Supplementary Material (ESI) for Chemical Communications

Viral MRI Contrast Agents: Coordination of Gd by Native Virions and Attchment of Gd Complexes by Azide-Alkyne Cycloaddition

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Electronic Supplementary Information (ESI)

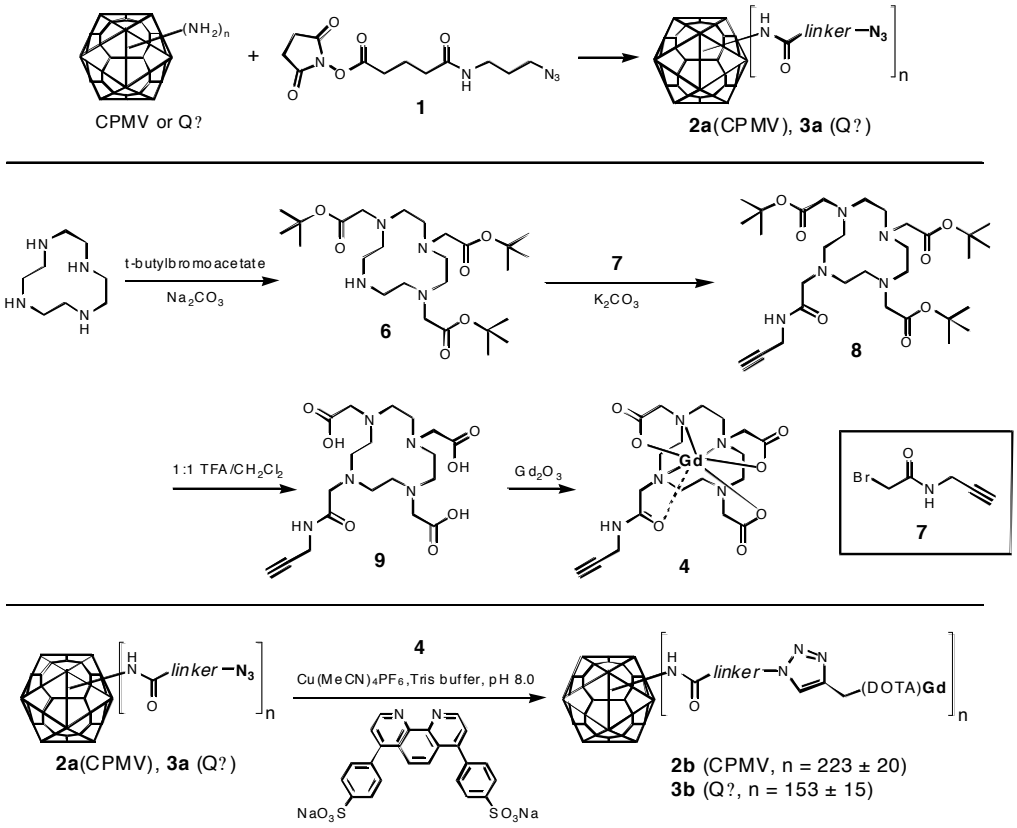
Materials and Instrumentation

Kits for Modified Lowry Protein Assay were purchased from Pierce. pQE-60 and M15 cells were purchased from Qiagen, while IPTG (dioxane-free, high purity) was obtained from Calbiochem. Metal standards for inductively coupled plasma optical emission spectrometry (ICP-OES) were purchased from Inorganic Ventures, Inc. All other chemicals were purchased from Acros Organics or Sigma-Aldrich and used as received. ^{1}H NMR spectra were measured at 200 MHz on a Varian Mercury 200 instrument. Mass spectra were taken using a HP 110 LC/MS spectrometer (model G1946A). UV-visible measurements were performed on a HP 845x UV-visible spectrophotometer. (ICP-OES) measurements were taken on a Varian VISTA AX CCD simultaneous spectrometer equipped with a Teflon nebulizer and sample uptake tubing. All standards and samples were spiked with an internal standard of yttrium at a final concentration of 10 ppm. FPLC analyses were performed with AKTA Explorer (Amersham Pharmacia Biotech) equipment, using Supersose-6™ size-exclusion columns. Ultracentrifugation was performed with a Beckman Optima L-90K Ultracentrifuge equipped with either SW41 or 50.2 Ti rotors. Magnetic resonance data were collected on a Bruker Avance 11.7T MRI (Bruker Biospin, Billerica MA), a Bruker Avance 4.7T (200 MHz) MRI, and a 1.5T (64 MHz) MRI imager (Magnetom SP400, Siemens Medical Systems, Erlangen, Germany).

