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Spin-label EPR Approaches to Protein Interactions

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2

HETERODIMER FORMATION OF MEMBRANE-FUSION E/K PEPTIDES STUDIED BY CONTINUOUS-WAVE EPR

2.1 Introduction

Membrane fusion is an essential process in living organisms. In eukaryotic cells, the early stage of fusion involves two membranes, each with a membrane-anchored SNARE protein^[1] (SNARE, soluble NSF attachment protein receptor; NSF = N-ethylmaleimide-sensitive factor). The mechanism of membrane fusion is still unknown^[2]. To investigate membrane fusion involving SNARE proteins, model systems are synthetically designed, which mimic the biological system. The building blocks are biologically inspired modules and consist of a membrane anchoring segment, a zipper segment, and a linker that connects the two segments (Figure 2.1a). To understand whether the final construct will be functional in membrane fusion, it is important to know how the components operate by themselves. In this study, we concentrate on the zipper segment, i.e., peptides that self-assemble into a coiled-coil^[3] complex, similarly to the zipper segment of SNARE proteins.

Inspired by the work of Litowski and Hodges^[4;5], we synthesized variants of the oligopeptides E and K, listed in Table 2.1. The E and K peptides are oppositely charged, due to the abundant glutamic acid (E) and lysine (K) residues, respectively. Figure 2.1b shows the ionic and hydrophobic

interactions, which are expected to stabilize the heterodimer. A tryptophan (W) and a tyrosine (Y) residue were incorporated to facilitate the use of UV-Vis spectroscopy to determine the concentration of the peptide. Under physiological conditions, peptide E adopts a predominantly random-coil conformation, while peptide K is predominantly α -helical^[6]. When mixed, peptides E and K are designed to twist around one another to form a coiled-coil^[4]. With only three heptad repeats, they are the shortest known coiled-coil pair, which assembles specifically into a stable heterodimer ($K_D \sim 10^{-7}$ M at 25 °C)^[4-6]. For our variants specifically, the E and K peptides form heterodimers in parallel fashion, with all of the residues participating in the coiled-coil^[7].

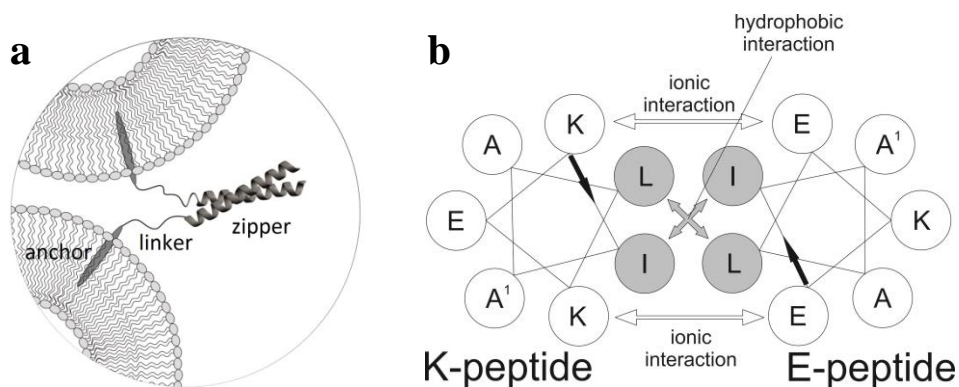


Figure 2.1: Schematic representations of: **a)** a membrane-fusion construct consisting of a zipper segment, a linker, and a membrane anchor; **b)** the K- and E-peptides in a helical wheel projection. The peptides propagate into the page from the N-terminus to the C-terminus. The repeating leucine (L) and isoleucine (I) residues form a hydrophobic face along both peptides^[8]. Their side chains interact with each other (grey arrows) in a “knobs-into-hole”^[5;9] manner, forming a continuous hydrophobic core. Ionic attractions between glutamic acid (E) and lysine (K) make the interaction selective.

