Sequence and Length Optimization of Membrane Active Coiled Coils for Triggered Liposome Release

Camilla Skyttner, Robert Selegård, Jakob Larsson, Christopher Aronsson, Karin Enander, Daniel Aili*

Division of Molecular Physics, Department of Physics, Chemistry and Biology, Linköping University, 581 83 Linköping, Sweden

KEYWORDS: Liposome, Membrane Active Peptide, Coiled Coil, Folding, Drug Delivery

ABSTRACT

Defined and tunable peptide-lipid membrane interactions that trigger the release of liposome encapsulated drugs may offer a route to improve the efficiency and specificity of liposome-based drug delivery systems, but this require means to tailor the performance of the membrane active peptides. In this paper, the membrane activity of a de novo designed coiled coil peptide has been optimized with respect to sequence and size to improve release efficiency of liposome encapsulated cargo. The peptides were only membrane active when covalently conjugated to the liposomes. Two amino acid substitutions were made to enhance the amphipathic characteristics of the peptide, which increased the release by a factor of five at 1 µM. Moreover, the effect of peptide...
length was investigated by varying the number of heptad repeats from 2 to 5, yielding the peptides KVC\textsubscript{2}-KVC\textsubscript{5}. The shortest peptide (KVC\textsubscript{2}) showed the least interaction with the membrane and proved less efficient than the longer peptides in releasing the liposomal cargo. The peptide with three heptads (KVC\textsubscript{3}) caused liposome aggregation whereas KVC\textsubscript{4} proved to effectively release the liposomal cargo without causing aggregation. The longest peptide (KVC\textsubscript{5}) demonstrated the most defined \(\alpha\)-helical secondary structure and the highest liposome surface concentration but showed slower release kinetics than KVC\textsubscript{4}. The four heptad peptide KVC\textsubscript{4} consequently displayed optimal properties for triggering the release and is an interesting candidate for further development of bioresponsive and tunable liposomal drug delivery systems.

INTRODUCTION

A vast number of different natural and synthetic membrane active peptides have been described that can affect lipid membrane integrity. These membrane active peptides are often short (< 40 amino acids), amphipathic and cationic.\textsuperscript{1} Many natural peptides demonstrate high affinities for lipid membranes, and further improvements in affinity can be made by conjugating various hydrophobic moieties to the peptides.\textsuperscript{2–4} The interaction of membrane active peptides with a lipid membrane typically results in membrane partitioning and pore formation or disruption of the lipid bilayer and formation of mixed peptide-lipid micelles, as well as conformational changes in the peptide.\textsuperscript{5} Small changes in the primary structure can result in a large difference in the peptide-lipid interaction.\textsuperscript{6}

Liposome-based drug delivery systems can improve the pharmacokinetics and biodistribution as well as reducing toxicity of pharmaceuticals.\textsuperscript{7} About twelve liposome-based drugs have been
approved for clinical use while many more are in various stage of clinical trials.\textsuperscript{8} The release of liposome-encapsulated drugs is vital for the drug molecule to become bioavailable and provide a therapeutic effect. This process is typically slow and relies on passive diffusion as well as liposomal degradation. Thus, it is of great interest to develop strategies for tunable and triggered drug release from liposomes in order to reliably achieve drug concentrations within the therapeutic window.\textsuperscript{9}

Due to the lipid membrane disruptive mechanisms of membrane active peptides, they can potentially be utilized to modulate the release of liposome encapsulated drugs. Al-Ahmady et al. showed that lipid-bilayer-embedded leucin-zippers could improve the release of a model drug from temperature responsive liposomes by exploiting the unfolding of the peptides caused by mild hyperthermia.\textsuperscript{10} Additionally, the primary structure of peptides conjugated to liposomes can be tailored to include recognition sites for enzymes typically overexpressed in the tumor microenvironment, such as matrix metalloproteinase 9 (MMP-9). Mallik and co-workers exploited MMP-9 responsive membrane active peptides to trigger the release of encapsulated species as a result of the proteolytic cleavage of the peptides.\textsuperscript{11,12} Membrane active peptides can thus be used as active and bioresponsive components in liposome-based drug delivery systems, and they should ideally be bound to the liposomes to ensure co-transportation and effective co-localization of peptides and liposomes. Conjugation of membrane active peptides to liposomes may, however, effect their membrane activity and means to optimize, modulate and trigger their effect on the drug release rate are thus central.

