Electronic Supplementary Information (ESI)

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

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1. Chemicals

CDTA monohydrate was purchased from Acros Organics (Geel, Belgium), PBS buffer from Life Technologies (Thermo Fisher Scientific, Schwerte, Germany) and organic solvents for HPLC from VWR (Langenfeld, Germany). All other chemicals were acquired from Sigma-Aldrich (Schnelldorf, Germany) and used without further purification.

No-carrier-added manganese-52g (n.c.a. ^{52g}Mn) was produced and isolated according to the procedure developed by our group.[1]

2. Materials and methods

γ-Spectroscopy was conducted with ORTEC γ-ray spectrometers (AMETEK GmbH, Meerbusch, Germany) which were energy and efficiency calibrated with certified radiation point sources (Co-60, Ba-133, Eu-152, Ra-226) from the Physikalisch-Technische Bundesanstalt (Braunschweig, Germany).

Radio-TLC measurements were carried out on 60F_{254} silica plates (Merck, Darmstadt, Germany) using 25% aq. NH_3/MeOH/H_2O 2:1:1 as mobile phase; radio-TLC were recorded using a Packard Instant Imager (Packard Instrument Company, Meridan, CT, USA). The radioactivity detection limit (LOD) was determined by serial dilution: LOD ≤ 3 Bq for an exposure time of 30 min.

Radio-HPLC was performed on a Merck Hitachi L-6000 pump, a Merck Hitachi L-4000 UV/VIS detector (Merck, Darmstadt, Germany) and a EG&G Ortec ACE NaI(Tl) radioactivity detector with photomultiplier (EG & G Ortec, Oak Ridge, TN, USA). The radioactivity detection limit was determined by serial dilution: LOD ≤ 0.3 kBq.

Mobile phase 1 (chromatograms in Fig. 2): Sørensen phosphate buffer/EtOH 99.5:0.5, pH 6.9); mobile phase 2 (for SE-HPLC): PBS/EtOH 99.5:0.5 (PBS = phosphate buffered saline, pH 7.4); mobile phase 3 (all other chromatograms): PBS/EtOH 99.5:0.5, pH 6.7.

RP stationary phase (for co-elution experiments of radioactive and non-radioactive Mn-CDTA): Synergi 4u Hydro RP 80Å, 250x4.6 mm (Phenomenex Inc., Aschaffenburg, Germany) with a flow rate of 0.8 mL/min; size-exclusion stationary phase (for blood serum analyses): Yarra 3 µm SEC-2000, 300x7.8 mm (Phenomenex Inc., Aschaffenburg, Germany) with a flow rate of 0.7 mL/min. UV detection wavelength for all measurements: 230 nm.

High resolution mass spectrometry was performed using a hybrid linear ion trap FT-ICR mass spectrometer LTQ-FT Ultra (Thermo Fisher Scientific, Bremen, Germany) equipped with a 7 T supra-conducting magnet. The electrospray ionisation (ESI) source was operated in the negative mode.

^{55}Mn concentrations in solution were determined by inductively coupled plasma mass spectrometry (ICP-MS) using an Agilent 7500 instrument with quadrupol mass analyser and collision cell (Agilent Technologies, Frankfurt am Main, Germany). The instrument was operated with helium as collision gas to minimize spectral interferences by cluster ions. Quantification was performed by external calibration using Rh as the internal standard.

For centrifugation a small 5415 C Centrifuge from Eppendorf (Hamburg, Germany) or a Heraeus Biofuge Stratos centrifuge (Thermo Fisher Scientific Inc., Schwerte, Germany) were used.

The *longitudinal relaxation times* of water protons (T_1) were measured at 20 MHz on a Bruker NMS 120 Minispec apparatus using the inversion recovery method (180° – τ – 90°). The temperature of the samples was set at 25.0 ± 0.2 °C and controlled with a circulating water bath.

