Supplementary Information

for

High kinetic inertness of a bis-hydrated Gd[III]-complex with a constrained AAZTA-like ligand

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Content:

I. Materials and Methods

1. *Materials*: The chemicals used for the experiments were of analytical grade. The concentration of the $MgCl_2$, $CaCl_2$, $SrCl_2$, $MnCl_2$, $Pb(NO_3)_2$, $Cd(NO_3)_2$, $ZnCl_2$, $CuCl_2$ and $LnCl_3$ solutions were determined by complexometric titration with standardized Na_2H_2EDTA and xylenol orange ($ZnCl_2$, $Pb(NO_3)_2$, $Cd(NO_3)_2$, $LnCl_3$), murexid ($CuCl_2$), *Patton & Reeder* ($CaCl_2$), methylthymolblue ($SrCl_2$) and Eriochrome Black T ($MgCl_2$) as indicator. The concentration of CyAAZTA was determined by pH-potentiometric titration in the presence and absence of a large (40-fold) excess of $CaCl_2$. The pH-potentiometric titrations were made with standardized 0.2 M KOH.

2. *Equilibrium measurements:* The protonation constants of CyAAZTA and the stability and protonation constants of metal complexes with CyAAZTA were determined by pH-potentiometric titration. The metal-to-ligand concentration ratio was 1:1 (the concentration of the ligand was typically 0.002 M). In calculating the equilibrium constants of the metal complexes, the best fitting of the KOH - pH data pairs, were obtained by assuming the formation of ML, MHL, MH_2L and MLH_{-1} complexes in the 1.7-12.0 pH range. The equilibrium constants were calculated with the program *PSEQUAD*.[1]

For the pH measurements and titrations, a *Metrohm 785 DMP Titrino* titration workstation and a *Metrohm-6.0233.100* combined electrode were used. Equilibrium measurements were carried out at a constant ionic strength (0.1 M KCl or KNO_3) in 6 mL samples at 25 °C. The solutions were stirred, and N_2 was bubbled through them. The titrations were made in the pH range of 1.7-12.0. KHphthalate (pH=4.005) and borax (pH=9.177) buffers were used to calibrate the pH meter. For the calculation of $[H^+]$ from the measured pH values, the method proposed by *Irving et al.* was used.[2] A 0.01M HCl or HNO_3 solution was titrated with the standardized KOH solution in the presence of 0.1 M KCl or KNO_3 ionic strength. The differences between the measured (pH_{read}) and calculated pH ($-log[H^+]$) values were used to obtain the $[H^+]$ at equilibrium. The ion product of water was determined from the same titrations (HCl/KOH or HNO_3/KOH) in the pH range of 11.5-12.0.

3. *Kinetic studies:* The kinetic stability of Gd(CyAAZTA) was determined by the rates of the exchange reactions taking place between Gd(CyAAZTA) and Cu^{2+} ions. The exchange reactions with Cu^{2+} ions were studied by spectrophotometry, following the formation of the Cu(CyAAZTA) complex at 300 nm with a *Cary 1E spectrophotometer* in a 1 cm quartz cuvette.

The concentration of Gd(CyAAZTA) was 2×10^{-4} M, while the concentration of Cu^{2+} was 20 to 40 times higher in order to guarantee the pseudo-first-order conditions. The temperature was maintained at 25°C and the ionic strength of the solutions was kept constant (0.1 M KCl). The exchange rates were studied in the pH range 2.1 – 4.5. In order to keep the pH values constant, dichloroacetic acid (pH range 2.1 – 2.5), chloroacetic acid (pH range 2.5 – 3.2), 1,4-dimethylpiperazine (pH range 3.1 – 4.1) and N-methylpiperazine (pH range 4.1 – 5.2) buffers (0.01 M) were used. The pseudo-first-order rate constants (k_d) were calculated by fitting the absorbance-time data to Eq. (1).

$$A_t = (A_0 - A_p)e^{-k_d t} + A_p \qquad (1)$$

where A_t, A_0 and A_p are the absorbance values at time t, at the beginning of the reaction and at equilibrium, respectively. The calculations were performed with the use of the program Micromath Scientist.[3]

