Supporting Information

A Cationic Gadolinium Contrast Agent for Magnetic Resonance Imaging of Cartilage

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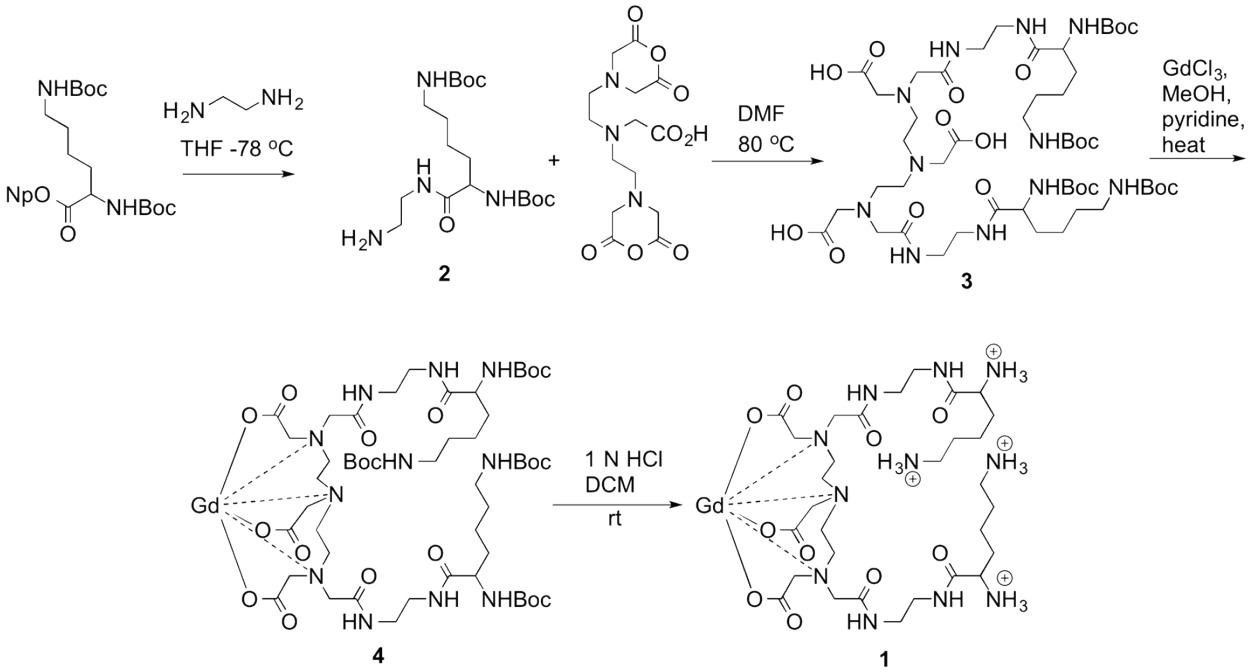
Experimental Section