3. Synthesis of $[Mn(CDTA)]^{2-}$ complexes

3.1. Synthesis of the reference compound $Na_2[^{55}Mn(CDTA)]$

CDTA monohydrate (100 mg, 0.269 mmol) was suspended in 2 mL water and 1M NaOH was added to allow full solubilization (pH ≈ 5.0-5.5). After addition of 500 µL of a 0.511 M $MnCl_2.4H_2O$ stock solution (0.95 eq.), the pH was adjusted to 5.5-6 by carefully adding small aliquots of 1M NaOH. The reaction mixture was stirred at RT for 30 minutes and the solvent was then removed *in vacuo* to yield the complex as a white solid. Molecular weight calculated for $C_{14}H_{18}MnN_2O_8$: 397.24. High-resolution MS (FT-ICR, negative), m/z: 398.05233 ($[M+H]^+$; calculated: 398.05274), 420.03537 ($[M+Na]^+$; calculated: 420.03468).

3.2. Radiosynthesis of n.c.a. $[[^{52g}Mn]Mn(CDTA)]^{2-}$

CDTA monohydrate (12 mg, 32 µmol) was dissolved in 1 M NaOAc buffer (0.5 mL, pH 6) in a glass vessel. A solution of $^{52g}MnCl_2$ (≈ 8 MBq) in 100 µL water was added and the mixture was stirred at RT and monitored by radio-TLC (see paragraph 3.4.; exposure time on the imager: 30 min). The reaction was complete within 30 min.

Note: To obtain a ligand-free sample of $[[^{52g}Mn]Mn(CDTA)]^{2-}$ for blood serum incubation tests, a low amount of ligand (0.5 mg, 1.33 µmol) was radiolabeled with ≈ 600-700 kBq $^{52g}MnCl_2$ in a total volume of 150 µL NaOAc buffer (1M, pH 6). This allowed for convenient HPLC purification using an analytical RP-column (Synergi 4u Hydro RP 80Å) and PBS/EtOH 99.5:0.5 (pH 6.7) as mobile phase. The pure radiocomplex (≈ 316 kBq) was recovered in approx. 2 mL eluent. This solution can be stored for several days at room temperature: no manganese was released from the complex after > 72 h as demonstrated by RP-HPLC (Fig. S1).

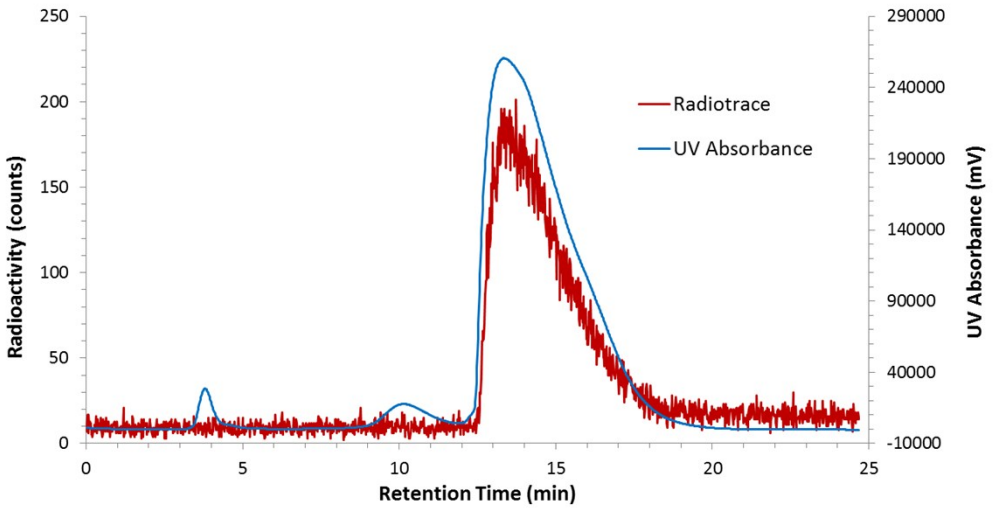


Fig. S1 RP-HPLC chromatogram of the HPLC-purified [[^{52g}Mn]Mn(CDTA)]$^{2-}$ fraction after > 72 h storage at RT co-injected with non-radioactive Mn-CDTA. If some manganese would have been released from the radiochelate, a radioactive signal would be observed at $R_t \approx 4$ min. The mobile phase used (PBS/EtOH 99.5:0.5, pH 6.7) was not the same as for the HPLC analysis depicted in Fig. 2 of the main text, which explains the lower retention time observed here for Mn-CDTA. The two low-intensity U.V. signals between $R_t \approx 3$-11 min were clearly attributed to impurities that are present in the co-injected non-radioactive Mn-CDTA sample. Thus, the above chromatogram also demonstrates the purity of the isolated n.c.a. [[^{52g}Mn]Mn(CDTA)]$^{2-}$ fraction.