*4. Relaxometric measurements: t*he water proton longitudinal relaxation rates as a function of the magnetic field strength were measured in non-deuterated aqueous solutions on a fast field-cycling Stelar SmarTracer relaxometer (Stelar s.r.l., Mede (PV), Italy) over a continuum of magnetic field strengths from 0.00024 to 0.25 T (corresponding to 0.01-10 MHz proton Larmor frequencies). The relaxometer operates under computer control with an absolute uncertainty in $1/T_1$ of ± 1%. Additional longitudinal relaxation data in the range 15-70 MHz were obtained on a Stelar Relaxometer connected to a Bruker WP80 NMR electromagnet adapted to variable-field measurements (15-80 MHz proton Larmor frequency). The exact concentration of Gd^{III} was determined by measurement of bulk magnetic susceptibility shifts of a *t*BuOH signal or by inductively coupled plasma mass spectrometry (ICP-MS, Element-2, Thermo-Finnigan, Rodano (MI), Italy). The ^1H T_1 relaxation times were acquired by the standard inversion recovery method with typical 90° pulse width of 3.5 μs, 16 experiments of 4 scans. The temperature was controlled with a Stelar VTC-91 airflow heater equipped with a calibrated copper–constantan thermocouple (uncertainty of ±0.1 °C). Variable-temperature ^{17}O NMR measurements were recorded on a Bruker Avance III spectrometer (11.74 T, 67.8 MHz for ^{17}O) equipped with a 5 mm probe and standard temperature control units. An aqueous solution of the complex containing 1.0% of the ^{17}O isotope (Cambridge Isotope) was used. The observed transverse relaxation rates were calculated from the signal width at half-height.

II. Equilibrium behavior of CyAAZTA

The protonation constants ($\log K_i^H$) of CyAAZTA and the stability constants of its metal complexes with divalent and trivalent metal ions were determined by pH-potentiometry. The $\log K_i^H$ values of CyAAZTA, defined by Eq. (1.) are listed and compared with those of AAZTA, DO3A and PCTA in Table S1.

$$K_i^H = \frac{[H_iL]}{[H_{i-1}L][H^+]} \qquad i=1, 2, 3, \dots 5 \qquad (1)$$

Table S1. Protonation constants of CyAAZTA, AAZTA, DO3A and PCTA (25°C)

I	CyAAZTA			AAZTA[b]			DO3A		PCTA[e]
	0.1M KCl	0.1M KCl[a]	0.1M KNO$_3$	0.1M KCl	0.1M KNO$_3$	0.1M (Me)$_4$NCl	0.1 M KCl[c]	0.1M (Me)$_4$NCl[d]	1.0 M KCl
$\log K_1^H$	10.48 (2)	10.63 (1)	9.90 (1)	11.23	11.18	11.35	11.99	11.59	11.36
$\log K_2^H$	6.43 (2)	6.47 (1)	6.39 (2)	6.52	6.50	6.61	9.51	9.24	7.35
$\log K_3^H$	4.23 (2)	4.27 (1)	4.35 (2)	3.78	3.78	3.89	4.30	4.43	3.83
$\log K_4^H$	2.76 (2)	2.71 (1)	3.29 (2)	2.24	2.22	2.40	3.63	3.48	2.12
$\log K_5^H$	1.68 (2)	1.98 (1)	2.17 (2)	1.56	1.68	2.12	1.84	–	1.29
$\Sigma \log K_i^H$	25.58	26.06	26.10	25.33	25.36	26.37	31.26	28.74	25.95

[a] Ref. 4; [b] Ref. 5; [c] Ref. 6; [d] Ref. 7; [e] Ref. 8

The comparison of the related protonation constants of CyAAZTA and AAZTA (Table S1) indicates that $\log K_1^H$ value for CyAAZTA is significantly lower, whereas $\log K_3^H$ for CyAAZTA is nearly half order of magnitude higher. The protonation sites of AAZTA were identified by a detailed NMR study.[5] By taking into account the similarities of CyAAZTA and AAZTA, it can be assumed that the protonation scheme of the two ligands are identical. Since the first protonation of AAZTA takes place by the involvement of all amino N donor atoms, the lower $\log K_1^H$ values of CyAAZTA could be explained by the presence of the *trans*-1,2-diaminocyclohexyl moiety which can modify the strength of the H-bond formed between the proton and the N-donor atoms. On the other hand, the slightly higher $\log K_3^H$ value of CyAAZTA might be accounted for the higher basicity of the carboxylate pendant arm.

The stability and protonation constants of Mg^{II}, Ca^{II}, Sr^{II}, Zn^{II}, Pb^{II}, Cd^{II} and Ln^{III}-complexes formed with CyAAZTA were investigated by pH-potentiometric method. The stability and

protonation constants of the metal complexes formed with CyAAZTA are defined by Eqs. (2) and (3).