Synthesis



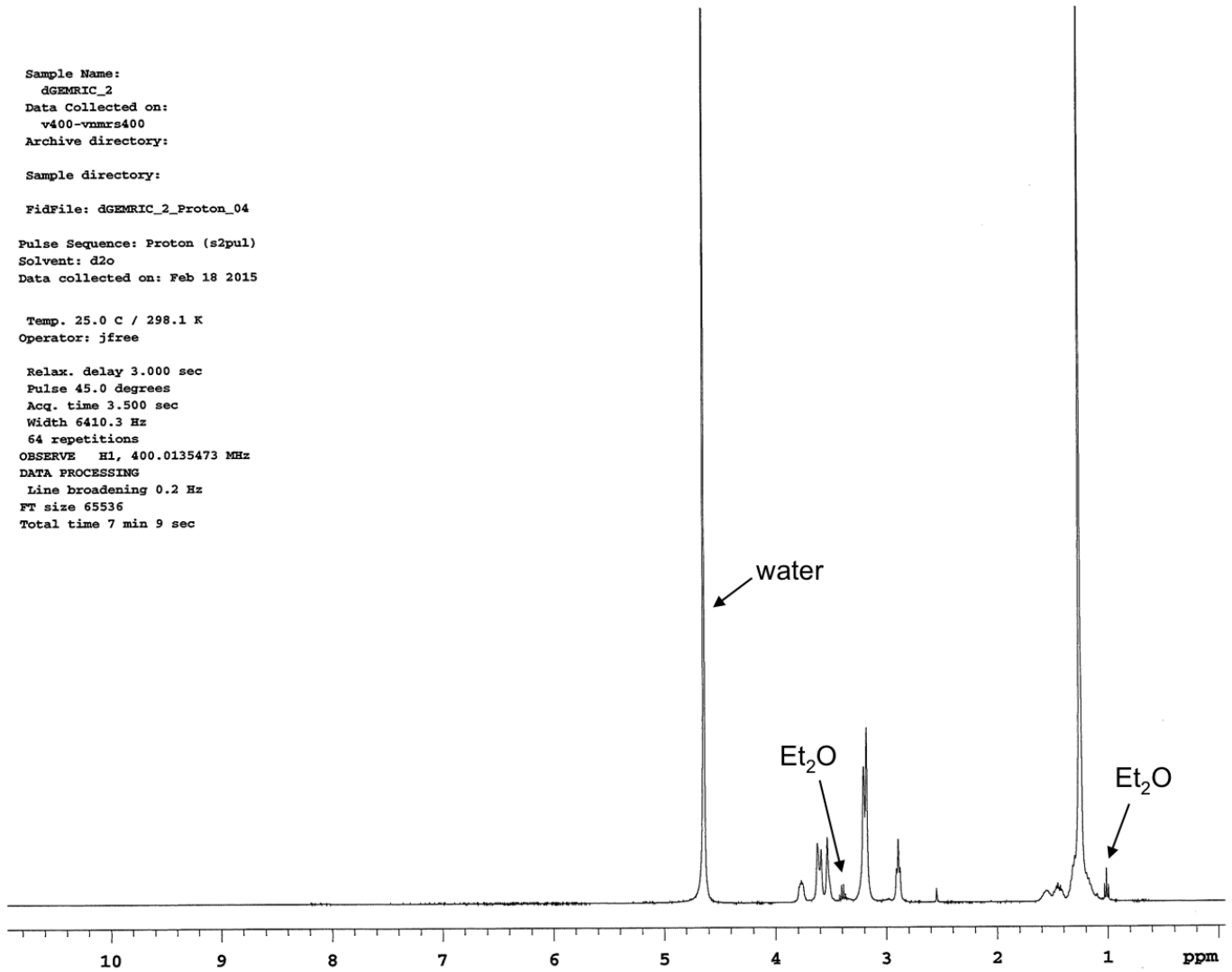
***Tert*-butyl 6-(2-aminoethylamino)-6-oxohexane-1,5-diyldicarbamate (2).** To a solution of dry THF (50 mL) at −78 °C, containing ethylene diamine (1 g, 16.7 mmol), was added Boc-Lys(Boc)-ONp (1.5 g, 3.3 mmol) dropwise over 1 h, dissolved in dry THF (10 mL). The reaction was allowed to proceed for 1 hr upon which it was taken up in ethyl ether, and washed with brine, and brine/1 N NaOH solution several times, until the yellow color is removed from the ether layer. The ether layer was dried over Na_2SO_4, filtered and volatiles evaporated. Compound **2** was purified by column chromatography (CH_2Cl_2/MeOH/NH_4OH 85:15:1) as a colorless oil that became a white foam when agitated with minimal diethyl ether under vacuum (75% yield). Spectral analysis agreed with previously reported literature values.[1, 2]

10-[(*Tert*-butoxycarbonyl)amino)-24-{10-[(*tert*-butoxycarbonyl)amino]-2,2-dimethyl-4,11,16-trioxo-3-oxa-5,12,15-triazaheptadecan-17-yl}-18,21-bis(carboxymethyl)-2,2-dimethyl-4,11,16-trioxo-3-oxa-5,12,15,18,21,24-hexaazahexacosan-26-oic acid (3). The procedure was adapted from Debroye et al.[3] Compound **2** (500 mg, 1.28 mmol) was added to a solution of dry DMF (1.0 mL), containing 2-{bis[2-(2,6-dioxomorpholino)ethyl]amino}acetic acid (357 mg, 0.57 mmol). The reaction was heated to 80 °C for 8 h. After cooling, the solution was precipitated into 20 mL ethyl ether (repeated twice), filtered and the filtrand was collected and dried, affording **3** in 93% yield. ^{1}H NMR (400 MHz, D_2O): δ = 1.26-1.51 (m, 44 H), 1.57-1.65 (m, 2 H), 1.68-1.78 (m, 2 H), 3.05 (t, *J* = 8.0 Hz, 4 H), 3.28-3.39 (m, 16 H), 3.70 (br s, 4 H), 3.75 (br s, 2 H), 3.78 (br s, 4 H), 3.89-3.96 (m, 2 H). ^{13}C NMR (100 Hz, D_2O): δ = 22.3, 27.6,