We have recently demonstrated that a \textit{de novo} designed 29 residue coiled coil peptide (KVC)\textsuperscript{13} was highly membrane active when covalently conjugated to a lipid membrane.\textsuperscript{14} The peptide-lipid conjugation was accomplished through a Michael addition reaction between the N-terminal Cys
residue in the peptide and maleimide-functionalized lipids (MPB-PE) mixed into POPC liposomes. The peptide-lipid conjugation resulted in a rapid peptide and maleimide concentration dependent permeabilization of the liposomes. In the absence of MPB-PE, or when excluding the Cys, no interactions between the peptides and the liposomes could be observed. The membrane partitioning was significantly reduced in the presence of complementary peptides (EI and EV) which were designed to heterodimerize with KVC and fold into coiled coils. This competitive interaction reduced the possibilities for KVC to interact with the lipid membrane. However, when introducing a third peptide (KI) that could outcompete KVC in the heterodimer, the membrane permeabilizing effect was reestablished. The possibility to dynamically tune, inhibit and activate release of compounds from liposomes are highly interesting in the development of more sophisticated liposome-based drug delivery systems. In addition, possibilities to introduce both specificity and triggered response are design features of high relevance in the development of many other therapeutic applications of membrane active peptides, including antimicrobial peptides.

Here we have investigated the effect of peptide length and sequence to further optimize KVC for potential drug delivery applications and to shed light on the mechanisms involved in the membrane partitioning processes. KVC was originally designed as a four heptad repeat coiled coil with an additional N-terminal Cys included to facilitate conjugation to lipids or polymers. Each heptad is a seven-amino acid repeat of hydrophobic (h) and polar (p) residues in a hpphpppp pattern, which is often seen in coiled coils. The repeat is commonly denoted as (abcdefg)_n where n is the number of repeats and a-g show the relative positions of the amino acids in a helical wheel diagram. In KVC, the hydrophobic residues (Leu and Val) are in positions a and d and a number of charged Lys in e and g gives the peptide a positive net charge (+5) at neutral pH. An asparagine (Asn) residue was incorporated in position a in the third heptad to promote formation of heterodimeric
structures with aligned and parallel orientation upon interaction with complementary peptides (EI and EV). In addition, a tryptophan (Trp) was incorporated in position $f$ in the third heptad as a chromophore. However, having a polar residue in the hydrophobic face and a highly hydrophobic residue in the hydrophilic face likely influences the membrane activity of the peptide. A new peptide, KVC$_4$, was thus synthesized where the Asn and Trp residues were replaced with a hydrophobic (Val) and a charged residue (Glu), respectively. Each substitution was done to match the residues found on the respective positions elsewhere in the peptide sequence, thus generating a peptide with four identical heptad repeats. As expected, this peptide showed improved membrane activity compared to KVC. Moreover, the effect of the size of the peptide on the membrane activity was investigated by altering the number of identical heptad repeats from two to five, thus eliminating the influence of single non-repeating residues. The resulting peptides, KVC$_2$, KVC$_3$, KVC$_4$, and KVC$_5$, were membrane active, but only when covalently conjugated to the liposomes. The peptides showed distinctly different kinetics in the release of liposome encapsulated 5(6)-carboxyfluorescein (CF), and KVC$_3$-KVC$_5$ outperformed KVC with respect to CF release efficiency, requiring lower peptide concentrations and shorter times to trigger the release. In contrast to KVC, KVC$_3$-KVC$_5$ adopted $\alpha$-helical secondary structure when conjugated to the liposomes, which indicate improved partition-folding coupling.

This work further indicates that the KVC-based peptides are highly interesting for design of liposome-based drug delivery systems with triggered and tunable release and highlights the importance of optimizing sequence and size to obtain peptides with desired abilities to modulate lipid membrane integrity.
RESULTS AND DISCUSSION

**Peptide sequence optimization.** To optimize the amphipathic characteristics of KVC and to facilitate the comparison of peptides with different number of heptad repeats the Asn in position 17 was replaced by Val, and Trp in position 22 was replaced by Glu. The new peptide, KVC₄, thus has four identical repeats of KVSALKE in addition to the N-terminal Cys. The Asn → Val substitution was in position b, located on the hydrophobic face, whereas the Trp → Glu substitution was in position g on the solvent exposed face of the folded α-helix (Figure 1).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>KVC</td>
<td>C KVSALKE KVSALKE KVSALKE KVSALKE</td>
</tr>
<tr>
<td>KVC₄</td>
<td>C KVSALKE KVSALKE KVSALKE KVSALKE</td>
</tr>
</tbody>
</table>

