Electronic Supplementary Material (ESI) for Physical Chemistry Chemical Physics

Supplementary Information

W-band pulse EPR distance measurements in peptides using Gd^{3+}-dipicolinic acid derivatives as spin labels

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Titration of 4MMDPA- mel-$C_{15}C_{27}$ with Gd^{3+}

To 50 µl of 0.01 mM 4MMDPA-mel-$C_{15}C_{27}$ with 5 µl of 2.2 mM arsenazo (1:22 peptide/arsenazo molar ratio), n aliquots of 21.6 µM $GdCl_3$ in DDW, at the required volume to keep $[Gd^{3+}]$=1.8 µM, were added. After each addition, the sample was vortexed intensively for one minute and then vortexed occasionally during the next 10 minutes. Then, UV-Vis absorption measurements were carried out on 1.5 µl of the total sample using UV-Vis Nanodrop® ND-1000 spectrometer. The spectra are shown in Fig. S1a. The originally bright pink arsenazo III solution turn light-to-dark purple with the formation of chelated arsenazo-Gd^{3+}. The addition of $GdCl_3$ was stopped when arsenazo III began turning purple-to-green, which indicates the presence of Gd^{3+} not coordinated to the 4MMDPA labeled peptide. The titration curve is shown in Fig. S1b and the compositions chosen for DEER measurements are indicated in the Figure.

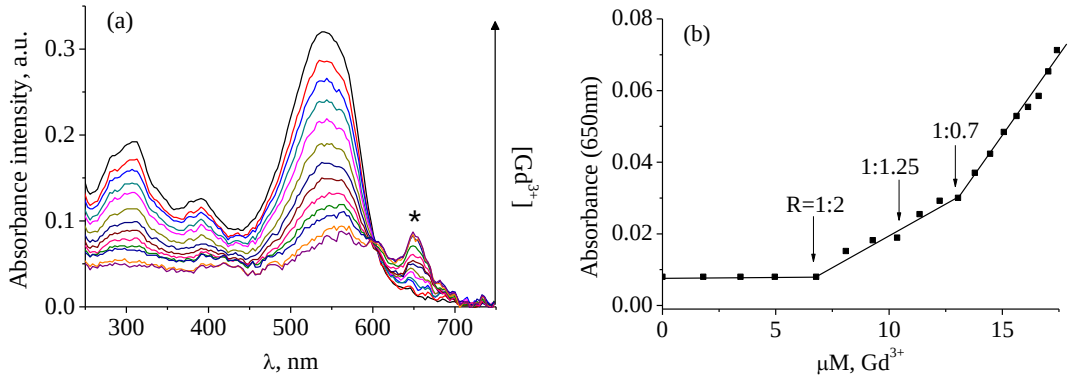

Figure S1: *(a)* UV-Vis absorbance spectra of 4MMDPA-mel$C_{15}C_{27}$ and arsenazo with gradual amounts of added $GdCl_3$ (The asterisk at 650 nm indicates the band of Gd^{3+}-arsenazo). *(b)* The corresponding plot of the UV-Vis absorbance intensity at 650 nm as a function of total [Gd^{3+}] added. The arrows indicate the molar ratios of [Gd^{3+}]/2[4MMDPA-mel$C_{15}C_{27}$] examined in this work.

Speciation of Gd^{3+}- $(4MMDPA)_{1-3}$ in solution

The 4MMDPA tag has only three coordination sites, therefore depending on the [Gd^{3+}]/[tag] ratio, complexes of Gd^{3+}-$(4MMDPA)_{0-3}$ can be formed. Using the literature reported room temperature equilibrium constants; $pK_{i(i=1-3)}$=8.74±0.01, 7.32±0.03, 5.77±0.03, respectively,[1] we calculated the relative concentration of each of the Gd^{3+}-$(4MMDPA)_n$ (n=0-3) complexes for a total concentration, (0.2 mM) of the ligand, [4MMDPA], as a function of R'=[Gd^{3+}]/[4MMDPA] (Fig. S2a). The chosen concentrations are typical for protein and peptide concentrations used in DEER measurements. These calculations show that for R'=1:1 the major component in solution is the n=1 complex but there is a significant contribution from both the n=0,2 complexes (~15% each). For R'=1:2 the contribution of the n=0 (free Gd^{3+}) is negligible, but the amounts of the n=1,2 complexes are comparable. For R'=1:0.7 there is approximately 40% free Gd^{3+} in the sample.

