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Supporting Information

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Long-Range Distances in Amyloid Fibrils of α-Synuclein from PELDOR Spectroscopy**

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SI1. Sample preparation

Protein expression and purification: Human α-synuclein (hAS) was expressed recombinantly in *E. coli* and purified as described before [1]. Double cysteine hAS mutants were generated using the Quik-Change site-directed mutagenesis kit (Agilent Technologies). The cysteine mutants were purified in the presence of 2 mM DTT. Directly before labeling with the spin label (1-oxy-2,2,5,5-tetramethyl-D-pyrroline-3-methyl)-methanethiosulfonate (MTSL, Toronto Research Chemicals Inc.) DTT was removed by size exclusion chromatography using Zeba spin desalting columns (7 kDa MWCO, Thermo Scientific). Free sulfhydryl groups were modified on ice for 1 hour with a 5 fold molar excess of MTSL. Excess MTSL was removed by gel filtration chromatography on a Superdex 75 16/60 column (GE Healthcare). Complete incorporation of MTSL was confirmed by mass spectrometry.

Aggregation in vitro (fibril growth): Samples were prepared and characterized as described previously.[2] Briefly, before aggregation, the wild type and MTSL-tagged double cysteine mutants were dialyzed against 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.02 % sodium azide. After dialysis the protein samples were centrifuged at +4 ^{0}C for 2 hours at 106000xg (TLA-100.3 rotor Beckman Coulter) to remove aggregates and filtrated through 0.22 μm ULTRAFREE-MC centrifugal filter units (Millipore). Wild type hAS was mixed with the respective MTSL-tagged mutant at ratios 10:1, 20:1, 30:1, 40:1 or 50:1 (mol/mol) and the total protein concentration was adjusted to 100 μM. The solutions of 500 μL volume each were incubated in parallel in 3 glass vials at 37 $^\circ$C and stirred with micro stir bars at 200 rpm for about 10 days using a multiple drive stirrer (Telesystem HP15S, Variomag, Daytona Beach, FL) until the concentration of fibrillized protein reached a steady state according to a control with stained ThioT fluorescence.[3] After aggregation the solutions were centrifuged for 30 min at room temperature at 106000xg, yielding gel-like pellets. The supernatants were discarded. Afterwards, the samples were washed with dialysis buffer and centrifuged again. This procedure was repeated twice. Subsequently, about 30 μL of D_2O was added to the gel-like samples. For CW EPR measurements, 15 μl was taken and filled into EPR capillaries. For DEER/PELDOR measurements at Q-band, 10 % (w/v) of deuterated glycerol was added to the samples as a cryoprotectant. Samples were transferred into 2-mm outer diameter Q-band EPR tubes and quick frozen in liquid nitrogen.

SI2. Experimental Section

PELDOR/DEER: Measurements were carried out using a Bruker ELEXSYS E580 spectrometer with Q-band capabilities. A standard four-pulse sequence was used with the pump pulse of 48 ns set at the resonance dip of the resonator and on resonant with the maximum of the nitroxide spectrum. The π/2 and π pulses of detection were set to 24 ns and 48 ns, and the frequency separation was usually 56 MHz. Experiments were performed at 20 K with repetition times between 10 and 20 ms and a video amplifier bandwidth of 20 MHz. Traces were acquired using a two-step phase cycle for baseline correction and were analyzed with DeerAnalyis2011 program.[6] Baseline subtraction was performed using a background from a 2nd order polynomial that was calibrated with a reference experiment on singly labelled mutants (Supporting Information). Deviation from the standard mono-exponential decay function is likely due to the particular non-homogeneous distribution of spin labels in fibrils along the fibril axis. The shape of the peak-pattern obtained from FT of the traces and comparison with the simulation did not give indication for orientational selectivity. Sample tubes contained about 15 μl fibrils in D_2O and 10% deuterated glycerol. The final spin concentration was ~ 10 - 20 μM.

Electron Microscopy: Samples were bound to a glow discharged carbon foil covered grid. After staining with 1% uranyl acetate, the samples were evaluated with a CM 120 transmission electron microscope (FEI, Eindhoven, The Netherlands). Pictures were taken with a TemCam 224A slow scan CCD camera (TVIPS, Gauting, Germany).

Fluorescence: For ThioT fluorescence measurements, a 5 µl aliquot was taken from the vials at different time of aggregation and added to 2.0 ml of 5 µM ThioT in 50 mM Na-Glycine, pH 8.2. The ThioT fluorescence was recorded at 480 nm with excitation at 445 nm (Varian CARY Eclipse).

