Electronic Supplementary Information

Synthesis of hexa aza cages, SarAr-NCS and AmBaSar and a study of their metal complexation, conjugation to nanomaterials and proteins for application in radioimaging and therapy †

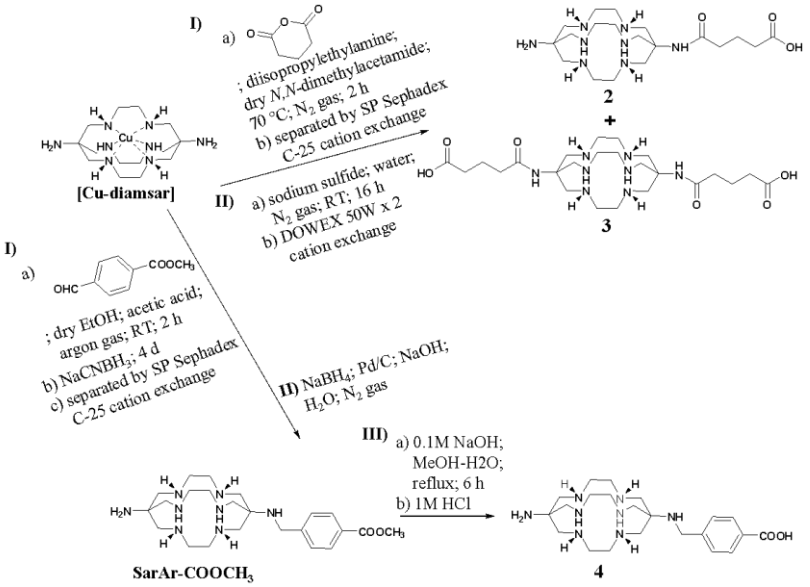
Eskender Mume,[a,b] Ali Asad,[c] Nadine M Di Bartolo,[a,b] Linggen Kong,[a] Christopher Smith,[a,d] Alan M Sargeson,[e] Roger Price[c] and Suzanne V. Smith*[a,b,f]

Centres for Antimatter-Matter Studies (CAMS) at the [a]Australian Nuclear Science and Technology Organisation (ANSTO), Menai, NSW 2234, Australia and the [b]Australian National University, Canberra, ACT 0200, Australia, [c]Radiopharmaceutical Production & Development (RAPID) Laboratory, Medical Technology and Physics, Sir Charles Gairdner Hospital, Perth, Australia, [d]Department of Chemistry, Faculty of Science, Chulalongkorn University, Pathumaw, Bangkok 10400, Thailand. [e]Research School of Chemistry, Australian National University, Canberra, ACT 0200. [f]Collider-Accelerator Department, Brookhaven National Laboratory, Upton NY 11973.

* *Corresponding author: suzanne @bnl.gov*

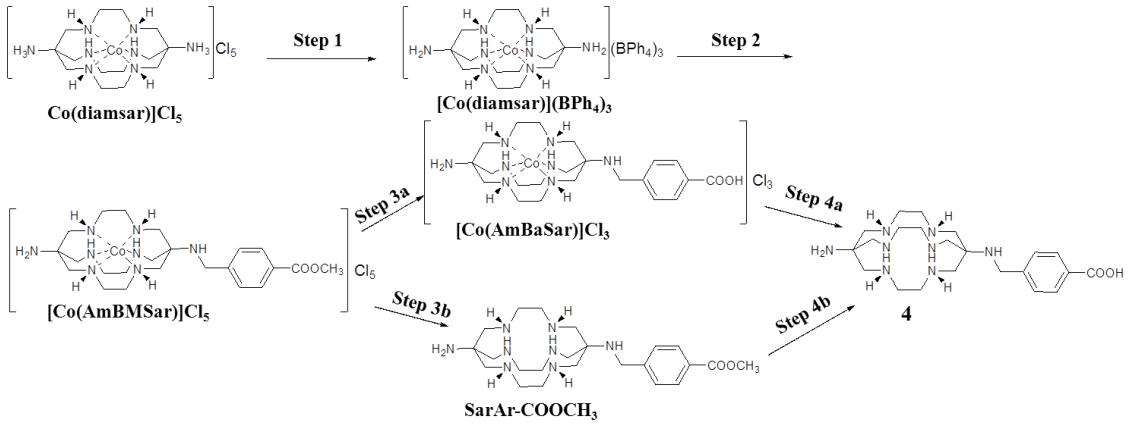
Table of Contents	

Scheme S1 below summarizes the strategy for the synthesis of bi-functional ligands **2, 3, 4**.



