

Probing the complex interaction between the pH-low insertion peptide and membrane bilayers

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Abstract

The pH-low insertion peptide (pHLIP) is a small pH responsive peptide with applications as a cancer therapeutic agent. pHLIP is the C-helix of bacteriorhodopsin (bR), which was found to be soluble in buffer yet interacts with model membranes. Moving forward, advances have been made to further understand the complex interactions that occur between pHLIP and the membrane using a myriad of biophysical techniques. From these studies, it was found that pHLIP interacts with the membrane in three distinct states: soluble in solution at neutral pH, associates with membranes as a random coil at neutral pH, and finally a transmembrane alpha helix at acidic pH. Taking advantage of the pH responsive properties, pHLIP has been utilized as a cancer therapeutic agent.

Although considerable work has been conducted with pHLIP, the precise understanding of the peptide-lipid interactions that occur are not completely understood. The plasma membrane, the primary target of pHLIP, is a diverse entity. Studies have shown that the plasma membrane consists of a multitude of lipid species that are distributed in an asymmetric fashion between the two leaflets. The lipids in the plasma membrane are not only diverse; they are also quite mobile. Lipids within the membrane can move both individually as well as collectively. pHLIP has been studied quite selectively in single component model membranes that do not include the characteristics described above.

Here, we studied how changes in the membrane composition affect the interactions between pHLIP and the lipids in the membrane. Using model membranes, we investigated how a symmetric distribution of a phospholipid with a phosphatidylserine (PS) headgroup influences the insertion of pHLIP. Taking the next step, we further mimicked the plasma membrane by distributing PS in an asymmetric fashion between the two leaflets. We found that both distributions affect the acidity needed for insertion of pHLIP. We also investigated how pHLIP influences

membrane dynamics by using neutron scattering techniques to probe collective membrane dynamics. We found that the inserted state of pHLIP changes collective membranes dynamics. Overall, this work advances our understanding of how pHLIP interacts with the lipid bilayer.

Table of Contents

<i>Chapter I: Introduction</i>	1
1.1 Origins of the pH low insertion peptide	2
1.2 Biophysical characterization of pHLIP.....	3
1.3 Relating pHLIP to its protein of origin, bR.....	7
1.4 pHLIP as a cancer therapeutic agent.....	8
1.5 Comparison of pHLIP to membrane active peptides	9
1.6 Physical properties of a lipid bilayer	11
1.7 The plasma membrane is asymmetric in character	16
1.8 Summary of conducted work	18
<i>Chapter II: Determination of the membrane translocation pK of the pH-low insertion peptide</i>	22
2.1 Abstract.....	24
2.2 Introduction	25
2.3 Materials and Methods	27
2.4 Results	32
2.5 Discussion.....	43
2.6 Acknowledgments	56
<i>Chapter III: The negative charge of the membrane has opposite effects on the membrane entry and exit of pHLIP</i>	57
3.1 Abstract.....	59
3.2 Introduction	60
3.3 Materials and Methods	62
3.4 Results	67
3.5 Discussion.....	70
3.6 Acknowledgements.....	78
<i>Chapter IV: PS membrane asymmetry influences the folding and insertion of a transmembrane helix</i>	79
4.1 Abstract.....	80
4.2 Introduction	81
4.3 Materials and Methods	84
4.4 Results	91
4.5 Discussion.....	101

4.6 Acknowledgments	108
<i>Chapter V: A transmembrane helix reduces the dynamics of membrane thickness fluctuations</i>	<i>109</i>
5.1 Abstract.....	110
5.2 Introduction.....	111
5.3 Materials and Methods.....	114
5.4 Results	124
5.5 Discussion.....	145
5.6 Acknowledgments	152
<i>Chapter VI: Conclusions and future directions</i>	<i>153</i>
6.1 Conclusions	154
6.2 Future Directions	156
<i>References</i>	<i>159</i>
<i>Vita</i>	<i>171</i>

List of Tables

Table 1. Variants of pHLIP.....	5
Table 2. Comparison of pHLIP to AMPs, CCPs, and other pH responsive peptides.	12
Table 3. Entry pK and slope values of the six methods.	36
Table 4. Rate Constants and normalized amplitudes of membrane insertion kinetics.	47
Table 5. The partition coefficient (K_p) values.	68
Table 6. Dynamic parameters obtained from the analysis of the neutron spin echo data from DOPC/DOPS/Chol bilayers in the presence and absence of pHLIP.	129
Table 7. Structural parameters obtained from both the joint and core-multi-shell model analysis of SANS/SAXS scattering data from DOPC/DOPS/Chol bilayers.....	139

List of Figures

Figure 1. The three states of pHLIP.....	5
Figure 2. The different modes of action in which AMPs cause cell death.	10
Figure 3. Chemical structure of the major lipid types found in the plasma membrane of cells.	14
Figure 4. Hierarchy of dynamics in lipid membranes.....	17
Figure 5. Asymmetry of the phospholipids.....	19
Figure 6. CD monitors helical formation during the membrane insertion of pHLIP.....	33
Figure 7. Three different analyses of W fluorescence pH titrations.	36
Figure 8. The pHLIP does not have a unique pK value.	37
Figure 9. Membrane exit of pHLIP yields similar pK_{CM} , pK_{SM} and pK_{FI} values.	38
Figure 10. Overlay of pHLIP membrane entry (black) and exit (grey) transitions analyzed via SM, CM, and FI.	39
Figure 11. NBD conjugates of pHLIP display different pK based on location.	41
Figure 12. NBD-pHLIP conjugate controls.	42
Figure 13. Intrinsic W, N_T -NBD, and C_T -NBD fluorescence display similar kinetics during pHLIP membrane insertion.	44
Figure 14. C_T - and N_T -NBD conjugates show no photo-bleaching at pH 8.	45
Figure 15. Stopped-flow fittings and residuals show that four exponential model agrees with the experimental data.	46
Figure 16. Qualitative comparison of fluorescence pK titrations with ssNMR pK_a titrations for the individual aspartate residues.	51
Figure 17. Tentative model showing the transition from State II to State III during the membrane insertion of pHLIP, highlighting the insertion step/s that each pK value reports on.	52
Figure 18. NBD lipid binding assay.....	68
Figure 19. Membrane entry and exit transitions.	69
Figure 20. Impact of POPS on pK values for pHLIP membrane entry (closed symbols) and exit (open symbols).	69
Figure 21. Reversible formation of the TM helix.	71
Figure 22. Reversibility of pHLIP.	74
Figure 23. Model of membrane entry and exit of pHLIP.	76
Figure 24. Fluorescence intensity characterization of the effect of PS on the membrane entry pK of pHLIP.....	76
Figure 25. Effect of the binding of pHLIP to lipid monolayers.....	77
Figure 26. Cyclodextrin mediated exchange used to generate asymmetric vesicles.	93
Figure 27. Annexin V-568 used to quantify the amount of PS in the outer bilayer leaflet.	94
Figure 28. Comparison of PS levels in the outer bilayer leaflet before (dark gray) and after (light gray) exchange.....	94
Figure 29. PS asymmetry is stable for days post exchange and in the presence of pHLIP.	95
Figure 30. PS membrane asymmetry alters pK_{CM} and pK_{FI}	97
Figure 31. PS membrane asymmetry has no influence on pK_{NBD}	98
Figure 32. PS membrane asymmetry has no substantial influence on the helical formation of pHLIP. ..	100
Figure 33. Influence of PS membrane asymmetry on the insertion of pHLIP.....	104
Figure 34. Inclusion of the charged lipid DOPS ensures unilamellar liposomes.	125
Figure 35. pHLIP responds in a pH dependent fashion in DOPC/DOPS/Chol vesicles.	125
Figure 36. q dependence of the decay rate, Γ , for protiated and tail deuterated samples.	128
Figure 37. Bending fluctuations are not influenced by the presence of pHLIP.....	130

Figure 38. State III pHLIP slows down the thickness fluctuation rate along with an increase in membrane viscosity.	132
Figure 39. pHLIP does not change the amplitude of the thickness fluctuations.	133
Figure 40. pH does not influence the measured thickness fluctuations.	133
Figure 41. The area compressibility modulus (K_A) is not altered by pHLIP.	134
Figure 42. Average bilayer thickness of DOPC/DOPS/Chol vesicles is not significantly different in the presence of transmembrane pHLIP as determined by SANS and SAXS.	136
Figure 43. Joint analysis determines the average bilayer thickness of DOPC/DOPS/Chol vesicles is not significantly different in the presence of State II pHLIP as determined by SANS and SAXS.	137
Figure 44. Core-multi-shell model analysis determines the average bilayer thickness of DOPC/DOPS/Chol vesicles is not largely different in the presence of pHLIP.	138
Figure 45. pHLIP does not change the bilayer structure.	140
Figure 46. State II pHLIP does not change the bilayer structure.	141
Figure 47. Membrane viscosity and fluidity investigated via fluorescent probes is not altered in the presence of pHLIP.	143
Figure 48. DCVJ responds to changes in the phase state of DMPC.	143
Figure 49. Membrane viscosity is influence by the concentration of Chol.	150
Figure 50. State III pHLIP leads to a suppression of membrane thickness fluctuations by way of increasing the membrane viscosity.	151

Chapter I: Introduction

1.1 Origins of the pH low insertion peptide

Membrane active peptides (MAPs) are an extensive group of peptides that are able to inhibit cell growth (1). Typically, MAPs fall into two categories: cell penetrating peptides (CPPs) and antimicrobial peptides (AMPs). However, other peptides have been identified that do not fall into these two categories (1). An additional group of peptides that could be categorized as MAPs are those that show pH responsive properties when interacting with the membrane (2-4). The pH-low insertion peptide (pHLIP) is a distinct member from this category. pHLIP is a pH responsive, 36-amino acid long peptide that contains six acidic residues and a negatively charged C-terminus (4).

Discovery of pHLIP came from refolding studies of bacteriorhodopsin (bR) (5). bR is a protein in *Halobacterium halobium* that functions as a light driven proton pump (6). Furthermore, bR contains a seven-helical structural motif, and its structure is known to atomic resolution (6, 7). bR has therefore been an attractive model protein to study protein folding. The protein can be refolded from a denatured state and still retain the ability to bind to its retinal chromophore and fold to its functional form to pump protons across the membrane (8). Further studies with bR began to investigate how the individual helices could stably fold across the membrane bilayer (9, 10). In these studies, the seven transmembrane (TM) spanning regions of bR were studied as individual peptides to evaluate their ability to form secondary structure within the lipid bilayer (9, 10). Out of the seven helices only one failed to form a stable secondary structure when presented with a lipid membrane (9, 10). The unique peptide was also soluble in solution as a random coil (10). This peptide corresponded to the C-helix of native bR and was termed BR-C (10). Using circular dichroism (CD), BR-C was shown to associate with lipid bilayers at neutral pH as a random coil and insert into lipid bilayers as a transmembrane (TM) α -helix at an acidic pH (10). Using a pH titration and monitoring the environment change of the native Trp residues, BR-C displayed a

midpoint of insertion (pK of insertion) of ~ 6.0 in 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) vesicles (10). This pH response property was associated with protonations of the four Asp and two Glu residues throughout the BR-C sequence (10).

1.2 Biophysical characterization of pHLIP

Moving forward, BR-C was renamed the pH-low insertion peptide (pHLIP) with the intended use as a cancer therapeutic agent but with the addition of a Cys residue for labeling purposes (11). Several additional alterations to the original pHLIP (BR-C) sequence have been made to create many pHLIP variants; however, only two will be of interest here (12) (Table 1). From pHLIP's discovery, a significant body of research has advanced our knowledge of this small pH responsive peptide. Initially pHLIP's ability to deliver cell impermeable cargoes, like anti-cancer drugs, to the cell interior was investigated (11). Later studies began to explore the peptide-lipid interactions that occur when pHLIP interacts with membranes. pHLIP displays solubility in aqueous solvents yet shows sensitivity to pH (4). pH sensitivity can be assayed by using native Trp fluorescence from the two Trp residues present in pHLIP (13, 14). Using a pH titration, pHLIP showed pH sensitivity in 16:0-18:1 PC (POPC) vesicles with a midpoint of insertion (pK of insertion) of ~ 6.0 , comparable to the previous report in DMPC (13). Using CD, the secondary structure of pHLIP was determined at neutral pH in the presence and absence of lipid bilayers as well as at acidic pH (>6) in the presence of lipid bilayers. pHLIP was mostly unstructured at neutral pH whether lipid bilayers were present yet formed an alpha helix at acidic pH in the presence of lipid membranes (4). The pH dependent interaction of pHLIP was thus characterized to include three different states. pHLIP is a soluble peptide in aqueous solution (State I), membrane adsorbed in the presence of phospholipid bilayers at neutral pH (State II), and finally a TM α -helix when the pH of the environment becomes acidic (State III) (Figure 1) (4). pHLIP's formation of the three characteristics states occur through protonations of the acidic residues in its sequence. The

seven acidic residues of pHLIP include four Asp groups, two Glu residues, and the C-terminus (15).

Protonation of two key Asp residues (Asp 14 and 25) was originally thought to be the driving force behind the pH sensitivity of pHLIP, however, further studies have shown that the additional acidic groups in pHLIP are also important (14-17). Protonation of the acidic groups increases the hydrophobicity of the peptide, leading to a favorable free energy of insertion. The energetics of the interaction in States II and III have also been characterized (13). pHLIP associating with the membrane in State II has a free energy change of -7.2 kcal/mol, whereas insertion and formation of State III has a free energy change of -1.8 kcal/mol (Figure 1) (13).

Several critical parameters describing the interaction of pHLIP with model membranes, including additional studies on the pK of insertion, have been elucidated. Determining the pK of insertion is a key parameter in understanding how pHLIP interacts with lipid bilayers. Further pK of insertion studies have looked at the effect of lipid acyl chain length, degree of lipid unsaturation in the acyl chain region, and the inclusion of cholesterol in the lipid bilayer (18). Vesicles prepared with the saturated lipid 14:0-PC had no influence on the pK of insertion compared to POPC (10). However, when sweeping the bilayer thickness by changing the lipid acyl chain composition from 14:1 PC to 22:1 PC, and including levels of cholesterol up to 20%, the pK of insertion varied as a function of chain length and cholesterol content compared to POPC (18). The pK of insertion first decreased from 14:1 PC to 20:1 PC, plateaued, and then increased with 22:1 PC (18). For 14:1-20:1 PC, increasing cholesterol concentrations led to additional decreases in the pK of insertion (18). Cholesterol had the opposite effect on the pK of insertion in 22:1 PC lipid (18). Lipid bilayer properties

Table 1. Variants of pHLIP.

Acidic residues are highlight in red and Trp residues are highlighted in blue.

	Sequence	Molecular Weight (Daltons)
BR-C	GG E QNPIY W ARY A D W LFTTPLLLL D LALLV D A E GT	4051.6
WTpHLIP	AA E QNPIY W ARY A D W LFTTPLLLL D LALLV D A E GT	4079.6
CCpHLIP	AA E QNPIY W ARY A D W LFTTPLLLL D LALLV D A E GT CG	4239.8

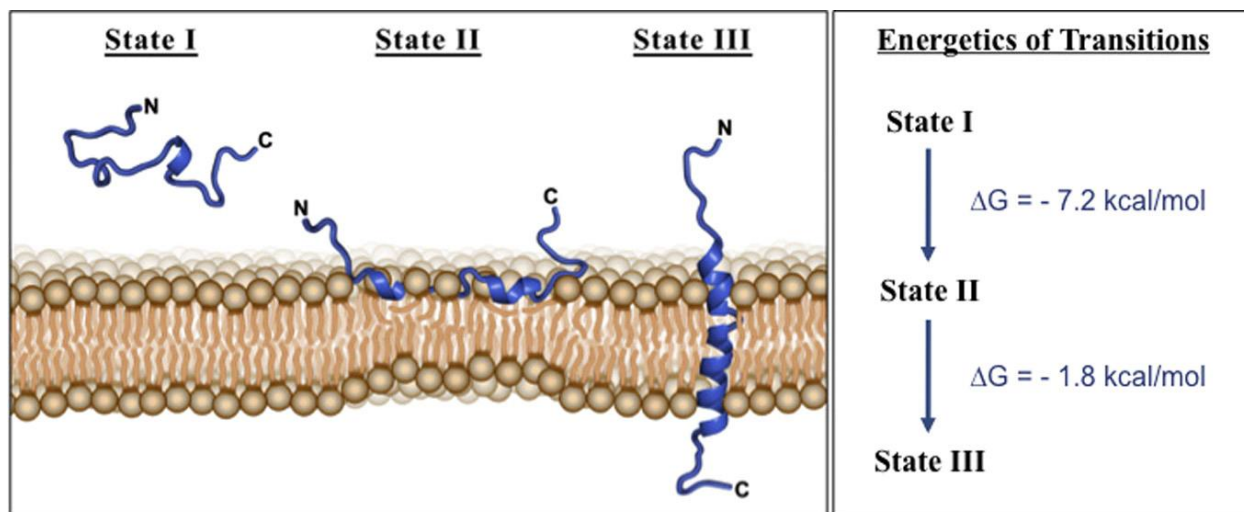


Figure 1. The three states of pHLIP.

