Supporting Information for:

A tailored multi-frequency EPR approach to accurately determine magnetic resonance parameters of Dynamic Nuclear Polarization agents: Application to AMUPol

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Detailed description of simulations

Parameters used in simulation of multi frequency EPR spectra

All EPR simulations were performed with Easyspin (version 5.0.0) [1]. For the simulations of frozen solution spectra, the ‘pepper’ function of Easyspin was used; the strategy to simulate the spectra at the three field/frequency combinations is described below. To account for anisotropic linewidths visible in the spectra, a hyperfine-strain model was used, a heuristic and, in EasySpin convenient model to include anisotropic linewidth variations, without postulating this as the mechanism of linewidth changes. Possible other options are for example G-strain. The parameters were: For the 275 GHz spectrum: 85.5 MHz, 22.5 MHz and 0 MHz in the x-, y- and z-direction, respectively. For 95 GHz the parameters where: 28.5 MHz, 7.5 MHz, and 0 MHz, and for 9 GHz no anisotropy of the lineshape was used. The component linewidths were mixtures of Gaussian and Lorentzian lines with full width at half maximum of 0.2 (Gaussian) and 0.65 (Lorentzian) mT (275 GHz); 0.6 and 0.05 mT (95 GHz); and 0.7 and 0.7 mT (9 GHz). In the simulations, a shift of the static magnetic field of maximally 1.3 mT was applied to compensate for the difference of the static magnetic field measured and at the sample. The error in the g-values of ± 0.00005 were determined from the 275 GHz spectrum by changing simulation parameters manually until differences with the experimental spectrum are noticeable, and the obtained values were used in the simulation of the remaining spectra without further changes.

Simulations of the liquid solution EPR spectra at 275 GHz were performed with the ‘chili’-function as an S = 1/2 spin system, in which the exchange interaction of the biradical is subsumed in the linewidth, as justified by the three line spectrum observed (see below). Best agreement was obtained with an anisotropic rotation model using 1.3 ns and 1.2 ns for the rotation correlation time in the x and y direction, and 2 ns in the z-direction. The component line was Lorentzian with a full-width-at-half-maximum of 0.2 mT. The observed peak-to-peak linewidth of this spectrum of 5 mT exceeds the J-coupling of 1.5 mT, in agreement with the three-line spectrum observed. To estimate, if the rotation correlation times agree with the size of the molecule, we use the Stokes-Einstein relation:

$$\tau_r = \frac{4\pi\eta\alpha^3}{3kT} \qquad \text{(S1)}$$

with the Boltzmann constant, k, and solvent viscosity, η ($\eta = 1.0 \cdot 10^{-2}$ $\mathrm{N \cdot s \cdot m^{-2}}$ for 60 % glycerol in water [2]), the measurement temperature, T, and, α, the molecular radius of 0.6 nm, obtained from the molecular weight of ca. 700 g/mol, and a density of 1. Using equation (S1), τ_r is 3 ns results, in good overall agreement with the experimental results above.

Simulations of the 9 GHz solution spectra were performed as a two-electron spin system, with the pepper routine of EasySpin with averaged G-, and hyperfine tensors. This is a workaround, as the ’chili’ routine, which can simulate motionally-averaged spectra, did not allow for two-electron-spin systems. We only very recently noticed that the latest version of EasySpin seems to accept a two-electron spin system (S = 1/2, S = 1/2) also in this routine, however, the EasySpin documentation indicates that only one electron spin, S = 1/2, can be used. As we did not systematically test this apparently new feature, we did not include it in the present simulation. For the spectrum at low viscosity (Fig S1c and Fig 2d), the dipolar tensor was assumed fully averaged, i.e. zero. For remaining conditions, see caption of Fig. S1.

