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Efficient bifunctional gallium-68 chelators for positron emission tomography: tris(hydroxypyridinone) ligands†

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A new tripodal tris(hydroxypyridinone) bifunctional chelator for gallium allows easy production of ^{68}Ga-labelled proteins rapidly under mild conditions in high yields at exceptionally high specific activity and low concentration.

The germanium-68/gallium-68 generator promises to make molecular imaging with positron emission tomography (PET) more widely available clinically, much as the molybdenum-99/technetium-99m generator did in the last century for single photon imaging, by providing a convenient, economic and reliable source of positron-emitting radionuclide without the need for a local cyclotron.[1] Gallium-68 has a half life of 68 min and a high positron yield (90%, 1.9 MeV).[2] To achieve its full potential, incorporation of this isotope into biomolecules for molecular imaging requires bifunctional chelators able to bind the Ga^{3+} ion rapidly under mild aqueous conditions, to give a complex with sufficient kinetic stability *in vivo* to withstand challenge from plasma transferrin, allowing imaging over several hours.

The current ''gold standard'' bifunctional ^{68}Ga chelator is the aminocarboxylate macrocycle DOTA (**1**, Fig. 1) and ^{67}Ga-DOTA bioconjugates have been reported to be stable in serum *in vitro* for at least 1250 h.[3] The most well known ^{68}Ga-DOTA bioconjugate is ^{68}Ga-DOTATOC, a somatostatin analogue used clinically for somatostatin receptor imaging.[4] Despite the high kinetic stability of the ^{68}Ga-DOTA complex, DOTA has several drawbacks as a ^{68}Ga chelator. Typical radiolabelling conditions entail heating (*e.g.* 95 °C) for up to 30 min at pH 4.6. The long radiolabelling time allows extensive decay during labelling and the high temperature and low pH are unsuitable for many proteins of interest for molecular imaging. Other chelators are being evaluated to circumvent some of these problems. The macrocycle NOTA (**2**) can be radiolabelled with ^{68}Ga at room temperature within 10 min at pH 3–5.5 and the complex has excellent stability in plasma.[5] Its derivative NODAGA (**3**) has been conjugated to proteins and radiolabelled with ^{68}Ga at pH 3.5-4 in 7 min.[6] Another promising chelator for ^{68}Ga is HBED (**4**), which forms a Ga complex with a very high logK of 38.5.[7] The derivative HBED-CC TFP (**5**) has been used for protein labeling in 80% yield by incubating at pH 4.1 for 5 min.[8] The acyclic ligand DTPA complexes gallium with high affinity but poor kinetic stability.[9] Derivatising the carbon backbone of DTPA can increase the stability of the ^{68}Ga complexes. Nevertheless these ^{68}Ga complexes undergo significant dissociation in serum.[10] Recently the acyclic H_2DEDPA (**6**) and a bifunctional

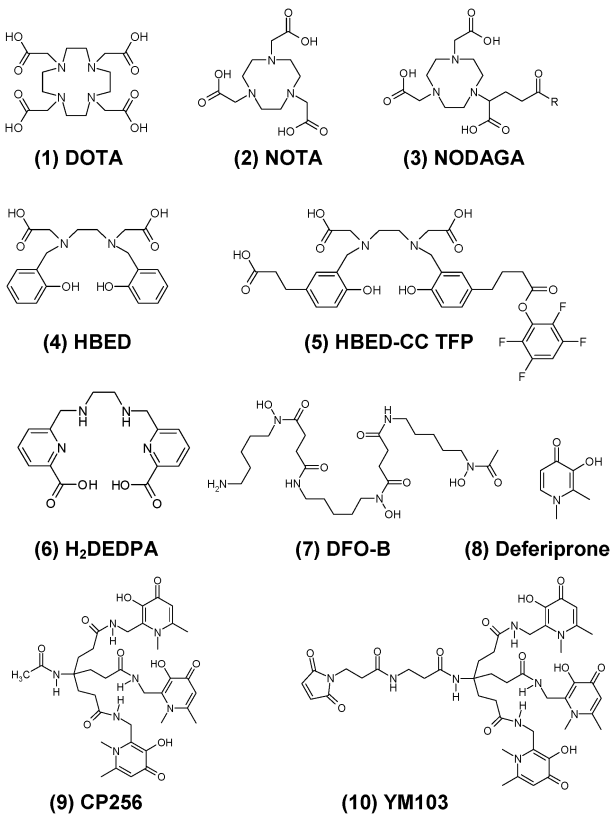

Fig. 1 Structures of some ^{68}Ga chelators.