3.3. Radiosynthesis of c.a. [[^{52g}Mn]Mn(CDTA)]$^{2-}$

CDTA monohydrate (12 mg, 32 µmol) and 0.1 equivalent of a 15 mM $^{55}MnCl_2.4H_2O$ stock solution (220µL, 3.2 µmol) were dissolved in 1 M NaOAc buffer (0.3 mL, pH 6) in a glass vessel. A solution of $^{52g}MnCl_2$ (≈ 2 MBq) in 50 µL water was added and the mixture was stirred at RT and monitored by radio-TLC (see paragraph 3.4.; exposure time on the imager: 30 min). The reaction was complete within 30 min.

3.4. Radio-TLC procedure for radiosynthesis monitoring

Radio-TLC was carried out on silica TLC plates (40 x 80 mm). 2x2 µL (or 3x2 µL) of the reaction solution were spotted on the baseline of the plate (about 1 cm above the lower end; blue spots in Figure S2).

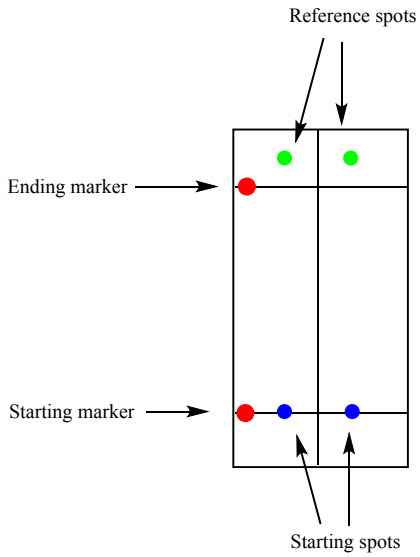


Fig. S2 Schematic picture showing TLC handling and spot placement.

After drying, the plate was transferred into a TLC chamber containing 2 mL of the mobile phase and left standing until the solvent has migrated until 1 cm below the end line. The plate is removed from the chamber and both the starting and the ending point were tagged with 2 µL of the reaction solution (red spots in Fig. S2). Additionally, reference points were spotted 0.5 cm above the solvent front in line with the original starting point (green spots in Fig. S2). After drying of these new spots, the plate was covered with Parafilm and measured on the Instant Imager for 30 min. In Fig. S3, a typical radio-TLC of an n.c.a. radiolabeling reaction (spotted 3 times) and the corresponding profile are shown. The absence of radioactive spots on the baseline of the TLC plate indicates that no free $^{52g}Mn^{2+}$ ions remained in the reaction mixture.

