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## Peptide Amphiphiles and their use in Supramolecular Chemistry

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### ***In vitro* and *In vivo* supramolecular biomembrane engineering using a lipidated coiled-coil motif**

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A simple and generic tool for the efficient formation of coiled coils at the surfaces of cells and zebrafish embryos is presented here. A pair of complementary coiled-coil-forming peptides, denoted E and K, were synthesized and conjugated to a cholesterol anchor. The resulting lipidated peptides entered into CHO cell membranes spontaneously *in vitro* and into the skin of zebrafish embryos *in vivo*. Addition of the acetylated complementary peptide resulted in efficient coiled coil formation at the surface of cells and zebrafish embryos. As the coiled coil unit acts as a molecular velcro, this supramolecular tool enables the modification of biomembranes with a wide range of molecular constructs. As a case study, the docking of liposomes to cell membranes and zebrafish skin was investigated. Liposomes bearing the complementary peptide efficiently docked at these biological interfaces through coiled coil formation. Importantly, this is the first time that *in vivo* membrane modification has been achieved. It is envisaged that this tool will aid the study of membrane processes by modulating the chemistry of these bio-interfaces. Furthermore, applications in the area of pharmacology and drug delivery such as high-throughput screening of drugs are expected.

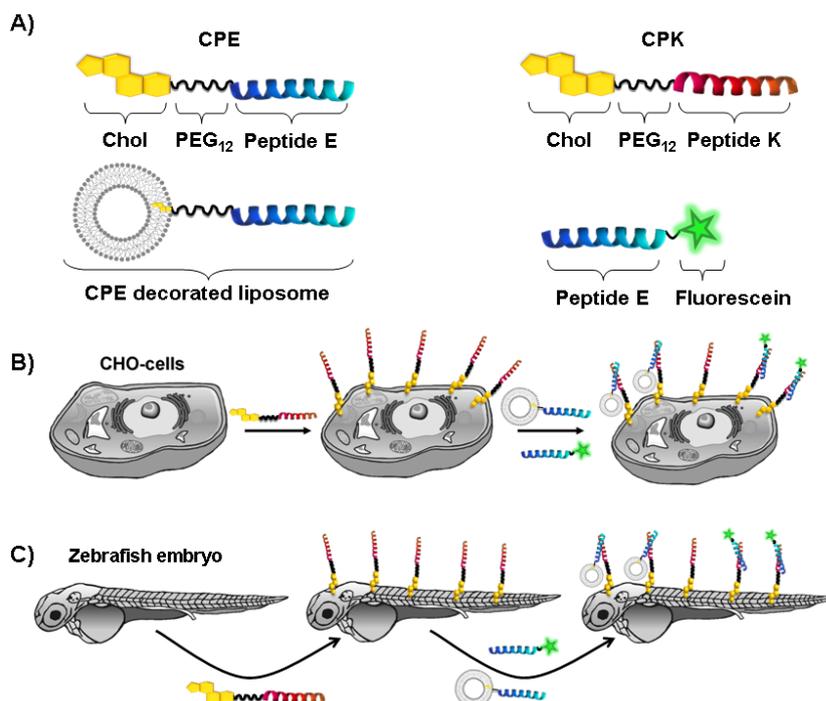
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**Introduction**

The molecular building blocks available in biological systems are able to self-assemble into well-defined structures in an extremely controlled manner. These structures need to be flexible and adaptive to the environment to enable them to carry out their function in a regulated manner. Therefore, nature uses multiple weak interactions (e.g. hydrogen bonding and van der Waals interactions) to act as the glue to hold these structures together. When many weak interactions cooperatively combine, relatively stable entities are produced, which retain the ability to ‘respond’ to external stimuli such as fluctuations in ion concentration, pH and temperature. For many years, nature has been a source of inspiration for supramolecular chemistry.<sup>1-4</sup> Here, scientists design relatively simple molecules which assemble into functional materials with well-defined properties, typically following the bottom up approach.<sup>5-8</sup> Recent progress has resulted in molecular systems which are responsive to multiple stimuli and are therefore highly controlled, emulating nature ever more closely. A relatively new branch of development is the application of supramolecular constructs in an *in vitro* and *in vivo* environment, to directly study and influence biological processes in live cells.

