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Evidence for Surface Recognition by a Cholesterol-Recognition Peptide

Masaru Mukai,[1] Kerney Jebrell Glover,[1] and Steven L. Regen[1,*]
[1]Department of Chemistry, Lehigh University, Bethlehem, Pennsylvania

ABSTRACT Two cholesterol recognition/interaction amino-acid consensus peptides, *N*-acetyl-LWYIKC-amide, and *N*-acetyl-CLWYIK-amide, have been coupled to exchangeable mimics of Chol (cholesterol) and Phos (1,2-dipalmitoyl-*sn*-glycerol-3-phospho-(1′rac-glycerol)) via disulfide bond formation. Equilibration between Chol and Phos via thiolate-disulfide interchange reactions has revealed that both peptides favor Chol as a nearest-neighbor in liquid-disordered (l_d) bilayers to the same extent. In contrast, no Chol- or Phos-recognition could be detected by these peptides in analogous liquid-ordered (l_o) bilayers. Fluorescence measurements of the tryptophan moiety have shown that both peptides favor the membrane-water interface. Taken together, these results provide strong evidence that the recognition behavior of the LWYIK motif is, fundamentally, a surface phenomenon but that partial penetration into the bilayer is also necessary.

Understanding how lipids and proteins interact with one another in cell membranes, and defining their time-averaged lateral organization, represent two major challenges presently facing cell biologists, biochemists, and biophysicists (1–4). Although it is widely believed that the two-dimensional structure of cell membranes plays a key role in the overall functioning of cells, it has proven difficult to characterize membrane organization at the molecular level even in the simplest of model systems.

One popular concept involving lipid-protein interactions that has emerged in recent years is based on the cholesterol recognition/interaction amino-acid consensus (CRAC) hypothesis (5–8). According to this hypothesis, those segments of an integral membrane protein that lie close to the membrane surface having the sequence (L/V)-$X_{1\text{-}5}$-(Y)-$X_{1\text{-}5}$-(K/R) have a special affinity toward cholesterol. Despite its popularity, experimental evidence in support of this hypothesis has been largely circumstantial.

We have recently begun a bottom-up approach to the study of the CRAC hypothesis, starting with the minimal CRAC peptide, LWYIK. This peptide was selected based on its simplicity and because it appears to play an important role in the fusion protein gp41 found in HIV-1 (7,8). Specifically, this highly conserved segment has been found to be a determinant for viral infection (7). In addition, there is significant evidence indicating that cholesterol in target cells is required for fusion with HIV virions. This finding strongly suggests that LWYIK plays a critical role in the fusion process through its ability to associate with cholesterol in membranes.

To investigate the cholesterol-recognition properties of LWYIK at the molecular level, we have employed the nearest-neighbor recognition (NNR) method. As discussed elsewhere, NNR measurements afford unique thermodynamic insight into the interactions between membrane components (9). In brief, two membrane components of interest (A and B) are converted into exchangeable, disulfide-based homodimers (AA) and heterodimers (AB) and are allowed to undergo monomer exchange in host liposomes via thiolate-disulfide displacement reactions. Nearest-neighbor recognition is then reflected by an equilibrium constant, K, that differs from 4.0 (random mixing) where $K = (AB)^2/(AA)(BB)$.

Recently, we have shown that LWYIK, having Cys attached to its C-terminus (i.e., Pep_c), favors association with an exchangeable sterol (Chol, cholesterol) over an exchangeable phospholipid (Phos, 1,2-dipalmitoyl-*sn*-glycerol-3-phospho-(1′rac-glycerol)) in the liquid-disordered (l_d) state (Fig. 1) (10). However, no such recognition by Pep_c could be detected in the liquid-ordered (l_o) state. Negative control experiments that were carried out with a non-CRAC peptide, VGVAPG (found in elastin), showed no nearest-neighbor preference for Chol or Phos in either the l_d or the l_o phases (10).

Here, we sought to test the hypothesis that the recognition behavior of LWYIK is largely confined to the membrane surface. In principle, if such recognition were a surface

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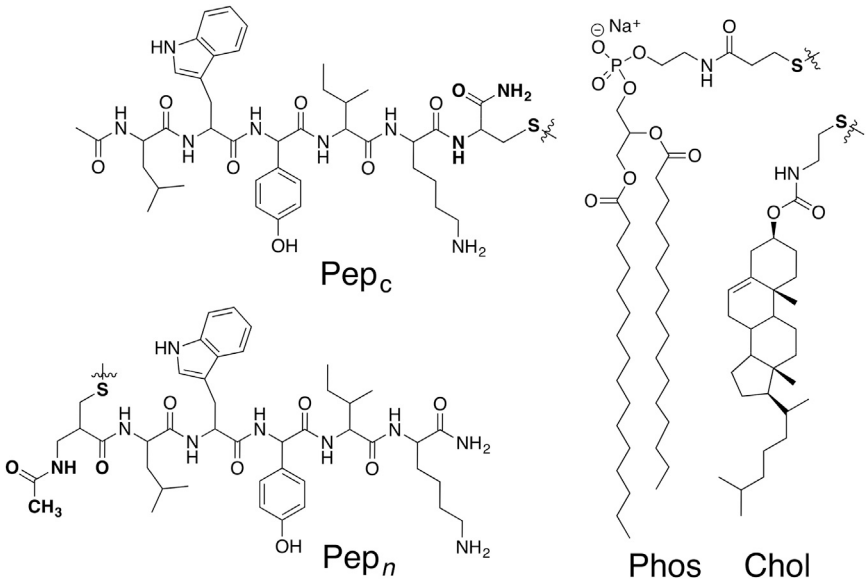

FIGURE 1 Structures of exchangeable lipids and peptides.

phenomenon where the CRAC motif wraps around the A-ring of the sterol nucleus, as has been previously proposed, then a similar degree of recognition would be expected when the peptide is attached via its N-terminus (Pep$_n$) as compared with this C terminus (Pep$_c$) (8). For both modes of attachment, the peptide's hydrophobic- and hydrogen bond-interactions with the A-ring should be similar. However, if the peptide were to extend into the bilayer, then different amino acids would be interacting with the same segments of a neighboring sterol nucleus, and, a different degree of recognition is expected.

To probe this question, we synthesized four lipid conjugates of LWYIK: i.e., {Pep$_c$-Phos}, {Pep$_c$-Chol}, {Pep$_n$-Phos}, and {Pep$_n$-Chol}, where each dimer bears a disulfide linkage (Fig. 1). These conjugates were readily prepared via thiolate-disulfide interchange between *N*-acetyl-LWYIKC-amide and *N*-acetyl-CLWYIK-amide with the activated lipids, **1** and **2** (Fig. 2).