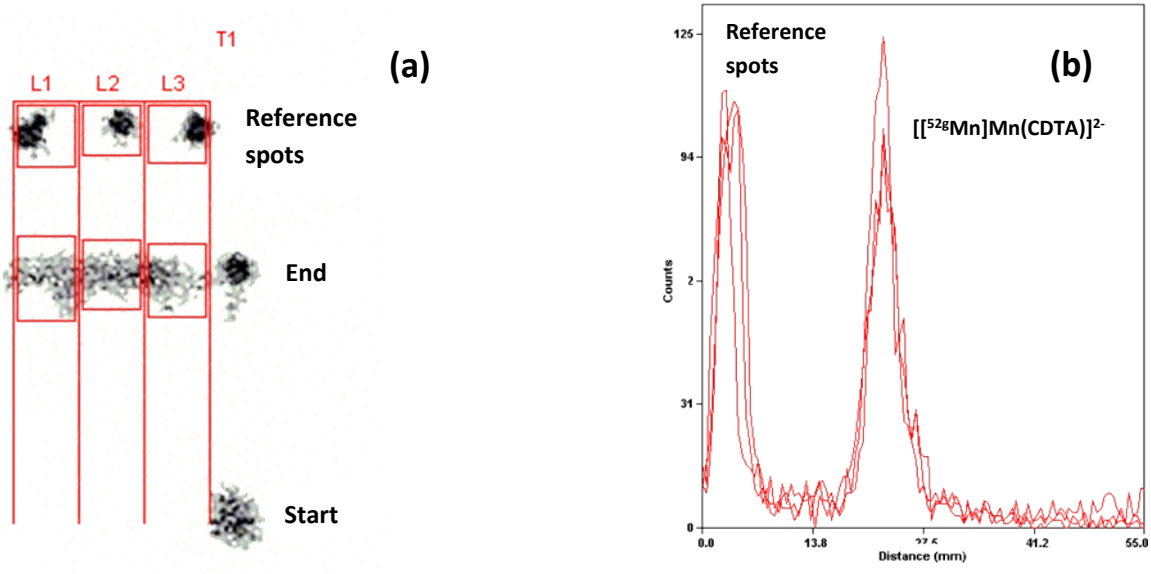


Fig. S3 (a) Radio-TLC of a $[[^{52g}Mn]Mn(CDTA)]^{2-}$ radiosynthesis mixture after 30 min (spotted 3 times); (b) corresponding radio-TLC profile.

The radiochemical yield (RCY) was determined, assuming that the spots observed after migration contain, in total, the same amount and especially the same ratio of activity as the reference spot (green in Figure S2) which was applied in equal volume on the plate after migration. Based on the radio-TLC profile (Figure S3b), the radiochemical yield was obtained by calculating the ratio between the radioactivity integral of the $[[^{52g}Mn]Mn(CDTA)]^{2-}$ spot and of the reference spot times 100. In the present case, the radiochemical yield was > 99%.

4. Stability monitoring in human blood serum (HBS)

A blood serum pool was prepared from whole blood samples of 4 adult humans (taken with NH_4-heparin test tubes from Sarstedt (Nümbrecht, Germany). The samples were centrifuged for 10 min with a frequency of 3000 min^{-1} at RT and the overlaying serum carefully removed with an Eppendorf pipette. The serum samples were mixed and stored at -20 °C until use.

4.1. Incubation of $^{52g}MnCl_2$ in HBS

To verify the propensity of Mn^{2+} ions to be bound by blood components, $^{52g}MnCl_2$ was incubated for 30 min and 2 days in HBS at 37 °C. For each time point, one Eppendorf vial was filled with HBS (0.5 mL) and spiked with $^{52g}MnCl_2$ (≈ 635 kBq and 1200 kBq, respectively). The vials were placed on an Eppendorf Thermomixer and gently shaken at 37 °C. After 30 min and 2 days, one incubation sample was diluted with 1 mL PBS and injected onto the SE-HPLC column. As shown by the chromatogram in Fig. S4a, part of the manganese was already taken up by macromolecules and smaller blood components after 30 min. After 2 days, all bound manganese seemed to be incorporated into larger molecular structures (Fig. S4b). The broad peak observed in both chromatograms around R_t ~ 20 min corresponds to free $^{52g}Mn^{2+}$ (Fig. S5).