Introduction of supramolecular systems into a biological environment allows for the placement of chemically tailored systems into, for example, cell membranes. This enables the modification or regulation of cellular behavior through artificial signals.<sup>9</sup> From a supramolecular chemistry point of view, two types of approach can be pursued to introduce chemical species into a cell membrane: 1) specific binding of guest molecules to membrane anchored biomolecules such as native proteins<sup>10</sup> or 2) non-specific labeling of membranes with the aid of hydrophobic and electrostatic interactions or through a chemical cross linker.<sup>11-13</sup> Lipidated peptides are particularly good candidates for application in biological systems as their aggregation behavior can be controlled by carefully balancing the hydrophobicity of the anchor and the hydrophilicity of the cargo,<sup>14</sup> which aids the incorporation of lipidated peptides into membranes. By employing a coiled coil motif as the peptide segment, a highly specific recognition system can be introduced into live cells. The coiled coil motif acts as “molecular Velcro” and can thus be used to link distinct molecular constructs together.<sup>15-20</sup> An example of specific labeling of proteins through coiled coil formation was recently supplied by Matsuzaki et al.<sup>21</sup> Surface modification through non-specific

binding of polymers to cell membranes has also been studied, for example by Ijiro et al.<sup>22</sup> Lipid grafted polymers adhered to cell membranes and could potentially act as a scaffold to which a wide range of functional moieties could be attached, thereby intervening in the chemistry of the cell. Furthermore, cationic graft copolymers have also been shown to interact electrostatically with cell membranes, resulting in chemically altered cell membranes.<sup>23</sup> Whereas all these examples illustrate that *in vitro* membrane functionalization is a highly rewarding strategy, examples of efficient *in vivo* strategies are currently still lacking. Therefore, it is our goal to transiently modify lipid membranes through specific supramolecular interactions in an *in vitro* and *in vivo* environment. This will enable studies of membrane related processes by modulating the chemistry at these interfaces. For this purpose, a pair of complementary coiled coil forming lipidated peptides E and K,<sup>15, 24, 25</sup> was used to specifically introduce a non-covalent and bio-orthogonal recognition motif to influence the chemistry of biological membranes (**Scheme 1**).



**Scheme 1.** Schematic representations of A) the lipidated E and K peptides, a CPE decorated liposome and the fluorescently labelled E peptide, B) anchoring of CPK into CHO cell membranes and subsequent coiled coil formation with fluorescently labeled E or liposomes decorated with E peptides and C) anchoring of CPK into the

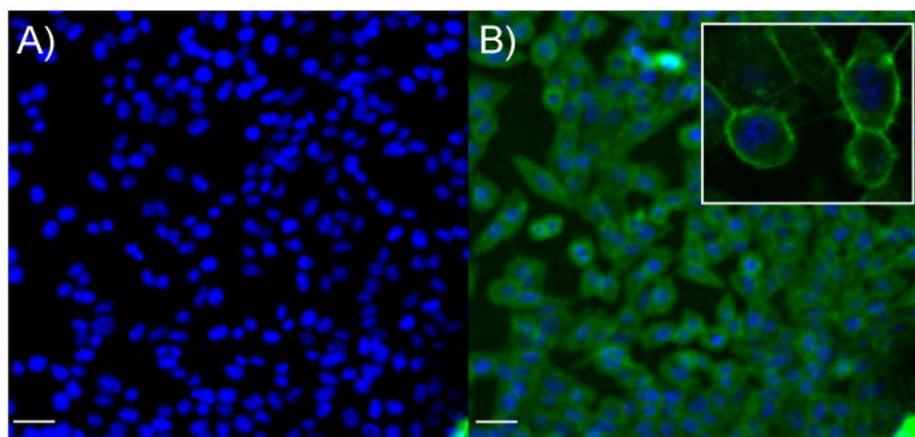
*skin of a zebrafish embryo and subsequent coiled coil formation with fluorescently labeled E or liposomes decorated with peptide E.*

Here, a generic supramolecular tool which allows us to rapidly and efficiently form coiled coil motifs at the surface of biological membranes was developed. This is of interest as a wide range of molecular constructs can be introduced to the surface of the cell through a specific coiled coil formation. Coiled coil forming peptides E [(EIAALEK)<sub>3</sub>] and K [(KIAALKE)<sub>3</sub>]<sup>26-29</sup> were first covalently conjugated to a PEG<sub>12</sub> spacer. Subsequently, a cholesterol moiety was coupled to the pegylated peptides yielding CPE and CPK (**Scheme 1A**).<sup>30</sup> The cholesterol moiety allows for the immediate insertion of the lipidated peptides into membranes through hydrophobic interactions and the PEG<sub>12</sub> moiety was incorporated to aid efficient molecular recognition between the peptide segments E and K. Recently, it was shown that upon the addition of micellar solutions of either CPE or CPK to plain liposomes, the lipidated peptides spontaneously inserted into liposomal membranes.<sup>30</sup> In the present study, CHO cell membranes and the skin of zebrafish embryos were modified with coiled coil forming peptides by the addition of a micellar solution of CPE or CPK resulting in immediate incorporation of these amphiphiles into the membranes. Subsequently, the complementary peptide was added, resulting in coiled coil formation. This system thus enables the docking of a wide variety of molecular constructs through coiled coil formation (**Scheme 1B and C**).