State I-In solution at neutral pH, pHLIP is a soluble, monomeric random coil. State II-In the presence of lipid membranes at neutral pH, pHLIP associates with the membrane in a mostly unstructured form. State III- As the pH of the environment becomes acidic, protonations occur in pHLIP leading to formation of an alpha helix and membrane insertion. The negative change in free energy during the transitions to State II and III show that pHLIP association and insertion are favorable interactions. Adapted from (19).

including saturation and acyl chain length can also influence the level of secondary structure pHLIP assumes in both State II and III (18). Increasing the acyl chain length from 14:1 to 22:1 phosphatidylcholine (PC) increased the level of helicity in State II (18). A similar effect was observed for State III; however, the addition of cholesterol also played a role in increasing the level of helicity (18).

The kinetics of membrane insertion have been studied using stopped-flow rapid mixing techniques including fluorescence and CD. In the presence of POPC lipid bilayers, pHLIP transitions from State II to State III via four intermediate steps, most likely involving protonations of acidic residues and folding of pHLIP (20, 21). From the stopped flow studies, it was determined that the insertion of pHLIP happens on the timescale of seconds to minutes (20, 21). During this transition, rapid formation of the alpha helix occurs within milliseconds before burial of the Trp residues, suggesting pHLIP first folds on the membrane surface, then inserts (20, 21).

Mutational studies have determined the importance of the two key acidic residues, Asp 14 and 25, along with Pro, a known helix breaker in proteins (22). The lack of a hydrogen atom on the backbone of the amide group of Pro prevents it to act as a hydrogen bond donor. Therefore, helical segments break when Pro is present (22). The replacement of Pro 20 with Gly led to formation of α -helix irrespective of pH, and membrane insertion occurred over a broad pH range (18). Replacement of either Asp 14 or 25 with Glu shifted the pK of insertion to ~ 6.5 , whereas replacement of both led to peptide aggregation at neutral pH (23, 24). Moving Asp 14 to position 13 decreased the pK of insertion to ~ 5.5 , whereas moving it to position 15 resulted in peptide aggregation (25). The movement of Asp 25 up or down the sequence also caused peptide aggregation (25). Using a dithionite quenching assay with a nitrobenzoxadiazole (NBD) labeled pHLIP, the directionality of insertion was determined. pHLIP unidirectionally crosses the lipid

bilayer with its C-terminus (11). This orientation in the membrane mimics the orientation of the C-helix in bR.

Using both Förster resonance energy transfer (FRET) and analytical ultra-centrifugation (AUC), pHLIP was found to be monomeric in State I, II, and III up to concentrations of 7 μ M (4). Being a monomeric peptide either on the surface or inserted into the membrane, pHLIP was assayed for membrane disruption via a leakage assay using either encapsulated 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and calcein dyes (4, 26). ANTS (427 Da) is a fluorescent dye that is quenched by p-xylene-bis-pyridinium bromide (DPX) (4). Calcein is a small, (623 Da) fluorescent dye that auto quenches its own fluorescence at high concentrations (26). No significant leakage of ANTS or calcein was observed, suggesting pHLIP does not disrupt the lipid bilayer in a significant fashion (4, 26). Furthermore, the ability of pHLIP to facilitate vesicle fusion was assayed by employing FRET (4). It was determined that pHLIP, in POPC model membranes, did not lead to vesicle fusion (4). This further suggests pHLIP does not affect the integrity of the membrane.

1.3 Relating pHLIP to its protein of origin, bR

pHLIP does display similarities with native bR. Mutational analysis of pHLIP determined that Pro 20 is a critical residue in the coil to helix transition pHLIP undergoes as a function of pH (14). pHLIP also showed sensitivity to mutations or repositioning of Asp 14 and 25 (14). Native bR contains several Pro residues within its sequence (27). Mutation of Pro 91 (Pro 20 in pHLIP) to Gly led to slower folding rates, but it did not influence the activity of the protein (27). Mutation of Asp 85 (Asp 14 in pHLIP) in native bR affected the regeneration of the retinal chromophore, leading to an influence of function (6). Changing the membrane thickness via different membrane lipids does not influence the ability of pHLIP to fold into the membrane or for bR to fold, bind retinal, and pump protons across the membrane (18, 28). Taken together, these data show that

pHLIP, just a single TM helix from bR, retains many characteristics common to native bR such as folding and function.

1.4 pHLIP as a cancer therapeutic agent

pHLIP has been assayed in detail to understand its applicability as a cancer therapeutic agent. Studies have included both *in vitro* and *in vivo* assays. Many diseased states including cancer, ischemic stroke, inflammation, and atherosclerotic plaques display local acidosis (29-32). Cancerous cells display local acidosis due to their heightened metabolism and poor blood perfusion within the tumor environment, among other factors (33). Due to its pH responsive property, pHLIP can specifically target cancerous cells; however, during this process, pHLIP will interact with healthy cells. Demonstrated with cell toxicity assays, pHLIP is not toxic to healthy cells (11). When encountering a cancerous cell, pHLIP can either deliver a drug cargo (up ~800 MW) or highlight the perimeter of the cell with a fluorescent label or a label that can be used with positron emission tomography (PET) or single-photon emission computed tomography (SPECT) (19, 34). Using fluorescently labeled pHLIP, several reports have used pHLIP to highlight acidic cancerous tissues (16, 35, 36). One specific example used the fluorescent label Cy5.5 conjugated to pHLIP to accurately highlight a tumor in a mouse model (36). Using this study, an analysis software was created to precisely find the tumor perimeter using a fluorescently labeled pHLIP (36). A multitude of cargoes have been successfully delivered to cells, showing pHLIP's ability to insert membrane impermeable cargoes to inhibit cell growth (11, 37, 38). One key example is the delivery of anti-miR-155 protein nucleic acid (PNA) to a mouse model with large B-cell lymphoma (39). Delivery was successful as inhibition of the tumor was observed in the mouse model showing pHLIP is an efficient cancer therapeutic agent (39).

pHLIP has also been shown to target other diseased states that display local acidosis (40, 41). *In vitro* studies showed that pHLIP specifically interacts with ischemic myocardium tissues

(41). The targeting was due to the pH responsive properties of pHLIP as a pH-insensitive variant, kVar7, was not able to localize to the diseased tissue (41). Moreover, pHLIP selectively localized to inflamed lung tissue in mice infected with influenza (40).

1.5 Comparison of pHLIP to membrane active peptides

Comparing pHLIP to MAPs, one can observe how pHLIP differentiates itself from CPPs and AMPs. Cell penetrating peptides are defined as a group of peptides that contain less than 40 amino acids, mostly being positively charged (42). Their principle mode of action is to translocate the cellular membrane and interact with intra-cellular targets causing cell death (43-45). Their overall positive charge enhances their ability to interact with cellular membranes decorated with negatively charged groups. CPPs can be used to deliver cargoes across the cell membrane such as proteins, peptides, and nanoparticles (45). Two well studied CPPs are *trans*-activator protein (Tat) and penetratin that display characteristic grouping them a CPPs (46, 47).

Antimicrobial peptides are defined as a group of peptides with a similar sequence length as CPPs that have a broad spectrum of activity against a variety of microbes (48, 49). AMPs are typically also positively charged (ranging from +2 to +9) and contain both hydrophilic and hydrophobic surfaces, making them amphipathic (48, 49). Different to CPPs, AMPs disrupt the integrity of the cellular membrane, typically via formation of a membrane pore. This can be achieved by three different modes: the barrel stave, the toroidal pore, or as a detergent (Figure 2) (49-51). A commonly studied AMP is alamethicin. Alamethicin's function is dependent on both the concentration of peptide on the membrane as well as changes in voltage, which lead to the formation of a barrel stave pore (52, 53).

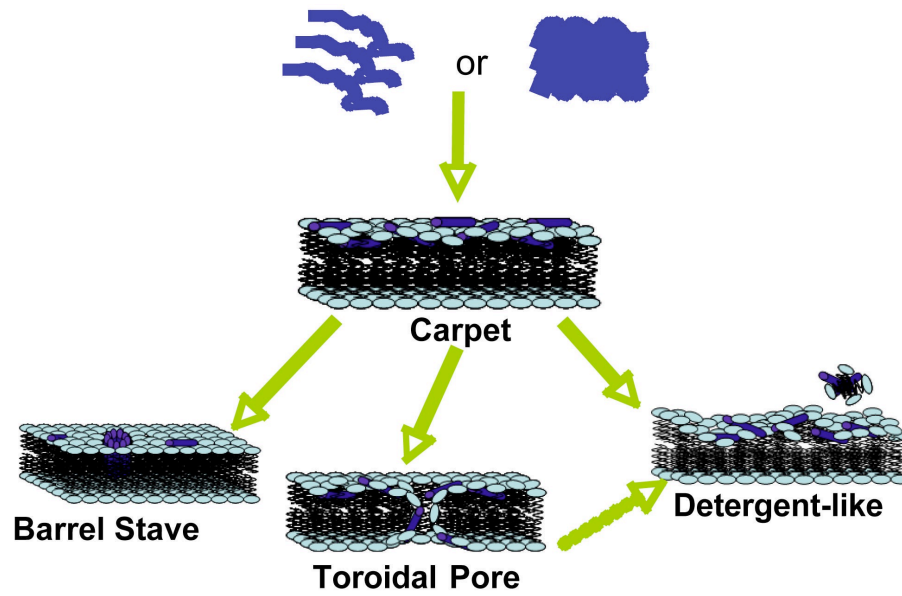


Figure 2. The different modes of action in which AMPs cause cell death.

AMPs are in solution as monomers or aggregates and then bind to cellular membranes to disrupt them. Adapted from (49).

Quite different to alamethicin and CPPs described here are synthetic peptides with pH-responsive properties. GALA, an amphipathic peptide, and the acidity-triggered rational membrane (ATRAM) peptide were designed to interact with membranes in a pH specific manner (2,3,52). GALA's mode of action is to cause bilayer destabilization which leads to apoptosis (2, 54), while ATRAM has displayed the ability to selectively target cancerous tissue with local acidosis *in vitro* (3).

Comparing pHLIP to the peptides mentioned above, one can see that pHLIP is different. Similar to CPPs, pHLIP shares the ability to delivery cargoes to the interior of the cell (16, 45). However, pHLIP includes many negatively charged residues and forms a stable TM α -helix that does not translocate the membrane. Both pHLIP and alamethicin share the similarity of being membrane inserted peptides; however, pHLIP does not disrupt the membrane (4, 52). pHLIP, GALA, and ATRAM all share the pH-responsive property allowing targeting of local acidity of an environment (3, 4, 54). pHLIP differentiates itself from GALA due to substantial leakage of membrane-encapsulated material induced in the presence of GALA and aggregation as part of the membrane insertion process, comparable to effects seen by alamethicin and other AMPs (2, 52, 53). pHLIP and ATRAM are quite similar; however, pHLIP targets more acidic/aggressive tumors due to its lower pK of insertion (3, 4). In the end, pHLIP is a unique peptide that shares characteristics with CPPs and AMPs yet contains its own distinctively diverse characteristics as concluded in Table 2.

1.6 Physical properties of a lipid bilayer

pHLIP's interactions with membranes have been primarily assayed by using phospholipids containing PC headgroups in single component systems. However, the

Table 2. Comparison of pHLIP to AMPs, CCPs, and other pH responsive peptides.

	Membrane Leakage	pH responsive	Overall charge	Cargo Delivery	Stable TM Helix
pHLIP	No	Yes	-	Yes	Yes
GALA	Yes	Yes	-	No	Yes
AMPs	Yes	No	+	No	Yes
CPPs	No	No	+	Yes	No

cellular plasma membrane is a lipid bilayer composed of two leaflets full of diverse lipid groups (55, 56). Three major classes of lipids contribute to biological membranes: phospholipids, cholesterol, and glycolipids (57). Phospholipids are the major class found in the plasma membrane and consist of a hydrophilic head group that is linked to two hydrophobic acyl chains (56, 57). Positioning of the phospholipids in the membrane has the hydrophilic head groups interacting with the aqueous solvent whereas the hydrophobic tails are sequestered away in the core of the membrane. Phospholipids can be classified into two groups that include glycerophospholipids species with different head groups [e.g. PC, phosphatidylserine (PS), and phosphatidylethanolamine (PE)] and phosphosphingolipids [e.g. sphingomyelin (SM)] (Figure 3) (57). PS and PE are the amino-containing glycerophospholipids whereas PC and SM are choline-containing glycerophospholipids and phosphosphingolipids, respectively. PS is the only one to contain a net negatively charged head group, while PE is the only one to cause packing stress within the bilayer due to the area of the headgroup compared to the acyl chains (56, 58). PC lipids contain an zwitterionic head group (58). Phospholipids found in the plasma membrane have diverse acyl chain lengths and degrees of unsaturation in each of the two acyl chains. The change in both the length and the level of saturation controls the phase behavior of the phospholipids. As a function of temperature, lipids can transition from a gel, highly viscous and condensed state, to a fluid, less viscous and condensed, state (58). Cholesterol, a highly rigid lipid composed mostly of ring structures, is found in high percentages in biological membranes and influences the rigidity and thickness of the membrane (Figure 3) (58, 59). Changes in acyl chain length, degree of unsaturation, and inclusion of cholesterol in the membrane also influence the static membrane thickness (60-62). For monounsaturated PC lipids, as the acyl chain length is increased, the static membrane thickness increases (63).

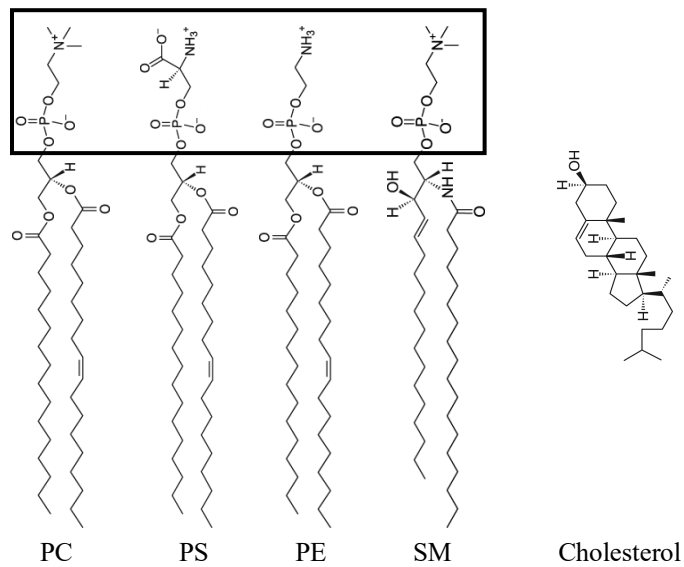


Figure 3. Chemical structure of the major lipid types found in the plasma membrane of cells.

Within the black box, the head groups are highlighted showing differences between them. PS is the only lipid species to contain a negatively charged headgroup. Cholesterol is largely different from the other lipids shown here as it is mostly composed of ring structures and has a short acyl chain.