$$K_{ML} = \frac{[ML]}{[M][L]} \tag{2}$$

$$K_{MH_iL} = \frac{[MH_iL]}{[MH_{i-1}L][H^+]} \qquad i=1,2 \tag{3}$$

The protonation and stability constants of the CyAAZTA complexes were calculated from the titration curves obtained at 1:1 metal to ligand concentration ratios. The titration data of CyAAZTA in the presence Zn^{2+}, Pb^{2+}, Cd^{2+} and Ln^{3+}-ions indicate base consuming process at pH>9. This process can be interpreted by assuming the hydrolysis of the metal ion, the coordination of OH^- ion/s characterized with $logK_{MLH-1}$ equilibrium constant (Eq. 4):

$$ML(OH^-) + H^+ \rightleftharpoons ML + H_2O \tag{4}$$
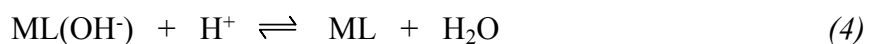
$$K_{MLH_{-1}} = \frac{[ML]}{[ML(OH^-)][H^+]}$$

The stability constants ($logK_{ML}$) of the CyAAZTA metal complexes are generally lower than those of the corresponding complexes of AAZTA, DO3A and PCTA (*Table 1* and *S2*). These data can be explained by the unfavourable preorganization of the donor atoms of CyAAZTA resulting in a lower suitability between the size of metal ions and the coordination cage of the ligand. Finally, CyAAZTA metal complexes, similarly to those of AAZTA, can be protonated at low pH values. In these complexes there is probably one or two weakly coordinated donor atom(s) (carboxylate-O), which can be protonated at pH values around 2 – 5.

Table 2. The stability ($\log K_{ML}$) and protonation ($\log K_{MHiL}$) constants of metal complexes formed with CyAAZTA, AAZTA, DO3A and PCTA ligands (25°C).

| Ionic strength	CyAAZTA 0.1 M KCl				AAZTA[a] 0.1 M KCl				DO3A[b] 0.1 M KCl		PCTA[c] 1.0 M KCl	
	$\log K_{ML}$	$\log K^H_{MHL}$	$\log K^H_{MH_2L}$	$\log K^H_{MLH_{-1}}$	$\log K_{ML}$	$\log K^H_{MHL}$	$\log K^H_{MH_2L}$	$\log K^H_{MLH_{-1}}$	$\log K_{ML}$	$\log K^H_{MHL}$	$\log K_{ML}$	$\log K^H_{MHL}$
Mg^{2+}	8.19 (1)	5.58 (6)	–	–	8.31	5.24	–	–	11.64	–	12.35	3.82
Ca^{2+}	12.38 (2)	4.00 (4)	–	–	12.76	3.34	–	–	12.57	4.60	12.72	3.79
Sr^{2+}	9.33 (2)	5.33 (6)	–	–	9.88	4.80	–	–	–	–	–	–
Zn^{2+}	17.09 (2)	4.17 (1)	2.83 (1)	9.43 (4)	18.01	3.87	2.36	11.25	21.57	3.47	20.48	3.10
Cu^{2+}	20.11[d]	4.24[d]	3.08[d]	9.62[d]	22.27[e]	3.93[e]	2.68[e]	10.79[e]	25.75	3.65	18.79	3.58
Pb^{2+} [f]	16.92 (3)	4.41 (2)	2.59 (3)	10.32 (5)	19.84	3.22	2.50	–	–	–	–	–
Cd^{2+} [f]	17.08 (4)	4.07 (3)	2.53 (3)	10.53 (2)	17.94	3.25	2.05	–	–	–	–	–

[a] Ref. 5; [b] Ref. 6, [c] Ref. 8; [d] Ref. 4; [e] Ref. 9, [f] in the presence of 0.1 M KNO$_3$ ionic strength