To determine whether Pep$_n$ is capable of favoring Chol over Phos, we carried out NNR measurements both in host membranes made from DPPC (1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine) and in ones made from DPPC

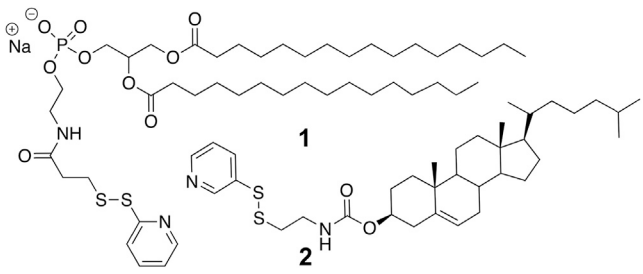

FIGURE 2 Activated forms of Phos and Chol.

that were rich in cholesterol, at 45°C. The former were in the l_d phase and the latter were in the l_o phase (11). The progress of the interchange was monitored by following the molar ratio of {Pep$_n$-Chol}/{Pep$_n$-Phos}, and also the formation of {Phos-Chol} as a function of time (Fig. 3). The latter confirms that monomer exchange has, in fact, occurred in the l_o phase (see the Supporting Material).

As discussed elsewhere, when three exchangeable monomers are involved in a NNR experiment (e.g., Pep$_n$, Phos, and Chol), three equilibria exist that are governed by Eqs. 1–3 (Fig. 4) (10). Here, the ratio K_1/K_2 (or selectivity, S) is a measure of the peptide's preference for associating with Chol (10). Our principal findings are summarized in Table 1. Based on its selectivity (S) values, the preference that Pep$_n$ has in becoming a nearest-neighbor of Chol in the l_d phase is virtually the same as that found with Pep$_c$. In addition, Pep$_n$ shows the same lack of selectivity

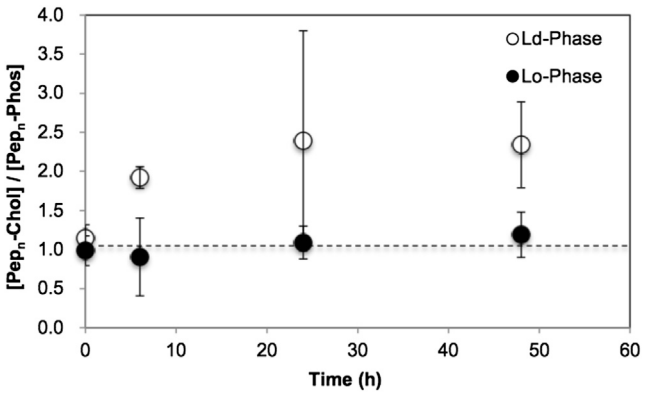

FIGURE 3 Plot of the molar ratio, {Pep$_n$-Chol}/{Pep$_n$-Phos}, as a function of time at 45°C in (●) cholesterol-rich and (○) cholesterol-poor bilayers.

$$\mathrm{Pep}_n\text{-}\mathrm{Pep}_n + \text{Chol-Chol} \overset{K_1}{\rightleftharpoons} 2\,\mathrm{Pep}_n\text{-Chol}$$

$$K_1 = \frac{[\mathrm{Pep}_n\text{-Chol}]^2}{[\mathrm{Pep}_n\text{-}\mathrm{Pep}_n]\,[\text{Chol-Chol}]} \quad (1)$$

$$\mathrm{Pep}_n\text{-}\mathrm{Pep}_n + \text{Phos-Phos} \overset{K_2}{\rightleftharpoons} 2\,\mathrm{Pep}_n\text{-Phos}$$

$$K_2 = \frac{[\mathrm{Pep}_n\text{-Phos}]^2}{[\mathrm{Pep}_n\text{-}\mathrm{Pep}_n]\,[\text{Phos-Phos}]} \quad (2)$$

$$\text{Phos-Phos} + \text{Chol-Chol} \overset{K_3}{\rightleftharpoons} 2\,\text{Phos-Chol}$$

$$K_3 = \frac{[\text{Phos-Chol}]^2}{[\text{Phos-Phos}]\,[\text{Chol-Chol}]} \quad (3)$$

$$S = \frac{K_1}{K_2} = \frac{[\mathrm{Pep}_n\text{-Chol}]^2[\text{Phos-Phos}]}{[\mathrm{Pep}_n\text{-Phos}]^2[\text{Chol-Chol}]} \quad (4)$$

$$2\,\mathrm{Pep}_n\text{-Phos} + \text{Chol-Chol} \overset{S}{\rightleftharpoons} 2\,\mathrm{Pep}_n\text{-Chol} + \text{Phos-Phos}$$

FIGURE 4 Dimer equilibria.

that $\mathrm{Pep_c}$ exhibits in the l_o phase. Thus, turning around the peptide has no significant influence on its selectivity properties.

To gain insight into the favored location of the LYWIK motif in the l_d phase, where Chol recognition was found, we examined the fluorescence of the tryptophan moiety. As previously shown, the λ_{max} value of W is very sensitive to the polarity of its microenvironment; i.e., aqueous, interfacial, and hydrophobic microenvironments are reflected by λ_{max} values of near 355, 345, and 335 nm, respectively (12).

Using host liposomes made from DPPC that were maintained at 45°C, the λ_{max} values for {Pep_c-Phos}, {Pep_c-Chol}, {Pep_n-Phos}, and {Pep_n-Chol} were 353 ± 0.09, 351 ± 0.01, 345 ± 0.77, and 335 ± 0.44 nm, respectively. These results reveal a floppy LWYIK segment that favors the membrane-water interface. Thus, the very wet W in {Pep_c-Phos} and {Pep_c-Chol} can be accounted for by a polar lysine group that separates the hydrophobic IYWL segment from the hydrocarbon interior of the membrane, thereby reducing hydrophobic interactions between them (Fig. 5) (13). The drier W groups that are found in {Pep_n-Phos} and {Pep_n-Chol} have their lysine group distal from the headgroup of the lipids. This positioning can allow for greater hydrophobic interactions between IYWL and the membrane interior; here K can hang out in the aqueous phase. Finally, the driest W that was found in