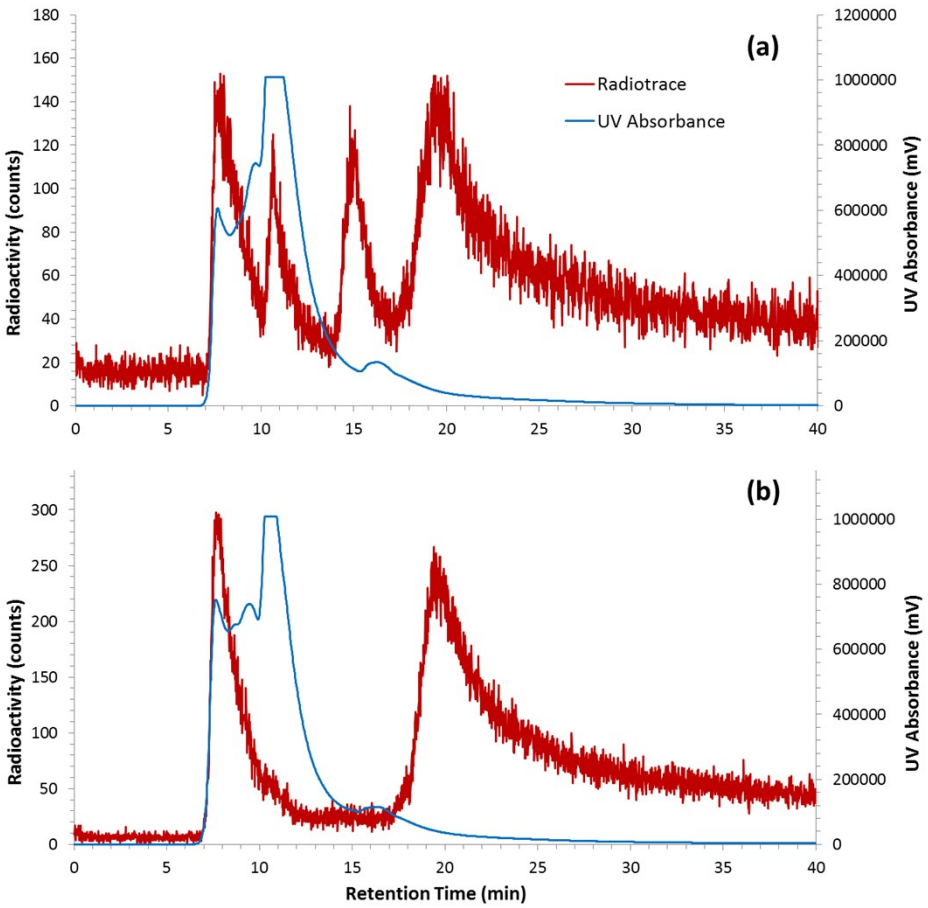


Fig. S4 UV and radioactivity SE-HPLC chromatograms obtained after incubating $^{52g}MnCl_2$ in HBS for (a) 30 min and (b) 2 days (37 °C). The radioactivity peaks between 7-17 min in (a) and 7-12 min in (b) correspond to $^{52g}Mn^{2+}$ bound by blood components.

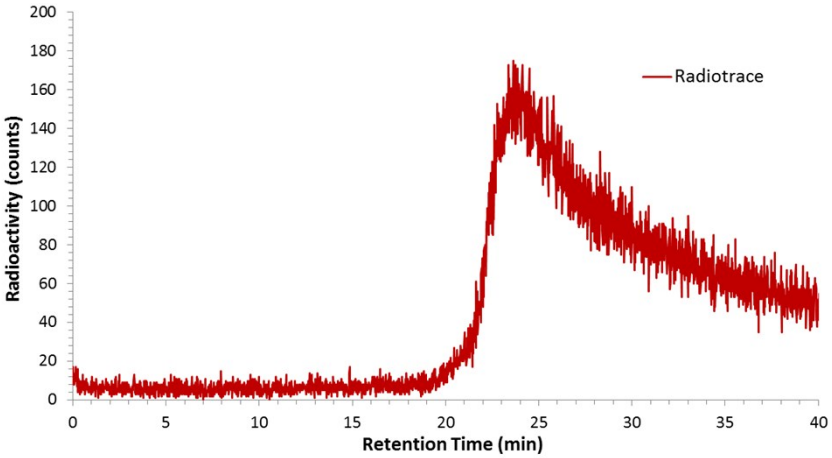


Fig. S5 SE-HPLC chromatogram of $^{52g}MnCl_2$. The retention time for $^{52g}Mn^{2+}$ is slightly lower than in Fig. S4, which is attributed to partial column overloading when blood samples are injected. Nonetheless, in both figures, the characteristic tailing confirms that this peak corresponds to free $^{52g}Mn^{2+}$.

