Rational Design of Protein-based MRI Contrast Agents

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Received

Running Title: Rational Design of Protein-based MRI Contrast Agents

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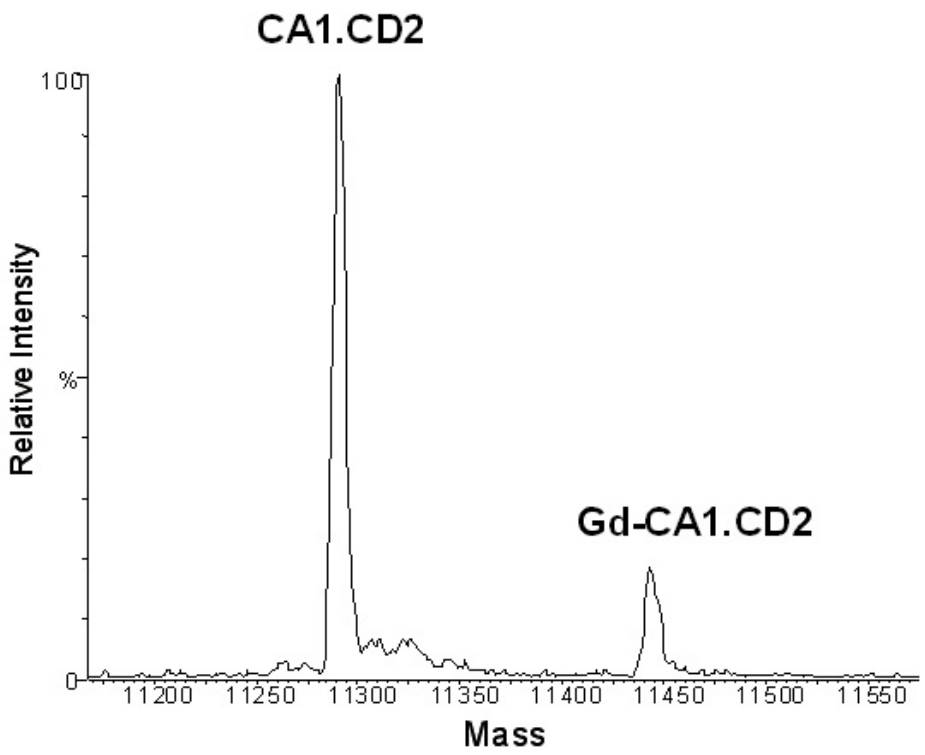
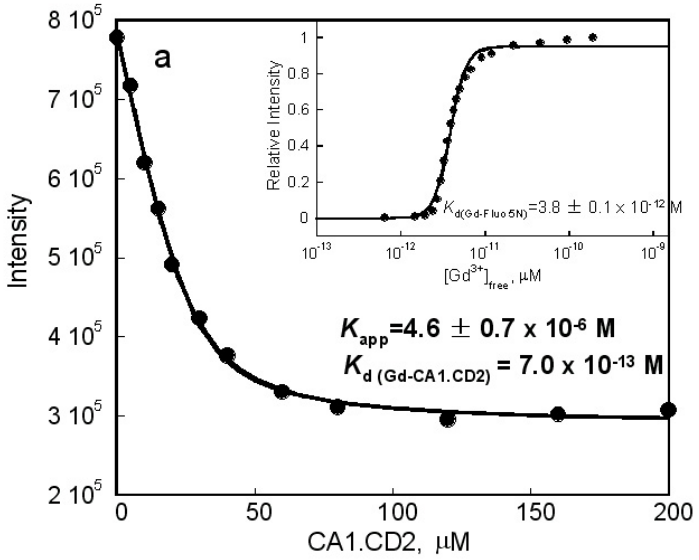
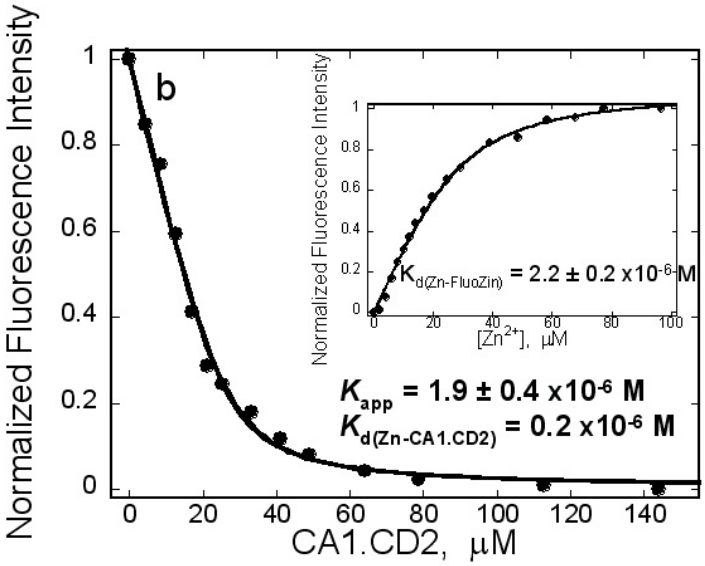


