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Radiolabelling with isotopic mixtures of $^{52g/55}$Mn(II) as straight route to stable manganese complexes for bimodal PET/MR imaging

Christian Vanasschen*‡, Marie Brandt‡, Johannes Ermert and Heinz H. Coenen
Institut für Neurowissenschaften und Medizin, INM-5: Nuklearchemie, Forschungszentrum Jülich GmbH, D-52425 Jülich, Germany
* Corresponding author
‡ Authors contributed equally

Radiolabelling using isotopic mixtures of $^{52g/55}$Mn(II) offers fast and easy access to new small molecule PET/MR tracers, composed of chemically identical reporting units. *Trans*-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA) was radiolabelled with carrier-added ^{52g}Mn(II) in >99% radiochemical yield, producing the first manganese-based bimodal PET/MR probe. The Mn-CDTA chelate was shown to be very stable to air oxidation and sufficiently inert to decomplexation in blood serum. Those data sparked our interest in functionalized CDTA ligands for the design of optimized PET/MR tracers.

The excellent spatial, submillimeter resolution obtained with magnetic resonance imaging (MRI) is a feature of tremendous value if added to positron emission tomography (PET), known for its remarkably high sensitivity. Thus, the highly synergistic combination of simultaneous PET and MRI holds great promise for functional molecular imaging of unprecedented quality. The increasing number of hybrid PET/MR scanners available, in addition to the introduction of new fully integrated whole-body prototypes,[1] has sparked the research interest in bimodal PET/MR probes. Such agents show great potential for biomarker-responsive imaging as well as for "PET-guided" high-resolution MRI.[2,3] In contrast to nanoparticulate PET/MR tracers,[4-11] surprisingly few publications are available regarding small-molecule agents.[12-15] This may be due to the challenging design of such tracers which is hampered by the enormous sensitivity difference between PET and MRI. Nanomolar *versus* millimolar concentration of imaging agent is required for PET and MRI measurements, respectively, making a 1-to-1 coupling of an MR probe to a PET tracer unreasonable for imaging applications. The only way to overcome this problem is to add tiny amounts of radionuclide to an MRI contrast agent, i.e. radiolabelling at very low molar activity (radioactivity/mole of MRI contrast agent).[2,16] However, in order to take full advantage of such a bimodal construct, both reporter molecules should ideally exhibit an identical biodistribution and pharmacokinetic behavior *in vivo*. It is therefore mandatory that the MR and PET reporters are chemically identical. So far, besides us,[17,18] only two research groups took these considerations into account for the development of small-molecule PET/MR probes.[12,14]

Here, an efficient approach towards novel PET/MR probes is reported, avoiding laborious ligand design or burdensome radiolabelling. It concentrates on the positron-emitter manganese-52g (^{52g}Mn) as an analogue of the paramagnetic MR reporter ^{55}Mn(II). As recently evidenced by high quality images, ^{52g}Mn appears to be a promising candidate for PET imaging ($t_{1/2}$ = 5.6 d, β^+-decay intensity: 29.6%, max. β^+-energy: 575 keV).[19,20] The production and purification of this radionuclide has already

been reported by us as well as several other groups.[19-24] Direct access to bimodal PET/MR tracers was considered *via* complexation of the isotopic $^{52g/55}$Mn(II) pair by a suitable chelator, eventually yielding an isotopically radiolabelled MR contrast agent.

In this study, radiolabelling of CDTA (*trans*-1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid) with an isotopic mixture of $^{55/52g}$Mn was carried out to yield the first manganese-based PET/MR tracer. Furthermore, the redox and blood serum stability of the corresponding $[Mn^{II}(CDTA)]^{2-}$ complex were assessed.