Scheme S1 Synthesis of **2**,**3** and **4** in the literature.

Two methods have been reported for the synthesis for **4**, they are summarized in **Scheme S2**.



Step 1 a) NaOH, H_2O, 30 min b) $NaBPh_4$, MeOH, 1 h, 85% yield	Step 2 a) Methyl 4-formylbenzoate, MeOH, AcOH, argon, reflux 72 h b) $NaHB(OAc)_3$, overnight c) Flash chromatography, Dowex 50-WX2 [H^+] cation exchange resin column, 39.1% yield
Step 3a a) MeOH-H_2O, K_2CO_3, reflux 5 h b) Dowex 50-WX2 [H^+] cation exchange resin column, 89% yield	Step 3b NaOH, $CoC1_2$, H_2O , NaCN, 70°C, N_2, overnight, 26% yield
Step 4a NaOH, $CoC1_2$, H_2O , NaCN, 70°C, N_2, overnight, 24.5% yield	Step 4b K_2CO_3, MeOH , reflux 6 h, 80.2% yield
Path 3a: Overall Reaction time= 102.5 h Cation exchange= 2 Yield= 7.4%	**Path 3b: Overall** Reaction time= 103.5 h Cation exchange= 1 Yield= 6.9%

Scheme S2 Two methods of synthesis of **4** reported in the literature.

The NMR spectra for SarAr-NCS is given below in **Fig S1** and **S2** and its electospray mass spectrum is given in **Fig S3**.

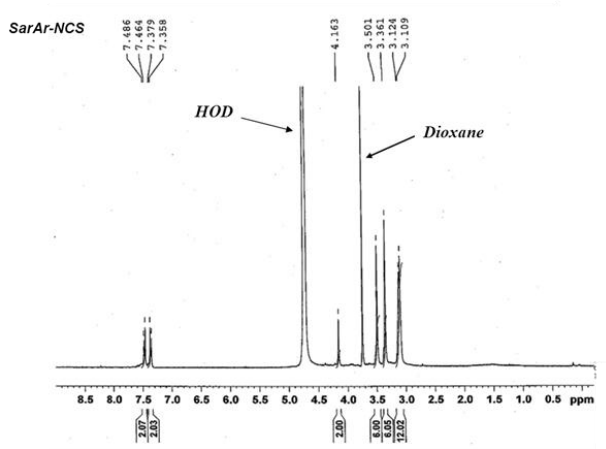


Fig. S1 ^{1}H NMR (D_2O) spectrum of SarAr-NCS (**5**).

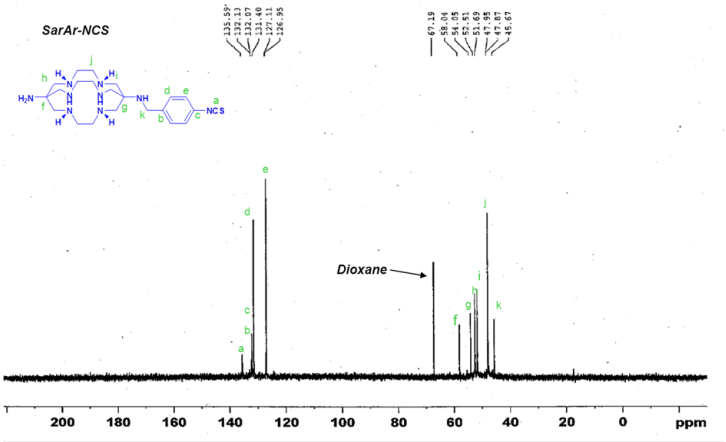


Fig. S2 ^{13}C NMR (D_2O) spectrum of SarAr-NCS (**5**).

