 0.7 cm. Tumoured mice were injected intravenously *via* the tail vein with 0.1 mL of sterile (0.22 μm) filtered ^{64}Cu–SarAr–B72.3 or ^{64}Cu–SarAr–B72.3-F(ab′)$_2$ prepared as previously described. Typical specific activity for ^{64}Cu–SarAr–B72.3 and ^{64}Cu–SarAr–B72.3-F(ab′)$_2$ was 3.8 ligands per mole of antibody and 1.0 ligands per mole of antibody, respectively. At the required time interval each animal was anaesthetised with CO_2 for cardiac puncture and then sacrificed by cervical dislocation (five animals per time point). The animal was dissected and the organs and selected tissues removed and weighed. Associated radioactivity was determined using the gamma counter. The percentage injected dose for each organ, as well as percentage injected dose per gram, was calculated.

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18 Abbreviations: **Sar** = 3,6,10,13,16,19-hexa-azabicyclo[6.6.6]icosane; **Diamsar** = 1,8-diamino-Sar; **SarAr** = 1-*N*-(4-aminobenzyl)-3,6,10, 13,16,19-hexa-azabicyclo[6.6.6]eicosane-1,8-diamine.
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