# Peptide Recognition of Cholesterol in Fluid Phospholipid Bilayers

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**S** *Supporting Information*

**ABSTRACT:** *N*-Acetyl-LWYIKC-amide, a cholesterol recognition/interaction amino acid consensus (CRAC) peptide, has been found capable of recognizing an exchangeable form of cholesterol in liquid-disordered ($l_d$) bilayers derived from 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC). In sharp contrast, such recognition is barely detectable in analogous cholesterol-rich, liquid-ordered ($l_0$) bilayers. These findings represent the first evidence for a peptide favoring a sterol as a nearest-neighbor in fluid bilayers. They also reveal that such recognition can be strongly dependent on the degree of compactness of the membrane.

Biological membranes are complex and dynamic assemblies that have two-dimensional structures, which are defined by lipid−lipid, lipid−protein, and protein−protein interactions.[1−3] While extensive studies of lipid−lipid interactions have led to the popular "lipid raft" hypothesis, only a primitive level of understanding currently exists for lipid−protein and protein−protein interactions.[4−9]

One model for lipid−protein interactions that has drawn special attention is based on the cholesterol recognition/interaction amino acid consensus (CRAC) hypothesis.[10,11] According to this model, those segments of a transmembrane protein that lie close the surface of a cell membrane, which conform to the sequence, (L/V)-X$_{1−5}$-(Y)-X$_{1−5}$-(K/R) [where X is one to five of any of the 20 naturally occurring amino acids], are expected to have an affinity toward cholesterol. Despite its popularity, it should be noted that virtually all of the evidence in support of this hypothesis has been circumstantial. No quantitative or even qualitative data currently exist, which shows that a CRAC peptide, or any other peptide, can favor cholesterol over a phospholipid as a nearest-neighbor in the physiologically relevant, fluid bilayer state.

A CRAC peptide that has been of special interest, in this regard, is LWYIK. Specifically, it has been postulated that this minimalistic CRAC motif plays a key role in the fusion protein, gp41, found in HIV-1.[12,13] Previous differential scanning calorimetry measurements of liposomal membranes have shown that *N*-acetyl-LWYIK-amide is more effective in inducing the segregation of cholesterol as compared with certain non-CRAC analogs.[13] Whether such segregation is driven by a favored affinity of this peptide toward cholesterol, however, was not established.

If CRAC segments do, in fact, have a special affinity toward cholesterol, they could serve as viable targets for therapeutic agents. Thus, by altering such interactions, the lateral organization and biological activity of CRAC-bearing membrane proteins should, in principle, be altered.

In the present work, we sought to test the ability of the LWYIK sequence to recognize cholesterol by use of the nearest-neighbor recognition (NNR) method. Previously, we showed that the NNR method could be used to probe the effects of lipidation of a dipeptide (GlyCys) with single chain surfactants on its partitioning between liquid-ordered and liquid-disordered regions of fluid bilayers.[14] As discussed elsewhere, NNR experiments involve exchangeable dimers that contain a bridging disulfide bond.[15,16] When allowed to undergo monomer exchange via thiolate-disulfide interchange, equilibrium mixtures are produced that reflect thermodynamically favored nearest-neighbors. In the present study, we have chosen **Phos**, **Chol**, and **Pep** (derived from *N*-acetyl-LWYIKC-amide) as our exchangeable monomers (Chart 1).