Figure S1. The ESI-TOF MS spectrum of Gd-CA1.CD2. The CA1.CD2 (10 μM) in 1 mM ammonium acetate (pH 7.0) was mixed with 20 μM of gadolinium chloride ($GdCl_3$). The ESI-TOF MS spectra of the complex were recorded by Q-TOF Micro Mass Spectrometer (Miscromass).


a
Intensity
CA1.CD2, μM
Relative Intensity
$K_{d(Gd\text{-}Fluo\ 5N)}$=3.8 ± 0.1 x 10^{-12} M
$[Gd^{3+}]_{free}$, μM
K_{app}=4.6 ± 0.7 x 10^{-6} M
$K_{d\ (Gd\text{-}CA1.CD2)}$ = 7.0 x 10^{-13} M


b
Normalized Fluorescence Intensity
CA1.CD2, μM
Normalized Fluorescence Intensity
$K_{d(Zn\text{-}FluoZin)}$ = 2.2 ± 0.2 x10^{-6} M
$[Zn^{2+}]$, μM
K_{app} = 1.9 ± 0.4 x10^{-6} M
$K_{d(Zn\text{-}CA1.CD2)}$ = 0.2 x10^{-6} M

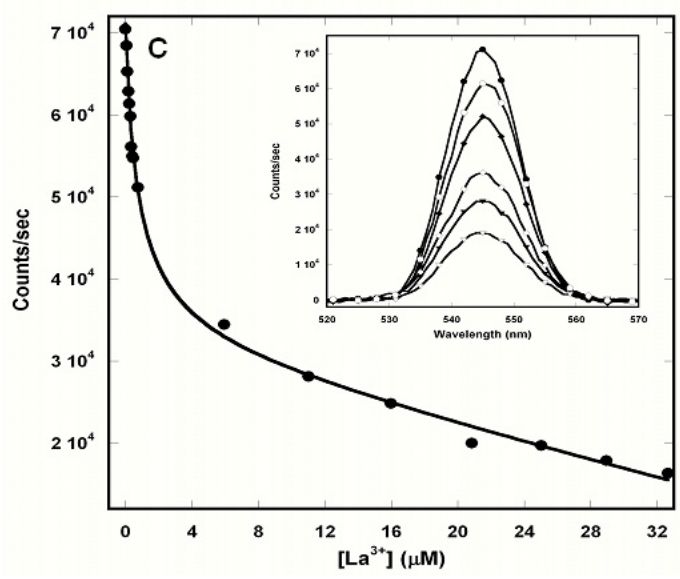

c
Counts/sec
$[La^{3+}]$ (μM)
Counts/sec
Wavelength (nm)