When the *in vivo* application of Mn(II) is intended, the potential toxicity of this transition metal needs to be considered with care. Although Mn(II) plays an essential role in biological systems, it is known to have acute toxic effects at higher concentrations (LD_{50} = 0.30-0.45 mmol.kg^{-1}, i.v. injection of $MnCl_2$ in rats).[25] Among others, Mn^{2+} is able to accumulate in the brain, causing a neurological disease known as "manganism".[26] Therefore, at the concentrations required for MR imaging (typically 0.05 – 0.10 mmol.kg^{-1}) the Mn^{2+} ion needs to be tightly bound within a complex to reduce the risk of *in vivo* release. Since certain Gd(III)-based MR contrast agents have recently been associated with the occurrence of a syndrome called nephrogenic systemic fibrosis (NSF), a renewed interest has emerged around Mn(II) complexes over the last five years.[27-33] The majority of these studies underline the difficulty in designing Mn^{2+} complexes combining sufficiently high thermodynamic stability and kinetic inertness, both prerequisites for *in vivo* use, with a fair T_1 contrast enhancement (or relaxivity, r_1). The latter requires the presence of at least one water molecule directly coordinated to the paramagnetic ion (hydration number, q, should be ≥ 1). So far, the Mn^{2+} chelate formed with the open-chain hexadentate ligand CDTA (Fig. 1) seems to be the best compromise between stability requirements and relaxivity (Table 1).[32] Therefore, the CDTA scaffold was considered for the preparation of novel $^{52g/55}$Mn(II)-based bimodal PET/MR tracers.

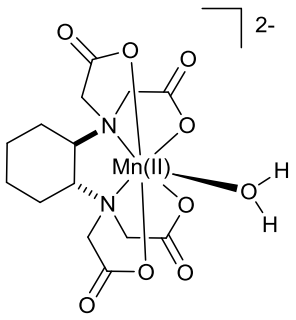

Fig. 1 $[Mn^{II}(CDTA)]^{2-}$ complex bearing one inner-sphere water molecule

Table 1 Relevant physicochemical parameters of $[Mn(CDTA)]^{2-}$ at 25 °C

Parameter		Literature value	Reference
Relaxivity	– r_1 ($mM^{-1}s^{-1}$) [a]	3.6	32
Hydration number	– q	0.9	34
Water exchange time	– τ_M (ns)	7.14	35
Stability constant	– $\log K_{ML}$ [b]	14.32 [c]	32
Dissociation half-life	– $t_{1/2}$ (h) [d]	12	32

[a] 20 MHz; [b] K_{ML} = [ML]/[M]x[L] (M = Mn^{2+}; L = CDTA); [c] Conditions : I = 0.15 M NaCl (note that values ranging from 14.70 to 17.43 were previously reported for the stability constant measured at 20°C)[36-38]; [d] Kinetic inertness is generally characterized by the dissociation half-life, $t_{1/2}$. Conditions: pH = 7.4, $[Cu^{2+}] = 10^{-5}$ M.

For the identification of Mn^{2+} complexes that are potentially suitable for *in vivo* imaging, thermodynamic stability and kinetic inertness are not the only parameters to be critically assessed. The redox stability of the Mn^{2+} ion within the chelate is also of prime importance since any oxidation to the lower-spin Mn^{3+} would lead to a decreased paramagnetism, i.e. a dramatic drop in relaxivity. It has been shown that some Mn(II) complexes are readily oxidized by simple contact with air, depending on the donor groups and the structure of the ligand.[39] On the other hand, such a tendency towards oxidation can be exploited to design redox-responsive MR contrast agents.[40] Here, paramagnetic $[Mn^{II}(CDTA)]^{2-}$ was easily prepared by mixing the commercially available ligand with $MnCl_2.4H_2O$ at pH 5.5-6. Formation of the desired chelate was confirmed by HRMS. The pH value for complexation appeared critical as CDTA is insoluble at pH < 5, while too basic conditions (pH > 8) lead to oxidation of Mn(II) or even its precipitation as $Mn(OH)_2$. The stability of $[Mn^{II}(CDTA)]^{2-}$ towards oxidation by air was assessed by monitoring its T_1 relaxivity over time (20 MHz, pH = 7.4, 25 °C). A relaxivity value of r_1 = 3.6 $mM^{-1}s^{-1}$ was obtained for both a freshly prepared complex solution and one stored for 7 months at room temperature (Fig. S8-S9, ESI†). This result is a proof of the redox stability of $[Mn^{II}(CDTA)]^{2-}$ under physiological conditions, confirming that the CDTA scaffold strongly favors the d^5 high-spin configuration of Mn^{2+}.

