Supplementary Material (ESI) for Chemical Communications
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Efficient bifunctional gallium-68 chelators for positron emission tomography: tripodal tris(hydroxypyridinone) ligands.

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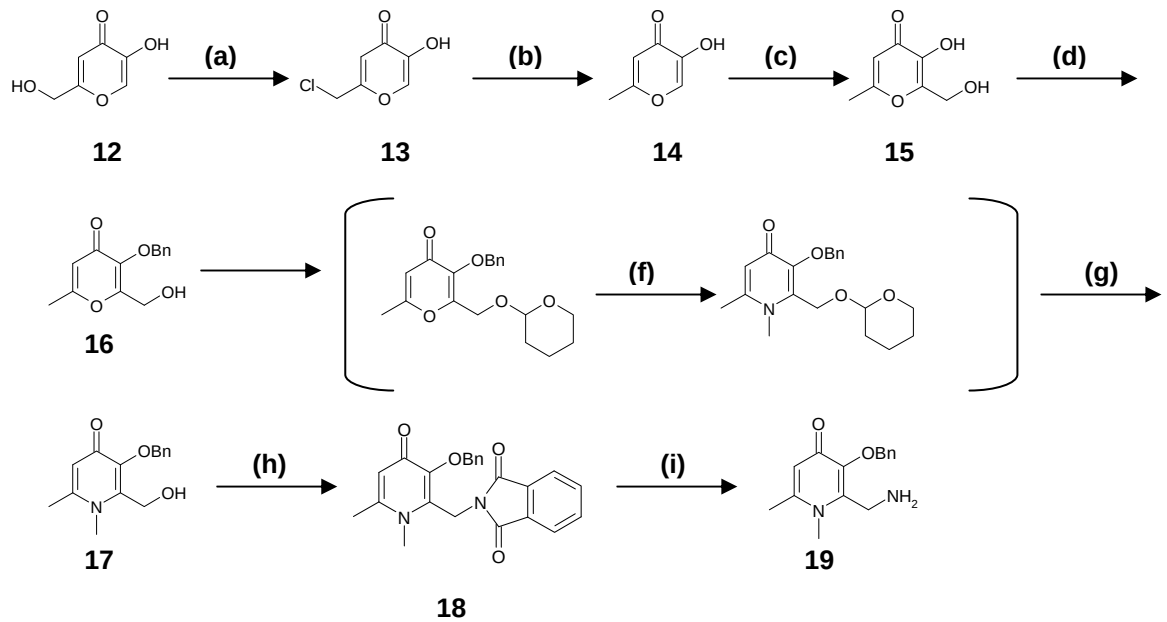
Supporting Information

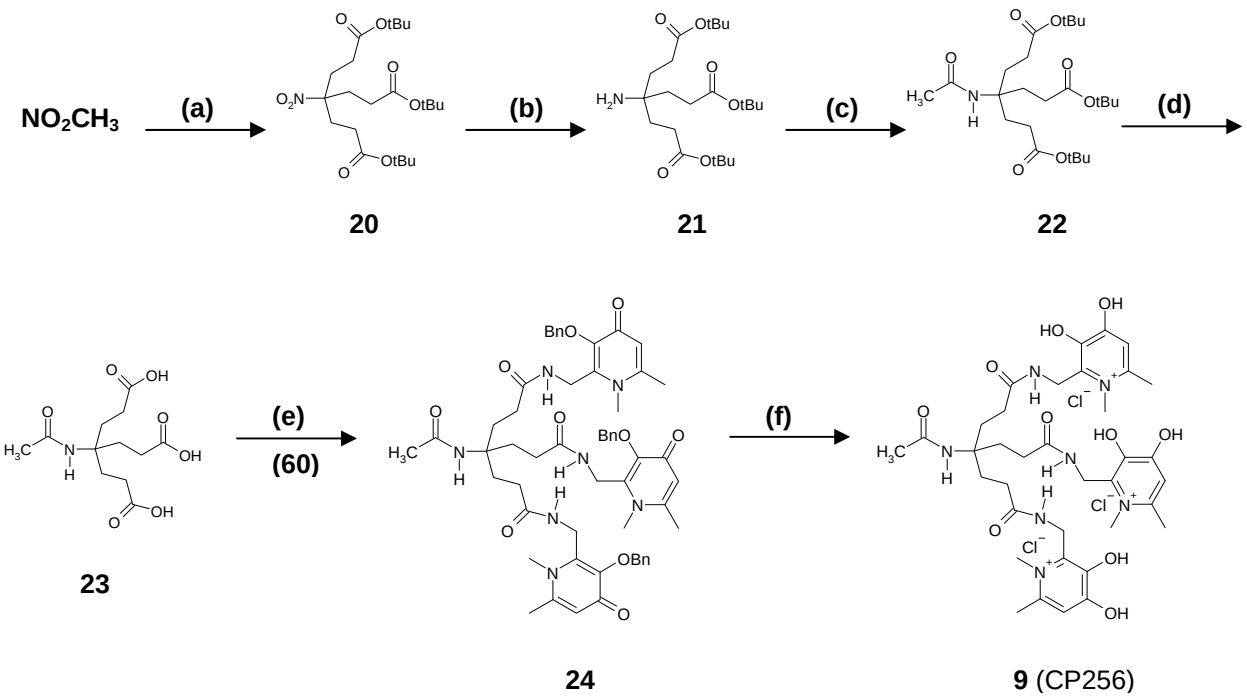
1. Chemical syntheses

Methods

CP256



Scheme S1. Synthesis of 2-amido-3-hydroxypyridin-4-one **19** and the intermediates **13-18**. Materials and methods: (a) $SOCl_2$, 25°C; (b) Zn/HCl, H_2O, 70°C; (c) HCHO, NaOH, H_2O, 25°C; (d) BnBr, NaOH, CH_3OH/H_2O, 70°C; (e) dihydropyran, p-toluenesulphonic acid, CH_2Cl_2, 25°C; (f) NH_2CH_3/H_2O, EtOH, 80°C; (g) HCl, EtOH, 80°C; (h) Ph_3P, DIAD, phthalimide, THF, 0-25°C; (i) NH_2NH_2/H_2O, EtOH, 80°C.



Scheme S2. Synthesis of tripodal chelator CP256 (**9**). Materials and methods: (a) Triton B, Tert-butyl acrylate, DME, 70°C; (b) T-1 Raney Ni, H_2, 25°C, EtOH; (c) CH_3COCl, Et_3N, CH_2Cl_2, 0-25°C, (d) HCOOH; (e) **19**, DCCI, HoBt, DMF 25°C; (f) Pd/C, H_2, HCl, CH_3OH, 25°C.

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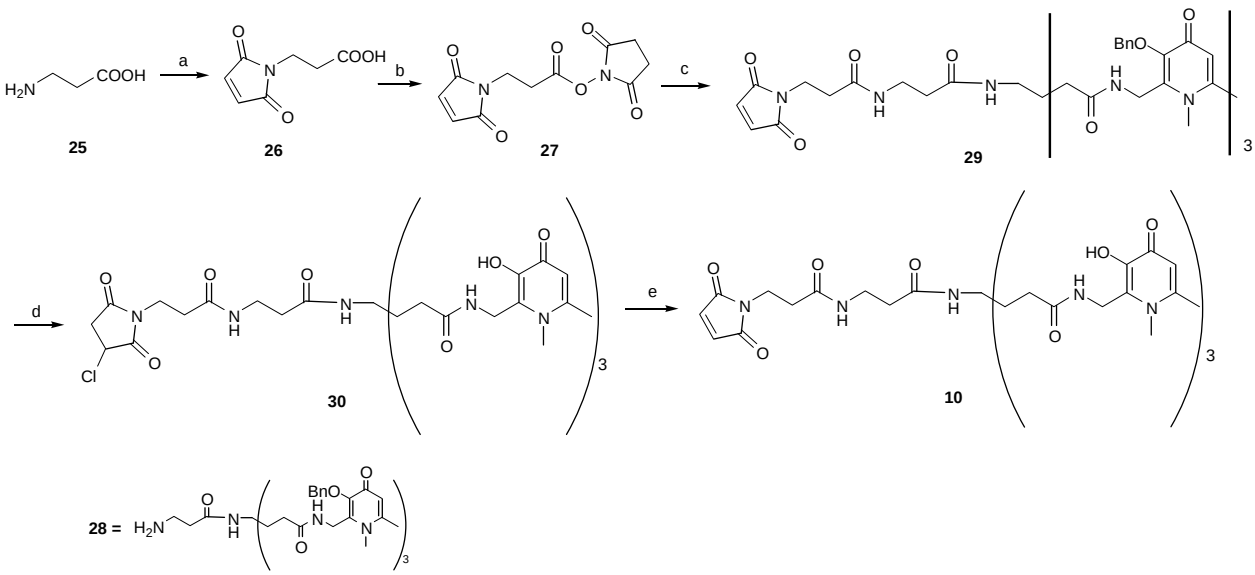


Scheme S3: Synthesis of maleimide linked hexadentate chelator **10**. a) maleic anhydride/DMF; b) DCC/NHS/DMF; c) **28**/DMF; d) BCl_3/DCM; e) Et_3N/DCM.

Scheme S1

Intermediates **13-16** [1], **17-19** [2] and **20-21** [3] and were synthesised by previously described literature methods.

Scheme S2

4-Acetylamino-4-(2-*tert*-butoxycarbonyl-ethyl)-heptanedioic acid di-*tert*-butyl ester (22)

To 5 ml CH_2Cl_2 was added 1 g of triester **21** (2.4 mmol, 1 equiv) and 0.3 g triethylamine (2.9 mmol, 1.2 equiv.) The solution was cooled to 0°C with an ice bath and 0.23 g acetyl chloride (2.9 mmol, 1.2 equiv) was added dropwise. The solution was stirred for a further 4 hours and allowed to warm slowly to room temperature. The solution was poured into a separating funnel and washed with 2 X 15 ml HCl (5% aqueous) and 1 X 15 ml concentrated NaCl. The solution was dried over Na_2SO_4 and the solvent was removed in vacuo. The residue was dissolved in minimal EtOAc and loaded onto a silica column, where it was purified by column chromatography with EtOAc and hexane (1:1) to give 0.79 g of a white solid in 72% yield.

^{1}H NMR (DMSO-d_6) σ 7.2 (s, 1H [CO**NH**]) 2.0 (m, 6H [CH_2-**CH_2**-COO-tbu]$_3$) 1.8 (s, 3H [**CH_3**-CO]) 1.7 (m, 6H [**CH_2**-CH_2-COO-tbu]$_3$) 1.35 (s, 27H [CH_2-CH_2-COO-**tbu**]$_3$)

m/z 458 $(M+H)^+$ 475 $(M+NH_4)^+$ [ES^+]

4-Acetylamino-4-(2-carboxy-ethyl)-heptanedioic acid (23)

1.5 g of triester **22** (3.28 mmol) was added to 20 ml formic acid (96%) and stirred for 4 hours. After this time the formic acid was removed by azeotropic distillation with 5 X 20 ml portions of toluene. The crude residue was recrystallised from EtOH and petroleum spirit to give 0.9 g of off-white crystals in 93% yield.

^{1}H NMR (DMSO-d_6) σ 12 (s, 3H [CH_2-COO**H**]) 7.2 (s, 1H [N-**H**]) 2.1 (m, 6H [CH_2-**CH_2**-COOH]$_3$) 1.8 (m, 6H [**CH_2**-CH_2-COO-tbu]$_3$) 1.7 (s, 3H [**CH_3**-CO])

m/z 290 $(M+H)^+$ [ES^+]

4-Acetylamino-4-{2-[(3-benzyloxy-1,6-dimethyl-4-oxo-1,4-dihydro-pyridin-2-ylmethyl)-carbamoyl]-ethyl}-heptanedioic acid bis-[(3-benzyloxy-1,6-dimethyl-4-oxo-1,4-dihydro-pyridin-2-ylmethyl)-amide] (**24**)

To 10 ml anhydrous DMF in a 100 ml round bottomed flask were added 0.4 g **23** (1.38 mmol, 1 equiv) 1.29 g **19** (4.98 mmol, 3.6 equiv) 0.67 g HOBt (4.98 mmol, 3.6 equiv) and 1.03 g DCCI (4.98 mmol, 3.6 equiv.) The mixture was stirred for 48 hours at room temperature under an atmosphere of N_2. The solution was filtered to remove the precipitated DCU. DMF was removed in vacuo. The residue was dissolved in methanol and purified by column chromatography (silica gel) with a mobile phase of 4:1 $CHCl_3/CH_3OH$. The fractions were combined, dried (Na_2SO_4) and the solvent was removed in vacuo to give 0.7 g of a white solid in 50% yield.