Figure S2. Measurement of metal binding constants. Gd^{3+} binding affinity (a) and Zn^{2+} binding affinity (b) of CA1.CD2 measured by dye competition assays. CA1.CD2 stock solution was gradually added into the 1:1 dye-metal complex to compete for the dye-bound metal ions. The insets show the titration curve for the dye indicators fluo 5N (a) and FluoZin (b), respectively. (c) La^{3+} binding affinity obtained by Aromatic residue sensitized Tb^{3+} luminescence energy transfer. The Tb^{3+} fluorescence of a protein-Tb^{3+} mixture decreases with the addition of La^{3+}. The La^{3+} concentrations are 0, 0.30, 0.76, 5.95, 11.03, and 28.95 μM from top to bottom. The Tb^{3+} fluorescence decrease competition was fitted (line) by a normal competition plus a nonspecific quenching effect (inset).

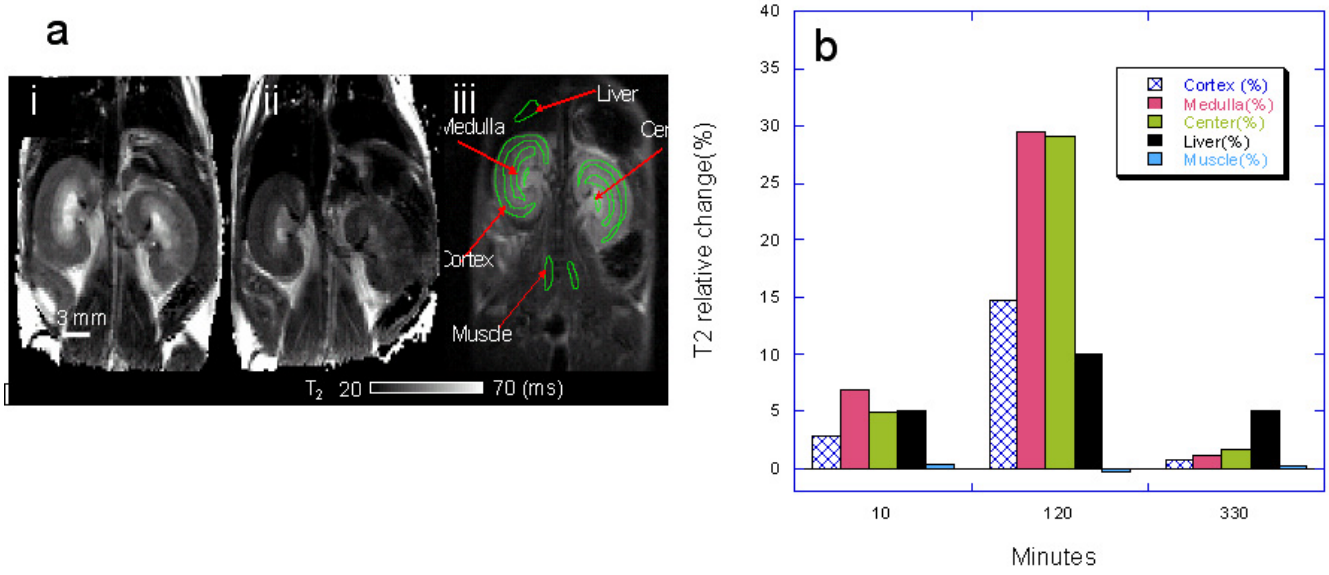


Figure S3. MRI imaging of CA1.CD2 at 9.4 T. (**a**) MR images of CD-1 mouse (26 g) (four mice were imaged) at 9.4T field using Gd-CA1.CD2 as a T_2-weighted contrast agent. The images were recorded pre- (i) and 2 hours post- (ii & iii) injection of 50 μl of 1.2 mM Gd-CA1.CD2 agent through the tail vein. MR images were recorded using a multi-echo Carr-Purcell-Meiboom-Gill (CPMG) sequence. The in-plane resolution is 0.2 x 0.3 x 1.0 mm. (**b**) The T_2 MRI relative intensity changes at kidney cortex (blue cross bars), kidney medulla (red bars), kidney center (green bars), liver (black bars), and muscle (blue bars) as a function of time (indicated). The MRI intensity in muscle at 10 minutes post contrast

administration was defined as 1. MRI intensities at other tissue sites were normalized to the muscle intensity.

