Synthesis of bis-spin labeled oligomers

**General Methods:**

Solid phase synthesis was performed in a 1.5mL disposable polypropylene reaction column, connected to a three-way valve equipped with vacuum and argon for mixing. Dichloromethane (DCM) used in coupling reactions was distilled over calcium hydride. Dry grade of dimethylformamide (DMF) from Aldrich was used for coupling. Diisopropylethylamine (DIPEA) was distilled under nitrogen sequentially from ninhydrin and potassium hydroxide and stored over molecular sieves. O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorphosphate (HATU) was obtained from Acros. All solid phase reactions were mixed by bubbling argon up through reactor, allowing for mixing and an inert atmosphere over the reaction. HPLC-MS analysis was performed on a Hewlett-Packard Series 1050 HPLC / 1100 electrospray MS instrument equipped with a Waters Xterra MS C₁₈ column (3.5μm packing, 4.6 mm x 100 mm) and a diode-array detector. Purification of final products was done on the same instrument.
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To five 1.5mL polypropylene solid phase peptide synthesis (SPPS) reaction vessel was added Rink Amide AM Resin (Novabiochem) (5mg, 3.15µmol loading). The resin was swollen for 1hr in dimethylformamide (DMF). The terminal Fmoc-protected amine was deprotected in 0.5mL of 20% piperdine/DMF for 40min. By measuring the absorbance at 301nm of a 1/100 dilution, the number of moles of Fmoc removed was calculated by using $\varepsilon = 7800 \text{ M}^{-1} \text{ cm}^{-1}$. The resin was washed with DMF, isopropanol, DMF, isopropanol, and DMF for 2min each.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 17.1 mg (31.5 µmol) of Fmoc-(Boc) pro4(2S4S) building block [Levins, C. G.; Schafmeister, C.
E. J. Org. Chem. 2005, 70, 9002-9008.] and 12.0 mg of HATU (31.5 μmol) in 160 μL of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 11.0 μL of DIPEA (63.0 μmol) was added to make the active ester. After 10 min activation time, an appropriate aliquot of the solution was added to each deprotected resin, and allowed to react for 30 min. The resin was then washed 3x 2 min with DMF. Double couplings allowed for quantitative acylation, as judged by subsequent Fmoc release in the same manner described above. This process of coupling/deprotection was repeated from three to seven additional times as needed to make the desired number of monomer units.

After the final nth (n=4-8) building block was attached and Fmoc group removed, the first spin label was attached. A solution of 29.0 mg (158 μmol) of 2,2,5,5-Tetramethyl-3-pyrrolin-1-oxyl-3-carboxylic acid and 60.0 mg of HATU (158 μmol) in 800 μL of 20% DCM/DMF was mixed using a micropipettor, after which 55.0 μL of DIPEA (315 μmol) was added to make the active ester. After 10 min activation time, an appropriate aliquot of the solution was added to each resin, and allowed to react for 30 min. After the second coupling, the resin was prepared for cleavage by washing with DMF, isopropanol, DMF, isopropanol, DCM, methanol, DCM, methanol, and DCM for 2 min each. The reactors were then put in vacuum tube, and dried, in vacuo, overnight.

The resins were cleaved in 1 mL of 2.5% water, 2.5% triisopropylsilane, in trifluoroacetic acid (TFA), with stirring for 2 hours. The solution was filtered away from the resin beads, and 2 x 1 mL was used to wash the beads. The solutions were pooled and the
solvent was removed under a stream of dry nitrogen. Residual solvent was removed, *in vacuo*, for one hour, yielding a colorless residue.

The cleaved products were dissolved in 125 µL of 20% piperidine/ N-methylpyrrolidinone (NMP). After 48hrs at room temperature, the products were precipitated by dripping into 2 mL of ether stirring in a 2.2mL polypropylene microcentrifuge tube. The precipitates were collected by centrifugation at 10000x g, 4°C, for 5min. The pellets were washed with 2mL of fresh ether, sonicated, and the centrifugation was repeated. The ether was removed and the pellets were allowed to dry. The pellets were dissolved in a solution of the active-ester of the spin probe, using identical amounts and volumes as above. This reaction was allowed to proceed for 30 min. The final, crude, *bis*-spin labeled oligomers were precipitated by dripping into 2 mL of ether stirring in a 2.2mL polypropylene microcentrifuge tube, washed and collected as above, with most of the excess spin-label conveniently being soluble in the ether. The precipitates were dissolved in 30µL of 30% acetonitrile in water with 0.1% trifluoroacetic acid. This crude material was purified on the analytical HPLC identified in the General Methods section. A small aliquot of each final product was re-injected on the LC-MS and confirmed by mass analysis. In all cases, the desired product was cleanly synthesized, with the only impurity being a small amount of HOAt.