The lipids that make up the membrane environment are not static but contain a variety of dynamics that include single lipid diffusion up to large scale collective membrane fluctuations (Figure 4) (64). Motions within a single lipid molecule can include rotations about an axis, acyl chain protrusions from the bilayer, and vibrations of atoms within the molecule (58, 65, 66). Within the plane of the bilayer, lipid movement occurs bidirectionally. Lateral diffusion within the plane of fluid bilayers occurs rapidly ($\sim 2 \times 10^{-8}$ cm²/sec) (67). Transbilayer lipid diffusion (also called “flip-flop”) refers to the movement of lipids from one leaflet to the other. Flip refers to outside-in diffusion while flop refers to inside-out diffusion of lipid molecules. Flip-flop occurs at a much slower rate compared to diffusion, with reported halftimes of minutes to hours in model and cellular membranes (68-70). Collectively, lipid membranes display different modes of motion that involve large portions of lipids in the membrane. Two different collective motions will be discussed here. The membrane bending modulus (κ) describes elastic out-of-plane motions that a lipid membrane experiences due to thermal undulations (Figure 4) (71). Thermal undulations of the membrane have been observed to occur at length scales greater than the membrane thickness (72). Bending motions are influenced by changes in membrane composition such as the acyl chain length, the phase state of the individual lipids, and the presence or absence of cholesterol (59, 60). Increasing the length of the acyl chain or decreasing the number of double bonds in the acyl chain increases κ (60). Raising the level of cholesterol in the membrane leads to an increase in κ except in the presence of monounsaturated acyl chains (60). Membrane thickness fluctuations (also referred to as peristaltic motions of the membrane) are described as excess dynamics that occur on top of undulation fluctuations (membrane bending) (64). Thickness fluctuations describe how the two leaflets of the membrane move independently of each other to cause dynamic changes in membrane thickness (Figure 4) (73, 74). Theoretical and experimental work has proposed thickness fluctuations to have a certain rate of motions as well as a length or amplitude of total

movement (73-75). The length scale on which thickness fluctuations occur is comparable to the average membrane thickness (75). This helped to suggest that these excess dynamics were fluctuations in membrane thickness (64). Membrane thickness fluctuations, both the rate and amplitude, have been observed experimentally to be sensitive to changes in the phase state of the lipid as a function of temperature (64, 75, 76). A comparison of three acyl chain saturated phospholipids 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) showed that acyl length had no influence on the rate or amplitude of thickness fluctuations above the melting temperature of the lipid (64). However, once in the gel state, thickness fluctuations are suppressed below the resolution of detection (64).

1.7 The plasma membrane is asymmetric in character

Not only are mammalian plasma membranes composed of many different lipids, studies have shown that the lipids within the membrane are asymmetrically distributed between the two leaflets (56, 77-79). Lipid synthesis primarily occurs in the endoplasmic reticulum membrane (79). During transport through other organelles and incorporation to the plasma membrane, the lipids are redistributed in an asymmetric fashion (56, 79). SM and PC are primarily located in the outer leaflet, while PS and PE are primarily located in the inner leaflet (Figure 5) (56). Cholesterol can be found in both membrane leaflets due to its rapid flip flop rate (half time ~1 sec) but is enriched in the inner leaflet (80, 81). The passive action of phospholipid flip-flop can dissipate asymmetry and is controlled by several mechanisms. Two ATP-dependent transporters can flip or flop the phospholipids back to their respective leaflets. The p-type aminophospholipid translocase selectively flips PE and PS with high head group specificity, and subgroups of the ATP binding cassette proteins flop lipids with low head group specificity (Figure 5)

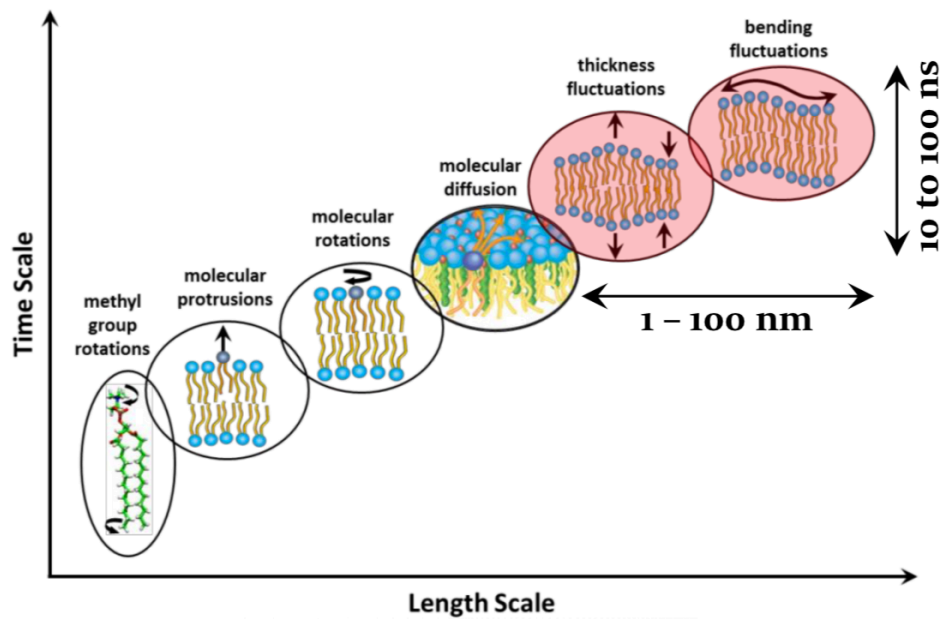


Figure 4. Hierarchy of dynamics in lipid membranes.

Membrane dynamics range from individual molecular motions such as rotation, diffusion, and vibration all the way to collective motions such as bending and thickness fluctuations. Adapted from (82).

(68, 83-86). Since the cell devotes ATP to actively maintain phospholipid asymmetry, it is thought to be an important feature for cellular function and viability (87). Lipid asymmetry can be lost during events such as apoptosis and blood coagulation through the action of the scramblase transporter where scrambling of the lipids occurs within the membrane (Figure 5) (88, 89). Once scrambling of the lipids occurs, PS presence on the outer leaflet of the cell is used as a recognition signal for apoptosis (90). This occurs through the action of Annexin V specifically binding to PS head groups, leading to further recognition and cell destruction (90). However, this property of the plasma membrane is not always a signal for cell death. PS presence in the outer leaflet of cancerous cells is a known occurrence (91). The loss of strict PS localization to the inner leaflet in cancerous cells is thought to be due to different reasons than for healthy cells, such as the generation of reactive oxygen species (ROS) or the hypoxic environment of the tumor (89). Hypoxia and ROS may cause concentration changes of Ca^{2+} , which could lead to the activation of the scramblase lipid transporter or cause oxidative stress on the p-type aminophospholipid translocase leading to its inhibition (89, 92). This altered localization of PS to the outer leaflet should signal apoptotic factors to destroy the cancerous cells; however, cancerous cells upregulate *BCL-2*, an anti-apoptotic regulator, and block the process of apoptosis (93).

1.8 Summary of conducted work

To this point, pHLIP has been described as a well characterized peptide that displays similarities to other MAPs as well as bR, its protein of origin. pHLIP research has uncovered many aspects of this peptide that can be related to cancer therapeutics and also to the general understanding of peptide:lipid interactions. Areas of research yet untouched in the pHLIP field will be introduced and examined to help push our understanding forward on this peptide and its intimate association with lipid bilayers that relate both to its role as a cancer therapeutic agent and

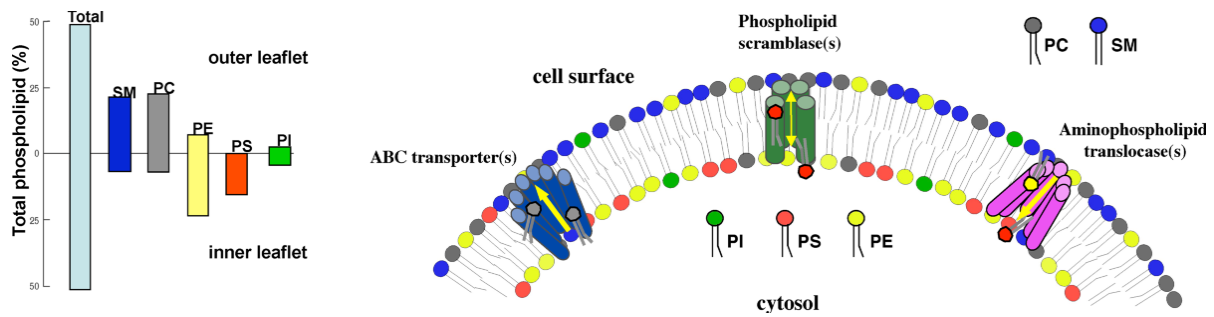


Figure 5. Asymmetry of the phospholipids.

(Left) Asymmetric distribution of the phospholipids as determined in mammalian plasma membranes. (Right) Depiction of lipid flip-flop and the mechanisms that either maintain (ABC transporters or aminophospholipid translocases) or dissipate (phospholipid scramblases) the asymmetry of the phospholipids. Adapted from (94).

a model TM peptide. To contribute to pHLIP's use as a cancer therapeutic agent, I have determined the insertion of pHLIP is adversely influenced by the presence of PS in the membrane and discovered the pK of translocation, the pH at which cargo is delivered. Using pHLIP as a model TM peptide, I have found that membrane PS asymmetry influences the insertion of pHLIP, and that membrane thickness fluctuations are suppressed in the presence of TM pHLIP.

Investigating the pHLIP literature, one will discover that a variety of methods have been used to investigate the pK of membrane insertion (10, 14, 18, 95). One might expect the pK of insertion values obtained from these different techniques to agree, however, that is not the case. Here, we compared the many different methods to determine the pK of insertion and found that each value represents different protonations of acidic residues in the sequence of pHLIP. So far, pHLIP has only been studied in the presence of PC model membranes (4, 10, 18). However, it is well known that the plasma membrane is not solely composed of PC but contains other lipids such as PS. Here, we aimed to understand how PS presence influences the insertion of pHLIP into model membranes. We found that the inclusion of PS leads to a decrease in the membrane insertion pK , causing pHLIP to only insert at more acidic pH values.

Comparable to pHLIP being primarily studied in the presence of PC phospholipids, pHLIP has been studied in only model membrane systems that contain a symmetric distribution of phospholipids (18, 95). The mammalian plasma membrane, the primary target of pHLIP, is not composed of a symmetrically distributed lipids but is known to be asymmetric (56). Here, we find that membrane asymmetry leads to an increase in the membrane insertion pK yet does not influence the folding of the peptide. As previous studies showed, typically pHLIP:lipid interaction studies involve determining the membrane effect on pHLIP. Using pHLIP to understand the effect of membrane proteins on bilayer physical properties has only been superficially investigated (18). Here, we investigate the effect of pHLIP on collective membrane fluctuations. We find that only

pHLIP in the TM state largely suppressed the rate of the thickness fluctuations, however, the amplitude is not influenced.

Overall, the studies here will provide a further understanding of how pHLIP both interacts with and influences lipid membranes. In addition, the results from this work will also contribute to understanding of the complex interactions that occur between peptides and membranes.

Chapter II: Determination of the membrane translocation pK of the pH-low insertion peptide

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Haden L. Scott and Justin M. Westerfield performed the research. Haden L. Scott, Justin M. Westerfield, and Francisco N. Barrera designed the research, analyzed the data, and wrote the manuscript.

2.1 Abstract

The pH-low insertion peptide (pHLIP) is a leading peptide technology to target the extracellular acidosis that characterizes solid tumors. The pHLIP binds to lipid membranes, and responds to acidification by undergoing a coupled folding/membrane insertion process. In the final transmembrane state, the C-terminus of pHLIP gets exposed to the cytoplasm of the target cell, providing a means to translocate membrane-impermeable drug cargoes across the plasma membrane of cancer cells. There exists a need to develop improved pHLIP variants to target tumors with greater efficiency. Characterization of such variants typically relies on determining the pK parameter, the pH midpoint of peptide insertion into the lipid bilayer. Here we report that the value of the pK can be strongly dependent on the method used for its determination. Membrane insertion of pHLIP involves at least four intermediate states, which are believed to be linked to the staggered titration of key acidic residues. We propose that some spectroscopic methods are influenced more heavily by specific membrane folding intermediates, and as a result yield different pK values. To address this potential problem, we have devised an assay to independently monitor the environment of the two termini of pHLIP. This approach provides new insights into the conformation pHLIP adopts immediately prior to the establishment of the transmembrane configuration. Additionally, our data indicate that the membrane translocation of the C-terminus of pHLIP, the folding step more directly relevant to drug delivery, occurs at more acidic pH values than previously considered. Consequently, such a pK difference could have substantial ramifications for assessing the translocation of drug cargoes conjugated to pHLIP.

2.2 Introduction

The pH-low insertion peptide (pHLIP) holds great promise for diagnostic and therapeutic applications in diseases characterized by acidosis. In particular, pHLIP has been shown to target different solid tumor types, and to translocate membrane-impermeable drugs across the plasma membrane into the cytoplasm of cancer cells (16, 19, 39, 96). Based on environmental factors, pHLIP can assume three different stable states: at neutral pH, pHLIP is unstructured in solution (State I), but in the presence of lipid bilayers it binds to the membrane surface (State II). Finally, a drop in pH causes pHLIP to insert across the membrane and fold into a transmembrane helix (State III) (4). The ability of pHLIP to target cancerous cells and deliver drug cargoes across the membrane is due to the extracellular acidity common to solid tumors (33, 96). Cancerous cells have a local extracellular pH roughly 0.5-1.0 pH units lower than healthy cells (pH 6.4-6.8 vs pH 7.2-7.4, respectively (33, 97). At physiological pH conditions pHLIP is believed to remain primarily surface-bound on the outer leaflet of the plasma membrane. Membrane insertion is precluded by the seven negative charges the peptide carries at this pH, consisting mostly of aspartic acid residues (14). However, the extracellular acidity of cancerous cells leads to the protonation of the acidic groups distributed along the sequence of pHLIP. As a result there is a large increase in peptide hydrophobicity that leads to pHLIP finding its energy minimum by inserting into the core of the lipid bilayer as a transmembrane α -helix (5). Membrane insertion is unidirectional, as only the C-terminus of the peptide crosses the bilayer and finds its way into the cytoplasm (4).

The key metric used for studying the membrane insertion of pHLIP is the apparent pK (also referred to as pH_{50} or pH dependence), which has been previously defined as the pH midpoint of peptide insertion into the lipid bilayer (14, 17, 98). The pHLIP contains two tryptophan residues (W9 and W15), which have been commonly used as reporters for the environmental changes occurring during membrane insertion. Such changes in tryptophan fluorescence have been

monitored primarily following fluorescence spectral maximum (SM, also referred to as λ_{max}) changes as a function of pH (4, 14, 18). The p*K* of insertion of pHLIP in POPC large unilamellar vesicles obtained by different laboratories using SM is 6.0-6.2 (4, 14, 17). Circular dichroism (CD) and tryptophan fluorescence intensity (FI) have also been used sparsely to obtain the p*K* of insertion (5, 95). However, a systematic comparison of the apparent p*K* values measured using different methods in identical conditions is lacking. As a result, it is not known if the different methods yield the same p*K* value.

Stopped-flow kinetic measurements have shown that the membrane insertion of pHLIP is not a two-state event. On the contrary, it is a complex process involving at least four intermediate conformations that precede the final transmembrane state (20, 21). The multi-step membrane insertion of pHLIP is expected to be controlled by the titration of seven acidic groups (4 D and 2 E side chains plus the C_t group). Importantly, a recent solid-state nuclear magnetic resonance (ssNMR) study on pHLIP shows that the four aspartic acid residues (D14, D25, D31 and D33) have clearly differentiated p*K*_a values as pHLIP inserts into the bilayer (17). Given the complexity of the membrane insertion, we wondered if using a single p*K* value could adequately describe the process. Furthermore, it has not been proven that this p*K* faithfully informs exclusively on the final membrane translocation step, when drug cargoes are transferred across the lipid membrane into the cytoplasm.

Recently, we have begun to use different analysis methods to further understand the complex insertion process of pHLIP (95). Here, we use six different approaches to measure the apparent p*K* of insertion of pHLIP. These include the development of an assay to independently study the environment of the N- and C- terminus via an extrinsic probe. Intriguingly, the different experimental methods did often yield different p*K* values. We use the recent ssNMR data to

interpret the pK results, and propose that the analysis methods can report on different step/s of the membrane insertion pathway of pHLIP.