## **Results and Discussion**

In order to establish coiled coil formation at the surfaces of Chinese hamster ovarian (CHO) cells, it was first confirmed that the cholesterol anchor entered into the membranes of the cells. After the addition of a 5 μM solution of fluorescein labeled CPE and incubation for a few minutes, confocal microscopy was used to image the treated cells. As expected, the cells showed highly fluorescent membranes, indicating that CPE was efficiently incorporated into the cell membrane (**Figure 1**). Surprisingly, some internalization of CPE was observed. A similar effect was observed by Irvine et al., who used a diacyllipid (C<sub>18</sub>) and cholesterol to anchor the oligonucleotides into cell membranes.<sup>31</sup> These lipidated oligonucleotides are able to anchor on the cell membrane in 30 min whereas lipidated peptides could anchor in less than 5 min. One explanation for this is that as oligonucleotides are more anionic as compared to the

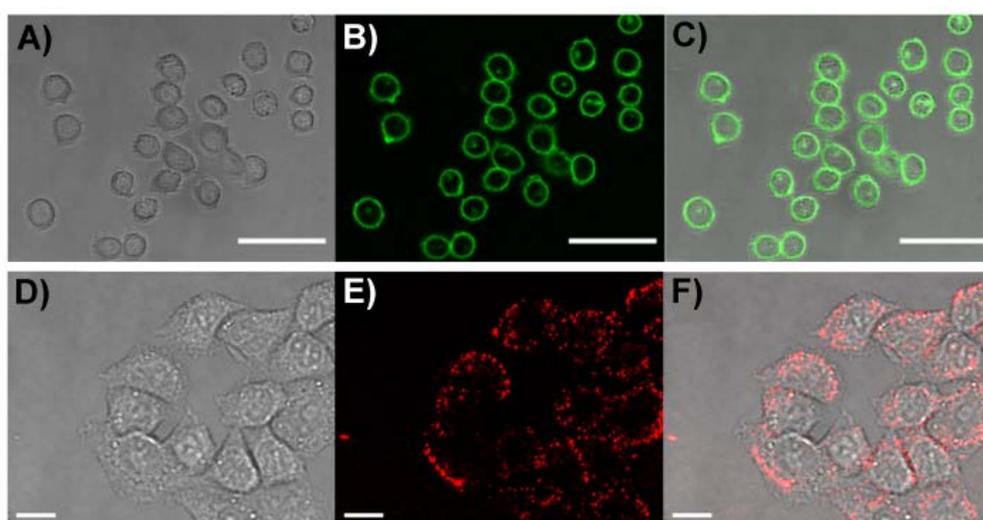
peptides used in this study, electrostatic repulsion might delay incorporation into the membrane. It was also found that incorporating a PEG<sub>24</sub> spacer between the oligonucleotide and lipid segments drastically reduced the insertion capability; a PEG<sub>12</sub> spacer did not hinder membrane insertion. Future work in our group will focus on these issues in order to have a better understanding of peptide amphiphile membrane insertion.



**Figure 1.** Confocal images of CHO cells. The cells were treated with the nuclear stain Hoechst for 30 min followed by incubation with fluorescently labeled CPE at 37 °C. Images were obtained A) 0 min and B) 10 min after the addition of fluorescent CPE (5  $\mu$ M). The inset shows a magnified image, revealing some internalization of the lipopeptide. The scale bar represents 25  $\mu$ m.

Subsequently, it was investigated whether the peptides at the CHO cell surface were accessible and capable of forming coiled coils with the complementary peptide. For this, cells were treated with a CPK solution of a final concentration of 5  $\mu$ M, which is the standard concentration for the lipidated peptides used throughout this study. A 5 minute incubation time was followed by washing with HBSS buffer to remove any unbound CPK. Next, a solution containing 2.5  $\mu$ M carboxyfluorescein labeled peptide E was added to the cells and incubated for 10 minutes. Three more washes with HBSS were performed to again remove any non-bound peptide E. Confocal imaging of the CHO cells revealed that the fluorescent signal was located at the cell membranes (**Figure 2**). In a control experiment the addition of CPK was omitted and as a result the cells showed no fluorescence (**Appendix, Figure S1**). These observations strongly suggest that the coiled coil formation between the membrane bound peptide E and peptide K is efficient. Additionally, the opposite route could also be taken, i.e.,

addition of CPE first, followed by treatment with fluorescent K peptides, which also yielded specific coiled coil formation, as observed by the fluorescence located at the membranes (**Appendix, Figure S2**). These results show that both CPK and CPE can be used to functionalize the CHO cell membrane. In contrast, the protein engineering approach only led to coiled coil formation when the engineered membrane protein contained peptide E, but not the other way around.<sup>32</sup> Furthermore, our system potentially allows for the modification of cell membranes of various cell lines without the aid of laborious protein engineering protocols. Additionally, our supramolecular method is fast and the necessary peptides can be synthesized within one day, as opposed to the protein engineering approach, where the incorporation of the modified receptor could take significantly more time.