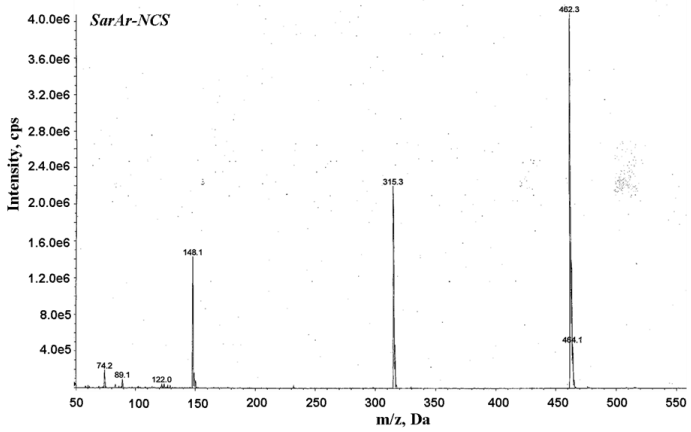


Fig. S3 Mass spectrum of SarAr-NCS (**5**) in positive ion mode.

Table S2 NMR Data for SarAr (**1**), AmBaSar (**4**) and SarAr-NCS **(5)**. ^{1}H NMR and ^{13}C NMR were recorded in D_2O; internal standard was dioxane; ^{1}H NMR (D_2O) δ: 3.75 (s); ^{13}C NMR (D_2O) δ: 67.2.

^{1}H NMR									
(1)	**(4)**	**(5)**	**(1)**	**(4)**	**(5)**	**(1)**	**(4)**	**(5)**
Shift (ppm)			*Multiplicity*			*Integration*		
2.92	3.20	3.12	s	s	s	12	12	12
3.00	3.40	3.36	s	s	s	6	6	6
3.10	3.50	3.50	s	s	s	6	6	6
3.58	4.20	4.16	s	s	s	2	2	2
6.81	7.60	7.37	d	d	d	2	2	2
7.13	8.10	7.46	d	d	d	2	2	2

^{13}C NMR												
(**4**)	*Shift*	170.3	130.4	130.1	129.5	57.6	54.0	51.6	51.1	47.4	47.2	45.2
(**5**)	*(ppm)*	135.6	132.1	132.1	131.4	127.0	58.0	54.1	52.5	51.7	47.9	45.7

Metal complexation studies.

The mobile phases and retention factor (R_f) for reactants and metal complexes for each ligand system is summarized in Table S1. Selected data from these metal complex reactions are given in **Fig. S4** to **S11**.

Table S1 Retention factor (R_f) and mobile phases used to separate the $^{64/nat}Cu^{2+}$, $^{57/nat}Co^{2+}$ or $^{65/nat}Zn^{2+}$ and each $^{64/nat}$Cu-Ligand, $^{57/nat}$Co-Ligand or $^{65/nat}$Zn-Ligand system.

Mobile phase	$^{64/nat}Cu^{2+}$ $^{57/nat}Co^{2+}$ or $^{65/nat}Zn^{2+}$	$^{64/nat}$Cu-Ligand $^{57/nat}$Co-Ligand or $^{65/nat}$Zn-Ligand
	R_f	R_f
0.1M sodium chloride: 0.1M EDTA: 9/1 v/v; used to separate $[^{64/nat}$Cu-SarAr-NCS$]^{2+}$, $[^{64/nat}$Cu-AmBaSar$]^{2+}$, $[^{57/nat}$Co-SarAr-NCS$]^{2+}$, $[^{57/nat}$Co-AmBaSar$]^{2+}$, $[^{65/nat}$Zn-SarAr-NCS$]^{2+}$ or $[^{65/nat}$Zn-AmBaSar$]^{2+}$ from free $^{64/nat}Cu^{2+}$, $^{57/nat}Co^{2+}$ or $^{65/nat}Zn^{2+}$	>0.9	<0.2

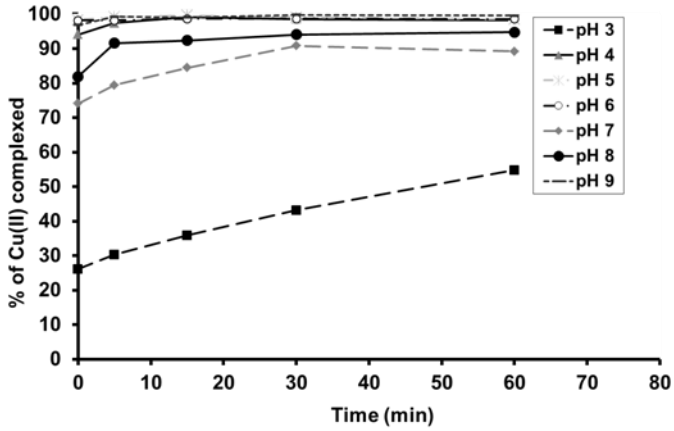


Fig. S4 Complexation of Cu(II) with **4** (AmBaSar) at 10^{-5} M over time at 23°C.