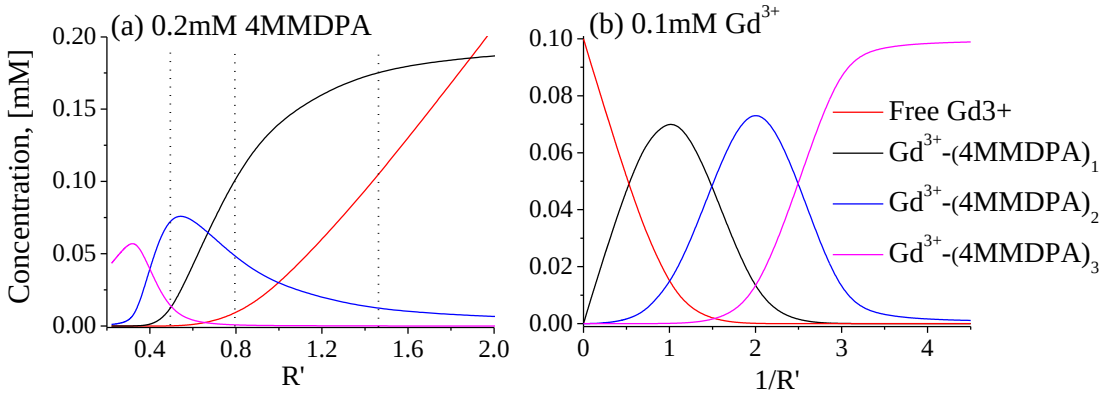

Figure S2: The calculated concentration of Gd^{3+}-$(4MMDPA)_1$, Gd^{3+}-$(4MMDPA)_2$ and Gd^{3+}-$(4MMDPA)_3$ and free Gd^{3+} in solution vs. (a) R', with [4MMDPA]=0.2 mM and *(b)* 1/R' with [Gd^{3+}]=0.1 mM. The dotted lines in figure *a* indicate the R values (1:2, 1:1.25, 1:0.7) for the labeled melittin examined in this work.

To verify the actual composition of the frozen solutions in terms of the Gd^{3+}-$(4MMDPA)_n$ complexes we prepared solutions of R'=[Gd^{3+}]/[4MMDPA] ratios of 1:100, 1:10, 1:5, 1:2, 1:1 and 1:0.5 and [Gd^{3+}] =0.1mM by dissolving $GdCl_3$ and the tag in D_2O (70%) /perdeuterated d_8- glycerol (30%). The speciation for such a system as a function of 1/R'=[4MMDPA]/[Gd^{3+}] is represented by the plots in Fig. S2b. Samples were vortexed and sonicated for one minute. These were characterized by W-band EPR and ^{1}H ENDOR (electron-nuclear double resonance).

The 10 K ED-EPR spectra of free Gd^{3+} (n=0) and the series listed above are depicted in Fig S3a,b. The spectra exhibit a clear broadening of the central transition and of the background due to all other transitions with decreasing R'. From this series we can isolate the spectra of free Gd^{3+} and of Gd^{3+}-$(4MMDPA)_3$ given by the spectrum of R'=1:100. The aquo Gd^{3+} spectrum was simulated with the ZFS parameters D=850 MHz and E=270 MHz with the D and E Gaussian distributions of 700 MHz and 130 MHz, respectively. The spectrum of the R'=1:100 sample was simulated with D=1800 MHz and E=180 MHz with the D and E distributions of 725 MHz and 60 MHz, respectively, as shown in Fig S4a,b.

We could not reproduce the spectra of R'=1:1 and 1:2 just by a superposition of the spectra of free Gd^{3+} and the 1:100 samples. This, and the comparison of the total width of the central transition of the spectra shown in Fig. S3, particularly the low field edge, indicate that spectra of Gd^{3+}-$(4MMDPA)_{1,2}$ are different and narrower than the spectrum of Gd^{3+}-$(4MMDPA)_3$, namely D is smaller.