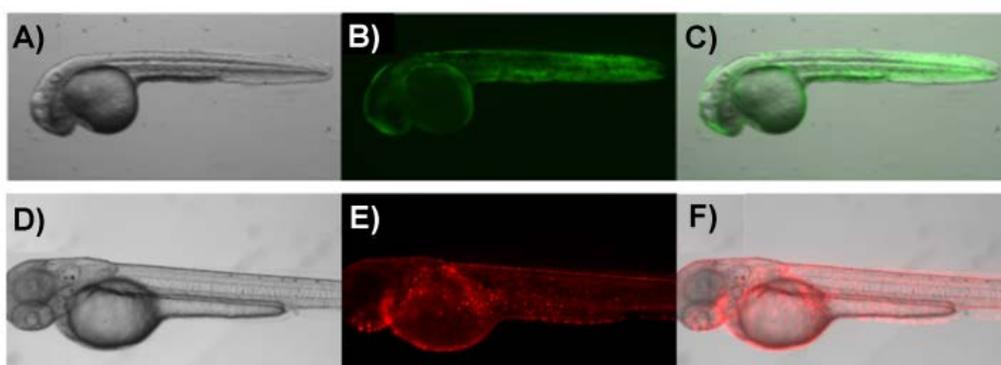
**Figure 2.** (A, B, C) Confocal microscopy images (A: bright field, B: fluorescence and C: overlay) indicating coiled coil formation between E and K at the surface of CHO cells. First, a 5  $\mu\text{M}$  solution of CPK in HBSS was added to the cells, 5 min incubation time. Subsequently, fluorescently labeled (carboxyfluorescein) E peptides (2.5  $\mu\text{M}$ ) were added and incubated for 5 minutes. Imaging took place after three washes with HBSS. Experiments were performed at 37  $^{\circ}\text{C}$ . The scale bar represents 50  $\mu\text{m}$ . (D, E, F) Confocal microscopy images (D: bright field, E: fluorescence and F: overlay) indicating docking of liposomes at the surface of CHO cells. First, a 5  $\mu\text{M}$  solution of CPK in HBSS was added to the cells, 5 min incubation time. Subsequently, CPE decorated liposomes (0.25 mM total lipid concentration and 1 mol% CPE) with 1 mol% DHPE-TR were added to the cells. All the experiments were performed at 37  $^{\circ}\text{C}$ . The scale bar represents 25  $\mu\text{m}$ .

These initial studies demonstrated that coiled coils can be formed at the surface of CHO cells by simply incubating a cholesterol peptide with the CHO cells and subsequently adding the complementary peptide. This system enables the docking of various molecules and nanoparticles to the cells. It was recently demonstrated that 100 nm liposomes can be immobilized at interfaces mediated by coiled coil formation between peptides E and K.<sup>33</sup> Therefore it was of interest to study whether liposome docking is also possible at a biological interface. Instead of adding a fluorescent peptide to the pre-treated cells, the complementary peptide was incorporated into liposomes (**Figure 2**). After membrane functionalization with CPK, fluorescent liposomes, containing 1 mol% DOPE-LR and 1 mol% CPE were incubated with the cells for 5-10 minutes. After 3 washes, confocal images of the cells were obtained (**Figure 2**). The images show increased fluorescence at the cell membranes. Furthermore, with the CPE liposomes by themselves, i.e. without the presence of peptide K at the cell membranes, no docking to the cells was observed (**Appendix, Figure S3**). This shows that large supramolecular assemblies can be docked onto membranes in an *in vitro* environment, using the orthogonal non-covalent coiled coil motif. These findings enable future applications such as biophysical studies or pharmaceutical research, for example adjuvant modified cells can be injected inside animals, which might lead to cell therapy applications.<sup>34</sup>

Preclinical studies are a required step in translating innovations from a laboratory setting to the clinic. Recent restrictions on animal testing have increased the demand for alternative animal models. Therefore, zebrafish have become an attractive model organism in recent years as they can be utilized as a tool for medical research bridging the gap between cell-based assays and mammalian models.<sup>35</sup> Due to its low-cost of husbandry, high reproductive capability, optical transparency of the embryos, and the ease of experimental manipulation, zebrafish might be a valuable tool in the development of novel drug delivery systems for human disease therapy.<sup>36</sup> Consequently, it was examined whether our coiled coil based system could also function in an *in vivo* zebrafish embryo environment. To the best of our knowledge, surface modification of zebrafish has not been studied before and therefore it was investigated whether our surface engineering system is functional in this *in vivo* environment (**Scheme 1C**). As the epidermis of zebrafish embryo consists only of

living cells, unlike terrestrial vertebrates in which dead, keratinized cells are present, it is possible to functionalize the whole organism.