III. ^{1}H-NMR spectra of Eu(CyAAZTA) and Yb(CyAAZTA)

^{1}H-NMR spectra were registered at 298K on a 400MHz (9.4T) JEOL spectrometer. Either in the case of Eu or Yb the number of detected resonances is consistent with the presence of just one diasteroisomeric form in which non-equivalent axial and equatorial protons can be distinguished.
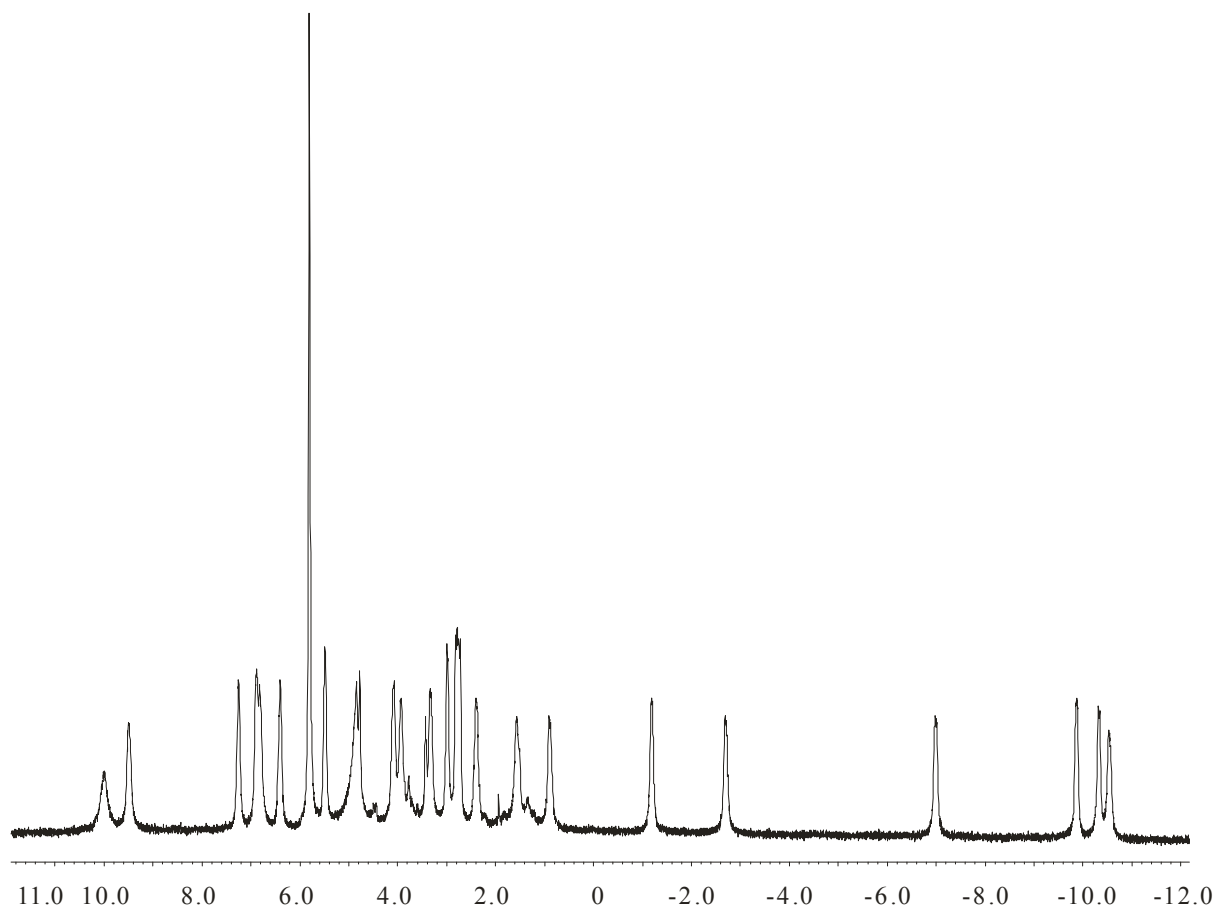
Fig. S1: ^{1}H-NMR spectrum of Eu(CyAAZTA) registered at 298K on a 400MHz (9.4T) spectrometer.

| 60.0 | 50.0 | 40.0 | 30.0 | 20.0 | 10.0 | 0 | -10.0 | -20.0 | -30.0 | -40.0 | -50.0 |
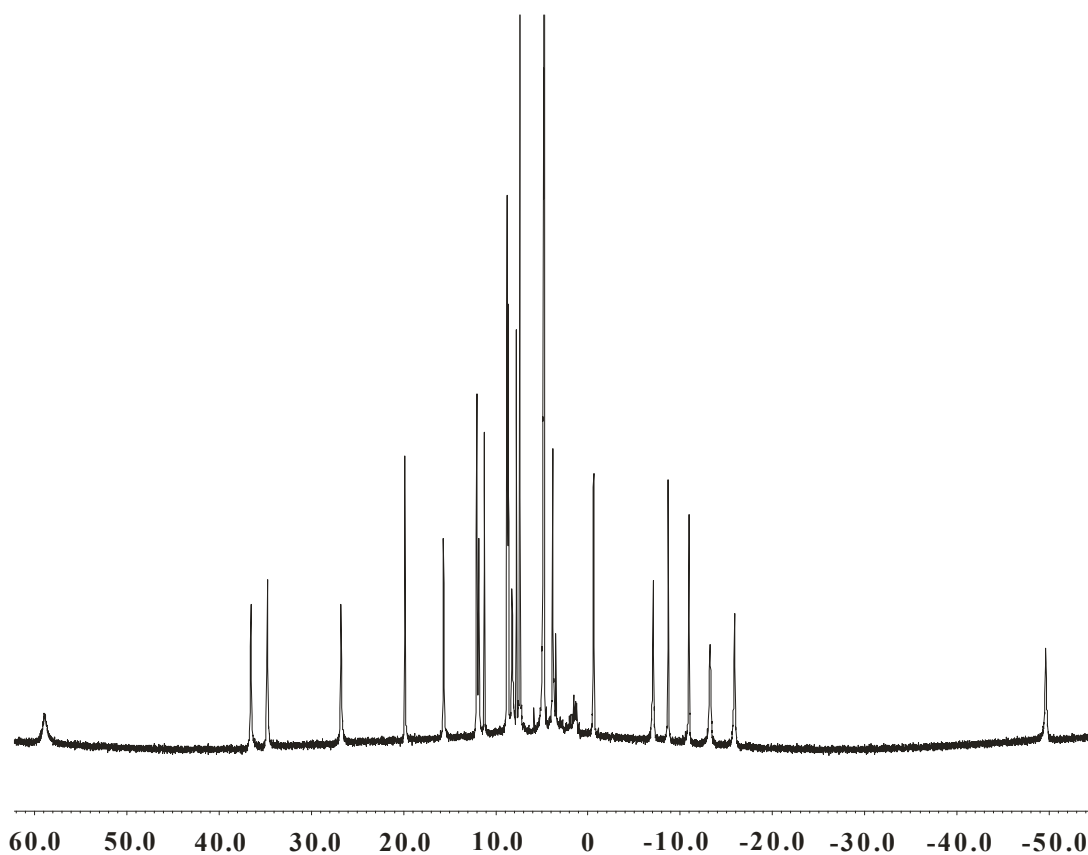
Fig. S2: ^{1}H-NMR spectrum of Yb(CyAAZTA) registered at 298K on a 400MHz (9.4T) spectrometer.