SI3. Optimization of spin dilution ratio between double-labeled mutant and wt

The dilution of the labeled protein with wild-type (wt) protein is essential for EPR distance measurements to reduce the intermolecular electron-electron couplings.[2] We performed a series of dilution experiments on fibrils assembled from double-labeled protein at position A90C and S42C (A90C/S42C) with various mixtures of wild-type protein. The dilution ratios of double-labeled protein vs. wt were prepared at 1:10, 1:20 and 1:40 in three replicates. The fibril characterization by ThioT fluorescence (Figure S2a) and electron microscopy (Figure S3) are shown. ThioT fluorescence measurements were plotted as a function of time and fitted to the sigmoidal curves as described by equation (1).[4]

$$f(t) = a + \frac{a+b}{1+\exp\left(\frac{t-t_{1/2}}{k}\right)} \qquad (1)$$

The parameters, $t_{1/2}$ and k, were acquired from the fit where $t_{1/2}$ is the time to 50% of maximal fluorescence, and $1/k$ is the apparent first-order rate constant for the growth of fibrils. The lag time, the initial time where a negligible change in ThioT fluorescence is observed, was calculated as ($t_{1/2}$-2k).

The X-band CW spectra at room temperature (Figure S1b) exhibit line shapes similar to the rigid limit case of isolated and immobilized spin labels. No sharp lines of free spin labels or free proteins are detectable. Moreover, also no broad components arising from exchange-coupled labels as detected in pure single mutants with MTSL[5] are visible.