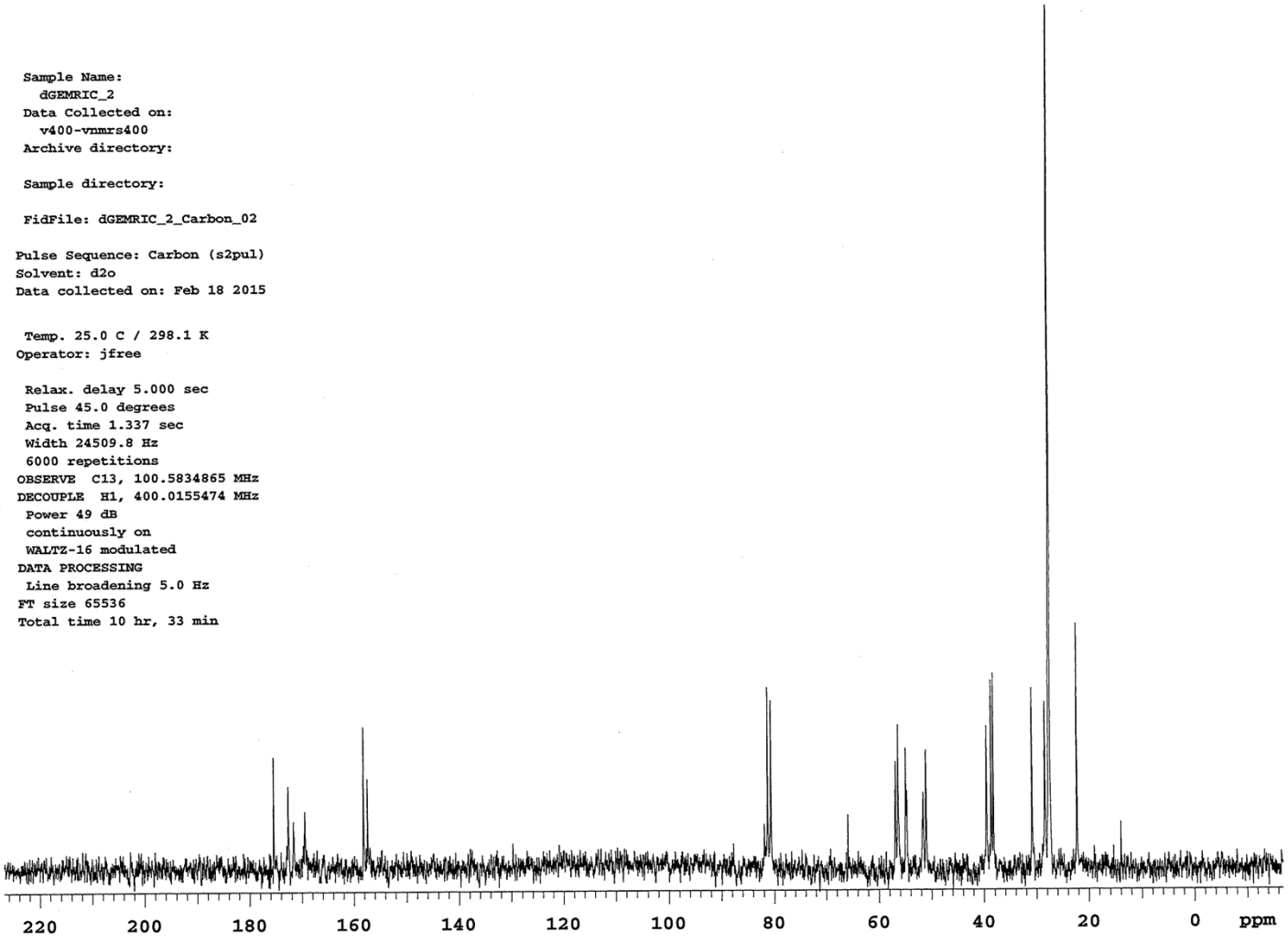
28.4, 30.9, 38.3, 38.7, 39.6, 51.1, 51.6, 54.8, 54.9, 56.4, 56.9, 80.7, 81.3, 157.4, 158.2, 169.4, 171.4, 172.6, 175.4. HRMS: *m/z* calcd for $C_{50}H_{91}N_{11}O_{18}$ $[M+H]^+$: 1134.6622; found: 1134.6636.

16-(*Tert*-butoxycarbonylamino)-2-[10-(*tert*-butoxycarbonylamino)-2,2-dimethyl-4,11,16-trioxo-3-oxa-5,12,15-triazaheptadecan-17-yl]-5,8-bis(carboxymethyl)-24,24-dimethyl-10,15,22-trioxo-23-oxa-2,5,8,11,14,21-hexaazapentacosane-1-carboxylic acid gadolinium chelate (4). Compound **3** (2 g, 1.4 mmol) was dissolved in dry pyridine (20 mL). To the solution was added dry MeOH until it turned clear, followed by the addition of $GdCl_3$ (480 mg, 1.8 mmol). The reaction was heated to reflux for 48 h. Upon cooling, the solution was precipitated into 200 mL ethyl ether, and filtered. The filtrand was dissolved in DI water and dialyzed for 4 days with frequent water changes in MW 500 tubing. The liquid was lyophilized affording **3** as white foam in 34% yield. (A large loss of material is observed during dialysis process, due to leakage of **3** through the membrane). HRMS: *m/z* calcd for $C_{50}H_{88}GdN_{11}O_{18}$ $[M+H]^+$: 1289.5625; found: 1289.5641.