2.3 Materials and Methods

Intrinsic tryptophan fluorescence spectroscopy. pHLIP (sequence: N₁-AAEQNPYIYWARYADWLFTTPLLLLDLALLVDADEGTCG-C₁) was synthesized using standard solid phase protocols (P3 Biosystems, Louisville, KY) and purified by reverse-phase high performance liquid chromatography (HPLC) to greater than 95% purity. A lyophilized peptide stock was dissolved in 10 mM sodium phosphate (NaPi) pH 8.0 buffer. 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) vesicles were prepared via extrusion through a 100-nm pore size membrane using a Mini Extruder (Avanti Polar Lipids, Inc., Alabaster, AL) in the same buffer to form large unilamellar vesicles. POPC and pHLIP were then incubated for one hour at room temperature, for a final lipid:peptide molar ratio of 200:1. Final peptide concentration was 1 μ M. Previous studies have shown that the C-terminal cysteine present in pHLIP does not cause disulfide-mediated dimerization under our experimental conditions (14). To perform a pH insertion titration, the pH of the different samples was adjusted by mixing with 100 mM stocks of sodium acetate, MES [2-(*N*-morpholino) ethanesulfonic acid)], or HEPES [(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)] buffers (25 μ L), to obtain the desired pH values. Final sample volume was 140 μ L. To perform a pH exit titration, pre-incubated pHLIP and POPC was acidified to pH 4 using 100 mM sodium acetate. This acidic sample was then mixed with the same buffer series as the insertion titration to obtain the exit titration. The final pH of each sample was measured using a 2.5 mm-bulb pH-electrode (Microelectrodes, Inc., Bedford, NH). Emission spectra were recorded using a Photon Technology International (Edison, NJ) Quanta Master fluorometer at room temperature with the excitation wavelength set to 280 nm, the emission wavelength range from 310 to 400 nm, and excitation and emission slits set to 3 nm. A previous

report has shown minimal Tyr contribution in the observed fluorescence signal with pHLIP for an excitation of 280 nm (20). Lipid blanks were subtracted in all cases. Data were analyzed by calculating the spectral center of mass (CM), with the following equation:

$$CM = \sum_1^n I_i \lambda_i / \sum_1^n I_i \quad (\text{Eq. 1})$$

where I_i is the fluorescence intensity measured at a wavelength λ_i . CM is used to monitor the environment of the two W residues, and information is extracted from the entire spectral range of the data (99, 100). The data were also analyzed by following the fluorescent emission intensity change at 335 nm, which is directly proportional to the population of molecular species present (101). For spectral maximum (SM) determination, curves were fit using a Lorentzian distribution to determine the wavelength of maximum intensity in each curve over the pH titration. CM, SM, and FI pH-titrations were then fitted to determine the pK using:

$$Signal = (F_a + F_b 10^{m(pH-pK)}) / (1 + 10^{m(pH-pK)}) \quad (\text{Eq. 2})$$

where F_a is the acidic baseline, F_b is the basic baseline, m is the slope of the transition, and pK is the midpoint of the curve, and signal is fluorescence or circular dichroism changes. Here the term pK is not being used in its strict sense, but it merely describes the apparent midpoint of the titration, as discussed elsewhere (95).

Circular Dichroism. Measurements were performed on a Jasco (Easton, MD) J-815 spectropolarimeter at 25°C. pHLIP was incubated with POPC vesicles (prepared as described earlier) in 10 mM NaPi pH 8.0 buffer for one hour. Afterwards, the pH was adjusted with 100 mM sodium acetate, MES, or NaPi (62.5 μ L) to a range of desired final pH values. Final sample volume was 250 μ L. The lipid:peptide molar ratio was 200:1 with a final peptide concentration of 7 μ M. Changes in helical content were reported by following the ellipticity at 222 nm. Spectra were

collected from 222 nm to 262 nm in 10 nm steps, and values at 222 nm were subtracted from values at 262 nm to correct for any changes in the baseline of each individual spectrum. By limiting the collected spectral range, the complete pH titration could be conducted in 5 hours while acquiring data with high accuracy (each sample was averaged 20 times using a scan rate of 100 nm/minute). Calculated molar ellipticity ($[\theta] = \theta/(10lcN)$) at 222 nm was plotted against measured pH, and the resulting sigmoidal transition was fitted using Eq. 2 to obtain the pK_{CD} .

NBD fluorescence assay. We conjugated the environmentally-sensitive dye NBD to the C-terminal cysteine in pHLIP using IANBD [N, N'-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine] (Thermo Fisher Scientific, Inc., Waltham, MA). For the conjugation of NBD to the N-terminus of pHLIP, we used instead NBD-X SE [succinimidyl 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino) hexanoate] (Anaspec, Inc., Fremont, CA). To this end, a pHLIP variant lacking a cysteine residue (N-AAEQNPYIYWARYADWLFTTPLLDDALLVDADEGT-C_t) was employed. After purification by size exclusion chromatography using a PD-10 column (GE Healthcare Bio-Sciences, Marlborough, MA) in 1 mM NaP_i buffer, pH 7.5, the conjugation was verified by MALDI-TOF and HPLC, and samples were aliquoted and lyophilized. For the experiment, samples were rehydrated in 10 mM NaP_i pH 8.0 at a final concentration of 0.8 μ M (the NBD extinction coefficient was 18,482 M⁻¹ cm⁻¹) and incubated with POPC vesicles (prepared as described earlier) for one hour. All experimental conditions, including lipid:peptide molar ratio and final salt concentration (19.3 mM), were identical to W fluorescence and CD experiments. The pH of each row of a 96-well plate was decreased using a series of 100 mM buffers (sodium acetate and NaP_i (14.3 μ L)). Final sample volume was 100 μ L. Fluorescence spectra were recorded at 25°C with excitation at 470 nm and an emission range of 520-600 nm using a Cytation 5 imaging plate reader (Biotek Instruments, Inc., Winooski, VT). The final pH of each well was measured. Fluorescence

emission intensity pH-titrations at 540 nm were then fitted to determine the $pK_{\text{Ct-NBD}}$ and $pK_{\text{Nt-NBD}}$ using Eq. 2.

Stopped-flow kinetic measurements. Lyophilized pHLIP and its two NBD conjugates were rehydrated in 10 mM NaPi pH 8.0 and incubated with POPC vesicles (prepared as described above) for one hour at a lipid:peptide molar ratio of 200:1. Peptide concentration in the incubation was 5 μM in the case of pHLIP and 0.2 μM in the case of the NBD conjugates. Final salt concentration was 55 mM for both pHLIP and both NBD conjugates. Spectral measurements were collected on a SX20 Stopped-Flow instrument with temperature control (Applied Photophysics, Inc., Surrey, United Kingdom). Pre-incubated peptide lipid samples (2 mL) at pH 8 were mixed with 100 mM sodium acetate pH 3.9 to obtain State III. In the case of NBD conjugates, pre-incubated samples were also run at pH 8 by mixing with 100 mM NaPi pH 8. For pHLIP, peptide only blanks were collected by mixing pHLIP with 100 mM NaPi pH 8, and the final pH was measured afterwards. The mixing volume was 120 μL at a 1:1 ratio with a dead time of ~ 1 ms. NBD conjugates were excited at 488 nm and fluorescence was collected through a 505 nm cut-off filter (excitation at 280 nm with a cutoff filter of 320 nm in the case of pHLIP fluorescence). After mixing, the concentration of the NBD conjugates was 0.1 μM , and the final pHLIP concentration was 2.5 μM . Data were collected over a 50 second interval with 4000 data points, repeated 30 times and averaged. The first 100 ms were not included in the fitting, since a small signal increase was observed in the controls in this time range. Slits were set to 2 nm. Gain was automatically set to the acidic sample containing inserted peptide. The first five injections were always discarded to ensure the previous sample was flushed out of the sample cell. Temperature was held at 25°C. POPC blanks were also collected by mixing POPC vesicles with 100 mM NaPi pH 8. For NBD conjugates, data for State II were subtracted from data for State III to correct for any potential photo-bleaching and light scatter from the vesicles. For W intensity experiments, pHLIP in

solution was subtracted from State III data. Subtracted data were analyzed as in (21), using the following equation:

$$\text{Signal} = A1 \times \exp(-k1 \times x) + A2 \times \exp(-k2 \times x) + A3 \times \exp(-k3 \times x) + A4 \times \exp(-k4 \times x) + F_o \text{ (Eq. 3)}$$

where A is the amplitude of the signal (fluorescence intensity), k is the rate constant in sec^{-1} , x is time, and F_o is the vertical offset. As described elsewhere (20), a four exponential model was required to adequately fit the data, due to the inability to find good agreement between the theoretical and experimental curves with less than four exponentials. Amplitudes were normalized to a total intensity range by summing the values only for the positive amplitude change in fluorescence.

Statistical analysis. Statistical analysis was performed on the pK values obtained to determine if a statistical difference existed. Analysis was performed using the SPSSv24 software (IBM Analytics, Armonk, NY). Two different multiple comparisons tests were used based on the homoscedasticity (2-sided Dunnett t-test) or heteroscedasticity (Dunnett T3) of the data. Comparison of all membrane entry pK values to SM were analyzed using a Dunnett T3 test. Comparison of C_i-NBD to FI and N_i-NBD pK was performed using a 2-sided Dunnett t-test. All membrane exit pK values were compared to SM with a 2-sided Dunnett's t-test. Both the 2-sided Dunnett t-test and the Dunnett T3 set one variable as the control to compare to all other treatment groups. $P \leq 0.05$ was considered significant for all tests.

2.4 Results

Circular Dichroism pK as a tool to monitor helical formation as a function of the membrane

insertion of pHLIP. Stopped-flow circular dichroism (CD) kinetic experiments of pHLIP show that upon acidification, helix formation starts at the membrane surface before membrane insertion, as reported by intrinsic tryptophan fluorescence intensity changes (20). The initial helix formation is followed by a multi-step insertion process resulting in the establishment of the final transmembrane state (20, 21). Figure 6A shows the CD spectra of pHLIP in the presence of POPC vesicles at pH 8 (State II, black trace), which corresponds to the largely unstructured conformation (with a minimum at ~ 200 nm) found before insertion starts. The conformational change leading to the transmembrane state (State III, gray trace) can be quantified by following the ellipticity change at 222 nm, the larger of the two minima characteristic of an alpha helix (102). We performed a titration between pH 4 and 8 and the change in ellipticity at 222 nm was monitored to determine the pK of the helical changes of pHLIP (Figure 6B), which is referred to thereafter as pK_{CD} , with a value of 6.03 ± 0.05 . The use of CD to determine the insertion pK allows us to characterize the ensemble of steps of helix formation occurring at the central part of the sequence. However, based on the previous stopped-flow data we reasoned that analyzing the pH-induced changes in the local environments of the two W residues could report on different steps of the insertion process.

Intrinsic tryptophan fluorescence reports changes in the local environment of the W residues

during membrane insertion. Tryptophan fluorescence is the method most commonly used to monitor the insertion of pHLIP into the hydrophobic core of membranes. The location of W residues is easily discernable between hydrophobic and hydrophilic environments due to the high sensitivity of the W fluorescence emission dipole to polarity (103). For this reason, environmental information can be extracted from the emission wavelength shift of the combined spectrum of residues W9 and W15 as the membrane insertion of pHLIP occurs. Specifically, analysis of the

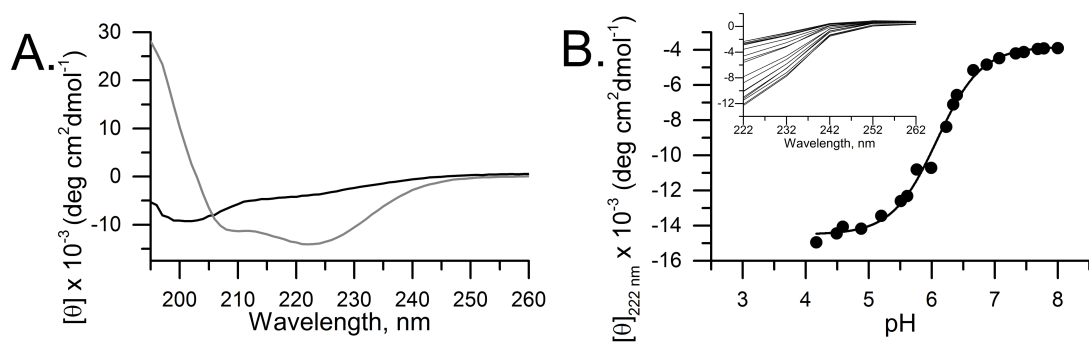


Figure 6. CD monitors helical formation during the membrane insertion of pHLIP.

(A) CD spectra showing the membrane surface-bound State II (black trace) and the inserted transmembrane State III (gray trace) of pHLIP in POPC vesicles. (B) The difference in molar ellipticity ($[\theta]$) between 222 nm and 262 nm was plotted against the pH to determine pK_{CD} , the midpoint of helical formation. Inset contains the titration CD spectra, showing that increasing acidity leads to α -helix formation, and thus more negative molar ellipticity at 222 nm.

emission spectrum shift used to obtain an apparent pK of insertion can be performed using a dual approach: calculating the fluorescence center of mass (CM) and the spectral maximum (SM), with SM being used to a greater extent (4, 14, 18, 95). The spectral CM provides wavelength-averaged information from the entire spectrum and is sensitive to changes in the shape and width of the emission peak (99, 104). On the other hand, fluorescence SM only reports changes in the spectral maximum, and is determined by fitting the spectral curve with a Lorentzian distribution, to obtain the peak emission wavelength. We also employed to obtain the apparent pK a third analysis method that has seen limited use, which consists of monitoring changes in the W fluorescence emission intensity (FI) (5, 95). A single wavelength, 335 nm, was chosen since it shows a large fluorescence intensity change from State II to State III.

We performed titrations of pHLIP in the presence of POPC vesicles and analyzed the fluorescence data using the three methods described above (Figure 7) and determined three pK values (Figure 8 and Table 3). Figure 7 shows the W fluorescence spectra and the sigmoidal titrations obtained with CM, SM, and FI analysis, which were used to determine pK_{CM} , pK_{SM} and pK_{FI} , respectively. Interestingly, whereas CM and SM display a similar pK of insertion (6.07 ± 0.08 and 6.22 ± 0.17 , respectively), the pK obtained monitoring the FI was ~ 0.5 pH units lower than both CM and SM. Correspondingly, the pH range where the titration occurred also differed when comparing FI to both CM and SM (arrows mark the approximate titration pH range in Figure 7B-D), in agreement with the sigmoidal FI curve being shifted to a lower pH range. Determination of the pK_{FI} was not dependent on the wavelength employed for the analysis (data not shown). Interestingly, the values of pK_{CM} and pK_{SM} overlap with pK_{CD} , with a value around ~ 6.1 , suggesting that these three parameters report on similar events. The different pK values obtained are compared in Figure 8.

The membrane insertion of pHLIP is a reversible process (20). We determined next whether the differences observed between the membrane entry pK values obtained with FI and the other methods were also found in the membrane exit. Accordingly, fluorescence experiments were performed to characterize the membrane exit. This process consists on the transition from the transmembrane State III to State II, obtained when pH is increased starting from the acidic endpoint of the membrane insertion transition (Figure 9A) (20, 95). The membrane exit titrations of pHLIP in the presence of POPC vesicles were analyzed via CM, SM, and FI changes (Figure 10). We observed that the pK_{SM} obtained for the membrane insertion and exit were close (6.22 ± 0.17 and 6.29 ± 0.08 , respectively). Similar results were also obtained for pK_{CM} (6.07 ± 0.08 and 6.21 ± 0.06 , respectively). However, the entry and exit transitions did not have the same midpoint when quantified using pK_{FI} . In fact, a clear hysteresis was observed in the FI data, since the membrane entry value was 5.68 ± 0.09 , while the membrane exit value was 6.15 ± 0.18 (Figure 10). Figure 9B illustrates that, in contrast to the membrane entry, the three analysis methods yielded similar exit pK values, with differences that were not statistically significant ($P > 0.05$), in contrast to the lower pK_{FI} observed for membrane insertion (Figure 4). The implications of this difference will be examined in the Discussion section.

There are at least two intrinsic caveats of employing W fluorescence to study pHLIP. One is the location of both W residues near the N-terminus. Since W fluorescence is sensitive to the local environment (103), this assay might have low sensitivity to the membrane translocation of the C-terminus of the peptide. The second limitation is that the W fluorescence reports on two local environments, those of W9 and W15, which are different, for example by having different titratable groups in their vicinities. To overcome these limitations, we decided to employ an extrinsic probe conjugated to either end of pHLIP.

Table 3. Entry pK and slope values of the six methods.