**Figure 1:** A) The primary structure of KVC and KVC₄ where the differences between the two peptides have been indicated with color coded letters. B) The 3D representation of the α-helical KVC and KVC₄ with the hydrophobic face (green) facing out from the paper plane and the N-terminal Cys at the bottom. C) The helical wheel diagram, drawn with DrawCoil,¹⁶ of KVC with arrows indicating the substitutions made to create KVC₄. D) All colors in this figure is according to the Lesk color scheme¹⁷ apart from Cys which is colored grey.
The rather small change in sequence was expected to improve the membrane activity of the peptide. The ability of the peptides to perturb the integrity of lipid bilayers was investigated using a 5(6)-carboxyfluorescein (CF) release assay using liposomes comprised of either 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) or POPC mixed with 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl)butyramide] (MPB-PE) at a molar ratio of 95:5. MPB-PE has a terminal maleimide moiety that can react with the thiol group in the Cys containing peptides through a Michael addition reaction, anchoring the peptides to the liposome surface (Figure 2A). In the absence of MPB-PE (0 mol% MPB-PE liposomes) the CF release caused by addition of KVC and KVC₄ was < 3 % and < 1 %, respectively (Figure 2B). This level of CF release was similar to the spontaneous leakage of CF (< 2 %, Figure S1) and consistent with previous studies of KVC and related peptides.¹⁴ In contrast, rapid and peptide concentration dependent release was observed from 5 mol% MPB-PE liposomes upon addition of the peptides. As expected KVC₄ proved more efficient than KVC in releasing encapsulated CF (Figure 2B). The CF release was triggered at significantly lower peptide concentrations for KVC₄ and the amount of CF release was about a factor of five higher than for KVC at a concentration of 1 µM.

Interestingly, the thermodynamic profiles obtained by isothermal titration calorimetry (ITC) indicated that the interaction of KVC₄ with the liposomes was slightly less exothermic than for KVC (Figure 2C-E). The interpretation of the ITC results was complicated by the fact that both covalent (Cys-maleimide) and multiple non-covalent peptide-lipid and peptide-peptide interactions were involved and appeared to occur on approximately the same time scale. The covalent conjugation of Cys alone to 5 mol% MPB-PE liposomes was exothermic (ΔH = -0.57 kcal/mol).¹⁴ Under identical conditions the change in enthalpy upon conjugation of KVC and KVC₄ to 5 mol% MPB-PE liposomes was lower than the corresponding Cys-MPB-PE interaction,
\[ \Delta H = -0.24 \text{ and } -0.16 \text{ kcal/mol, respectively.} \] This indicates that except for the Cys-MPB-PE interaction the peptide-lipid interactions were endothermic, which was further confirmed by the lack of interactions between KVC or KVC\textsubscript{4} and 0 mol\% MPB-PE liposomes (Figure 2E and Figure S2). Conjugation was thus a prerequisite in order for the peptides to interact with the lipid membrane and likely facilitated an entropy driven membrane partitioning process. The difference in net charge between KVC (+5 at pH 7.4) and KVC\textsubscript{4} (+4 at pH 7.4) due to the replacement of Trp for Glu in KVC\textsubscript{4} may contribute to the difference in the calorimetric heat traces between the two peptides but differences in membrane partitioning and peptide oligomerization and folding are also contributing factors considering the large differences in CF release for the two peptides.

**Figure 2:** A) Schematic illustration of the conjugation of Cys-containing peptide to MPB-PE-containing liposomes. B) Normalized CF release 2 h after addition of 0.1-10 \( \mu \text{M} \) KVC (circles) or KVC\textsubscript{4} (triangles) to liposomes with 5 mol\% (filled symbols) or 0 mol\% (empty symbols) MPB-PE. The total lipid concentration was 40 \( \mu \text{M} \). C) ITC heat traces of 5 mol\% MPB-PE liposomes (30 mM lipids) titrated into 150 \( \mu \text{M} \) KVC\textsubscript{4}. D) ITC of 5 mol\% MPB-PE liposomes (30 mM lipids)
titrated into 150 µM KVC. E) Enthalpies for the interactions in C, D and for liposomes with 0 mol% MPB-PE (30 mM lipids) and KVC or KVC₄. Legend as in B.

**Optimization of peptide length.** The peptides KVC₂, KVC₃, KVC₄ and KVC₅, have 2, 3, 4, and 5 identical heptad repeats of the sequence KVSAŁKE, respectively (Figure 3A), and were designed and synthesized in order to investigate the effects of the number of residues on peptide membrane activity. All peptides have a single N-terminal Cys residue to enable conjugation to MPB-PE containing liposomes. Theoretically, the unfolded contour length of the shortest peptide, KVC₂, is 5.5 nm when assuming a contour length per amino acid of 3.65 Å.¹⁸ This is almost enough to span a POPC lipid bilayer with a thickness of 4 nm,¹⁹ not taking into account the potential steric hindrance caused by peptide-conjugation. If folded into α-helix and assuming a theoretical amino acid distance of 1.5 Å,²⁰ the length of KVC₂ would be 2.25 nm. The corresponding estimated unfolded contour lengths of KVC₃, KVC₄ and KVC₅ are 8 nm, 10.6 nm, and 13.1 nm, respectively, and the size when folded would be 3.3 nm, 4.4 nm and 5.4 nm, respectively.