In the present work we assess to what extent mobility information from room-temperature spin-label EPR^[10;11] can be used to study dimer formation of the E/K peptides. To do so, a cysteine residue was introduced and the peptide was coupled to an MTSL label^[12] (Figure 2.2). All investigated peptides are listed in Table 2.1. We use the abbreviations SL-K for the K peptide with the spin label (SL) attached at the N-terminus, and E-SL and K-SL for the E and K peptides, respectively, when the spin label is attached at the C-terminus. An accompanying study shows that the spin label does not change the secondary structure of the peptides, nor that it disturbs the self-assembly of the E/K peptide pair^[7]. Advantages of spin-label EPR are that heterodimer formation can be detected *in situ* and in the presence of membranes.

Table 2.1 Peptide sequences

peptide	sequence
E-SL	Ac-(EIAALEK) ₃ -GYC(SL)-NH ₂
K-SL	Ac-(KIAALKE) ₃ -GWC(SL)-NH ₂
SL-K	Ac-C(SL)-(KIAALKE) ₃ -GW-NH ₂
E	Ac-(EIAALEK) ₃ -GY-NH ₂
K	Ac-(KIAALKE) ₃ -GW-NH ₂

C(SL) = cysteine with MTSL attached, Ac = acetyl

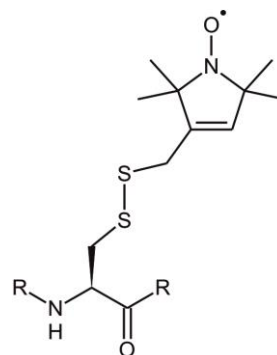


Figure 2.2 Chemical structure of the spin-label MTSL attached to a cysteine residue in a peptide.

In this study we use two approaches. We mix the spin-labeled peptide with its unlabeled partner peptide (e.g. E-SL with K), expecting a mobility decrease upon heterodimer formation. As a control, we mix the spin-labeled peptide with its unlabeled twin peptide (e.g. E-SL with E). Any mobility change due to unspecific interaction or viscosity changes should be revealed by the latter

experiment. In the second approach, we investigate samples in which both partners are labeled, to detect potential spin-spin interaction owing to the close approach of the spin labels.

We show that heterodimer formation can be detected by the mobility change in EPR. The absence of spin-spin interaction in the SL-K:E-SL pair and E-SL:K-SL pair puts a structural constraint on the heterodimer: a minimal distance of 0.8 nm between the two electron spins. The present work paves the road for future EPR studies on the peptides E and K integrated into more complex systems.

2.2 Material and methods

The synthesis of the peptides listed in Table 2.1 was done by Tingting Zheng (Supramolecular & Biomaterials Chemistry group at Leiden Institute of Chemistry) and has been described elsewhere^[7].

EPR measurements

The cw-EPR measurements were performed at 9.8 GHz using an ELEXSYS E 680 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) equipped with a rectangular cavity. All spectra were recorded at a microwave power of 0.63 mW with a field sweep of 15 mT and 2048 field points. Field modulation at a frequency of 100 kHz was employed with an amplitude of 0.04 mT. The measurement time was 20 minutes per sample. The time constant was 2.56 ms with a conversion time of 5.12 ms. The temperature was 293 ± 1 K.

Solutions were contained in 50 μ L micropipettes (BLAUBRAND[®] intraMARK) with an inner/outer diameter of 0.80/1.50 mm. Samples were prepared in phosphate buffered saline (PBS), pH 7.4. The measurements done are summarized in three categories: a sample of (i) labeled peptide, 150 μ M, and mixtures of (ii) labeled peptide with non-labeled peptide, both 100 μ M, and (iii) labeled peptide with labeled peptide, both 100 μ M. Peptide concentrations for category (i) and (ii) were based on UV-Vis absorption. For category (iii) spin concentrations were used (see below). A quantitative analysis of the spin-label concentration was made by double integration of the EPR spectrum and comparison to the spectrum of a reference sample with known spin concentration. Based on this analysis, we found that more than 80% of the peptides E-SL, K-SL, and SL-K gave an EPR signal, i.e., were effectively spin labeled.

To check whether the peptide influences the solution viscosity, we also measured a sample of the spin label (MTSL) alone and in the presence of 200

μM of peptide E. Similarly, peptide E-SL was measured at concentrations between 100 μM and 200 μM in increments of 25 μM .