	CM	SM	FI	CD	N _t -NBD	C _t -NBD
pK	6.07 ± 0.08	6.22 ± 0.17	5.68 ± 0.09	6.03 ± 0.05	5.64 ± 0.06	5.24 ± 0.17
Slope						
(m)	1.55 ± 0.50	2.16 ± 0.74	2.34 ± 0.38	1.4 ± 0.17	4.65 ± 0.63	1.38 ± 0.35

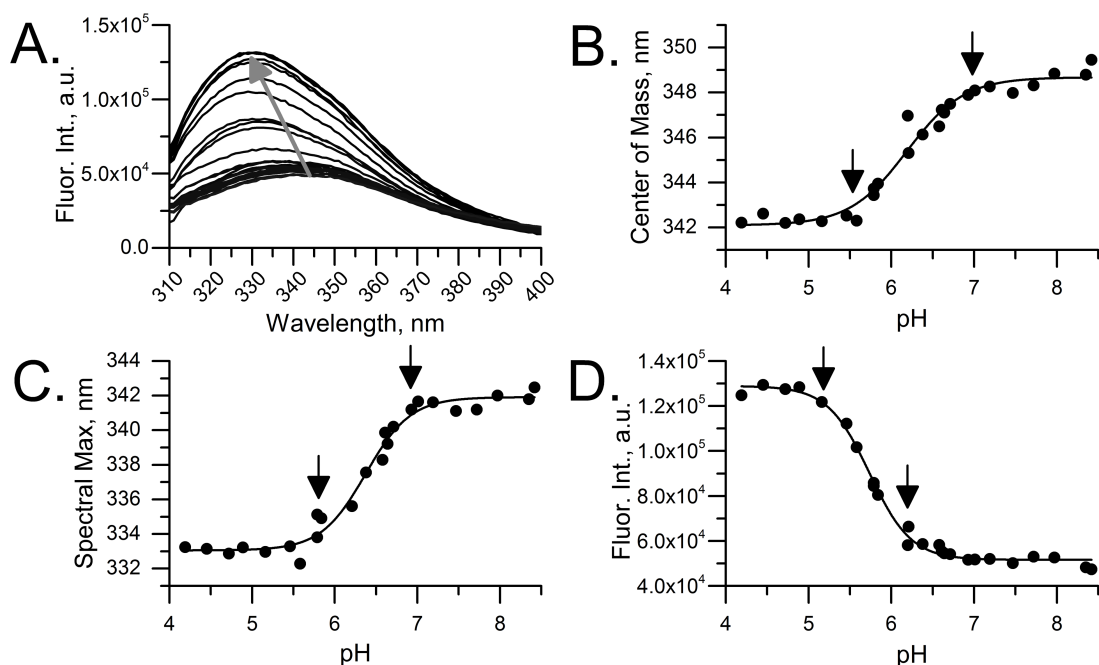


Figure 7. Three different analyses of W fluorescence pH titrations.

(A) Sample acidification (gray arrow) results in an increase in W fluorescence intensity and spectral blue shift. A similar pK was obtained by following changes in CM (B) and SM (C) upon acidification. (D) W intensity changes were also analyzed by following the fluorescence emission intensity (FI) at 335 nm. Lines in B-D show the fits of Eq. 2 to the data. Black arrows mark the approximate pH values for the start and end of the titrations.

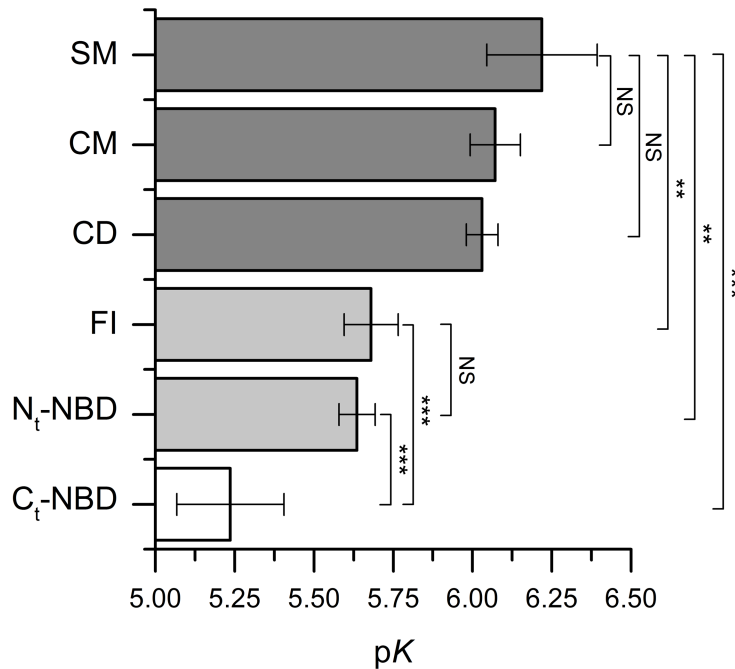


Figure 8. The pHIP does not have a unique pK value.

Bars correspond to the pK determined by W fluorescence spectral maximum (SM), spectral center of mass (CM), or intensity (FI), by circular dichroism (CD), or by NBD conjugated to the N-terminus (N_t-NBD) or a C-terminal C residue (C_t-NBD) of pHIP. Mean values are shown, for experiments repeated 3-6 times, and error bars are the standard deviation. Color shading represents groups with no statistical difference. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$; NS; no significance.

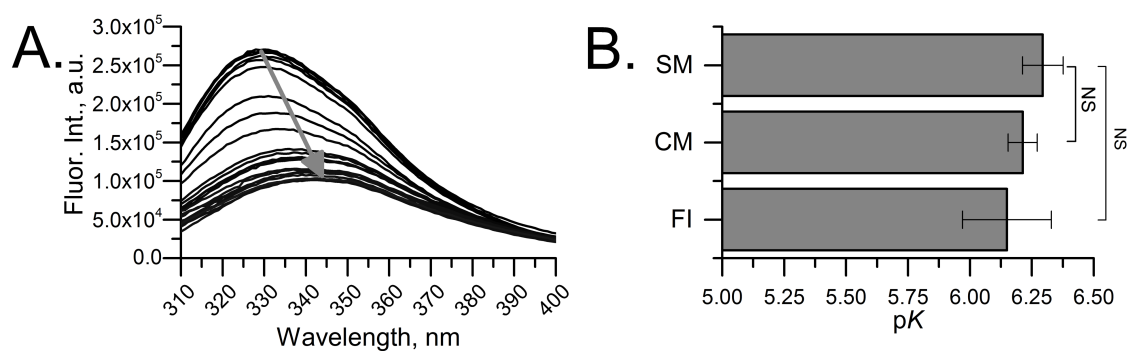


Figure 9. Membrane exit of pHLIP yields similar pK_{CM} , pK_{SM} and pK_{FI} values.

(A) The increase in pH (gray arrow) results in a decrease in the tryptophan fluorescence intensity and a red shift in spectral maximum in the emission spectra. (B) The three membrane exit pK values determined by W fluorescence (CM, SM, and FI) are not statistically significant ($P > 0.05$). NS; no significance. Error bars are the standard deviation.

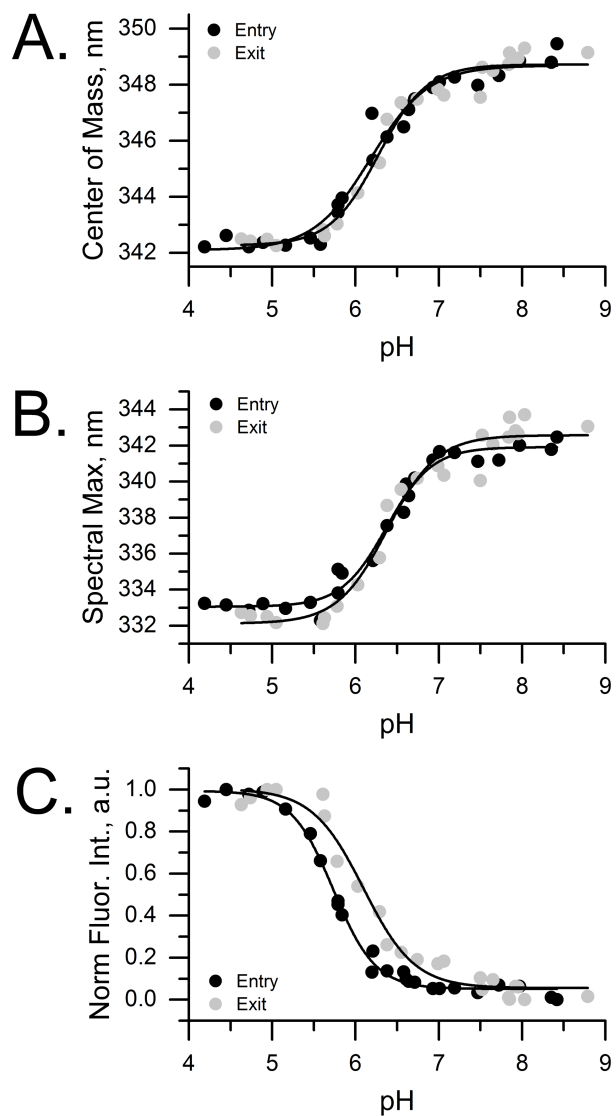


Figure 10. Overlay of pHLIP membrane entry (black) and exit (grey) transitions analyzed via SM, CM, and FI.

(A-B) Membrane entry and exit titrations analyzed via SM and CM show overlap. (C) The membrane entry and exit titrations analyzed via FI display hysteresis, observed as incomplete reversibility between entry and exit.

Use of site-specific NBD labeling to monitor each terminus. We next conjugated the NBD dye to either the N-terminal amino group of pHLIP, or to a cysteine residue located close to the C-terminus, the end that translocates across the bilayer. Our aim was to obtain pK values that report on the local environment of each terminus of pHLIP. NBD is a pH-insensitive probe that responds to changes in hydration altering its fluorescence emission intensity (105-109). We performed pH titrations in the presence of POPC vesicles with the NBD conjugated to a C-terminal cysteine residue of pHLIP (pHLIP-NBD) or to the N-terminus (NBD-pHLIP) (Figure 11). We observed that the insertion pK of NBD-pHLIP (pK_{Nt-NBD}) was 5.64 ± 0.06 . This value was similar to pK_{FI} , which reports on changes of the W residues located at the N-terminal half of pHLIP. Strikingly, the pK of pHLIP-NBD (pK_{Ct-NBD}) was 5.24 ± 0.17 , significantly more acidic than any other pHLIP pK values reported here (Figures 8 and 11C) or elsewhere. As a control, fluorescence spectra of the two NBD conjugates of pHLIP were collected in buffer (Figure 12). No significant shift in fluorescence SM was observed between the conjugates. This suggests that the different neighboring residues of NBD present at either end of pHLIP do not significantly influence the NBD fluorescence. As a result, we propose that the NBD signal is not influenced by local effects but is primarily reporting on global conformational changes of pHLIP. As a result, our data suggest that the translocation of the C-terminus occurs at more acidic pH values than initially considered.

Next we performed stopped-flow kinetic experiment in the presence of POPC vesicles that monitored W, N_t -NBD, and C_t -NBD fluorescence intensity changes. The objective of this experiment was two-fold. It served as a control to ensure that the conjugation of the NBD dye to either end of pHLIP did not alter the membrane insertion mechanism. However, more importantly, it allowed gaining new mechanistic understanding of the membrane insertion process. Rapid mixing of acidic buffer with pre-incubated peptide-lipid samples led to the transition from

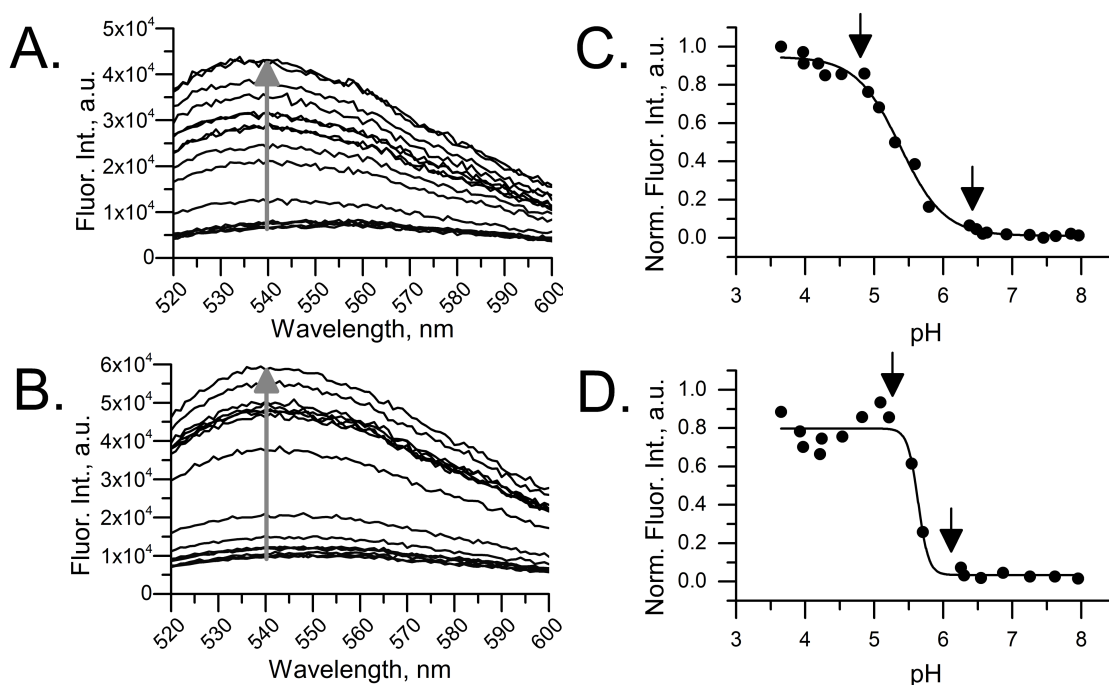


Figure 11. NBD conjugates of pHLIP display different pK based on location.

(A-B) Representative emission spectra of pHLIP-NBD (A) and NBD-pHLIP (B) showing an increase in fluorescence intensity as acidity is increased (gray arrows). (C-D) Normalized fluorescence intensity at 540 nm plotted versus pH to determine $pK_{\text{Ct-NBD}}$ (C) and $pK_{\text{Nt-NBD}}$ (D). Lines in C and D show the fits of Eq. 2 to the data. Black arrows mark the approximate pH values for the start and end of the titrations.

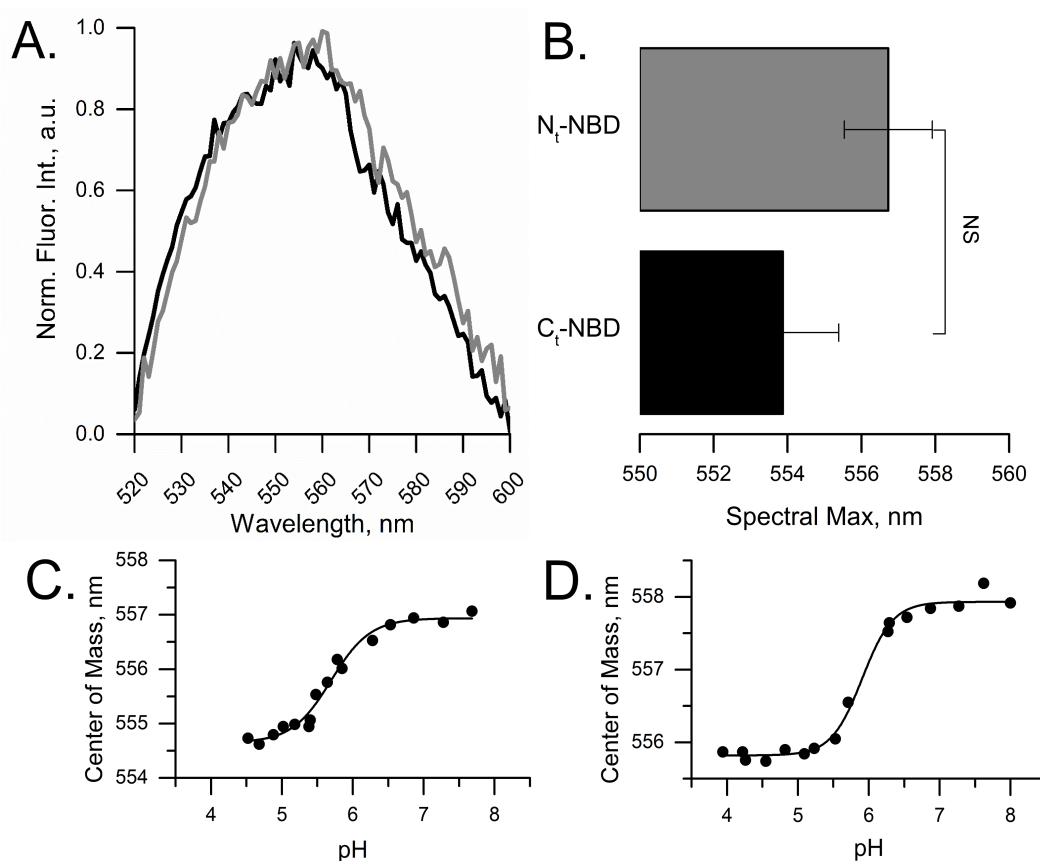


Figure 12. NBD-pHLIP conjugate controls.