Calculations of DNP enhancements

DNP simulations conducted in the context of the present study followed our earlier publication Mance *et al.* [3] with the exception that an additional term was added to the Hamiltonian representing the the exchange coupling J_{ab} between electrons a and b, resulting the total time-dependent Hamiltonian

$$\begin{aligned}\hat{H}(t) = {} & \Delta\omega_a\hat{S}_{az} + \Delta\omega_b\hat{S}_{bz} + \omega_n\hat{I}_z + \omega_1\{\hat{S}_{ax}+\hat{S}_{bx}\} \\ & +D_{ab}\{2\hat{S}_{az}\hat{S}_{bz} - \hat{S}_{ax}\hat{S}_{bx} - \hat{S}_{ay}\hat{S}_{by}\} \\ & +A_{zz}\hat{S}_{az}\hat{I}_z + A_{zx}\hat{S}_{az}\hat{I}_x + A_{zy}\hat{S}_{az}\hat{I}_y \\ & +J_{ab}\{\hat{S}_{ax}\hat{S}_{bx} + \hat{S}_{ay}\hat{S}_{by} + \hat{S}_{az}\hat{S}_{bz}\}\end{aligned}$$

As in Ref. [3] , $\Delta\omega_a$ and $\Delta\omega_b$ represent the electron-Zeeman interaction in the rotating frame (with $\Delta\omega_{a,b}=\omega_{a,b}-\omega_{mw}$ and ω_{mw} is equal to the microwave frequency). In addition, ω_n stands for the nuclear-Zeeman interaction, ω_1 is the field strength of the microwave, D_{ab} the dipolar electron-electron interaction and A_{zz}, A_{zx} and A_{zy} are the secular and pseudo-secular hyperfine interaction terms. As in ref. [3] we assumed that dipolar and HFI tensor to be collinear and oriented by the Euler angles (0, π/4, 0) relative to the principal axis system (PAS) of the G tensor. For each timestep of a single rotor period, we calculated the relaxation rates and interactions after rotating from the principal axis system (PAS) to the laboratory frame. After a single rotor period was completed, an evolution super-operator can be defined which can be applied to the initial density matrix to calculate subsequent rotor-cycles.

Table S1 Compete set of parameters used in DNP enhancement simulations

Parameter	**TOTAPOL**	**AMUPol estimated [3,4]**	**AMUPol measured (present work)**
Gxx	2.00988	2.00988	2.0094
Gyy	2.00614	2.00614	2.0061
Gzz	2.00194	2.00194	2.00205
D^{dip}/MHz	23	35	53
J/MHz	0	0	43
T_{1e} / μs	9	60	60
T_{2e} / μs	0.3	0.8	0.8

As discussed before, this approach enables us to determine the behavior of MAS-DNP at high magnetic fields and characterize the relative performance of the MAS-DNP polarizing agents. We attribute the observed dependence of the enhancement to electron nuclear polarization transfer occurring at CE level anti-crossings upon spinning of the sample [5]. The transfer efficiency at such "rotor events" is proportional [6] to the square of the electron-electron spin-spin interaction, which is considerably larger for AMUPol than TOTAPOL (being a combination of J and **D**). The effect of this interaction is less pronounced at lower fields, where the DNP effect is higher (i.e., it tends to saturate at the value of ≈ 660, the ratio of the electron and proton gyromagnetic ratios) but becomes manifest at higher fields (i.e., as the overall enhancements decreases). For similar reasons, the relative performance of the two biradicals under study is not reproduced by the calculation when local nuclei with large hf-values, i.e. nuclei close to the unpaired electrons, are considered (as the overall enhancement gets close to saturation). However, the experimentally observed behavior is found in the calculation when remote nuclei are considered. The distinction between the local and remote nuclei has been discussed in our previous publication [3].