The radionuclide ^{52g}Mn was produced by irradiation of ^{nat}Cr with protons in the energy range from 16.9 to 8.2 MeV.[24] When using cation-exchange chromatography, a successful separation of n.c.a. ^{52g}Mn from macroscopic amounts of ^{nat}Cr could only be achieved after an extensive removal of chloride ions.[24] To avoid this time consuming step, an improved separation method based on Amberlite CG400 resin has recently been developed within our group (Buchholz *et al.*, *Radiochimica Acta*, accepted for publication). By this means, 99.5% of n.c.a. ^{52g}Mn are recovered in 2-3 mL of 3 M HCl with a chromium content below 0.02%. Radiolabelling with ^{52g}Mn was carried out by adding ≈ 8 MBq $^{52g}MnCl_2$ to a 64 mM CDTA solution in sodium acetate buffer (pH 6), producing the

corresponding radiotracer $[[^{52g}Mn]Mn(CDTA)]^{2-}$ with > 99% radiochemical yield (RCY). No uncomplexed $^{52g}Mn^{2+}$ ions remained after 30 min stirring at RT, as demonstrated by radio-TLC monitoring of the n.c.a. reaction (mobile phase: 25% aq. NH_3/H_2O/MeOH; R_f $^{52g}Mn^{2+}$ = 0; R_f $[[^{52g}Mn]Mn(CDTA)]^{2-}$ ≈ 0.95, ESI†). To our knowledge, DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) is the only other polyaminocarboxylate ligand which has been labelled with ^{52g}Mn so far.[41] Under the same conditions, carrier-added (c.a.) $[[^{52g}Mn]Mn(CDTA)]^{2-}$ was prepared by addition of 0.1 equivalent $^{55}MnCl_2$ carrier to the reaction mixture. Again, labelling with > 99% RCY was completed within 30 min (radio-TLC). The identity of the radioactive complexes formed was confirmed by RP-HPLC analysis using a C18 column with polar endcapping (Fig. 2). Interestingly, the HPLC conditions developed here (mobile phase: Sörensen phosphate buffer/EtOH 99.5:0.5, pH 6.9) enable to quantitatively separate the excess of CDTA ligand from the labelled complex. This is of major importance for the *in vivo* evaluation of c.a. $[[^{52g}Mn]Mn(CDTA)]^{2-}$ as bimodal PET/MR tracer.

