Electron paramagnetic resonance approaches to study biologically relevant reactions: examples from amyloid aggregation to enzymes

Passerini, L.

Chapter 2.
Identifying the Elusive Dimerization Product Interfering with Methylsulfonato-group Labelling of Cysteines in Proteins

This chapter will be submitted to Chemistry-A European Journal: L. Passerini, R. Dekkers, K.B.S.S. Gupta, M. Overhand, M. Huber. “Identifying the elusive dimerization product interfering with methylsulfonato-group labelling of cysteines in proteins”, to be submitted.

2.1 Introduction

The methanethiosulfonate (mts)-group is an often used functional group to couple an mts-label to a thiol-group, often from a cysteine of a protein. The mts-group is popular because of its high reactivity and selectivity. This group enables to selectively label proteins with spin- or fluorescent-labels. Often, labelling is the first step to obtain structural or biochemical information, because it opens up a way to apply a variety of techniques in biochemical research, such as electron paramagnetic resonance (EPR), super-resolution-microscopy-techniques, or *Förster*-resonance-energy-transfer (FRET) based distance measurements. Spin labeling is universally applied to investigate proteins by EPR or paramagnetic NMR [1, 2]. It enables structure determination by nanometer-distance constraints by EPR methods [3-6] or the elucidation of protein dynamics or protein-partner interactions, in vitro or in cells [7-13].

Yet the reaction has a major disadvantage: under standard conditions, when cysteine groups of proteins are labelled with the well-known spin label MTSL ((1-Oxyl-2,2,5,5-tetramethyl-Δ-3-pyrroline-3-methyl) methanethiosulfonate) [14], a side reaction occurs that limits the reaction yield. A characteristic EPR signal shows that the side product is a molecule in which two nitroxide radicals are coupled, i.e. a nitroxide-biradical. To suppress this reaction, the MTSL concentrations has to be kept below 200 µM [15], which limits protein concentrations to 20 µM at the typical 10:1 excess of spin label.

In spite of four decades of site-directed-mutagenesis spin labelling, pioneered by the Hubbell group [10, 11], the underlying reaction is not known. Here, we study the formation of the

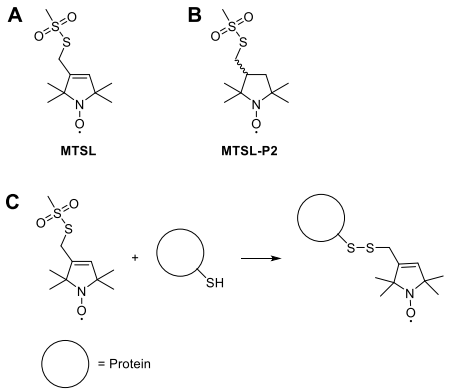

Figure 1. Structure of methylsulfoxide spin label (MTSL) investigated (A), MTSL-P2 (B), and labelling-reaction scheme of the reaction of the label with a cysteine in the protein (C).

side-product under typical spin-labelling conditions for the most-used variants of the methanethiosulfonate spin label (Fig. 1A and 1B) and determine the structure of the biradical-side product by a combination of EPR and mass spectrometry, solving a puzzle that was plaguing labelling reactions for the last four decades.

2.2 Materials and methods

Description of experiment

MTSL ((1-Oxyl-2,2,5,5-tetramethyl-Delta-3-pyrroline-3-methyl)Methanethiosulfonate) and MTSL-P2 ((1-Oxyl-2,2,5,5-tetramethylpyrrolidin-3-yl) methyl methanethiosulfonate) were purchased from Toronto Research Chemicals. Stock solutions were prepared in DMSO, MTSL concentration 24 mM, and stored at -20 °C. The samples were prepared by diluting the stock solutions in 10 mM TRIS-HCl buffer, pH 7.4, for a final MTSL concentration of 100 µM. Incubation was performed on a thermomixer (Eppendorf, Thermomixer comfort) at a temperature of 24°C and shaking at 1000 rpm. A volume of 20 µL was extracted at selected time points for EPR measurement. TCEP (tris(2-carboxyethyl)phosphine) was purchased from Sigma Aldrich. To test reduction, a five molar excess of TCEP was added to the incubating solution, the reaction was allowed to proceed for 30 minutes before EPR measurement. The EPR signal intensity (double integral) was measured before adding TCEP and after the end of the incubation time to be sure that no radical decay occurred after addition of TCEP and during the incubation time. For preparation of the ESI-MS samples, the stock in DMSO was diluted in ammonium acetate (NH_4Ac) buffer, pH 7.4, and incubated in the same conditions (24 °C, 1000 rpm). Dimer formation and effect of TCEP were checked with EPR measurements before ESI-MS analysis.

EPR measurement conditions

EPR spectra were acquired on an EMX EPR spectrometer (Bruker, Rheinstetten, Germany). Glass micropipettes of a volume of 50 µL (Blaubrand Intramark, Wertheim, Germany) were filled with 20 µL of sample for each measurement. Measurement conditions were: microwave frequency: 9.88 GHz, modulation amplitude: 0.2 mT, modulation frequency: 100 KHz, power: 0.63 mW, time constant: 10.24 ms, conversion time: 10.24 ms. All measurements were performed at room temperature.