4.2. Incubation of [[^{52g}Mn]Mn(CDTA)]$^{2-}$ in HBS

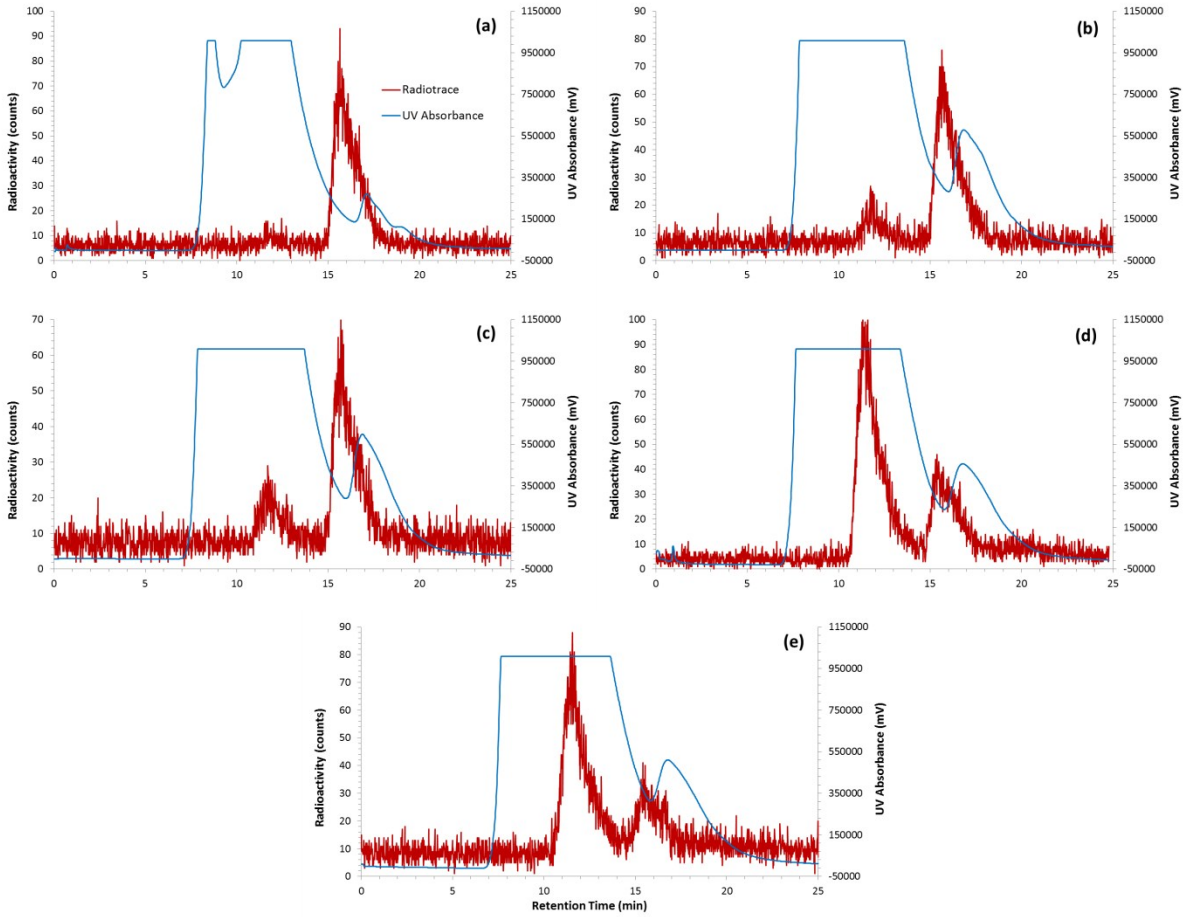
To be able to monitor the stability of a radiocomplex in blood serum, it is of prime importance to work with a pure, ligand-free sample of the latter. Indeed, any trace of non-labelled ligand could "re-chelate" free radiometal ions that were released from the complex. In addition, free ligand may also chelate Cu^{2+} ions present in blood serum, thereby eliminating one potential source of radiocomplex dissociation. For those reasons, pure n.c.a. [[^{52g}Mn]Mn(CDTA)]$^{2-}$ was isolated by RP-HPLC (see paragraph 3.2) before starting the incubation experiments.

4.2.1. First trials : acetonitrile precipitation and centrifugal filtration

Before SE-HPLC was proven to be the method of choice for stability monitoring, isolation of [[^{52g}Mn]Mn(CDTA)]$^{2-}$ from the blood samples was attempted by acetonitrile precipitation of proteins as well as by their removal using centrifugal filtration (Amicon Ultra-0.5 mL centrifugal filters 3K and 30K, Merck, Darmstadt, Germany). In both cases, gamma-counting indicated that around 10 to 30% of the total radioactivity was found within the protein fraction, potentially suggesting that some $^{52g}Mn^{2+}$ was released from the complex and bound by the blood macromolecules. Surprisingly, with each method, this same result was obtained regardless of the incubation time in blood serum. Given the absence of any significant increase of "protein-bound"-radioactivity over time, which would be expected for a decomplexation process, those data appeared inconclusive or even misleading.