16,20-Diamino-5,8-bis(carboxymethyl)-2-{2-[2-(2,6-diaminohexanamido)ethylamino]-2-oxoethyl}-10,15-dioxo-2,5,8,11,14-pentaazaicosane-1-carboxylic acid gadolinium chelate HCl salt (4). To a solution of **3** (1 g, 0.78 mmol) in dry DCM (15 mL) was added 4 N HCl in dioxane (5 mL). The reaction was allowed to proceed for 48 h, following which the volatiles were evaporated under vacuum. Subsequently, the residue was coevaporated with THF (3 × 30 mL) in order to remove residual HCl, and dried under high vacuum for 24 h. Finally, the residue was dissolved in MeOH (15 mL) and precipitated into ethyl ether (300 mL), and filtered. The solid was collected and dried, affording **1** as white powder in quantitative yield. HRMS: *m/z* calcd for $C_{30}H_{56}GdN_{11}O_{10}$ $[M+H]^+$: 889.3538; found: 889.3546.



^{1}H NMR spectrum for the 10-[(*tert*-butoxycarbonyl)amino]-24-{10-[(*tert*-butoxycarbonyl)amino]-2,2-dimethyl-4,11,16-trioxo-3-oxa-5,12,15-triazaheptadecan-17-yl}-18,21-bis(carboxymethyl)-2,2-dimethyl-4,11,16-trioxo-3-oxa-5,12,15,18,21,24-hexaazahexacosan-26-oic acid.



^{13}C NMR spectrum for the 10-[(*tert*-butoxycarbonyl)amino]-24-{10-[(*tert*-butoxycarbonyl)amino]-2,2-dimethyl-4,11,16-trioxo-3-oxa-5,12,15-triazaheptadecan-17-yl}-18,21-bis(carboxymethyl)-2,2-dimethyl-4,11,16-trioxo-3-oxa-5,12,15,18,21,24-hexaazahexacosan-26-oic acid.

HPLC Analysis

HPLC analysis was performed on Varian ProStar HPLC pump HPLC instrument with a Optilab DSP Interferometric Refractometer or Ranin Dynamax UV-1 Absorbance Detector at the characteristic gadolinium absorbance peak of 275 nm, equipped with a Hamilton Company reverse phase [HxSil C18, 5 µm, 4.6 x 250 mm] column, using water:acetonitrile gradient as mobile phase.

Cell Maintenance of NIH3T3 Murine Fibroblast Cells for Toxicity Studies

NIH3T3 murine fibroblast cells were maintained in Dulbecco's Modified Eagle Media supplemented with 10% bovine calf serum and 1% penicillin/streptomycin. Cells were maintained in a humidified atmosphere at 37 °C and 5% CO_2. Subconfluent cells were harvested and seeded on 96 well plates at 20,000 cells/well for use in *in vitro* cytotoxicity studies.

In vitro cytotoxicity study

NIH3T3 fibroblasts were allowed a 4 & 24 hour exposure to concentrations of 1.0 and 0.1 mM gadopentetate and Gd(DTPA)Lys_2, and 2.0 and 0.2 mM lysine in phosphate free DMEM (Catalog #: 11971-025, Life Technologies, Carlsbad, CA). Cells were maintained in a humidified atmosphere at 37 °C and 5% CO_2. After exposure, cell viability was tested using a colorimetric MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] cell proliferation assay (Sigma, St. Louis, MO) and absorbance read at 490 nm on a Beckman-Coulter AD 340 Plate Reader (Brea, CA). Cell viability in each well was calculated as the percentage of the positive control absorbance.

Bovine Osteochondral Plugs

Bovine osteochondral plugs (7 mm diameter; n = 3/group; 9 total) were cored with a diamond tipped coring bit (Part # 102080, Starlite Industries, Rosemont, PA) from the tibia and femur of freshly slaughtered 1-2 year old cows (Research 87, Boylston, MA).

MRI Parameters and Scanning Procedure

Each set of bovine plugs was potted in Poly(methyl methacrylate) and edges sealed with Krazy Glue (Elmer's Products, Columbus, OH). The plugs were immersed in 50 mL solutions of 0.1 mM and 1 mM Magnevist and 0.1 mM Gd(DTPA)Lys_2 (balanced to 400 ± 10 mOsm with sodium chloride) and continuously imaged on an 8.5 T magnetic resonance microimaging system using a 30 mm birdcage radiofrequency coil (Bruker Corporation, Billerica, MA). To measure the T1 relaxation parameter a Rapid Acquisition with Relaxation Enhancement (RARE) pulse sequence was used with the following parameters: repetition time = 40-5000 ms, effective echo time = 8 ms, echo train length = 2, number of averages = 1, acquisition matrix = 150 × 150, field-of-view (FOV) = 30 × 30 mm^2 yielding an in-plane resolution of 0.2 × 0.2 mm. A central 1.0 mm thick imaging slice perpendicular to the cartilage surface was acquired taking approximately 16.5 minutes per scan.