Results of liquid solution EPR on AMUPol

To determine the isotropic magnetic resonance parameters, in particular the exchange interaction J, liquid-solution EPR was measured at room temperature (see Fig. S1). Spectra at 9 GHz where measured under two conditions: in the DNP solution – high-viscosity, 50 % glycerol, Fig. S1 b - and in buffer – low viscosity, less than 1 % glycerol, Fig. S1c and Fig. 2d main text. The spectrum measured under low viscosity conditions has significantly narrower lines (Figure S1c and Fig. 2d, main text) than that at high viscosity (Fig. S1b). Although under the low viscosity conditions, the lines are much narrower than in the spectrum shown in S1b, there are tell-tale signs of incomplete averaging of **G**- and **A**-tensor anisotropies: The slight difference in the intensities of equivalent lines, i.e. of lines that are symmetrically displaced about the central line at B_0 of 351.43 mT. In the fast-motion limit, these lines should have equal intensity. The simulation, red line in Fig. S1c, assumes fast rotation, see below, and therefore cannot fully reproduce line intensities experimentally observed. A nitroxide biradical with negligible J would have three equal-intensity lines, separated by A_{iso}, the isotropic hyperfine coupling constant of the electron spin with the ^{14}N nucleus of the nitroxide. Therefore, the multiline spectrum (Fig. S1c) is direct evidence that J has a finite value, the magnitude of which we determine as described below. A similar spectrum was published very recently in a study, in which 9 GHz-liquid solution EPR spectra measured under conditions corresponding to the low-viscosity spectrum, where shown for a series of bi-nitroxide DNP agents.[7] That study was limited to liquid-solution 9 GHz EPR [7], and therefore only the spectral lineshape, A_{iso}, i.e. the average of the **A**-tensor principal values, and the J-coupling can be compared (see below). The A_{iso} 16.50 G, i.e. 46.2 MHz, compared to 52.77 MHz in the present study (see Table 1 main text), deviates by 13 % from the value obtained in the present study, J, 15.80 G, i.e 44.3 MHz only deviates by 3 % from the value of 43 MHz found in the present study. The deviation in A_{iso} could partly be due to the difference in solution conditions, and may partly reflect the lower sensitivity of the solution 9 GHz EPR spectrum to the hyperfine parameters. Evidently, the experiments reported in [7] cannot reveal the G-tensor parameters and the dipolar interaction, to which the DNP enhancements are sensitive.

In the 275 GHz, solution EPR spectrum (Fig. S1 a), obtained in the high-viscosity DNP solution, the anisotropy of the G- and hyperfine tensors is only partially averaged, and the resulting spectrum is well-represented by anisotropic rotation with rotation correlation times in the order of 2 ns (see Materials and methods), using G- and A-tensor parameters obtained from the simulation of the frozen solution spectra. Eventual residual dipolar interaction and J are subsumed under the linewidth of the spectrum.

Strategy for simulation of multi-frequency EPR spectra

Simulations were performed with a uniform set of parameters for the spectra at all field/frequency combinations, and for frozen and liquid solution. The **G**-, **A**- and **D**-tensor parameters were determined relying mostly on the frozen solution spectra obtained at 95 and 275 GHz. The isotropic exchange interaction parameter J was derived from the low-viscosity spectrum shown in Fig. 2d, main text, and Fig. S1c and was kept fixed in all other simulations, assuming that J does not depend on the temperature and the solvent.

Tensor directions

Initially, the principal axes of the **G-,** hyperfine (**A**) and dipolar-interaction (**D**) tensors were kept collinear, with the unique axis of the axial **D**-tensor ($\mathbf{D}_{\mathbf{par}}$) collinear with the x-axis. The resulting 275 GHz EPR spectrum, which is most sensitive of all three used EPR field/frequency combination, is shown in Fig. S2 as the blue simulation. To improve the agreement with the g_{xx}-feature, a rotation of the g-tensor in the x-y-plane was performed. Tilting $\mathbf{D}_{\mathbf{par}}$ by 50° away from g_{xx} towards g_{yy}, the red spectrum in Fig. S2 is obtained, which agrees much better with the experimental one. This rotation also improved the agreement with the lower frequency EPR spectra. The difference between the red and blue simulations in Fig, S2 nicely illustrates the sensitivity of the 275 GHz EPR spectra to details of the electron-electron spin interaction and especially the tensor directions. To illustrate the error bounds of the simulation, we note that tilt angles of 10° result in significant changes in the simulation. Therefore, the confidence level of the Euler angles used is conservatively estimated in the range of $\pm 10^{o}$, emphasizing the advantage of these very high-field experiments. Overall, the sensitivity of spectra to relative tensor directions depends strongly on the angles themselves and on the magnetic resonance parameters of the paramagnetic center in question, and therefore cannot be generalized. The combination with the lower frequency EPR spectra provides uniqueness of the fit.

In summary, the multi-frequency EPR approach used in the present study results in a robust set of simulation parameters that reflect the essential magnetic resonance parameters of AMUPol.