IV. Kinetic studies of the transmetallation reactions between Gd(CyAAZTA) and Cu^{2+}

The rates of the transmetallation reactions between Gd(CyAAZTA) and Cu^{2+} (Eq. (5)) were studied by following the formation of CuL with spectrophotometry at 300 nm in the pH range $2.1 - 4.5$ (25°C, 0.1 M KCl):
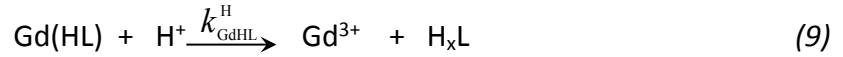
$$Gd(CyAAZTA)^{-} + Cu^{2+} \rightleftharpoons Cu(CyAAZTA) + Gd^{3+} \qquad (5)$$

The kinetic studies were carried out in the presence of an excess of Cu^{2+}, thus the rate of transmetallation could be expressed as follows:

$$-\frac{d[GdL]_t}{dt} = \frac{d[CuL]_t}{dt} = k_d[GdL]_t \qquad (6)$$

where $[GdL]_t$ and $[CuL]_t$ are the concentrations of the complexes and k_d is the pseudo-first-order rate constant. According to the kinetic data, the rates of the exchange reactions are determined by the dissociation of the Gd(CyAAZTA) complex followed by the fast reaction between the free CyAAZTA ligand and Cu^{2+} ions

The dissociation reactions of Gd(CyAAZTA) might take place by the spontaneous dissociation of GdL (Eq. (7)) and GdL (Eq. (8)), the proton assisted dissociation of and Gd(HL) species (Eq. (9)) characterized by the rate constants k_0, k_{GdHL} and k^H_{GaHL}, respectively.

$$GdL \xrightarrow{k_0} Gd^{3+} + H_xL \qquad (7)$$

$$Gd(HL) \xrightarrow{k_{GdHL}} Gd^{3+} + H_xL \qquad (8)$$

$$Gd(HL) + H^+ \xrightarrow{k^H_{GdHL}} Gd^{3+} + H_xL \qquad (9)$$

By considering all the possible reaction pathways for the dissociation of Gd(CyAAZTA), the dissociation rate of Gd(CyAAZTA) can be described by Eq. (10):

$$-\frac{[GdL]_t}{dt} = k_0[GdL] + k_{GdHL}[Gd(HL)] + k^H_{GdHL}[Gd(HL)][H^+] \qquad (10)$$

Considering the total concentration ($[GdL]_t=[GdL]+[Gd(HL)]$) and K_{GdHL} protonation constant of Gd(CyAAZTA) (Eq. (3)), the k_d rate constant can be expressed by Eq. (11):

$$k_d = \frac{k_0 + k_1[H^+] + k_2[H^+]^2}{1 + K_{GaHL}[H^+]} \qquad (11)$$

The rate constants, k_0, $k_1=k_{GdHL} \times K_{GdHL}$ and $k_2=k^H_{GdHL} \times K_{GdHL}$ characterize the spontaneous and proton-assisted dissociation of Gd(CyAAZTA), respectively. For the calculation of k_0, k_1 and k_2, the k_d values presented in Figure 1 have been fitted to Eq. (11) with the use of the K_{GdHL} protonation constants of Gd(CyAAZTA) obtained by pH-potentiometry.