^{1}H NMR (DMSO-d_6) σ 8 (t, 3H [CO**NH**]) 7.3 (m, 15H [CH_2-**Bnz**]) 7.15 (s, 1H [CO**NH**]) 6.2 (s, 3H [**5-H** pyridinone]) 5.05 (s, 6H [**CH_2**-Bnz]) 4.3 (d, 6H [CONH-**CH_2**-pyridinone]) 3.35 (s, 9H [6-**CH_3** pyridinone]) 2.2 (s, 9H [N-**CH_3** pyridinone]) 2.0 (m, 6H [**CH_2**-CH_2-CONH-pyridinone]) 1.75 (m, 6H [CH_2-**CH_2**-CONH-pyridinone]) 1.7 (s, 3H [**CH_3**-CONH-tripod])

m/z 1010 $(M+H)^+$ 1032 $(M+Na)^+$ [ES^+]

4-Acetylamino-4-{2-[(3-hydroxy-1,6-dimethyl-4-oxo-1,4-dihydro-pyridin-2-ylmethyl)-carbamoyl]-ethyl}-heptanedioic acid bis-[(3-hydroxy-1,6-dimethyl-4-oxo-1,4-dihydro-pyridin-2-ylmethyl)-amide] tris hydrochloride salt (**9**)

0.64 g of **24** (0.63 mmol, 1 equiv) was dissolved in 40 ml CH_3OH in a thick walled hydrogenation flask. 2.3 ml of HCl (1.25 M in CH_3OH) was added to the solution. 0.2 g of Pd/C catalyst was added and the solution was hydrogenated for 18 hours (room temperature, 40 psi.) The flask was removed with care and the solution filtered to remove Pd/C. The solvent was removed in vacuo. The residue was redissolved in minimal methanol. The product (**9**) was precipitated with acetone. Once the precipitate had settled, the supernatant was removed and the precipitate was washed with cold diethyl ether. This process was repeated 3 times and solvent removed in vacuo to give 0.4 g of the tris hydrochloride salt of **9** as a white solid in 74% yield.

Elemental analysis. Found: C, 48.21; H, 5.98; N, 10.04. $C_{36}H_{52}N_7O_{10}Cl_3$ requires C, 50.92; H, 6.17; N, 11.55%);

^{1}H NMR (DMSO-d_6) σ 8.8 (t, 3H [CO**NH**]) 7.25 (s, 1H [CO**NH**]) 7.1 (s, 3H [**5-H** pyridinone]) 4.5 (d, 6H [CONH-**CH_2**-pyridinone]) 3.8 (s, 9H [6-**CH_3** pyridinone]) 2.55 (s, 9H [N-**CH_3** pyridinone]) 2.1 (m, 6H [**CH_2**-CH_2-CONH-pyridinone]) 1.8 (m, 6H [CH_2-**CH_2**-CONH-pyridinone]) 1.7 (s, 3H [CH_3-CO**NH**-tripod])

^{13}C NMR (DMSO-d_6) σ 21.0 (**C**H_3), 24.0 (**C**H_3CO), 29.8 (C**C**H_2CH_2), 30.5 (CCH_2**C**H_2), 35.2 (NH**C**H_2), 39.2 (N**C**H_3), 57.2 (NH**C**), 113.2 (**C**-5H in pyridinone), 139.4 (**C**-2 in pyridinone), 143.5 (**C**-3 in pyridinone), 148.8 (**C**-6 in pyridinone), 161.1 (**C**-4 in pyridinone), 169.4 (CH_3**C**O), 173.8 (**C**ONH).

m/z 740.3631 $(M+H)^+$ (10% rel. abundance) 370.6843 $(M+2H)^{2+}$ (100% rel. abundance) HRMS [ES^+]

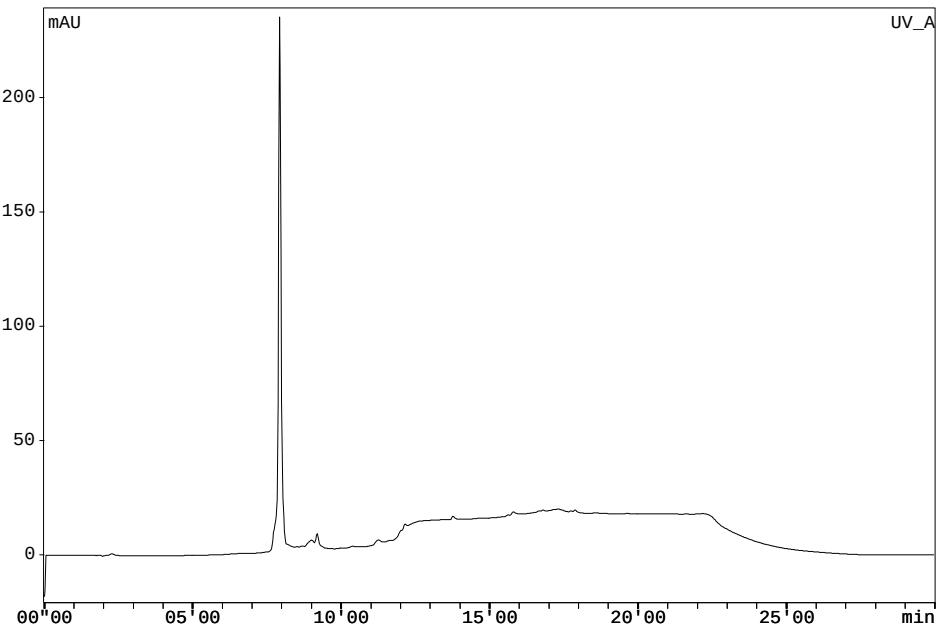


Figure S1. HPLC trace of CP256 ligand.

Scheme S3

The synthesis of compound **28** has been reported previously. [4]

Succinimido 3-maleimidopropanoate (27)

A solution of β-Alanine (1.8 g, 20 mmol) and maleic anhydride (2 g, 20 mmol) in DMF (30 mL) was stirred at room temperature for 3h. After the solid was completely dissolved, N-hydroxysuccinimide (NHS; 2.3 g, 20 mmol) and dicyclohexylcarbodiimide (DCC; 4.6 g, 24 mmol) was added into the solution and the mixture was stirred at room temperature overnight. The solution

was filtered and the precipitate was washed with water (100 ml) and dichloromethane (100 ml). The filtrate was collected and the organic layer was washed with 3x50 ml 5% $NaHCO_3$ and one time with brine. The organic layer was dried with Na_2SO_4 and dichloromethane removed under reduced pressure to obtain a white solid (55%). This solid does not need further purification and can be directly used for next coupling step. ^{1}H NMR ($CDCl_3$, 400MHz): δ 2.82 (s, 4H, two succinimido-CH_2), 3.02 (t, J=7.0 Hz, 2H, $COCH_2$), 3.94 (t, J=7.0 Hz, 2H, NCH_2), 6.74 (s, 2H, two CH).

Synthesis of compound 29:

The activated acid **27** (6 mmol) and amine **28** (5 mmol) in anhydrous DMF (50 ml) was stirred at room temperature for one day. The solvent was removed under reduced pressure and the residue was purified on a silica gel column using chloroform:methanol (8:2) as an eluent to afford a white foam (81%).

^{1}H NMR ($CDCl_3$, 400MHz): δ 2.05 (brs, 6H, CH_2), 2.15 (s, 9H, CH_3), 2.23 (brs, 2H, CH_2), 2.29 (brs, 6H, CH_2), 2.41 (t, J=6.6 Hz, 2H, CH_2), 3.32 (brs, 2H, CH_2), 3.36 (s, 9H, CH_3), 3.73 (t, J=6.6 Hz, 2H, CH_2), 4.35 (s, 6H, CH_2), 4.87 (s, 6H, CH_2), 6.10 (s, 3H, CH), 6.65 (s, 2H, CH), 6.76 (brs, 1H, NH), 7.23-7.30 (m, 15H, ArH), 7.72 (brs, 3H, NH). ^{13}C NMR ($CDCl_3$, 100MHz): 20.81 (CH_3), 30.70 (CH_2), 31.71 (CH_2), 34.43 (CH_2), 34.62 (CH_2), 34.82 (CH_2), 36.31 (CH_3), 36.68 (CH_2), 57.99, 73.42 (CH_2), 118.25 (CH), 128.30 (CH), 128.52 (CH), 128.58 (CH), 134.23 (CH), 136.97, 141.05, 146.35, 148.04, 170.43, 170.66, 171.70, 173.10, 173.23.

ESI-MS: 1190.40 $(M+1)^+$

Synthesis of compound 30:

The benzyl protecting compound **29** in anhydrous DCM under N_2 was added boron trichloride (12 equiv) in ice-bath and the mixture was stirred at room temperature for 2 days. The excess BCl_3 was quenched by adding MeOH and followed by 1 mL concentrated hydrochloric acid. The mixture was evaporated to obtain brown solid, which dissolved in methanol and precipitated with the addition of acetone. This procedure was repeated three times to afford white solid (72%).

^{1}H NMR (DMSO-d_6, 400MHz): δ 1.75-1.84 (m, 6H, CH_2), 2.08-2.13 (m, 6H, CH_2), 2.21 (t, J=7.1 Hz, 2H, CH_2), 2.32 (t, J=7.5 Hz, 2H, CH_2), 2.57 (s, 9H, CH_3), 2.85 (dd, J=4.1, 8.3 Hz, 1H, one H of maleic CH_2), 3.16 (q, J=6.5 Hz, 2H, CH_2), 3.34 (dd, J=8.5, 18.3Hz, 1H, one H of maleic CH_2), 3.58 (t, J=7.5Hz, 2H, CH_2), 3.89 (s, 9H, CH_3), 4.57 (d, J=5.0 Hz, 6H, CH_2), 4.99 (dd, J=4.1, 8.5 Hz, 1H

maleic CH), 7.28 (s, 3H, CH), 7.30 (brs, 1H, NH), 8.09 (t, J=5.6 Hz, 1H, NH), 8.92 (t, J=5.1 Hz, 3H, NH).

^{13}C NMR (DMSO-d_6, 100MHz): δ 20.56 (CH_3), 29.26, (CH_2), 29.98 (CH_2), 32.67 (CH_2), 34.71 (CH_2), 35.27 (CH_2), 35.58 (CH_2), 35.83 (CH_2), 39.00 (CH_2), 39.05 (CH_3), 49.82 (CH), 56.79, 112.73 (CH), 139.90, 142.69, 148.55, 159.59, 169.12, 170.08, 173.18, 173.36, 173.44.

ESI-MS: 956.27 $(M+1)^+$.

HRMS: Calcd for $C_{44}H_{59}ClN_9O_{13}$ $(M+1)^+$, 956.3921; Found, 956.3953.

Synthesis of compound 10:

Compound **30** (20 mg) in DCM (20 mL) was added excess Et_3N and the mixture was stirred at room temperature overnight. After evaporation to remove the solvent, the residue was loaded on a solid phase extraction column by dissolving in 1ml 0.1% formic acid solution. The column was firstly washed with 0.1% formic acid (10 mL) to remove triethylamine hydrochloride salt, followed by elution with methanol (10 mL). The fraction of the methanol solution was concentrated and dried by vacuum oven to yield a white powder (80%).

^{1}H NMR (CD_3OD, 400MHz): δ 1.94-2.03 (m, 6H, CH_2), 2.20-2.29 (m, 6H, CH_2), 2.32 (t, J=6.3 Hz, 2H, CH_2), 2.38 (s, 9H, CH_3), 2.42 (t, J=7.0 Hz, 2H, CH_2), 3.31 (q, J=6.7 Hz, 2H, CH_2), 3.66 (s, 9H, CH_3), 3.69 (t, J=7.1Hz, 2H, CH_2), 4.58 (s, 6H, CH_2), 5.25 (s, 3H, NH), 6.29 (s, 3H, CH), 6.81 (s, 2H, CH).

^{13}C NMR (CD_3OD, 100MHz): δ 21.03 (CH_3), 30.11, (CH_2), 31.48 (CH_2), 35.43 (CH_2), 35.69 (CH_2), 36.02 (CH_2), 37.21 (CH_3), 37.49 (CH_2), 59.17, 63.70 (CH_2), 114.58 (CH), 129.59, 132.25, 135.57 (CH), 147.38, 148.87, 171.12, 172.26, 172.98, 173.33, 175.89.

ESI-MS: 920.33 $(M+1)^+$.

HRMS: Calcd for $C_{44}H_{58}N_9O_{13}$ $(M+1)^+$, 920.4154; Found, 920.4146. (100 % rel. abundance).

Equipment and consumables

All chemicals were purchased from Sigma Aldrich and Acros Organics and were reagent grade or better. Solvents were purchased from Fisher Scientific, Sigma Aldrich and VWR. NMR solvents were purchased from GOSS Scientific and NMR tubes were manufactured by Wilmad. Silica gel for column chromatography were purchased from Merck. Solid phase extraction column was a Phenomenex Strata C18-E, 500 mg size, 3 mL volume.

Samples were dried in a vacuum oven (Gallenkamp, rated to ≤ 250°C) connected to a vacuum pump (BOC-Edwards.) Samples were hydrogenated on a Parr hydrogenator (room temperature) at 40 psi with automatic shaking. Infrared Spectra were acquired on a Perkin Elmer Spectrum one FTIR using KBr disks. The software used was called "Spectrum."

^{1}H-NMR spectra were acquired on a 400 MHz Ultra-sheild magnet (Bruker) and with XWin-NMR software. Spectra were analysed on MestReNova sotware version 5.3 (Mestrelab Research). Mass spectrometry samples were submitted to the EPSRC Mass Spectrometry Centre, Swansea or the Mass Spectrometry facility, King's College, London. Samples at EPSRC were run on a Waters ZQ4000 quadrupole mass spectrometer (ESI), a Finnigan MAT 95 XP high resolution double focusing mass spectrometer (EI, CI) or a Micromass Quattro II quadrupole mass spectrometer (EI). Samples at King's College were run on a Thermofisher LCQ DECA XP ion trap mass spectrometer. High resolution LC-MS was performed at the Division of Imaging Sciences, King's College, London on an Agilent 6520 Accurate-Mass Q-TOF LC/MS connected to an Agilent 1200 HPLC system with vacuum degasser, quaternary pump, variable wavelength detector and autosampler.