None of the peptides induced any increase in CF release when exposed to liposomes with 0 mol% MPB-PE liposomes (Figure 3B). However, all peptides triggered increased membrane permeability and CF release from 5 mol% MPB-PE liposomes albeit to a different extent and with different kinetics (Figure 3C). The extent of the release was substantially lower for KVC₂ than for KVC₃-KVC₅, which all showed fairly similar amount of CF being released at the highest peptide concentrations (5-10 µM) after 120 min incubation. On the other hand, large differences in the release kinetics were observed for the different peptides. The fastest CF release was obtained for KVC₄, followed by KVC₅, KVC₃, and KVC₂, Figure 4. After 20 min incubation with 10 µM
peptide, the CF release was 1.4 %, 25.2 %, 65.1 % and 35.7 % for KVC₂, KVC₃, KVC₄ and KVC₅, respectively (Figure 3D-G). KVC₄ thus outperformed also the longer KVC₅ with respect to CF release rate. None of the peptides released more than 80 % of the encapsulated dye within the 2 h timeframe of the measurement. Worth noting is that even though KVC₃ was not as efficient as KVC₄ and KVC₅ in increasing the permeability of the liposomes it proved better than the original KVC (Figure 2B). The CF release of liposomes incubated with 2 µM KVC and KVC₃ resulted in a release of about 29 % and 47 %, respectively.

**Figure 3:** A) Primary structure of KVC₂-KVC₅. B) CF release of 0-10 µM KVC₂-KVC₅ with 40 µM 0 mol% or C) 5 mol% MPB-PE liposomes. D-G) The CF release kinetics in B during 0-120 min for D) KVC₂, E) KVC₃, F) KVC₄ and G) KVC₅.
KVC\textsubscript{2} was the peptide that caused the least changes in membrane permeability. Since the contour length of KVC\textsubscript{2} is just about enough to span the lipid membrane, the conjugation of the peptide via the N-terminal Cys can to a larger extent than for the longer peptides impose steric hindrance that influences the possibilities of forming a transmembrane pore.

The association of membrane active peptides to liposomes can, in addition to pore formation, lead to disruption of the bilayer and formation of peptide-lipid micelles, or liposomal aggregation. Dynamic light scattering (DLS) was utilized to probe the effect of peptide conjugation on the size of the liposomes. No changes in hydrodynamic radius were seen upon addition of KVC\textsubscript{2}-KVC\textsubscript{5} to liposomes with 0 mol\% MPB-PE and only minor increases, indicative of conjugation, were obtained for KVC\textsubscript{2}, KVC\textsubscript{4} and KVC\textsubscript{5} upon addition to 5 mol\% MPB-PE liposomes (Figure 4). However, addition of 10 \textmu M KVC\textsubscript{3} to 5 mol\% MPB-PE liposomes resulted in a bimodal size distribution with a very broad distribution around 80 nm and an additional size distribution at around 1-1.5 \textmu m, indicating aggregation. The fact that aggregation only occurred upon addition of KVC\textsubscript{3} and not for the shorter or longer peptides suggests that the conjugated KVC\textsubscript{3} is long enough to form inter-liposomal homodimers, causing bridging aggregation, and short enough not to immediately partition into the lipid bilayer since this process would compete with homodimerization. A similar effect was recently observed for a related peptide, KIC, which caused massive aggregation above a threshold concentration when conjugated to liposomes.\textsuperscript{14}
Figure 4: A) DLS hydrodynamic radius of liposomes with 5 mol% MPB-PE incubated with 1 and 10 µM KVC<sub>2</sub>-KVC<sub>5</sub> and B) 0 mol% MPB-PE incubated with 1 and 10 µM KVC<sub>2</sub>-KVC<sub>5</sub>. The total lipid concentration was 40 µM in all samples.