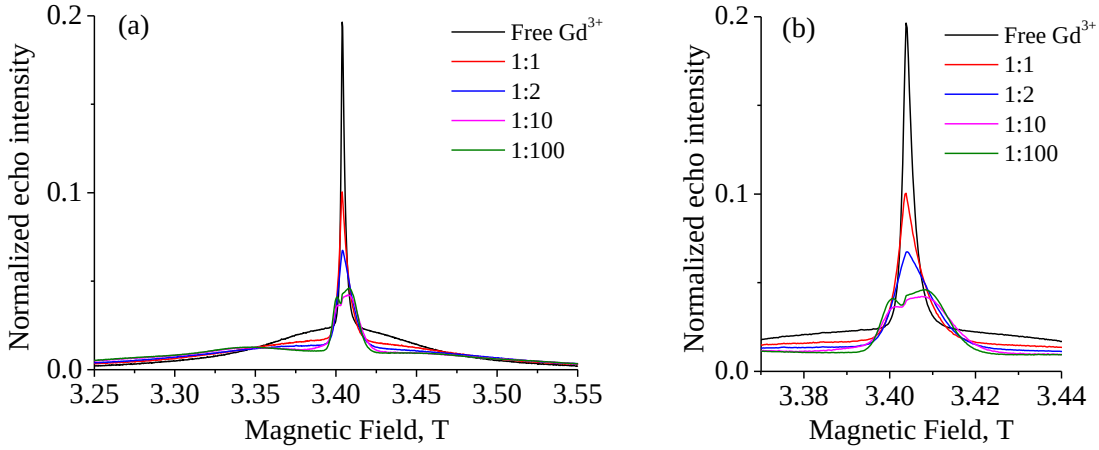

Figure S3: 10 K W-band ED-EPR spectra of frozen solutions of Gd^{3+}-(4MMDPA) as a function of [Gd^{3+}]/[4MMDPA]. *(a)* Full scale and *(b)* the region of the central transition. The spectra were normalized by equalizing the area under the curve to unity.

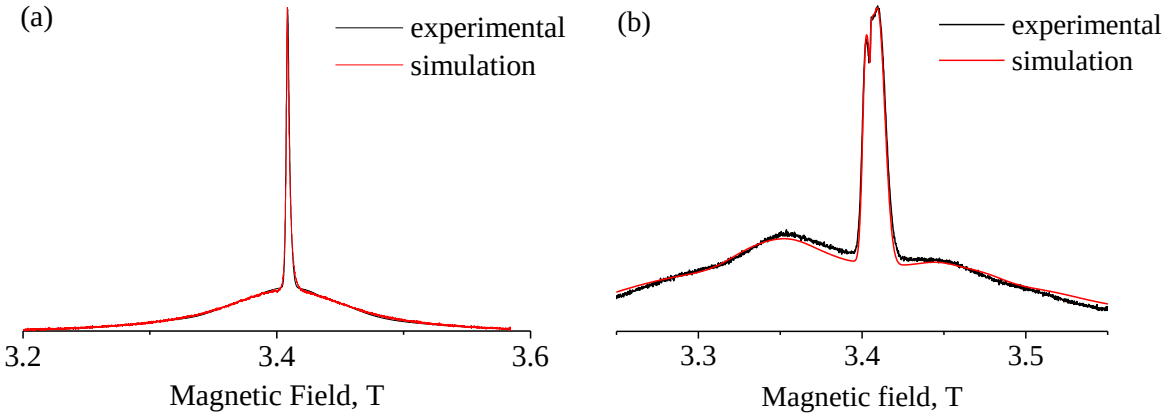

Figure S4: 10 K W-band ED-EPR spectra of *(a)* aquo Gd^{3+} and *(b)* Gd^{3+}-$(4MMDPA)_3$ (R'=1:100) and the corresponding simulations obtained from Easyspin [2] and the parameters listed in the text.

ENDOR measurements

In further attempts to determine the composition of the frozen samples subjected to the DEER measurements in terms of the number of 4MMDPA ligands that binds to Gd^{3+} we have performed ^{1}H ENDOR measurements that count the number of water ligands, m.[3] Measurements were performed with the magnetic field set to the maximum of the Gd^{3+} |-1/2>↔|+1/2> central transition. The ^{1}H ENDOR spectra were measured using the Mims ENDOR sequence

$\pi/2 - \tau - \pi/2 - T - \pi/2 - \tau -$ echo, with an RF pulse applied during the time interval T. The duration of the $\pi/2$ pulses was 12.5 ns, τ was 130 ns and t_{RF} = 30 μs. All ENDOR spectra were recorded using the random acquisition mode,[4] with one shot for each RF point and the repetition time was 1 ms. The data accumulation time varied from 20 min to 2 hr depending on the S/N. All ENDOR measurements were carried out at 10 K.