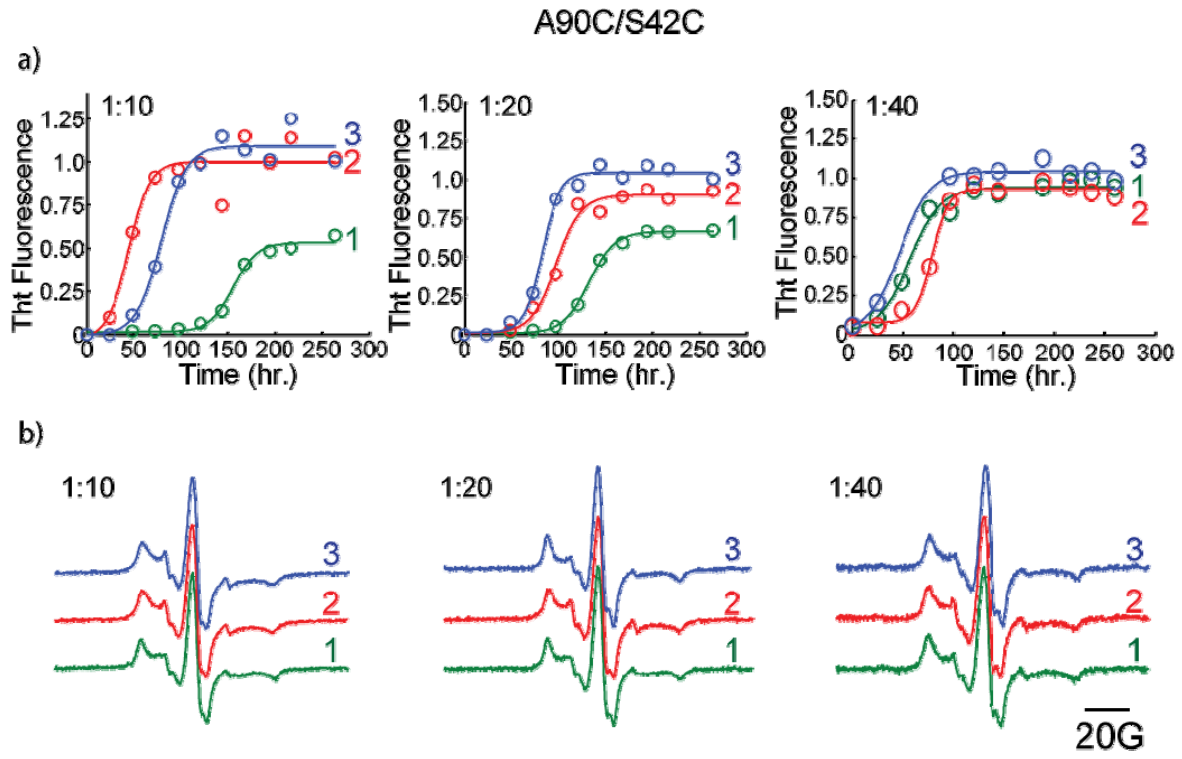


Figure S1. Aggregation curves measured by ThioT fluorescence **(a)** and 9 GHz-CW EPR spectra at room temperature **(b)** of samples prepared from the double labeled mutant of A90C/S42C diluted in wt. The ratio of A90C/S42C:wt was varied at 1:10 (left), 1:20 (middle), and 1:40 (right). Each dilution was prepared in three batches as indicated by 1 (green), 2 (red), and 3 (blue) curves. CW spectra of fibrils were obtained at 2 mW microwave power and at field modulation of 5G at 100 kHz of modulation frequency over a scan range of 200 G.