(A) The spectral maxima of the two NBD conjugates of pHLIP were measured in buffer to study the level of solvent accessibility of NBD at each terminus of pHLIP. Experiments were conducted as described in the NBD fluorescence method paragraph, but in the absence of POPC vesicles. (B) The data show no statistical difference ($P=0.11$) between the different linkage locations of NBD based on an independent sample t -test with unequal variances (NS; no significance). CM analysis was used to determine the pK of insertion in the presence of POPC vesicles for (C) C_t-NBD (5.64 ± 0.06) and (D) N_t-NBD (5.99 ± 0.13). A lower pK of insertion value was observed when determined by CM compared to FI. The difference is attributed to the non-linearity of the CM determination method.

State II to State III (Figure 13). As reported previously, W fluorescence initially increased and then saturated (20, 21). Interestingly, while both NBD conjugates also showed an initial signal increase, it was followed by a significant signal decrease (Figure 13). We do not suspect the decrease to result from photo-bleaching as control experiments showed that the signal was essentially flat at pH 8 (Figure 14). Kinetic traces for W, N_T-NBD, and C_T-NBD changes were fitted with four exponential terms (fitting residuals can be found in Figure 15). Table 4 shows that our control experiment monitoring W changes yielded results similar to previous reports (20, 21). Importantly, we obtained overall similar rate constant values for both NBD conjugates of pHLIP, suggesting that the presence of the dye does not have a significant effect on the insertion kinetics.

2.5 Discussion

The pK of insertion is often the only parameter used to describe the pH-responsiveness of peptides developed for targeting acidosis (110-114). Thus, progress in the field can be hindered if the molecular event/s the pK reports on is/are not solidly defined. Here we perform a systematic analysis that directly compares the pH dependence of pHLIP obtained employing several methods in identical conditions, including a new approach that employs an extrinsic fluorescent probe. Our data suggest that there is not a single pK value for pHLIP. On the contrary, significantly different values were obtained when employing an array of common measurements. Furthermore, we report that the use of extrinsic probes can be used to determine the pH range where specific regions of pHLIP undergo an environmental change during the membrane insertion process. Figure 8 shows the different pK values obtained employing the six different spectroscopic approaches. The pK values can be classified in three groups, shown with different color shading. The highest value corresponds to pK_{CM} , pK_{SM} and pK_{CD} . These three approaches yield values whose differences are not statistically significant ($P > 0.05$), with an average pK of ~6.1.

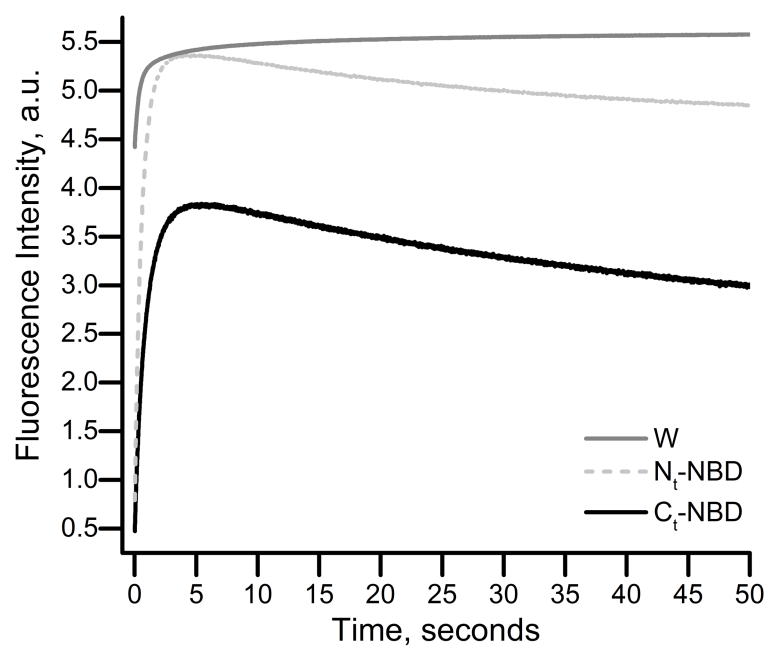


Figure 13. Intrinsic W, N_t-NBD, and C_t-NBD fluorescence display similar kinetics during pHLIP membrane insertion.

Average spectra of W (dark gray), N_t-NBD (dashed light gray), and C_t-NBD (black) in POPC.

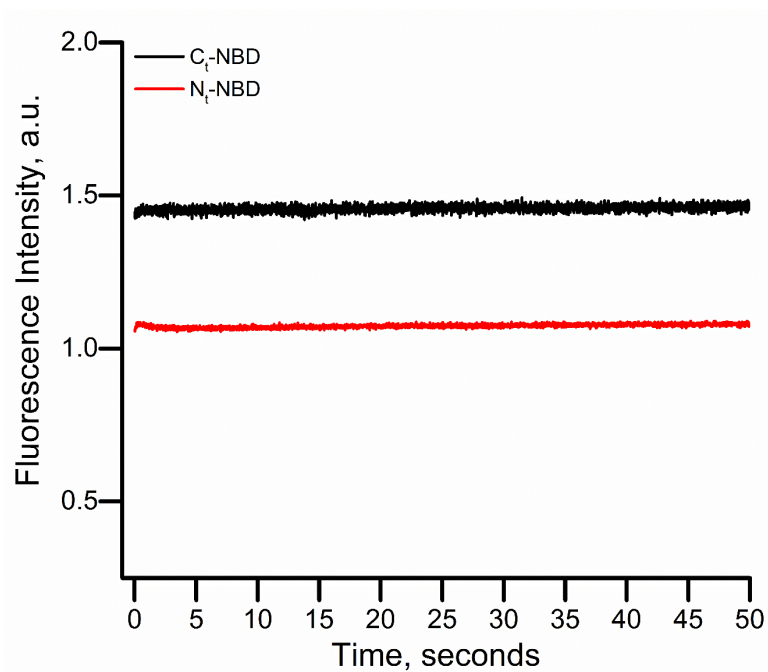


Figure 14. C_t- and N_t-NBD conjugates show no photo-bleaching at pH 8.

Signal of both C_t- (black) and N_t-NBD (red) conjugates remains flat during the duration of the stopped-flow experiment at pH 8 (State II). No change in signal suggests that no photo-bleaching is detected during the 50 second excitation of the fluorophores.

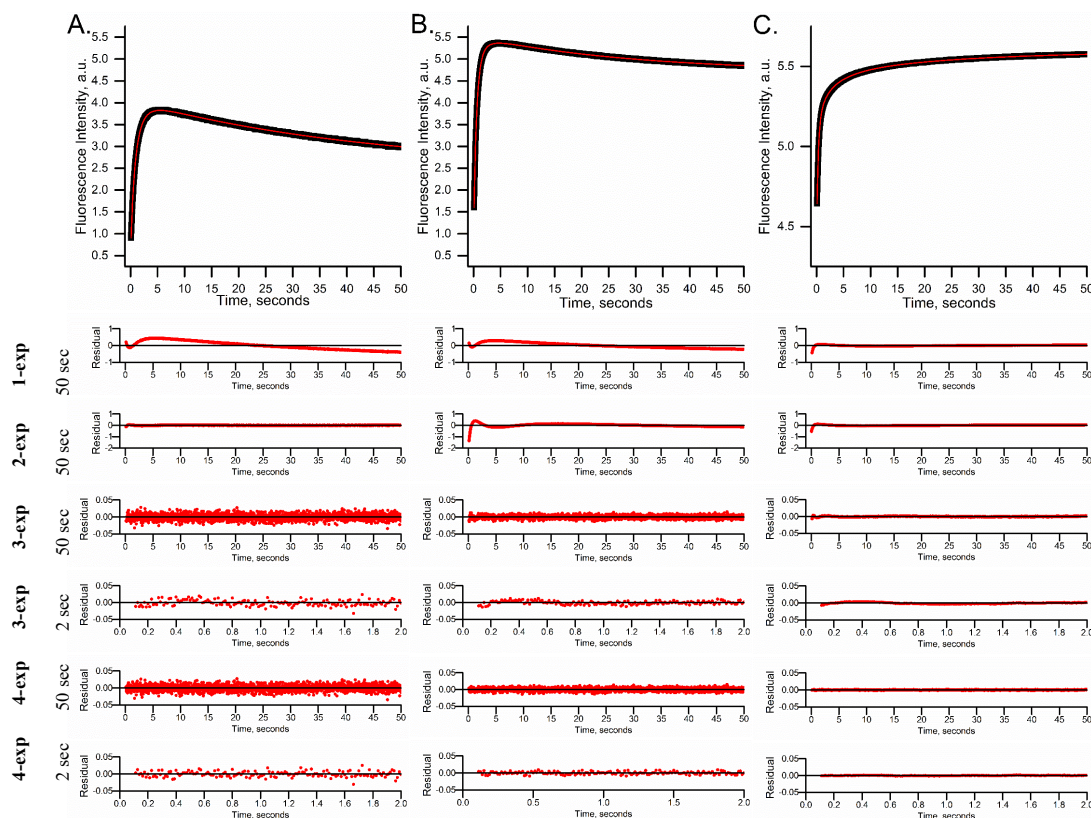


Figure 15. Stopped-flow fittings and residuals show that four exponential model agrees with the experimental data.

Average stopped-flow data for C_T -NBD (A), N_T -NBD (B), and W (C) fit using a four-exponential model (red line). Below each fitting are shown the residual plots for 1-4 exponential model fits (top to bottom). Additional panels show magnification of the first 2 seconds of the 3- and 4-exponential fits. Satisfactory fittings were only obtained employing four exponential terms, as evidenced by the observed initial negative signal and positive signal at 0.4-0.5 seconds in all the 3-exponentials fits. A previous report used a three-exponential model to analyze the kinetic data. While residuals for three exponential steps showed little deviation, we believe that four exponentials better represent the number of protonation steps (17, 21).

Table 4. Rate Constants and normalized amplitudes of membrane insertion kinetics.

	k_1^{-1} , sec	k_2^{-1} , sec	k_3^{-1} , sec	k_4^{-1} , sec
	A1, %	A2, %	A3, %	A4, %
N _i -NBD	0.13 ± 0.04	0.50 ± 0.21	1.55 ± 0.74	34.47 ± 16.8
	12.41 ± 6.29	51.97 ± 8.30	35.62 ± 14.24	-22.74 ± 3.80
C _i -NBD	0.27 ± 0.12	0.86 ± 0.24	2.27 ± 1.17	55.80 ± 20.78
	11.26 ± 11.47	58.10 ± 10.28	30.64 ± 17.98	-43.91 ± 5.83
Trp	0.20 ± 0.06	1.03 ± 1.08	3.47 ± 2.43	24.04 ± 18.72
	31.32 ± 6.24	42.95 ± 14.70	11.90 ± 4.90	13.84 ± 8.67

Next, pK_{FI} and pK_{Nt-NBD} group for an average pK value of ~ 5.6 . Interestingly, these two parameters employ probes (W and NBD) that are located near or at the N-terminus of pHLIP. Finally, pK_{Ct-NBD} reports on the most acidic pK value, ~ 5.2 . Strikingly, the pK_{Ct-NBD} value is ~ 0.9 units lower than pK_{SM} , the typical value used in the literature (4). This is a large pH difference (108), but the use of NBD as a tool to determine the pK of pHLIP insertion is a novel approach. Previously, NBD-pHLIP conjugates were used to determine the directionality of the membrane insertion of pHLIP and to test the ability of pHLIP to translocate cargoes (4, 34). The translocation of different cargo molecules by pHLIP WT and variants has been previously studied. These reports indicate that cargo molecules do not affect the pH-dependent insertion, as determined by monitoring W fluorescence changes (21, 34, 37). We attempted to collect W fluorescence data with our pHLIP-NBD conjugate but signal was reduced beyond reliable detection in the presence of NBD. Significant W signal loss in the presence of NBD has been observed previously and suggested to result from FRET from W to NBD occurring in specific conditions (115, 116). Previous work has shown that pHLIP translocation is not affected by the conjugation of NBD at either terminus (34).

The method commonly used to determine the membrane insertion pK of pHLIP has been the SM analysis of W fluorescence (4, 14, 18, 95). Here, we find that the pK obtained by analyzing SM and CM, is significantly higher than the one obtained monitoring FI changes. The pK is shifted ~ 0.5 units and the differences are statistically significant ($P \leq 0.005$) (Figure 8). However, caution must be exercised when rationalizing the differences. The shift in pK could result from the methods reporting on different steps of the membrane insertion process. However, an alternative possibility is that the shift is a consequence of the intrinsic differences of each analysis method. White, Fleming, Ladokhin, and co-workers have discussed the problems associated with relying on tryptophan spectral blue-shift analysis (101, 117), since spectral center of mass and spectral maximum changes do not linearly depend on the amount of folded protein or peptide (101, 117).

This deviation from linearity can cause one to erroneously follow membrane insertion and not accurately report on the acquisition of final folded state (101). Conversely, W fluorescence intensity at a fixed wavelength shows a linear relationship with the amount of folded species. The linear nature of FI changes thus allows a more accurate determination of the amount of peptide in the final folded state (101).