The interaction and partitioning of similar membrane active peptides with lipid membranes often result in conformational changes due to the amphipathic character of the folded peptides. However, despite being found to partition into the membrane and trigger CF release when conjugated to liposomes, KVC was not found to fold. Circular dichroism (CD) spectroscopy of KVC<sub>2</sub>-KVC<sub>5</sub> indicated that all peptides except KVC<sub>5</sub> existed as random coils in solution (Figure 5A-D). The free KVC<sub>5</sub> in PB showed some tendencies to adopt an α-helical conformation, likely due to homodimerization which tends to be more prominent the longer the peptide. In contrast to KVC, changes in secondary structure were seen for all the KVC<sub>2</sub>-KVC<sub>5</sub> peptides when conjugated to 5 mol% MPB-PE liposomes. The longer the peptide, the larger the change in secondary structure and the more pronounced the increase in helicity (Figure 5E-H). In comparison, when incubating the peptides with 0 mol% MPB-PE liposomes no such changes were observed (Figure 5I-L). The secondary structures of the peptides incubated with 0 mol% MPB-PE
liposomes did not differ significantly from peptides in buffer without liposomes. KVC$_2$ showed only minor structural changes upon conjugation to 5 mol\% MPB-PE liposomes which combined with its relatively slow CF release indicate weak partition-folding coupling. KVC$_5$ showed the highest helicity with a mean residue molar ellipticity at 222 nm (MRE$_{222\text{nm}}$) of -9 $\times$ 10$^3$ deg cm$^2$ dmol$^{-1}$ res$^{-1}$. The folding was also faster for KVC$_5$ than for the other peptides. The rather low MRE$_{222\text{nm}}$ values obtained for all peptides was possibly due to scattering caused by the high concentration of liposomes. However, since the lipid concentration was identical in all samples the MRE$_{222\text{nm}}$ values were comparable. Only a small increase in helicity was seen after the first spectrum was recorded 10 minutes after conjugation of KVC$_5$ to 5 mol\% MPB-PE liposomes was initiated (Figure 5H). In contrast, for both KVC$_3$ and KVC$_4$ the structural changes were not as fast and dramatic, and the helicity increased over time for both peptides. The MRE$_{222\text{nm}}$ decreased from about -2.0 and -3.4 $\times$ 10$^3$ deg cm$^2$ dmol$^{-1}$ res$^{-1}$ to -4.9 and -5.7 $\times$ 10$^3$ deg cm$^2$ dmol$^{-1}$ res$^{-1}$ for KVC$_3$ and KVC$_4$, respectively, over a period of 24 hours. Since the CF release occurred on a time scale of less than 60 minutes for both KVC$_3$ and KVC$_4$ under these conditions, this indicates that the peptides may contribute to the permeabilization of the membrane even though not fully folded. This was also the case for KVC as previously demonstrated.$^{14}$ However, one must keep in mind that the CD spectra are linear combinations of the secondary structures for all the peptides in the sample, including those in the solution and peptides that had been conjugated to, but not yet partitioned into, the lipid bilayer. The CD data, however, clearly indicates that a large fraction of the KVC$_3$-KVC$_5$ peptides are folded when conjugated and the more efficient CF release seen for these peptides compared to KVC can thus likely be attributed to a more efficient partition-folding coupling.
Figure 5: CD spectra of 50 µM KVC$_2$-KVC$_5$ A-D) without liposomes, E-H) with 5 mol% MPB-PE liposomes and I-L) with 0 mol% MPB-PE liposomes. All samples were incubated in PB and spectra were recorded after 10 min, 1.5 h and 24 h. The total lipid concentration was 1 mM.

Surface plasmon resonance (SPR) was utilized to investigate both the surface concentration of peptides conjugated to the liposomes and their accessibility to interactions with the complementary peptide E1 (Figure 6A). Liposomes with 5 mol% MPB-PE were immobilized on a Biacore L1 chip and subsequently exposed to injections of KVC$_2$-KVC$_5$ followed by injections of E1. The SPR response after injection of KVC$_2$-KVC$_5$ increased as a function of peptide length, both as a result
of increasing molecular weight of the peptides and the amount of any peptide bound to the liposomes. KVC₂ gave the lowest response and KVC₅ the highest (Figure 6B). By normalizing the responses with respect to both the molecular weight of the peptides and the immobilization level of the liposomes the number of peptides per lipid could be calculated (Figure 6C). Interestingly, whereas both KVC₂ and KVC₃ were found to bind to the liposomes at a level corresponding to the maximum surface concentration of MPB-PE, also considering the possibility of translational movement of lipids from the inner to the outer lipid leaflet, the surface concentration of both KVC₄ and KVC₅ exceeded that of MPB-PE. A similar effect was previously seen for a related de novo designed peptide, JR2KC, and indicates that peptides can bind non-covalently to the lipid membranes once the membrane permeabilization process has been initiated.³³ To further study the accessibility of the membrane bound peptides for competing interactions, the complementary peptide EI was introduced to KVC₂-KVC₅-functionalized liposomes. EI is designed to heterodimerize with KVC and fold into a coiled coil,¹³ and was also found to fold in the presence of KVC₄ and KVC₅ (Figure S3). EI is Glu rich with a net charge of -5 at neutral pH and has previously been shown not to interact with liposomes.¹⁴ EI did, however, bind extensively to liposomes with conjugated KVC₂ (Figure 6D), likely as a result of electrostatic interactions considering the opposite charge of the two peptides (+2 for KVC₂ and -5 for EI). The ratio of EI to KVC₂ was about 1:1, which indicates that KVC₂ did not partition into the membrane to any larger extent, in agreement with the CD and CF release data. Conjugated KVC₃ and KVC₄ were both interacting with EI but to a lesser extent than KVC₂, which indicates that their hydropic and charged residues are less available for interactions due to membrane partitioning. It is not possible to determine whether only about 50% of the peptides were embedded in the membrane or if the process was reversible and that the injection of EI caused the peptides to associate with EI instead
of interacting with the lipid membrane. A coiled coil peptide with a similar sequence as KVC_3, (KIAALKE)_3, designed by Alexander Kros et al., was also shown to interact with the lipid membrane and adopt a distinct \( \alpha \)-helical secondary structure when tethered to the lipid membrane by means of peptide-lipidation. In addition, when introducing liposomes functionalized with a complementary peptide, (EIAALEK)_3, peptide heterodimerization occurred, resulting in liposome fusion, indicating that the interaction of the (KIAALKE)_3 peptide with the lipid membrane was reversible. However, for the KVC_2-KVC_5 peptides, when increasing peptide length, the peptide-lipid interactions became more pronounced. Hence, no association of KVC_5 and EI could be observed, further indicating that this peptide was fully and irreversibly partitioned in the membrane under these conditions.