Simulation of EPR spectra

Simulations of cw-EPR spectra were done with EasySpin^[13], a software package for MATLAB (The Mathworks, Natick, MA, USA). The function *Garlic* was combined with the isotropic rotation model. The spin system was defined by tensors $\vec{\bar{g}} = [g_{xx} \ g_{yy} \ g_{zz}] = [2.0078 \ 2.0058 \ 2.0023]$ and $\vec{\bar{A}}_N = [A_{xx} \ A_{yy} \ A_{zz}] = [5.99 \ 5.99 \ 36.38]$ MHz. The hyperfine tensor $\vec{\bar{A}}_N$ derives from the interaction of the electron spin with the ^{14}N ($I = 1$) nucleus. A second component was added (5%) to account for the satellite lines due to coupling of the electron spin with ^{13}C ($I = 1/2$) nuclei in natural abundance. For this fraction $\vec{\bar{A}}_C = [A_{xx} \ A_{yy} \ A_{zz}] = [6.63 \ 6.63 \ 6.63]$ MHz was used. Within a series of simulations concerning one particular labeled peptide (e.g. E-SL, E-SL:E, or E-SL:K), the lineshape parameters were kept constant. The simulated spectrum was adjusted to the experimental one varying the rotation-correlation time. We used visual inspection to make the simulated spectrum resemble the experimental spectrum.

Rotation-correlation time

We assume that the line shape of the EPR spectrum, described by the rotation-correlation time τ_r derives from a combination of the local mobility of the spin label ($\tau_{r,\text{local}}$) and overall peptide motion $\tau_{r,\text{peptide}}$

$$\frac{1}{\tau_r} = \frac{1}{\tau_{r,\text{peptide}}} + \frac{1}{\tau_{r,\text{local}}} \quad (2.1)$$

To calculate the rotation-correlation time of the peptide $\tau_{r,\text{peptide}}$, the Stokes-Einstein equation

$$\tau_{r,\text{peptide}} = \frac{\eta V}{k_B T} \quad (2.2)$$

is used, where η is the solution viscosity, for water 1.00 mP·s, k_B is the Boltzmann constant, and T is the temperature, in this work: 293 ± 1 K. The volume V of the E and K peptides is described by cylinders with a length of 3.9 nm and a diameter of 1.1 nm. The volumes are 3.7 nm^3 for the monomeric peptide and 7.4 nm^3 for the heterodimer. From equation 2.2, $\tau_{r,\text{peptide}} = 0.92 \text{ ns}$ for a monomeric peptide and $\tau_{r,\text{peptide}} = 1.83 \text{ ns}$ for a heterodimer are obtained.

Averaging of dipole-dipole interaction

For a system containing two unpaired electron spins, the dipole-dipole interaction is averaged by molecular tumbling if

$$\omega_{dd} < \frac{2\pi}{\tau_r}. \quad (2.3)$$

The dipole-dipole coupling between two spins is proportional to the inverse cube of the distance^[14]

$$\omega_{dd}(\theta, r) = \frac{2\pi g_1 g_2}{g_e^2} (3 \cos^2 \theta - 1) \frac{52.04}{r^3} [\text{MHz nm}^3]. \quad (2.4)$$

See Section 1.4 for the clarification of the symbols used in equation 2.4.

For $\tau_r = 1.83$ ns an upper limit of $\omega_{dd} = 546 \cdot 10^6$ rad/s results, which corresponds to a distance of 0.8 nm.

2.3 Results

Figure 2.3 shows the EPR spectrum of E-SL:E superimposed on the spectrum of E-SL:K. The spectra are superimposed such that the middle one of the three EPR lines overlaps optimally. The high-field line in the spectrum of E-SL:K is broadened compared to E-SL:E. A similar feature is observed in the spectra of the samples in which K-SL or SL-K are mixed with their partner peptides (data not shown).

Control experiments show that the spectrum of the free spin label is not influenced by the presence of different concentrations of peptide (see Material and methods). Also, the spectral lineshape of peptide E-SL does not change within the signal-to-noise ratio for samples where the concentration of E or E-SL is varied.