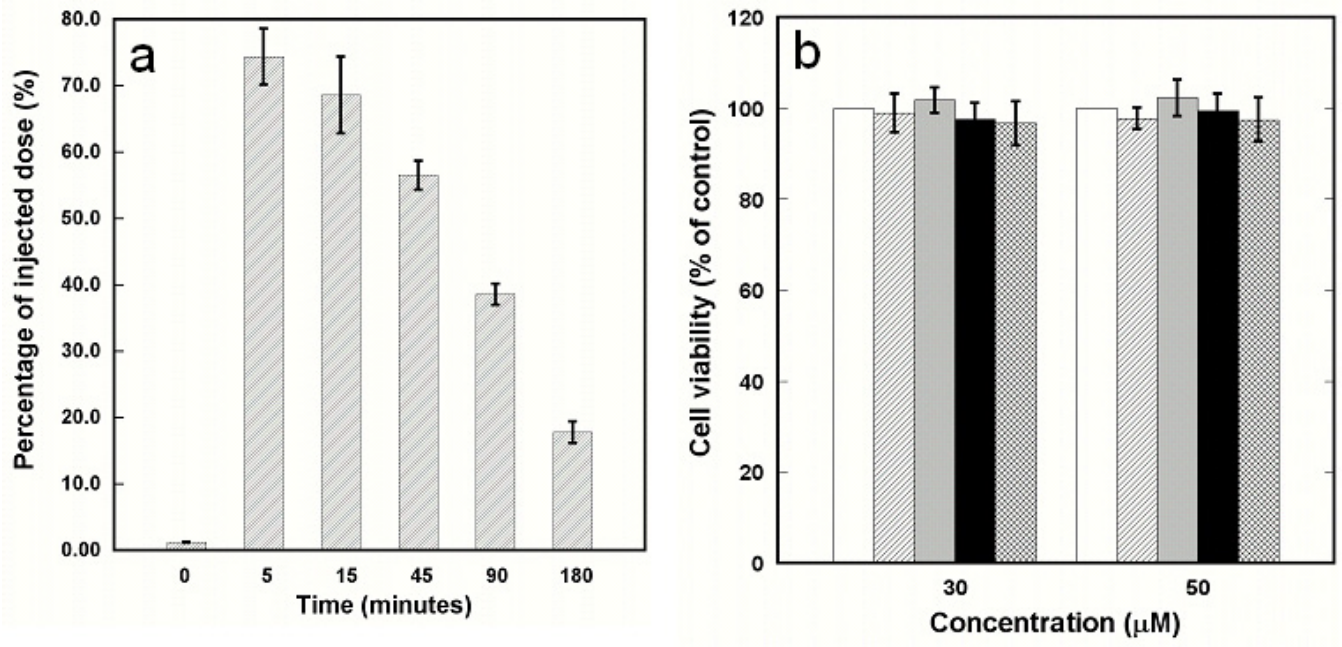


Figure S4. Detection of CA1.CD2 in serum and cytotoxicity of CA1.CD2. (**a**) Sandwich ELISA detection of CA1.CD2 in mouse blood using OX45 and PabCD2. PabCD2 was used as the capture antibody and OX45 was used as the detection antibody in the sandwich-ELISA experiments. Serum samples were obtained from test mice at 0, 0.5, 1.0, 2, and 3 hours post injection (tail vein) of Gd-CA1.CD2 (~1.0 μmole/kg). The amounts of CA1.CD2 are expressed as percentages of the injected dose. Calculations are based on the assumption that the total blood volume is 8% of each individual mouse body weight. (**b**) The cytotoxicity of designed protein Gd-CA1.CD2. The SW480 cells were grown under standard conditions ($1x10^4$ cells in 100 μl medium). The cells were treated by addition of wild type CD2 (strip bars), CA1.CD2 (grey bars), Gd-CA1.CD2 (black bars), and Gd-DTPA (cross bars) with concentrations of 30 μM (left panel) or 50 μM (right panel). The open bars are controls where cells were treated with PBS buffer. The cells were incubated with the treatments for 48 hours. The cells were then subjected for MTT assay. The results were presented as percentages of viable cells using the cells

that were treated with buffer alone (filled red bars) as a reference (100%). The cell lines SW620 and HEK293 were similarly examined. The error bars in (a) and (b) are standard deviations of four measurements or four animals (n=4).