Propagation and Isolation of Virus Particles

CPMV particles were produced in cowpea plants and isolated using previously published procedures.[1] The K16M Qβ coat protein gene was cloned into the vector pQE-60 and expressed under IPTG control in M15 cells. After expression, collected cells were lysed by sonication and lysozyme treatment and then centrifuged to remove insoluble cell components. Virus was precipitated from the resulting supernatant using 8% PEG 8000. Following further centrifugation, the isolated pellet was resuspended in 0.1M potassium phosphate pH 7.0. Virus was then purified by ultracentrifugation through 10-40% sucrose gradients. A more detailed procedure for expression and isolation of Qβ particles will be presented elsewhere, and may be obtained from the author. Note that we find that gradient ultracentrifugation purification is more reliable than size-exclusion "spin columns" previously employed (and still used for preliminary cleanup in some cases). CPMV concentrations were determined by absorbance at 260 nm (0.1 mg/mL virus sample gives an absorbance of 0.8). Qβ concentrations were determined using the Modified Lowry Protein Assay.[2]

Figure S1. Synthetic manipulations.



*Synthesis of Gd(DOTA) alkyne complex **4** (Figure S1)*

N-Propargylbromoacetamide (7). Aqueous NaOH (0.1 M, 40 mL) was layered on top of a solution of propargylamine (4.0 mL, 58.3 mmol) in 200 mL CH_2Cl_2. Bromoacetyl bromide (16.3 mL, 187 mmol) was carefully added by syringe into the CH_2Cl_2 layer, causing the immediate formation of a white precipitate. The reaction was then stirred vigorously for 2.5 hours at room temperature, during which time the precipitate redissolved. The reaction solution was transferred to a separatory funnel with excess CH_2Cl_2 and water. The aqueous layer was isolated and extracted with CH_2Cl_2 (2 x 100 mL). All organic fractions were combined and washed with 50 mM Na_2CO_3 (3 x 100 mL). The resulting solution was dried over $MgSO_4$ and reduced to dryness by rotary evaporation, yielding a brown oil. Storage overnight under vacuum at room temperature produced a tan solid (3.6 g, 35%). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 6.76 (br, 1H, NH), 4.09 (dd, 2H, $NHCH_2$), 3.90 (s, 2H, CH_2), 2.29 (t, 1H, C-H); ESI-MS (9:1 CH_3CN/H_2O w/ 0.1% TFA) 176 ($M+H^+$, 100%), 198 ($M+Na^+$, 62)

*Compound **6***. In a 500 mL flask, cyclen **5** (4.09 g, 23.7 mmol), $NaHCO_3$ (6.22 g, 74.0 mmol), and t-butylbromoacetate (10.5 mL, 71.1 mmol) were dissolved in 400 mL CH_3CN (HPLC grade). The mixture was stirred at room temperature for 48 hours and then filtered through a frit and the trapped solids were washed with CH_3CN (15 mL). The filtrate was evaporated to dryness and the residual solid was broken up with a spatula and then treated with excess diethyl

ether (400 mL). An insoluble white solid was isolated by filtration, and was washed with copious amounts of diethyl ether (100mL). Drying in a vacuum oven at 55ºC gave a yield of 7.5 g (54%).[3] ^{1}H NMR ($CDCl_3$, 200 MHz) δ 10.0 (br, 2H, NH_2), 3.39 (s, 4H, CH_2), 3.30 (s, 2H, CH_2), 3.11 (s, 4H, CH_2), 2.92 (s, 12H, CH_2), 1.48 (s, 27H, $C(CH_3)_3$); ESI-MS (9:1 CH_3CN/H_2O w/ 0.1% TFA) *m/z* 516 ($M+H^+$, 100%), 538 ($M+Na^+$, 10)

*Compound **8***. Compound **6** (1.48 g, 2.48 mmol), **7** (1.29 g, 7.33 mmol), and K_2CO_3 (2.78 g, 20.1 mmol) were placed in a 500 mL flask and the mixture was then dissolved in CH_3CN (HPLC grade, 150 mL). The reaction mixture was heated at reflux for 48 hours, cooled to room temperature, and the solvent was removed by rotary evaporation. CH_2Cl_2 (50 mL) was added, the mixture was filtered through filter paper, and then evaporated to dryness, yielding crude **8** as a golden oil, which was taken on to the next step without purification. ESI-MS (9:1 CH_3CN/H_2O w/ 0.1% TFA) 610 ($M+H^+$, 100%), 632 ($M+Na^+$, 90).