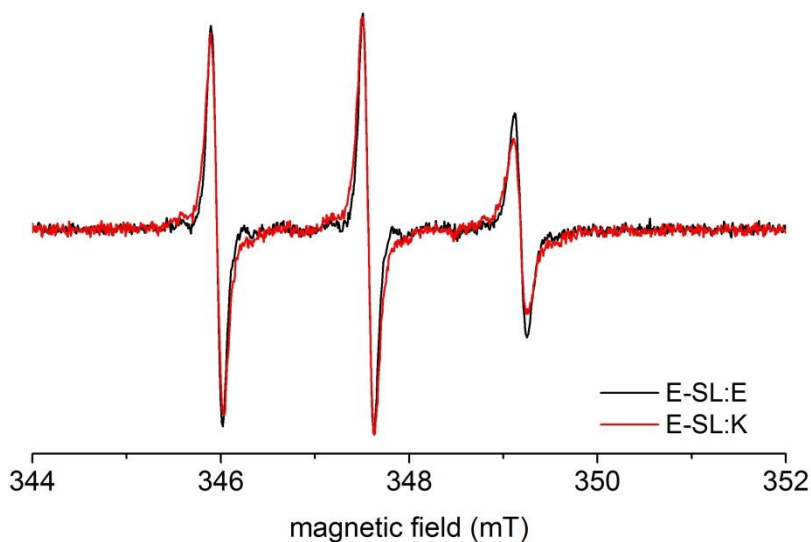


Figure 2.3: The room temperature cw-EPR spectrum of E-SL:E (in black) superimposed on E-SL:K (in red).

In total nine combinations of peptides were measured (see Table 2.2). Simulations were performed with a model of isotropic rotation. The simulated spectra agree well with the experimental spectra, i.e., within the noise amplitude. The exception is the simulated spectrum of SL-K:E. Here the amplitude of the low-field line even in the best-matched simulation was 7% larger and the high-field line was 28% broader than the experimental spectrum. The parameters obtained by the simulations are the rotation-correlation times (τ_r) and linewidths given in Table 2.2.

Table 2.2 Parameters used for the simulation of EPR spectra.

simulation	linewidth ^a (mT)	τ_r ^b (ps)
E-SL	0.11 – 0.02	158
E-SL:E	0.11 – 0.02	186
E-SL:K	0.11 – 0.02	307
K-SL	0.10 – 0.04	169
K-SL:K	0.10 – 0.04	181
K-SL:E	0.10 – 0.04	236
SL-K	0.09 – 0.07	197
SL-K:K	0.09 – 0.07	200
SL-K:E	0.09 – 0.07	269

^a The spectral lines were best described using a mixture of Gaussian and Lorentzian lineshapes. The first and second value correspond to the width of the Gaussian and Lorentzian line, respectively.

^b The rotation-correlation time was determined with an error of ± 15 ps.

Considering the three samples with E-SL, the τ_r values of E-SL and E-SL:E agree within the experimental error, whereas the τ_r of E-SL:K is significantly larger. The same is true for samples containing K-SL and SL-K. The increase in τ_r is largest for E-SL, i.e., from 158 (E-SL) to 307 ps (E-SL:K), and

smallest for K-SL. Amongst the heterodimers, τ_r is largest for E-SL:K (307 ps) and smallest for K-SL:E (236 ps).

Figure 2.4 shows the overlay of the spectrum of E-SL:K-SL and the suitable reference spectrum. Similarly for E-SL:SL-K (Figure 2.5). The spectra of samples in which both partners are labeled are identical within the noise to their respective reference spectra.