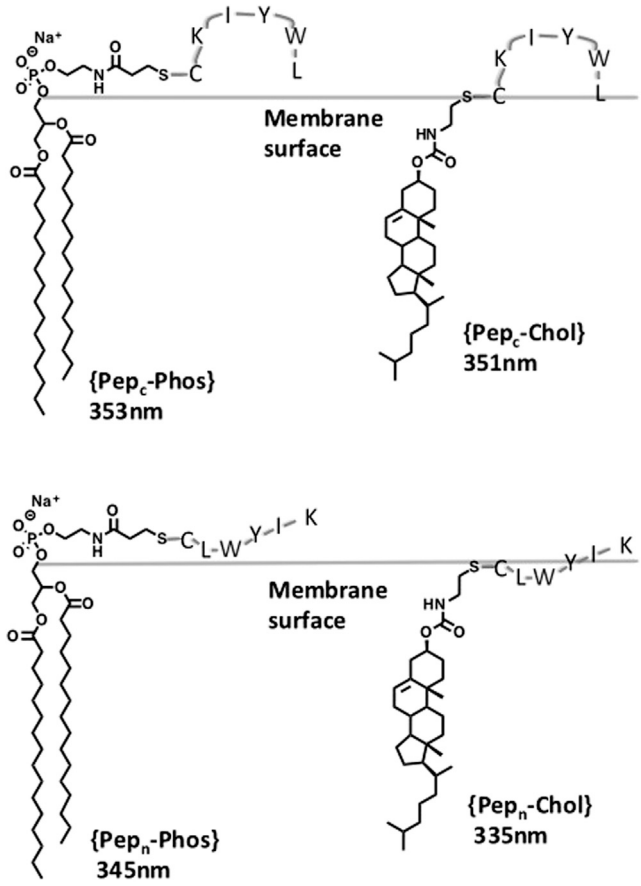

FIGURE 5 A stylized illustration showing four lipid-peptide conjugates at a water/membrane interface with hypothetical peptide conformations.

{Pep_n-Chol} is a likely result of having a distal lysine group and having the peptide attached to Chol, which can penetrate deeper into the membrane.

The fact that turning around LWYIK does not alter its selectivity and that it favors the membrane surface constitutes strong evidence that its recognition behavior is, fundamentally, a surface phenomenon. The fact that such recognition occurs only in the l_d phase further implies that partial penetration into the bilayer is necessary. From a biological standpoint, these results provide a firm basis for believing that CRAC motifs, typically found in the juxtamembrane region of proteins, play an important role in defining the protein's lateral organization.

TABLE 1 Recognition of Chol by Pep_n and Pep_c

Phase	Peptide	K_1	K_2	K_3	R[a]	S
l_d	Pep_n	0.68 ± 0.25	0.10 ± 0.04	2.39 ± 0.83	2.34 ± 0.55	7.01 ± 3.99
l_o	Pep_n	0.67 ± 0.25	0.85 ± 0.49	9.23 ± 4.22	1.19 ± 0.29	0.79 ± 0.54
l_d[b]	Pep_c	2.08 ± 0.71	0.23 ± 0.07	3.05 ± 1.15	2.50 ± 0.37	8.89 ± 4.08
l_o[b]	Pep_c	0.21 ± 0.12	0.26 ± 0.07	7.40 ± 1.55	0.95 ± 0.45	0.81 ± 0.78

[a]Molar ratio of Pep_n-Chol/Pep_n-Phos or Pep_c-Chol/Pep_c-Phos.
[b]Data taken from Mukai et al. (10).

SUPPORTING MATERIAL

Supporting Materials and Methods, one scheme, seven figures, and six tables, are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(16)30284-3.

AUTHOR CONTRIBUTIONS

M.M. carried out all NNR studies; K.J.G. recorded fluorescence measurements; and S.L.R. designed the research and wrote the article.

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Supplemental Information

Evidence for Surface Recognition by a Cholesterol-Recognition Peptide

Masaru Mukai, Kerney Jebrell Glover, and Steven L. Regen

Evidence for Surface Recognition by a Cholesterol-Recognition (CRAC) Peptide

Masaru Mukai, Kerney Jebrell Glover and Steven L. Regen*

Department of Chemistry, Lehigh University, Bethlehem, Pennsylvania 18015, United States

Supporting information

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1. General information

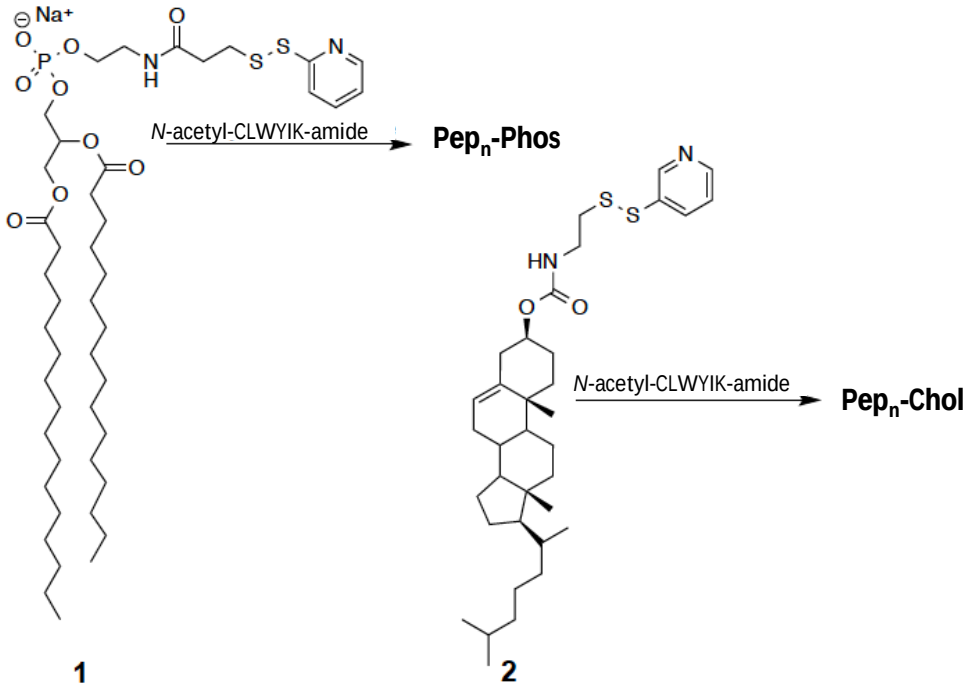
To determine the exchangeable dimer content in the nearest-neighbor recognition reactions, HPLC analysis was used with a 5 μm, 4.6 × 250 mm ultra-sphere 5 ODS column. The instrument used was a Waters Alliance HPLC system consisting of a Waters 717 plus auto sampler, a Waters 1525 binary HPLC pump and a Waters 2487 Dual λ Absorbance detector. ^{1}H NMR spectra were recorded on a Bruker Avance 500 MHz instrument. All mass spectral measurements were performed by an Agilent LC-TOF high resolution TOF analyzer at the University of California-Riverside. Deionized water was purified by a Millipore Milli-Q filtering system having one carbon and two ion-exchange stages.