*Compound **9***. Compound **8** was dissolved in 30 mL of 1:1 CH_2Cl_2/TFA and stirred overnight. Solvent was removed by rotary evaporation and the resulting oil was resuspended in MeOH (20 \ mL) and again evaporated to dryness. This cycle was repeated twice more, and then three times with CH_2Cl_2. The crude oil was redissolved in a minimal volume of MeOH and then treated with excess diethyl ether, yielding crude **9** as a hygroscopic tan solid (1.38 g).[4] ESI-MS (9:1 CH_3CN/H_2O w/ 0.1% TFA) 442 ($M+H^+$, 100%), 221.5 ($M+2H^+$, 85).

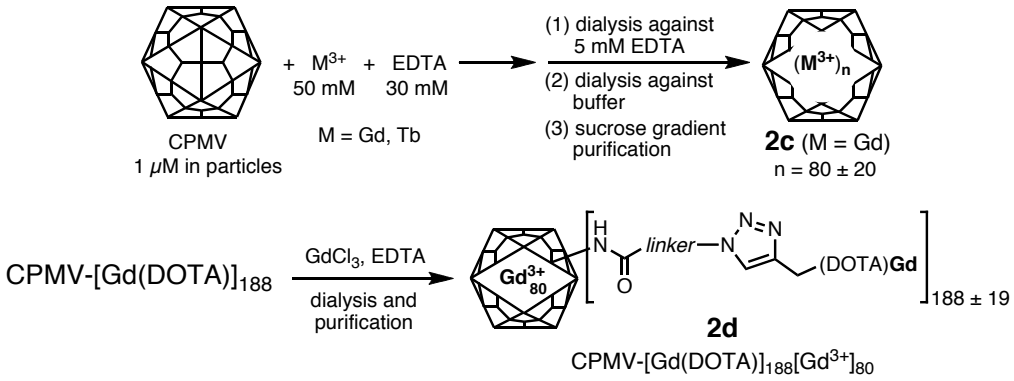
*Gd complex **4***. Ligand **9** (0.97 g, 2.2 mmol) and Gd_2O_3 (478mg, 1.32 mmol) were heated in 25 mL H_2O at reflux overnight. The mixture was cooled to room temperature and filtered through a layer of Celite and sand. The water was removed by rotary evaporation and the resulting oil was dissolved in a minimal volume of MeOH. Addition of excess diethyl ether produced an off-white solid (722 mg, 1.21 mmol).[5] ESI-MS (9:1 CH_3CN/H_2O w/ 0.1% TFA) 597($M+H^+$, 100%), 299 ($M+2H^+$, 85).

*Bioconjugation to CPMV and Qβ (particles **2b** and **3b**)*.

The *N*-acylated azide particles **2a** and **3a** were prepared by reaction of CPMV and Qβ with *N*-hydroxysuccinimide ester **1**[6] by previously published procedures.[7] These virus particles decorated with terminal azides were then subjected to the CuAAC process,[6] as follows. Under a nitrogen atmosphere in an inert-atmosphere glove box, **2a** (final concentration of 2 mg/mL, 21.4 μM in protein subunits) and **4** (1 mM) were dissolved in degassed 0.1M Tris buffer, pH 8.0. A 100 mM stock of $Cu(CH_3CN)_4OTf$ was prepared in degassed CH_3CN and a 100 mM stock of sulfonated bathophenanthroline ligand in degassed 0.1M Tris buffer, pH 8.0. A 1:2 mixture of $Cu(CH_3CN)_4OTf$ and ligand was prepared and added to achieve a final concentration of 1 mM Cu^+ and 2 mM ligand. For the Qβ variation, the reagents used were **3a** (2 mg/mL, 140 μM in protein subunit), **4** (2.9 mM), $Cu(CH_3CN)_4PF_6$ (715 μM), and bathophenanthroline ligand (1.4 mM). The reactions were sealed, brought out of the glove box, and agitated gently on a rotisserie mixer overnight. Each reaction was conducted on 1 mL of total solution in 2 mL eppendorf tubes; larger-scale reactions were performed by multiplying the number of these tubes, rather than by using larger reaction volumes. After reacting overnight, individual reactions were combined and the virus particles were purified on 10-40% sucrose gradients followed by ultrapelleting, with a second gradient/ultrapelleting step if required to completely remove all of the excess small molecules. The number of attached complexes per virion was calculated by

