Supporting Material for

Coiled-coil formation on lipid bilayers - implications for docking and fusion efficiency

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Materials and Methods

Materials

All chemicals were of HPLC grade and used without further purification. DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), POPE (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine) and MCCDOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[4-(p-maleimidomethyl)cyclohexane-carboxamide]) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Fluorescently labeled lipids Texas Red DHPE (Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt) and Oregon Green 488 maleimide were purchased from Invitrogen (Darmstadt, Germany) and sulforhodamin B was obtained from Sigma-Aldrich (Seelze, Germany). All chemicals for peptide synthesis, *i.e.* Fmoc-protected α-amino acids, rink amide MBHA resin LL, coupling reagents HBTU (2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate), and DIEA (N,N-Diisopropylethylamine) were purchased from Novabiochem (Darmstadt, Germany).

Peptide synthesis

Peptides E3Cys (Ac-$(EIAALEK)_3$GWGGGC-NH_2), *i*-E3Cys (Ac-$(KELAAIE)_3$GWGGGC-NH_2), K3Cys (Ac-WG$(KIAALKE)_3$GGGGC-NH_2), and *i*-K3Cys (Ac-WG$(EKLAAIK)_3$GGGGC-NH_2) were synthesized manually using Fmoc-protected α-amino acids (1). Peptides were amidated at C- and acetylated at N-terminus, cleaved from resin

using TFA (trifluoroacetic acid) and purified by RP-HPLC (L-6200A Intelligent Pump and L-4200 UV/Vis Detector, Merck-Hitachi, Darmstadt, Germany). Therefore, Grace Vydac C18 columns run with a linear gradient (solvent A: H_2O / AcCN / TFA 99:1:0.1; solvent B: H_2O / AcCN / TFA 10:90:0.05) were used. Peptides were characterized by ESI-MS analysis (Apex IV, Bruker Daltonics, Billerica, MA, USA). The purity determined by RP-HPLC of resulting peptides was 88-94 %.

Vesicle preparation, bilayer formation, and *in situ* coupling reaction with peptides

Lipid stock solutions (c_{lipid} = 1-10 mg/mL) were prepared in chloroform and transformed into lipid films by removal of the solvent in a nitrogen stream followed by 3 h drying in vacuum. Multilamellar vesicles (MLVs) were produced by dissolving the dried lipid films in buffer reaching a final concentration of 1 mg/mL. In all experiments except for ATR-IR spectroscopy, PB 6.8 (50 mM Na_2HPO_4, pH = 6.8) was used as a buffer solution. MLVs were either transformed into small unilamellar vesicles (SUVs) by sonication (50 W, 0.4 s Puls, 30 min) in a vessel resonator (Sonoplus HD 2070, Bandelin, Berlin, Germany) or transformed into large unilamellar vesicles (LUV) by extrusion (LiposoFast Extruder, Avestin, Ottawa, Canada). Vesicle size was determined by DLS (dynamic light scattering, see Castorph *et al*. (2)), which showed that size distribution of SUVs is centered at a diameter of 30-50 nm. Solid supported membranes (SSMs) were formed by spreading SUV on a hydrophilized glass surface at temperatures above the phase transition of the used lipids. Covalent coupling of peptides to maleimide bearing lipids of the preformed SSMs was achieved by incubating the membrane with a peptide concentration of $c \approx 50$ µM at room temperature for 1-3 hours in PB 6.8 followed by thorough rinsing with buffer (1).

CD (circular dichroism) spectroscopy

Prior to CD spectroscopy, concentration of peptides dissolved in PB 6.8 was estimated by UV-Vis absorption spectroscopy at 280 nm (3). CD measurements were carried out using a JASCO-810 spectrometer (Gross-Umstadt, Germany). Spectra were accumulated (3×) and averaged (data range λ = 190 - 250 nm; scanning speed 10 nm/min; data pitch 0.1 nm). For better visibility the spectra shown in this paper were smoothed, while analysis of K_D values was carried out with unsmoothed data. Fitting of spectra to determine the secondary structure elements of the peptides was accomplished using DichroWeb, an online analyses database for CD spectra (4, 5). For dilution experiments to obtain K_D values, 60-100 data points at fixed wavelength were collected and averaged (6).

