A paramagnetic contrast agent with myeloperoxidase-sensing properties.

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Synthesis of compounds L₁ and L₂.

To a solution of freshly prepared DTPA-bis(anhydride) (0.357g, 1mmol) in dry DMF (30mL) containing 2.2 eq. of dry Et₃N, serotonin (L₁ synthesis) or tyramine (2.2 eq.) (L₂ synthesis) were added, and the resulting mixture was stirred at room temperature for 48h. Upon completion, the solvent was removed in vacuo and the crude mixture was solubilised in 50mL of 5% NaHCO₃ aqueous solution (50mL). The basic solution was washed twice with 25mL of methylene chloride and evaporated again to give a white powder. The previous powder was solubilised in MeOH and precipitated by slow acetone diffusion. This procedure was repeated three times to afford compounds 1 and 2 with yields of 87% and 73% respectively.

**Compound 1:** ¹H NMR (DMSO-d₆, 400 MHz), δ (ppm): 2.61 (4H, t, J=7.2Hz), 2.82 (4H, t, J=7.2Hz), 3.06 (4H, m), 3.17 (4H, s), 3.19 (4H, s), 3.21-3.23 (4H, m), 3.39 (2H, s), 6.71 (4H, d, J=8.4 Hz), 7.02 (4H, d, J=8.4Hz), 9.37-9.39 (2H, m). FAB-MS: 632, [M+H]+.

**Compound 2:** ¹H NMR (DMSO-d₆), δ (ppm): 2.70 (4H, t, J=7.6Hz), 3.10 (4H, m), 3.21 (4H, s), 3.24 (4H, s), 3.29-3.31 (4H, m), 3.46 (2H, s), 6.56 (2H, dd, J₁=6.4 Hz, J₂=2.2 Hz), 6.93 (2H, d, J=2.1 Hz), 7.02 (2H, d, J=2.1 Hz), 7.09 (2H, d, J=8.6 Hz), 8.42-8.45 (2H, m), 10.05 (2H, s). FAB-MS: 710, [M+H]+.

Synthesis of the Ln(III) complexes.

0.08 mmol of either compound 1 or 2 were dissolved in 20 mL of distilled water containing 1% of citric acid. LnCl₃ anhydrous (1.05 eq.) was added and the solution brought to pH 7 with 0.5M NaOH solution. The mixture was stirred in dark and under argon for 72 h. After this period, the mixture was lyophilized and resolubilised in 1.5 mL of distilled water. The complexes were isolated by HPLC (Protein and Peptide C-18 column, Vydac, s/n e950622-2-1) using a gradient of acetonitrile in water.

MALDI-TOF.
Solutions 50mM of L₁-Eu³⁺ and L₂-Eu³⁺ were incubated in the presence of either HRP or MPO (20 iU) and 10mM H₂O₂ at 40°C for 2 hours. The solutions were then freeze-dried and solubilised using a mixture 0.1-50-50 v/v TFA-acetonitrile-deionised water. 1µL of these solutions were deposited onto MALDI plates and 0.5µL of 10mg/mL DHB solution was added. MALDI-TOF acquisitions were performed in linear mode with delayed extraction mode and positive polarity. In preliminary experiments a molecular weight range from 400Da to 10000Da was used for scanning. Final spectra were acquired in a range from 400Da to 10000Da. A minimum of 400 laser shots was required.

**Stock solutions for relaxivity measurements.**

Stock solutions of compounds L₁-Gd³⁺ and L₂-Gd³⁺ were prepared by dissolving the corresponding complexes in DPBS buffer solution (pH 7.4) and were kept at -4° in the dark. Every stock solution was titrated by using ICP-MS data. Myeloperoxidase was purchased from Biodesign Intl. (Saco ME, USA) and was used as received; MPO activity was measured as described by Klebanoff, S. J.; Waltersdorph, A. M.; Rosen, H.; Methods Enzymol. Vol. 105, p 399-403.

**MR imaging and relaxometry parameters:**

Relaxation times at 0.47 T were measured using Bruker NMS-120 Minispec spectrometer by using an inversion recovery sequence, with a TR=5T₁, and 15 TI points evenly spaced up to 5T₁.

Relaxation times at 1.5 T were measured on 1.5 T GE Signa MRI scanner utilizing an inversion recovery sequences with TR=2000 ms, TI=50, 75, 100, 150, 200, 250, 300, 350, 400, 500, 625, 750, 1000 ms. A fixed-area region of interest was then drawn to obtain the signal intensity of the samples.