determining the gadolinium concentration by ICP-OES and comparing it to the virus concentration. The integrity of product particles was verified in every case by size exclusion chromatography using a Superose 6 gel column, which showed no particle decomposition.

Figure S2. Attachment of lanthanide ions to virus capsids.



*Coordination of Gd^{3+} to CPMV Structure (particles **2c**)*

CPMV in the normal storage buffer (0.1 M phosphate, pH 7.0) was dialyzed into HEPES buffer (0.1 M, pH 7.0), establishing a virus concentration of approximately 5 mg/mL (53 μM in protein, 0.90 μM in virions). A solution of $GdCl_3$ and EDTA (50 mM Gd^{+3}, 30 mM EDTA) in HEPES buffer was added to the CPMV solution, providing a ratio of Gd^{+3} to CPMV of approximately 10,000:1. A white gelatinous precipitate formed immediately; without CPMV such a precipitate is not observed, and so the precipitate is assumed to be some form of aggregated virus. The mixture was placed in a dialysis bag (molecular weight cut-off 10,000) and gently agitated in a 1 L solution of 10 mM EDTA, 0.1 M HEPES, pH 7.0 for 24 hours, causing the precipitate to gradually dissolve to give a clear colorless solution. The dialysis procedure with EDTA was repeated once more for another 24 hours. The dialysis bag was then transferred to 1 L of pure HEPES buffer (0.1 M, pH 7.0) for 12 hours and then to phosphate buffer (0.1 M, pH 7.0) for 12 hours to further remove excess and weakly bound gadolinium ions and EDTA complexes. The resulting virus samples were purified on 10-40% sucrose gradients. The sucrose band containing the CPMV-[Gd^{3+}] (**2c**) was removed and centrifuged at 42,000 rpm to obtain the virus as a colorless pellet. The pellet was dissolved in phosphate buffer (0.1 M, pH 7.0) at a concentration of 5-10 mg/mL. Virus particle integrity was verified by size exclusion chromatography using a Superose 6 gel column. Gadolinium loadings were obtained by determining the gadolinium concentration in the Gd-CPMV solution using ICP-OES and comparing it to the virus concentration. Values of 60-100 metal ions per virus particle were observed.

*Composite CPMV particle **2d** (Figure S2)*

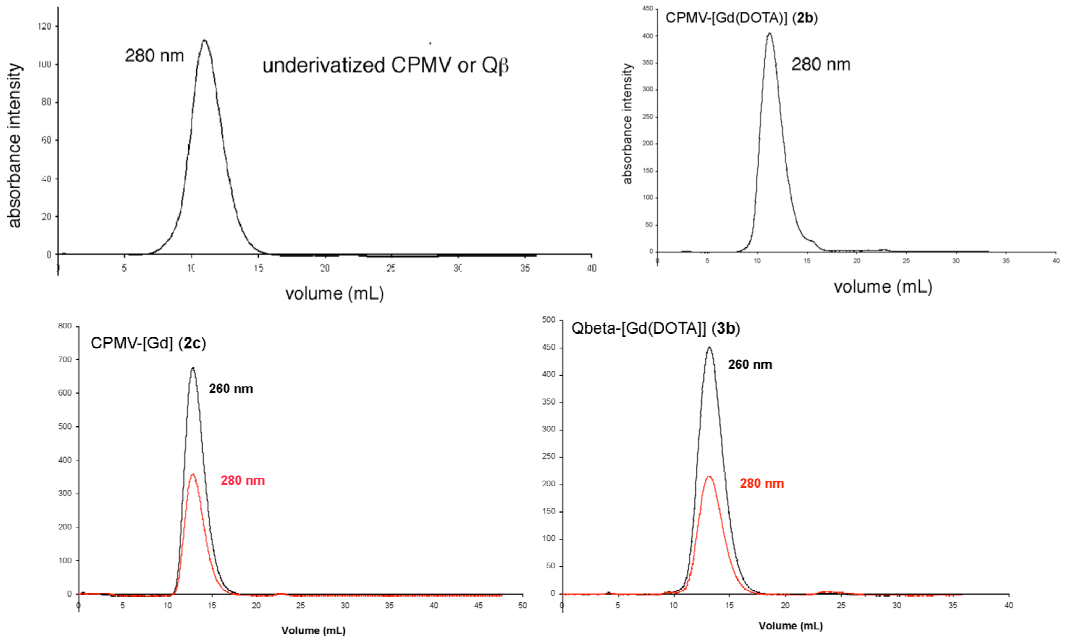
Using the procedure described for **2b** and **3b**, CPMV-[Gd(DOTA)] was prepared and found to display 188 complexes per virion. Gd^{+3} was then introduced as described above for **2c**. Approximately 70% of the starting virus particles were recovered, and no viral decomposition in the purified product was detected by size exclusion chromatography. Gadolinium loadings were