Table S1. Kinetic parameters obtained from ThioT fluorescence for the A90C/S42C mutant diluted in wt.

A90C/S42C mutant:wt	ThioT					
	Lag time (hours)			$t_{1/2}$ (hours)		
	(1)	(2)	(3)	(1)	(2)	(3)
1:10	131	22	53	156	45	80
1:20	106	72	64	133	98	83
1:40	26	63	17	57	79	47

Distance measurements as a function of dilution are shown in Figure S2. Specifically, for the samples at 1:10 dilution only one out of three revealed a substantial dipolar oscillation. For the 1:20 dilution, two out of three and finally all three batches for 1:40 dilutions lead to observable dipolar oscillation (Fig. S2 a,b).

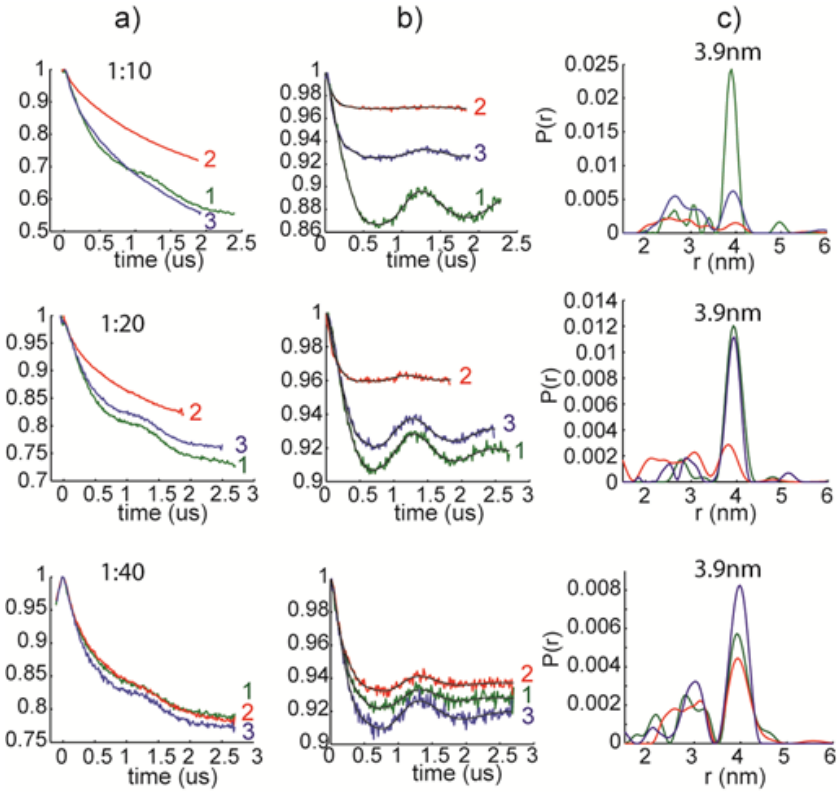
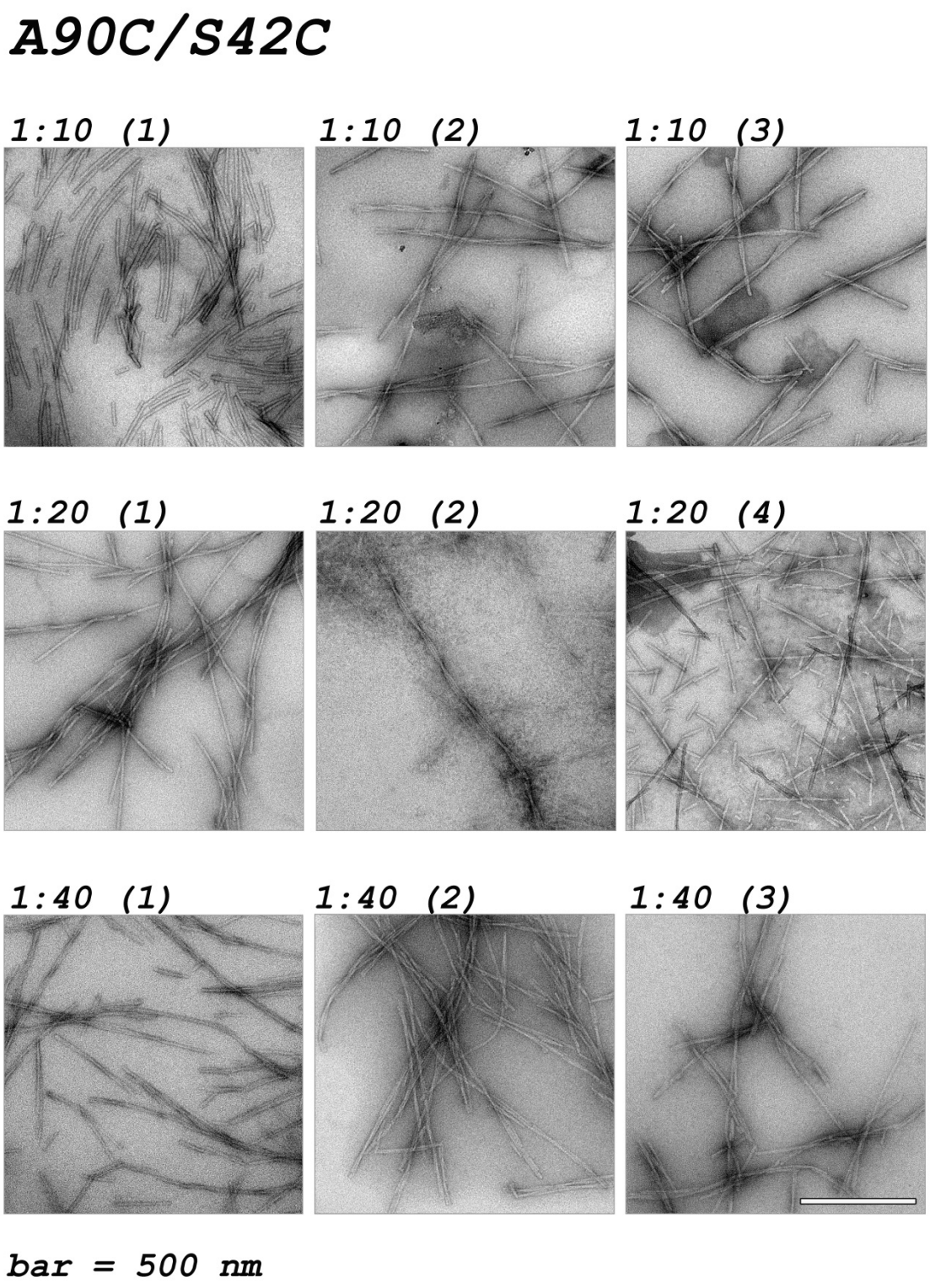