**Chart 1**
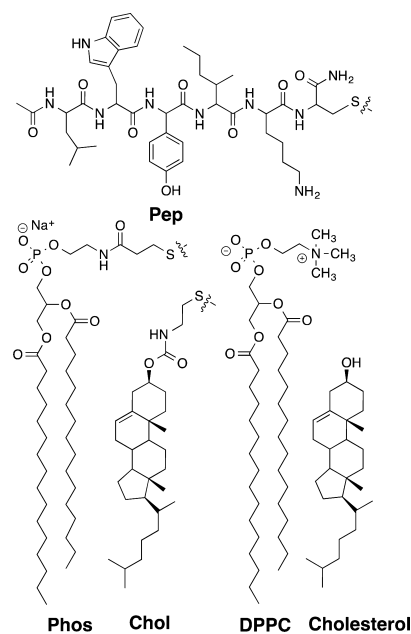


As discussed elsewhere in detail, despite the presence of a net negative charge and a disulfide bridge, **Phos** has proven to be an excellent mimic 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) as well as the corresponding phosphatidylglycerol.[15] Similarly, **Chol** has proven to be an excellent mimic
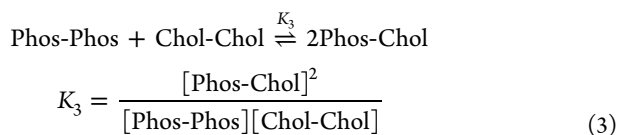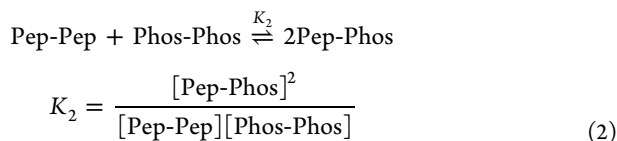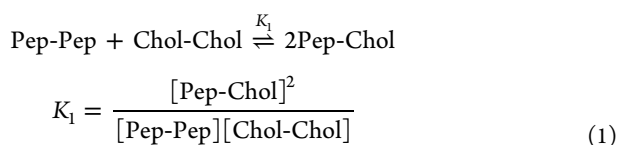
of cholesterol.[15] When three different monomers are employed in an NNR experiment, product mixtures are governed by three independent equilibria.[17] Thus, for membranes containing **Phos**, **Chol**, and **Pep**, the three equilibria are governed by eqs 1, 2, and 3. Here, the ratio $K_1/K_2$ represents a measure of the peptide's preference or selectivity, $S$, in associating with **Chol**. In the present study, we have used host membranes derived from 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and cholesterol to create liquid-disordered ($l_d$) and liquid-ordered ($l_0$) states.[18] The latter is commonly used as a model for "lipid rafts", and the former is commonly used as a model of the surrounding "sea" of phospholipids.

Dimers **Pep-Phos** and **Pep-Chol** were synthesized as shown in Scheme 1. Thus, reaction of **1** and **2** with *N*-acetyl-LWYIKC-

**Scheme 1**
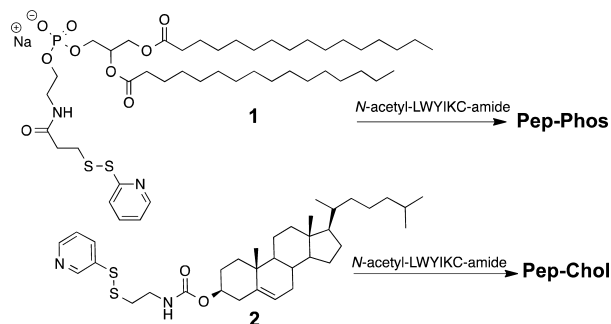
amide afforded **Pep-Phos** and **Pep-Chol**, directly. Methods that were used to prepare **Phos-Chol**, **Phos-Phos**, and **Chol-Chol** were similar to those previously described.[14]

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

$$K_1 = \frac{[\text{Pep-Chol}]^2}{[\text{Pep-Pep}][\text{Chol-Chol}]} \tag{1}$$

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

$$K_2 = \frac{[\text{Pep-Phos}]^2}{[\text{Pep-Pep}][\text{Phos-Phos}]} \tag{2}$$

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

$$K_3 = \frac{[\text{Phos-Chol}]^2}{[\text{Phos-Phos}][\text{Chol-Chol}]} \tag{3}$$

$$S = \frac{K_1}{K_2} = \frac{[\text{Pep-Chol}]^2[\text{Phos-Phos}]}{[\text{Pep-Phos}]^2[\text{Chol-Chol}]} \tag{4}$$