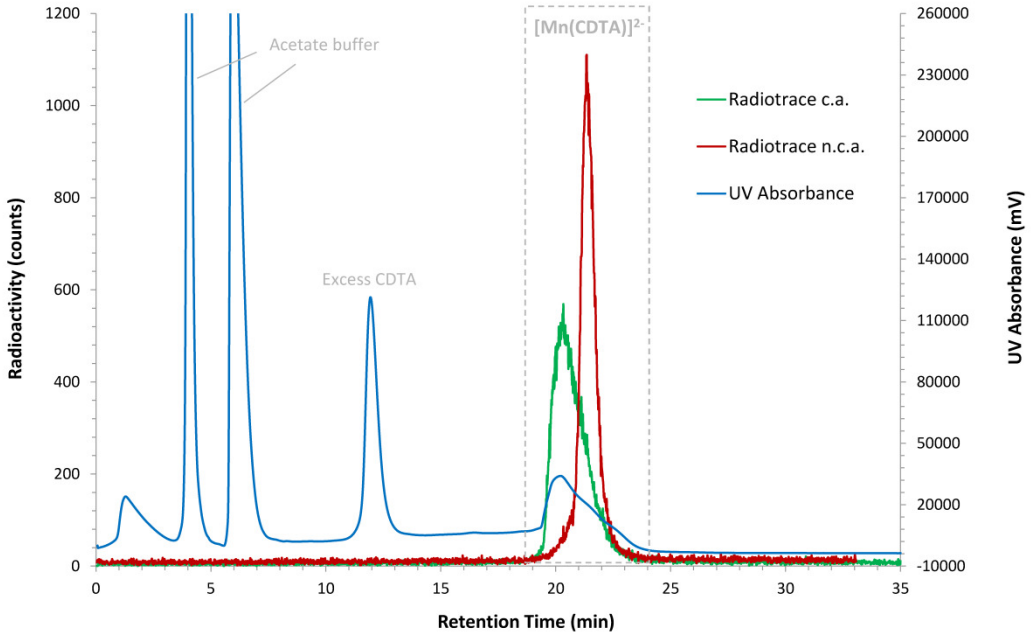

Fig. 2 Overlayed HPLC chromatograms of n.c.a. and c.a. $[[^{52g}Mn]Mn(CDTA)]^{2-}$. The UV signal (blue) and the peak obtained for the c.a. radiocomplex (green) have exactly the same retention time (R_t = 20.3 min). A very slight shift in retention time is observed for the n.c.a. chelate (R_t = 21.3 min; red). This is explained by the absence of macroscopic amounts of carrier which also results in a narrower peak. The intense UV peaks at ≈ 4 min and ≈ 7 min correspond to sodium acetate buffer signals, whereas the peak at about 12 min represents excess CDTA, both being present in the radiolabelling mixture.

These results highlight how accessible bimodal PET/MR tracers become when an isotopic $^{55/52g}Mn^{2+}$ mixture is used, leading to a blend of chemically equivalent PET and MR probes. Such chemical

equivalence has already been reported for two PET/MR small molecule bimodal probes. In 2010, a bimodal pair composed of an ^{18}F-labelled, pH-responsive, Gd(III)-based MR reporter and its non-radioactive ^{19}F-counterpart was published.[14] A few years later, a Gd(III)-DOTA monoamide trimer whose central TRAP ligand (TRAP = 1,4,7-triazacyclononane-1,4,7-triphosphinate) was loaded with a mixture of the positron-emitter ^{68}Ga and inactive ^{69}Ga was designed.[12] In this context it should also be mentioned that a small molecule dual-modal SPECT/MR tracer composed of chemically equivalent reporter units has been proposed (^{123}I for SPECT, Gd^{3+} for MR).[42] In all three cases, however, the approach required an additional molecular structure/group in order to combine the MR reporter (Gd^{3+}) and the PET/SPECT radionuclide in a same molecular entity. In addition, the non-radioactive analogues, incorporating $^{19}F/Gd^{3+}$, $^{69}Ga^{3+}/Gd^{3+}$ and $^{127}I/Gd^{3+}$, had to be prepared independently and then mixed with the radioactive tracer to obtain a bimodal formulation.

So far, the stability of $[Mn^{II}(CDTA)]^{2-}$ in blood serum has only been evaluated qualitatively through relaxivity measurements on incubated samples of the paramagnetic chelate or approximated by theoretical calculations.[32] Yet, incubation of the ^{52g}Mn-radiocomplex in human blood serum (HBS) may allow an accurate quantitation of the amount of ^{52g}Mn being released from the chelate over time. For this purpose, HPLC-purified n.c.a. $[[^{52g}Mn]Mn(CDTA)]^{2-}$ was incubated in HBS for 1, 3, 5, 18 and 24 hours at 37 °C. Preliminary tests have shown, that isolation of blood proteins by precipitation or centrifugal filtration techniques do not enable to quantify potentially released ^{52g}Mn (ESI†). More reliable results were obtained using size exclusion-HPLC (SE-HPLC). With this technique, the ability of proteins present in HBS to bind manganese(II) ions was demonstrated in $^{52g}MnCl_2$ incubation experiments (Fig. S4, ESI†). The SE-HPLC chromatogram obtained for a blood sample spiked with n.c.a. $[[^{52g}Mn]Mn(CDTA)]^{2-}$ and incubated for 5 h is depicted in Fig. 3a. Biomacromolecules and other blood components give rise to a very broad signal between ≈ 7-20 min (U.V. channel) whereas two narrower radioactivity peaks are eluted around 12 and 16 min. Based on its retention time, the signal at ≈ 12 min can be ascribed to ^{52g}Mn(II) that was released from the complex and bound by blood molecules, most likely albumin and some globulins.[43] The peak at 16 min, on the other hand, may correspond to intact $[[^{52g}Mn]Mn(CDTA)]^{2-}$ as suggested by the co-elution observed at this same retention time for the radiocomplex and its non-radioactive analogue on the size-exclusion column (Fig. 3b). This was further confirmed by peak co-elution observed for all incubation samples co-injected with "cold" Mn-CDTA on RP-HPLC (R_t ≈ 15 min, Fig. S7, ESI†). The presence of only one radioactivity signal also revealed the absence of any additional small-molecule manganese complex that may have been formed with chelating blood components like citric acid, L-lactic acid or amino acids.