V. Relaxometry

The inner sphere contribution to r_1 is determined by the number of bound water molecules and their rate of exchange ($k_{ex} = 1/\tau_M$), the molecular rotational correlation time (τ_R), and the electronic relaxation times ($T_{1,2e}$) of Gd^{3+}. The outer sphere term depends on $T_{1,2e}$, the relative diffusion coefficient between the complex and the water molecules (D) and their distance of closest approach, a. The ^{17}O R_2 data depend primarily on $T_{1,2e}$, the hyperfine $Gd–^{17}O_{water}$ coupling constant $A_O/\hbar$, τ_M and q. Information on q and $A_O/\hbar$ are derived from the temperature dependence of $\Delta\omega$ (Figure S3). Additional relevant parameters are those associated with the electronic relaxation times $T_{1,2e}$, $i.e.$ the trace of the square of the zero-field splitting tensor, Δ^2; the correlation time describing the modulation of the zero-field splitting, τ_V, and its activation energy, E_V; the

enthalpy of activation, $\Delta H_M{}^\#$, for the water exchange process. A reasonable estimate of some of the relaxation parameters is typically done during the fitting: q was fixed to 2; the distance between the metal ion and the protons of the bound water molecule, r, was fixed to 3.1 Å; a was fixed to 4 Å and D to 2.24 and 3.1×10^{-5} cm^2 s^{-1} at 298 and 310 K, respectively. The typical values of 1.0 and 1.6 kJ·mol^{-1} were assigned to the activation energy for the modulation of the zero-field splitting interaction (E_V) and to the rotational motion of the complex (E_R). At 310 K the relaxometric parameters obtained by fitting the NMRD profiles are the following: $^{20}r_1 = 7.9$ mM^{-1} s^{-1}, $\Delta^2 = (2.6 \pm 0.2) \times 10^{19}$ s^{-2}; $\tau_V = 28 \pm 2$ ps; $k_{ex} = (14.5 \pm 0.3) \times 10^6$ s^{-1}; $\tau_R = 63 \pm 1$ ps.
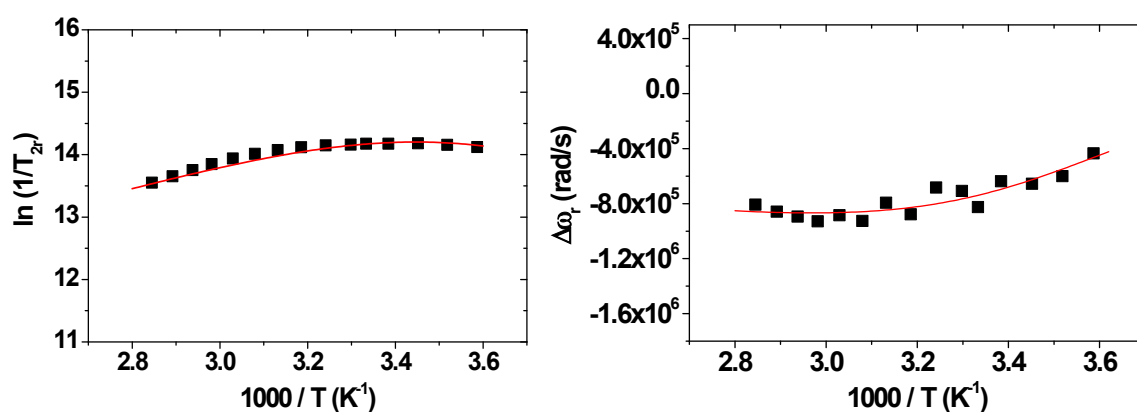

Figure S3. Temperature dependence of the reduced water ^{17}O NMR transverse relaxation rates (left) and chemical shifts (right) at 11.75 T and pH = 7 for a 20 mM solution of Gd(CyAAZTA).
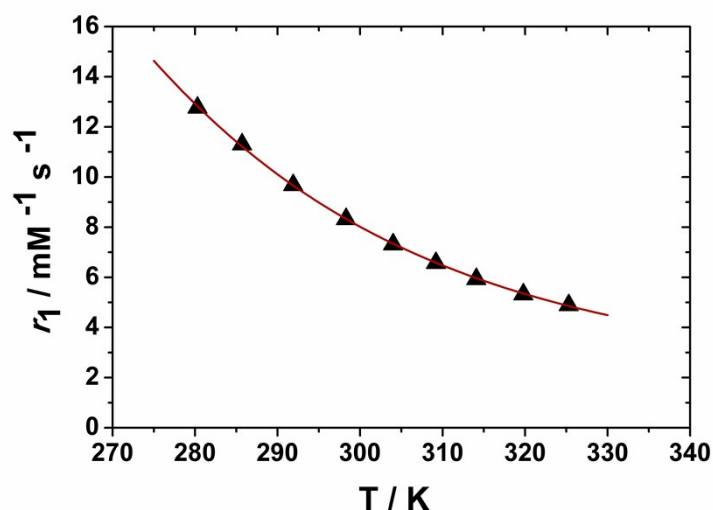