Isotopic distributions were calculated using Molecular Weight Calculator version 6.46. The resulting graph data were extracted and processed in Microsoft Excel 2003.

HPLC analysis of the CP256 ligand was performed on Agilent 1200 series HPLC with quadrupole pump, vacuum degasser, variable wavelength detector (14 µl standard loop, set to 254 nm) and manual syringe injector (Rheodyne 7725i). The HPLC column was an Agilent Eclipse XDB-C18 (5 µm, 4.6 mm X 150 mm) with a guard column. The acquisition and analysis software was Gina Star version 5.01 (Raytest). Flow rate was 1 ml/min.

Time (min)	**A (%)**	**B (%)**
0	95	5
2	95	5
15	5	95
20	5	95
25	95	5

Table S1. Gradient for HPLC analysis of CP256 ligand.

2. High resolution LC-MS analysis of CP256, Ga-CP256 and Fe-CP256

Results

Molecular formulas and calculated monoisotopic masses

CP256: $C_{36}H_{49}N_7O_{10}$ (M = 739.3541)

Ga-CP256: $C_{36}H_{46}N_7O_{10}Ga$ (M = 805.2562)

Fe-CP256: $C_{36}H_{46}N_7O_{10}[^{56}Fe]$ (M = 792.2656)

Sample	LC retention time (mins)	Species	m/z (calculated)	m/z (found)	Relative abundance (%)
CP256	5.1	$(M + H)^+$	740.3614	740.3618	10
		$(M + 2H)^{2+}$	370.6843	370.6858	100
Ga-CP256	5.3	$(M + H)^+$	806.2635	806.2635	70
		$(M + Na)^+$	828.2454	828.2444	25
		$(M + 2H)^{2+}$	403.6354	403.6366	100
		$(M + H + Na)^{2+}$	414.6263	414.6268	50
		$(M + 2Na)^{2+}$	425.6173	425.6186	25
Fe-CP256	5.4	$(M + H)^+$	793.2728	793.2741	55
		$(M + Na)^+$	815.2548	815.2559	15
		$(M + 2H)^{2+}$	397.1401	397.1424	100
		$(M + H + Na)^{2+}$	408.1310	408.1320	45
		$(M + 2Na)^{2+}$	419.1220	419.1230	22

Table S2. LC-MS results of CP256 and its Ga and Fe complexes.

Isotopic distribution modelling

Isotopic distribution for $(C_{36}H_{46}N_7O_{10}Ga + H)^+$	
m/z: Calculated/(found)	% Relative abundance: Calculated/(found)
806.2635/ (806.2635)	100.00/(100.00)
807.2635/ (807.2655)	42.59/(41.73)
808.2635/ (808.2634)	77.28/(76.99)
809.2635/ (809.2653)	30.34/(29.20)
810.2635/ (810.2670)	7.56/(7.36)
Isotopic distribution for $(C_{36}H_{49}N_7O_{10} + 2H)^{2+}$	
m/z: Calculated/(found)	% Relative abundance: Calculated/(found)
370.6843/ (370.6858)	100.00/(100)
371.1843/ (371.1869)	42.64/(51.38)
371.6843/ (371.6887)	10.93/(14.26)

Isotopic distribution for $(C_{36}H_{46}N_7O_{10}Fe + H)^+$	
m/z: Calculated/(found)	% Relative abundance: Calculated/(found)
791.2775/ (791.2779)	6.33/(5.47)
792.2775/ (792.2768)	2.69/(2.92)
793.2775/ (793.2741)	100/(100)
794.2775/ (794.2765)	44.72/(42.20)
795.2775/ (795.2782)	12.14/(10.88)

Table S3. Calculated and experimental isotopic distributions of Ga-CP256 and Fe-CP256 and the CP256 ligand.

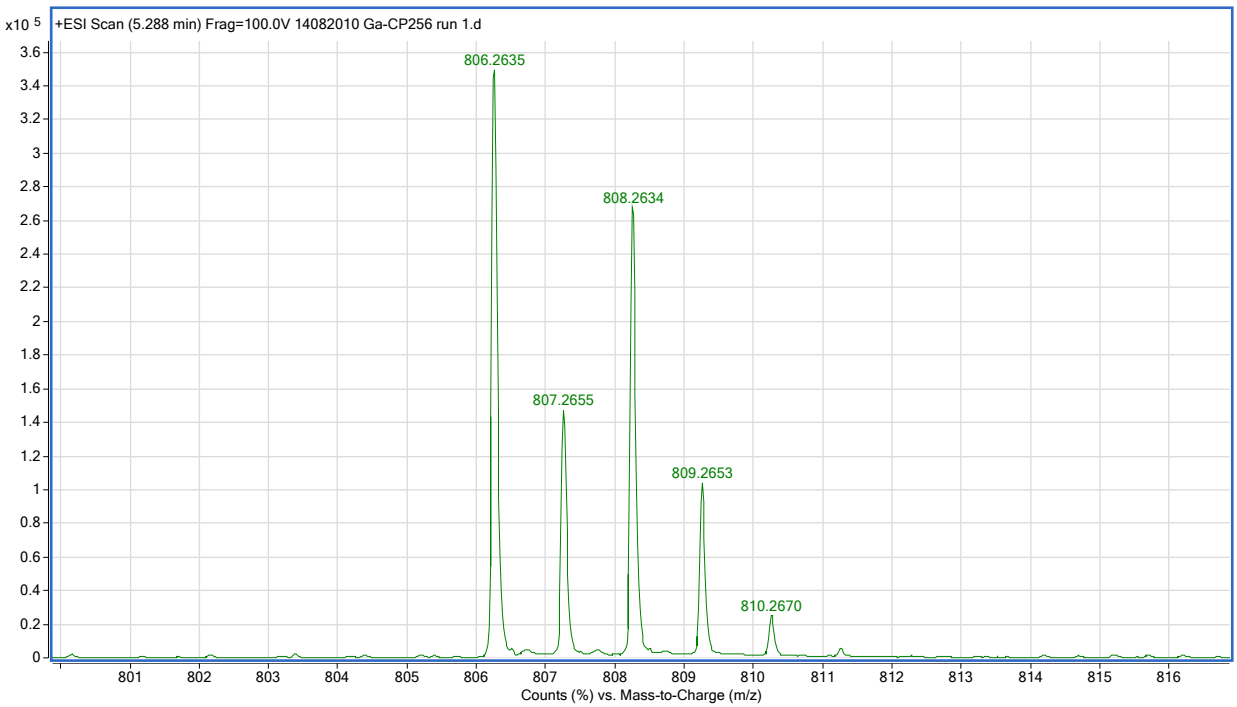


Figure S2. Mass spectrum showing $(M + H)^+$ where M = Ga-CP256

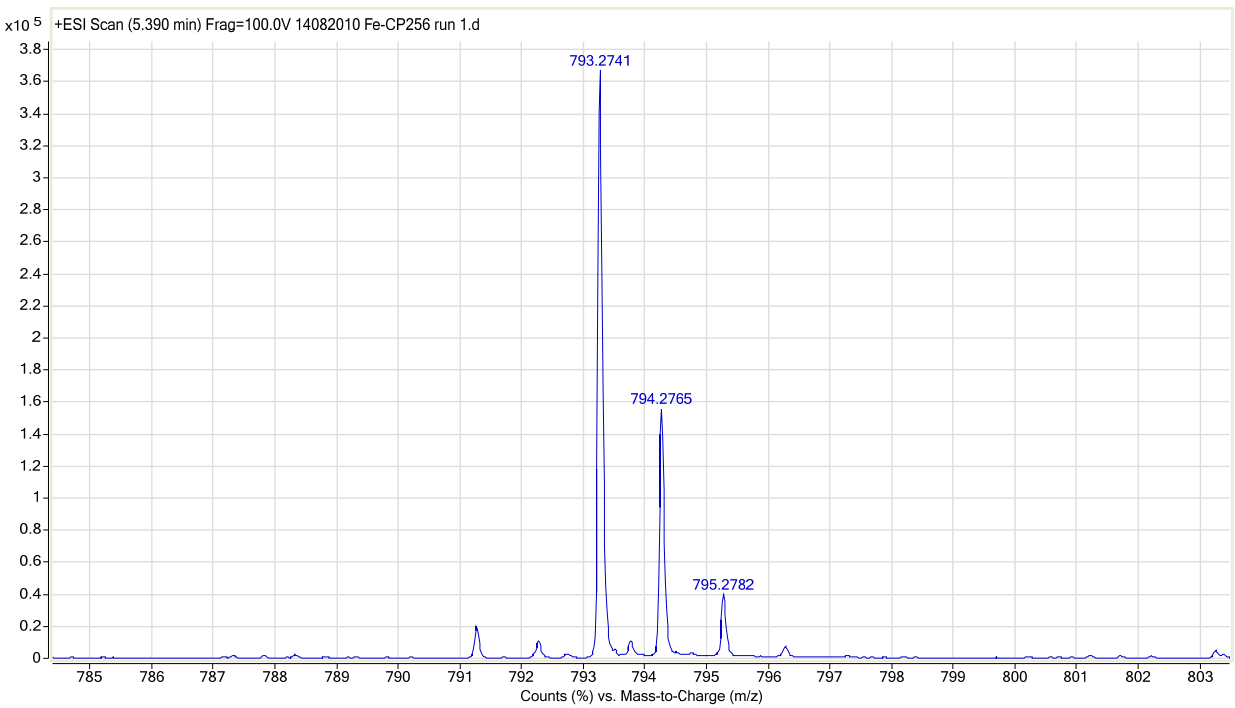


Figure S3. Mass spectrum showing $(M + H)^+$ where M = Fe-CP256

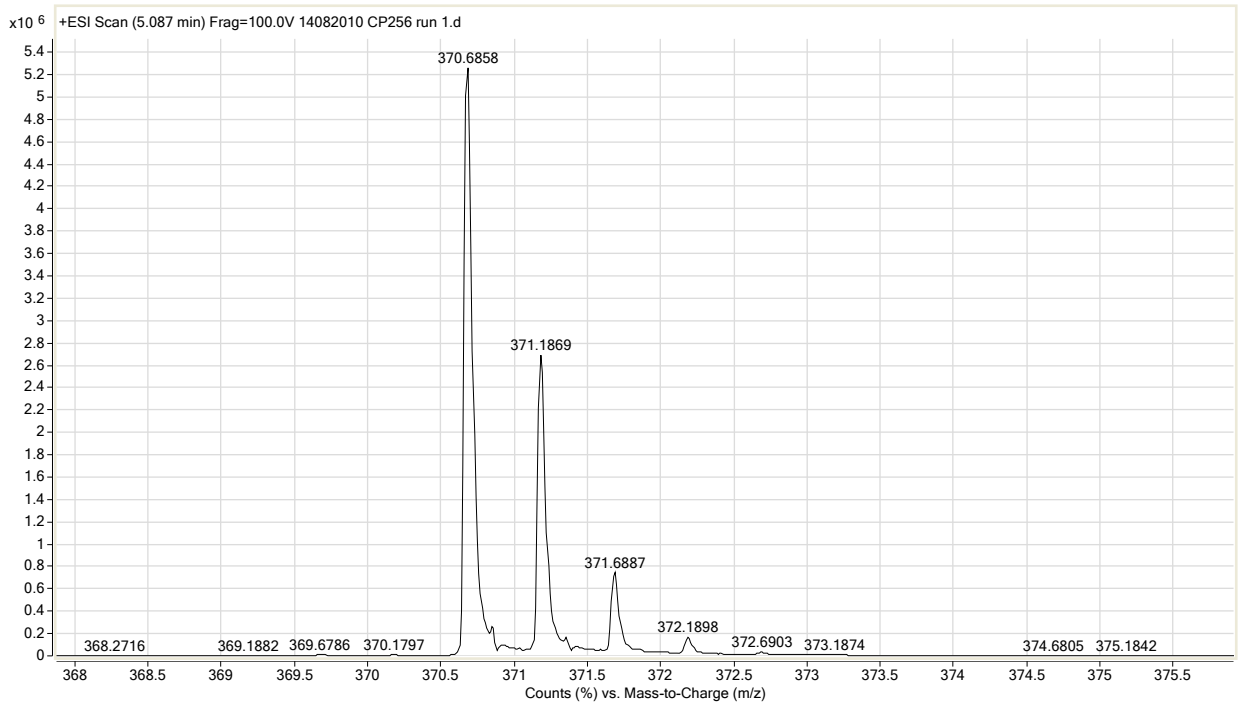


Figure S4. Mass spectrum showing $(M + 2H)^{2+}$ where M = CP256

Methods

Preparation of complexes:

Ga-CP256

1 mg CP256 was dissolved in 1 ml methanol to give a ligand concentration of 1.18 mM (based on tris HCl of CP256 with RMM of 848.) This solution was added to 0.3 mg $Ga(NO_3)_3$ (RMM 255, anhydrous basis) to give a 1:1 metal to ligand stoichiometry at a concentration of 1.18 mM.

Fe-CP256

The Fe^{3+} complex was produced in an analogous procedure to the Ga^{3+} complex, but with $Fe(NO_3)_3.9H_2O$ (RMM = 386) as the metal source.