EPR spectra simulation

MATLAB (R2019a, The MathWorks, Inc., Natick, MA,USA) and the EasySpin package (5.2.35)[16] were used for simulations of the EPR spectra. Spectra were simulated as the sum

of monoradical and biradical nitroxides using the "pepper" function with isotropic g and hyperfine tensors, since biradical spectra can only be simulated by "pepper". Parameters used for the simulation can be found in table 1. The contribution (weight) of the monomer and biradical components to the simulation was adjusted to match the experimental spectral shape. Errors were determined by manually adjusting the contributions of the components until the simulation were visibly deviating from the experimental lineshape.

ESI-MS

ESI-MS samples were prepared as described in the "description of experiment" section above. Measurements were performed with an Q-Exactive HF Orbitrap (Thermo Scientific) equipped with an electrospray ion source (ESI), injection of 2 µl of a 1 µM solution via Ultimate 3000 nano UPLC (Dionex) system , with an external calibration (Thermo Scientific). Source voltage of 3.5 kV, capillary temperature 275 °C, no sheath gas, Resolution = 240.000 at m/z=400. Mass range m/z=160-2000 or until a maximum of 6000. Eluents used: MeCN:H_2O (1:1 v/v) supplemented with 0,1% formic acid.

2.3 Results

In a typical spin-labelling reaction the protein is incubated with a ten-fold excess of spin label in a suitable aqueous buffer solution using reaction times between two and 18 hours, either at room temperature or at 4 °C. Here we mirror these conditions, by studying the reaction systematically in the absence of the protein.

Figure 2 shows the EPR spectrum of (1-Oxyl-2,2,5,5-tetramethyl-Δ-3-pyrroline-3-methyl) methanethiosulfonate (MTSL) after nine hours of incubation in an aqueous solution (buffer), such as the conditions normally used for protein-spin labelling. The spectrum observed is the superposition of two components, shown separately in figures 2B and 2C. One is the classical three-line spectrum (fig. 2B) of nitroxide radicals at room temperature. The splitting into three lines arises from hyperfine interaction of the unpaired electron with the ^{14}N nucleus of the N-O bond. The second component is a five-line spectrum (fig. 2C), that originates from two coupled nitroxide radicals [17], which we will refer to in the following as biradical. A five-line pattern is observed, if the exchange interaction (J) is significantly larger than the hyperfine interaction A_{iso} ($J>2\ A_{iso}$)[17-19]. In table 1, the relevant spectral parameters are given, which are obtained from spectral simulations (see Materials and methods).

Spectra were acquired in time intervals over the course of two days. Figure 3 shows the relative contribution of the MTSL biradical and the monomer component as they develop over time. The biradical contribution increases over time and a maximum of 55% in the biradical component is observed after nine hours of incubation, followed by a decrease to a value of 23% after 51 hours of incubation. The total radical concentration, obtained from the

double integration of the EPR spectra, shows an initial decrease in the number of spins in the sample during the first three hours of incubation, possibly due to decay of the radical. Then the number of spins in the sample reaches a plateau and remains constant for the entire incubation time (green points in figure 3).

After a fair amount (43 %) of biradical had formed in the solution, a mild reductant, specific for disulfide bridges, TCEP (tris(2-carboxyethyl)phosphine), was added to the incubating MTSL solution in a five-fold excess relative to MTSL. This abolishes the biradical component in the EPR spectrum completely, see fig. 4A, suggesting that the biradical contains a disulfide bond.

To obtain information on the structure of the dimer, we used mass spectrometry (electron-spray-ionization mass spectrometry, ESI-MS). Mass spectrometry of the MTSL sample after three hours of incubation shows a base peak at 466 m/z. Simulation of the peak gives the element composition of the

species: $C_{19}H_{34}O_5N_2S_3$. Upon addition of TCEP to the sample, the 466 m/z peak disappears, and a new base peak of 267 m/z appears, corresponding to the oxidized TCEP. The peak of 267 m/z confirms that TCEP

reacts with a disulfide bond. Another new peak at 249 m/z is observed after reaction of the dimer with TCEP, possibly a breakdown product of the reaction between the MTSL dimer and TCEP, however, this peak could not be assigned to a specific chemical structure. Mass spectra are shown in fig. SI 5 and in table 2 the base peaks observed are listed.

For the analogous compound, MTSL-P2, similar results to MTSL were observed. In fig. 4B the EPR spectrum obtained after three hours incubation is shown. Also in this case, the spectrum originates from a superposition of the three-line-nitroxide spectrum with a biradical spectrum of two coupled nitroxides. For MTSL-P2, the shape of the biradical spectrum differs from that of MTSL. The spectrum can be simulated with J = 71 MHz, see table 1. This value of J, however, does not reproduce accurately the experimental spectrum, as the peaks at 345 and 356 mT in the simulation are not observed in the spectrum (see fig SI1). The time course of the reaction (fig. SI 4) closely parallels that of MTSL. Also in the case of MTSL-P2, addition of TCEP completelely abolished the biradical component in the EPR spectrum, see fig. 4 B.