One day-old zebrafish embryos were pre-treated with a solution of CPK in egg water. Next, fluorescently labeled peptide E was added and incubated with the zebrafish embryos for 30 minutes, followed by 3 washes with egg water prior to imaging. Fluorescent stereomicroscope imaging revealed that the skin of the embryos was highly fluorescent (**Figure 3A-C**). This is a strong indication that efficient coiled coil formation was accomplished *in vivo*. Furthermore, the control experiment in which the embryos were not pre-treated with CPK showed no significant fluorescence (**Appendix, Figure S4**). This strongly indicates that CPK efficiently decorated the skin of the zebrafish and that the peptide was available for coiled coil formation with its complementary peptide. Similar to *in vitro* experiments, functionalizing zebrafish embryos with CPE followed by incubation with fluorescently labeled K peptide was also attempted. Surprisingly, this alternative route did not yield efficient coiled coil formation on the skin of the zebrafish embryos, which is in contrast to the cell-based study. Fish are known to have a different lipid composition than terrestrial vertebrates, as they contain high levels of polyunsaturated fatty acids.<sup>37, 38</sup> Presumably, this lipid composition favors the positively charged K peptide over the negatively charged E peptide. However, further studies are necessary to elucidate the difference in the behavior of the peptides at the surface of CHO cells versus zebrafish embryos.



**Figure 3.** (A, B, C) Coiled coil formation between CPK and fluorescently labelled E peptides on the skin of zebrafish embryos, as indicated by fluorescent stereomicroscope images of 1 day-old zebrafish embryos. The embryos were pre-treated with 5 $\mu$ M CPK in egg water, followed by incubation with 2.5  $\mu$ M complementary fluorescent peptide E for 30 minutes at 34°C (A: bright field, B:

fluorescence, C: overlay). (D, E, F) Docking of liposomes on the skin of zebrafish embryos through coiled coil formation. Fluorescent stereomicroscope images of 2 day-old zebrafish embryos pre-treated with 5 $\mu$ M CPK in egg water followed by incubation with 0.25 mM liposomes bearing 1 mol% CPE and 1 mol% DHPE-TR on the membrane for 30 minutes at 34°C (A: bright field, B: fluorescence, C: overlay). Embryos are shown in lateral view, anterior to the left.

As a final investigation, docking of liposomes, as previously demonstrated for the CHO cells, could also be achieved *in vivo*, on the skin of 2 day-old zebrafish embryos was studied. In each group, embryos were selected and these embryos were first incubated with CPK in egg water for 30 min at 34 °C. After 30 min. embryos were washed and then incubated with 1 mol % CPK decorated fluorescently labeled liposomes for 30 min at 34 °C. Upon the addition of fluorescent E decorated liposomes to zebrafish that were pre-treated with CPK, the skin lit up brightly, revealing the docking of liposomes to the skin of the embryos (**Figure 3D-F**). Control experiments in which no CPK was administered displayed no significant fluorescence, showing that liposome docking can be attributed to the specific coiled coil interactions between peptides E and K (**Appendix, Figure S5**). As zebrafish have been intensively studied as a model for various infectious diseases,<sup>39, 40</sup> this study enables future studies in which infected zebrafish embryos are treated using therapeutic liposomes controlled by supramolecular coiled coil interactions. Liposomes contain both hydrophilic and hydrophobic regions which enables a wide range of drug encapsulation possibilities, which could be controllably released into the zebrafish embryos. Therefore, in future studies a variety of therapeutic substances will be screened, to investigate their effect on a range of diseases.<sup>41</sup>

## Conclusions

In conclusion, a generic tool to modify the surface of live cells and zebrafish embryos in a fast and efficient manner was designed. By using a supramolecular approach, employing an orthogonal coiled coil forming peptide pair, specific interactions between peptides E and K lead to coiled coil formation at the membrane of CHO cells and the skin of zebrafish embryos. This method can be used to dock a wide range of materials at a high local concentration to CHO cells and zebrafish embryos, which was demonstrated by liposome docking *in vitro* and *in vivo*. This generic method

holds much promise as an elegant and fast tool in supramolecular biomembrane engineering and drug delivery. It also enables biophysical studies of biological processes occurring at membranes. In future work, the aim is to introduce specificity into the system. This might be achieved by constructing mixed micelles containing our lipidated peptides and lipidated aptamers. The aptamers can be designed to attach to specific cell types. In this way, it might be viable to deliver the lipidated peptides to a specific cell type. Subsequent addition of liposomes that are decorated with the complementary peptide should give cell specific liposome docking.

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## **Experimental section**

### *Materials*

The Fmoc-protected amino acids were purchased from Novabiochem. The Sieber Amide resin was purchased from Agilent Technologies. Fmoc-NH-PEG<sub>12</sub>-COOH was purchased from IRIS Biotech. DOPE, cholesterol and DOPC were obtained from Avanti Polar Lipids. Cholesteryl hemisuccinate and 5,6-Carboxyfluorescein were obtained from Sigma-Aldrich. 8 well plate Lab-tek was purchased from Thermo Scientific, USA. DMEM media was obtained from PAA-the cell culture company. 3-aminobenzoic acid (Tricaine) was purchased from Sigma-Aldrich.