The aquo complex of Gd^{3+} (n=0) has a total of 9 water ligands (m=9)[5] whereas for the Gd^{3+}-$(4MMDPA)_n$ complexes we get for n=1,2,3 m=6,3,0 respectively. Figure S6a shows the ^1H ENDOR spectra of solutions of Gd^{3+}-(4MMDPA) with various R's. These spectra are normalized according to the ENDOR effect, E, that is defined according to:

$$E = \frac{I(RF_{on}) - I(RF_{off})}{I(RF_{off})}$$

where $I(RF_{on})$ and $I(RF_{off})$ are the echo intensity with RF on and off. In Fig. 6b,c we plot E at ±2.4 MHz and ±1.3 MHz, corresponding to the $\frac{1}{2}A_{\parallel}$ and $\frac{1}{2}A_{\perp}$ features of the M_S=±1/2 manifolds. Taking into account that under the same experimental conditions E is proportional to m, the y scale on the right shows the averaged m values for each sample. These plots show that indeed for R'=1:10 and 1:100 the sample contains mainly Gd^{3+}-$(4MMDPA)_3$ because m~1-2. For R'=1:2 and 1:1 we obtained m=4.7 and m=5.5 respectively. If we use the relative amounts of each Gd^{3+}-$(4MMDPA)_n$ species for R'=1:2 from Fig. S2b and assume the same lineshape for the n=1 and 2 complex, a weighted average yields m=3 which does not agree with the experimental value. Alternatively, to obtain m=4.7 we find that the linewidth ratio of the n=1,2 complexes, $\Delta H_2/\Delta H_1$, should be ~7.84. This ratio is far too large and inconsistent the spectra shown in Fig. S3. The width of the central transition of free Gd^{3+} (n=0) is 29 G and for the n=3 complex it is 160 G. Since 29 G< ΔH_2 and ΔH_1 <160 G the above ratio is considerably overestimated. To reduce this ratio the concentration of the n=1 complex has to increase. Similarly, for the R'=1:1 sample m=5.5 cannot be obtained from the relative concentrations obtained from Fig. S2 and the contribution from free Gd^{3+} must increase.

The calculated speciation curves used in above arguments were derived from room temperature equilibrium constants. Our samples however, were rapidly frozen from room temperature and the equilibrium constants that are relevant for our experimental conditions are probably those of just above the freezing temperature, and may well be different than those determined at room temperature. Moreover our solutions include 30% glycerol which may have an effect as well.