MRI Image Processing

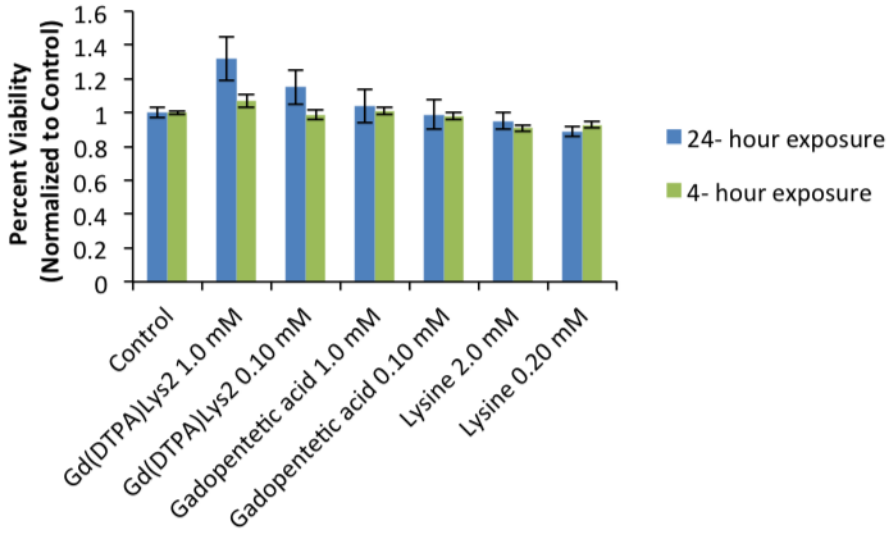
Cartilage and solution object maps were created and used to obtain serial T1 relaxation times with MRI Mapper (BIDMC, Brookline, MA) and Matlab2012b (MathWorks, Natick, MA).

Statistics

ANOVA and Tukey's Test were processed using Prism 6 (Graphpad Software Inc, La Jolla, CA).

Results

MTS Assay



SI Figure 1. Cytotoxicity after 24 and 4 hour exposures to 3T3 fibroblasts (n=3/group). Both **1** Gd(DTPA)Lys_2 and gadopentetic acid were found to be non-toxic (>90% viability) at 1.0 mM and 0.10 mM concentrations. Lysine (2.0 mM and 0.20 mM) was used as a negative control.

HPLC analysis of Gd(DTPA)Lys$_2$ (1)

Refractive index detector:

The results indicate that only one molecular species is present in the product. (blue = blank; green = **1**)

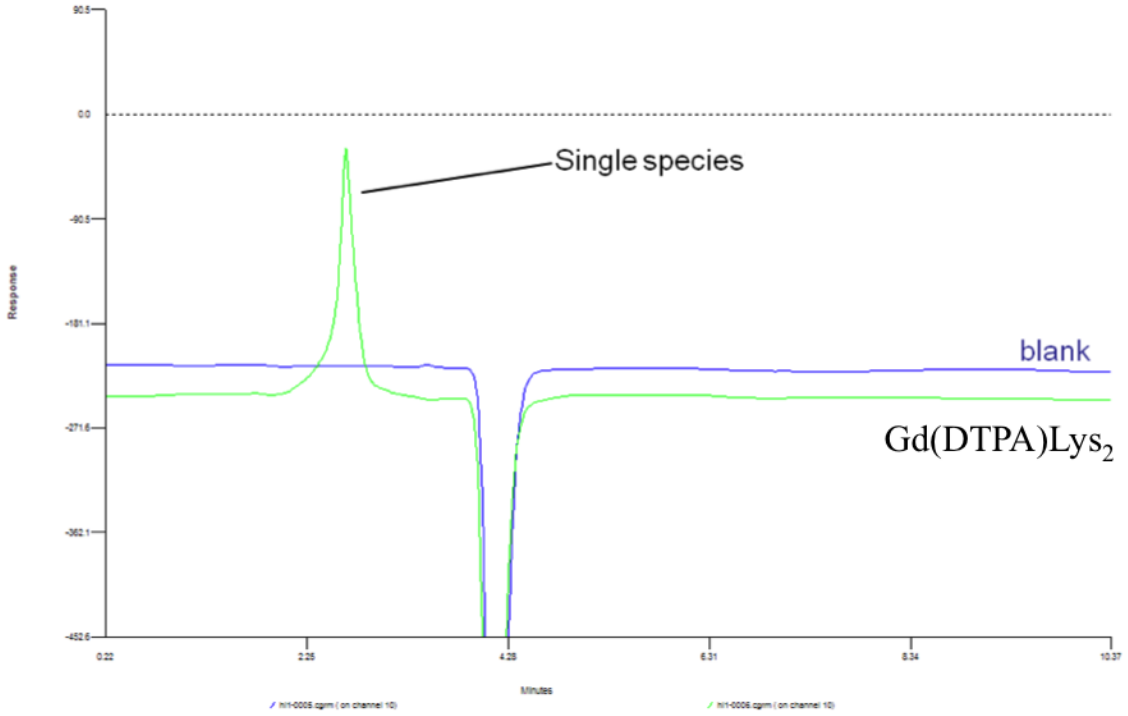


Figure SI-2a. HPLC Characterization by refractive index.