## **General methods**

### *Peptide synthesis*

The cholesterol modified peptides were synthesized as described elsewhere in **chapter 4**. Synthesis of fluorescently labeled peptides was performed on rink amide resin. After initial Fmoc deprotection using 20% piperidine in DMF, Fmoc-Lys(boc)-COOH was coupled, using 4 eq. of DIC and 4 eq. of HOBt, for 1 hour. Next, the side chain Boc group was removed using TFA/ DCM (50:50 v/v) for 30 mins. The resin was then neutralized using DIPEA/NMP (10:90 v/v) for 10 mins (3x). 5(6)-Carboxyfluorescein (Sigma Adrich) was coupled using 4 eq. of DIC and 4 eq. of HOBt dissolved in DMF and coupling was performed overnight. The resulting fluorescently labeled resin was used to synthesize fluorescently labeled acetylated peptide E and K along with fluorescently labeled CPE using standard Fmoc chemistry procedure described earlier.<sup>30</sup>

### **Peptide and liposome sample preparation**

Lipid (DOPC/DOPE/CH, molar ratios 2:1:1, total concentration 1 mM in CHCl<sub>3</sub>) and peptide (50 μM in 1:1 CHCl<sub>3</sub>/MeOH) stock solutions were prepared. Peptide samples were prepared by taking the appropriate amount of peptide stock, evaporating the solvent over a stream of air, addition of HBSS and sonication for 1 min at 55°C. Liposomes were prepared by using appropriate amounts of lipid and peptide stock solutions evaporating the solvent over a stream of air, addition of HBSS and sonication for 1 min at 55°C.

### **CHO cell culturing and experiments**

Chinese hamster Ovary (CHO) cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM, PAA-The Cell Culture Company), supplemented with 10% fetal calf serum (iron supplied), 2% of L-glutamine, 1% penicillin and 1% streptomycin. The cells were cultured with 5% CO<sub>2</sub> at 37 °C. The medium was refreshed every two days and the cells were passaged by trypsinization at 70% confluence. For fluorescence assays, the cells were transferred and grown on an 8-well slide (Lab-Tek, Thermo scientific). One washing step with HBSS was performed. Subsequently, the 5 µM CPK solution was added and left to incubate for 5 minutes at 37 °C. Next, the liquid was removed and a single washing step was performed. Afterwards, either fluorescently labeled E peptide (2.5 µM) or 250 µM fluorescently labeled E decorated liposomes were added to the cells and left to incubate for 5-10 minutes. Subsequently, the microscope slide was transferred to a Leica SPE confocal microscope and images were obtained within 1 h. In the CPE incorporation experiment, nuclei were stained with 100 ng/mL Hoechst 33342 in DMEM for 30 min. 5 µM fluorescently labeled CPE was added to the wells and cells were imaged over 10 min using an automated Nikon TiE2000 confocal microscope.

### **Zebrafish embryo experiments**

Zebrafish (*Danio rerio*) were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (<http://ZFIN.org>). After harvesting embryos from single crosses they were grown at 28°C in egg water (60µg/ml Instant Ocean sea salts). The stage of embryo was selected based on the auto fluorescence levels. At one day post fertilization dechorionated embryos were incubated with the peptides added to the egg water. The experimental group was pre-treated in 5µM CPK for 30 minutes at 34°C. Next, CPK was replaced with 2.5 µM complementary fluorescent E peptide and incubated for another 30 minutes at 34°C. Embryos in the control group were exposed only to 2.5 µM complementary fluorescent E peptide. Following fluorescent peptides were washed away three times with egg water; embryos were anesthetized with 200 µg/mL buffered 3-aminobenzoic acid (Tricaine) and imaged with a Leica M205FA stereo fluorescent microscope. Docking of liposomes on the biological membranes was also studied using 2 day-old zebrafish embryos as an *in vivo* system. 5 µM solution of CPK in egg water was added to the embryos for 30 minutes incubation at 34°C. After subsequent washing,

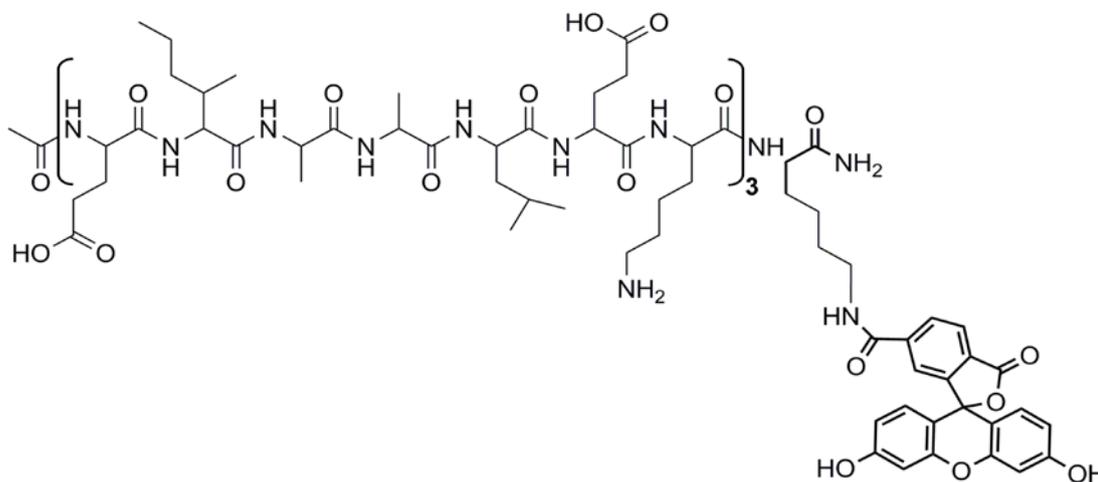
0.25 mM liposomes containing 1 mol% fluorescent DHPE-TR and 1 mol% CPE were added to the embryos for another 30 minutes at 34°C. Washing and imaging was performed as mentioned above.