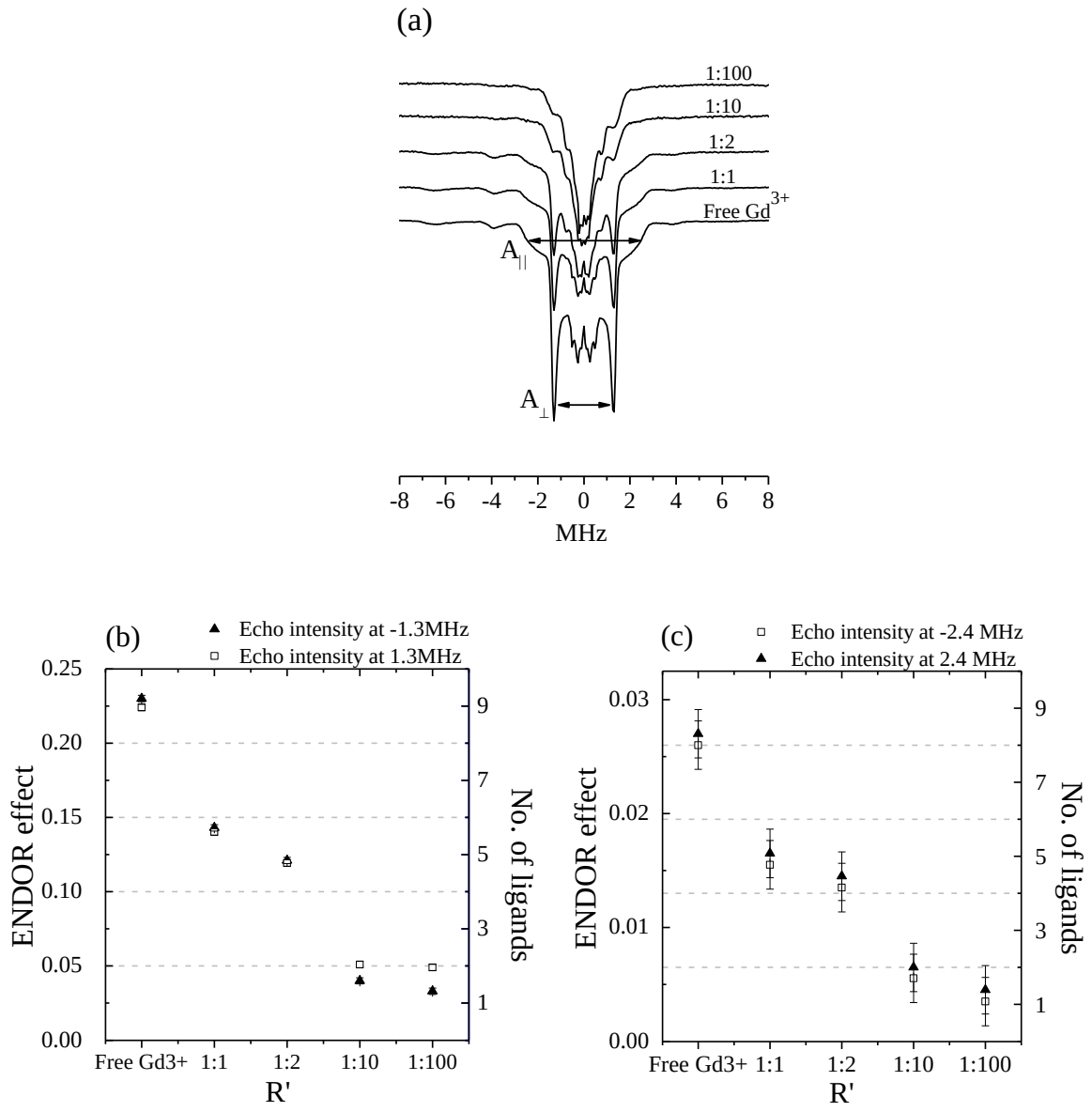

Figure S5: *(a)* Mims ENDOR spectra of solutions of Gd^{3+}-4MMDPA with R'=1:100, 1:10, 1:2, 1:1 and free Gd^{3+}. The water hyperfine couplings corresponding to the $M_S=\pm1/2$ are indicated. *(b)* The ENDOR effect at ±1.3 MHz (error bars are in the same size of the symbol) and *(c)* at ±2.4 MHz and m as a function of R'.

From the EPR and ENDOR measurements we conclude that the room temperature binding constants obtained do not describe the speciation in frozen solutions well enough and the binding constants are lower at the temperature at which the samples freezes. Nonetheless, these curves are useful as guidelines and give idea on the compositions of these solutions.

In principle, simulations of the EPR spectra should provide the relative contributions of the various complexes. Such a simulations, however, involve a rather large number of parameters, D and E and their distributions for the n=1 and n=2 complexes (a total of 8 parameters) along with the relative contributions of the n=0-3 complexes for each R'. This is not a trivial task and it is beyond the scope of this work.

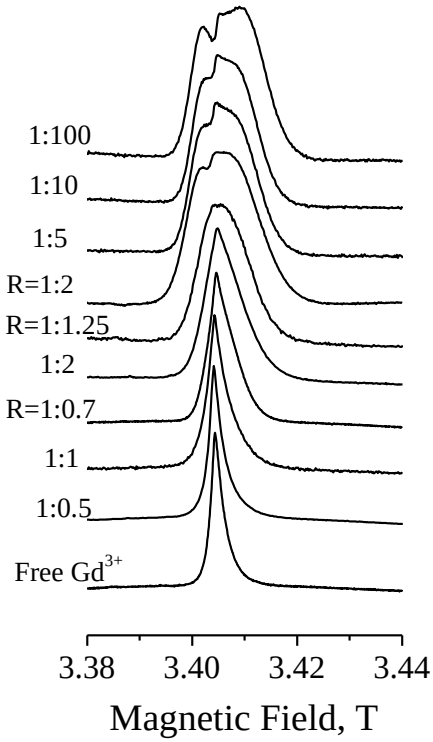

Figure S6: Comparison of the 10 K W-band ED-EPR spectra of solutions of Gd^{3+}-4MMDPA with R'=1:100 ,1:10, 1:5, 1:2, 1:1, 1:0.5 and 0.1 mM free Gd^{3+}, in D_2O/30% glycerol, and of Gd^{3+}-4MMDPA-mel$C_{15}C_{27}$, where R=1:2 (25 K), 1:1.25 (10 K) and 1:0.7 (25 K). Here [Gd^{3+}] was 0.1, 0.16, 0.28 mM, respectively, and [4MMDPA-mel$C_{15}C_{27}$]=0.2 mM.