2. Experimental procedures

2.1 Synthetic procedures

The exchangeable dimers were prepared as shown in Scheme SI-1. The exchangeable dimer **Chol-Chol, Chol-Phos** and **Phos-Chol** were prepared as described elsewhere.[1,2] Compounds **1** and **2** were prepared as described elsewhere.[2,3] **Pep_c-Phos** and **Pep_c-Chol** were prepared as described elsewhere.[4]

Scheme SI-1



Compound Pep_n-Phos: The peptide monomer *N*-acetyl-CLWYIK-amide (58 mg, 66.9 µmol, GenScript, Corp., Piscataway, NJ) in DMF (0.5 mL) was added dropwise to a solution of compound **1** (43 mg, 70 µmol) in 1 mL of $CHCl_3$ (1 mL). The resulting mixture was stirred overnight at room temperature under an atmosphere of argon. The crude product was purified by column chromatography (silica gel, $CHCl_3$: MeOH : H_2O = 2/1/0.1, *v/v/v*) affording typical yields of ca. 40% of **Pep_n-Phos** having ^{1}H NMR ($CDCl_3$: d-methanol = 2 : 1, *v/v* , 500 MHz, 22.5 °C) δ ppm: 0.81-093 (m, 18 H, Leu-δC*H*$_3$, lle-γC*H*$_3$, lle-δC*H*$_3$, $CH_2CH_2(CH_2)_{12}CH_3$), 1.33-1.75 (m, 50 H, lle-γC*H*$_2$, $CH_2CH_2(CH_2)_{12}CH_3$), 1.35-2.03 (m, 13 H, any-Cβ*H*, Leu-γC*H*, Ile-γC*H*$_2$, Lys-γC*H*$_2$, Lys-δC*H*$_2$, $CH_2CH_2(CH_2)_{12}CH_3$), 2.03 (s, 3 H, *Acetyl*), 2.28 (br q , 4 H, $CH_2CH_2(CH_2)_{12}CH_3$), 2.49-2.66 (br m, 2 H, C=OC*H*$_2$CH$_2$S), 2.69-3.02 (m, 7 H, C=OCH$_2$C*H*$_2$S, any-Cβ*H*, Lys-εC*H*$_2$, overlap DMF peak), 3.41 (t, 2 H, ^3J = 5.5 Hz, C*H*$_2$N), 3.36-3.45 (m, 2 H, any-Cβ*H*), 3.83-4.09 (m, 6 H, C*H*$_2$OPOC*H*$_2$, C*H*$_2$CH, any-αC*H*), 4.09-4.19 (m, 2 H, any-αC*H*), 4.32-4.30 (m, 1 H, any-αC*H*), 4.37 (br , 1 H, C*H*$_2$CH), 4.04-4.53 (m, 2 H, any-αC*H*), 5.21 (s, 1 H, overlap water peak ,CH_2C*H*), 6.41-6.58 (m, 3 H, Tyr-*Ar*, $(CH_2)_2$N*H*), 6.95 (d, 2 H, ^3J = 8.4 Hz, Tyr-*Ar*), 7.04 (t, 1 H, ^3J = 7.3 Hz, Typ-*Ar*), 7.08-7.17 (m, 2 H, Typ-*Ar*), 7.33 (d, 1 H, ^3J = 7.3 Hz, any amide), 7.35-7.41 (m, 2 H, Typ-*Ar*), 7.48 (d, 1 H, overlap $CHCl_3$, each amide), 7.57 (d, 1 H, ^3J = 5.1 Hz, any amide), 7.82 (d, 1 H, ^3J = 8.1 Hz, any amide), 8.45 (d, 1 H, ^3J = 4.4 Hz, any amide) and HR-ESI MS for $C_{83}H_{138}N_{10}O_{17}PS_2$ ([M-Na]$^-$) Calculated: 1641.9426; Found: 1641.9395.

Compound Pep_n-Chol: The peptide monomer *N*-acetyl-CLWYIK-amide (76 mg, 120 µmol, GenScript, Corp., Piscataway, NJ) in DMF (1 mL) was added drop wise to solution of compound **2** (100 mg, 120 µmol) in $CHCl_3$ (1 mL). The resulting mixture was stirred overnight at room temperature under an atmosphere of argon. The crude product was purified by column chromatography (silica gel, $CHCl_3$: MeOH : H_2O = 3/ 1/0.1, *v/v*) affording typical yields of ca. 40% of **Pep_n-Phos** having ^{1}H NMR ($CDCl_3$: d-methanol = 2 : 1, v/v , 500 MHz, 22.5 °C) δ ppm: 0.64 (s, 3H, Cholesterol-13C *methyl*), 0.84-1.60 (m, 34H, any cholesterol, Leu-δC*H*$_3$, lle-γC*H*$_3$), 0.84-1.60 (m, 21H, any cholesterol, any-Cβ*H*, lle-δC*H*$_3$, lle-γC*H*$_2$, Leu-γC*H*, Ile-γC*H*$_2$, Lys-γC*H*$_2$, Lys-δC*H*$_2$), 1.60-2.06 (m, 12 H, any cholesterol, *Acetyl*, any-Cβ*H*), 2.19-3.05 (m, 10H, Cholesterol-4C*H*$_2$, any-Cβ*H*, SCH$_2$C*H*$_2$, Lys-εC*H*$_2$), 3.05-3.26 (m, 3 H, SC*H*$_2$CH$_2$ any-

Cβ*H*,), 3.03-3.28 (m, 2 H, any-Cβ*H*), 3.83-4.02 (m, 2 H, any-αC*H*), 4.09-4.26 (m, 3 H, any-αC*H*), 4.55-4.69 (br, 1 H, any-αC*H*, overlap methanol), 5.31 (s, 1 H, Cholesterol-6C*H*), 6.72 (d, 2H, ^3J = 7.3 Hz, Tyr-*Ar*), 7.04 (s, 1 H, Typ-*Ar*), 6.92 (d, 2 H, ^3J = 7.3 Hz, Tyr-*Ar*), 7.00-7.19 (m, 3 H, Typ-*Ar*), 7.32-7.51 (m, 4 H, overlap $CHCl_3$, Typ-*Ar*, any-*amide*), 7.56 (d, 1 H, ^3J = 5.4 Hz, any *amide*), 7.75 (d, 1 H, ^3J = 6.6 Hz, any *amide*) and HR-ESI MS for $C_{73}H_{113}N_{10}O_{10}S_2$ ($[M+H]^+$) Calculated: 1353.8077; Found: 1353.8116

2.2 Nearest-neighbor recognition (NNR) experiments

Thin films of lipid were prepared by evaporating a chloroform solution containing 0.15 μmol [**Pep$_n$-Chol**], 0.15 μmol [**Pep$_n$-Phos**] and varying amounts of DPPC and cholesterol under a stream of argon. After drying the thin film overnight under reduced pressure (0.4 mm Hg), 2.0 mL of a 10 mM Tris-HCl buffer (10 mM Tris, 500 mM NaCl, 2 mM NaN_3, 1 mM EDTA, pH = 7.4), a 60 μL aliquot of 1.68 μM monesin and dithiothreitol (15 μL of a 19.8 mM solution in pH 7.4 Tris buffer, 1 eq. with respect to disulfide content) were added to each of the dried films. The mixtures were then vortexed every 5 min for 30 s over a time span of 30 min with intermittent incubation at 60 °C. Following this, the dispersions were subjected to six freeze/thaw cycles (liquid nitrogen/60 °C water bath). The vesicle dispersions (2 mL) were heated to 45 °C, and oxygen was removed by purging with argon. A sufficient amount of 0.1 M NaOH (63 μL) was added to bring the pH to 7.4 (after adjusting the temperataure to 45 °C) to start the exchange reaction.