**Figure 6:** A) Schematic illustration of the SPR experimental setup with immobilized liposomes on a Biacore L1 sensor surface and the subsequent injections of KVC_n and EI. B) SPR sensorgrams showing the immobilization of KVC_2-KVC_5 to pre-immobilized 5 mol% MPB-PE liposomes to a Biacore L1 sensor surface and the sequential injection of the complementary peptide EI. 0 mol%
MPB-PE liposomes were used as a reference and treated identically, and the responses were subtracted from the data shown in B. C) Amount of KVC$_2$-KVC$_5$ per lipid when conjugated to 5 mol\% MPB-PE liposomes and D) the amount of EI bound to KVC$_2$-KVC$_5$-functionalized liposomes expressed as EI per KVC$_2$-KVC$_5$.

CONCLUSIONS

In summary, a systematic approach to optimize the membrane activity of the coiled coil peptide KVC was investigated. As a first step, the amphipathic characteristics of KVC were improved by replacing Asn in position 17 by Val and Trp in position 22 by Glu, rendering the peptide KVC$_4$. KVC$_4$ was found to trigger release of liposome encapsulated CF faster and more efficiently than KVC when conjugated via an N-terminal Cys to POPC liposomes containing the maleimide lipid MPB-PE. In the absence of MPB-PE no CF release or peptide-lipid interactions occurred for neither of the peptides. ITC confirmed that the peptide-lipid membrane interactions were exothermic but occurred only because of the Cys-maleimide conjugation. To further investigate the mechanisms involved and the dependence on peptide length of the membrane activity, the number of heptad repeats was varied from two to five. Irrespectively of length, conjugation led to release of encapsulated CF. The CF release triggered by KVC$_3$-KVC$_5$ was more rapid than for KVC and KVC$_2$, and required lower concentrations. The most rapid release was obtained for KVC$_4$. In contrast to KVC and KVC$_2$, KVC$_3$-KVC$_5$ adopted a defined $\alpha$-helical conformation when conjugated, further supporting a more definite partition-folding coupling. The CF release efficiency could not solely be predicted by analyzing folding of peptides conjugated to liposomes, since the competitive interaction of peptide homodimerization also contributed to the folding.
Thus, there were no clear evidence that folding of the peptides was a prerequisite for the increase in membrane permeability, though it proved highly likely that the conjugated amphipathic peptide probably folded upon interaction with the lipid bilayer. Interestingly, conjugation of KVC₃ resulted in liposome aggregation whereas for the other peptides only minor changes in liposome size were observed upon conjugation. The conjugated peptide to MPB-PE ratio increased from about 1:1 for KVC₂-KVC₄ to about 2:1 for KVC₅. The high liposome surface concentration obtained for KVC₅ was likely an effect of both conjugation of peptide oligomers and recruitment of non-conjugated peptides to sites where the lipid bilayer integrity was disrupted. In addition, whereas KVC₂, and to a certain extent also KVC₃ and KVC₄, could interact with a complementary peptide after conjugation, KVC₅ did not which indicates that it was efficiently and irreversibly buried in the lipid bilayer or already folded into oligomers. A comparison of the properties of KVC₂-KVC₅ and the parameters investigated in this paper are summarized in Table 1, and clearly show the non-trivial effects of varying peptide length on peptide-lipid membrane interactions. Difficulties to rationally predict the performance of designed membrane active peptides highlight the need of systematic studies to optimize peptide sequences and function. For further work on triggered release, KVC₄ is an attractive candidate since it induced rapid membrane permeabilization and efficient CF release without causing liposome aggregation. The highly defined intermolecular interactions involved in oligomerization and folding of the coiled coils used here will also facilitate further development of strategies for bioresponsive and tunable liposome content release, which is requirement for practical implementation in drug delivery systems.