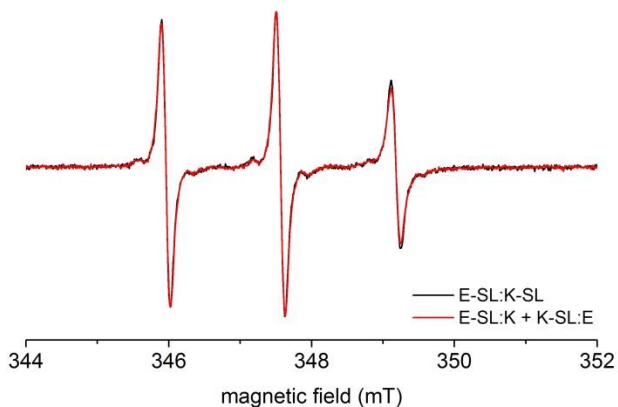


Figure 2.4: The spectrum of E-SL:K-SL (in black) superimposed on the sum of the spectra of E-SL:K and SL-K:E (in red).

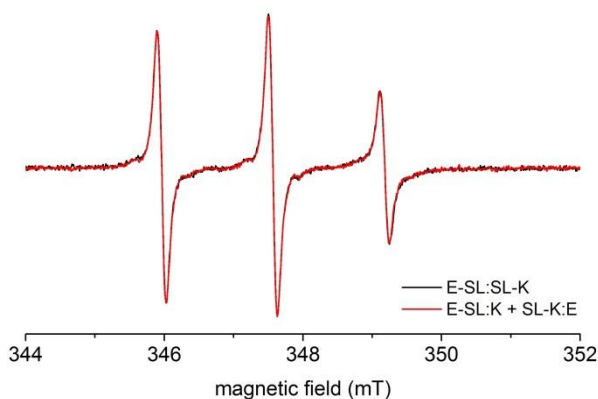


Figure 2.5: The spectrum of E-SL:SL-K (in black) superimposed on the sum of the spectra of E-SL:K and SL-K:E (in red).

2.4 Discussion

To investigate heterodimer formation in the E/K peptides we determined the mobility of the spin label in a set of combinations of these peptides (Table 2.2). In almost all cases, an isotropic rotational model was sufficient to simulate the mobility of the spin label showing that neither monomers nor dimers have preferential axes of rotation. A significant increase of τ_r is found in all cases where the heterodimers are formed, irrespective of the position of spin-label attachment (N- or C-terminus, E or K peptide), showing that mobility measurements by cw-EPR provides a valid method to detect dimer formation in the E/K peptides. A set of control experiments shows that the peptides do not significantly influence the τ_r via viscosity changes of the solution. Significant changes in τ_r between a spin labeled peptide in the absence and presence of a non-labeled peptide are therefore considered to be caused by peptide-peptide interaction.

How do the observed τ_r values relate to the rotation of the peptide? The measured τ_r values are significantly smaller than those expected for the rotation of the peptide itself, i.e., 0.92 ns for peptide K and 1.83 ns for the heterodimer (see Material and methods). Using equation 2.1, the contribution of peptide rotation ($\tau_{r,\text{peptide}}$) to τ_r is in the order of 20%, revealing that τ_r is largely determined by local mobility, i.e., rotation of the nitroxide about the single bonds joining it to the peptide and/or the mobility in the peptide backbone (Figure 2.2).

Consequently, the τ_r changes reveal that the local mobility decreases when heterodimers are formed. The local-mobility change is largest for the C-terminus of the E peptide, and also somewhat larger for the N-terminus of the K peptide than for the C-terminus of K. A possible explanation for the larger τ_r

change in the E peptide compared to the K peptide is that the E peptide, which is partially random coil in solution, has to convert to an α -helix conformation when the heterodimer is formed. The partial random-coil conformation of the E peptide could offer more flexibility to the nitroxide before heterodimer formation, making the total change in τ_r larger than for the K-peptide.

For none of the combinations of spin-labeled peptides (Figures 2.4 and 2.5) spin-spin interactions were observed, showing that spin labels are too far apart to have either exchange or dipolar interaction. Exchange interaction (J) manifests itself as line broadening or in the occurrence of extra lines in the EPR spectrum if $J \geq A_N/2$, in our case at distances < 0.5 nm. Dipolar interaction could be observed if the dipolar interaction ω_{dd} is sufficiently large not to be averaged by molecular tumbling, which for our labels is at distances < 0.8 nm. The absence of any such effect on the spectra of the peptide partners, where both C-termini are labeled (E-SL:K-SL) or where the E-C-terminus and K-N-terminus (E-SL:SL-K) are labeled, shows that the spin labels are separated by more 0.8 nm.