SPR (surface Plasmon resonance)

SPR (SR7000DC, Reichert Life Sciences, Seefeld, Germany) spectroscopy measurements were done on a chip, coated with a dense thiol-derivatized bioinert hydrogel matrix consisting of carboxymethyldextran (THC1000M, XanTec Bioanalytics, Düsseldorf, Germany). K-peptides were immobilized on the surface by disulfide coupling. Subsequently, E-peptides

dissolved in PB 6.8 were added in different concentrations (1-50 µM) and the rates of adsorption k_{on} and desorption k_{off} were determined for each concentration separately. All dissociation constants $K_D = k_{off}/k_{on}$ were calculated from the kinetic data of >4 different peptide concentrations.

Ellipsometry

Experiments were performed using an imaging ellipsometer EP3-SW from Accurion (Goettingen, Germany) as described previously (7, 8). Measurements were carried out in PB 6.8 employing a closed fluid chamber. For the *in situ* coupling reaction of E peptides to maleimide functionalized lipids embedded in a solid supported membrane, a peptide concentration range of 100-120 µM was used, while for the coupling reaction of K peptides 50-60 µM were found to be sufficient. Changes in layer thickness are determined measuring the angle *del*, which is for thin dielectric layers linearly related to the height (1 ° *del* ≈ 0.9 nm).

ATR-IR (attenuated total reflection infrared) spectroscopy

Peptides used for IR measurements were lyophilized 5× from 0.05 M HCl to replace trifluoroacetate counterions at the peptide backbone with chloride ions (9). All experiments were carried out in D_2O containing 50 mM NaCl. Spectra were acquired using a Vertex 70 FTIR (Bruker Optics, Ettlingen, Germany) spectrometer equipped with a MCT detector with a resolution of 2 cm^{-1}. Peptide-functionalized SSM were measured in a closed fluid chamber using a ZnSe ATR-IR crystal coated with a thin layer of Si (BioATRII, Bruker Optics, Ettlingen, Germany).

FRAP (fluorescence recovery after photobleaching)

For FRAP experiments, bilayers were spread on MatTek Dishes (Ashland, MA, USA) in PB 6.8 (50 mM Na_2HPO_4, pH = 6.8) and functionalized *in situ* with the corresponding peptides. For determination of membrane mobility, SSM containing 1 mol% BODIPY were used. Peptide mobility was determined using coiled-coil forming peptides labeled after binding to already coupled lipopeptides with Oregon Green 488 maleimide covalently to the residual cysteine moiety. This reaction was carried out *in situ* with a fluorophor concentration of 30 µg/mL. FRAP experiments were carried out using an upright confocal laser scanning microscope (CLSM) equipped with a water immersion objective with 63× magnification (LSM710, Zeiss, Jena, Germany). Time-elapsed CLSM images were analyzed with a program written in Igor Pro (Wavemetrics, Tigard, OR, USA) to obtain lateral diffusion constants (10, 11).

Fusion assays using fluorescence spectroscopy

Fluorescence spectroscopy was carried out with a FluoroMax-4 (Horiba Scientific, Unterhaching, Germany). For Texas Red self-quenching experiments (12), SUV were prepared by sonication with a composition of DOPC/Texas Red/MCCDOPE 88:10:2 and DOPC/MCCDOPE 98:2, respectively. Vesicle population 1 (equipped with Texas Red) was functionalized with K-peptides. E-peptides were covalently bound to population 2. For content mixing LUVs were prepared by extrusion (100 nm) in HEPES 7.4 (20 mM HEPES, 150 mM KCl) with a composition of DOPC/POPE/MCCDOPE 70:28:2. One vesicle population contained sulforhodamin B (SRB) in a self-quenching concentration of 20 mM (13). Population 2 was prepared in HEPES 7.4 with isoosmolar salt concentration. Excess peptide and SRB in surrounding buffer was removed by column chromatography using sephadex NAP-25 columns (illustra, GE Healthcare, Solingen, Germany).