Figure S2. PELDOR traces before (a) and after (b) background subtraction of double-labelled mutant A90C/S42C diluted in wt at ratios 1:10, 1:20, and 1:40. Three sample batches were aggregated in parallel, i.e., 1 (green), 2 (red) and 3 (blue). The corresponding distance distributions in (c) indicate the most probable distance at 3.9 nm. Exp. parameters: shots per point (SPP) = 50, shot repetition time (SRT) = 10 ms, acquisition time = 24-36 hours. T = 20 K.

Figure S3. Electron microscopy pictures of fibrils prepared from doubly labelled A90C/S42C mutant diluted in wt at different ratios.



SI4. Distance measurements on the singly labeled A90C mutant and on the A90C/A53C, A90C/V55C and A90C/V63C double-labeled mutants.

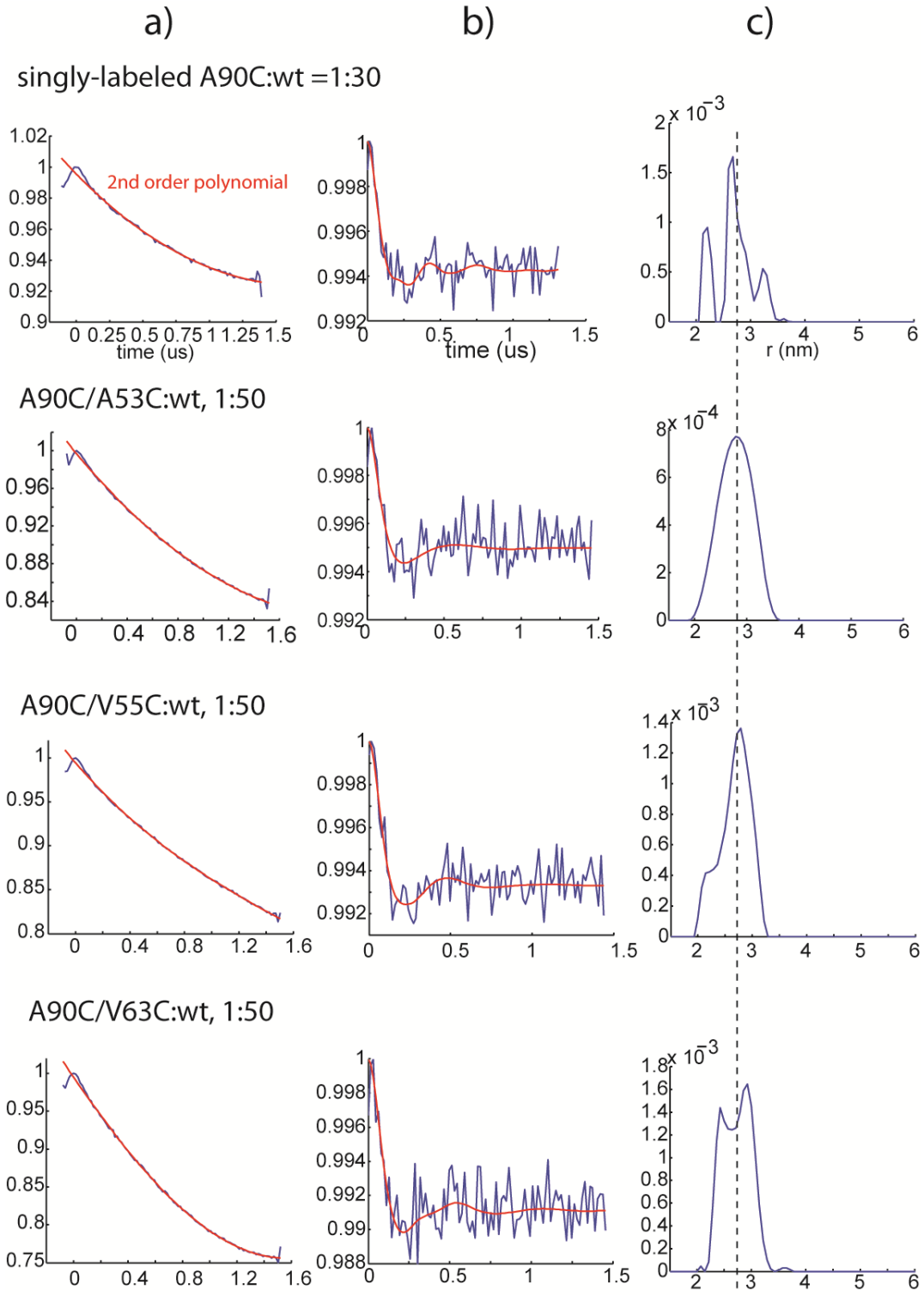


Figure S4: Four-pulse PELDOR traces before (a) and after (b) background subtraction of a singly labeled mutant at A90C diluted in wt at 1:30 ratio. The background subtracted DEER signal is fitted to a second-order polynomial function (red line, a). The corresponding distance distributions are shown in (c). The dash line represents the distance at 2.8 nm. The origin of this distance is unknown.

SI5. Distance measurements of mutants A90C/T54C and A90C/T64C on three different samples.

The distance measurements of the mutant sA90C/T54C and A90C/T64C show reproducible distances among different sample preparation as displayed below.