Table S1. Summary of Animal Clinical Pathology Profiles

	Test mice[a]	Control mice[b]	Normal rang[c]
Urea Nitrogen (mg/L)	26.0 ± 0.2[d]	27.0 ± 0.2	18 - 31
Total Urine Protein (g/L)	5.2 ± 0.2	5.3 ± 0.2	5.9 – 10.3
Total Bili (mg/L)	0.4 ± 0.2	0.5 ± 0.2	0.3 – 0.8
Direct Bili (mg/L)	0.0	0.0	
ALT (U/L)	49.0 ± 0.2	72.0 ± 0.2	44 - 87
ALP (U/L)	115.0 ± 0.2	302.0 ± 0.2	43 - 71
AST (U/L)	280.0 ± 0.2	219.0 ± 0.2	101 - 214
GGT (U/L)	0.0	-3.0	

[a]Four mice per group were injected with Gd-CA1.CD2 at dose of 4.0 μmole/kg. All clinical chemistry parameters are measured on an Olympus AU 400 analyzer by MU Research Animal Diagnostic Laboratory (details see Material and Methods).

[b]Control group mice were injected with 50 μl of phosphate buffered saline pH = 7.4.

[c]Normal range values are from Quesenberry, K.E. and J.W. Carpenter; Ferrets, Rabbits, and Rodents Clinical Medicine and Surgery; W.B. Saunders: Philadelphia, 2003.

[d]The standard deviations of measurements with four animal (n=4).

Computational simulation of contrast agent relaxivities[1]

The simulation of r_1 and r_2 as a function of magnetic field strength is performed based on supplementary equations 1 and 2:

$$r_1 = \frac{cq}{55.5}\frac{1}{T_{1m} + \tau_m} \qquad (1)$$

$$r_2 = \frac{cq}{55.5}\frac{T_{2m}^{-2} + \tau_m^{-1}T_{2m}^{-1} + \Delta\omega_m^2}{\tau_m((\tau_m^{-1} + T_{2m}^{-1})^2 + \Delta\omega_m^2)} \qquad (2)$$

In these equations, the water coordination number, q, is assumed to be 1 and the agent concentration is 0.001 M; τ_m is the dwelling time of the coordination water; and $\Delta\omega_m$ is the chemical shift difference between the bound and free water. Since $\Delta\omega_m^2$ is much smaller than other components, equation 2 is simplified to supplementary equation 3 and used in this simulation:

$$r_2 = \frac{cq}{55.5}\frac{1}{T_{2m} + \tau_m} \qquad (3)$$

T_{im} is determined by dipole-dipole (DD) and scalar or contact (SC) mechanisms as shown in supplementary equation 4:

$$\frac{1}{T_{im}} = \frac{1}{T_i^{DD}} + \frac{1}{T_i^{SC}} \qquad i = 1, 2 \qquad (4)$$

Because the contribution from T_i^{DD} is much greater than that of T_i^{SC}, only the former is used in the simulation, which is obtained using supplementary equations 5 and 6:

$$\frac{1}{T_1^{DD}} = \frac{2}{15}\frac{\gamma_I^2 g^2 \mu_B^2}{r_{GdH}^6} S(S+1)(\frac{\mu_0}{4\pi})^2[\frac{7\tau_{c2}}{1+\omega_s^2\tau_{c2}^2} + \frac{3\tau_{c1}}{1+\omega_I^2\tau_{c1}^2}] \qquad (5)$$

$$\frac{1}{T_2^{DD}} = \frac{1}{15}\frac{\gamma_I^2 g^2 \mu_B^2}{r_{GdH}^6} S(S+1)(\frac{\mu_0}{4\pi})^2[\frac{13\tau_{c2}}{1+\omega_s^2\tau_{c2}^2} + \frac{3\tau_{c1}}{1+\omega_I^2\tau_{c1}^2} + 4\tau_{c1}] \qquad (6)$$