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derivative have shown promise as $^{67/68}$Ga chelators, radiolabelling at pH 4.5 in 10 min.[11]

The similarity in coordination chemistry between Fe^{3+} and Ga^{3+} suggests the use of siderophore-like chelators for gallium. Indeed the siderophore Desferrioxamine-B (DFO-B, **7**) was used previously as a bifunctional chelator for $^{67/68}$Ga [12] but blood clearance of ^{68}Ga-DFO-Octreotide in patients is slow.[13] Deferiprone (**8**) is a bidentate 3-hydroxy-4-pyridinone (HPO) that is used clinically for the treatment of iron overload. Deferiprone and its analogues are effective scavengers of ^{67}Ga with the ability to remove ^{67}Ga bound to transferrin.[14] Hexadentate tris-HPO derivatives have been developed for Fe^{3+} and Al^{3+} sequestration[15] and have recently been shown to bind gallium with high affinity.[16] However, these ligands cannot be derivatised for bioconjugation, and they are conformationally ill-suited to the formation of mononuclear six-coordinate complexes. Here we evaluate the use of CP256 (**9**), a powerful hexadentate HPO Fe^{3+} chelator which overcomes both these limitations,[17] for radiolabelling with gallium radioisotopes. We report its radiolabelling properties with ^{68}Ga ($\leq$ 5 min at room temperature and at mild pH, $\sim$6.5) in comparison with those of other chelators widely used for this purpose, and the stability of its ^{67}Ga complex in biological media (see ESI† for further information). We also report the synthesis of its bifunctional maleimide derivative YM-103 (**10**) and its use to label a thiol-containing protein C2Ac with ^{68}Ga as a potential PET imaging agent for cell death.

CP256 was found to complex natural-abundance gallium rapidly at neutral pH and room temperature. Mass spectrometry showed the presence of only a 1 : 1 complex at m/z 740.3631 for singly protonated Ga-CP256 and 370.6865 for the doubly protonated species. CP256 is designed to form neutral, six-co-ordinate complexes with 3+ metals such as Fe^{3+} and Ga^{3+} and these results indicate such a species. No multinuclear complexes were detected, and only a single species was detected by HPLC. Radio-HPLC analysis after 1 min incubation of CP256 with ^{67}Ga-citrate showed a single radioactive peak with a retention time matching that of the cold Ga-complex. A side-by-side comparison was made of the ^{68}Ga-chelating efficiency of CP256 with that of other established Ga chelators (DOTA, NOTA and HBED), using instant thin layer chromatography (ITLC) at progressively lower chelator concentration, using the same ^{68}Ga generator (IGG100, Eckert & Ziegler, Germany) eluate for each ligand (Fig. 2). A radiochemical yield (RCY) of ^{68}Ga-CP256 of 98–100% was achieved at a ligand concentration of 10 μM after 5 min, pH 6.5 at room temperature. At 1 μM, the RCY was 73%. In contrast, the RCY of ^{68}Ga-NOTA (at room temp) and ^{68}Ga-DOTA (with heating at 100 °C for 30 min at pH 4.4) fell to approximately 80% and 95% respectively at 10 μM and 4% and 7% at 1 μM (Fig. 2). The RCY of ^{68}Ga-HBED dropped from 96–100% at 10 μM and above to 36% at 1 μM. Further data supporting the comparative labelling efficiency of CP256 are provided in the ESI.†

Incubation of ^{67}Ga-CP256 in human serum for 4 h at 37 °C showed no evidence of protein binding or release of ^{67}Ga when analysed by size exclusion chromatography. To provide a more stringent test of resistance to transchelation with transferrin, the complex was incubated with a 130-fold excess of

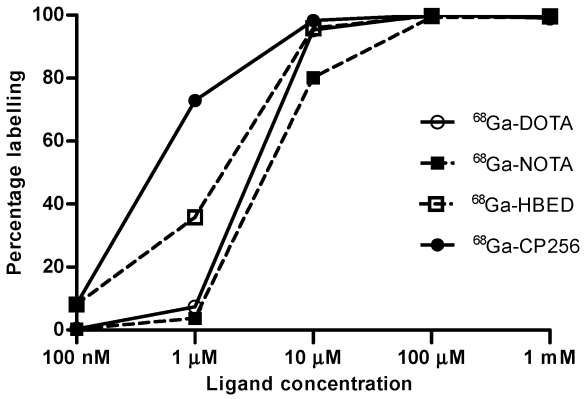

Fig. 2 Radiolabelling yield *versus* ligand concentration for ^{68}Ga-DOTA (pH 4.4, 30 min, 100 °C), ^{68}Ga-NOTA (pH 3.6, 10 min, room temp), ^{68}Ga-HBED (pH 4.6, 10 min, room temp) and ^{68}Ga-CP256, (pH 6.5, 5 min, room temp). All experiments were conducted with the same batch of ^{68}Ga eluate. All radiolabelling buffers were 0.2 M acetic acid/sodium acetate.

