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Supplemental Information

Evidence for Surface Recognition by a Cholesterol-Recognition Peptide

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

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Supporting information

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1. General information

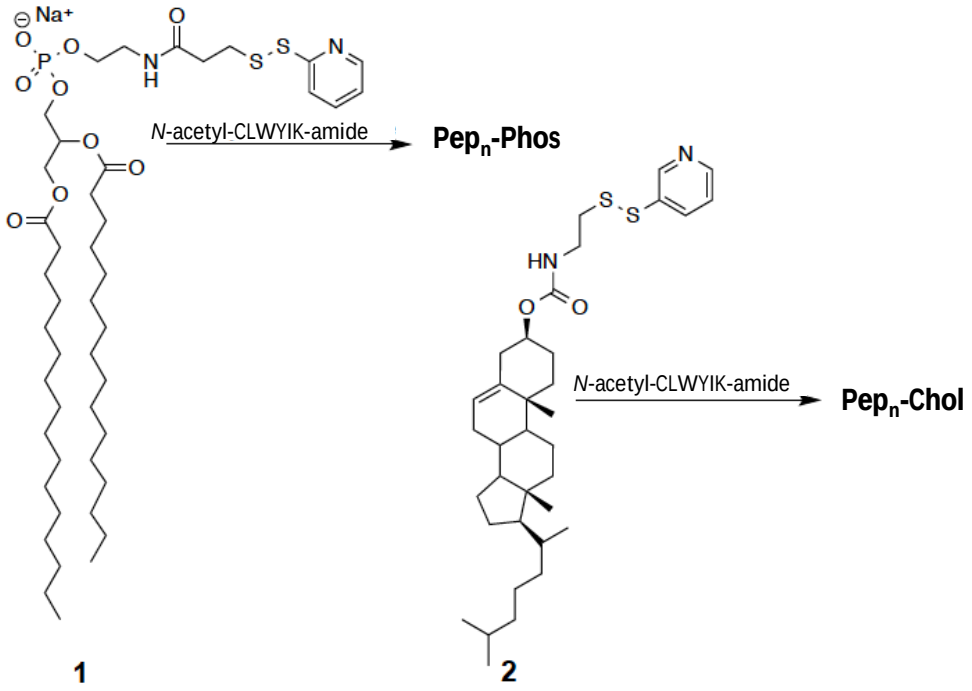
To determine the exchangeable dimer content in the nearest-neighbor recognition reactions, HPLC analysis was used with a 5 μm, 4.6 × 250 mm ultra-sphere 5 ODS column. The instrument used was a Waters Alliance HPLC system consisting of a Waters 717 plus auto sampler, a Waters 1525 binary HPLC pump and 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