Supporting information - Results

CD-Spectroscopy of antiparallel coiled-coils heterodimers

Dissociation constants of antiparallel coiled-coil dimers were determined with CD spectroscopy in solution. The general procedure was essentially identical to the analysis of parallel coiled-coil dimers mentioned in the article. CD spectra (see Fig. S1 *A*) reveal a predominately α-helical structure of dimers which decreases upon dilution. These dilution experiments allowed us to determine the K_D values (see Fig. S1 *B*, *C*).

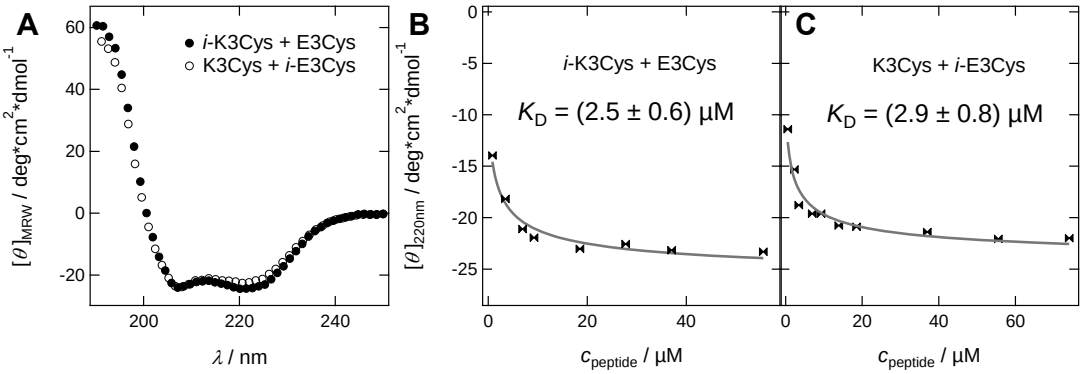

FIGURE S1 (*A*) Spectra of antiparallel coiled-coil dimers composed of *i*-K3Cys and E3Cys (filled circles) or K3Cys and *i*-E3Cys (open circles), respectively. Amount of α-helix was determined to be 79 % (filled circles) and 73 % (open circles), respectively. (*B*) Concentration dependence of $[\theta]_{MRW}$ at 220 nm of heterodimeric coiled-coil was used to determine K_D for E3Cys and *i*-K3Cys (fit is shown as grey line); (*C*) Concentration dependence of $[\theta]_{MRW}$ at 220 nm of heterodimeric coiled-coil was used to estimate K_D for *i*-E3Cys and K3Cys (fit is shown as grey line (6)).

Dissociation constants determined by SPR

With the SPR technique, not only the dissociation constant K_D was determined, but also the on- and off-rates of the binding process, k_{on} and k_{off} (see table S1). Association of peptides into a coiled-coil structure can be best described by a double exponential function in which we took the faster rate to describe the association (τ_a), while dissociation of the dimer can be fitted with a monoexponential function (τ_d). The corresponding rates and dissociation constants are computed according to Eq. S1-S3:

$$k_{off} = 1/\tau_d$$

$$k_{on} = \frac{(\tau_a)^{-1} - k_{off}}{c_{peptide}} \qquad \text{(S1, S2 and S3)}$$

$$K_D = k_{off} / k_{on}$$

TABLE S1 Thermodynamic data determined from SPR spectroscopy: Double-exponential fit to association phase provides τ_a (faster time scale) and k_{on}, monoexponential fit to dissociation upon exchanging the peptide solution with pure bufferprovides k_{off}. K_D are computed according to eq. S3.