We decided to perform additional experiments to investigate if the pK differences observed between FI and spectral shift methods (SM and CM) could be due to the latter improperly following the folding of pHLIP as membrane insertion occurs. Since the membrane insertion of pHLIP is a reversible process (20, 95), we sought to investigate if the fluorescence pK difference observed for the membrane insertion was also observed for the membrane exit. We rationalized that if the difference between pK_{CM} and pK_{SM} and pK_{FI} was due to intrinsic properties of the analysis methods, we might expect to see a similar pK difference in the membrane entry and exit. Conversely, Figure 9B shows that there is not a significant difference in pK exit values of SM, CM, and FI ($P > 0.05$). However, the membrane exit of pHLIP follows a different pathway with less kinetic intermediates than the membrane insertion (20), which complicates comparing the two processes. As a result, we cannot rule out that the values of pK_{CM} and pK_{SM} might have an artefactual contribution. Thus we suggest that FI is the most accurate intrinsic fluorescence parameter to determine the pK . Conversely, CD also shows a linear response during peptide folding (118), and thus pK_{CD} would not be affected by the potential problems of the other members of the high pK group (pK_{CM} and pK_{SM}). Since pK_{CD} (helical formation) was significantly higher than pK_{FI} (W burial), we suggest that the two parameters are reporting on different aspects of the multi-step membrane insertion/folding of pHLIP. Others have reported deviations between linear spectroscopic methods when determining folding midpoints (119-121). This difference often

indicates that the folding/unfolding is a multi-step process, as is the case for pHLIP (119, 121). Conversely, perfect overlap of the midpoint region generally suggests a two-step process (119).

pK_{CD} as the midpoint of helical formation. We wanted to investigate the relationship between the different pK values (Figure 8) and the separate steps of the folding/membrane insertion of pHLIP. Interestingly, the pK_a values of D14, D25, D31, and D33 of pHLIP have been recently determined by ssNMR in POPC vesicles (17). Comparison between each of the three pK groups and the individual ssNMR pK_a titrations is highly informative (Figure 16). As a representative of the group with a higher pK we selected SM, since it is the parameter most commonly employed to obtain the pK of pHLIP. We observed overlap between the SM sigmoidal curve and the ssNMR curves for the titrations of D25, D31 and D33. As a result there is a good agreement between pK_{SM} (6.22 ± 0.17) and the value obtained by averaging these three independent pK_a values (6.30 ± 0.21) (17). On the other hand, the overlap with D14 was not clear (Figure 16A). Interestingly, the curve of pK_{SM} nicely overlaps with the pK_a of D33 (6.34 ± 0.21). The agreement led us to speculate that CD (and potentially also SM and CM) might report on the ensemble of the membrane insertion intermediates resulting from the titration of residues D33, D31, and D25. Our data thus suggests that there is a correlation between the protonation of D33, D31, and D25 and transmembrane helical formation, as reported by pK_{CD} (17, 102). Figure 17 displays the proposed steps required for the insertion of pHLIP. The cartoon also shows at what point each aspartate group titrates into the protonated state, and highlights the insertion step/s that each pK value reports on.

pK_{FI} as the midpoint of N_t conformational changes. We observed a less complete overlap between the ssNMR curves and the FI titration, as a result of the acidic shift in the pK value for FI

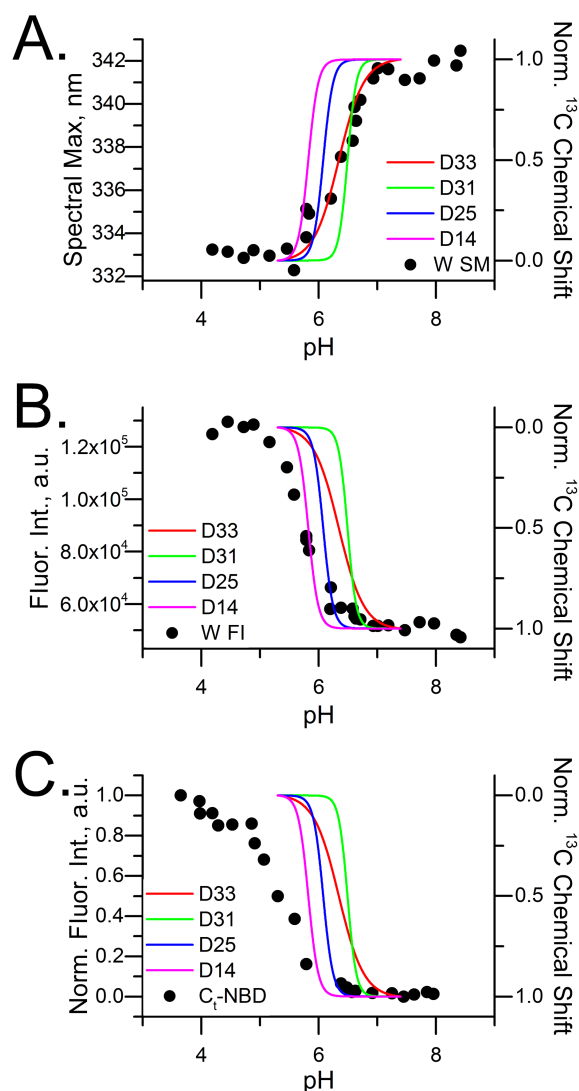


Figure 16. Qualitative comparison of fluorescence pK titrations with ssNMR pK_a titrations for the individual aspartate residues.

The individual titrations for D14 (magenta), D25 (blue), D31 (green) and D34 (red) obtained by Qiang and co-workers (17) are overlaid with the fluorescence data obtained following tryptophan SM (A) and FI (B), and pHLIP-NBD (C). The ssNMR data were represented by the fitting to the Boltzmann equation (17). Normalized chemical shifts are shown for a better comparison, and values were inverted in panels B and C to align with the fluorescence data changes.

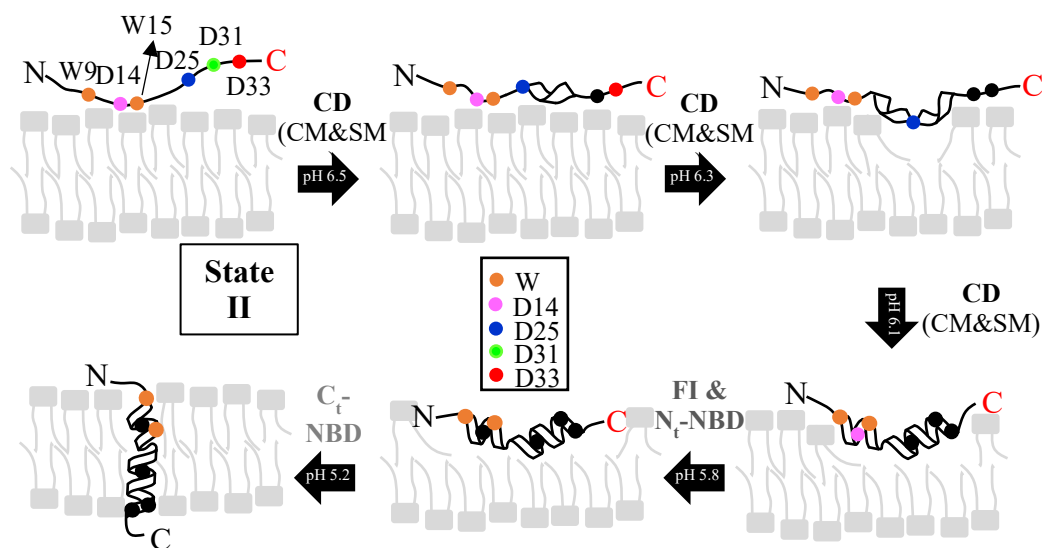


Figure 17. Tentative model showing the transition from State II to State III during the membrane insertion of pHLIP, highlighting the insertion step/s that each pK value reports on.

The black arrows represent the transitions between each step in the membrane insertion process, with the corresponding pH midpoints. The change of the D residues from colored to black represents the protonation. CD, and potentially SM and CM, reports on the insertion process encompassing D33, D31, and D25, and are then shown labeling the first three transitions. FI and N_t-NBD follow the protonation of D14, and to a lesser extent D25 and D33 (not shown in figure). Finally, C_t-NBD reports on the last insertion step, where the C-terminal end translocates across the bilayer.

(and NBD-pHLIP) (Figure 16B). In this case, there is no clear overlap with the titration of D31, and only partial overlap with the D33 and D25 titrations. However, the pK_{FI} of 5.68 ± 0.09 is within the error to the pK_a of D14, 5.82 ± 0.08 (17). We hypothesize that pK_{FI} reports primarily on the partial local burial of the N-terminus of pHLIP, which is expected to be controlled by the protonation of D14 (Figure 17) (17). This is supported by the similar values of pK_{FI} , which reports on the environment of W9 and W15, and pK_{Nt-NBD} , which is informed by the N-terminus (Figure 8). Interestingly, we observed hysteresis when FI was used to compare the two directions of the process: membrane entry (insertion) and exit. Hysteresis was not observed in the case of SM or CM (Figure 12). The existence of hysteresis might not be surprising, since the intermediate steps populated in both directions of the folding pathway are different. During the membrane exit of pHLIP, the N-terminal region might change environment concomitantly with helix unraveling, and explain why the three pK values in Figure 9 are similar. This is supported by stopped-flow CD data indicating that the pHLIP helix starts to unfold within the membrane before membrane exit occurs (20).

pK_{Ct-NBD} as the translocation midpoint. The rate-limiting step in the formation of the transmembrane state of pHLIP is the translocation of the C-terminus (17, 20). For therapeutic applications drug cargoes are conjugated to the C-terminus. Its unidirectional translocation enables pHLIP to be an effective drug delivery agent to acidic tissues such as solid tumors (4, 16). It has been recently suggested that tryptophan fluorescence changes are not capable of detecting the final translocation of the C-terminus of pHLIP across the bilayer (17). Here we address this potential shortcoming and study the membrane insertion of pHLIP using a C-terminal NBD probe. Interestingly, the pK obtained using this probe, pK_{Ct-NBD} , is more acidic than any of the individual aspartic acid pK_a values determined via ssNMR (Figure 16C) (17). Due to the location of NBD at position 37, it is interesting to compare the pHLIP-NBD curve with the titration of the closest

residue studied by ssNMR, D33. Although both are essentially C-terminally located, $pK_{\text{Ct-NBD}}$ is ~ 1.0 pH unit lower than the pK_a of D33 (17). Additionally, D33 displays a much broader titration compared to aspartates 14, 25, and 31, encompassing a larger pH range in the titration event, similarly to $pK_{\text{Ct-NBD}}$. We speculate that the differences in slope might result from long-range effects of the yet unresolved titrations of E34 and/or the C_t group affecting the cooperativity of the D33 titration (122). It would not be surprising if E34 and the C_t had the most acidic pK_a values, since they are located in the most polar region of the sequence. As a result, water accessibility would be less hindered, and their pK_a values might be closer to those typically found in solution (~ 3.7 and ~ 4.3 for C_t and E, respectively (123)), however, it is unknown the effect of titration of E34 and the C_t on the each pK . Additional ssNMR determination of the pK_a of E34 and the C_t group is needed to shed light on this hypothesis. We suggest that the C-terminal NBD group might be sensitive to the titration of such neighboring acidic groups, to report on the translocation of the C-terminal end of pHLIP that results in the acquisition of the final transmembrane state (Figure 17). We suggest that a new pK term might be needed to adequately describe this step, and we refer to it here as the pK of translocation, which is significantly more acidic than the well-established pK of insertion.

Figure 17 also shows the proposed location of the N- and C-terminus at each step, based on the stopped-flow data (Figure 13). As expected for a kinetic study of the full transition from State II to State III, the three parameters employed here reported on all the intermediates. However, the differences observed for NBD conjugates compared to W provided clues on the location of the peptide termini. As reported previously, the W intensity increased in all steps, as W9 and W15 were progressively buried into the membrane (Figure 7) (20, 21). During the first insertion steps, intensity increased for the two NBD conjugates, indicating that both the N- and C-termini buried into the membrane (Figure 17). Strikingly, the NBD intensity later decreased in both cases. This

suggests that as the transmembrane configuration is being acquired both the N- and C-termini move from a membrane-buried state to a more hydrated final location. Interestingly, comparing the relative magnitude of the NBD decreases can inform on the conformational changes needed for formation of the final transmembrane state. In particular, our data suggest that both peptide termini are transiently buried in the membrane. The larger C_t-NBD decrease is expected to result from the NBD leaving the core of the membrane and gaining hydration as translocation is completed. However, the N_t-NBD signal decrease was unexpected, and it suggest that the N_t of pHLIP transiently explores a hydrophobic position of the membrane. This situation, expected to be energetically unfavorable, is reversed when pHLIP insertion is completed and the final transmembrane state is established.

Our results show that significantly different pH dependence values are obtained when employing different methods to study the membrane insertion of pHLIP. As a result caution must be exercised in interpreting pK data. This is particularly important when discussing pHLIP and other peptides to translocate a drug cargo linked to the inserting end (111). We propose that the common pK_{SM} parameter is primarily influenced by intermediate steps that precede translocation of the C_t. As a result, we suggest that SM does not accurately report on translocation of the C_t, and the pK_{SM} value would be higher than the pH midpoint of cargo translocation. More precise cargo translocation information might be obtained instead employing pK_{C_t-NBD} , which we propose informs on the pK of translocation occurring at more acidic pH values. The use of this parameter might be used as a guide in the design of pHLIP variants that efficiently target the extracellular pH of tumors. Understanding which step in the folding/insertion transition a particular analysis method reports on, is critical to evaluate the cancer targeting ability of peptides which undergo acidosis-dependent transmembrane translocation.

2.6 Acknowledgments

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Chapter III: The negative charge of the membrane has opposite effects on the membrane entry and exit of pHLIP

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3.1 Abstract

The pHLIP peptide targets acidic diseases such as cancer. The acidity of the environment causes key aspartic acids in pHLIP to protonate, causing the peptide to insert into membranes. Here we investigate how the negative charge of the membrane influences how pHLIP enters and exits the lipid bilayer. We found that electrostatic repulsion affected differently the membrane entry and exit of pHLIP for negatively charged membranes. As a consequence, a large hysteresis was observed. We propose this is not a consequence of structural changes, but results from local changes in the environment of aspartic acids, shifting their pK values.

3.2 Introduction

The full extent of the complexity of tumors as organs has only recently been recognized. Tumors not only contain cancer cells, but also a number of seemingly healthy, specialized cells are found in the tumor microenvironment (124). The composition of the tumor microenvironment is severely influenced by metabolites pumped out of tumor cells. Malignant cells have an altered metabolism compared to healthy cells. The Warburg effect describes the increased rate of aerobic glycolytic metabolism of cancer cells. This effect, together with the faulty vasculature of tumors, leads to the accumulation of anaerobic metabolites, such as lactic acid, in the tumor microenvironment. The ensuing microenvironment acidity is a characteristic generally found in solid tumors (125). Since acidity is a general property of solid tumors, it holds great promise as a targeting principle to design technologies to fight cancer. A leading technology to target tumor acidity is the pHLIP (pH-Low Insertion Peptide) (126). The pHLIP is soluble in aqueous solution, but when membranes are made available it binds to them in a disordered conformation (5). The nature of the membrane interaction of pHLIP is controlled by pH. Therefore, acidification prompts the peptide to gain helical conformation and insert across the membrane, establishing a transmembrane (TM) helix (127). The pH-sensing properties of pHLIP lead to its accumulation in multiple types of murine tumors, including melanoma, prostate carcinoma and lymphoma (126, 128), as well as in human tumor biopsies (129).

The physical properties of the lipid bilayer impact the interaction of pHLIP with lipid membranes. For example, the structure pHLIP adopts at the membrane surface has been proposed to be influenced by the bilayer elastic bending modulus (130). The level of acidity required for pHLIP to insert into the membrane is a key property for tumor-targeting. It was found that different levels of acidity are required for pHLIP to insert into lipid vesicles of different composition. Cell studies suggested that this is due to differences in the fluidity of the membrane (130). Several differences exist between the membranes of healthy and tumor cells, one being its charge (131).

Thus, cells of the tumor and its vasculature can contain significant amounts of the negatively charged lipid phosphatidylserine (PS) exposed in the outer monolayer of the plasma membrane (132). Conversely, healthy mammalian cells lack PS at their surface (133). The pHLIP has a strong negative net charge at physiological pH. It is then plausible that electrostatic repulsion can occur between the negative charges found in pHLIP and the membrane of tumor cells. However, the effect of charge on the targeting of pHLIP is not known. Here, we investigate the impact of membrane charge on the membrane affinity and insertion of pHLIP.

The pHLIP technology has been applied for preclinical diagnostics and therapeutics in a large diversity of tumors (126). In all cases, the route of administration of pHLIP has been parenteral. After injection, pHLIP circulates in the blood until it reaches an acidic area such as a tumor, where it accumulates by inserting into the plasma membrane of cancer cells. The first step in the insertion process consists of the binding to the surface of the cell, followed by membrane insertion as a TM helix. Here, we have recapitulated these two separate events by studying the binding and insertion of pHLIP in large unilamellar vesicles of different composition.

3.3 Materials and Methods

Liposome Preparation. Large unilamellar vesicles were prepared by extrusion. POPC and POPS were purchased from Avanti Polar Lipids, Inc. in powder form. Chloroform was added to the powder to obtain a stock concentration of 25 mg/mL for POPC and 10 mg/mL for POPS. Aliquots were taken from this stock in set volumes depending on the needs of the experiment. The samples were first dried under a stream of Argon gas and then dried under a vacuum overnight. All samples were rehydrated using 10 mM NaPi pH 8 buffer, except CD experiments that used 1 mM NaPi pH 8 buffer, and the NBD-binding assay, which used 10 mM HEPES pH 8 buffer. The samples were vortexed until the entire dried lipid had dissolved and then centrifuged for one minute. Extrusion was then performed using a Mini-Extruder (Avanti) making 30-100 passes (depending on the concentration of the sample) through a 100-nm pore size membrane.

Peptide Preparation and Labeling. Peptide was purchased from P3 Biosystems in a lyophilized form. Purity was checked to be greater than 95% by HPLC. Peptide was taken out in aliquots and rehydrated using the appropriate buffer for the experiment. Concentration of the peptide was checked using a Cary Series 100 UV-VIS spectrophotometer (Agilent Technologies) to obtain the absorbance (A) and then calculated using the Beer-Lambert Law with a molar extinction coefficient (ϵ) of $13,940 \text{ M}^{-1} \text{ cm}^{-1}$. Samples were diluted to the correct concentration for each experiment. A C_t-Cys pHLIP was labeled with the thiol-reactive nitrobenzoxadiazole (IANBD amide) (Life Technologies) following the protocol described by the supplier. Free dye was removed using size exclusion chromatography with a PD-10 column (GE Healthcare), and the purity of the sample was checked using MALDI-TOF.