obtained by determining the gadolinium concentration using ICP-OES and comparing it to the virus concentration.

FPLC of Viral Particles

All virus conjugates described here exhibited size-exclusion FPLC traces identical to those below (Figure S3), which are characteristic of intact, non-aggregated particles.

Figure S3. Representative size-exclusion FPLC analyses of virus-Gd conjugates.



Relaxivity Measurements

The samples were analyzed in 1 ml Eppendorf tubes, stored at 4°C but equilibrated to room temperature for imaging. They were placed vertically in the magnet with a piece of foam to hold the tubes immobile. For each sample, T1 imaging and multi echo T2 weighted phantom imaging were performed, using a standard circular RF coil. T1 datasets were obtained with spin echo sequence with the following parameters: 1-3 transverse slices (thickness 2 mm; interleaved 2 mm); field of view (FOV) 2 cm, matrix 256 X 256, TR/TE (250, 500, 1000, 2000, 3000, 5000/50 ms) for 200 and 500 MHz, TR/TE (270, 500, 1000, 2000, 3000, 5000/20 ms) for 64 MHz data, with 4 averages. The spin echo T2 sequence used the following parameters: 20 slices (thickness 2 mm; interleaved 2 mm), FOV 3cm, matrix 256 X 256, TR/TE (2850/20 ms at 200 MHz, TR/TE (4697/10 ms at 500 MHz) with 2 averages.

An example of the T1 images obtained is shown in Figure S4. T1 images were transferred to an imaging analysis program (Cheshire™, PAREXEL International Corp.) for region of interest (ROI) analysis. A circular ROI was drawn on the first image of the imaging data for each sample and the mean signal intensity (SI) value was extracted. The ROI was then propagated to each increasing TR. Wherever possible the receiver gain was kept constant or a SI correction factor was applied. The data were sorted by sample and increasing TR; the T1 was calculated by fitting the SI derived data (tolerance=1e-4, stepsize iterations=100) into a two-parameter equation single exponential curve (SigmaStat, Systat Software Inc., Point Richmond, CA).

Figure S4. An example of T1 images obtained at 500 MHz; Gd(DTPA) and **2c** containing identical concentrations of Gd (23 μM).