Equipment and consumables

Samples were analysed by high resolution LC-MS. The HPLC system was an Agilent 1200 system with vacuum degasser, quaternary pump, variable wavelength detector (14 µl standard loop), and an autosampler. The mass spectrometer was an Agilent 6520 Accurate-Mass Q-TOF LC/MS. Samples were acquired using Agilent Masshunter workstation acquisition software (B.02.01) and

data was analysed using Agilent Masshunter Qualitative analysis software (B.02.00). The HPLC column used was an Agilent Zorbax SB C18 cartridge column (2.1mm X 30mm, 3.5 µm.) Following chromatography, the column eluant was split, with 90% passing to the VWD and 10% passing to the mass spectrometer. The gradient was operated at 0.5 ml/min and the mobile phase was a gradient using mass spectrometry grade reagents: A = H_2O + 0.1% formic acid (Fluka, 34673) and B = methanol + 0.1 % formic acid (Fluka, 34671). The gradient is given in Table S3. Spectra were acquired using electrospray ionisation in positive mode using reference mass correction with hexakis(1H,1H,3H-tetrafluoropropoxy)phosphazine (C_{18} H_{18} N_3 O_6 P_3 F_{24}, m/z = 922.0098 $[M + H]^+$). The cone voltage was 100V. Isotopic distributions were calculated using Molecular Weight Calculator version 6.46.

Time (min)	**A (%)**	**B (%)**
0	95	5
1	95	5
10	5	95
20	5	95
23	95	5
30	95	5

Table S4. Gradient for LC-MS analysis of CP256 and the Fe^{3+} and Ga^{3+} complexes.

3. HPLC of non-radioactive Ga-CP256 and ^{67}Ga-CP256 complexes.

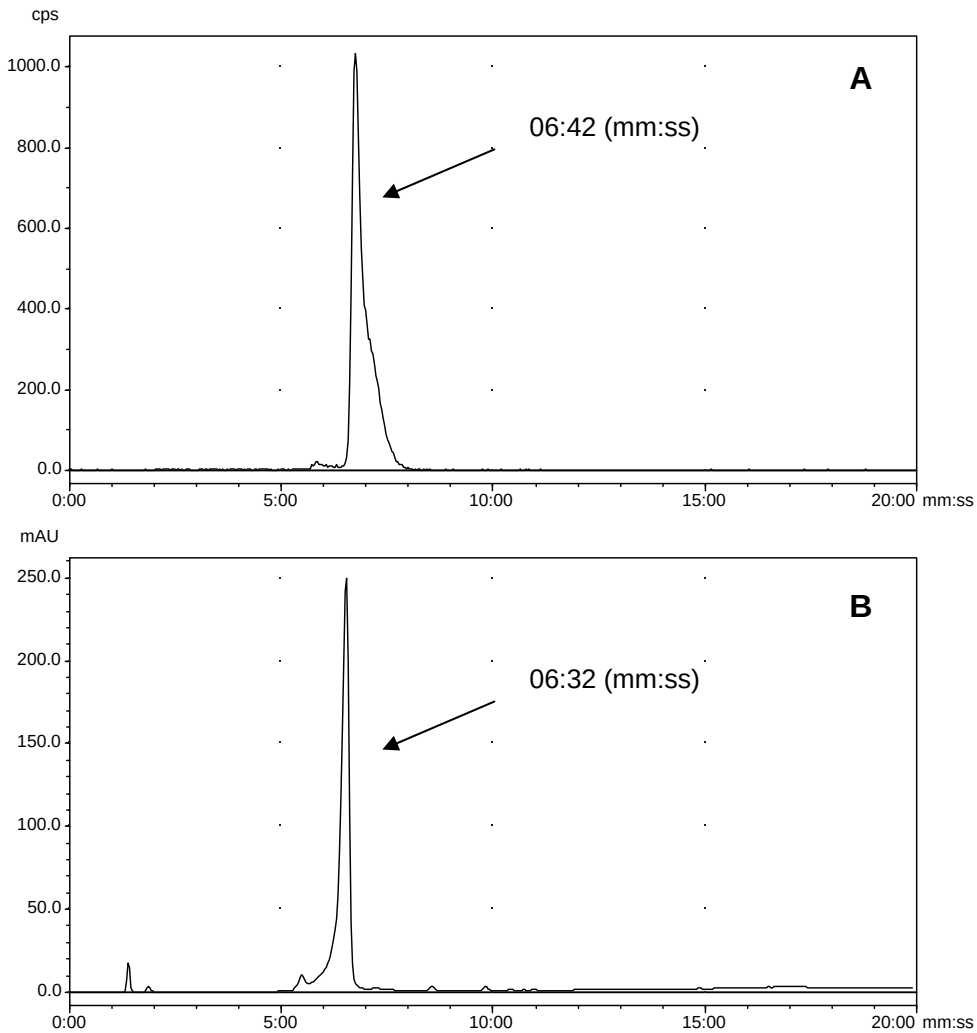


Figure S5. HPLC traces showing ^{67}Ga-CP256 complex (A) and non-radioactive Ga-CP256 complex (B) The slight difference in retention times is due to the length of tubing between UV and radioactivity detectors. (80 cm tube length, 0.5 mm OD, leading to a volume in the tubing of 0.16 ml and a time delay of ~ 9-10 seconds).

Preparation of Radioactive ^{67}Ga-CP256 complex for HPLC

A 0.1 mg/ml solution of CP256 in PBS was prepared. To 100 µl of this solution was added 10 µl (~1 MBq) ^{67}Ga-citrate solution. After 1 min 20 µl of this solution was withdrawn with an HPLC syringe and injected into the HPLC.

Equipment, gradient and conditions.

HPLC analysis was carried out on an Agilent 1200 series HPLC with quadrupole pump, vacuum degasser, variable wavelength detector (14 µl standard loop, set to 254 nm), gamma detector (Bioscan FC3600) and manual syringe injector (Rheodyne 7725i). The HPLC column used for all analyses was an Agilent Eclipse XDB-C18 (5 µm, 4.6 mm X 150 mm) with a guard column. The analytical software used for radio-TLC and radio-HPLC acquisition and analysis was Laura 4.0.2.75 (Lablogic).

Linear gradient of 10% B at 0 minutes to 90% B at 15 minutes. (A = 0.1% TFA in H_2O, B = 0.1% TFA in methanol.) The flow rate was 1 ml/min.

^{67}Ga for radiolabelling was ^{67}Ga-citrate solution for injection (Mallinckrodt Medical) 74 MBq/ml (at ref. date; contains 6.5 mM sodium citrate, 135 mM NaCl, 0.9% Benzyl alcohol)

4. Protocol for ^{68}Ga radiolabelling for titration studies

Results

Ligand conc.	Radiochemical yield (%)			
	DOTA	**HBED**	**p-SCN-Bn-NOTA**	**CP256**
1 mM	99.0	99.6	99.2	100.0
100 µM	100.0	99.7	99.3	100.0
10 µM	95.3	95.9	80.1	98.3
1 µM	7.4	35.8	3.7	72.9
100 nM	0.4	8.1	0.3	8.3

Table S5. Radiolabelling yields (determined by ITLC) for ^{68}Ga-complexes.

Methods

Preparation of ligands.

1 mM solutions of DOTA, CP256, HBED and p-SCN-Bn-NOTA were prepared in H_2O. These solutions were successively diluted tenfold from the 1 mM solutions to produce solutions with concentrations of 100 µM, 10 µM, 1 µM and 100 nM. 100 µl aliquots of each of these 5 solutions (including the 1 mM solution) were placed into separate 1 ml plastic tubes. The samples were then lyophilised, resulting in 5 tubes for each ligand containing between 10 pmol and 100 nmol ligand.

Purification of ^{68}Ga from ^{68}Ge/^{68}Ga generator.

The ^{68}Ge/^{68}Ga generator was eluted with 6 ml 0.1 N HCl, and the 2 ml containing the peak radioactivity was taken for purification, as follows.

A SAX column (Chromafix, Macherey Nagel) was equilibrated with 2 ml H_2O and dried with 10 ml air through a syringe. To 2 ml of the generator eluate (60 MBq in 0.1 ml HCl) was added 1.5 ml of a 36% solution of HCl to give a ~4 M solution with regard to HCl. The 3.5 ml eluate was loaded onto the SAX column at a rate of 2 ml/min and the column dried with 10 ml air. 100% of the radioactivity was retained by the column. The column was washed with 300 µl of 5 M NaCl

solution, with 3 MBq (5%) of the total activity eluted from the column. The column was then eluted with 250 µl H_2O. 28 MBq (45%) of the radioactivity eluted and the solution pH was ~3.

Buffering of purified ^{68}Ga for radiolabelling

0.2 M acetate buffers of varying pH were produced by mixing 0.2 M sodium acetate and 0.2 M acetic acid solutions for radiolabelling the different chelators. Buffer preparation is shown in Table S6.

Ligand	**pH required**	**% 0.2 M sodium acetate (vol)**	**% 0.2 M acetic acid (vol)**
DOTA	~ 4.6	49	51
HBED	~ 4.6	49	51
p-SCN-Bn-NOTA	~ 3.6	8	92
CP256	~ 7	100 (pH 7.7)	0

Table S6. Details for producing acetate buffers of varying pH.

Once the relevant pH acetate buffer had been prepared for each ligand a 500 µl aliquot was added to 50 µl of the purified ^{68}Ga (~5.6 MBq). 100 µl aliquots of the radiolabelled buffer were then added to each of the 5 tubes containing between 10 pmol and 100 nmol for each ligand to give final solutions radiolabelled with ~1 MBq in 100 µl with concentrations between 100 nmol and 1 mM. Radiolabelling conditions are given in Table S7.

Ligand	**Temperature**	**Radiolabelling time (mins)**	**Measured solution pH**
DOTA	100°C	30	4.4
HBED	21°C	10	4.2-4.4
p-SCN-Bn-NOTA	21°C	10	3-3.5
CP256	21°C	5	6.5

Table S7. Radiolabelling conditions for ^{68}Ga-complexes.

Once radiolabelling was completed, 3 µl of the radiolabelling solution was taken from each tube and spotted onto separate ITLC strips (90 X 10 mm, origin 15mm). These strips were then developed simultaneously in separate TLC tanks (15 ml centrifuge tubes containing 1.6 ml mobile phase). Once the mobile phase had reached ~80 mm, the strips were removed, allowed to dry at room temperature and imaged using autoradiography. ITLC conditions are given in Table S8.

Ligand	**Stationary Phase**	**Mobile Phase**	**R_f (Complex)**	**R_f (unchelated ^{68}Ga)**
DOTA	ITLC-SG	Phosphate	0.7-1	0-0.2
HBED	ITLC-SG	Phosphate	0.8-1	0-0.2
NOTA	ITLC-SG	Phosphate	0.6	0-0.2
CP256	ITLC-SA	Citrate	0	1

Table S8. ITLC conditions for ^{68}Ga-complexes.

Phosphate mobile phase was 0.4 M sodium phosphate (monobasic) pH 4.
Citrate mobile phase was made up from a combination of 0.1 M trisodium citrate and 0.1 M citric acid to make a buffer at pH 5.

For the CP256 incubations, to determine that the species at R_f 0 was ^{68}Ga-CP256 and not precipitated ^{68}Ga, a standard of ^{68}Ga in the pH 7 acetate buffer was run on ITLC-SA in the 0.1 M citrate buffer system. ^{68}Ga migrated to the solvent front with only 2.5 % of the total radioactivity remaining at the baseline.

Equipment and consumables

Radioactive samples were measured using a CRC-25R dose calibrator (Capintec). ITLC analyses were carried out using autoradiography, which was performed on a Perkin Elmer Cyclone Plus Phosphorimager System. Samples were developed in an autoradiography cassette on a 12.5 X 25.2 cm film. The results were analysed using Optiquant version 5 (2006).
All general reagents and consumables were purchased from Sigma-Aldrich or from Fisher Scientific. ITLC-SG and ITLC-SA strips were purchased from Varian. p-SCN-Bn-NOTA was purchased from Macrocyclics (Dallas, USA), HBED was purchased from Strem Chemicals and

DOTA was purchased from Sigma Aldrich. 5 M NaCl solution was purchased from Sigma Aldrich. SAX cartridges (SAX SPEC, Chromafix 30-PS-HCO_3, 45mg) were purchased from Macherey-Nagel, Germany. $^{68}Ge/^{68}Ga$ generator was an Eckert & Ziegler IGG100, 1.1 GBq nominal activity. The pH of buffers was determined using a pH 0-14 glass electrode (Denver Instruments) connected to a Denver Ultrabasic pH meter. The pH meter was calibrated using mercury free buffer solutions, pH 4.00, 7.00 and 10.00 (Reagecon). The pH of radioactive samples was determined using pH strips with ranges of 0-14 (Fisher), 3.8-5.5 (Fluka) and 6.0-8.1 (Fluka).