Figure S4. Temperature dependence of the longitudinal water proton relaxivity at 20 MHz and pH = 7.
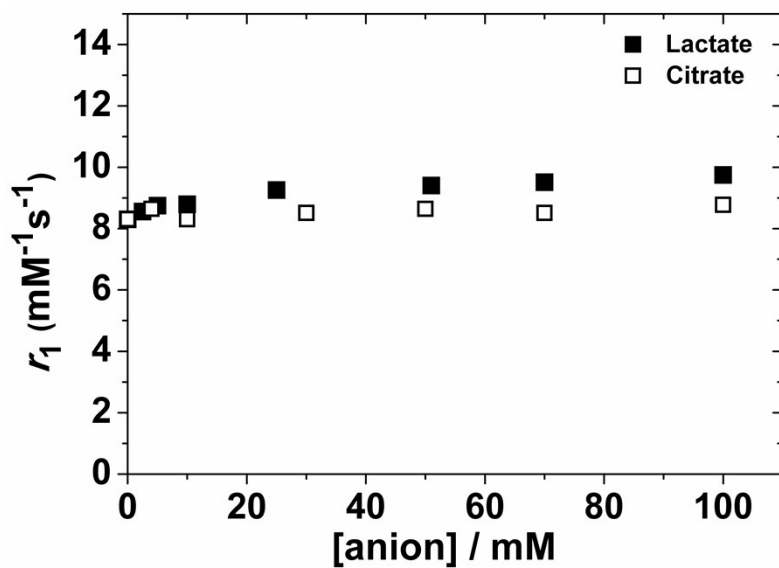
Figure S5. Variation of the longitudinal water proton relaxivity of Gd(CyAAZTA) at 20 MHz, 298 K and pH = 7 as a function of increasing concentrations of lactate and citrate anions.
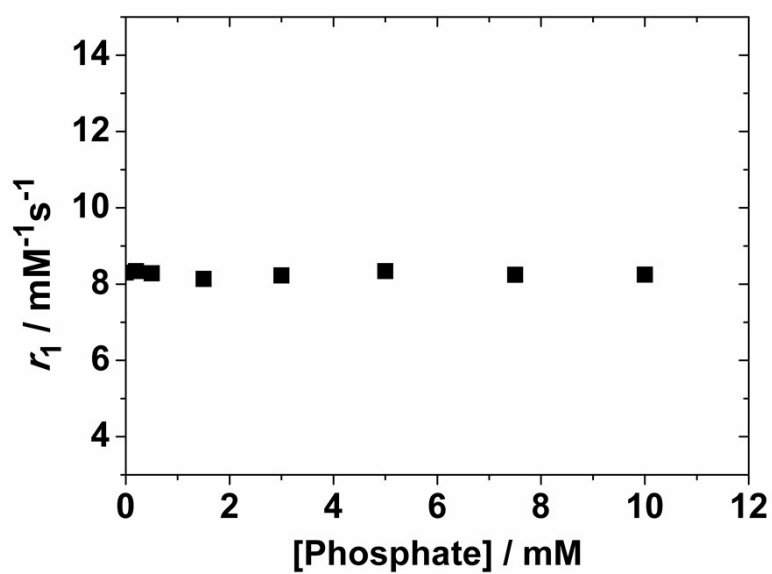