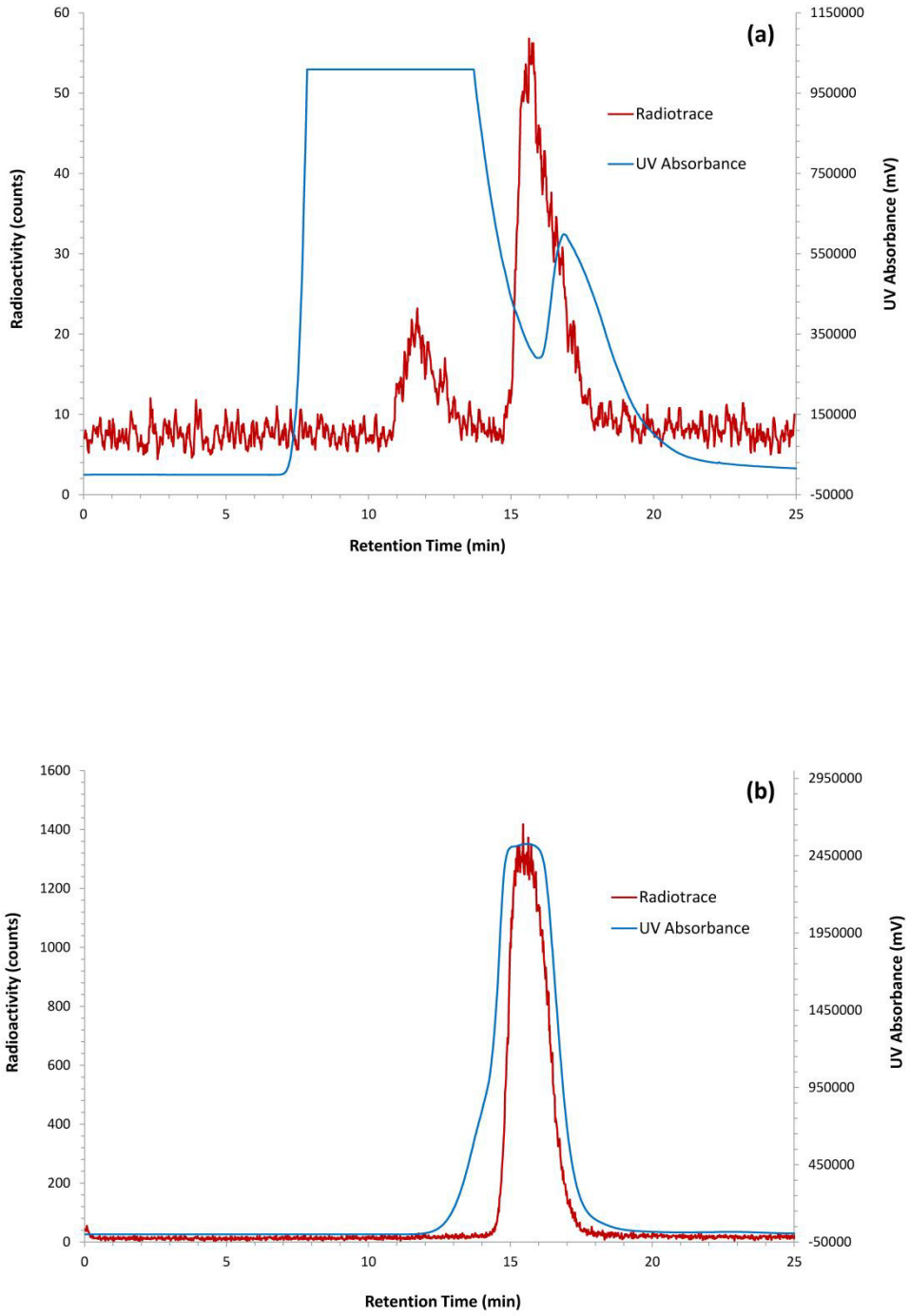

Fig. 3 (a) SE-HPLC chromatogram obtained for [[^{52g}Mn]Mn(CDTA)]$^{2-}$ after 5 h incubation in HBS at 37 °C. The radiotrace corresponds to a simple moving average curve (order = 5). The U.V. signal generated by blood macromolecules was higher than the upper detection limit of the detector, R_t ≈ 7-15 min. (b) SE-HPLC chromatogram of [[^{52g}Mn]Mn(CDTA)]$^{2-}$ co-injected with non-radioactive Mn-CDTA.