**Reverse phase HPLC chromatograms of compounds 1-5.**

Reverse phase C$_{18}$ high performance liquid chromatography monitored at 254 nm (5-95% AcN, 30 min, 0.05% formic acid).
1
4mer calculated (M+H) = 933.4
ES-MS m/z of 1 = 934.0
$t_R = 10.9$ min

2
5mer calculated (M+1H) = 1071.4
ES-MS m/z of 2 = 1072.1
$t_R = 11.0$ min
6mer calculated (M+1H) = 1209.5
ES-MS m/z of 3 = 1210.2

\[ t_R = 10.9 \text{ min} \]

7mer calculated (M+1H) = 1347.5
ES-MS m/z of 4 = 1348.2

\[ t_R = 10.7 \text{ min} \]
5
8mer calculated (M+1H) = 1485.6
ES-MS m/z of 5 = 1486.2

$t_R = 10.5$ min
**Electron Spin Resonance Spectroscopy**

**Materials and methods**

**Sample preparation**

For ESR experiments, 0.2 mM of the double labeled molecules were dissolved in 70% buffer (50 mM phosphate buffer, pH 7.4, 200 mM NaCl, 3 mM NaN₃, 1 mM EDTA) and 30% glycerol. Each sample (~10 µl) was placed in a ~1.5 OD mm. pyrex capillary tube and flash frozen in liquid nitrogen immediately before insertion into the cavity.

**FT-ESR Spectroscopy**

The ESR experiments were performed using Bruker EleXsys E580 CW/FT X-band ESR spectrometer equipped with a Bruker X-band ER 4118X-MS2 split ring resonator for compounds 1, 3 and 4 (n=4,6 and 7), and 4118X-MS3 for compound 2 and 5 (n=5 and 8). Both resonators provided identical results for the same measurement. The temperature was controlled by an Oxford ITC605 temperature controller and an ER 4118CF gas flow cryostat. All experiments were performed at the temperature of 80 K.

Four-pulse DEER experiments were obtained with a resonator Q ≥ 100 and an ASE TWTA with an output power of 1 KW. The pulse sequence for generating the dipolar time evolution data is shown in figure S1. Two-step phase cycle was used for baseline correction. The observer frequency, \( \nu_A \), was set at the central field of the spin label peak.

![Figure S1. Four-pulse DEER sequence.](image)
which is around 9.5-9.6 GHz, and the pump frequency, $\nu_B$, was set at 70 MHz higher. The length of the $\pi/2$ and $\pi$ pulses was 24 and 48, respectively. The interpulse delays were 200 ns for $\tau_1$, and 2200 ns for $\tau_2$. The increment of time $T$ after the second pulse was 16 ns for 128 points. For each step of the phase cycle 500 averages were collected at a repetition rate of 1 KHz. The acquisition time used for each sample was roughly 24 hours.

**Data Analysis**

The 4-pulse DEER time domain data were processed before acquiring the distance distribution functions. The background decay due to intermolecular interaction was subtracted by fitting the last 75% of the data with a first order polynomial function. For compounds 3-5 ($n=6-8$) the time domain data was smoothed by hamming function and zerofilled to 512 points before analyzing the data. Fourier Transform of the processed data provided the frequency spectra.

Figure S2. a) 4-Pulse DEER time traces of the polymers at $n=4$ to 8 after background subtraction (black solid lines), the corresponding data fitting using Tikhonov regularization method (red dash lines), b) Fourier Transform of the DEER time traces.
The time domain data was analyzed using DEERAnalysis 2004 program which is freely available on the web [www.mpip-mainz.mpg.de/~jeschke/distance.html]. The distance distribution functions were obtained using the Tikhonov regularization method with the regularization parameter of 4.0 for compounds 1-2 (n = 4-5), and 50.0 for compounds 3-5 (n = 6-8). In this analysis no constraints on the maximum possible distance was imposed. The experimental and simulated time domain signal and spectra are shown in Figure S2 and the distribution functions are shown in Figures S3-S7. The data for compound 5 (n=8) was also fit with the program from Chiang, et al using regularization parameter determined by L-curve criterion [Chiang, Y.-W.; Borbat, P. P.; Freed, J. H. J. Magn. Res. 2005, 172, 279-925] and both programs provided the same result.

The distribution functions for compounds 1-2 (cf. Figures S3-S4) appear to have physically unreasonable lobes on the upper end (large r-values). These are possibly due to the fact that the Tikhonov procedure is mathematically ill-posed in the presence in the noise. The use of a better regularization parameter of 10, obtained by using an L-curve criterion, was successful in suppressing these lobes.