Aliquots (400 μL) were withdrawn as a function of time and the exchange reactions were quenched by adding 25 μL of 8.3 M acetic acid and vortexing. These aliquots were quickly frozen, using liquid nitrogen and stored at -20 °C until HPLC analysis was carried out. For HPLC analysis, to each thawed aliquot was added 2000 μL of $CHCl_3$/MeOH (2:1 v/v) and Aldrithiol-2 (2,2-dipyridyldisulfide, 74 μL of a 10 mM solution in $CHCl_3$). The tubes were vortexed, centrifuged, and the aqueous phases removed using a Pasteur pipette. The organic phase was then concentrated under reduced pressure using a Savant SVC-100 SpeedVac concentrator equipped with a cold trap and vacuum pump (~1 hr at ~ 0.4 Torr). The residual compounds were dissolved in 60 μL of $CHCl_3$ and 140 μL of the HPLC mobile phase for analysis of [**Pep$_n$-Chol]** and [**Pep$_n$-Phos]**. The solution was divided into two tubes (each 100 μL).

The concentrations of [**Chol-Chol]**, [**Phol-Phos]** and [**Chol-Phos]** were analyzed by HPLC using a Symmetry C_{18} 5 μm reversed phase column. The analysis was done in an isocratic mode using a mobile phase consisting of 760 mL of EtOH, 120 mL of deionized H_2O, 100 mL of hexane, and 10 mL 1 M aq. $N(n\text{-}Bu)_4OAc$. The flow-rate was 0.9 ml/min, the column temperature was 31 °C, and detection was done at 203 nm.

The concentrations of [**Pep$_n$-Chol]** and [**Pep$_n$-Phos]** were analyzed by HPLC using a Symmetry C_{18} 5μm reversed phase column. The analysis was done in an isocratic mode using a mobile phase consisting of 890 mL of EtOH, 100 mL of deionized H_2O, 1 mL of

TFA, and 10 mL 1 M aq. $N(n\text{-}Bu)_4OAc$. The flow-rate was 0.9 ml/min, the column temperature was 31 °C, and detection was done at 280 nm.

In this study, liposomes that were rich in cholesterol were prepared from DPPC/cholesterol/Pep_n-Phos/Pep_n-Chol (57.5/37.5/1.25/1.25, mol/mol/mol/mol; cholesterol-poor analogs were made from DPPC/Pep_n-Phos/Pep_n-Chol (95.0/1.25/1.25, mol/mol/mol).