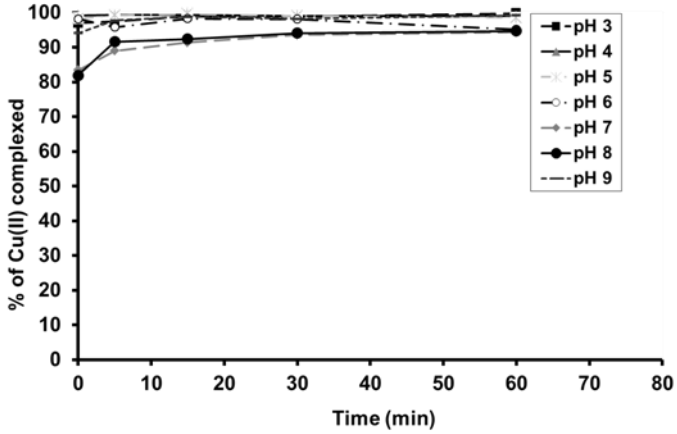


Fig. S5 Complexation of Cu(II) with **4** (AmBaSar) at 10^{-4} M over time at 23°C.

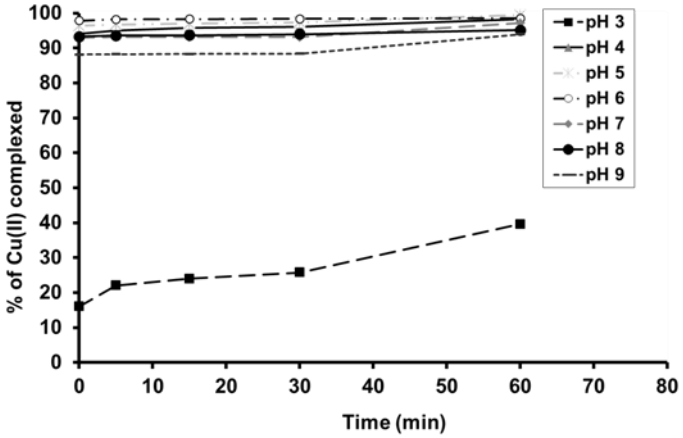


Fig. S6 Complexation of Cu(II) with **5** (SarAr-NCS) at 10^{-5} M over time at 23°C.

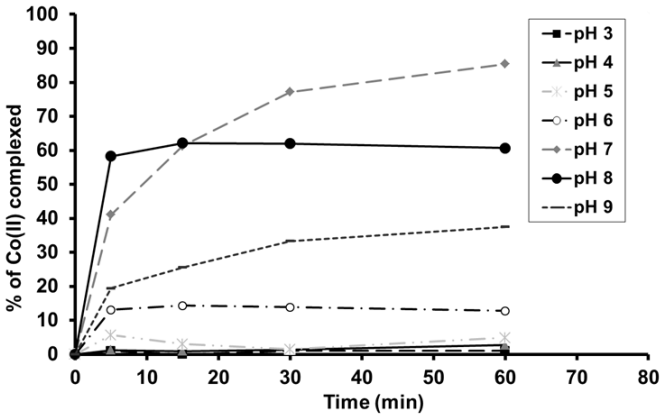


Fig. S7 Complexation of Co(II) with **4** (AmBaSar) at 10^{-5} M over time at 23°C.

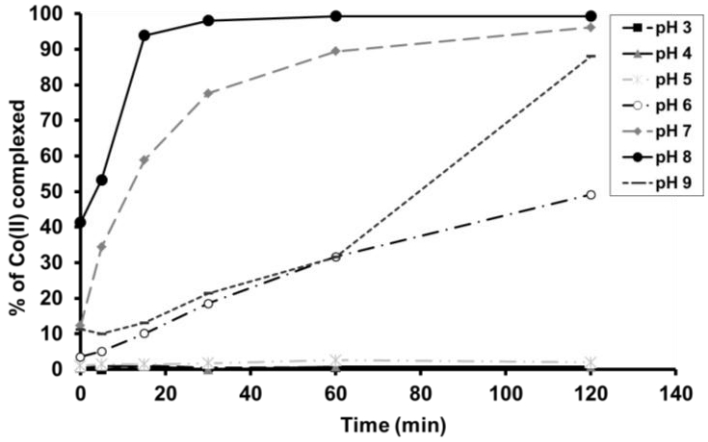


Fig. S8 Complexation of Co(II) with **5** (SarAr-NCS) at 10^{-5} M over time at 23°C.