	K3Cys + E3Cys	***i*-K3Cys + *i*-E3Cys**	**K3Cys + *i*-E3Cys**	***i*-K3Cys + E3Cys**
k_{on} / $M^{-1}s^{-1}$	21600 ± 12600	7900 ± 4300	12300 ± 8700	8900 ± 4700
k_{off} / s^{-1}	(8.4 ± 1.5) $\times 10^{-2}$	(1.3 ± 0.2) $\times 10^{-3}$	(4.1 ± 0.8) $\times 10^{-3}$	(6.8 ± 1.1) $\times 10^{-3}$
K_D / µM	0.5 ± 0.3	2.3 ± 1.8	0.5 ± 0.3	1.2 ± 1.0

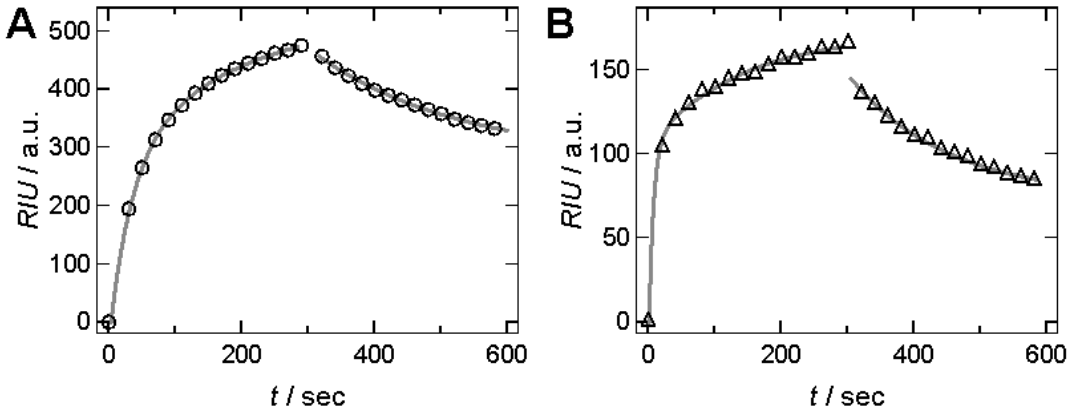

FIGURE S2 Association (t = 0-300 s) and dissociation (t = 300-600 s) of E-peptides coupled to immobilized K-peptides on a hydrogel monitored with SPR. Different concentrations of added peptide were used to compute K_D values. Here one kinetic measurement is shown exemplarly. Association phase was fitted by a double exponential function (k_{on}), while peptide dissociation was fitted a by mono exponential function (k_{off}). Fits are shown as grey lines. (*A*) *i*-E3Cys added to immobilized K3Cys in a concentration of 15 µM (open circles). k_{on} = (1.2 ± 0.9) × 10^4 $M^{-1}s^{-1}$; k_{off} = (4.1 ± 0.8) × 10^{-3} s^{-1}; K_D = k_{off} / k_{on} = (0.5 ± 0.3) µM. (*B*) E3Cys added to immobilized *i*-K3Cys in a concentration of 15 µM (open triangles). k_{on} = (8.9 ± 4.7) × 10^3 $M^{-1}s^{-1}$; k_{off} = (6.8 ± 1.1) × 10^{-3} s^{-1}; K_D = k_{off} / k_{on} = (1.2 ± 1.0) µM.

Monitoring *in situ*-coupling reaction and coiled-coil formation using Ellipsometry

In situ coupling of peptides to a solid supported lipid bilayer (SSM) through maleimide chemistry was monitored by time-resolved ellipsometry as previously described (1). The principal angle *del* determined by this method is proportional to layer thicknesses for sufficiently thin dielectric layers (h < 30 nm) (7). Absolute height changes resulting from peptide coupling and coiled-coil formation on a spreaded SSM (see Fig. S3) were computed from *del* assuming a refractive index of 1.5 for the peptides.