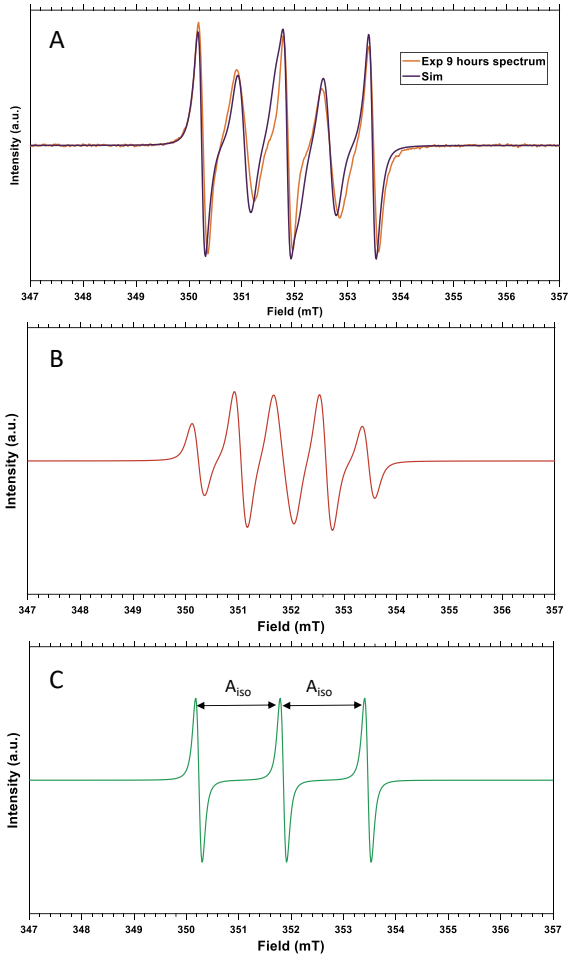

Figure 2. Formation of the MTSL biradical monitored by EPR. A) Spectrum, after nine hours of incubation in aqueous buffer. Purple: experimental spectrum, black: total simulation, orange: biradical component, green : mono-radical component. B) Biradical component from A). C) Mono-radical component. EPR spectra recorded as first derivative of absorption.

Mass spectra of the MTSL-P2 solution after three hours of incubation show a base peak at 470 m/z and, as observed for MTSL, addition of TCEP causes the disappearance of the 470 m/z peak and appearance of the peak corresponding to oxidized TCEP, at 267 m/z.

The similar behaviour of MTSL and MTSL-P2 with respect to dimer formation, the reaction with TCEP, and the base peaks of the mass spectra of the dimers observed, indicates that the same chemical dimerization reaction is happening for both labels.

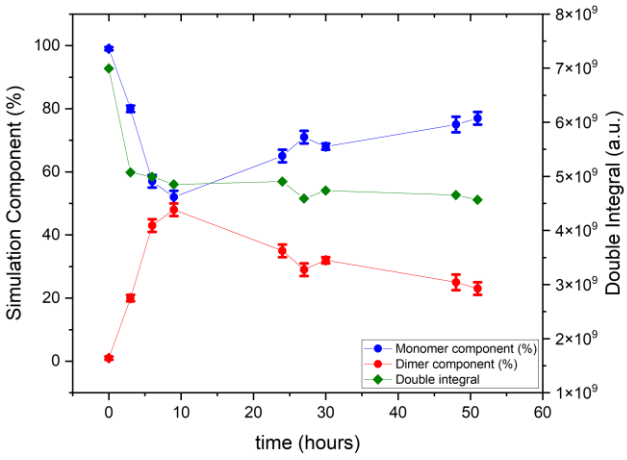

Figure 3. Development of mono- and biradical components for MTSL over time. Blue: monoradical contribution, red: biradical contribution, green: total intensity of the EPR spectrum (double integral). For details about error estimation, see Materials and methods.

Table 1. Parameters used for simulation of mono- and biradical EPR spectral components of MTSL and MTSL-P2. For details, see Materials and methods

	Monomer		Biradical		
	g_{iso}	A_{iso} (MHz)	g_{iso}	A_{iso} (MHz)	J (MHz)
MTSL	2.0059	45.3	2.0059	45.3	600
MTSL-P2	2.0059	45.3	2.0059	45.3	71

Table 2. Mass spectrometry results: base peaks observed in the mass spectra of MTSL and MTSL-P2 after 24 hours incubation and after TCEP addition to the sample. Mass spectra are shown in S.I. For details, see Materials and methods

	During incubation			After TCEP addition	
	Mass**		Sum formula	Mass	
MTSL	466.16210	100%	$C_{19}H_{34}O_5N_2S_3$	267.06245*	100%
MTSL-P2	470.19340	100%	$C_{19}H_{38}O_5N_2S_3$	267.06240*	100%

*oxidized TCEP. ** contain extra O-atom because of cysteine oxidation [20]

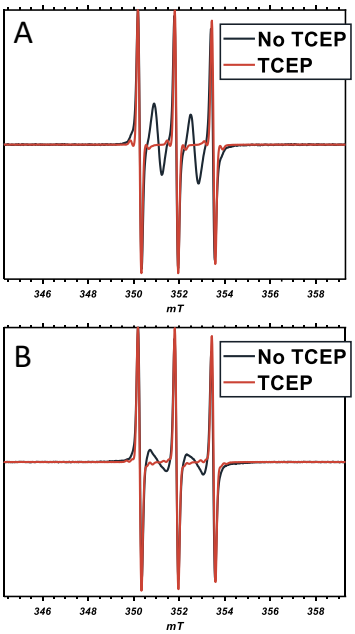

Figure 4. Reductant TCEP (tris(2-carboxyethyl)phosphine) abolishes biradical component. Effect of TCEP on biradical component of MTSL (A) and MTSL-P2 (B). Black: spectrum after three hours of incubation, orange: spectrum obtained after addition of excess TCEP.