Conformation of AMUPol according to simulations

Because of the simulation approach chosen (see 'Strategy for spectral simulations' above), the nitroxide bonds in the two radical fragments are parallel to each other (collinearity of the g_{xx} principal axes) and they are chemically equivalent (same principal values of **G**– and **A**-tensors). The two nitroxides have the same orientation with respect to the D-tensor principal axes.

We find that the parallel component of the dipolar interaction tensor, $\mathbf{D_{par}}$ is tilted away from the g_{xx} direction, by 50° towards the g_{yy} axis. The orientation of $\mathbf{D_{par}}$ between g_{xx} and g_{yy} is compatible with the nitroxides pointing away from each other, suggesting an extended conformation of the biradical.

As we did not test how sensitive the simulations are to deviations from this model, we cannot determine whether the nitroxide rings could also be orthogonal, as was suggested for optimum DNP enhancement, as described in [8], for example.

The value of D corresponds to a distance between the spins of 12.5 Å, in good agreement with the DFT, resp. MD optimized structures of AMUPol reported by Sauvee *et al.* [4, 7][1]. Although all simulations are performed with a single conformation, we cannot exclude that some of the deviations of experiment and simulation derive from a distribution of conformations of AMUPol, similar to previous findings for TOTAPOL[8]. Given that the deviations are small in the case of AMUPol, there is no need to include multiple conformations explicitly, as was done in the sophisticated simulation approaches described by Hu *et al.*[8] and Gafurov *et al.*[9].

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[1] Since reference [7] appeared very recently, and gives only limited information about the structure obtained by MD calculations we could not include the parameters given there explicitly in the simulations.

A combination of quantum mechanical calculations and EPR simulation will be the most promising approach to improve the simulations even further, which we are planning in the future. Comparison with the conformation obtained from MD calculations [4] and more recent data presented in [7] would be useful once the structure is made available to the public.