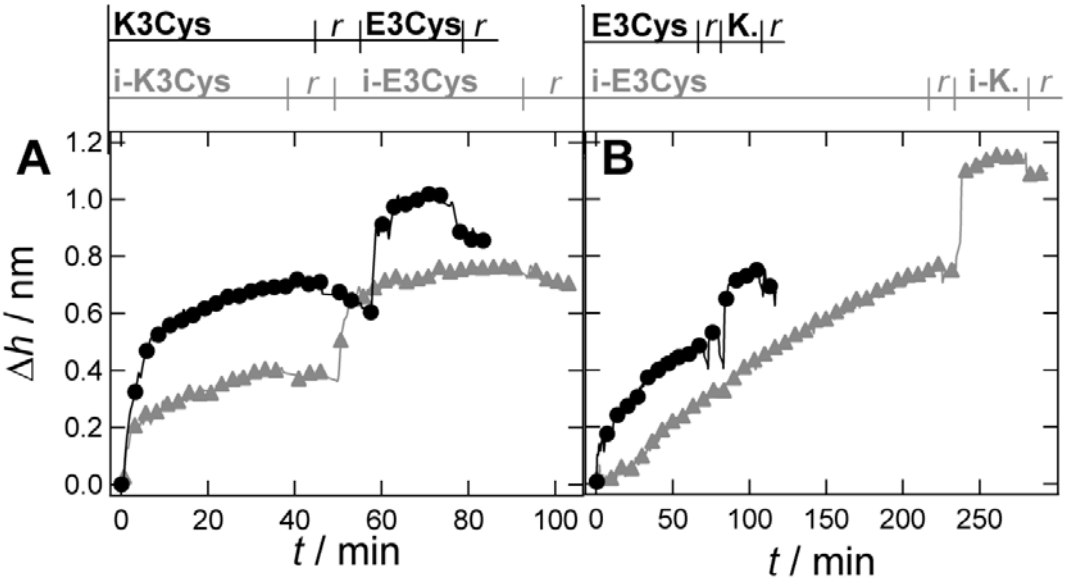

FIGURE S3 Layer thicknesses of peptides calculated from ellipsometry measurements of *in situ* coupling reaction to a DOPC/MCCDOPE 97:3 bilayer followed by coiled-coil formation. Spreading of bilayer is not shown, h_{SSM} is set to zero. (*A*) K-peptides were attached covalently to MCCDOPE lipids, followed by rinsing (indicated above with *r*), E-peptides were added for coiled-coil formation, followed by rinsing (black solid circles: LP-K3Cys + E3Cys; grey solid triangles: LP-*i*-K3Cys + *i*-E3Cys). (*B*) E-peptides were attached covalently to MCCDOPE lipids, followed by rinsing (indicated above with *r*), K-peptides were added for coiled-coil formation (K3Cys = K., *i*-K3Cys = i-K.), followed by rinsing (black solid circles: LP-E3Cys + K3Cys; grey solid triangles: LP-*i*-E3Cys + *i*-K3Cys).

Prior to the measurement, a bilayer consisting of DOPC/MCCDOPE 97:3 was deposited on a Si-wafer. The calculated layer thickness for the formed SSM from ellipsometry was $h_{SSM} \approx$ 3.5 - 3.7 nm, which is in good agreement to reported values (14). The actual process of bilayer spreading is not shown here; the addition of peptide solution for *in situ* coupling reaction is used as starting point instead. In all curves shown in Fig. S3, formation of both lipopeptides as well as coiled-coil structures is clearly visible via a discrete height increase (see table S2). Rinsing induces a decrease of layer thickness, especially for the last rinsing steps after heterodimerization, at which no covalent bonds are created and stable coiled-coil structures are formed. All measurements display roughly the same height difference of 0.7-0.8 nm after coiled-coil formation. With 3 mol% MCCDOPE in the bilayer, no complete surface coverage by peptide coupling is achieved; hence this value rather reflect the incomplete surface coverage and should not be mistaken as the actual size of the coiled-coil complexes.

TABLE S2 Absolute height differences for *in situ* coupling reaction ($\Delta h_{\text{lipopeptide}}$), coiled-coil formation ($\Delta h_{\text{cc-peptide}}$) and final rinsing ($\Delta h_{\text{rinsing}}$) on a SSM consisting of DOPC/MCCDOPE 97:3.