$$2\text{Pep-Phos} + \text{Chol-Chol} \overset{S}{\rightleftharpoons} 2\text{Pep-Chol} + \text{Phos-Phos}$$
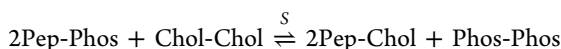
In our NNR experiments, 1/1 mixtures of **Pep-Phos**/**Pep-Chol** were incorporated into host membranes, which were prepared as multilamellar liposomes and maintained a 45 °C, pH 7.4.[14] Using established procedures, thiolate-disulfide interchange reactions were then initiated via partial reduction of the dimers with dithiothreitol.[14] The progress of the exchange was monitored by withdrawing aliquots and analyzing the dimer composition by reversed phase HPLC. In Figure 1 are shown plots of the molar ratio of **Pep-Chol**/**Pep-Phos** and
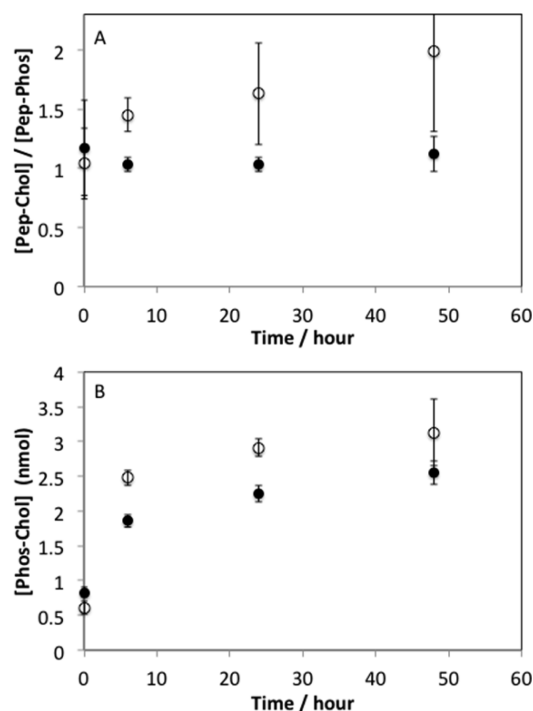
**Figure 1.** Plot of (A) molar ratio of **Pep-Chol**/**Pep-Phos** and (B) the formation of **Phos-Chol** as a function of time at 45 °C in (●) cholesterol-rich and (○) cholesterol-poor bilayers. Cholesterol-rich vesicles were made from DPPC/cholesterol/**Pep-Phos**/**Pep-Chol** with a molar ratio of 57.5/37.5/1.25/1.25. Cholesterol-poor vesicles were made from DPPC/**Pep-Phos**/**Pep-Chol** having a molar ratio of 95.0/1.25/1.25.

the formation of **Phos-Chol** as a function of time, the latter confirming that monomer exchange has, in fact, occurred in the $l_0$ phase. Despite the error in these measurements, cholesterol recognition by the peptide is indicated in the $l_d$ phase (with a **Pep-Phos**/**Pep-Chol** ratio greater than 1) but not in the $l_0$ phase. For the latter, the peptide appears to be evenly distributed between phospholipid and sterol.

Table 1 gives the equilibrium quantities of **Pep-Chol**, **Pep-Phos**, **Phos-Phos**, **Chol-Chol**, **Phos-Chol**, and **Pep-Pep**. Based on these values, the calculated values for $K_1$, $K_2$, and $K_3$ in these two phases are shown in Table 2.

To ensure that these exchange reactions allow for equilibrium states to be reached, we have also carried out an NNR experiment in the $l_d$ phase, starting with the heterodimer, **Phos-Chol**, using an excess of *N*-acetyl-LWYIKC-amide in the buffer. As expected, the $K_3$ value was virtually the same as that found, starting with a 1/1 mixture of **Pep-Phos**/**Pep-Chol**; i.e., $K_3 = 1.55$.