### Characterization of fluorescently labeled peptides using HRMS

1. Ac-(EIAALEK)<sub>3</sub>K-Carboxyfluorescein

Chemical Formula: C<sub>131</sub>H<sub>201</sub>N<sub>27</sub>O<sub>41</sub> [2808.45]

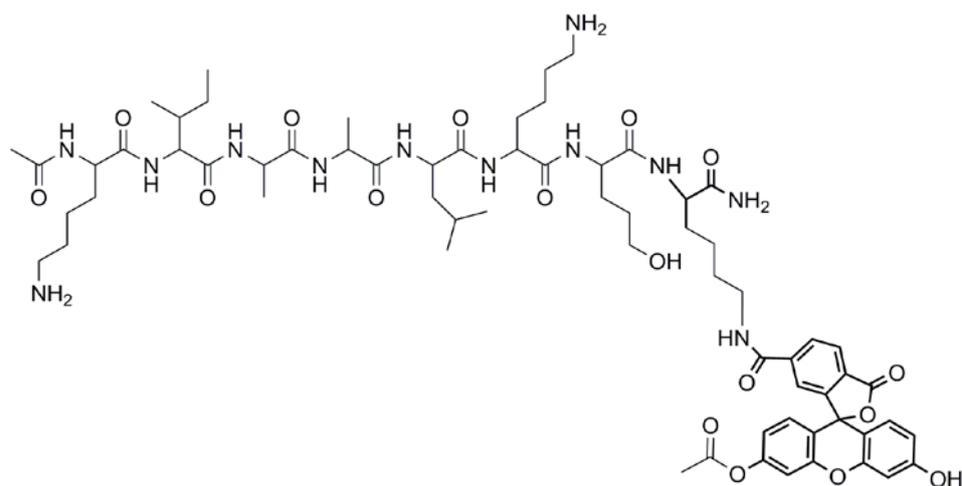
HR-MS: Calcd. [1405.225, M+2H]<sup>2+</sup>, Found 1405.22



2. Ac-(KIAALKE)<sub>3</sub>K-Carboxyfluorescein

Chemical Formula: C<sub>136</sub>H<sub>218</sub>N<sub>30</sub>O<sub>36</sub> [2808.45]

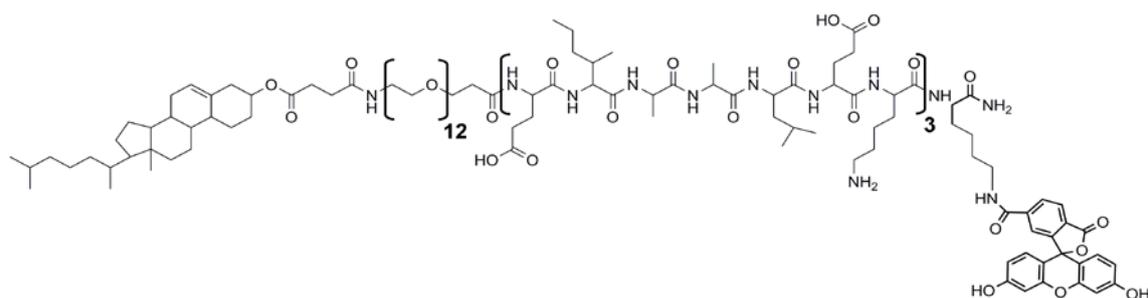
HR-MS: Calcd. [1424.81, M+2H+OAc]<sup>2+</sup>, Found 1424.817



3. Cholesterol-PEG<sub>12</sub>-(EIAALEK)<sub>3</sub>K-carboxyfluorescein

Chemical Formula: C<sub>187</sub>H<sub>300</sub>N<sub>28</sub>O<sub>56</sub> [3834.15]