UV/VIS detector

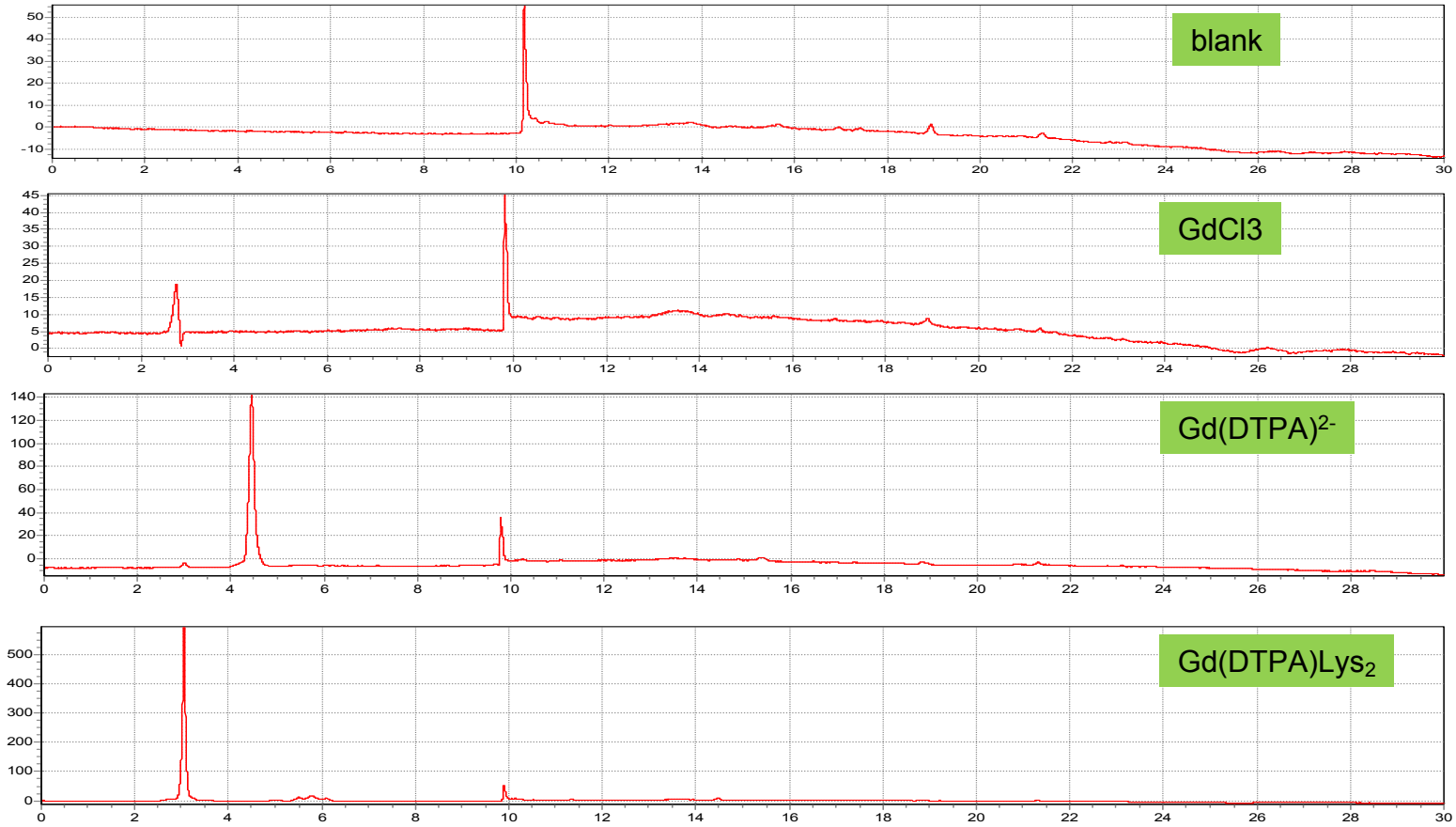


Figure SI-2b. HPLC Characterization by UV detection at 275 nm.