5. Protocol for ^{67}Ga radiolabelling for titration studies

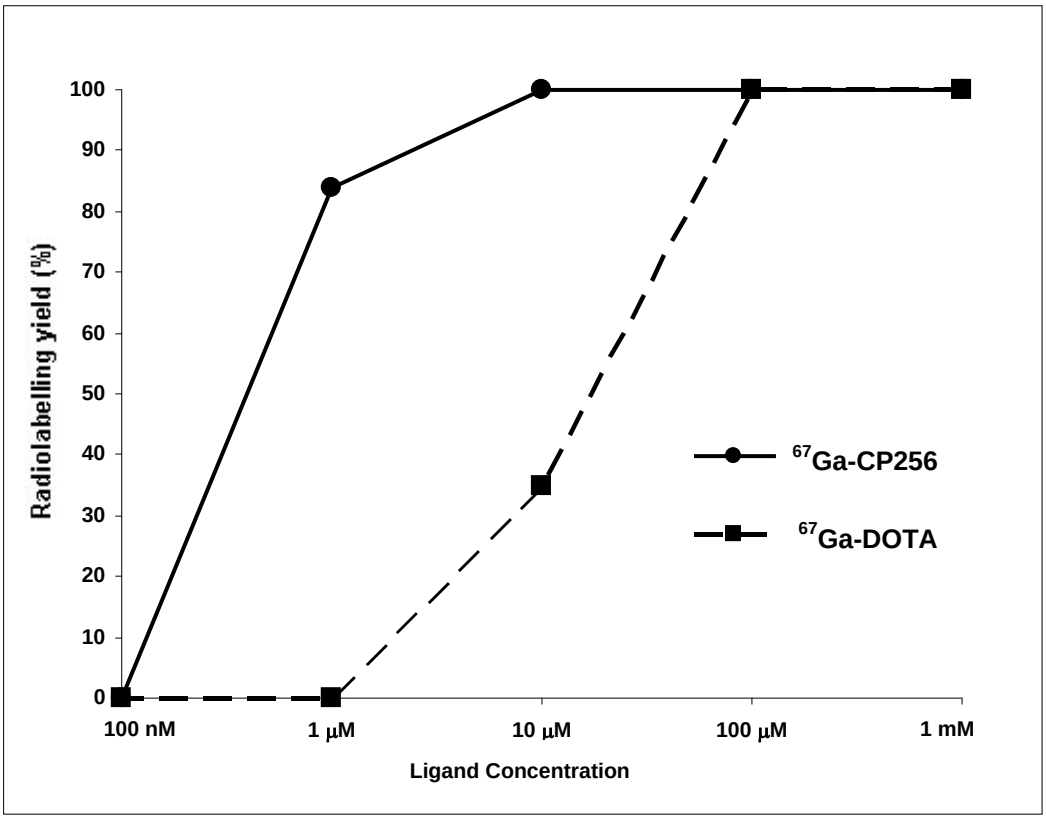


Figure S6. Results from ^{67}Ga labelling of CP256 and DOTA. Results are comparable with those of the ^{68}Ga experiment in the main communication.

Ligand Concentration	Radiotracer labelling yield	
	CP256	DOTA
1 mM	100	100
100 µM	100	100
10 µM	100	35
1 µM	84	0
100 nM	0	0

Table S9. Radiolabelling yields for ^{67}Ga-DOTA and ^{67}Ga-CP256 in tabulated form.

Complex	Stationary Phase	R_f (Complex)	R_f (unchelated ^{67}Ga)
^{67}Ga-DOTA	Silica gel	0.4	0 & 0.8-1
^{67}Ga-CP256	Silica gel	0	1

Table S10. TLC results for radiolabelling experiments with ^{67}Ga-citrate. A standard of ^{67}Ga-citrate using the same TLC system as for ^{67}Ga-DOTA showed ^{67}Ga-citrate to have Rf values of 0 & 0.8-1.

Methods

Radiolabelling

1.25 mM stock solutions of each ligand were prepared in PBS (CP256) or acetate buffer, pH 4.6 (DOTA) The stock solution was diluted 10 fold by successive addition of the radiolabelling buffer to create extra solutions with ligand concentrations of 125 μM, 12.5 μM, 1.25 μM and 125 nM. Eighty microlitres of each solution was added to a plastic microcentrifuge tube and radiolabelled with 20 μl of ^{67}Ga-Citrate (1.5 MBq) to produce radiolabelled solutions with final ligand concentrations of 1 mM, 100 μM, 10 μM, 1 μM and 100 nM. A metal heater block (containing oil in the wells) was used to heat the ^{67}Ga-DOTA solution.

Ligand	Labelling buffer	Labelling temperature	Labelling time	Measured pH
DOTA	Acetate	80°C	45 min	4.6
CP256	PBS	25°C	1 min	6.6

Table S11. Radiolabelling conditions for ^{67}Ga-DOTA and ^{67}Ga-CP256.

TLC analysis

Silica TLC plates were spotted with 1 μl of radiolabelled complex at the origin, which was marked at 5 mm in pencil. Spots were allowed to dry on silica gel TLC plates but not for ITLC-SG strips. The plates were placed in a developing chamber (100 ml wide necked sample bottle) filled with a 3 mm depth of the mobile phase. Stationary phases and mobile phases are listed in Table S12. The TLC plate was developed until the solvent front had reached a distance of up to 65 mm. The plates

were removed from the developing chamber and dried in an oven set at 80°C. Radiochemical yields were determined using either a TLC scanner or by electronic autoradiography.

Radiotracer	**Mobile phase**	**Stationary phase**
^{67}Ga-CP256	Methanol/citric acid (0.5 M)/ammonium formate (10% w, v) [40/20/40]	Silica gel
^{67}Ga-DOTA ^{67}Ga-citrate	Sodium citrate (aq) [0.3 M]	Silica gel

Table S12. TLC conditions for ^{67}Ga-DOTA and ^{67}Ga-CP256.

Equipment and consumables

TLC analyses were carried out on a Mini-Scan TLC scanner with FC3600 detector and γ detector probe (laura 4.0.2.75 software) or on a Packard Instant Imager (electronic autoradiography) with Imager version 2.05 software. The gas mixture was 1% isobutane, 2.5% carbon dioxide and 96.5% argon (Air products).

Acetate buffer solution pH 4.6 (Sigma Aldrich 31048-1L).

6. Measuring stability in human serum

Results

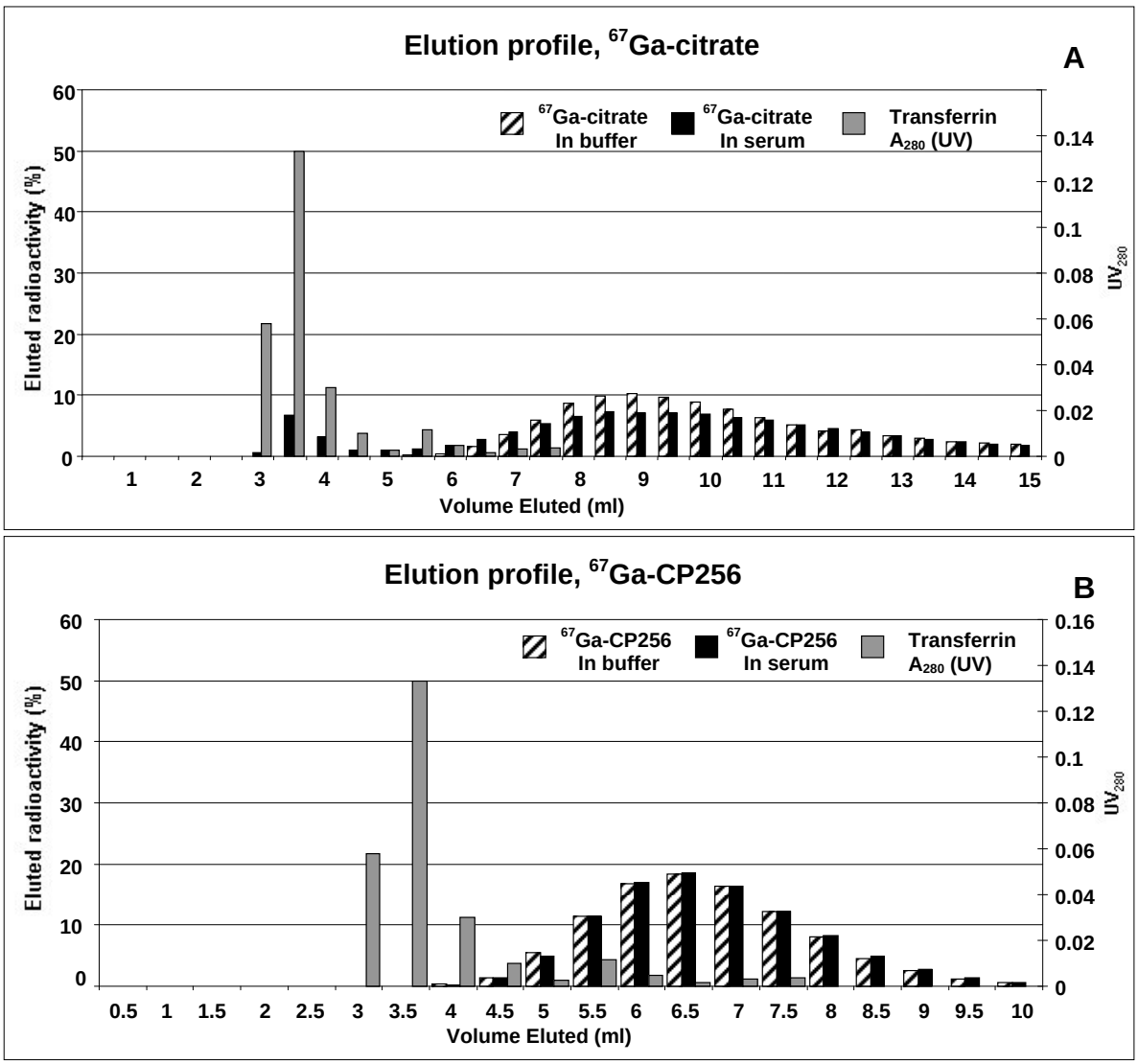


Figure S7. Gel filtration on PD10 column. Elution profile of ^{67}Ga-citrate complex (A) after incubation with human serum after 4 hours and (B) ^{67}Ga-CP256 after incubation with human serum for 4 hours. The UV elution profile of apo-transferrin is also shown.

Methods

Radiolabelling of ^{67}Ga-CP256 for serum stability

A solution of 0.1 mg/ml CP256 was prepared in PBS. An aliquot of 10 µl ^{67}Ga-citrate was added to 100 µl ligand. After 5 minutes, QC was performed by HPLC using the same gradient system as for the non-radioactive Ga-CP256 complex.

Incubation of tracers.

Two 200 µl aliquots of human serum were incubated at 37^{o}C in a metal heater block. To one serum incubation was added 20 µl of ^{67}Ga-CP256 and to the second incubation was added ^{67}Ga-citrate as a standard.

Aliquots of 20 µl of both tracers were incubated at 37^{o}C in 200 µl PBS buffer as a reference.

Gel filtration using a PD10 column

For ^{67}Ga-CP256: PD10 columns were equilibrated with 25 ml PBS buffer. After 4 hours, 20 µl of ^{67}Ga-CP256 (incubated in serum) was added to the top of the column, which was then eluted with PBS buffer. The PD10 columns were eluted with 15 ml in 30 X 0.5 ml fractions, collected in plastic microcentrifuge tubes. All tubes were counted in a gamma counter. The standard elution profile of ^{67}Ga-CP256 (in PBS buffer) from a PD10 column was determined by loading 20 µl of the ^{67}Ga-CP256 stock onto a PD10 column. Elution and gamma counting was the same as for the incubation in serum.

This protocol was repeated for the ^{67}Ga-citrate incubation.

Equipment and consumables

TLC and ITLC analyses were carried out on a Mini-Scan TLC scanner with FC3600 detector and γ detector probe. The same probe and detector from the same system was also connected to the HPLC system, an Agilent 1200 series with quadrupole pump, degasser, UV detector and manual syringe injector, which was fitted with a 20 µl loop. The HPLC column used for all analyses was an Agilent Eclipse XDB-C18 (5 µm, 4.6 mm X 150 mm) with a guard column. The analytical software used for radio-TLC and radio-HPLC was Laura 4.0.2.75 (Lablogic). Radioactive samples were counted for 10 seconds (window 101-110) on a 1282 Compugamma Gamma Counter (LKB Wallac) using Ultroterm software. UV spectrophotometry of apo-transferrin was performed on a

Cary UV spectrophotometer with Cary WinUV software. The wavelength was set at 280 nm. The dose calibrator used for measuring radioactivity in samples was a CRC-25R (Capintec).

All general reagents and consumables were purchased from Sigma-Aldrich or from Fisher Scientific. Specialist chemicals and consumables were purchased as follows: Acetate buffer solution pH 4.6 (Sigma Aldrich 31048-1L); human serum (Sigma Aldrich H4522); human apo-transferrin (Sigma Aldrich T5391-10MG); Sartorius Vivaspin 500 30 kDa MWCO filters (Fisher Scientific FDP-875-025B); PD10 columns (GE Healthcare 17-0851-01); ^{67}Ga-citrate solution for injection (Mallinckrodt Medical) 74 MBq/ml (at ref. date; contains 6.5 mM sodium citrate, 135 mM NaCl, 0.9% Benzyl alcohol); glass TLC plates (silica gel 60, F_{254}, 250 mm X 750 mm) (Merck); ITLC-SG strips (Pall Sciences). p-SCN-Bn-NOTA was purchased from Macrocyclics (Dallas, USA.)