	LP-K3Cys + E3Cys	**LP-*i*-K3Cys + *i*-E3Cys**	**LP-E3Cys + K3Cys**	**LP-*i*-E3Cys + *i*-K3Cys**
$\Delta h_{\text{lipopeptide}}$ / nm	0.59 ± 0.11	0.40 ± 0.11	0.47 ± 0.11	0.63 ± 0.11
$\Delta h_{\text{cc-peptide}}$ / nm	0.47 ± 0.11	0.45 ± 0.11	0.29 ± 0.11	0.47 ± 0.11
$\Delta h_{\text{rinsing}}$ / nm	-0.32 ± 0.11	-0.08 ± 0.11	-0.05 ± 0.11	*n.a.*

Normalization of fluorescence spectroscopy fusion assays

Fusion assays are normalized to a one-to-one mixture of vesicles corresponding to100 % fusion. Hence, we assume that one K-decorated and fluorescently labeled vesicle fuses with exactly one E-functionalized unlabeled liposome. This results in a dilution of the fluorophor in the shell of the liposome or in its interior. Assuming, that the two interacting liposomes possess the same size, membrane mixing results in a twofold dilution, since the membrane area of the new vesicle doubles. While the membrane area is related to r^2, the volume is related to r^3, which means that the volume enclosed by the fused vesicles increases by a factor of $2^{3/2} \approx 2.8$.

As a consequence, starting with 10 mol% Texas Red DHPE as the membrane dye for lipid mixing and 20 mM SRB for content mixing, respectively, 100 % fusion results in 5 mol% Texas Red DHPE and 7 mM SRB, respectively.

To determine the concentration-dependent quenching of Texas Red DHPE in SUVs, a series of vesicles were prepared with an amount of fluorescent dye ranging from 1-10 mol%. Afterwards, the fluorescence intensity was measured before and after disruption of vesicles. This way, a quenched and unquenched, maximal intensity could be determined. Plotting the quencher concentration, in this case Texas Red DHPE, against $F_{\text{max}} / F_{\text{quenched}} - 1$ results in a Stern-Volmer plot (see Fig. S4). In the concentration range, which is interesting for our experiments, the $F_{\text{max}} / F_{\text{quenched}} - 1$ increases linearly with quencher-concentration.

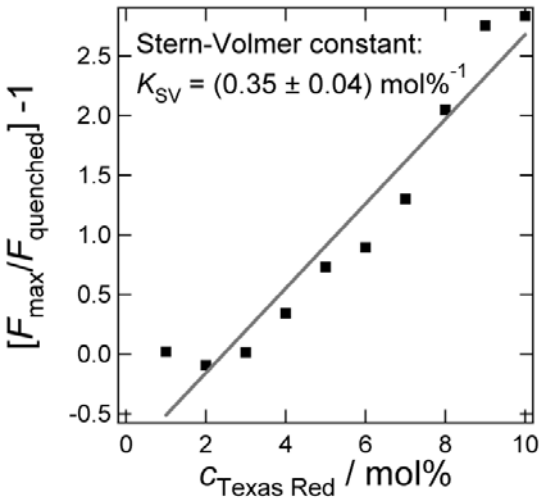

FIGURE S4 Stern-Volmer plot of Texas Red DHPE quenching in DOPC vesicles. $F_{quenched}$ denotes fluorescence intensity of intact vesicles, F_{max} the intensity after disruption of SUV with detergent (SDS, c = 0.2%). Points correspond to the measured data and the grey line represents a linear fit. Stern-Volmer constant K_{SV} was determined from the slope.

Using the Stern-Volmer equation (Eq. S4), a quenching constant K_{SV} can be determined which is dependent on the quencher concentration [Q].

$$\frac{F_{max}}{F_{quenched}} - 1 = K_{SV} \cdot [Q] \qquad \text{(S4)}$$

For SRB self-quenching in SUV, a Stern-Volmer constant is published to be $K_{SV} = 990\ M^{-1}$ (15). Finally, using the concentrations for the diluted fluorophor and the given K_{SV}, the ratio $F_{max}/F_{quenched} - 1$ after 100 % fusion can be calculated.