4.2.2 Size-exclusion HPLC

The stability of n.c.a. [[^{52g}Mn]Mn(CDTA)]$^{2-}$ in HBS at 37 °C was evaluated after 1, 3, 5, 18 and 24 h.[a] For each time point, two Eppendorf vials were filled with HBS (0.5 mL) and spiked with a purified fraction of the radiocomplex (100 µL, ≈ 16 kBq). All 10 Eppendorf vials were placed on an Eppendorf Thermomixer and gently shaken at 37 °C. At each time point, 1 sample was diluted with 1 mL PBS and injected twice into the SE-HPLC column: radioactivity signals eluted after ≈ 12 and 16 min correspond, respectively, to protein-bound ^{52g}Mn and [[^{52g}Mn]Mn(CDTA)]$^{2-}$ (Fig. S6). The second sample was loaded onto an Amicon Ultra-0.5 mL 3K centrifugal filter and centrifuged for 30 min at 14000 rpm to get rid of the proteins. The obtained filtrate was then co-injected with non-radioactive Mn-CDTA onto a RP-HPLC column. As shown on Fig. S7 for an 18 h incubation sample, co-elution of U.V. and radioactivity signals unequivocally confirms that the radioactivity peak observed at R_t ≈ 16 min in SE-HPLC corresponds to intact [[^{52g}Mn]Mn(CDTA)]$^{2-}$. Moreover, the absence of any other radioactive signal in the RP chromatogram underlines that no other small molecule manganese complexes were formed with blood components.



[a] As no significant difference in urinary excretion was observed 1 h post-injection of n.c.a. and c.a. [[^{54}Mn]Mn(CDTA)]$^{2-}$ in mice, the n.c.a. and c.a. ^{52g}Mn chelates are expected to behave very similarly *in vivo* (*D. Fornasiero, J. C. Bellen, R. J. Baker and B. E. Chatterton, Invest. Radiol., 1987,* ***22****, 322-327).*

Fig. S6 SE-HPLC chromatograms obtained for [[^{52g}Mn]Mn(CDTA)]$^{2-}$ samples after different incubation times in HBS at 37 °C: (a) 1 h, (b) 3 h, (c) 5 h, (d) 18 h, (e) 24 h. Non-smoothed, original radiotraces are shown. Although two consecutive HPLC runs were performed at each time point, only one chromatogram is shown here.

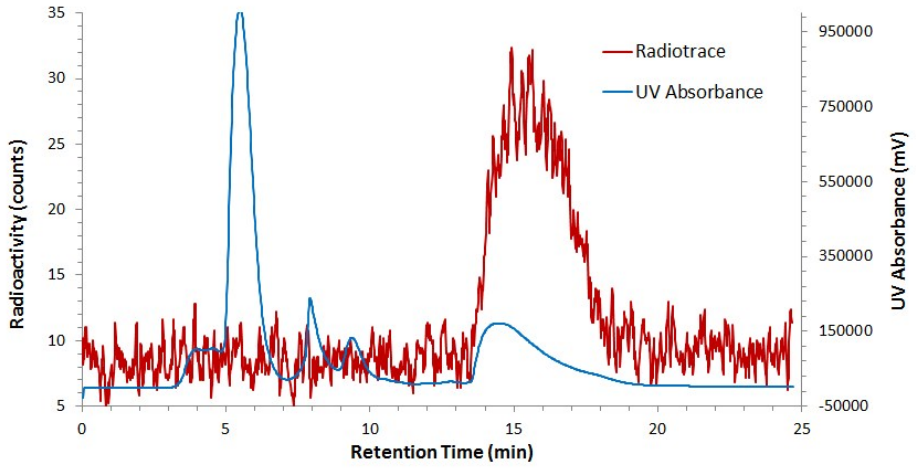


Fig. S7 RP-HPLC chromatogram of the co-injection of [[^{52g}Mn]Mn(CDTA)]$^{2-}$ incubated 18 h in blood serum and of non-radioactive Mn-CDTA. The radiotrace corresponds to a simple moving average curve (order = 5). The mobile phase used for HPLC analysis (PBS/EtOH 99.5:0.5, pH 6.7) was not the same as for the chromatograms depicted in Fig. 2 of the main text which explains the lower retention time observed here for Mn-CDTA. The U.V. signals between 4 and 10 min may be attributed to non-macromolecular blood components that could not be eliminated by centrifugal filtration.