Uptake of Contrast Agents Normalized to Bath

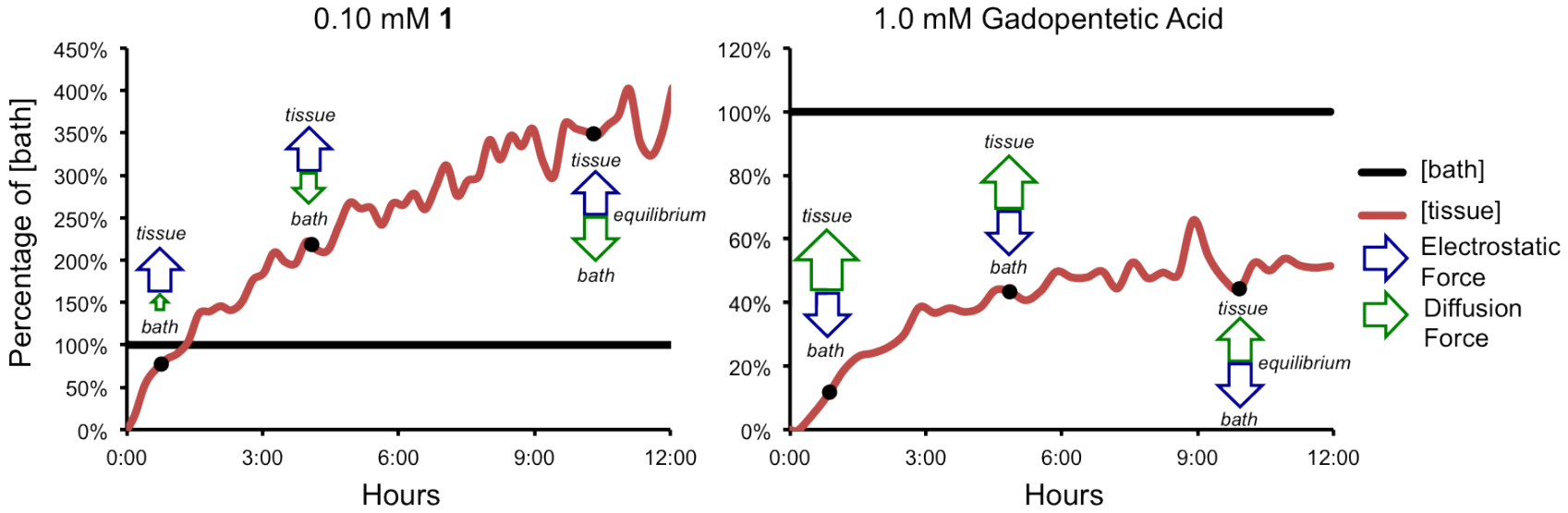


Figure SI-3. Uptake of 0.10 mM **1** (left) and 1.0 mM gadopentetic acid (right), from the *bath* (black) into the *tissue* (red). Size of arrows for electrostatic force (blue arrow) and diffusion force (green arrow) indicate magnitude and the arrows point toward direction of force (either towards bath or tissue). For **1**, an attractive electrostatic force (right facing blue arrow) continuously drives **1** from the bath towards the tissue and a diffusion force initially drives **1** from the bath towards the tissue (when the [bath] is greater than the [tissue]) and then drives **1** from the tissue back to the bath until equilibrium is reached. For gadopentetic acid, a diffusion force continuously drives gadopentetic acid from the bath towards the tissue (because the [bath] is always greater than the [tissue]) and a repulsive electrostatic force (left facing blue arrow) drives gadopentetic acid from the tissue back to the bath until equilibrium is reached. Error bars excluded for clarity.

Curve Fitting to the Exponential Decay Equation $f(t)= \alpha\, e^{-t/\tau} + \beta$

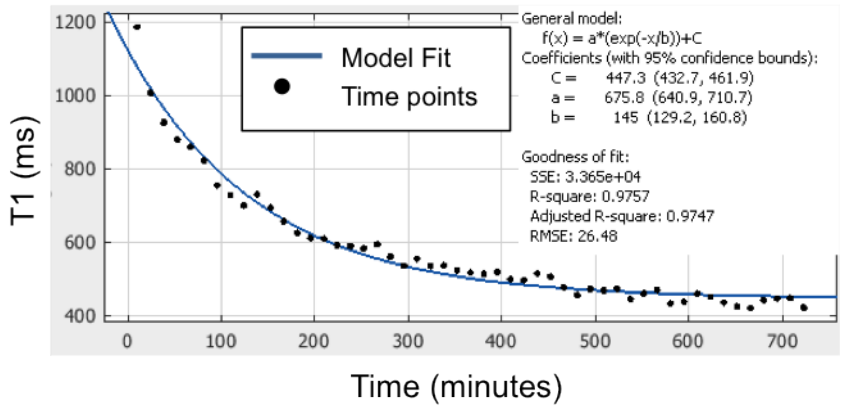


Figure SI-4a. Average T1 value of cartilage as 0.10 mM **1** diffuses into the osteochondral plugs (n=3). The time points were fit to an exponential decay curve.

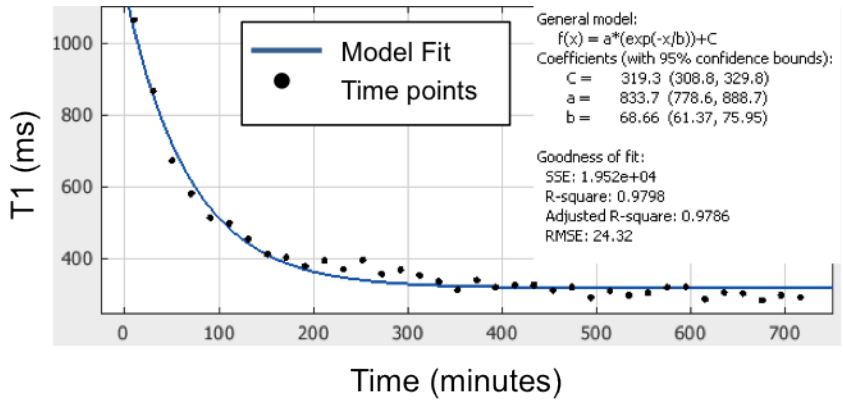


Figure SI-4b. Average T1 value of cartilage as 1.0 mM gadopentetic acid diffuses into the osteochondral plugs (n=3). The time points were fit to an exponential decay curve.