2.4 Discussion

In this study, we investigate an unwanted side reaction that interferes with spin labelling of cysteine-proteins. The goal of the study was to understand the reaction and determine the product. To do so, we investigated conditions of product formation for two different spin labels, MTSL and MTSL-P2 (Fig. 1A and B) and propose structures for the products. For both compounds, we show that the product of the side reaction is a nitroxide-dimer, in which the two nitroxide moieties are covalently linked in a reaction that irreversibly converts the mts-group of the MTSL molecule. a.

Within the first nine hours of incubation about 55% of the MTSL is converted to biradical. The biradical contribution to the spectra remains constant (MTSL-P2) or decays after the initial growth period (MTSL). We attribute the decay in the latter case to the breakage of the biradical product or to a biradical decay, i.e. one of the nitroxides on the biradical becomes diamagnetic.. The amount of biradical formed in these experiments is higher than one could

expect, considering that in spin-labelling reactions the concentration of MTSL can be twice as large [15] as in the experiments described here, and also often longer reaction times are used. In the experiments presented here, however, the cysteine-bearing protein (see Fig. 1) is absent. We suggest that in standard spin-labelling reactions the mts-group reacts faster with the electron-rich cysteine SH-group of the protein than with another mts-group (see below), explaining that in protein-labelling dimer formation occurs at significantly higher MTSL concentrations than observed here.

Dimer structure

The EPR spectrum of the biradical contribution shows that two nitroxide moieties interact, and the magnitude of the exchange coupling (J) suggests effective through-space or through-bond coupling of the two nitroxide groups [17]. The biradical of MTSL-P2 has a smaller J than MTSL, suggesting a weaker nitroxide-nitroxide interaction than in the case of MTSL. As described in the SI, some spectral features do not agree with the precise J-value used suggesting that the difference in J could also be due to differences in the biradical-conformational dynamics, as described for other nitroxide biradicals before [17, 19]. Therefore, the difference in the EPR spectra of MTSL and MTSL-P2 cannot be further interpreted. Generally, the exchange interaction cannot easily be related to molecular structure, therefore in order to derive the structure of the dimer, additional information is needed. Chemical evidence comes from the reactions tested here: The pyrroline double bond cannot be involved in the dimerization reaction, because the biradical-rate-of formation and -yield for MTSL is similar to that of MTSL-P2, even though MTSL-P2 lacks this double bond. Also, for MTSL and MTSL-P2, the product must have a reducible disulfide bond, as the biradical EPR spectrum is abolished by TCEP. According to literature, TCEP is selective for disulfides, and does not reduce the nitroxide group [21]. Our experiments confirm this (see Materials and methods), proving that the disappearance of the biradical spectrum is not caused by partial nitroxide reduction in the biradical.

The proposed structure of the dimer (figure 5) agrees with the base peaks from mass spectrometry. Consequently, the asymmetric dimer shown in figure 5 fulfills all the criteria stated above and can be explained by the reaction pathway shown in figure 5.

The reaction starts with the nucleophilic attack of a carbanion at the mesyl group of one MTSL molecule on the mesylated sulfur atom of a second MTSL molecule. The reaction proceeds with the mesyl-group of the second MTSL as the leaving group, forming the hypothesized product. This nucleophilic substitution can be explained by the fact that the methanesulfoxide-group is relatively acidic, while methanesulfinate is known to be an excellent leaving group. The reaction leads to a product that has the expected chemical

properties and agrees with the sum formula from mass spectrometry of the reaction product.

Given that the reaction mechanism does not involve a radical step, the reaction is likely to occur also in other labelling reactions, thus also when fluorescent labels are attached to a cysteine-bearing protein by a methanethiosulfoxide group, yet it was never reported. The reasons could be that fluorescent labels are more often linked by other groups, like the maleimide group, and that the labelling is often carried out at lower concentrations than spin-labelling reactions. Also, there would be no clear signature of a dimer, unlike the biradical in EPR. Ultimately, any dimer product would be removed in the final labelling step