7. Measuring stability in human apo-transferrin solution:

Results

Size exclusion chromatography

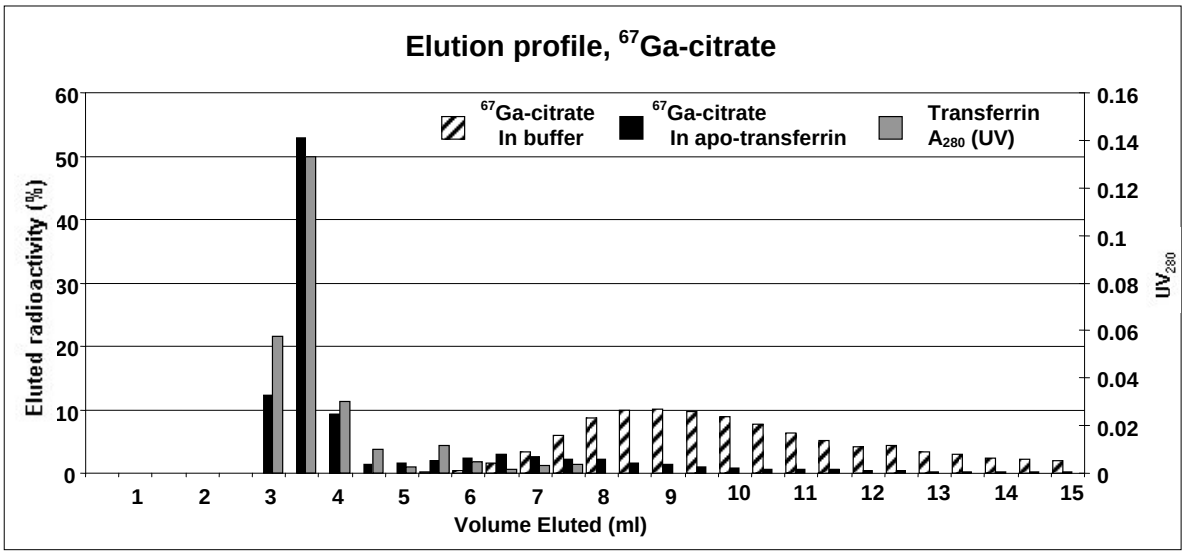


Figure S8. Gel filtration on PD10 column. (A) Elution profile of ^{67}Ga-citrate complex after incubation with apo-transferrin after 4 hours incubation. The UV elution profile of apo-transferrin is also shown.

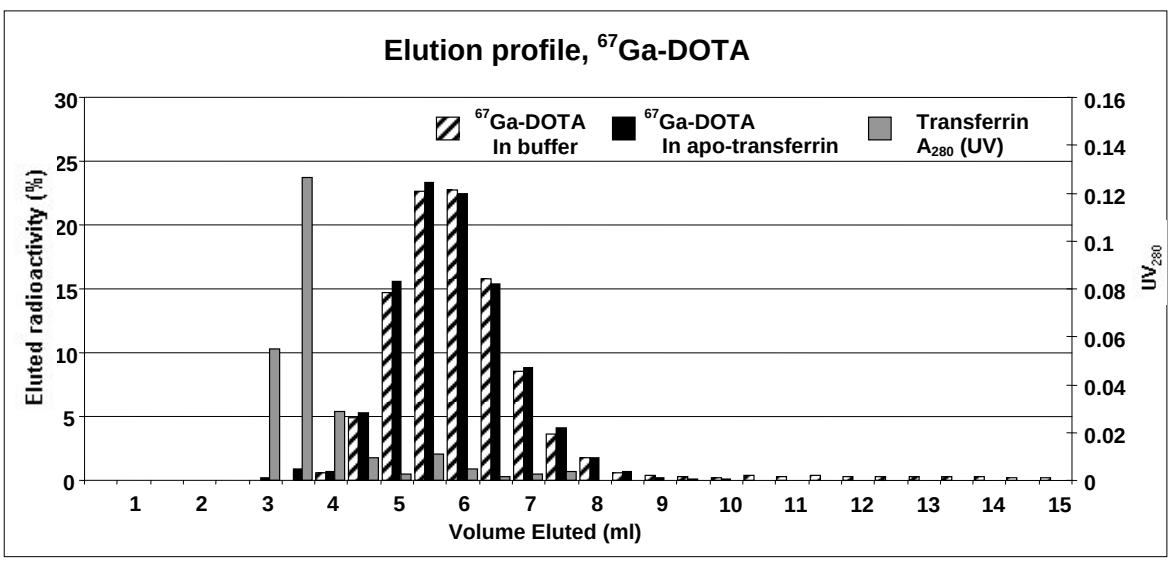


Figure S9. Gel filtration elution profile of ^{67}Ga-DOTA complex after 4 hours incubation in apo-transferrin solution; 100 μM DOTA radiolabelling concentration.

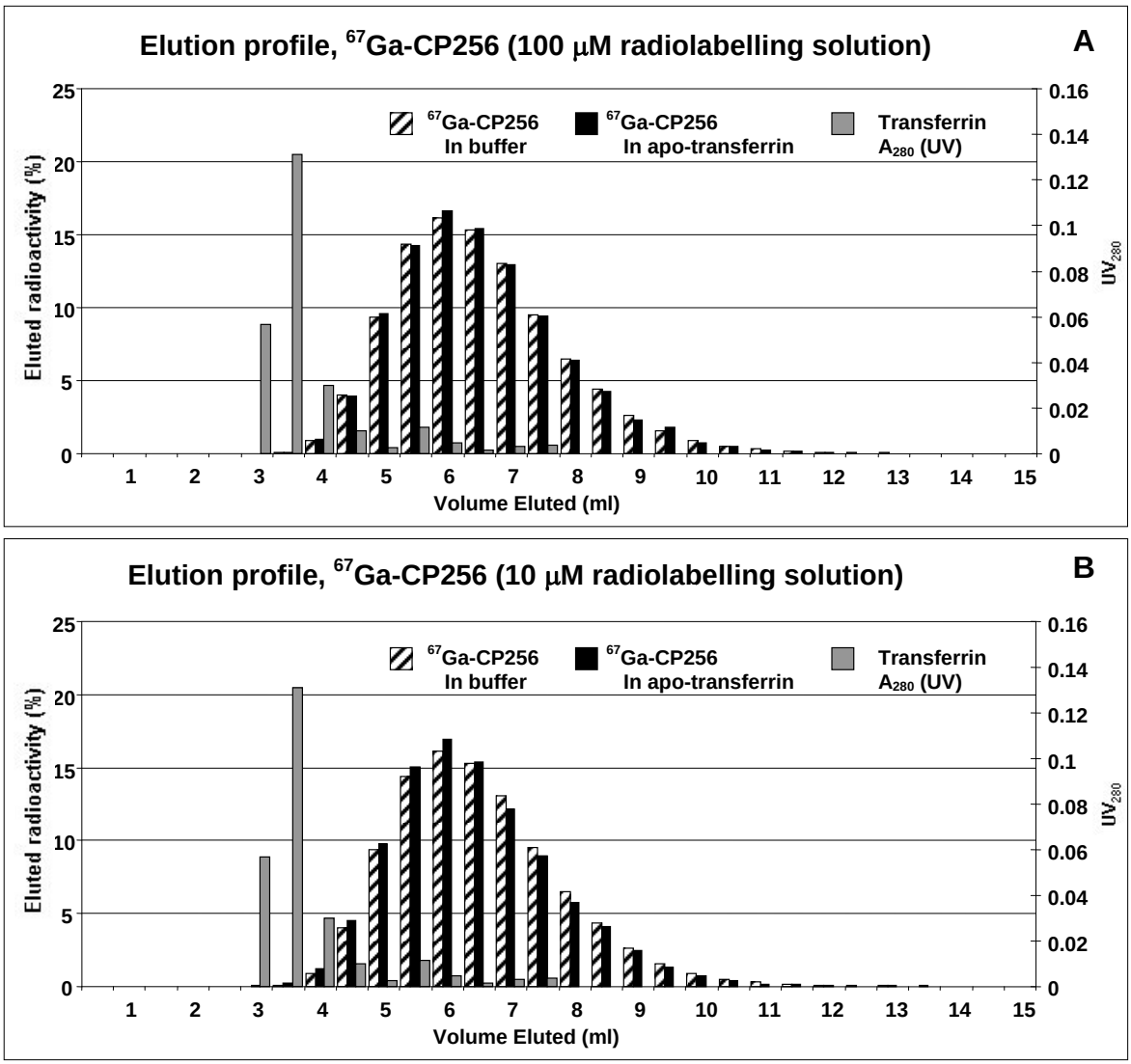


Figure S10. Gel filtration on PD10 column. Elution profile of ^{67}Ga-CP256 complex after 4 hours incubation in apo-transferrin solution (A) 100 μM radiolabelling solution (B) 10 μM radiolabelling solution.

Measuring stability using 30 KDa MWCO filters

Complex	% Radioactivity retained by 30 KDa MWCO filter	
	In reference buffer (after 4 hours)	**In apo-transferrin solution (after 4 hours)**
^{67}Ga-citrate (6.5 mM)	1.7 ± 0.5	73.0 ± 4.1
^{67}Ga-CP256 (10 μM)	3.5 ± 1.3	3.9 ± 0.8
^{67}Ga-CP256 (100 μM)	4.9 ± 0.5	3.8 ± 0.5
^{67}Ga-DOTA (100 μM)	1.5 ± 1.7	2.6 ± 0.8

Table S13. Retention of ^{68}Ga-complexes on MWCO filters in apo-transferrin vs control samples in buffer.

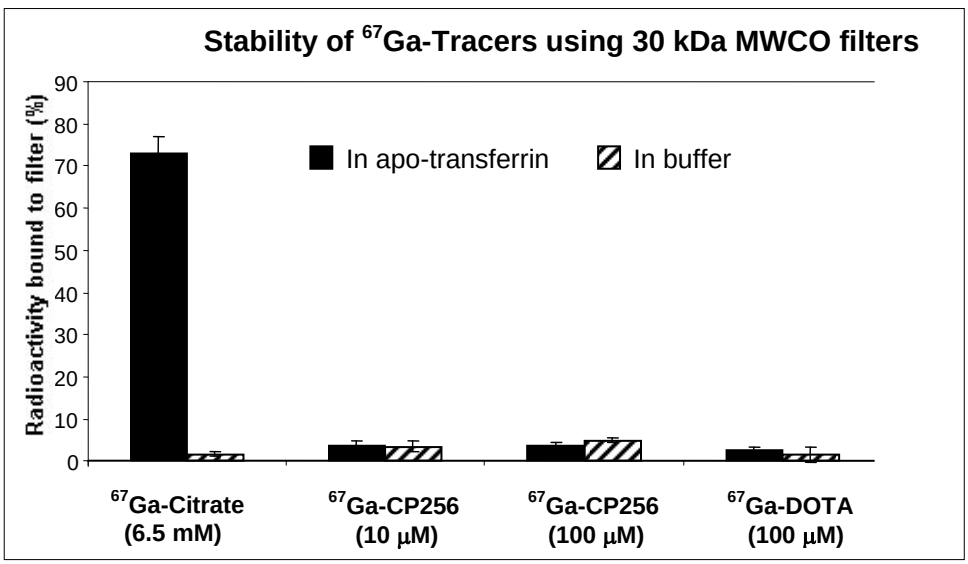


Figure S11. Binding of ^{67}Ga-citrate, ^{67}Ga-CP256 and ^{67}Ga-DOTA to 30 kDa MWCO filters. (Graphical representation of results from Table S8.)

Methods

Preparation of apo-transferrin solution

50 mM TRIS-HCl buffer (pH 7.5) was prepared by dilution of a 1 M stock solution. Sodium bicarbonate was added to a concentration of 25 mM. A 2.5 mg/ml (32 μM) solution of apo-transferrin was prepared in this buffer.

Preparation of ^{67}Ga-tracers

Solutions of ^{67}Ga-CP256 and ^{67}Ga-DOTA were radiolabelled at ligand concentrations of 100 μM. A 10 μM CP256 solution was also prepared for Radiolabelling conditions can be found in Table S14.

A 10 μl aliquot of each radiolabelled tracer was added to 30 μl of an appropriate buffer to neutralise the pH to between 7 and 7.5 and . This buffer was 2 M TRIS-HCl (pH 7.5) for DOTA. For preparations of ^{67}Ga-CP256 and ^{67}Ga-citrate the buffer was PBS. Details can be found in Table S15.

Ligand	Labelling buffer	Labelling temperature	Labelling time	Measured pH
DOTA	Acetate	80°C	40 min	4.6
CP256	PBS	25°C	1 min	6.6

Table S14. Radiolabelling conditions for ^{67}Ga-complexes used for stability measurements.

Ligand	Ligand conc. after radiolabelling	Radiolabelling buffer	Dilution buffer	Final ligand conc.
DOTA	100 μM	Acetate	TRIS	25 μM
CP256	100 μM	PBS	PBS	25 μM
	10 μM	PBS	PBS	2.5 μM

Table S15. Dilution and neutralisation conditions for radiolabelled complexes.

For tracer incubations in apo-transferrin

Equilibration and elution of PD10 columns was the same as for the serum incubations with the exception that 25 mM sodium bicarbonate was added to the PBS elution buffer. The standard elution profiles of ^{67}Ga-citrate, ^{67}Ga-CP256 and ^{67}Ga-DOTA were determined by adding 20 µl of each tracer that had been incubated in the reference buffer (50 mM TRIS-HCl containing 25 mM sodium bicarbonate) and eluting the columns as described above.

To calculate the percentage of total eluted activity for each 0.5 ml fraction

Total eluted radioactivity for each column was calculated by adding together the counts for each of the 30 fractions. Eluted activity for each fraction was then calculated by the following equation:

$$\frac{\text{Counts per fraction}}{\text{Total counts (for all 30 fractions)}} \times 100$$

Determination of tracer stability using 30 kDa MWCO microcentrifuge filters

PBS (100 µl, containing 25 mM bicarbonate) was applied to the top section (filter section) of the tube. The incubation solution (5 µl) for each tracer (after 4 hours) was added and the tube was spun in a microcentrifuge at 10,000 g for 8 minutes. Another 100 µl PBS was applied to wash and the tube was spun again at 10,000 g for a further 8 minutes. The filter section of the tube was removed and counted separately from the tube in a gamma counter. Percentage binding of the radioactivity to the MWCO filter was calculated using the following equation:

$$\frac{\text{Counts from filter section}}{\text{Counts from filter section + counts from tube section}} \times 100$$

The analysis was done in triplicate after 4 hours for the apo-transferrin incubations. The analysis was done 6 times (6 tubes) for the incubations in the 50 mM TRIS-HCl reference buffer. Average values were taken as described above.