Table 1: Summary of effect of peptide length on peptide-lipid interactions.
<table>
<thead>
<tr>
<th></th>
<th>KVC₂</th>
<th>KVC₃</th>
<th>KVC₄</th>
<th>KVC₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conjugation-dependent peptide-lipid interaction</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>CF release</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>+ Total release after 2 h</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Rate</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Discrete system (no aggregation)</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Folding upon conjugation</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Conjugation efficiency (KVC₅ / MPB-PE)</td>
<td>0.8 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>1.3 ± 0.2</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Heterodimerization (Ei / KVC₅)</td>
<td>1.2 ± 0.4</td>
<td>0.5 ± 0.5</td>
<td>0.6 ± 0.2</td>
<td>0.0 ± 0.3</td>
</tr>
</tbody>
</table>
MATERIALS AND METHODS

General. The lipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1,2-dioleoyl-sn-glycero-3-phophoethanolamine-N-(4-(p-maleimidophenyl)butyramide) (MPB-PE) were purchased from Avanti Polar Lipids (Alabaster, USA). Sensor chip L1 was purchased from GE Healthcare (Uppsala, Sweden). All other chemicals were purchased from Sigma Aldrich (Saint Louis, USA).

Peptide synthesis were performed on a Quartet automated peptide synthesizer (Gyros protein technologies) using standard fluroenylmethoxycarbonyl (Fmoc) chemistry. The sequences of KVC, KVC2-KVC5 are shown in Figure 1A and 3A. The sequence of EI was EIAALEKEIAALEKENAALEWEIAALEK. KVC and EI were synthesized as described previously. The peptides KVC2-KVC5 were synthesized on a 50 µM scale using a Rink-amide resin (Sigma Aldrich, 0.47 mmol/g) as solid support. Each amino acid coupling was performed with a fivefold excess of Fmoc protected amino acid (Iris Biotech GmbH) and O-(6-chlorobezotriazol-1-yl)-N, N, N-tetramethyluronium hexafluorophosphate (HCTU) (Iris Biotech GmbH) as activator and a tenfold excess of DIPEA as base. Fmoc deprotection after each amino acid coupling were accomplished using piperidine (20% in DMF, v/v, Applied biosystems). N-terminal acetylation was performed after final Fmoc deprotection by treatment with acetic anhydride in DMF (1:1, v/v). The peptides were cleaved from the solid support by exposure to a solution of trifluoroacetic acid (TFA), ethanedithiol, water and triisoprolylsilane (94:2.5:2.5:1, v/v/v/v) for 2 hours followed by filtration and evaporation of the solvent. The crude peptides were precipitated in cold diethylether twice before being purified using a 30 min aqueous gradient of acetonitrile (10-50%) with 0.1% TFA on a semi-preparative HPLC-system (Dionex) (Figure S4A). Peptide identity was confirmed by
MALDI-TOF MS using α-cyano-4-hydroxy-cinnamic acid as matrix (Figure S4B). Peptide concentrations were estimated from dilutions of dry weight where all peptides where handled in the same manor to minimize the difference in error between the samples.

**Liposome preparation** was done by thin film hydration with the tailored lipid composition of MPB-PE an POPC in the molar ratios 0:100 and 5:95 resulting in 0, and 5 mol% MPB-PE liposomes. Evaporation of the solvent was done under a flow of nitrogen followed by vacuum desiccation overnight. Liposomes were formed by rehydration of the dried lipid film with 0.01 M PBS (pH 7.4) and 10 min incubation followed by 1 min vortexing. To decrease the size polydispersity the liposomes were extruded 21 times through a 100 nm polycarbonate membrane using a Mini Extruder (Avanti Polar Lipids, USA). For encapsulation of CF in liposomes, the dry lipid film was instead rehydrated with 50 mM CF (self-quenching concentration) in 10 mM PB and 90 mM NaCl adjusted to pH 7.4. The liposome preparation followed as described above with an additional final step of removing the non-encapsulated CF by gel filtration using a PD-Mini/Miditrap G-25 column (GE Healthcare, Singapore) eluated with 0.01 M PBS buffer.