In an alternative approach the ESR data was also inverted using the Tikhonov regularization method with limits imposed on the maximum allowable distance. Both methods yielded virtually identical distance distribution functions for compounds 1-5 (see below, Figures S3-S7).
Modeling

Molecular dynamics simulations were carried out using the Amber94 force field [Cornell, W. D.; Cieplak, P.; Bayly, C. I.; Gould, I. R.; Merz, K. M.; Ferguson, D. M.; Spellmeyer, D. C.; Fox, T.; Caldwell, J. W.; Kollman, P. A. J. Am. Chem. Soc. 1995, 117, 5179-5197]. Five-nanosecond in vacuo molecular dynamics simulations at 300 Kelvin were carried out on each compound 1-5. Histograms of the distance between the two nitrooxide nitrogens were calculated and overlaid on the population distributions determined by ESR.

Figures S3-S7 show the distance distribution functions obtained from molecular dynamics and from ESR. For compounds 1 and 2 molecular dynamics predicts a significant population of conformers with interspin distances shorter than 2 nm (cf. Figures S3 and S4). These conformers are unlikely to be sampled by the DEER experiments due to the use of pump pulses of 48 ns. Limitations in the excitation bandwidth of this pulse are expected to suppress the distance distribution function below 2 nm [ Martin, R. E.; Pannier, M.; Diederich, F.; Gramlich, V.; Hubrich, M.; Spiess, H. W. Angew. Chem., Int. Ed. 1998, 37, 2833-2837], which possibly accounts for the discrepancy between experiment and dynamics in the lower wings of the distribution function.
Figure S3. Overlay of the distance between nitroxide nitrogens calculated from a 5 nanosecond Amber molecular dynamics simulation at 300 K of the n=4 labeled scaffold (black) on the distance distribution determined from ESR for the same molecule.

Figure S4. Overlay of the distance between nitroxide nitrogens calculated from a 5 nanosecond Amber molecular dynamics simulation at 300 K of the n=5 labeled scaffold (black) on the distance distribution determined from ESR for the same molecule.
Figure S5. Overlay of the distance between nitrooxide nitrogens calculated from a 5 nanosecond Amber molecular dynamics simulation at 300 K of the n=6 labeled scaffold (black) on the distance distribution determined from ESR for the same molecule.

Figure S6. Overlay of the distance between nitrooxide nitrogens calculated from a 5 nanosecond Amber molecular dynamics simulation at 300 K of the n=7 labeled scaffold (black) on the distance distribution determined from ESR for the same molecule.
Figure S7. Overlay of the distance between nitroxide nitrogens calculated from a 5 nanosecond Amber molecular dynamics simulation at 300 K of the n=8 labeled scaffold (black) on the distance distribution determined from ESR for the same molecule.

The mean distance and standard deviation ($\sigma$) were calculated from the distribution functions using first and second moment as defined by Equations [S1] and [S2]

$$<r> = \frac{\sum_{r_1}^{r_2} r \cdot P(r)}{\sum_{r_1}^{r_2} P(r)}$$  \hspace{1cm} [S1]$$

$$\sigma = \sqrt{\frac{\sum_{r_1}^{r_2} r^2 \cdot P(r)}{\sum_{r_1}^{r_2} P(r)} - <r>^2}$$  \hspace{1cm} [S2]$$

where $P(r)$ is distance distribution function, $r$ is distance, $r_1$ and $r_2$ is the lower and upper range in the distribution function and $\sigma$ is the standard deviation. The results are summarized in Table 1.
Within experimental resolution, the estimates of $\bar{r}$ from dynamics are in reasonable agreement with the experiments for $n=4$-$7$. Note, that for $n=4$ and $n=5$ the experimental value of the average inter nitroxide distance is shifted to higher values possibly because the conformer with $r$ less than 20 Å are inadequately sampled by the DEER experiment (see above). However, molecular dynamics overestimates the mean distance for $n=8$ by ~3.5 Å. Also, the molecular dynamics distributions overestimate the internitroxide distance for long scaffolds (by as much as ~7 Å for $n=8$, cf. Figures S3-S7).

In Figure S8 we compare the experimental data for $n=8$ with a simulated DEER data based on the probability distribution predicted by molecular dynamics. It is evident that the experimentally derived distribution function yields a better fit compared to the simulated data based on molecular dynamics results.
Figure S8. Overlay of the DEER time traces (a) and frequency spectra (b) of experimental data (red), ESR data fit (dashed black), and simulated DEER signal using the P(r) derived from molecular dynamics (blue) for n = 8 scaffold.

Snapshots from the dynamics simulations for n=4 (Figure S9) and n=8 (Figure S10) are presented to illustrate the flexibility of these molecules according to the dynamics simulations. We are currently investigating the addition of explicit solvent and parameterization of the force field for these molecules.
Figure S9. The superposition of the last 500 picoseconds of the 5 nanosecond molecular dynamics simulation for the n=4 compound 1. The central diketopiperazine ring was aligned for each structure and the nitrooxide N-O atoms are rendered as solid spheres.
Figure S10. The superposition of the last 500 picoseconds of the 5 nanosecond molecular dynamics simulation for the n=8 compound 5. The central diketopiperazine ring was aligned for each structure and the nitrooxide N-O atoms are rendered as solid spheres.