Relaxation times and DEER control measurements.

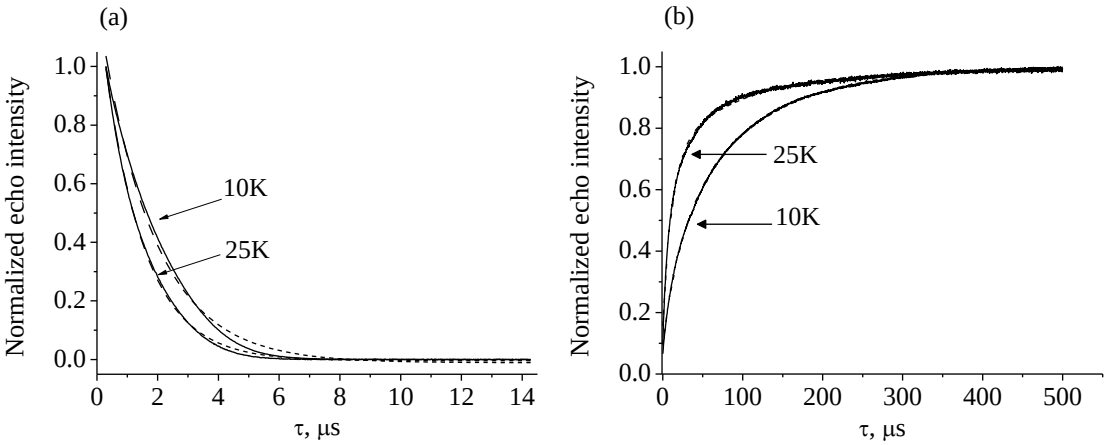

Fig. S7: *(a)* W-band two pulse echo decay of Gd^{3+}-4MMDPA-mel$C_{15}C_{27}$ with R=1:1.25, measured at 10 K and 25 K and their exponential fit *(dashed)* using $V(t) = e^{-2\tau/T_M}$ where T_M(10 K)=3.6 μs and T_M(25 K)=2.6μs. *(b)* Saturation recovery time domain traces of the same sample at the same temperatures their bi-exponential fit *(dashed)* using $V(t) = (1 - Ae^{t/T_{1a}} - Be^{t/T_{1b}})$ where A=0.3, B=0.6, T_{1a} (10 K)=22 μs, T_{1b}(10 K)=100 μs, T_{1a} (25 K)=91 μs and T_{1b} (25 K)= 12 μs.

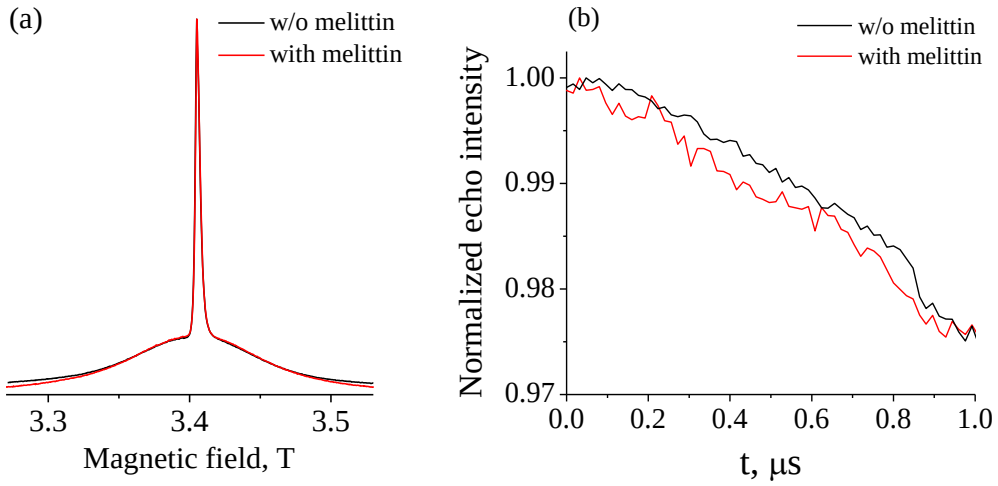

Fig. S8: *(a)* 25 K W-band ED EPR spectra of 0.2 mM Gd^{3+} solution in D_2O/glycerol with 0.4 mM native melittin (red) and w/o (black), and *(b)* the corresponding W-band four pulse DEER traces.

Literature

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