The data obtained at 0.47 T and 1.5 T were fitted by using the equation $I_x = I_0 \ast (1-2\exp(T/T_1))$ to obtain T₁, where $I_x$ is the signal obtained at the time point T₁, with I₀ (total signal or magnetization) and T₁ being fitted values.
Matrigel Implantation:

MPO/Matrigel imaging experiments: Matrigel™ (Ewing’s sarcoma basement membrane matrix extract, Beckton-Dickinson), was used to implant and immobilize human MPO and glucose oxidase in the right extremity of each mouse for MR imaging. The left extremity implant mixture contained no enzyme and served as an internal control. 400 µL of Matrigel™ (Beckton-Dickinson) mixture and MEM (Cambrex) were injected into the thighs of each mouse one hour prior to imaging. The right thigh mixture also contained 15 U of MPO(3 iU/mg protein) and 4 iU of glucose oxidase (Calbiochem), while the left thigh mixture contained no enzyme to serve as an internal control.

MR imaging:

MR imaging experiments were performed by using 1.5 T GE Signa MRI unit. For all MR imaging experiments, pre-contrast T2- (TR=2000, TE=100, ETL=8, NEX=4, fast spin echo sequence) and T1- weighted images (TR=500, TE=11, NEX=4, spin echo sequence) with fat saturation were initially performed to locate the implants. The substrates (0.3 mmol/kg) were then injected via the tail vein of the mouse. The mouse was immediately imaged after the injection of the contrast agent using multiple T1-weighted sequences with fat saturation for at least 3 hours.
Substrate binding to plasma components:

Human plasma was extracted from healthy donors by the use of Polymorphprep (Accurate Chemicals) and centrifugation according to the manufacturer’s instructions. Aliquots of 3 mM solution of compound L₁-Gd³⁺ was mixed with aliquots of L₁⁻¹¹¹In as a tracer, with or without human plasma and with and without MPO/H₂O₂. These solutions were loaded on Bio-Rad Biospin P-6 minicolumns and plasma was separated according to the method suggested by the manufacturer. Radioactivity retained in the columns and the eluted material were counted separately in 1480 Wizard gamma counter (Perkin-Elmer) and the fraction of radioactivity bound to proteins was calculated (n=3 independent experiments).

Dialysis experiment:

Solution A: 1mL of a solution 0.5 mM of compound L₁-Gd³⁺ in PBS buffer containing 5.5 mM of glucose was supplemented with MPO (0.5 iU), GOX (0.25iU)-glucose.

Solution B: 1mL of a solution 0.5 mM of compound L₁-Gd³⁺ prepared in the presence of 40mg/mL of HSA.

Solution C: 1mL of a solution 0.5 mM of compound L₁-Gd³⁺ in PBS buffer containing 5.5 mM of glucose and 40mg/mL of HSA was supplemented with MPO (0.5 iU), GOX (0.25iU)-glucose.

The solutions were incubated at 40°C for 18 hours. After the incubation period, 400 µL of these solutions were injected into dialysis cassettes with 10.000 Mw pore cut-off (Pierce, Slide-A-Lyzer 10K). The samples where dialyzed with two changes in PBS buffer after 4 hours and then overnight. The contents of the cassettes was removed and lyophilised. The lyophilisate was re-solubilised with 400 µL of buffer. The same procedure was applied to 400 µL of the initial solution prior dialysis. 200µL aliquots of the previous solutions were submitted to gadolinium content analysis.

SPECT-CT imaging.

Indium-111 labelled L₁ was prepared by trans-chelation from¹¹¹In-oxiquinoline complex. To prepare L₁⁻¹¹¹In, 50 µg DTPA-diSer (1 mg/ml) were mixed with 1 mCi¹¹¹In-oxiquinoline solution in 0.7-1.0 ml saline and incubated for 2 h under argon. Trans-chelation and purity was controlled by HPLC (Discovery C-18 column, 25x3 mm, Supelco) using 0-55% gradient of acetonitrile in water.

80-100 µCi of L₁⁻¹¹¹In in 200 µL of saline was injected intravenously into three mice prepared in the same manner as in the MPO/Matrigel MR imaging experiments. The animals were imaged 3 hours after injection on a high resolution X-SPECT/CT system (Gamma Medica). Six hours after injection, the animals were sacrificed and biodistribution was determined in major organs. The % dose accumulated per gram of major organs was determined by using the 1480 Wizard gamma counter (Perkin-Elmer).
A pie-graph showing biodistribution of the residual radioactivity (expressed as % injected dose/g) in animals 6 hours after the intravenous administration. Median values obtained in n=3 animals are shown.