Non-specific interaction of soluble peptides with lipid bilayers

Two kinds of control experiments were carried out to quantify the impact of non-specific interaction of peptides added to membranes in the absence of lipopeptides. One, in which we added E and K peptides to neat phospholipid bilayers (PC) and second set of experiments in which we added an increasing amount of maleimide functionalized lipids to the bilayer to check for linearity in peptide coupling. We used both ATR-IR and ellipsometry to quantify non-specific adsorption on the membrane. Both E-peptides, E3Cys and *i*-E3Cys, showed no interaction with pure DOPC (see Fig. S5 *A*). However, K-peptides adhere transiently to plain DOPC bilayers (see Fig. S5 *C*). We attribute this effect to the opposed net charges of the peptides. E-peptides are negatively charged, while K-peptides carry a net charge of +3. Addition of the corresponding E-peptide reverses the effect in contrast to the covalent coupling. Moreover, the kinetics of adsorption is considerably slower than in the case of specific in situ coupling. This leads to the conclusion that coiled-coil formation in solution is thermodynamically favored in comparison to electrostatic interaction of K-peptides to phospholipids.

Interestingly, we found that non-specific adsorption of K-peptides is absent if small amounts of MCC-DOPE are present in the bilayer (see Fig. S5 *B*). We varied the amount of the receptor lipid MCCDOPE within the SSM from 1 mol% to 10 mol% resulting in a linear increase of the apparent height of the thin film after addition of K-peptides. Almost no adsorption is observed for 1 mol% of MCC-DOPE. Extrapolating the linear response to zero MCC-DOPE suggest that non-specific adsorption, even of the K-peptide, is negligible. This experiment also indicates that our ellipsometry response is linear with respect to peptide coverage.

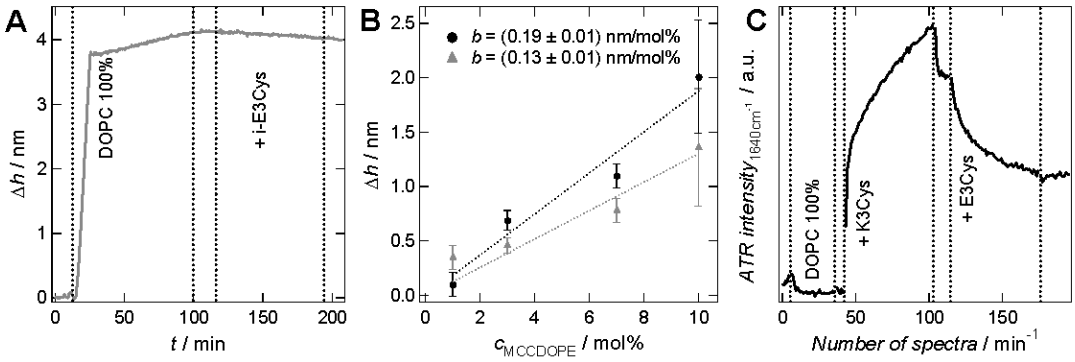

FIGURE S5 (*A*) Layer thicknesses calculated from ellipsometry measurements of bilayer spreading (DOPC 100%) with subsequent addition of *i*-E3Cys. Before and after each step sample was rinsed with buffer (PB 6.8). (*B*) Layer thickness of K3Cys (black dots) and *i*-K3Cys (grey triangles) added to SSM containing different amounts of receptor lipid MCCDOPE. Presented values *b* are the slopes of the shown linear fit functions (dotted lines). (*C*) Time course of ATR-IR flow cell measurement. Plot shows intensity of amide I band at 1640 cm^{-1}. First, bilayer was formed on ATR-crystal (DOPC 100%) afterwards K3Cys was added to the setup followed by the addition of E3Cys. Before and after each step sample was rinsed with D_2O containing 50 mM NaCl.

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