3. NMR spectra of compounds

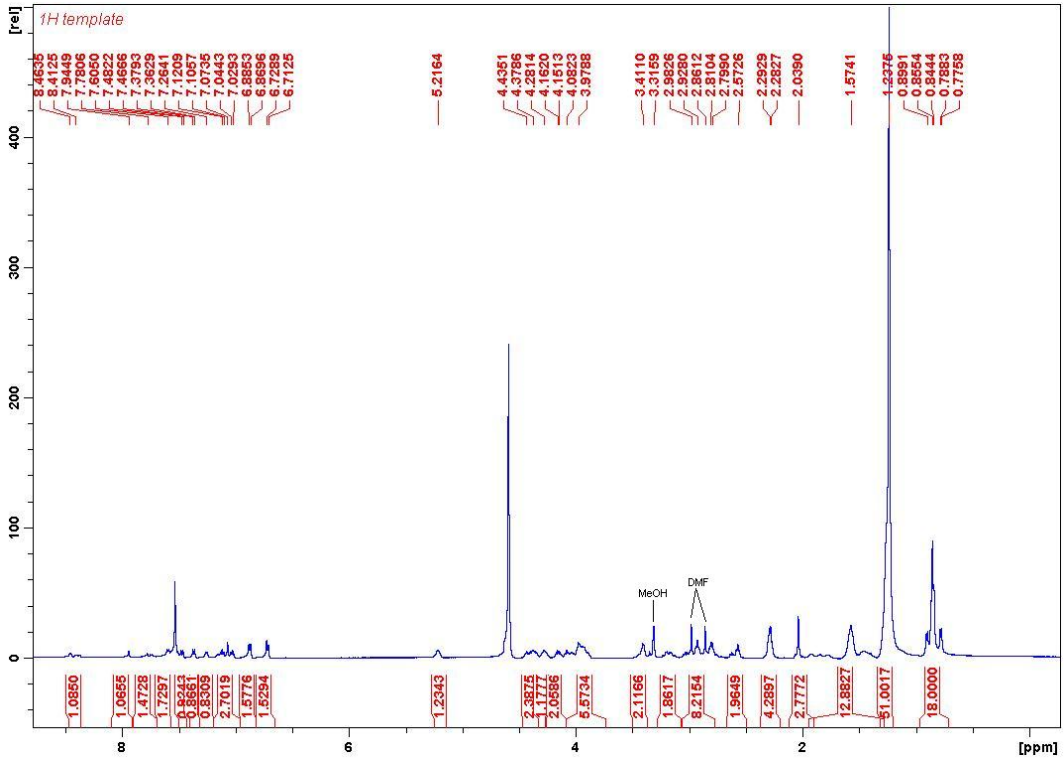


Figure SI-1: ^{1}H NMR spectrum of **Pep$_n$-Phos**

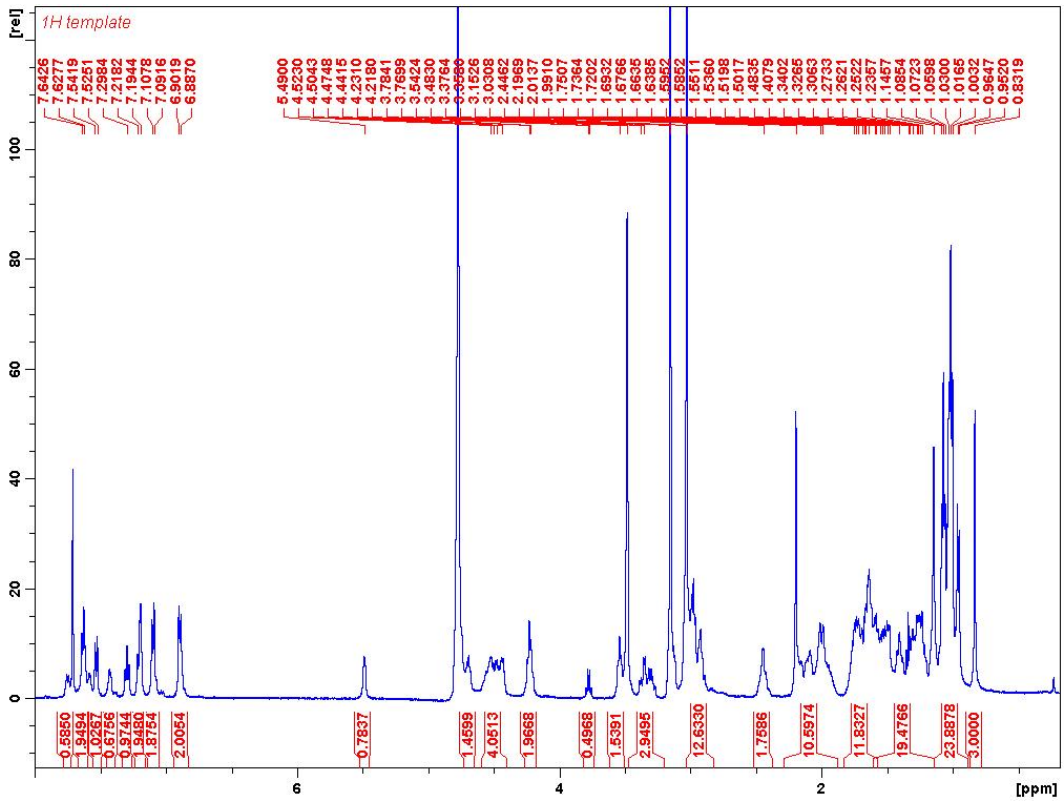


Figure SI-2: ^{1}H NMR spectrum of **Pep$_n$-Chol**

4. Calibration of chromatographic system

The chromatographic system was calibrated by injecting various known amounts of the specific dimers in eluent and analyzed by HPLC. The system was found to respond as follows: for **[Phos-Phos]**, 478140×*n***[Phos-Phos]**-518=Signal (R^2=0.9984); for **[Chol-Chol]**, 591890×*n***[Chol-Chol]**-90867=Signal (R^2 = 0.9988); for **[Chol-Phos]**, 533520×*n***[Chol-Phos]**-12637=Signal (R^2 = 0.9988) ; for **[Pep$_n$-Phos]**, 2279499×*n***[Pep$_n$-Phos]**+78797=Signal (R^2 = 0.9990) ; for **[Pep$_n$-Chol]**, 290097×*n***[Pep$_n$-Chol]**+33510=Signal (R^2 = 0.9992). Where signal is the area of the peaks for the dimers and *n***[Phos-Phos]**, *n***[Chol-Chol]**, *n***[Chol-Phos], n[Pep$_n$-Phos], n[Pep$_n$-Chol]** are the number of nmol of the dimers. Calibration data for **[Pep$_n$-Phos]**, **[Pep$_n$-Chol]** are given in Table SI-2 and the calibration curves are shown in Figure SI-3. Details for the calibration of **[Chol-Chol], [Chol-Phos], [Phos-Phos]** were shown in previous publications.[4,5]

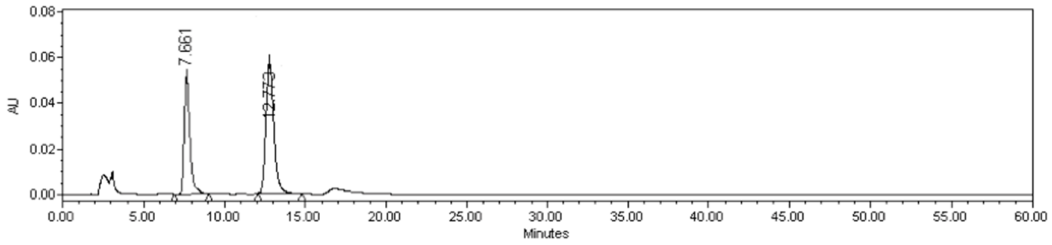


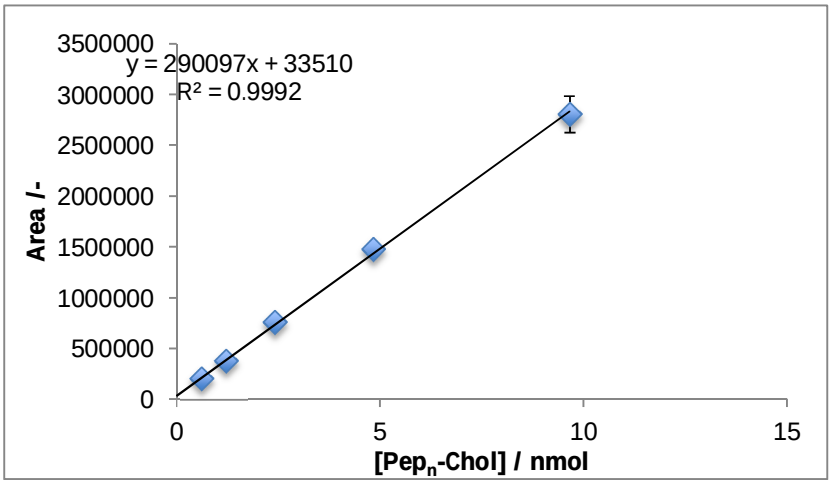
Figure SI-3: A sample chromatogram of **Pep$_n$-Chol** (Retention time: 7.66 min) and **Pep$_n$-Phos** (Retention time: 12.77 min).

Table SI-1: Data for calibration curves of **Pep_n-Chol**.

nmol	Area		
	run1	run2	run3
9.65	2995492	2650169	2779992
4.82	1458704	1505427	1465802
2.41	742155	770430	747029
1.21	388958	405141	355959
0.60	201200	230850	200446
0	12222	-12841	-11727

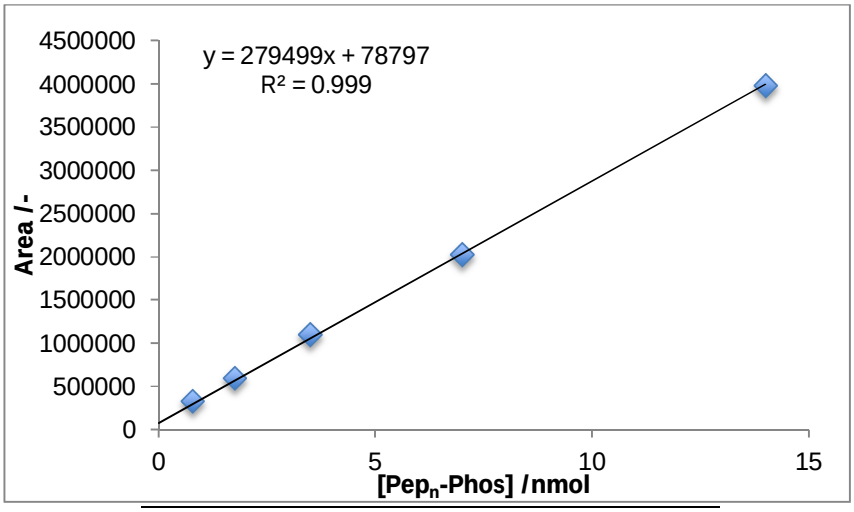
Table SI-2: Data for calibration curves of **Pep_n-Phos.**

nmol	Area		
	run1	run2	run3
14.0	3944933	4038905	3964580
7.0	2007459	2030981	2025342
3.5	1086574	1149249	1062515
1.8	590421	626501	568251
0.78	363124	314176	317288
0	127	-12744	1050