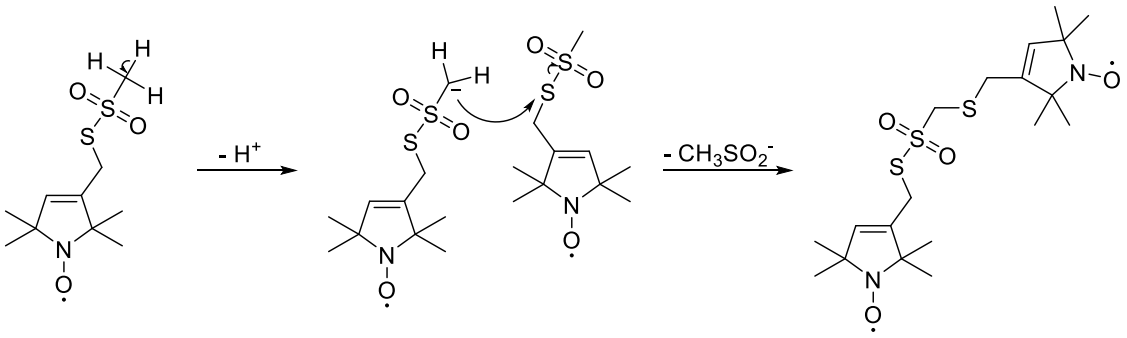

Figure 5 Proposed reaction mechanism for MTSL and structure of the dimer product (right). Note: The additional oxygen-atom mass of the base peak (table 2) is attributed to an cysteine oxidation .

when unreacted label is removed from the protein.

The question that remains is if the side reaction can be suppressed or avoided in spin-labelling reactions. The disulfide linkage of the dimer makes this unlikely as breaking the disulfide bond of the dimer by a reductant would also decouple already coupled spin label from the protein, so it would not be useful.

In conclusion, we show that the unwanted biradical reaction that hampers spin-labelling reactions of proteins is an irreversible process, in which the unwanted product is formed in high yields. We propose a structure for this product that agrees with the mass obtained from mass spectrometry and the chemical properties observed in this study. So after four decades of mystery we have elucidated the reaction that causes the formation of the biradical product in spin-labelling reactions of proteins. We show that this biradical is a covalently bound dinitroxide, linked via a disulfide bond.

Supplementary information

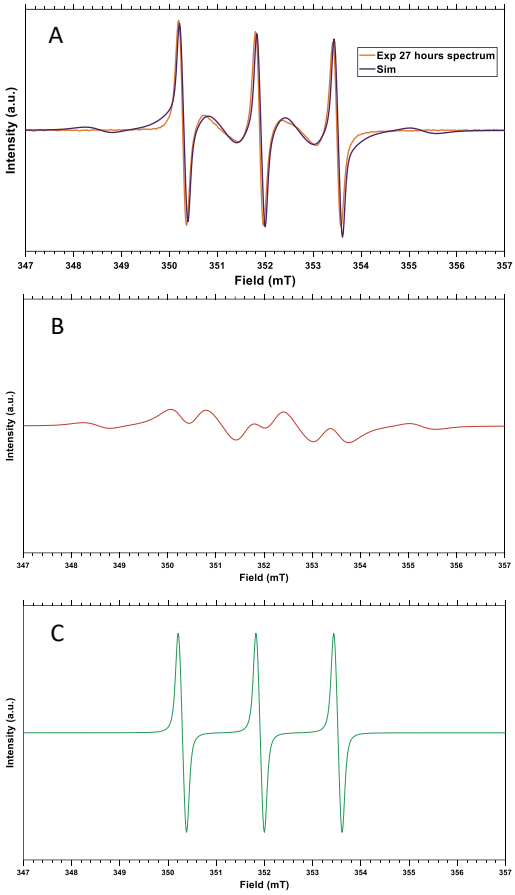

Figure SI.1. Formation of MTSL-P2 biradical monitored by EPR. EPR spectrum after incubation of MTSL-P2 in aqueous buffer. A) Spectrum, after nine hours. Purple : experimental spectrum, black : total simulation, orange : biradical component, green: mono radical component. B) Biradical component from A). C) Monoradical component. EPR spectra recorded as first derivative of absorption. The value of J used was selected solely to reproduce the spectrum between 350 and 354 mT. The simulation shows additional lines at 348 and 356 mT that are not experimentally measured, suggesting the J value used is not accurate.