The SE-HPLC radiotraces measured after 1 h, 5 h and 24 h incubation of the radiocomplex in HBS show the expected increase of the amount of ^{52g}Mn released from the chelate over time (Fig. 4). The percentage of intact [[^{52g}Mn]Mn(CDTA)]$^{2-}$ complex at each time point was determined by integration of the peak areas of the radioactivity signals in Fig. 4. The corresponding values are listed in Table 2.

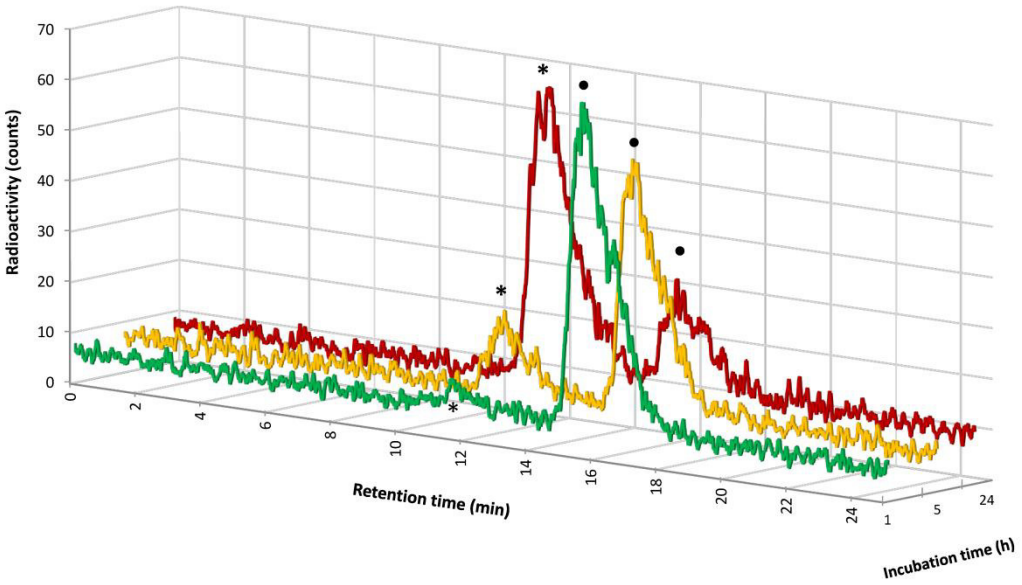

Fig. 4 SE-HPLC chromatograms obtained for [[^{52g}Mn]Mn(CDTA)]$^{2-}$ after 1h (green), 5 h (orange) and 24 h (red) incubation in HBS at 37 °C. Signals at ≈ 12 min correspond to protein-bound ^{52g}Mn (*) and peaks at ≈ 16 min can be ascribed to intact complex (●). For the sake of clarity, 3 h and 18 h data as well the U.V. signal corresponding to each radiotrace are not shown here. Individual radiotraces correspond to simple moving average curves (order = 5; chromatograms showing all measured data can be found in Fig. S6, ESI†). Although two consecutive HPLC runs were performed at each time point, only one chromatogram is shown here.

Table 2 Amount of intact [[^{52g}Mn]Mn(CDTA)]$^{2-}$ determined by SE-HPLC after incubation in HBS at 37 °C

Incubation time (h)	Intact radiocomplex (%) [a]
1	≥ 95 [b]
3	87 ± 5
5	78 ± 4
18	32.5 ± 0.4
24	28.2 ± 0.4

[a] Values correspond to the average of two independent HPLC runs. [b] No error value is given as the protein-bound ^{52g}Mn fraction could be detected but not quantified accurately.