5. Relaxivity measurements

The relaxivity (r_1, mM^{-1}s^{-1}) is the longitudinal water proton relaxation rate ($1/T_1$) enhancement obtained with a 1 mM concentration of paramagnetic metal complex:

$$r_1 = \frac{\left(1/T_1\right) - \left(1/T_{1,water}\right)}{[paramagnetic\ agent]}$$

The relaxivity value for [Mn(CDTA)]$^{2-}$ was determined by plotting the reciprocal longitudinal relaxation time, corrected for the water contribution ($1/T_1 - 1/T_{1,water}$), as a function of the complex concentration (range : 0.3 – 2 mM; samples were prepared by dissolving the complex in HEPES buffer, pH 7.4). In practice, the T_1 values were measured on a Bruker NMS 120 Minispec apparatus (20 MHz, 25 °C) using the inversion recovery method (180° – τ – 90°) by averaging 3 data points obtained at 10 different τ values. The slope of the obtained least square fit corresponds to the relaxivity of the complex.

The manganese concentration of each sample was measured by ICP-MS.

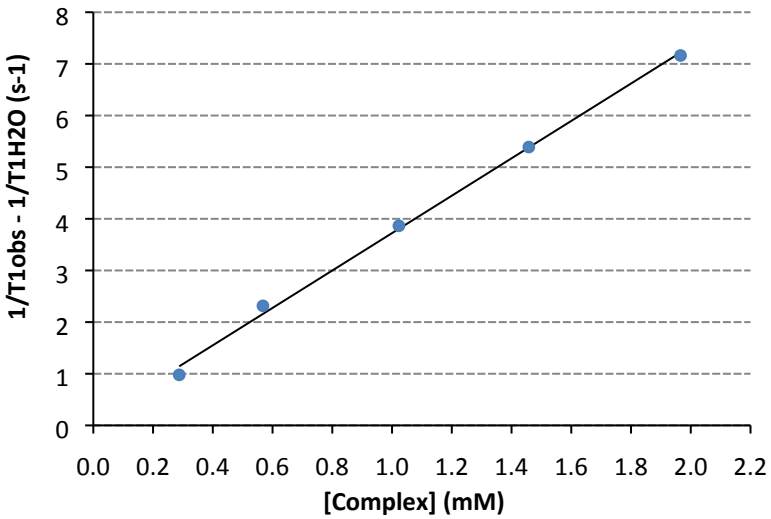


Fig. S8 Relaxivity determination of a freshly dissolved $[Mn(CDTA)]^{2-}$ sample (20 MHz, 25°C, pH = 7.4).

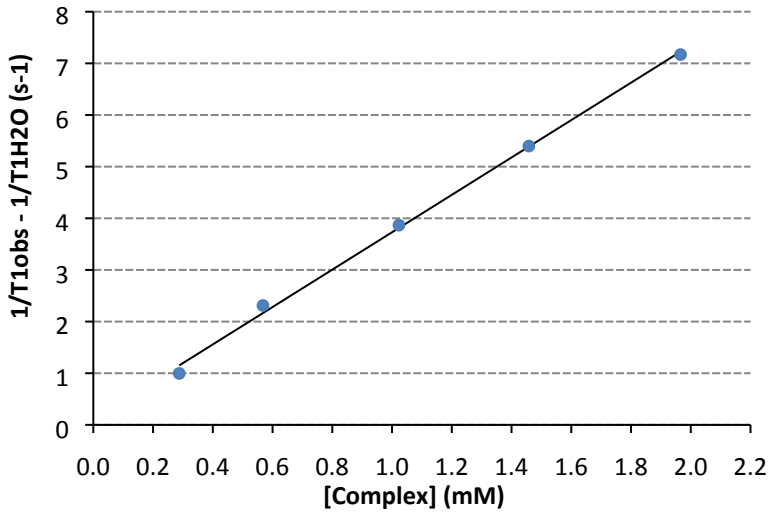


Fig. S9 Relaxivity determination of a $[Mn(CDTA)]^{2-}$ solution that was stored for 7 months at room temperature (20 MHz, 25°C, pH = 7.4).

6. References

1. M. Buchholz, I. Spahn, B. Scholten and H. H. Coenen, *Radiochim. Acta*, 2013, **101**, 491-499.