Figures

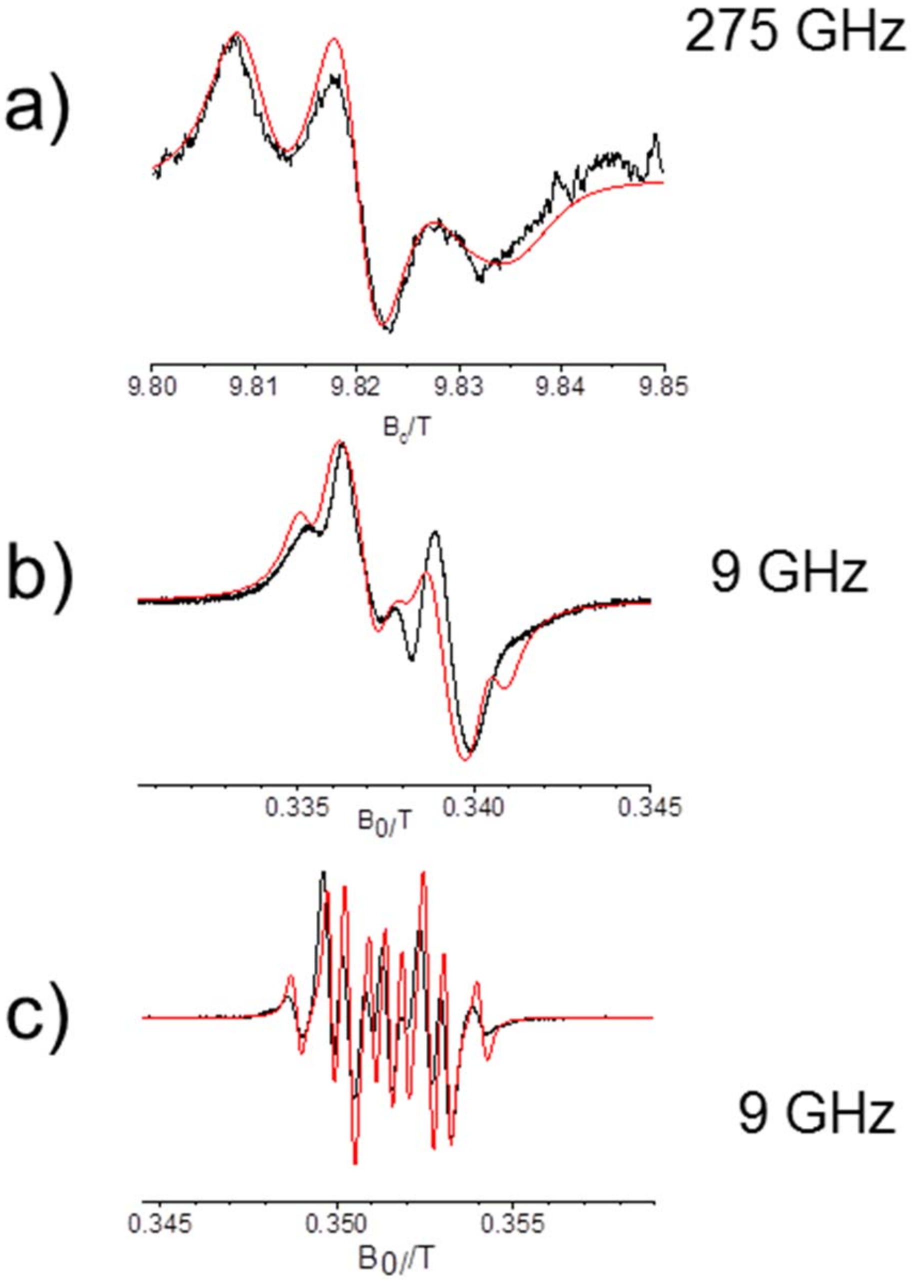

Figure S1 Room temperature EPR spectra of AMUpol at a) 275 GHz and b) 9 GHz in D_8-glycerol/D_2O/H_2O mixture with a volume ratio of 60/30/10 to illustrate the room-temperature spectrum in the solvent of DNPand c) at 9 GHz in water with less than 2 % residual glycerol. (The spectrum in c) is also shown in Fig. 2d)) Black: experimental. Red simulation. Simulation of the spectrum shown in b) performed with the Easyspin function pepper and reduced **D**-tensor parameters: $D_{xx} = -21.2$ $D_{yy} = +10.6$ $D_{zz} = +10.6$ MHz

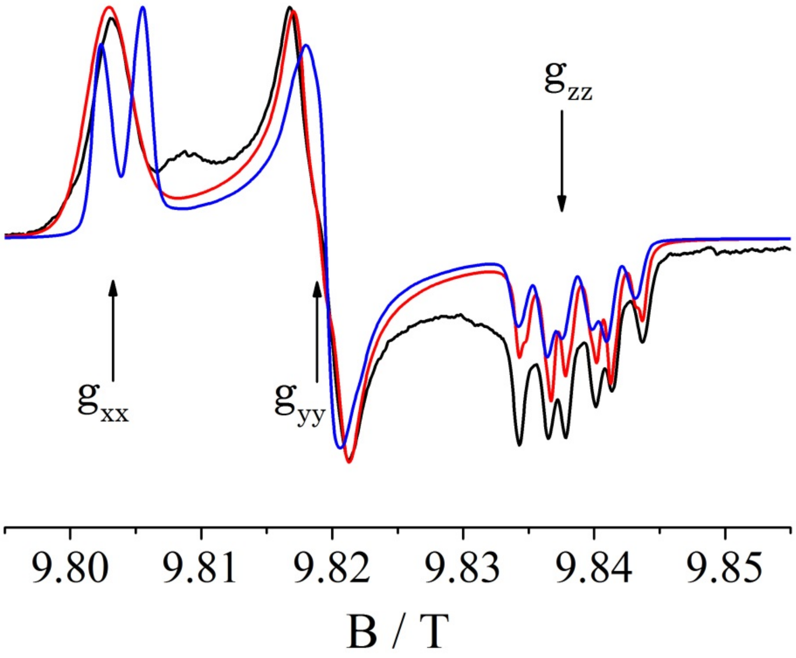

Fig. S2 Sensitivity of the 275 GHz EPR spectrum to **D**-tensor properties. Shown in black is the EPR spectrum of AMUPol in frozen solution at 275 GHz, for conditions, see Fig. 2 main text and Materials and methods (SI). Two simulations are shown in red and blue. Red: Parameters as described in the text. Blue: Same D-tensor principal values as red, but tensor axes rotated in the g_{xx}-g_{yy} plane (see text).

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