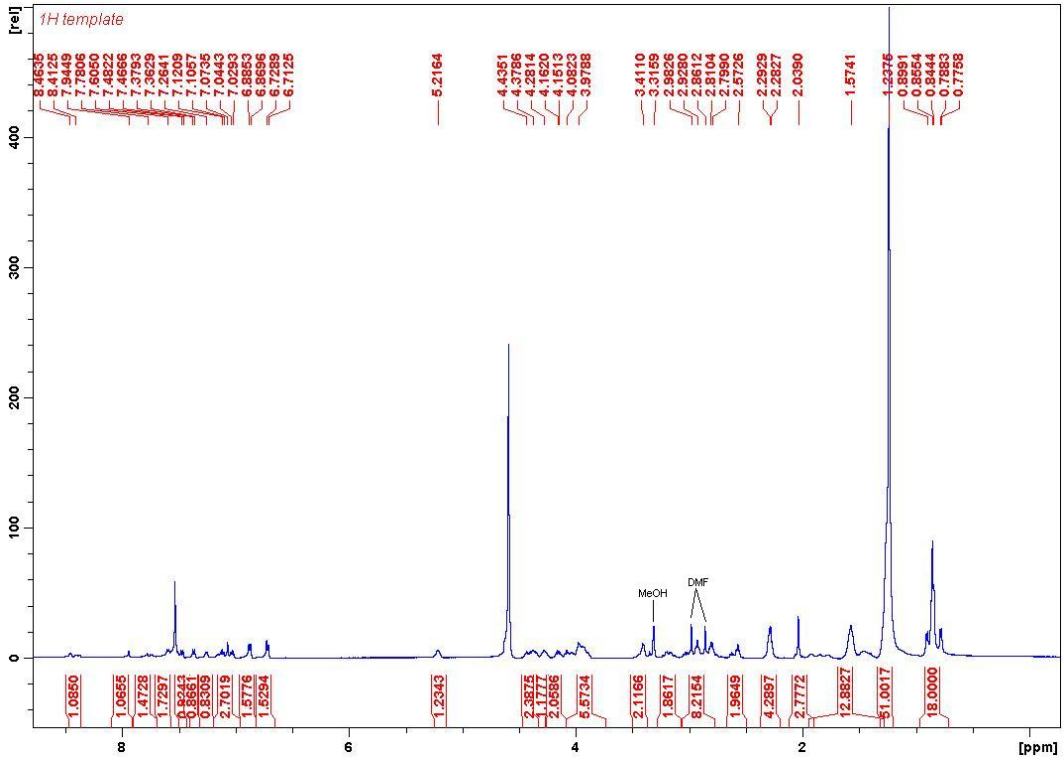


Figure SI-1: ^{1}H NMR spectrum of **Pep$_n$-Phos**

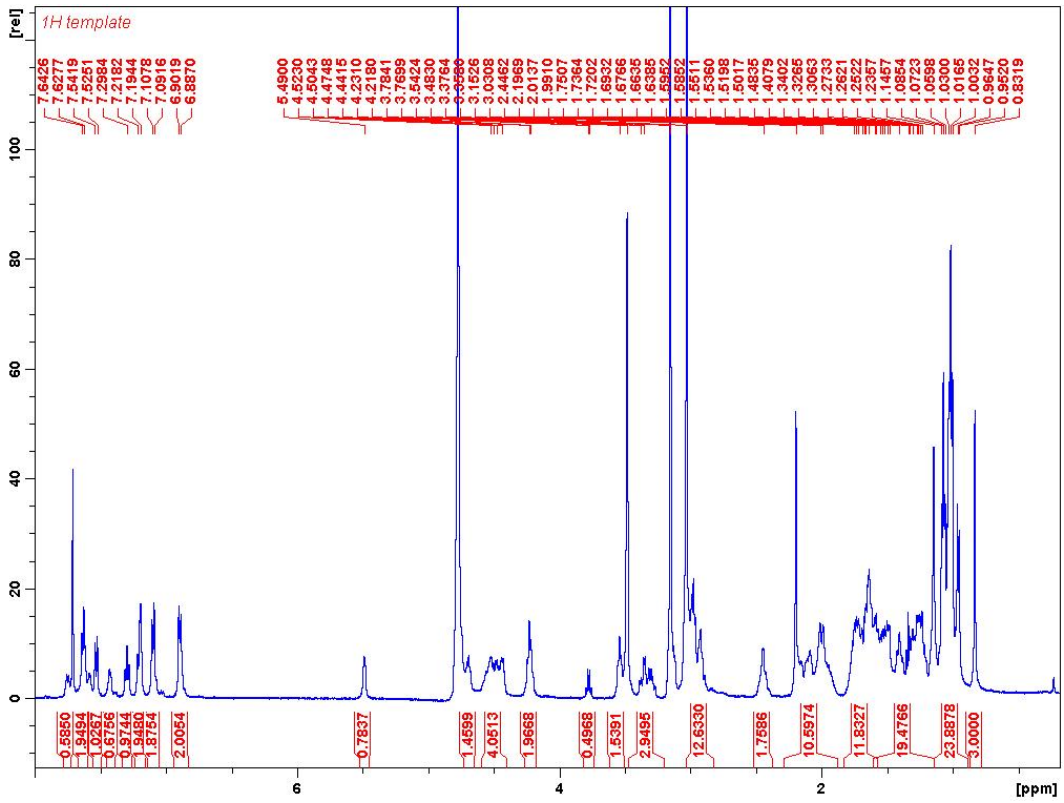


Figure SI-2: ^{1}H NMR spectrum of **Pep$_n$-Chol**

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]