Diffusion out after exposure to 0.1 mM 1 and 1.0 mM $Gd(DTPA)^{2-}$

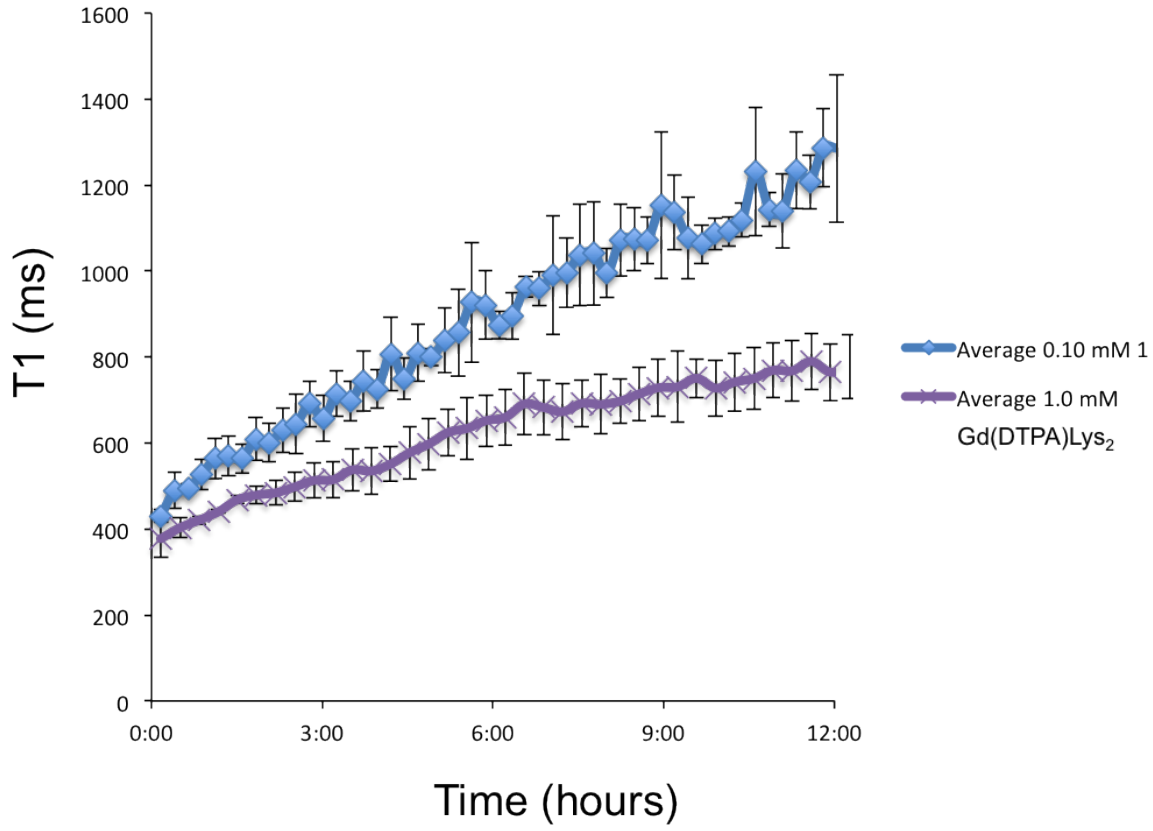


Figure SI-5. Diffusion out after exposure to 0.1 mM **1** and 1.0 mM $Gd(DTPA)^{2-}$

References

1. D. Appelhans, V. Stastny, H. Komber, D. Voigt, B. Voit, P. Lhotak and I. Stibor, *Tetrahedron Lett.*, 2004, **45**, 7145-7149.
2. S. Baer, J. Nigro, M. P. Madej, R. M. Nisbet, R. Suryadinata, G. Coia, L. P. T. Hong, T. E. Adams, C. C. Williams and S. D. Nuttall, *Org. Biomol. Chem.*, 2014, **12**, 2675-2685.
3. E. Debroye, S. V. Eliseeva, S. Laurent, L. Vander Elst, S. Petoud, R. N. Muller and T. N. Parac-Vogt, *Eur. J. Inorg. Chem.*, 2013, DOI: Doi 10.1002/Ejic.201300196, 2629-2639.
4. S. W. Bligh, A. F. Drake and P. J. Sadler, *Eur. J. Biochem.*, 1989, **181**, 223-224.
5. J. H. Kang, S. Choi, W. Lee and J. K. Park, *J. Am. Chem. Soc.*, 2008, **130**, 396-+.
6. L. Telgmann, M. Sperling and U. Karst, *Anal. Chim. Acta*, 2013, **764**, 1-16.