Equipment and consumables.

The equipment and consumables were the same as for part 6, with the following additions:

Samples were centrifuged using an Eppendorf centrifuge 5424 microcentrifuge.

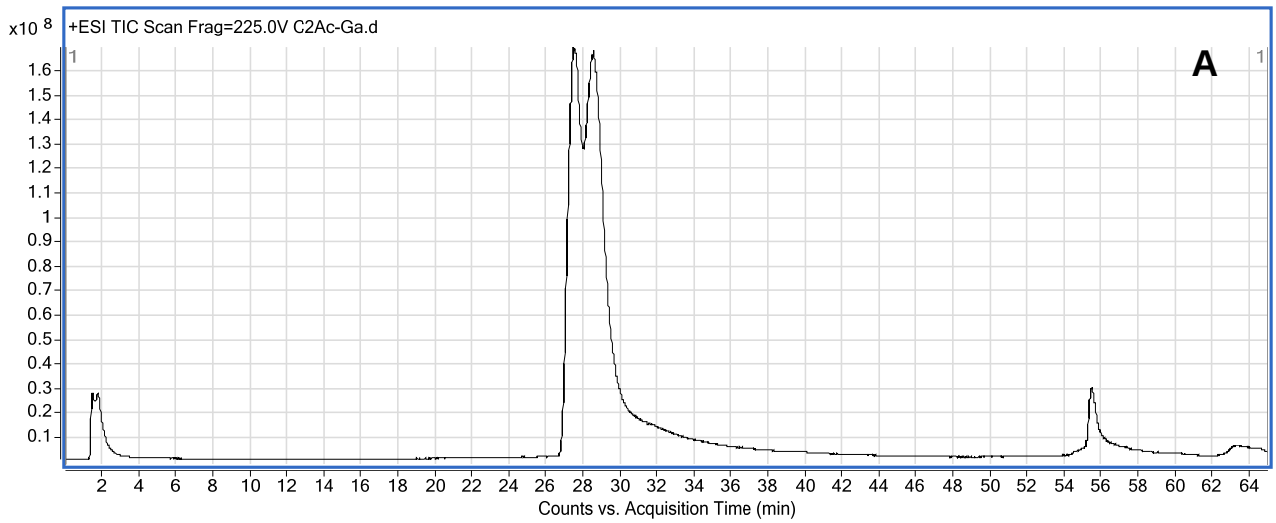
Sartorius Vivaspin 500 30 kDa MWCO filters were purchased from Fisher Scientific (FDP-875-025B).

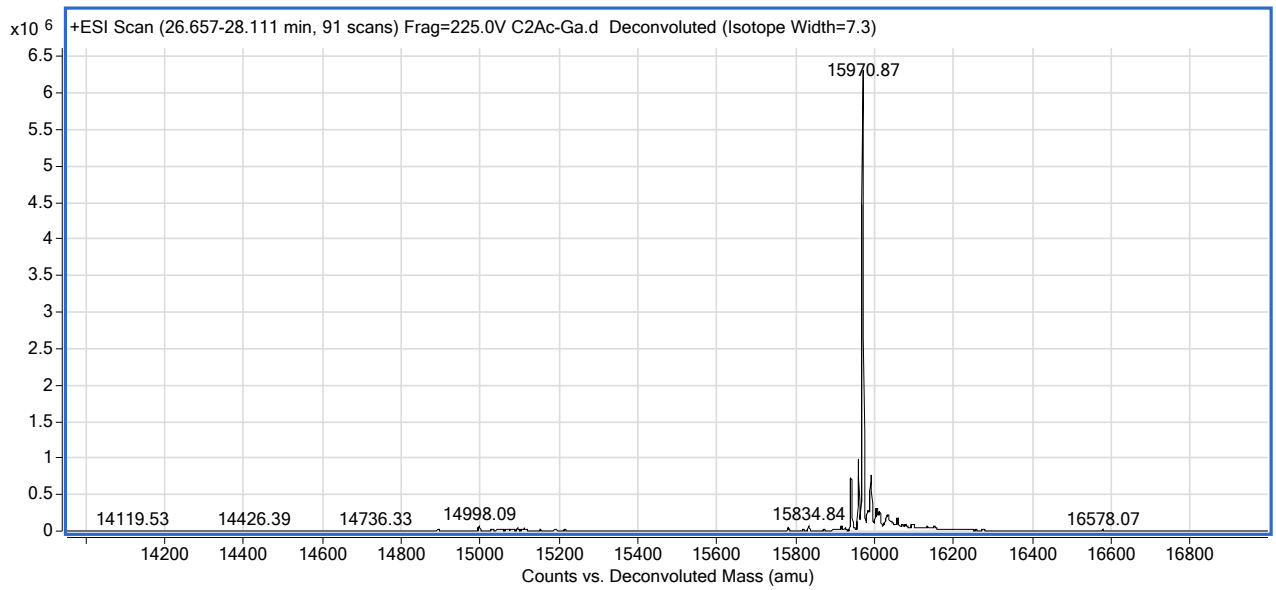
8. Conjugation of YM-103-chlorosuccinimide or YM-103 with C2Ac protein, radiolabelling and in vitro functional assay.

Results

Conjugation

C2A is a small protein that recognises phosphatidylserine displayed on cells undergoing apoptosis. It has been engineered with a cysteine residue to facilitate site-specific labelling with thiol-reactive agents. [5] The protein was conjugated with CP256 using either YM-103 or the chlorosuccinimide analogue (11) (see figure S16). The same product was obtained for both conjugation reactions. The average molecular weight of C2A is 14997. The found average molecular weight of the C2Ac-YM-103 conjugate was 15970.87, which corresponds to the C2Ac-YM-103 conjugate with an additional iron atom and without 3 hydrogen atoms, as shown in figure S12. C2Ac and the C2Ac-YM-103 conjugate elute during LC-MS with two peaks between 26.6 and 30 minutes. The first peak, extracted as a mass spectrum between 26.6 and 28.1 minutes (figure S12, A) contains the C2Ac-YM-103 conjugate (figure S12, B) and the second, between 28.1 and 29.6 minutes (figure S12, C) contains a mixture of the C2Ac-YM-103 conjugate and unmodified C2Ac (with a mass of 14997.40).


x10 8
+ESI TIC Scan Frag=225.0V C2Ac-Ga.d
A
Counts vs. Acquisition Time (min)


x10 6
+ESI Scan (26.657-28.111 min, 91 scans) Frag=225.0V C2Ac-Ga.d Deconvoluted (Isotope Width=7.3)
15970.87
14119.53
14426.39
14736.33
14998.09
15834.84
16578.07
Counts vs. Deconvoluted Mass (amu)

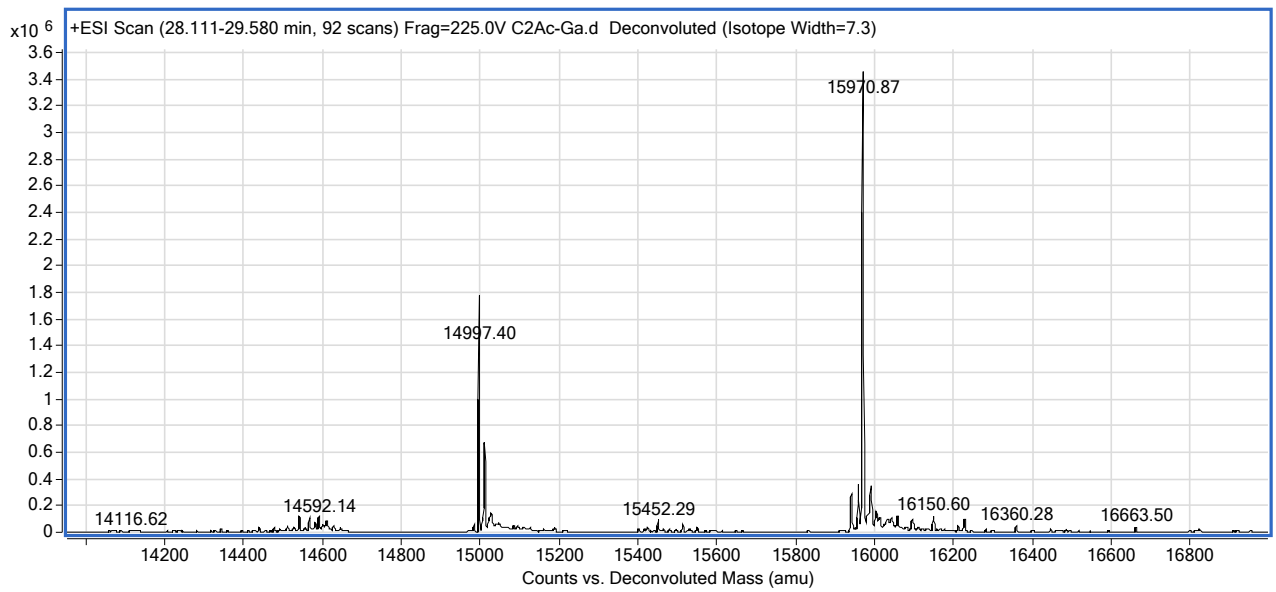

x10 6
+ESI Scan (28.111-29.580 min, 92 scans) Frag=225.0V C2Ac-Ga.d Deconvoluted (Isotope Width=7.3)
15970.87
14997.40
14116.62
14592.14
15452.29
16150.60
16360.28
16663.50
Counts vs. Deconvoluted Mass (amu)

Figure S12. Positive mode ESI mass spectrum showing (A) total ion chromatogram, (B) deconvoluted mass spectrum from 26.6-28.1 minutes containing C2Ac-YM-103, (C) deconvoluted mass spectrum from 28.1-29.6 minutes containing a mixture of C2Ac and C2Ac-YM103.

Radiolabelling

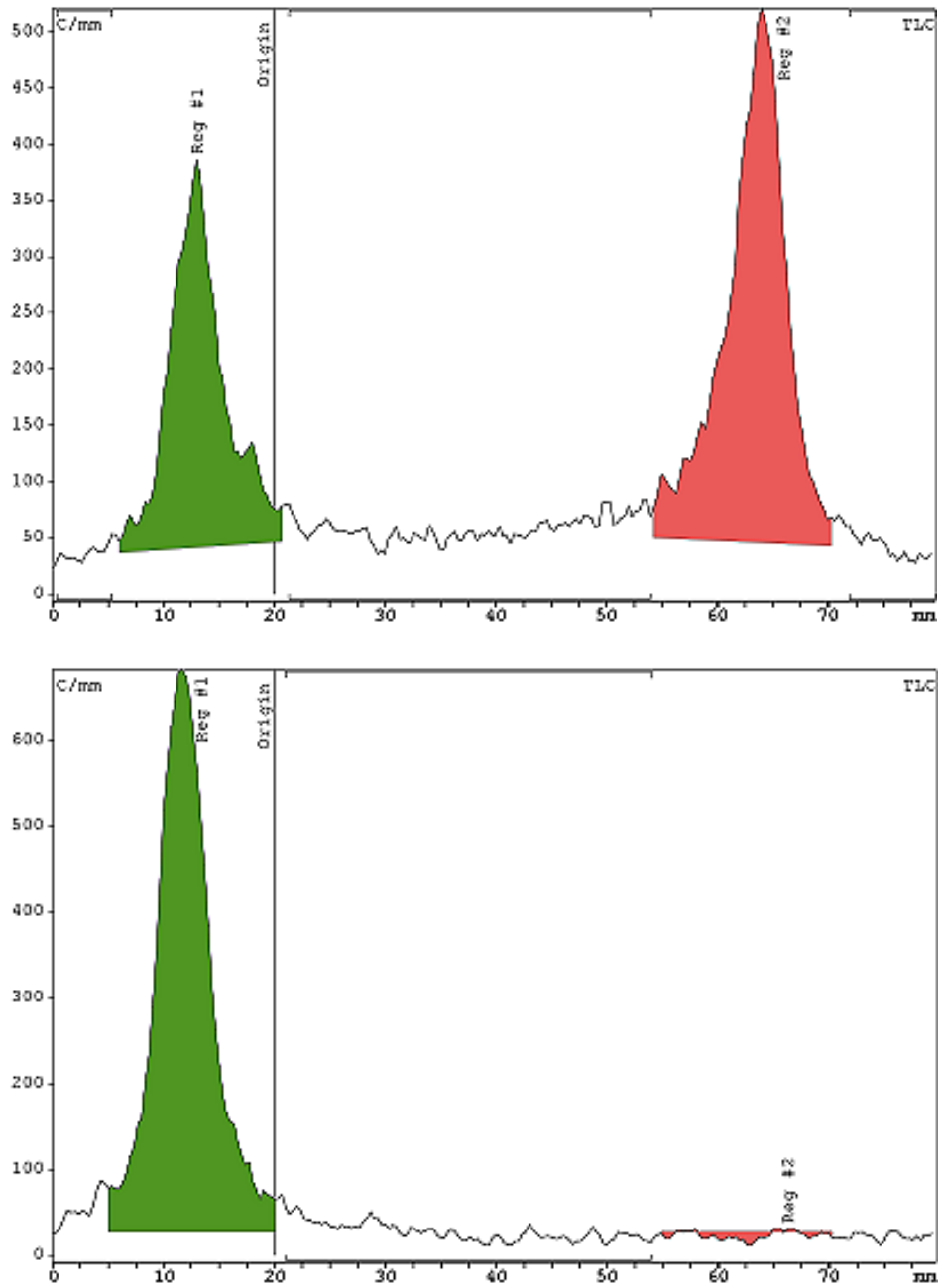


Figure S13. Radio-ITLC chromatograms of (A) ^{68}Ga-C2Ac and (B) ^{68}Ga-C2Ac-YM-103 conjugate. Radioactivity at R_f 0 (10 mm) is radiolabelled protein and free, unchelated ^{68}Ga at R_f 1 (65 mm).