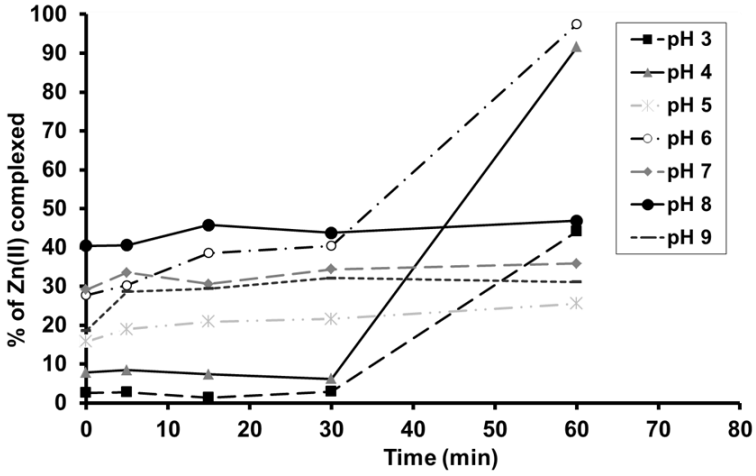


Fig. S9 Complexation of Zn(II) with **4** (AmBaSar) at 10^{-5} M over time at 23°C.

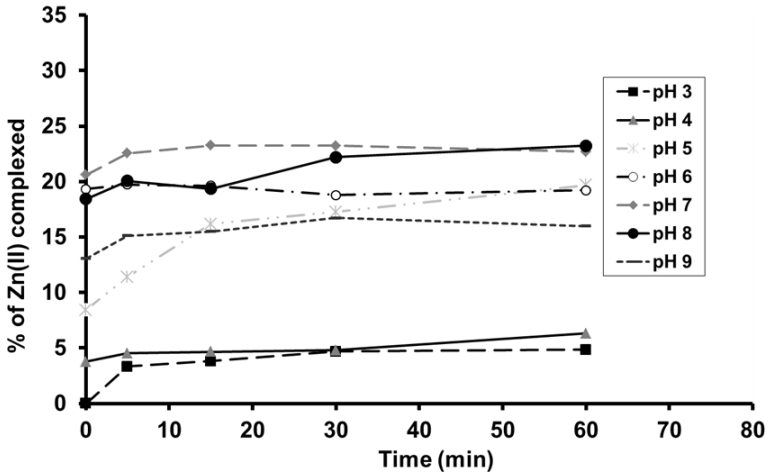


Fig. S10 Complexation of Zn(II) with **5** (Sarar-NCS) at 10^{-5} M over time at 23°C.

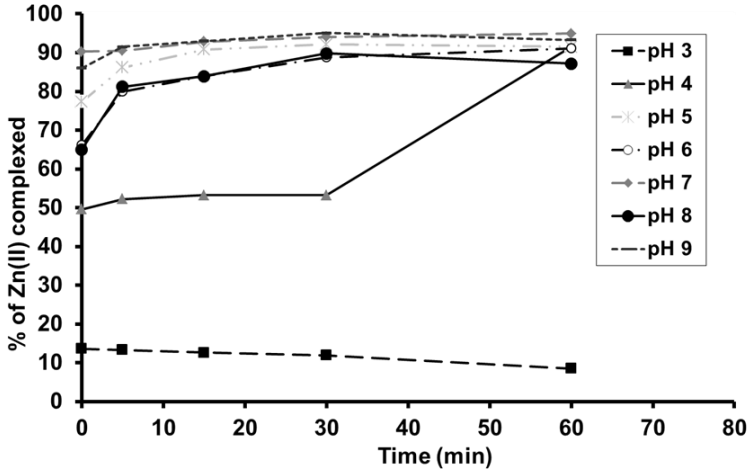


Fig. S11 Effect of concentration Zn (II) complexation by **4** [AmBaSar] at 10^{-4} M over time at 23°C.