The following values are used in the calculation: the gyro-magnetic constant for proton γ_I, 2.675×10^8 $T^{-1}s^{-1}$; g, 2.0; Bohr magneton μ_B, 9.274×10^{-24} J T^{-1}; S, 7/2; permeability of vacuum μ_0, 1.257×10^{-6} N A^{-2}; and the distance between the Gd^{3+} and proton r_{GdH}, 3.0×10^{-10} m (normally 2.7 – 3.3 Å). The

frequency of proton ω_I equals to the γ_I multiplied by the magnetic field while the frequency of electron ω_s is 658-fold of ω_I. The τ_{ci} is determined by the rotational correlation time τ_R, the water dwelling time τ_m, and T_{ie} as shown in supplementary equation 7:

$$\frac{1}{\tau_{ci}} = \frac{1}{\tau_R} + \frac{1}{\tau_m} + \frac{1}{T_{ie}} \qquad i = 1, 2 \qquad (7)$$

T_{ie} is related to the electron frequency ω_s as well as the τ_v (correlation time of splitting) and Δ^2 (mean square zero field splitting energy) of the Gd^{3+} as in supplementary equations 8-10:

$$\frac{1}{T_{1e}} = 2C\left(\frac{1}{1+\omega_s^2\tau_v^2} + \frac{4}{1+4\omega_s^2\tau_v^2}\right) \qquad (8)$$

$$\frac{1}{T_{2e}} = C\left(\frac{5}{1+\omega_s^2\tau_v^2} + \frac{2}{1+4\omega_s^2\tau_v^2} + 3\right) \qquad (9)$$

Where

$$C = \frac{1}{50}\Delta^2\tau_v\{4S(S+1) - 3\} \qquad (10)$$

Various combinations of τ_R (1 ps, 10 ps, 100 ps, 1 ns, 10 ns, and 100 ns), τ_m (1 ps, 10 ps, 100 ps, 1 ns, 10 ns, and 100 ns), τ_v (1 and 10 ps), and Δ^2 (10^{17}, 10^{18}, 10^{19}, and 10^{20} s^{-2}) have been proposed for the calculation of magnetic field-dependent relaxivities under magnetic field strengths ranging from 0.001 MHz to more than 1000 MHz. For small molecules such as DPTA with τ_R at hundreds of ps level, the relaxivity is < 10 $mM^{-1}s^{-1}$ no matter how the other parameters are adjusted. On the other hand, for the contrast agents with τ_R at 10 ns level, such as the CD2 derivatives in our study, the relaxivity can reach a much higher level by adjusting other parameters such as the τ_m.

Toxicity of contrast agent Gd-CA1.CD2

The protein contrast agent Gd-CA1.CD2 did not exhibit acute toxicity at the MRI imaging dose (~2.4 mole/kg), as demonstrated by the fact that all MR imaged mice that received the contrast agent (>10) behaved normally and remained healthy (sacrificed five days after agent injection). The effects of Gd-CA1.CD2 on liver enzymes (ALT, ALP, AST), serum urea nitrogen, bilirubin, and total protein from CD-1 mice 48 hours post-contrast injection were negligible compared to the control mice (Table S1). In addition, no cytotoxicity was observed in tested cell lines, SW620, SW480 and HEK293 that were treated with 50 μM Gd-CA1.CD2, by MTT assay (Supplementary Fig. 3b). Based on the preliminary characterization of toxicity, we conclude that the protein contrast agent is relatively safe.

Reference

(1) Toth, E.; Helm, L.; Merbach, A. E. ***Contrast Agents I: Magnetic Resonance Imaging W. Krause, Ed.*** **2002,** ***221,*** **61-102.**