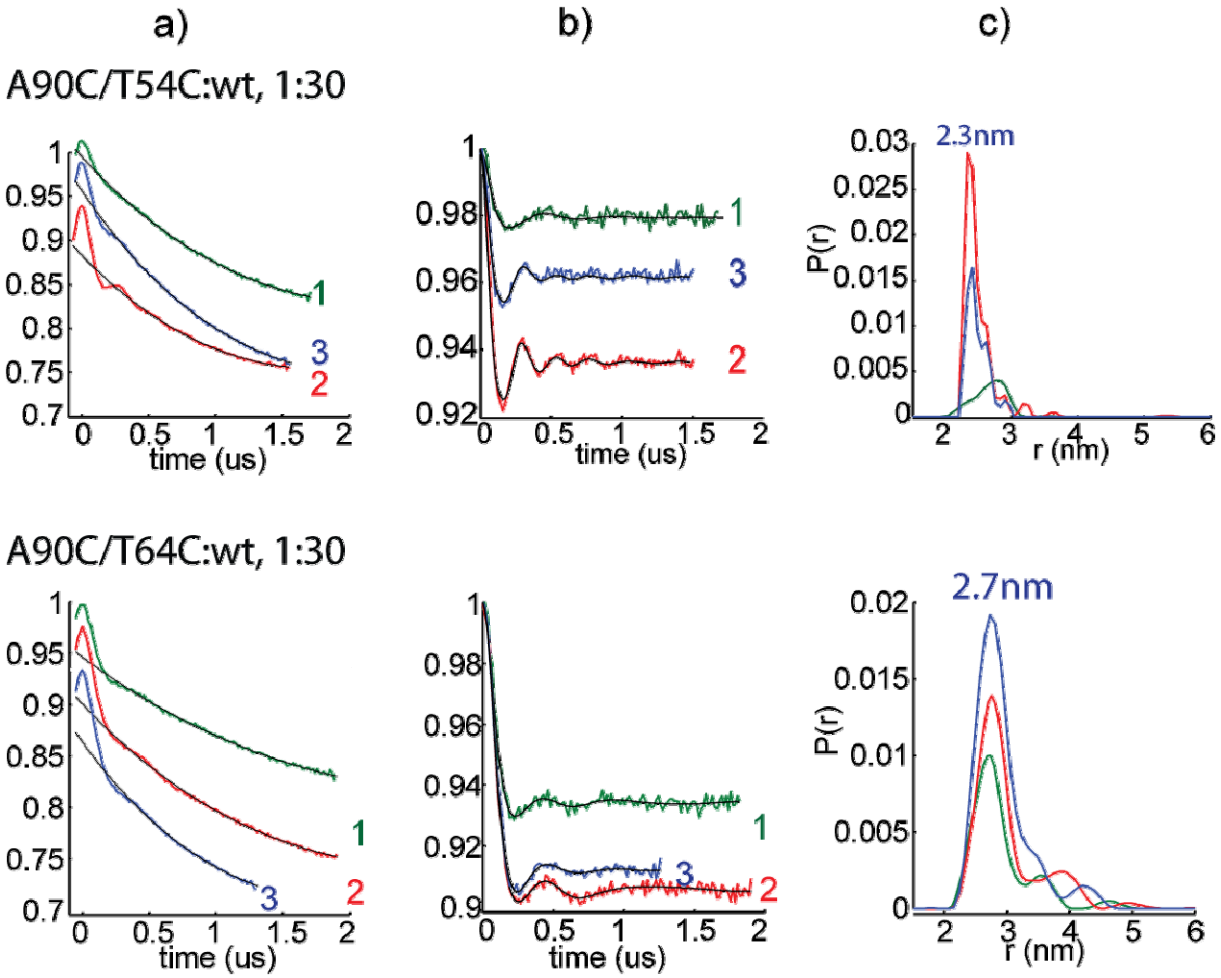


Figure S5: Four-pulse PELDOR traces before (a) and after (b) background subtraction of double-labeled mutants A90C/T54C (top) and A90C/T64C (bottom). The corresponding distance distributions are shown in (c).

SI6. Fibril characterization of double-labeled mutants diluted in wt (room temperature CW spectra and EM images)

The optimized ratio of double-labeled protein in wt for all other mutants was prepared at either 1:30 or 1:50 and the fibrils were characterized by CW-EPR and electron microscopy.