	Slope	Intercept
Parameter	290097	33510
Std. Dev	4958	22556
R^2	0.9953	69687

Figure SI-4: Calibration curves for **Pep$_n$-Chol**



	Slope	Intercept
Parameter	279499	78797
Std. Dev	2607	17194
R^2	0.9986	53279

Figure SI-5: Calibration curves for **Pep$_n$-Phos**

5. Data for NNR measurements

Table SI-3: Concentration of exchangeable dimers after 48-hour reaction and resulting equilibrium constants (K_1, K_2 and K_3), selectivity (S), and ratio (R: **Pep_n-Chol**/**Pep_n-Phos**) in the liquid-disordered state (l_d).[a]

	Area					
	run1	run2	run3	run4	Average	Std. dev.
Phos-Phos	541315	711129	583180	634588	617553	88469
Phos-Chol	846178	1035772	800494	1026648	927273	124759
Chol-Chol	442521	555770	439926	478180	479099	66146
Pep_n-Chol	343834	480894	537629	434773	449283	99633
Pep_n-Phos	197600	276950	262724	216011	238321	42308
	l_d phase (nmol)					
	run1	run2	run3	run4	Average	Std. Error[a]
Phos-Phos	1.13	1.49	1.22	1.32	1.29	0.20
Phos-Chol	1.61	1.97	1.52	1.94	1.76	0.20
Chol-Chol	0.90	1.26	0.90	0.96	1.00	0.22
Pep_n-Chol	1.07	1.54	1.73	1.57	1.48	0.20
Pep_n-Phos	0.42	0.70	0.65	0.75	0.63	0.12
Pep_n-Pep_n[b]	2.84	3.73	2.88	3.25	3.18	0.41
K_1=	-	-	-	-	0.68	0.25
K_2=	-	-	-	-	0.10	0.04
K_3=	-	-	-	-	2.39	0.83
Ratio=	-	-	-	-	2.34	0.55
S=	-	-	-	-	7.01	3.99

[a] Standard errors for concentrations were calculated from the standard deviation of the Y value average and the standard errors of the slope and intercept of the calibration curve.[4]

[b] **Pep_n-Pep_n** amounts were estimated using mass balance, and represent maximum values.

Table SI-4: Concentration of exchangeable dimers after 48-hour reaction and resulting equilibrium constants (K_1, K_2 and K_3), selectivity (S), and ratio (R: **Pep_n-Chol/Pep_n-Phos**) in the liquid-ordered state (l_o).[a]

	Area					
	run1	run2	run3	run4	Average	Std. dev.
Phos-Phos	266162	204759	403853	403853	291591	101954
Phos-Chol	1327009	1058385	1698367	1698367	1361254	321362
Chol-Chol	526755	-	673023	673023	522096	153310
Pep_n-Chol	318788	402852	556355	632420	425998	120463
Pep_n-Phos	315504	376152	467213	539011	386290	76361
	l_o phase (nmol)					
	run1	run2	run3	run4	Average	Std. Error[a]
Phos-Phos	0.56	0.43	0.85	0.85	0.67	0.25
Phos-Chol	2.51	2.01	3.21	3.21	2.73	0.20
Chol-Chol	1.04	-	1.29	1.29	1.21	0.27
Pep_n-Chol	1.16	1.46	1.80	2.06	1.62	0.20
Pep_n-Phos	1.10	1.31	1.39	1.64	1.36	0.28
Pep_n-Pep_n[b]	2.86	-	3.74	3.74	3.24	0.51
K_1=	-	-	-	-	0.67	0.25
K_2=	-	-	-	-	0.85	0.49
K_3=	-	-	-	-	9.23	4.22
Ratio=	-	-	-	-	1.19	0.29
S=	-	-	-	-	0.79	0.54

[a] Standard errors for concentrations were calculated from the standard deviation of the Y value average and the standard errors of the slope and intercept of the calibration curve.[4]
[b] **Pep_n-Pep_n** amounts were estimated using mass balance, and represent maximum values.

Table SI-5: Data for equilibration in liquid-disordered state at 45 °C

time (h)		Concentration (nmol)					
		run1	run2	run3	run4	average	std.
0 h	**Pep_n-Chol**	3.70	3.99	4.52	-	1.14 [a)]	0.17 [a)]
	Pep_n-Phos	3.01	3.91	3.82	-		
	Phos-Chol	0.66	0.63	0.67	-	0.65	0.02
6 h	**Pep_n-Chol**	3.29	3.57	3.82	-	1.92 [a)]	0.14 [a)]
	Pep_n-Phos	1.78	1.98	1.80	-		
	Phos-Chol	1.40	1.39	1.34	-	1.38	0.03
24 h	**Pep_n-Chol**	0.97	2.49	-	-	2.39 [a)]	1.41 [a)]
	Pep_n-Phos	0.72	0.75	0.70	-		
	Phos-Chol	2.35	1.53	1.83	-	1.9	0.41
48 h	**Pep_n-Chol**	1.07	1.54	1.73	1.57	2.34 [a)]	0.55 [a)]
	Pep_n-Phos	0.42	0.70	0.65	0.75		
	Phos-Chol	1.61	1.97	1.52	1.94	1.76	1.61

a) [Pep_n-Chol] / [Pep_n-Phos]