To test for possible contributions due to electrostatic forces, similar NNR reactions were also carried out in 500 mM instead of 150 mM NaCl. In this case, the values of $K_1$, $K_2$, and $K_3$ were found to be sensitive to the salt concentration, but the preference for **Pep** associating with **Chol** is clearly evident in the $l_d$ phase. No significant lipid recognition by the peptide could be detected in the $l_0$ phase. It is also noteworthy that the values of $K_3$ are now similar to what we have previously observed in these same two phases in the absence of peptide.[19]

In preliminary experiments, a series of negative control experiments were also carried out in which LWYIK was replaced with a non-CRAC (**nPep**) peptide sequence, VGVAPG, found in elastin. In this case, no selectivity could

**Table 1. Equilibrium Dimer Distributions**[a]

| Phase | Pep-Chol | Pep-Phos | Phos-Phos | Chol-Chol | Phos-Chol | Pep-Pep |
|-------|----------|----------|-----------|-----------|-----------|---------|
| $l_0$ | 3.39 ± 0.21 | 3.03 ± 0.36 | 1.76 ± 0.40 | 1.06 ± 0.28 | 2.56 ± 0.17 | 4.10 ± 0.28 |
| $l_d$ | 0.78 ± 0.15 | 0.39 ± 0.11 | 3.58 ± 0.75 | 1.96 ± 0.54 | 3.30 ± 0.48 | 7.19 ± 1.34 |

[a]Values listed are in nmol, as determined by HPLC using suitable calibration curves and are averages derived from three independent experiments ±1 SD. Values for **Pep-Pep** were estimated based on the mass balance and are maximum values.

**Table 2. Recognition of Chol by Pep**[a]

| Phase | NaCl (mM) | $K_1$ | $K_2$ | $K_3$ | $R$[b] | $S$ |
|-------|-----------|-------|-------|-------|--------|-----|
| $l_d$ | 150 | 0.043 ± 0.022 | 0.006 ± 0.004 | 1.55 ± 0.70 | 1.99 ± 0.68 | 7.20 ± 5.83 |
| $l_0$ | 150 | 2.64 ± 0.79 | 1.28 ± 0.43 | 3.53 ± 1.31 | 1.12 ± 0.15 | 2.07 ± 0.93 |
| $l_d$ | 500 | 2.08 ± 0.71 | 0.23 ± 0.07 | 3.05 ± 1.15 | 2.50 ± 0.37 | 8.89 ± 4.08 |
| $l_0$ | 500 | 0.21 ± 0.12 | 0.26 ± 0.07 | 7.40 ± 1.55 | 0.95 ± 0.45 | 0.81 ± 0.78 |

[a]Equilibrium dimer distributions that were used to calculate $K_1$, $K_2$, and $K_3$ using 500 mM NaCl are listed in the Supporting Information. [b]**Pep-Chol/Pep-Phos** ratio.

be detected in either the $l_d$ or $l_0$ phase; i.e., the **nPep-Chol/nPep-Phos** ratios were 1.16 ± 1.20 and 1.14 ± 0.86, respectively (Supporting Information).

The present findings are significant because they represent the first evidence for a peptide favoring cholesterol over a phospholipid as a nearest-neighbor in the physiologically relevant, fluid bilayer state. They also indicate that such recognition can be sensitive to the compactness of the membrane. Thus, our findings imply that penetration, or at least partial penetration, of LWYIK into the hydrocarbon interior of the membrane is important for such recognition. In a broader context, NNR measurements of this type provide an opportunity for interrogating CRAC as well as non-CRAC motifs in ways that have not previously been possible. In this regard, it should now be possible to test the veracity of the CRAC hypothesis.

Studies aimed at gaining insight into peptide structure–cholesterol recognition relationships via NNR measurements and molecular dynamics simulations are currently in progress.

## ■ ASSOCIATED CONTENT

### ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b08132.

Methods used for chemical synthesis and carrying out NNR reactions (PDF)

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### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

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