Figure S6. Variation of the longitudinal water proton relaxivity of Gd(CyAAZTA) at 20 MHz, 298 K and pH = 7 as a function of increasing concentrations of phosphate anion.
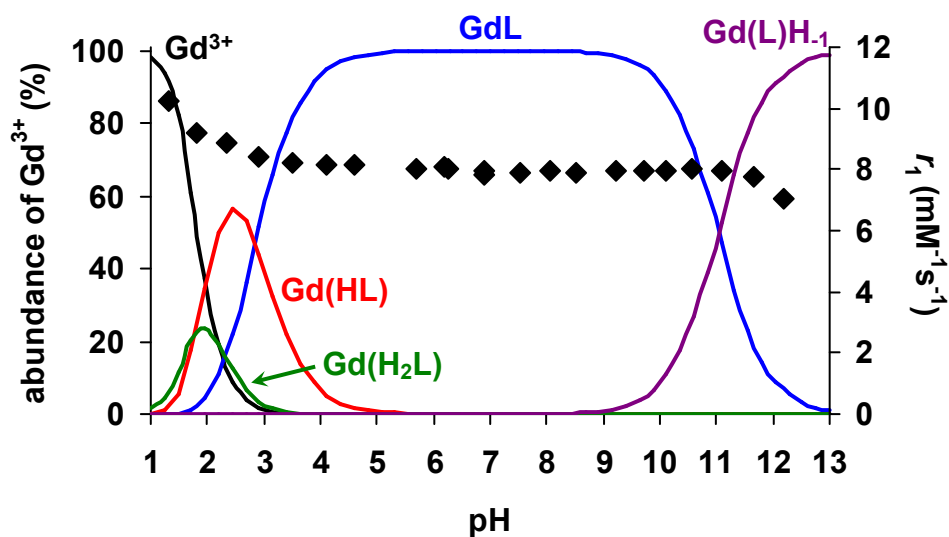
Figure S7. Relaxivity values and the species distribution of the Gd^{3+} - CyAAZTA system as a function of pH ($[Gd^{3+}]=[CyAAZTA]=1.0$ mM, 0.1 M KCl, 25°C).
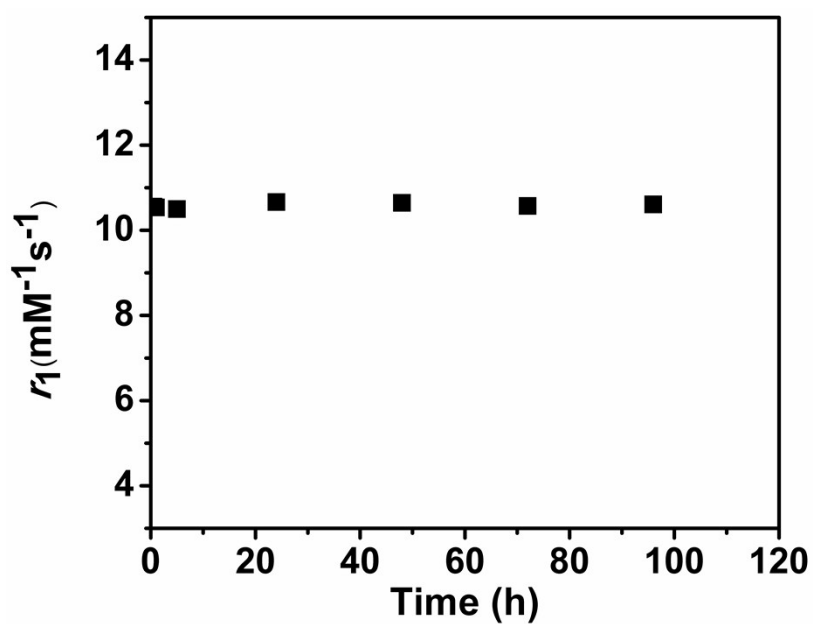
Figure S8. r_1 values of Gd(CyAAZTA) in Seronorm™ at 20 MHz and 298 K as a function of time.
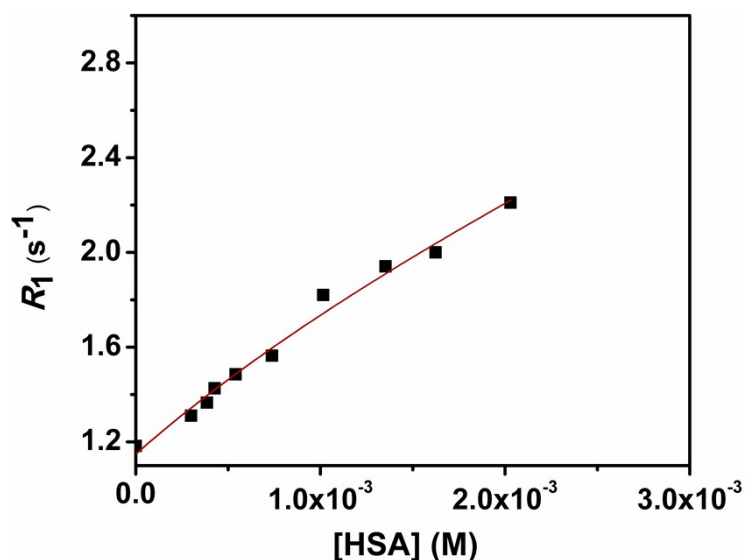
Figure S9. Water proton relaxation rate of a 0.097 mM aqueous solution of Gd(CyAAZTA) as a function of increasing amounts of HSA (20 MHz, 298 K).

VI. References

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