Interestingly, the degradation rate of the complex correlated quite well with the dissociation half-life obtained by Kálmán *et al.* in a transmetallation study performed with the paramagnetic complex ($t_{1/2}$ = 12 h; pH 7.4, $[Cu^{2+}] = 10^{-5}$ M).[32] Our finding that ≈ 22% of the radiocomplex dissociated after 5 hours incubation has to be counterbalanced by the short blood elimination half-life expected for a hydrophilic compound like $[Mn(CDTA)]^{2-}$ (around 90 min for Gd-DOTA).[44] Most notably, biodistribution studies carried out with n.c.a. and c.a. $[[^{54}Mn]Mn(CDTA)]^{2-}$ in mice suggested that approx. 85% of the injected dose are excreted by glomerular filtration 1 hour after injection.[45] Additionally, it has to be emphasized that a significantly less inert manganese chelate, Mn-DPDP (Teslascan® or Mangafodipir; DPDP = *N,N'*-dipyridoxylethylenediamine-*N,N'*-diacetate-5,5'-bisphosphate) was approved for clinical use by the FDA in 1997 and routinely applied for the MR imaging of liver lesions.[46,47] As a more recent example, CMC-001, a formulation of non-complexed $MnCl_2$, alanine and Vitamin D3 currently undergoes phase III clinical trials for MR visualization of liver and bile.[48] None of the above contrast agents have been associated with increased toxicity.

While there is no doubt that ligands like NOTA or DOTA form manganese chelates that would be less prone to partial decomplexation *in vivo* than Mn-CDTA, such structures do not possess a coordinated water molecule (q = 0), resulting in very poor relaxivity values.[49] This leaves no space for the design of high relaxivity or biomarker-responsive PET/MR probes. To the best of our knowledge, Mn-CyP3A is the only other monoaquated Mn complex being at least as kinetically stable as Mn-CDTA.[50]

In conclusion, the capability in producing and purifying the positron-emitter $^{52g}Mn^{2+}$ offers fast and easy access to small molecule PET/MR tracers without recourse to elaborated multi-ligand constructs or multiple-step (radio)labelling.[12,14,15] The first manganese-based PET/MR probe, carrier-added $[[^{52g}Mn]Mn(CDTA)]^{2-}$, was obtained by simple addition of an isotopic $^{55/52g}Mn^{2+}$ mixture to a solution of the CDTA ligand. The chemical equivalence of the PET and MR reporting units composing this bimodal tracer ensures identical *in vivo* behavior. In addition to its high thermodynamic stability and kinetic inertness,[32] $[Mn(CDTA)]^{2-}$ was shown to be extremely stable towards oxidation as well as sufficiently inert to decomplexation in blood serum. Based on those promising data, the synthesis of CDTA ligands bearing functional groups for further derivatization is currently in progress. Such structures open the door to target-specific, biomarker-responsive or high relaxivity bimodal tracers for *in vivo* applications. In the latter context, the positron-emitter ^{51}Mn ($t_{1/2}$ = 46.2 min, β^+-decay intensity: 96.9%, max. β^+-energy: 2185 keV) will advantageously replace ^{52g}Mn in PET studies given its more favorable decay properties: a significantly shorter half-life, in much better accordance with the fast clearance of small molecules, and a higher positron abundance which reduces the exposure to

gamma radiation.[22,51] Thus, the here performed as well as the planned studies may contribute to pave the way for a greater acceptance of dual-modal probes in hybrid PET/MR imaging.

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Textual abstract

Radiolabelling using isotopic mixtures of $^{52g/55}$Mn(II) offers fast and easy access to new small molecule PET/MR tracers, composed of chemically identical reporting units. *Trans*-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA) was radiolabelled with carrier-added ^{52g}Mn(II) in >99% radiochemical yield, producing the first manganese-based bimodal PET/MR probe. The Mn-CDTA chelate was shown to be very stable to air oxidation and sufficiently inert to decomplexation in blood serum. Those data sparked our interest in functionalized CDTA ligands for the design of optimized PET/MR tracers.

Novel isotopic $^{52g/55}$Mn(II) complexes as basis for bimodal PET/MR imaging