HR-MS: Calcd. [1279.05, M+3H]<sup>3+</sup>, Found 1279.059

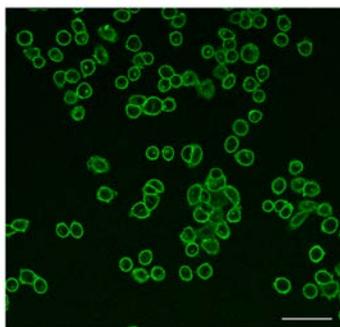


**Appendix: supplementary information**

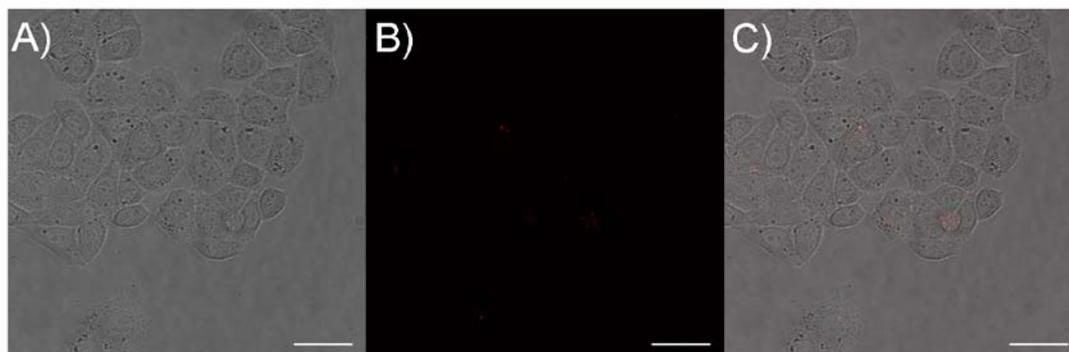
**Confocal images of CHO cells**



**Figure S1.** Confocal microscopy images (A: bright field, B: fluorescence and C: overlay) indicating that peptide E by itself does not bind to the cell membranes. Here, only fluorescently labeled (carboxyfluorescein) E peptides were added and incubated for 5 minutes with the CHO cells. Imaging took place after three washes with HBSS. Experiments performed at 37 °C. The scale bar represents 50  $\mu\text{m}$ .

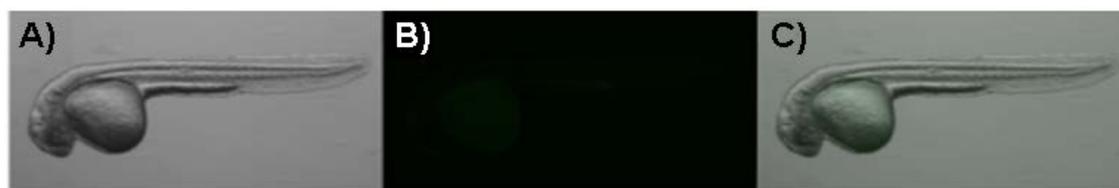


**Figure S2.** Confocal microscopy image of CHO cells, showing that the addition of CPE first, followed by fluorescently labelled K resulted in efficient coiled coil formation. First, 5  $\mu\text{M}$  CPE (5  $\mu\text{M}$ ) was added to the cells for 10 min. Subsequent addition of fluorescently labeled K peptides (2.5  $\mu\text{M}$ ). The image reveals that this reverse route also yields fluorescence located at the cell membranes, indicating that coiled coil formation occurred efficiently. All the experiments were performed at 37 °C. The scale bar represents 50  $\mu\text{m}$ .

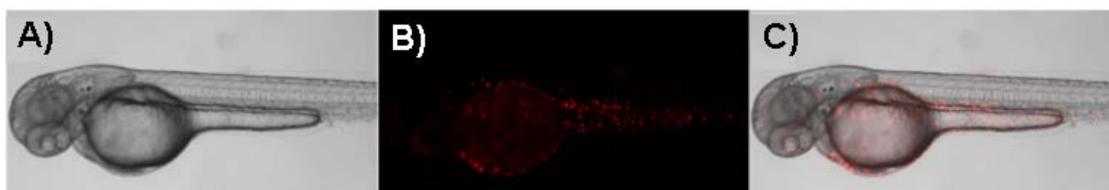


**Figure S3.** Confocal microscopy images (A: bright field, B: fluorescence and C: overlay) showing that E decorated liposomes by themselves do not dock onto CHO cells. CPE liposomes (0.25 mM total lipid concentration) with 1 mol% DHPE-TR were added to the cells. All the experiments were performed at 37 °C. The scale bar represents 50  $\mu\text{m}$ .

#### Stereo microscope images of zebrafish embryos



**Figure S4.** Control zebrafish embryos exposed only to 2.5  $\mu\text{M}$  complementary fluorescent E (A: bright field, B: fluorescence, C: overlay). Embryos are shown in lateral view, anterior to the left.



**Figure S5.** Control group of zebrafish embryos which was incubated only with 0.25 mM liposomes with 1% CPE and 1% TR for 30 minutes at 34°C (A: bright field, B: fluorescence, C: overlay). Embryos are shown in lateral view, anterior to the left.