In a parallel dimer, the shortest distance is expected for the E-SL:K-SL pair, in which both spin labels are at the C-terminus. However, even in this situation the distance could be substantial. The helical-wheel projection shows that if the C-terminal residues that follow the third heptad repeat (K-SL: -GWC-SL) complete a full turn, the cysteine residue would position at the site of the first alanine residue (A¹) of the K peptide (Figure 2.1b), respectively the E peptide, i.e., at opposite faces of the helix. Assuming a helix diameter of 1.1 nm and a linker length of 0.5 nm, a distance of ~ 3.2 nm results, which is significantly larger than the distance to which the liquid-solution measurements we performed are sensitive. Therefore, the absence of spin-spin interaction is consistent with the model shown in Figure 2.1b. The present results do not enable us to exclude an anti-parallel arrangement of the heterodimer.

Paramagnetic NMR and Förster resonance energy transfer experiments do provide such evidence^[7].

We can exclude that oligomers are formed in which peptides cover the termini of their partners. Such an arrangement would block spin label motion and lead to correlation times in the order of oligomer rotation.

In conclusion, we find small but significant changes in the mobility of the spin label under conditions where heterodimers are formed. These *in situ* measurements confirm heterodimer formation in solution, showing that the E/K peptides form a complex. The same approach can be applied to the full construct in a vesicle environment enabling the detection of complex formation in the fully assembled fusion construct.

References

- [1] T. Weber, B.V. Zemelman, J.A. Mcnew, B. Westermann, M. Gmachl, F. Parlati, T.H. Sollner, J.E. Rothman, SNAREpins: Minimal machinery for membrane fusion. *Cell* **92** (1998) 759-772.
- [2] R. Jahn, Some classic papers in the field of membrane fusion - a personal view. *Nature Structural & Molecular Biology* **15** (2008) 655-657.
- [3] P. Burkhard, J. Stetefeld, S.V. Strelkov, Coiled coils: a highly versatile protein folding motif. *Trends in Cell Biology* **11** (2001) 82-88.
- [4] J.R. Litowski, R.S. Hodges, Designing heterodimeric two-stranded alpha-helical coiled-coils - Effects of hydrophobicity and alpha-helical propensity on protein folding, stability, and specificity. *Journal of Biological Chemistry* **277** (2002) 37272-37279.
- [5] D.A. Lindhout, J.R. Litowski, P. Mercier, R.S. Hodges, B.D. Sykes, NMR solution structure of a highly stable de novo heterodimeric coiled-coil. *Biopolymers* **75** (2004) 367-375.
- [6] H.R. Marsden, A.V. Korobko, E.N.M. van Leeuwen, E.M. Pouget, S.J. Veen, N.A.J.M. Sommerdijk, A. Kros, Noncovalent triblock copolymers based on a coiled-coil peptide motif. *Journal of the American Chemical Society* **130** (2008) 9386-9393.
- [7] T. Zheng. Dissertation, Leiden University, *to be published*.
- [8] J.M. Mason, K.M. Arndt, Coiled coil domains: Stability, specificity, and biological implications. *Chembiochem* **5** (2004) 170-176.
- [9] F.H.C. Crick, The Packing of Alpha-Helices - Simple Coiled-Coils. *Acta Crystallographica* **6** (1953) 689-697.
- [10] L.J. Berliner, *Spin labeling: Theory and Applications*, Academic Press, New York, 1976.
- [11] N.M. Atherton, *Principles of electron spin resonance*, Ellis Horwood, Chichester, 1993.
- [12] 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** (1982) 450-455.
- [13] S. Stoll, A. Schweiger, EasySpin, a comprehensive software package for spectral simulation and analysis in EPR. *Journal of Magnetic Resonance* **178** (2006) 42-55.
- [14] G. Jeschke, Distance measurements in the nanometer range by pulse EPR. *Chemphyschem* **3** (2002) 927-932.