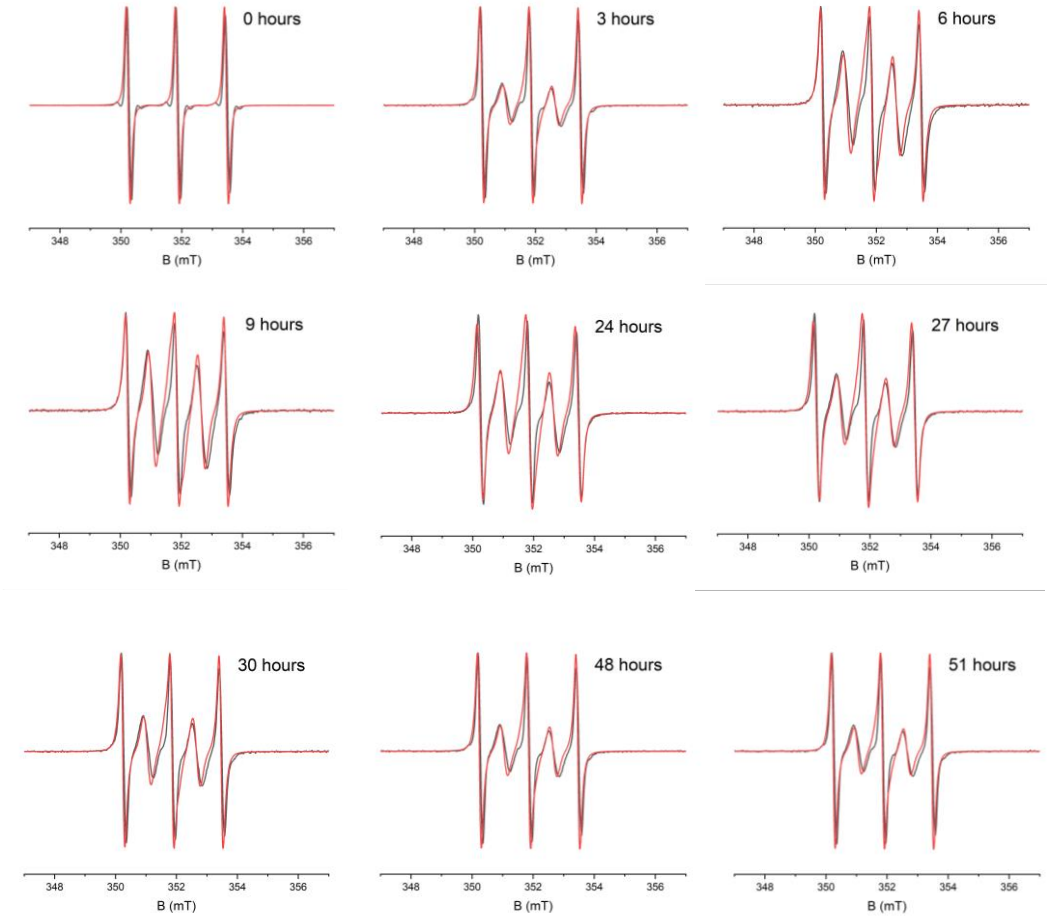

Figure SI.2. EPR spectra of MTSL acquired at different time points. Black: experimental spectrum. Red: simulation.

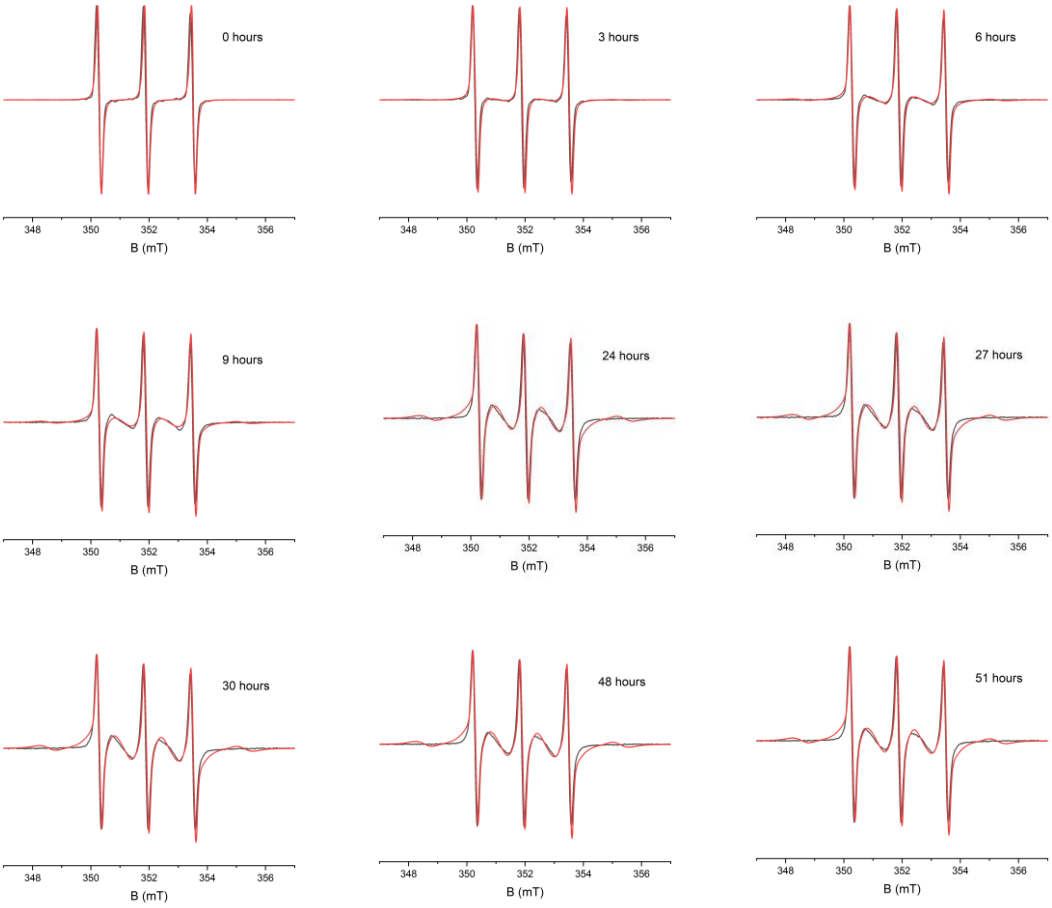

Figure SI.3. EPR spectra of MTSL-P2 acquired at different time points. Black: experimental spectrum. Red: simulation.