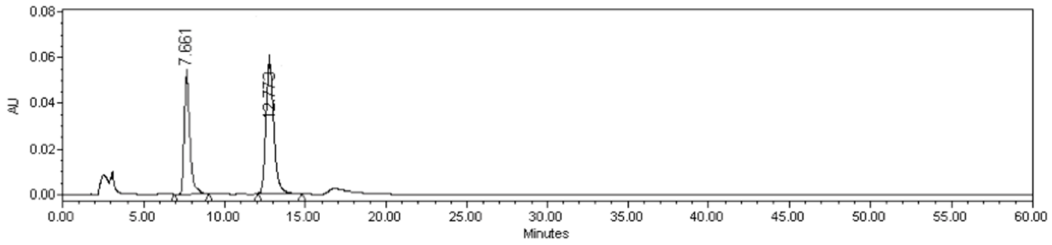


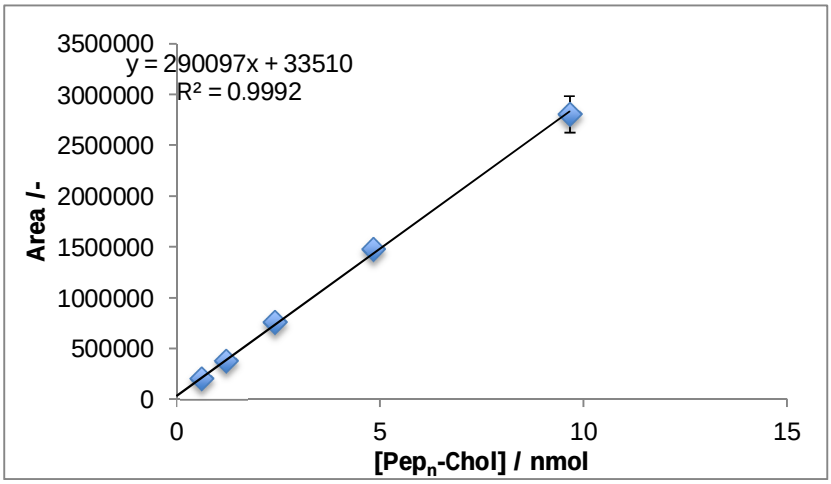
Figure SI-3: A sample chromatogram of **Pep$_n$-Chol** (Retention time: 7.66 min) and **Pep$_n$-Phos** (Retention time: 12.77 min).

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

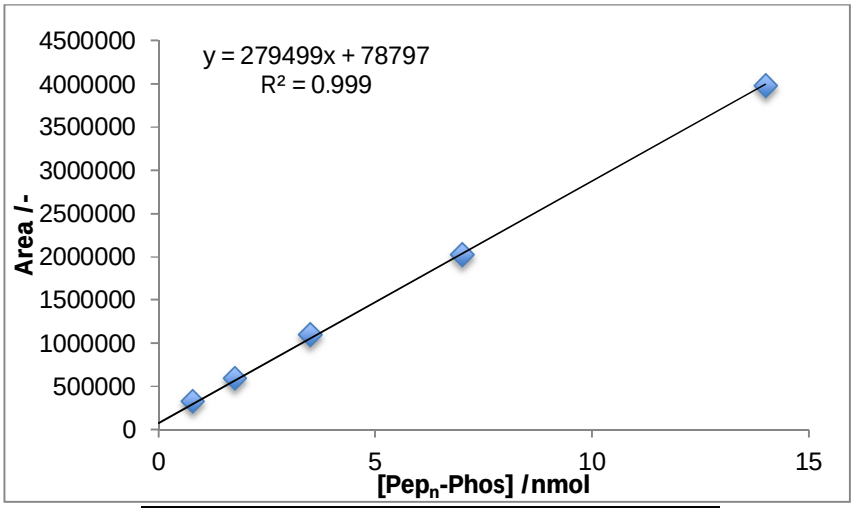
Table SI-2: Data for calibration curves of **Pep_n-Phos.**