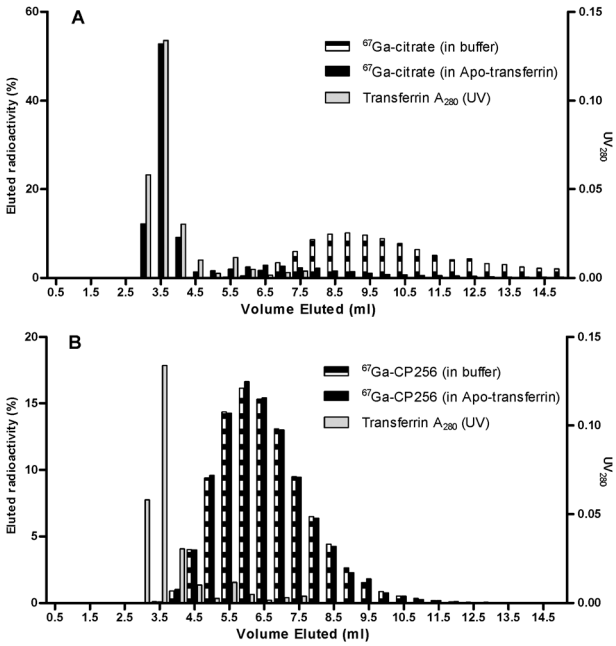

Fig. 3 Size exclusion elution profiles of (A) ^{67}Ga-citrate and (B) ^{67}Ga-CP256 in buffer and apo-transferrin after 4 h incubation.

apotransferrin (a 260 fold excess in terms of Ga-binding capacity since there are two metal binding sites per transferrin molecule) in the presence of bicarbonate at 37 °C. Again no transchelation was observed (Fig. 3), whereas significant transchelation by transferrin was observed with ^{67}Ga-citrate as the control.

The outstanding radiolabelling kinetics of ^{68}Ga-CP256 and *in vitro* stability observed for ^{67}Ga-CP256 encouraged us to synthesise a bifunctional derivative for protein labelling. The maleimide derivative **10** was chosen to confer site-specificity on conjugation to proteins containing an engineered free cysteine residue. To evaluate the potential of **10** for ^{68}Ga labelling of biomolecules, the protein C2Ac was selected. C2Ac is an analogue of C2A (the phosphatidylserine (PS)-binding domain of synaptotagmin I, an amphipathic protein

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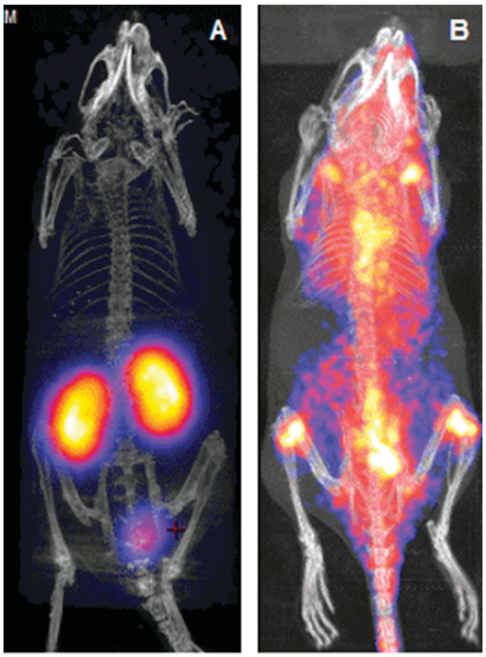

Fig. 4 PET-CT images of mice 90 min after intravenous injection of (A) ^{68}Ga-YM-103-C2Ac and (B) unchelated ^{68}Ga.

which binds to PS in a Ca^{2+}-dependent manner and is being investigated as an imaging agent for cell death) into which a cysteine residue has been engineered[18] for purposes of bioconjugate synthesis. Incubation of **10** (or its HCl addition product, which is also formed as an intermediate during the synthesis of **10**, see ESI†) with C2Ac produced the bioconjugate YM-103-C2Ac whose electrospray mass spectrum showed the incorporation of a single molecule of **10**. The conjugate (20 μg, 1 mg ml^{-1}, 63 μM) was incubated with freshly-eluted ^{68}Ga in 0.5 M ammonium acetate buffer, pH ~5.5, giving quantitative labelling after 5 min as determined by TLC.

The radiolabelled conjugate showed retention of calcium-dependent binding to PS in a red blood cell binding assay *in vitro*. A PET imaging study was therefore carried out in a normal mouse (Fig. 4A).‡ After 90 min post-injection the ^{68}Ga was located almost exclusively in the kidney, with some excretion to the bladder, in contrast to the distribution throughout the whole mouse observed (Fig. 4B) when uncomplexed ^{68}Ga was injected. This demonstrates that ^{68}Ga is not released from the conjugate *in vivo* during the imaging period. We conclude that the tris(hydroxypyridinone) ligand CP256 is an excellent chelator for gallium and can be radiolabelled with gallium isotopes more quickly, with higher yield, under milder conditions and to significantly higher specific activity than the currently established chelators DOTA, NOTA and HBED. The complex is extremely stable in serum and when challenged with excess apotransferrin. The maleimide derivative **10** can be conjugated site-specifically to cysteine residues and the resulting bioconjugate is efficiently labelled with ^{68}Ga to high specific activity. It is likely to become the bifunctional chelator of choice for labelling of sensitive proteins with ^{68}Ga.

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Notes and references

‡ Animal studies were carried out in accordance with UK Research Councils' and Medical Research Charities' guidelines on Responsibility in the Use of Animals in Bioscience Research, under a UK Home Office licence and were approved by the King's College London Local Ethics Committee.

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