Radiolabeling Silica Particles using SarAr-NCS.

The synthesis of the silica particle was prepared via using an oil in water emulsion. Detail description is given in the main manuscript. The particle size determined by TEM is given in **Table S2**. **Fig. 4** in the main manuscript summaries the results from the radiolabeling of the silica particle and it is this data that was used to determine the number of SarAr-NCS conjugated to the particles available for complexation of the Cu(II).

Table S2 Size range and peak diameter of silica particles.

Size range	67-121 nm
Peak diameter	94.0 nm

Table S2 Data used to calculate number of available SarAr-NCS sites on silica particle.

Mass (mg)	1.454
Density (g/mL)	1.30
Diameter (nm)	94.0
Cu^{2+} saturated (nmole)	30.0
SarAr-NCS conjugated at saturation	$1.81x10^{16}$
Volume of total particles (cm^3)	1.12×10^{-3}
Volume of each particle (cm^3)	4.35×10^{-16}
Number of particles	$2.57 x10^{12}$
Number of SarAr-NCS per particle	$7.02x10^{3}$
Number of ligand per mg particle	$1.24x10^{16}$

Radiolabeling B72.3 Antibody using SarAr-NCS.
Effect of time and molar ratio of reactants was invesitgated and summarized in **Table S3** and **S4** respectively.

Table S3 Effect of time on formation of Sarar_NCSN-B72.3 immunoconjugate.

Reaction Condition	Value
B72.3 concentration (mg/mL)	10.0
Molar ratio of SarAr-NCS: B72.3	10.0
Reaction time (min)	15, 30 and 120
pH	8 (0.1 M sodium phosphate buffer)

The following matrix of reaction conditions was employed to determine the optimum concentration of antibody and ligand for the conjugation reaction.

Table S4 Effect of reactant concentration on formation of SarAr-NCSN-B72.3 immunoconjugate.

Reaction Condition	Value
B72.3 concentration (mg/mL)	1.0, 2.5, 5.0 and 10.0
Molar ratio of SarAr-NCS: B72.3	3, 6 and 12
Reaction time (min)	30
pH	8 (0.1 M sodium phosphate buffer)

At the specified time intervals, an aliquot of the reaction mixture was removed, purified, radiolabeled with ^{64}Cu. The radiolabeled SarAr-immunoconjugate was characterized by size exclusion of HPLC-SG and ITLC-SG.

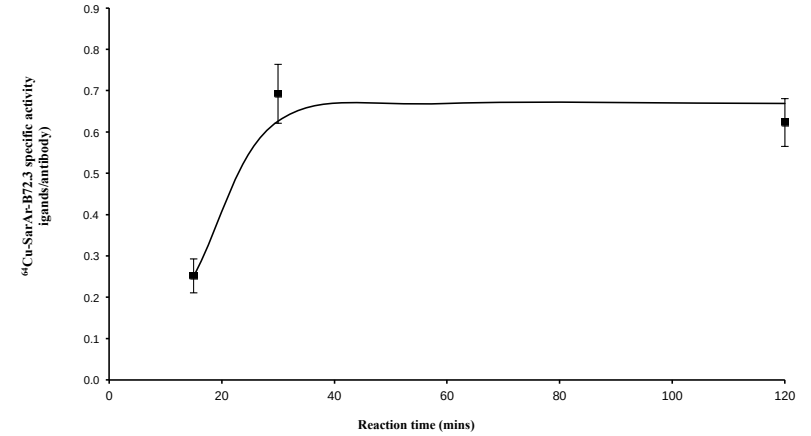


Figure S12 Effect of reaction time on SarAr NCS conjugation to B72.3.[a]

[a](phosphate buffer pH 8, 37°C, [B72.3] = 10 mg/mL, B72.3:SarAr-NCS = 1:10).

Radioimmunoassay

The immunoreactivity of the radiolabelled antibody was determined by a modified method of Lindmo *et al*[1] and described in detail in Di Bartolo *et al* 2006 reference 9. Typically bovine submaxillary mucine (BSM as antigen) or glycine (Gly as control) was serially diluted in triplicate onto Immulon 2 microtitre plates (8 lg 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-NCS–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. 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.

[1] T. Lindmo, E. Boven, F. Cuttitta, J. Fedorko and P. A. Bunn, Jr., *J. Immunol. Methods*, 1984, **72**, 77.