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]**

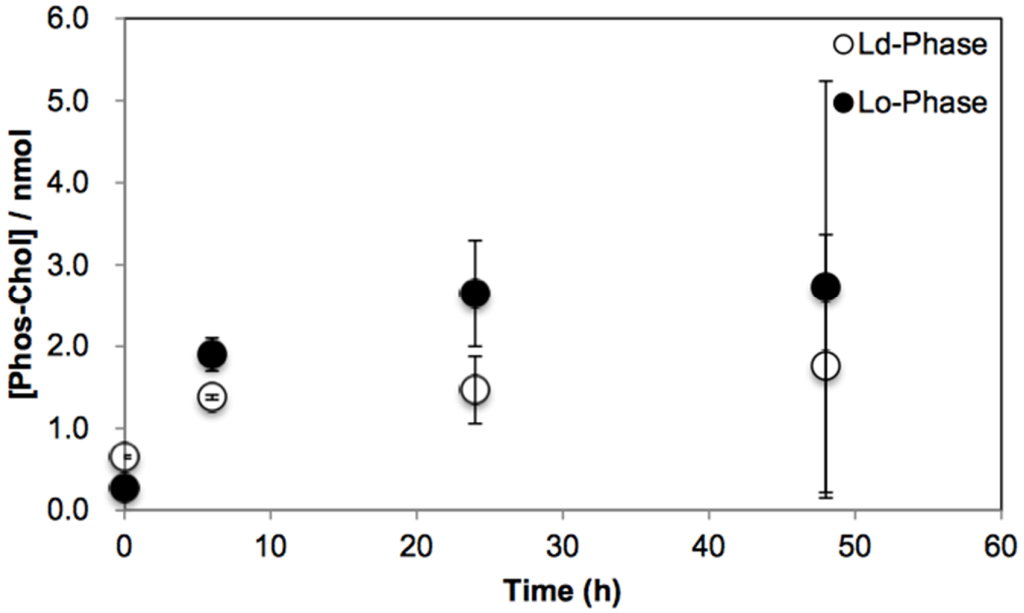


Figure SI-6. Plot of the formation of {Phos-Chol} as a function of time at 45 °C in (●) cholesterol-rich and (○) cholesterol-poor bilayers. Cholesterol-rich vesicles were made from DPPC/cholesterol/Pep_n-Phos/Pep_n-Chol with a molar ratio of 57.5/37.5/1.25/1.25. Cholesterol-poor vesicles were made from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

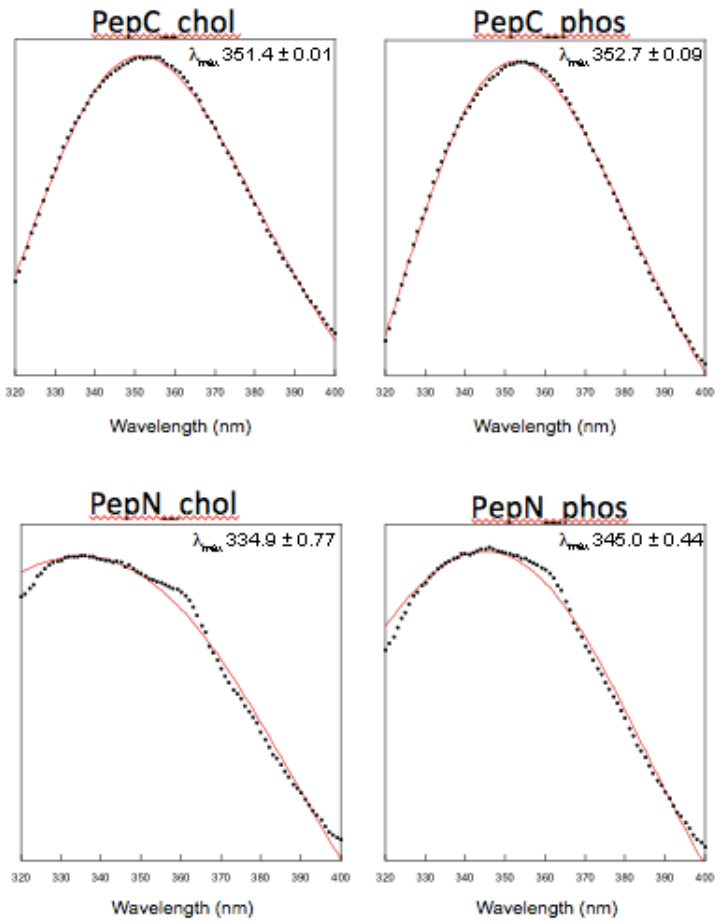


Figure SI-7 Tryptophan emission spectra with excitation at 295 nm of CRAC peptide conjugated compounds in DPPC liposomes at 45°C. Data points are in black and the Fit line is shown in red.

7. References

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