Table SI-6: Data for equilibration in liquid-ordered state at 45 °C

time (h)		Concentration (nmol)					
		run1	run2	run3	run4	average	std.
0 h	**Pep_n-Chol**	10.10	10.40	10.30	-	0.99 [a)]	0.02 [a)]
	Pep_n-Phos	10.30	10.30	10.43	-		
	Phos-Chol	0.46	0.13	0.40	-	0.27	0.19
6 h	**Pep_n-Chol**	1.31	2.10	1.38	-	0.91 [a)]	0.50 [a)]
	Pep_n-Phos	0.95	2.26	2.03	-		
	Phos-Chol	1.76	1.80	2.13	-	1.90	0.20
24 h	**Pep_n-Chol**	1.19	1.57	1.38	-	1.09 [a)]	0.21 [a)]
	Pep_n-Phos	1.04	1.43	1.33	-		
	Phos-Chol	2.36	2.18	1.15	-	1.90	0.65
48 h	**Pep_n-Chol**	1.16	1.46	1.80	2.06	1.19 [a)]	0.29 [a)]
	Pep_n-Phos	1.10	1.31	1.39	1.64		
	Phos-Chol	2.51	2.01	3.21	3.21	2.73	2.51

a) **[Pepn-Chol] / [Pepn-Phos]**

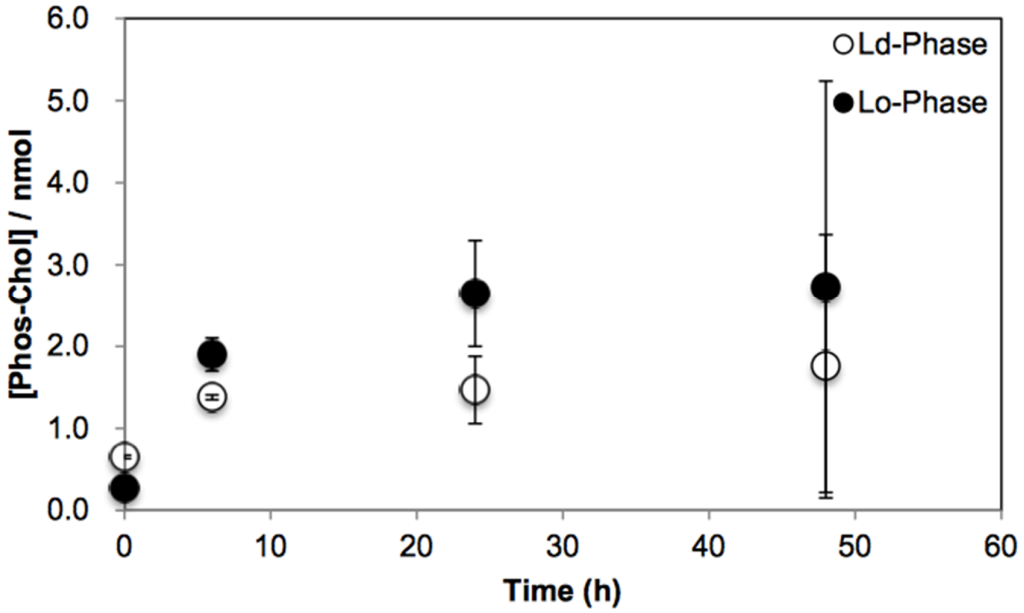


Figure SI-6. Plot of the formation of {Phos-Chol} as a function of time at 45 °C in (●) cholesterol-rich and (○) cholesterol-poor bilayers. Cholesterol-rich vesicles were made from DPPC/cholesterol/Pep_n-Phos/Pep_n-Chol with a molar ratio of 57.5/37.5/1.25/1.25. Cholesterol-poor vesicles were made from DPPC/Pep_n-Phos/Pep_n-Chol having a molar ratio of 95.0/1.25/1.25.

6. Fluorescence experiments

Thin films of lipid were prepared by evaporating a chloroform solution containing 0.3 μmol of peptide conjugate (Pep_n-Chol, Pep_n-Phos, Pep_c-Chol or Pep_c-Phos) and 11.4 μmol of DPPC under a stream of argon. After drying the thin film overnight under reduced pressure (0.4 mm Hg), 2.0 mL of a 10 mM Tris-HCl buffer (10 mM Tris, 150 mM NaCl, 2 mM NaN_3, 1 mM EDTA, pH = 7.4) was added to each of the dried films. The mixtures were then vortexed every 5 min for 30 s over a time span of 30 min with intermittent incubation at 60 °C. Following this, the dispersions were subjected to six freeze/thaw cycles (liquid nitrogen/60 °C water bath) and extruded 15 times through a 100 nm pore diameter polycarbonate filter (Nuclepore, Whatman Inc.) using argon at a pressure of ~100 psi. Steady-state fluorescence emission spectra were acquired at 318 K using an Varian Eclipse fluorescence spectrophotometer (Agilent, Santa Clara, CA) using a 1 × 0.1 cm quartz cuvette. The excitation wavelength was 295 nm which selectively excites tryptophan residues. Both excitation and emission slits were set to 5 nm. The emission spectrum was measured from 310-400 nm with a scan speed of 2 nm/s and 1.0 nm data point increments, averaging 16 scans. The λ_{max} values were determined by fitting the data to a log-normal distribution using Igor Pro 6.3.7.2 (WaveMetrics, Inc., Lake Oswego, OR).

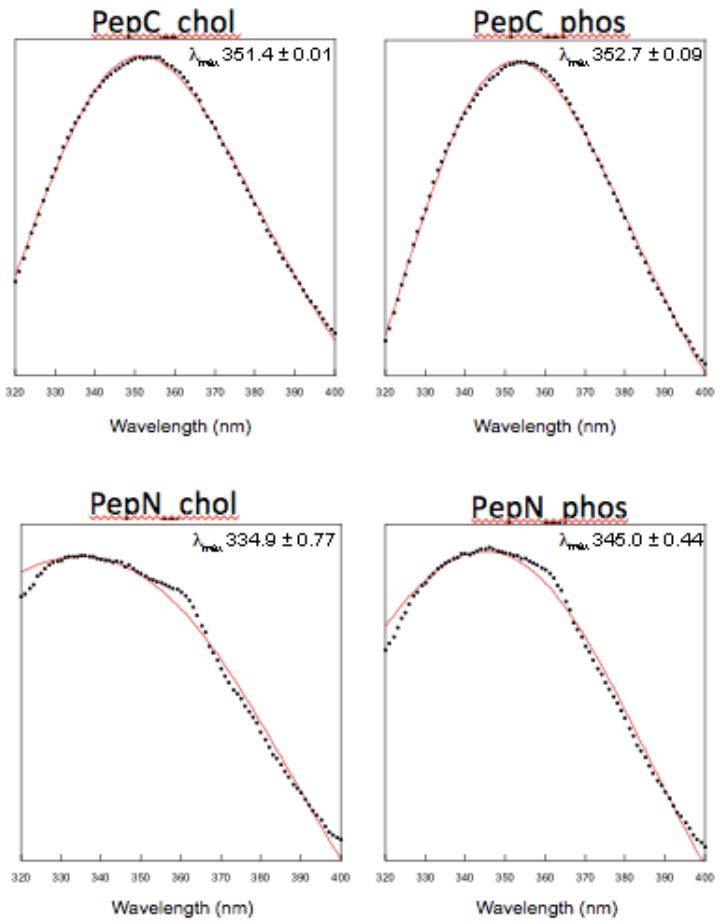


Figure SI-7 Tryptophan emission spectra with excitation at 295 nm of CRAC peptide conjugated compounds in DPPC liposomes at 45°C. Data points are in black and the Fit line is shown in red.

7. References

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