Figure S13 (B) shows that after 5 minutes incubation with ^{68}Ga, the C2Ac-YM-103 conjugate was radiolabelled in 100% yield, compared with unmodified C2Ac (figure S13 (A)), which showed some free ^{68}Ga. After 15 minutes of incubation with ^{68}Ga, the radiolabelled ^{68}Ga-C2Ac-YM-103 conjugate was eluted eluted from a gel filtration column. Figure S14 shows that the radiolabelled C2Ac-YM-103 elutes in the fractions where a protein of this size elutes in a PD10, whereas ^{68}Ga-C2Ac elutes as a wide band, indicating dissociation of ^{68}Ga and instability of the radiolabelling.

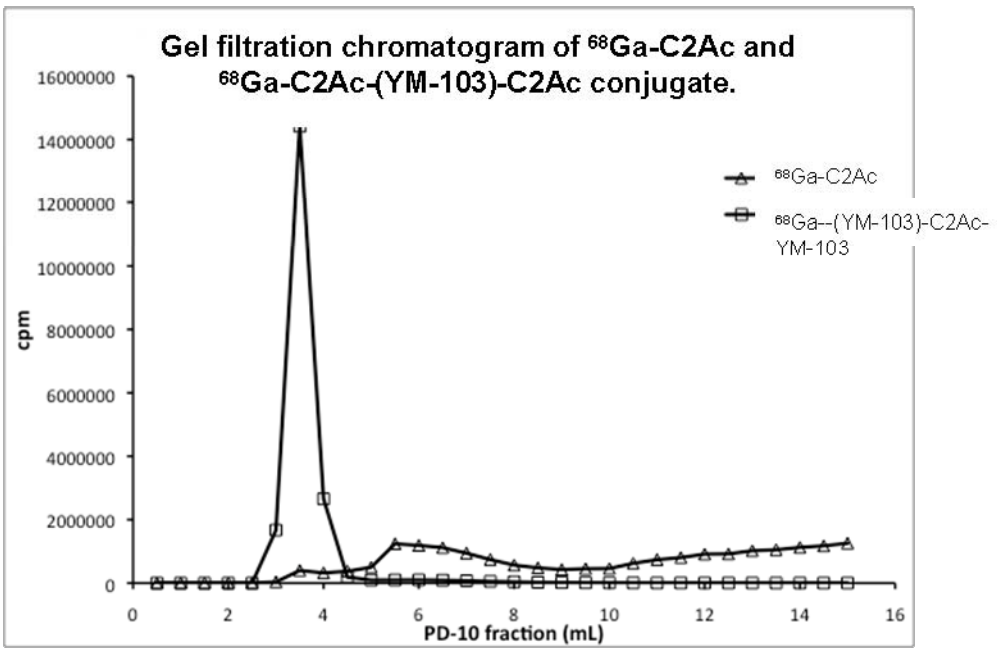


Figure S14. Gel filtration chromatogram (PD-10) showing radiolabelling of C2Ac and the C2Ac-YM-103 conjugate.

In vitro functional asssay on ^{68}Ga-C2Ac-YM-103.

The ^{68}Ga-labelled C2Ac-YM-103 conjugate showed specific calcium dependent binding to phosphatidylserine (PS) in a red blood cell binding assay, as shown in figure S14. This indicates that the protein is still functional, despite the maleimide conjugation. The EC_{50} = 1.15 mM Ca^{2+}. The R value was 0.99718.

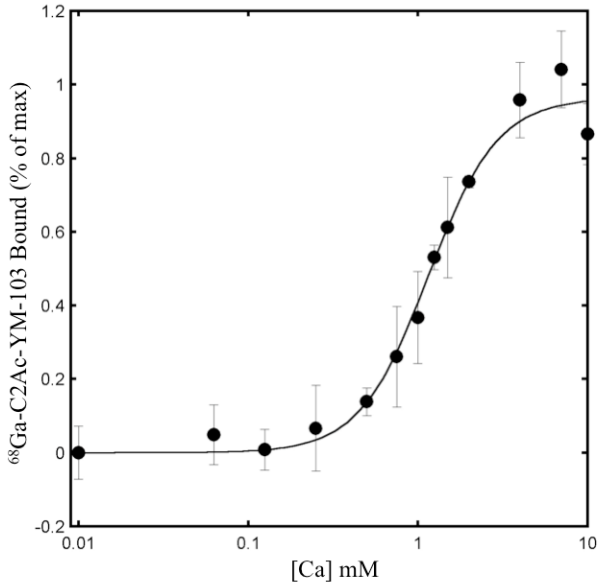
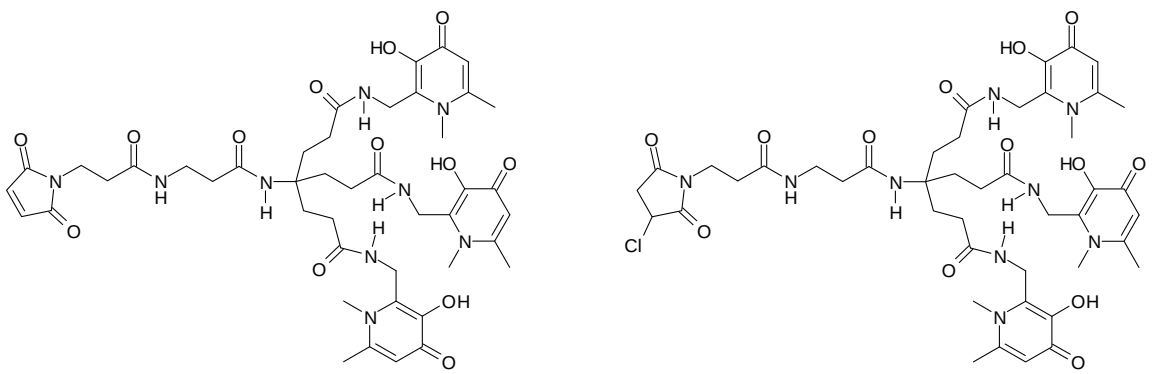


Figure S15. The ^{68}Ga-labelled C2Ac-YM-103 conjugate shows specific calcium dependent binding to phosphatidylserine (PS) in a red blood cell binding assay.



10 (YM-103) **11**

Figure S16. YM-103 (10) and chlorosuccinimide derivative (11).

Methods

Conjugation of C2Ac and YM-103.

C2Ac [5] (2 mg in a solution of 2mg/mL) was treated with a 3:1 molar excess of YM-103 (10) or its chlorosuccinimide derivative (11) (see figure S16) for 4 hours at room temperature. The mixture was purified using a PD-10 column pre-equilibrated with PBS + Chelex and analysed by LC-MS.

^{68}Ga radiolabelling of C2Ac and C2Ac-YM-103 conjugate.

100 µg of C2Ac or C2Ac-YM-103 at a concentration of 1 mg/mL was used. 200 µL of preconcentrated ^{68}Ga was added to the conjugate to give a final volume of 300 µl. At 5 minute intervals, 2 µL of the mixture was spotted on ITLC-SG and the chromatogram developed using 0.1 M citrate buffer as the mobile phase.

Red Blood Cell assay

The binding of radiolabelled ^{68}Ga-C2Ac-YM-103 to PS on red blood cells (RBC) was performed according to a literature method.[6] A commercial preparation of preserved human RBC was obtained from Beckman-Coulter (High Wycombe, UK). Calcium titrations of RBC were performed in a buffer of 50 mM HEPES-sodium, pH 7.4, 100 mM NaCl, 3 mM NaN_3, with 1 mg/mL BSA as carrier protein. Reactions were prepared with a final concentration of 1 nM ^{68}Ga labelled C2Ac-YM-103 and calcium; RBC were then added, and the reaction (1 mL) was incubated for 8 min at RT. The cells were then centrifuged (3 min at 9000 rpm the supernatant was removed, and the cells were resuspended in 1 ml assay buffer containing the same concentration of calcium used during the incubation step. The cells were centrifuged again, the supernatant was removed, and the pellet was resuspended in 0.7 ml assay buffer plus 10 mM ethylenediaminetetraacetic acid (EDTA) to release ^{68}Ga labelled C2Ac-YM-103 bound in a calcium-dependent manner. After centrifugation to remove the RBC, the released labelled ^{68}Ga-C2Ac-YM-103 in the supernatant was measured using a gamma counter. The EC_{50} was calculated as described in the literature using the equation $Y = [Ca]^N/([Ca]^N + EC_{50}{}^N)$ where $Y = B/B_{max}$, B is the observed amount of radiolabelled protein bound at a given calcium concentration, and B_{max} is the concentration of radiolabelled protein bound at saturating calcium concentrations. Curve fitting was performed using a nonlinear curve fit by a routine based on the Levenberg-Marquardt algorithm using Kaleidagraph (Synergy Software, Reading, US).

Equipment.

The Radio-TLC analysis was preformed on a miniGITA star TLC scanner with Gina Star software version 4.07 (Raytest). Samples were centrifuged using an Eppendorf centrifuge 5424 microcentrifuge. Gamma counting was performed on a 1282 Compugamma Gamma Counter (LKB Wallac)

Time (min)	A (%)	B (%)
0	95	5
5	95	5
30	52.8	47.2
35	10	90
36	95	5
40	95	5

Table S16. HPLC conditions for analysis of C2Ac and C2Ac-YM-103 conjugate by LCMS.

The LC-MS system was the same as that in section 2. The fragmentor voltage was 225 V, the column used was an Agilent Zorbax SB C18 cartridge column (2.1mm X 30mm, 3.5 μm) and the mobile phase consisted of A: H_2O + 0.05% TFA and B: 70% acetonitrile + 0.045% TFA.

9. In vivo imaging studies of bioconjugate

Results

PET scanning in mice with Ga-68 C2A conjugate and free ^{68}Ga

PET images taken at 1.5 h after injection of the conjugate into mice showed rapid clearance of radioactivity from blood and tissues with uptake exclusively in kidney with a small amount of activity excreted into the bladder (figure 4 (A), main publication). Radioactivity in explanted tissues confirmed almost all radioactivity was present in the kidneys (Figure S17). This contrasts with the images obtained with injection of "free" ^{68}Ga in which activity was seen widely distributed throughout the tissues, as shown in Figure S17.

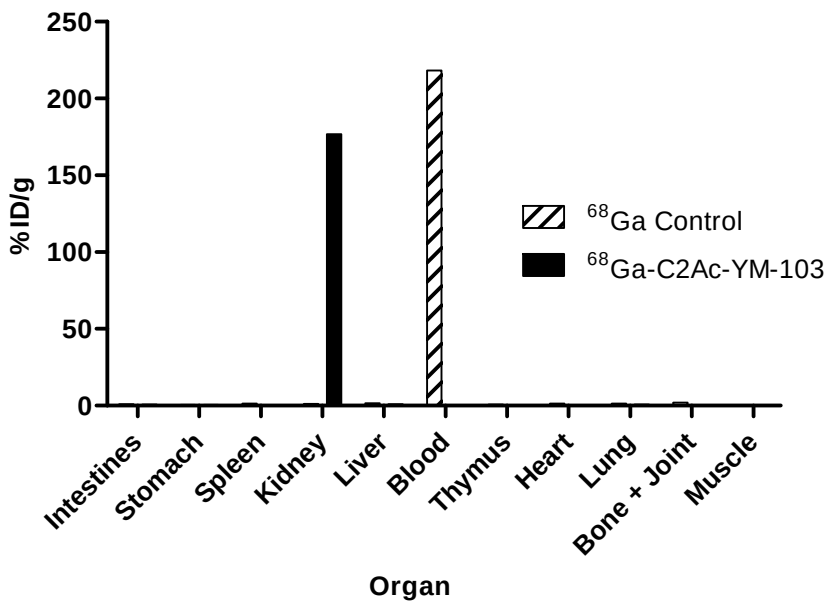


Figure S17. showing percentage of injected radioactivity per gram in mouse tissues 90 min post injection for ^{68}Ga-C2Ac-YM-103 and unchelated ("free") ^{68}Ga control.

Methods

PET scanning in mice with ^{68}Ga-C2Ac-YM-103.

The ^{68}Ga labelled C2A conjugate, synthesised as described above, or unchelated ^{68}Ga in acetate buffer, was filtered through a 0.22 μm filter an injected into C57B/6 mice (n = 4, 10 MBq in 100 μl for each animal). PET/CT scans were acquired 90 min post injection using a NanoPET/CT scanner (Bioscan, Paris, France) with PET acquisition time 1800 s, coincidence relation: 1-3. Image reconstruction: OSEM with SSRB 2D LOR, energy window: 400-600 keV, filter: Ramlak cutoff 1, number of iterations/subsets: 8/6.

Animals were sacrificed at 90 min and explanted organs counted in a gamma counter to determine biodistribution.

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