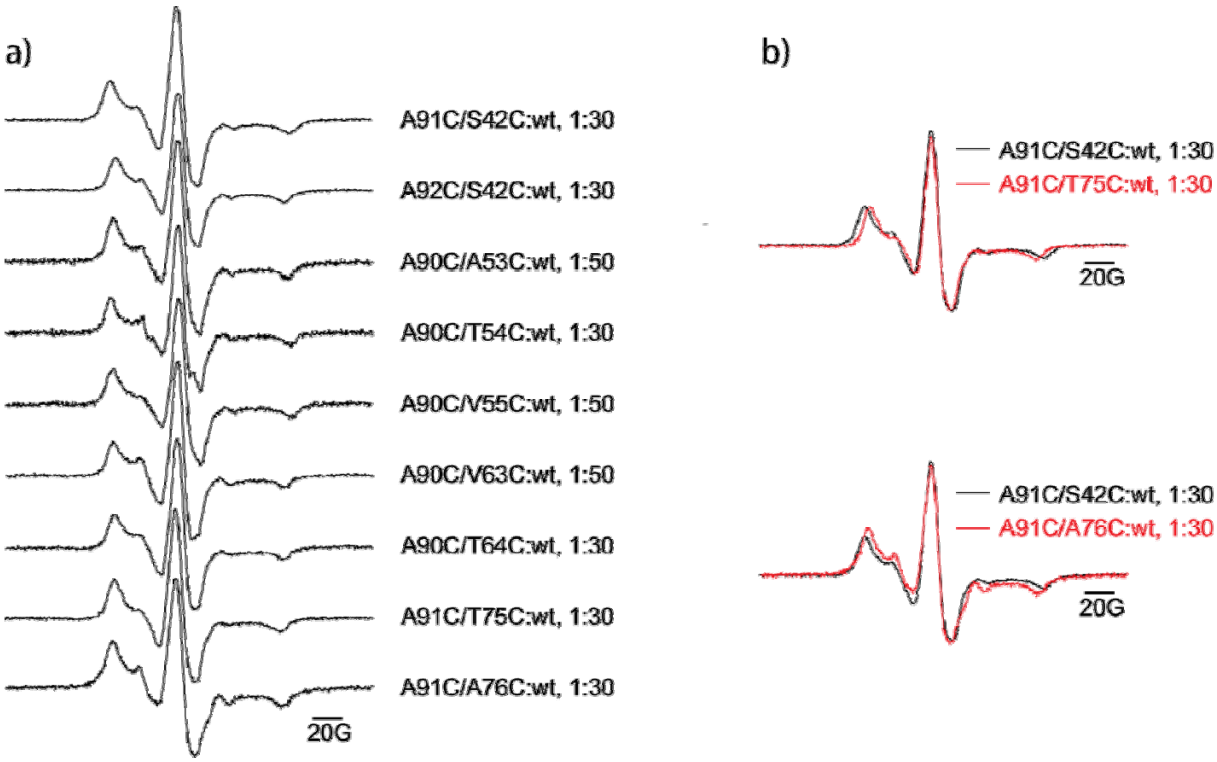


Figure S6. (a) 9 GHz-CW EPR spectra of the doubly labeled mutants diluted with wt protein at room temperature, (b) comparison of CW spectra between mutants A91C/S42C (black) and A91C/T75C (red, top), and A91C/A76C (red, bottom). The DEER experiment in A91C/T75C and A91C/A76C showed a mean distance at 2.2 nm. The central line widths of the CW spectra (b) are superimposable indicating that there is no additional electron-electron coupling due to shorter distances in mutants A91C/T75C and A91C/A76C. We note that the hyperfine splitting in the EPR spectrum of both mutants is slightly reduced as compared to the A91C/S42C mutant. This is likely due to enhanced hydrogen bonding of the spin label at positions 75 and 76 that reduces the spin density on the nitrogen. CW spectra of fibrils were obtained at 2 mW microwave power and at field modulation of 5G at 100 kHz of modulation frequency over a scan range of 200 G.