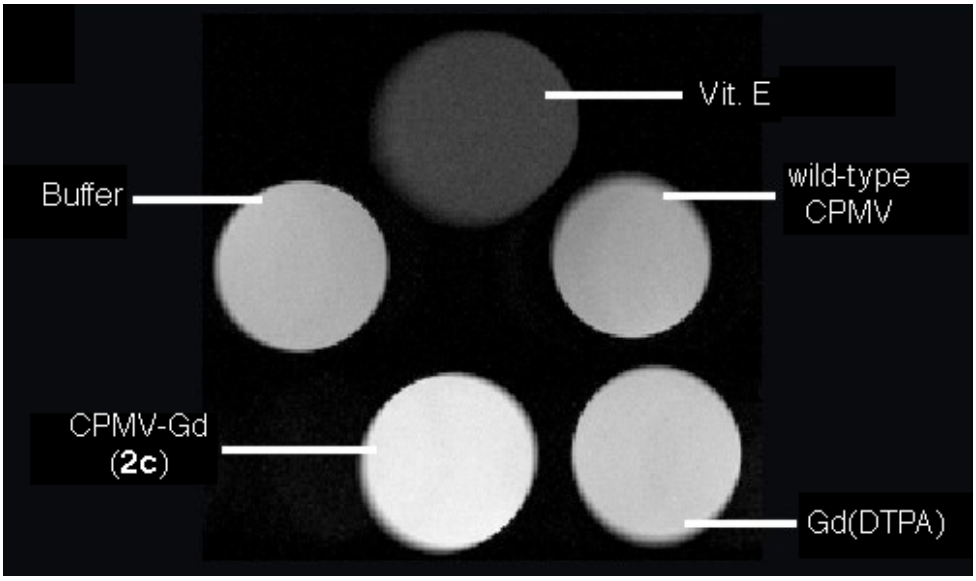
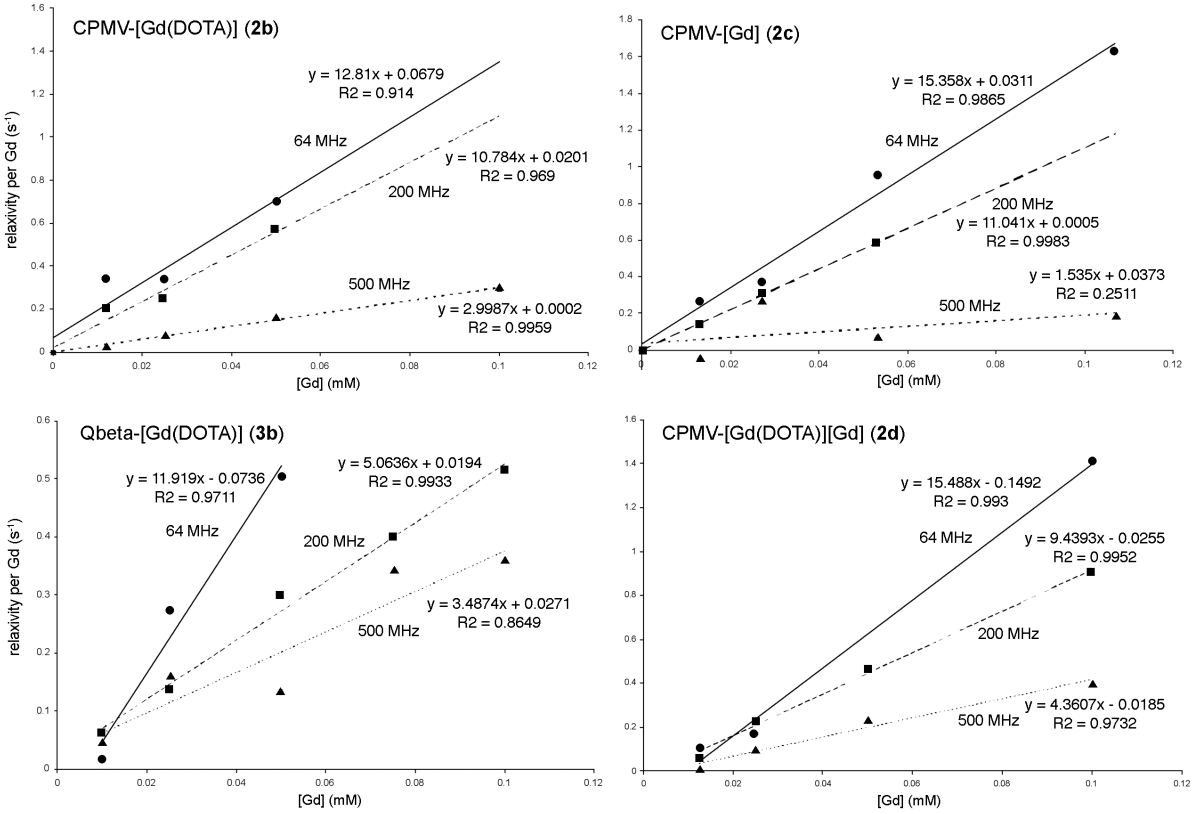


Figure S5. Summary of T1 data obtained for Gd-labeled virus particles as a function of field strength.



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