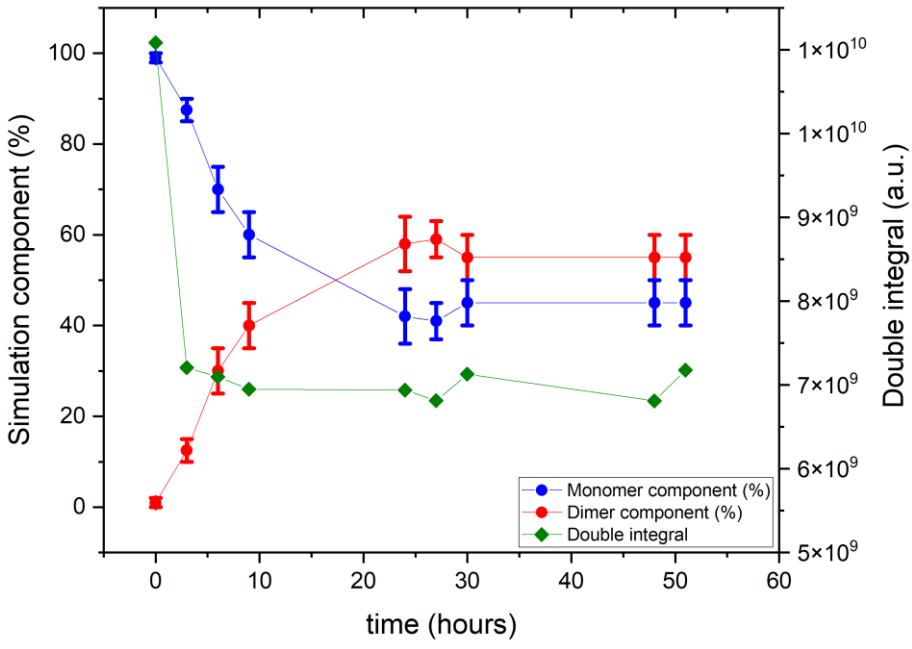

Figure SI.4. Development of MTSL-P2 biradical component over time. Contribution of the mono- and biradical component over the 51 hours of incubation. Blue : monoradical contribution, red : biradical contribution, green : total intensity of the EPR spectrum (double integral value). For details about error estimation, see materials and methods. Given that the simulation of the MTSL-P2 biradical spectrum gives additional lines that are not experimentally observed, the contribution measured through simulation of the biradical for MTSL-P2 is overestimated, and the monomer contribution is underestimated as a consequence.

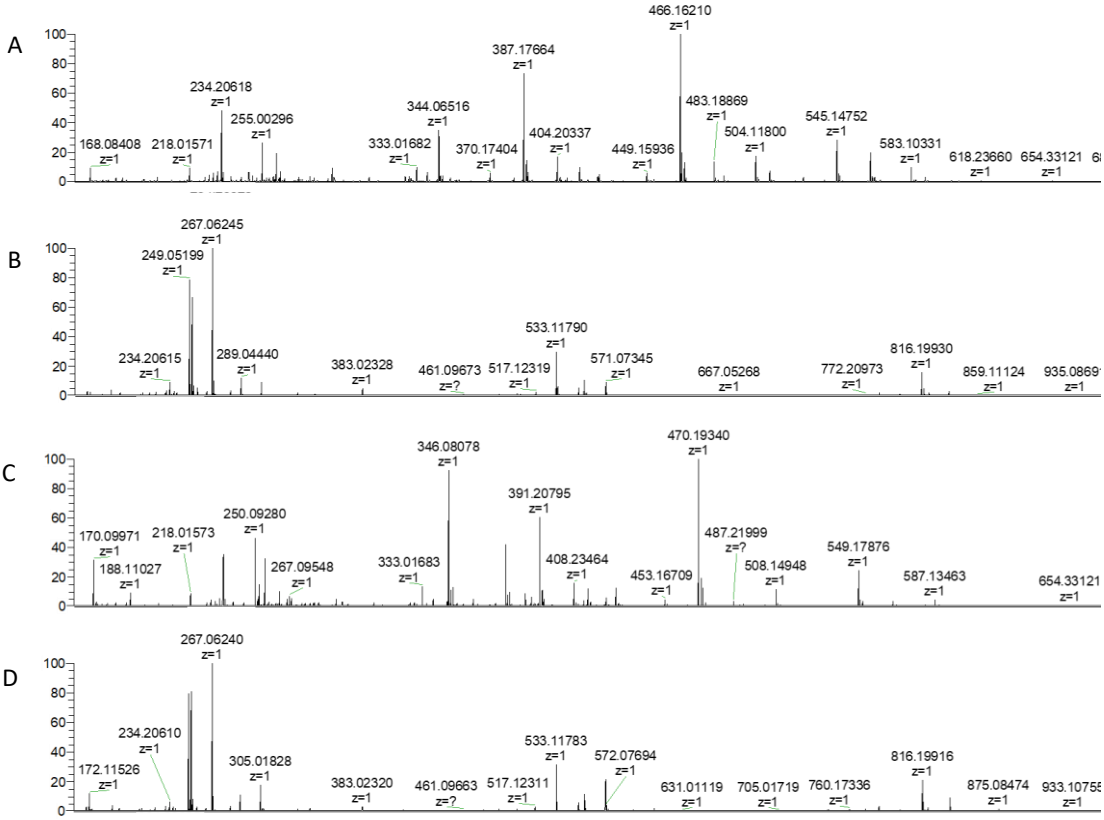

Figure SI.5. MS-ESI spectra of MTSL and MTSL-P2. A): MTSL after three hours incubation. B): MTSL after three hours incubation and reaction with TCEP. C): MTSL-P2 after three hours incubation. D): MTSL-P2 after three hours incubation and reaction with TCEP.