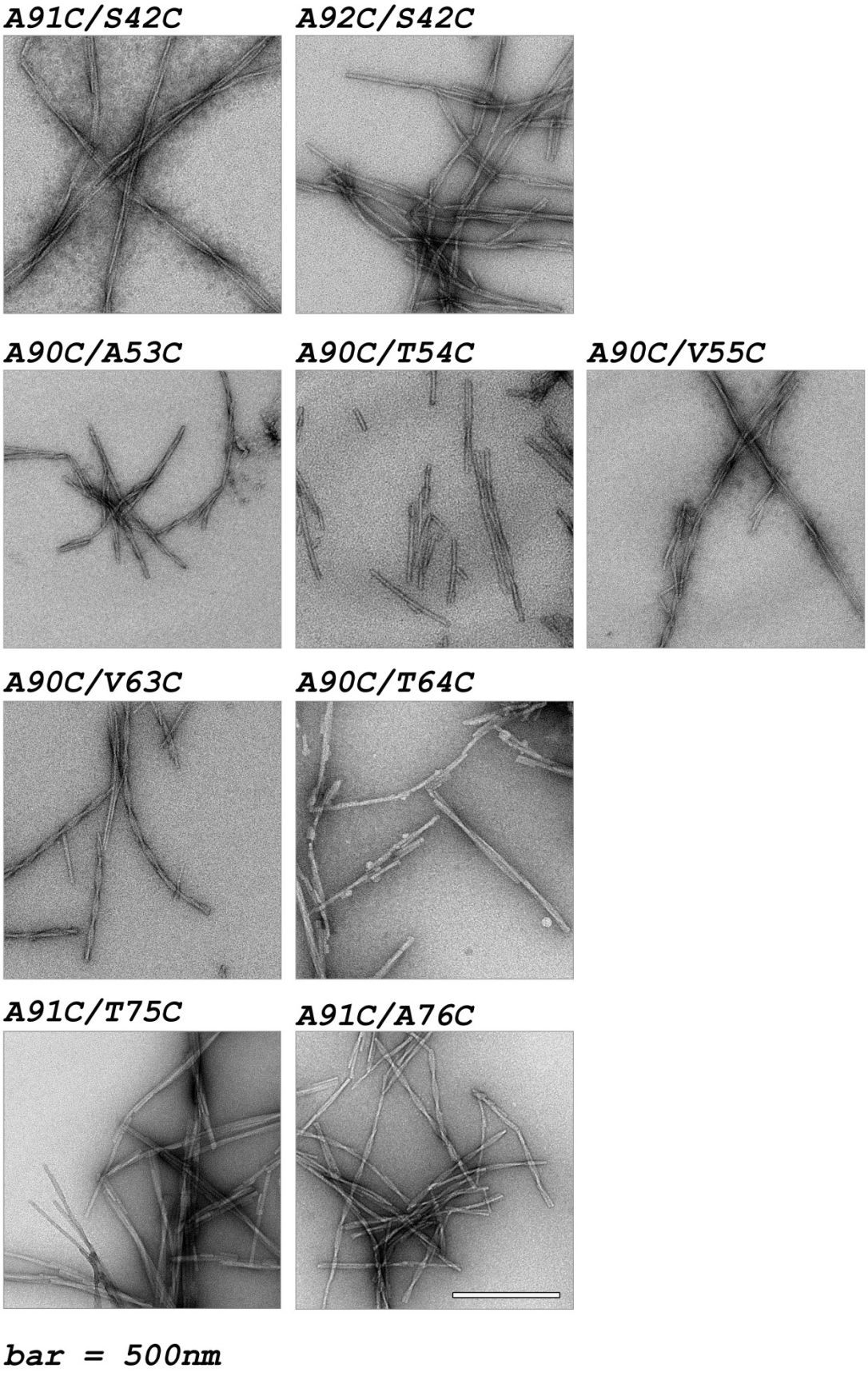


Figure S7. Electron microscopy pictures of fibrils prepared from doubly labeled mutants diluted in wt in ratio 1:30.

SI7. Vectorial information on spin label location from two distances to neighbour labels within β-sheets

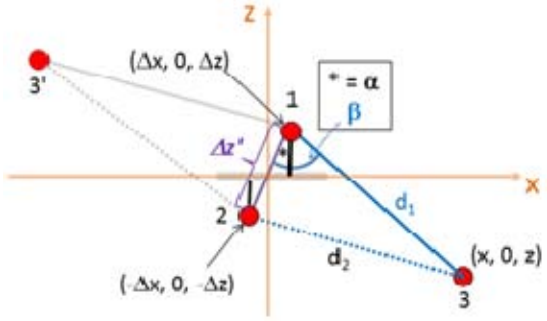


Figure S8. Schematic arrangement of a distant spin label with respect to two adjacent ones in a β-strand (red dots). In a model approximation, side chains of adjacent labels (black bars) lie in a plane perpendicular to the plane of the β-strand (grey bar). Distances d_1 and d_2 form a triangle with the vector Δz'. The intersection of Δz' with the direction of the β-strand (x-axis) defines the origin of the coordinate system. The inclination of Δz' *vs* the z axis is given by the angle α, whereas $\beta \angle \Delta z', d_1$. The angles α and β are given by:

$$\cos\alpha = 2 \cdot \Delta z / \Delta z'; \cos\beta = \left(d_1^2 + \Delta z'^2 - d_2^2\right) / \left(2 \cdot d_1 \cdot \Delta z'\right);$$

An intermediate frame (x', z') is defined with z' along the direction of Δz'. The symmetry-related solution (3') is immediately obtained in this frame from the mirror coordinates (+x', -x') given by eq. 2 (main text).

References

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