**Carboxyfluorescein leakage assays** were done by using liposomes with CF encapsulated at self-quenching concentration. Leakage of CF was seen as an increased fluorescence over time. This was measured ($\lambda_{ex} = 485$ nm and $\lambda_{em} = 520$ nm) with a fluorescence plate reader (BMG Fluostar Galaxy, Germany). The result was presented as percentage of CF released calculated as:

\[
\% \text{ CF release} = \frac{(F-F_0)}{(F_{tot}-F_0)} \times 100
\]

where $F_0$ was the initial fluorescence intensity measured before addition of any peptide, $F$ was the fluorescence intensity at any specific time point and $F_{tot}$ was the maximum fluorescence corresponding to full release of CF which was achieved by addition of 1 % Triton X-100. All
samples were prepared using 0.01 M PBS buffer (pH 7.4), a total lipid concentration of 40 µM, a final total volume of 250 µL and a sample size of 9 in a 96-well plate. Each peptide addition was of the same volume resulting in final peptide concentrations of 0, 0.1, 0.2, 0.5, 1, 2, 5 and 10 µM and CF release was measured every 2 min for 2 h.

**Isothermal titration calorimetry** (ITC) was done using a Microcal PEAQ-ITC (Malvern, United Kingdom). Liposomes with a total lipid concentration of 30 mM was used containing either 0 mol% or 5 mol% MPB-PE. The liposomes were titrated into 150 µM KVC or KVC₄ in 3 µL injections each 5 min. The area of the peaks corresponding to each injection were used in order to plot and evaluate the kcal/mol injectant. All samples were diluted in 0.01 M PBS buffer (pH 7.4).

**Dynamic light scattering (DLS)** was carried out on a Zetasizer Nano ZS90 (Malvern, United Kingdom). The hydrodynamic radius of liposomes incubated with peptides were determined by averaging at least 30 cycles of 10 s runs with a minimum 150 kcps count rate. The results were fitted to the cumulant model using the Zetasizer software. Liposome and peptide concentrations were chosen to correspond to that of the CF leakage assays. In these experiments 0, 1 and 10 µM KVC₂-KVC₅ was added to liposomes with 0 or 5 mol% MPB-PE (total lipid concentration 40 µM) in 0.01 M PBS (pH 7.4) at 25 °C. Data were recorded 0, 2 and 24 h after addition of peptides to the liposomes.

**Circular dichroism spectroscopy** was carried out using a Chirascan (Applied Photophysics, United Kingdom) CD spectrometer using a 1 mm cuvette in the wavelength range 197-280 nm. Each sample was prepared in 0.01 M PB buffer (pH 7.4). Peptide concentrations were 50 µM in all samples and the total lipid concentration was 1 mM when incubated with liposomes, resulting
in a Cys:maleimide ratio of 1:1 in the samples including liposomes with 5 mol% MPB-PE. All samples where incubated at RT and measured after 10 min, 1.5 h and 24 h.

**Surface plasmon resonance (SPR)** data was acquired using a Biacore 3000 instrument (GE Healthcare, Sweden) and L1 sensor chips. The flowrate was 10 μL/min and PBS was used as the running buffer. To minimize unspecific electrostatic binding to the sensor chip, the removal of negative charges in the dextran matrix was done by injection of 10 min 250 μM EDC and 50 μM NHS followed by 3.5 min 1 mM ethanolamine. Each measurement started with a wash with two 30 s injections of isopropanol mixed with 50 mM NaOH (2:3) followed by a 15 min injection at 5 μL/min of 0 or 5 mol% MPB-PE liposomes at a lipid concentration of 0.5 mM. To remove loosely bound liposomes, running buffer was subsequently flowed at 100 μL/min for 5 min, followed by a 30 s injection of 10 μM NaOH. The peptide injections were always 10 min using a peptide concentration of 200 μM and the post-injection phase was monitored for 10 min. The peptide injection order was always KVC₂, KVC₃, KVC₄ or KVC₅ and then EI. The measurement ended with a 2x30 s Isopropanol:50 mM NaOH 2:3 regeneration step.

**ASSOCIATED CONTENT**

**Supporting Information.** CF release from 0 and 5 mol% MPB-PE liposomes, ITC of KVC₄ and KVC with 0 mol% MPB-PE liposomes, CD spectra of KVC₂-KVC₅ and EI, HPLC traces and mass spectra of KVC₂-KVC₅

**AUTHOR INFORMATION**
Corresponding Author

*E-mail: daniel.aili@liu.se

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

ACKNOWLEDGMENT

The authors kindly acknowledge the financial support from the Swedish Research Council (VR), grant number 2017-04475, and the Swedish Cancer Foundation, grant number CAN 2017/430, the Swedish Government Strategic Research Area in Materials Science on Functional Materials at Linköping University (Faculty Grant SFO-Mat-LiU No. 2009-00971). During this study CS was enrolled in the graduate school Forum Scientium.
REFERENCES


KVC_n = C-(KVSALKE)_n
Heptad repeats (n)

n = 2
n = 3
n = 4
n = 5

Full release of liposomal cargo

No release of liposomal cargo