References

[1] P.S.N. Ishita Sengupta, and Christopher P. Jaroniec, Protein Structure Determination with Paramagnetic Solid-State NMR Spectroscopy, Acc. Chem. Res. 46 (2013) 2117-2126.
[2] D.S. Joel R. Gillespie, Characterization of long-range structure in the denatured state of staphylococcal nuclease. I. paramagnetic relaxation enhancement by nitroxide spin labels, Journal of Molecular Biology 268(1) (1997) 158-169.
[3] K. M. Salikhov, and A. M. Raitsimring, The Theory of Electron Spin-Echo Signal Decay Resulting from Dipole-Dipole Interactions between Paramagnetic Centers in Solids, jOURNAL OF MAGNETIC RESONANCE 42 (1981) 255 - 276.
[4] M. Pannier, S. Veit, A. Godt, G. Jeschke, H.W. Spiess, Dead-time free measurement of dipole-dipole interactions between electron spins. 2000, J Magn Reson 213(2) (2011) 316-25.
[5] G. Jeschke, DEER distance measurements on proteins, Annu Rev Phys Chem 63 (2012) 419-46.
[6] S.K. Milov AD, Shchirov MD, Use of the double resonance in electron spin echo method for the study of paramagnetic center spatial distribution in solids, Fiz. Tverd. Tela (Leningrad) 23 (1981) 975-982.
[7] L.C.a.W.L. Hubbell, A new spin on protein dynamics, TRENDS in Biochemical Sciences 27(6) (2002) 288-295.
[8] Y.E. Nesmelov, D.D. Thomas, Protein structural dynamics revealed by site-directed spin labeling and multifrequency EPR, Biophys Rev 2(2) (2010) 91-99.
[9] F. Torricella, A. Pierro, E. Mileo, V. Belle, A. Bonucci, Nitroxide spin labels and EPR spectroscopy: A powerful association for protein dynamics studies, Biochim Biophys Acta Proteins Proteom 1869(7) (2021) 140653.
[10] A.P. Todd, J. Cong, F. Levinthal, C. Levinthal, W.L. Hubbell, Site-directed mutagenesis of colicin E1 provides specific attachment sites for spin labels whose spectra are sensitive to local conformation, Proteins 6(3) (1989) 294-305.
[11] W.L. Hubbell, A. Gross, R. Langen, M.A. Lietzow, Recent advances in site-directed spin labeling of proteins, Current Opinion in Structural Biology 8(5) (1998) 649-656.
[12] A. Bonucci, O. Ouari, B. Guigliarelli, V. Belle, E. Mileo, In-Cell EPR: Progress towards Structural Studies Inside Cells, Chembiochem 21(4) (2020) 451-460.
[13] S. Kucher, C. Elsner, M. Safonova, S. Maffini, E. Bordignon, In-Cell Double Electron-Electron Resonance at Nanomolar Protein Concentrations, J Phys Chem Lett 12(14) (2021) 3679-3684.
[14] L.J. Berliner, J. Grunwald, H.O. Hankovszky, K. Hideg, A novel reversible thiol-specific spin label: Papain active site labeling and inhibition, Analytical Biochemistry 119(2) (1982) 450-455.
[15] E. Bordignon, EPR Spectroscopy of Nitroxide Spin Probes, eMagRes2017, pp. 235-254.
[16] S. Stoll, A. Schweiger, EasySpin, a comprehensive software package for spectral simulation and analysis in EPR, J Magn Reson 178(1) (2006) 42-55.

[17] S.S. Eaton, L.B. Woodcock, G.R. Eaton, Continuous wave electron paramagnetic resonance of nitroxide biradicals in fluid solution, Concepts Magn Reson Part A Bridg Educ Res 47A(2) (2018).
[18] M. Abe, Diradicals, Chem Rev 113(9) (2013) 7011-88.
[19] G.R. Luckhurst, Alternating linewidths. A novel relaxation process in the electron resonance of biradicals, Molecular Physics 10(6) (1966) 543-550.
[20] A. Scherer, X. Yao, M. Qi, M. Wiedmaier, A. Godt, M. Drescher, Increasing the Modulation Depth of Gd(III)-Based Pulsed Dipolar EPR Spectroscopy (PDS) with Porphyrin-Gd(III) Laser-Induced Magnetic Dipole Spectroscopy, J Phys Chem Lett 13(47) (2022) 10958-10964.
[21] E.B. Getz, M. Xiao, T. Chakrabarty, R. Cooke, P.R. Selvin, A Comparison between the Sulfhydryl Reductants Tris(2-carboxyethyl)phosphine and Dithiothreitol for Use in Protein Biochemistry, Analytical Biochemistry 273(1) (1999) 73-80.