Fluorescence spectroscopy. Peptides were prepared by dissolving in 10 mM NaPi pH 8 buffer and then incubated with POPC or POPC/POPS (POPS concentrations of 1, 2.5, 5, 6.25, 10, and 30%) lipids prepared in the same buffer for one hour, resulting in a lipid to peptide ratio of 200:1.

The final peptide concentration was 1 μ M. The pH of the samples was adjusted using 100 mM concentrations for the indicated buffers (sodium acetate, pH 4.1-5.35: MES, pH 4.5-6.6: HEPES, pH 7.04-8.41; sodium carbonate, pH 9.05). Experiments using NaCl had a final NaCl concentration of 150, 400, or 800 mM in the samples. Blanks were subtracted in all cases. Final pH of the samples was measured after recording the spectra. Emission spectra were recorded using a Photon Technology International Quanta Master fluorometer at room temperature with the excitation wavelength set to 280 nm, the emission wavelength range from 310 to 400 nm, and the slits set to 3 nm. Data were analyzed by calculating the spectral center of mass (CM) with the following equation:

$$CM = \sum_1^n I_i \lambda_i / \sum_1^n I_i$$

where I_i is the fluorescence intensity measured at a wavelength λ_i . This is used to monitor the two tryptophans' environment during the experiment, extracting information on the entire wavelength range (100, 134). The data were also analyzed by following the fluorescent intensity change at 335 nm. Center of mass and fluorescent intensity pH-titrations were then fitted to determine the pK, using Eq. 1:

$$(F_a + F_b 10^{m(pH-pK)}) / (1 + 10^{m(pH-pK)})$$

where F_a is the acidic baseline, F_b is the basic baseline, m is the slope of the transition, and pK is the midpoint of the curve. The pK curve graphs were fitted to Eq. 2:

$$F_o + (F_{max}) \times (x / (K_d + x))$$

where F_o is the initial fluorescent signal, F_{max} is the maximum fluorescent signal, and x is the

percentage of PS. This was used to determine the midpoint of PS ($\sim K_d$). Fitting was done using OriginLab software.

Circular dichroism. Samples were prepared the same way as in the fluorescence experiments (1 mM NaPi was used to rehydrate the peptide). POPC and POPC/POPS 90/10 samples were used. The lipid to peptide ratio was 200:1. The final peptide concentration was 5 μ M. Samples were pH-corrected using 10 mM NaPi pH 8, 5 mM sodium acetate pH 3.9, or 25 mM NaPi pH 8.5. The final pH of the samples was measured after the recording of the spectra. CD spectra were acquired using a Jasco J-815 spectropolarimeter interfaced with a Peltier system. Spectra were recorded at 25°C using a 2 mm cuvette, the scan rate was 50 nm/min, and 20-40 accumulations were performed. Raw data were converted into mean residue ellipticity according to $[\Theta] = \Theta / (10lcN)$ where Θ is the measured ellipticity, l is the path length of the cell in cm, c is the protein concentration, and N is the number of amino acids. Appropriate blanks were subtracted in all cases.

Dynamic Light Scattering. DLS was used to measure fluorescence exit and CD reversibility samples to ensure intactness of vesicles at the end of the measurements. Samples were filtered using a 200 nm filter and then measured using a Wyatt DynaPro NanoStar instrument. For the analyses, the MW-R model was set to globular proteins and the Rg model was set to sphere. Samples were measured at 25°C. Our results show that vesicle size and integrity were not disrupted as a consequence of increasing or decreasing sample pH.

NBD-Binding Assay. Peptides were prepared by adding 470 μ L of H₂O to lyophilized samples of pHLIP-NBD in 10 mM NaPi, to make stock concentration of 10.9 μ M. Lipids were prepared as described above. POPC and POPC/POPS 70/30 lipid samples were used. Peptide was mixed with various lipid concentrations (0 - 8×10^{-4} M) to give a final peptide concentration of 2.5 μ M for all samples. Blanks were subtracted in all cases. Measurements were performed with a Photon

Technology International Quanta Master Fluorometer at 25°C with excitation wavelength set to 470 nm, emission wavelength range from 520 to 580 nm, the emission slits width was 10 nm, the excitation slits width was 6 nm, the emission polarizers were set to 90°, and the excitation polarizers were set to 0° to minimize the light scattering contribution from the vesicles. Data were analyzed by calculating the center of mass and by monitoring the fluorescent intensity change at 540 nm. Data were fitted using Eq. 3:

$$(F_o + F_{max} \times ((K_p \times x) / (55.3 + K_p \times x)))$$

where F_o is the initial fluorescent signal, F_{max} is the change in fluorescent signal, x is the concentration of lipid in M, and 55.3 is the molar concentration of water. This was used to determine the K_p of binding. Fitting was done using OriginLab software. ΔG was calculated using the equation:

$$\Delta G = -RT \cdot \ln(K_p)$$

where R is the gas constant, T is the temperature in Kelvin, and K_p is the partition coefficient.

Monolayer Preparation. Monolayer experiments were performed using a KSV NIMA Langmuir trough. The trough was filled with 10 mM NaPi pH 8 and then a lipid-chloroform mixture was spread onto the air/water interface. Lipid stock concentration was 0.4 mg/mL. After the trough was prepared, peptide insertion experiments were performed. This was done by first compressing the barriers at 1 mm/min to a target surface pressure. After the target pressure was reached, the area was set constant. Next we injected the peptide into the subphase by immersing a pipette tip under the barrier and then slowly injected the peptide into the subphase, being careful not to disrupt the monolayer. The final concentration of pHLIP in the subphase was 500 nM.

OCD measurements. To form the bilayer, a lipid monolayer was first deposited on a demountable quartz cuvette (Starna) using the Langmuir–Blodgett method. The second monolayer was formed by vesicle fusion on top of the monolayer. Previously, slides were thoroughly cleaned by sequential sonication in 10 min cycles in Contrad 70 (5% in water), 2-propanol, acetone, and 2-propanol. The slides were then placed in piranha solution for 7 min and then rinsed and stored in water. To deposit the monolayers, two slides were stacked together and vertically immersed into the subphase (10 mM sodium phosphate, pH 7.8) of a KSV NIMA trough. The lipid solution was prepared in chloroform and was spread at the air/water interface of the trough. Before the monolayer was compressed to 30 mN/m, chloroform was allowed to evaporate for 30 min. The monolayers were deposited by raising the slides out of the subphase at a rate of 2 mm/min. The Langmuir-Blodgett monolayer was then incubated with a peptide/lipid solution at pH 4 (10 mM sodium acetate). The peptide/lipid solution consisted of 10 μ M pHLIP and 0.5 mM lipid vesicles that were sonicated for 15 min. Fusion was allowed to be completed overnight under 100% humidity. After removing the excess vesicles, four slides were carefully washed with pH 4 buffer and then stacked to form two cuvettes filled with buffer. The OCD spectrum was measured on a Jasco J-815 spectropolarimeter and lipid background subtracted.

3.4 Results

We first studied the acquisition of the surface-bound state by comparing the binding affinity of pHLIP to vesicles of the zwitterionic POPC and the negatively charged POPC/POPS in a molar ratio of 70/30. We hypothesized that binding of pHLIP to POPS-containing vesicles would be hindered, due to the negative charge that phosphatidylserine carries on its head group. The fluorescence intensity of a pHLIP-NBD complex was monitored to assess the binding affinity to the different lipid headgroups. Strikingly, we found that pHLIP displayed similar affinity for both lipid headgroups (Figure 18 and Table 5). The obtained partition coefficients, $2.1 \pm 0.4 \times 10^5$ and $1.6 \pm 0.5 \times 10^5$ for POPC and POPC/POPS, respectively, result in a very similar binding ΔG : -7.3 ± 0.2 and -7.1 ± 0.3 kcal/mol. Unexpectedly, this indicates that the negative charge of POPS has no effect on pHLIP binding, suggesting that the affinity to the negatively charged vesicles is not reduced due to electrostatic repulsion. This suggests that pHLIP might bind with similar affinity to the surface of healthy and tumor cells decorated with PS.

The high specificity of pHLIP to accumulate in tumor cells arises from its pH-dependent membrane insertion. However, the effect of the membrane charge on the membrane insertion of pHLIP is not known. It has been established that the membrane entry pK of pHLIP in POPC vesicles is 6.0 (135). To address whether the negative charge that POPS carries alters the entry pK, pH titrations were performed in POPC vesicles with increasing POPS content. We obtained pH-titrations by monitoring the changes in the fluorescence spectrum center of mass (CM), which reports changes in the *skewness* of the spectrum (100). Our results (Figure 19 and Figure 20) demonstrated that the presence of POPS caused a sharp decrease in the entry pK, until saturation occurred at ~5% POPS with a pK ≈ 5.6 (Figure 20). The overall content of PS in the plasma membrane of mammals is around 10% (133). Consequently, the observed pK decrease was obtained for physiological levels of PS.

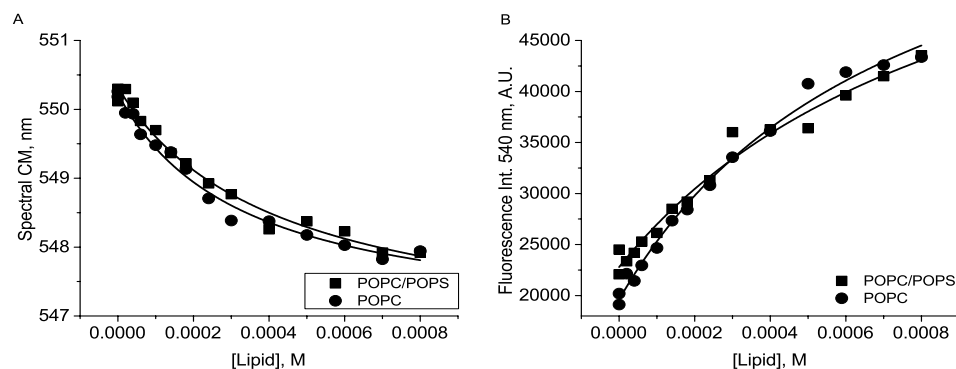


Figure 18. NBD lipid binding assay.

Overlay of pHLIP-NBD binding assays demonstrating no change in binding affinity between 30% POPS (squares) and 0% POPS (circles) in POPC. Curves were fitted to Eq. 3 (black line). pHLIP-NBD was incubated with various lipid concentrations ($0 - 8 \times 10^{-4}$ M) to give a final peptide concentration of $2.5 \mu\text{M}$ for all samples.

Table 5. The partition coefficient (K_p) values.

Partition coefficient (K_p) values obtained by monitoring emission spectra center of mass (CM) and fluorescence intensity at 540 nm (FI) from Figure 18. Errors are the standard deviation of the mean.

Lipid	Method	K_p value	Error
POPC	FI	1.1×10^5	9.8×10^4
	CM	2.1×10^5	4.4×10^4
POPC/POPS (70/30)	FI	2.5×10^5	2.4×10^5
	CM	1.6×10^5	5.1×10^4

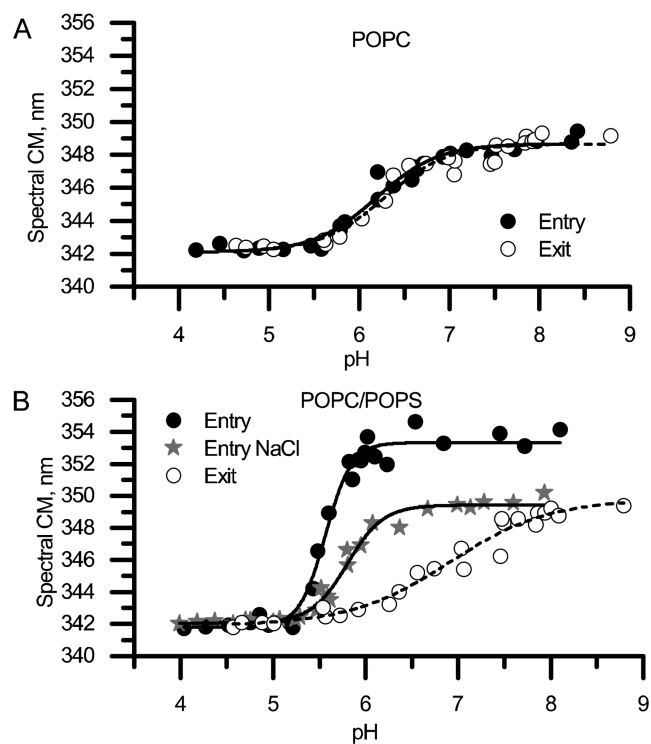


Figure 19. Membrane entry and exit transitions.

Acidification leads pHLIP to insert into the membrane (entry). After a subsequent pH raise, pHLIP leaves the membrane core (exit). (A) POPC and (B) POPC/POPS 90/10. Stars show how 0.4 M NaCl affects entry in POPC/POPS 95/5.

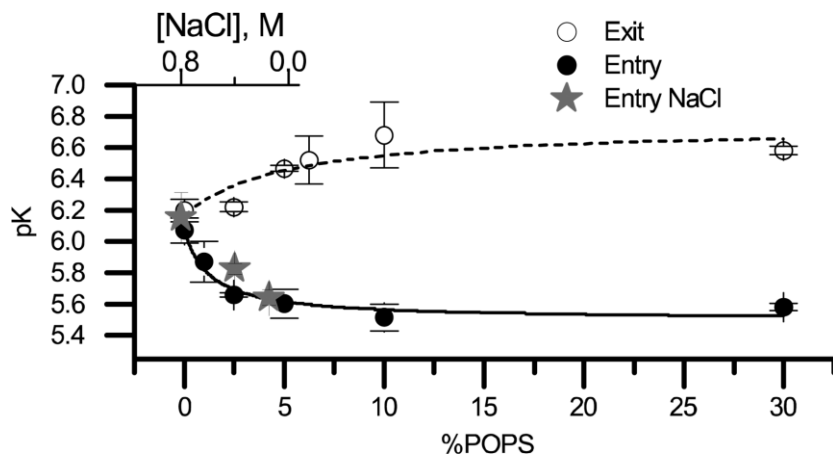


Figure 20. Impact of POPS on pK values for pHLIP membrane entry (closed symbols) and exit (open symbols).

The role of electrostatic interactions is assayed varying NaCl concentrations on the membrane entry experiments with 95/5 POPC/POPS vesicles.

Aiming to understand why POPS decreased the membrane entry pK, we performed two modalities of circular dichroism (CD) experiments. CD experiments of pHLIP in vesicles, performed at basic and acidic pH, showed that the presence of POPS does not affect the conformation of pHLIP compared to POPC (Figure 21). At pH 8, pHLIP binds to the periphery of either vesicle type in a random coil conformation. On the other hand, the CD spectra of pHLIP at acidic pH showed the characteristics of an α -helix: minima at 208 and 222 nm and a shoulder with positive ellipticity at <200 nm. The presence of POPS did not influence these spectral features, indicating that POPS does not affect the α -helix formation. However, it is conceivable that POPS might be affecting the alignment of the helix with respect to the membrane plane. To gain information about the orientation of the helix, we performed oriented CD (OCD) measurements with hydrated stacked bilayers (see insets in Figure 21). We observed for both lipids similar spectral features (maximum at ~195 nm and broad minima between 210-225 nm), typically observed for a TM helix (136). The combination of the two types of CD experiments demonstrated that pHLIP still forms a TM helix in the presence of negatively charged lipids.

3.5 Discussion

The value of the pK of ionizable groups can be severely influenced by the properties of the environment. The three main factors are the Born effect (dehydration), charge-charge interactions and hydrogen bonds (137). Aspartate residues exposed to water have pK values close to 3.9, while those shielded from water typically have higher values. The decrease in the membrane entry pK obtained for POPS suggests that, in the peripheral state of pHLIP, the aspartate residues are more hydrated when the membrane contains POPS. Insights pertaining to the peripheral state of pHLIP can be gained by examining the degree of insertion in the bilayer of the two tryptophan residues of pHLIP. This information can be used to assess if the negative charge of POPS affects the location of pHLIP on the membrane surface compared to POPC. Figure 19 shows that the

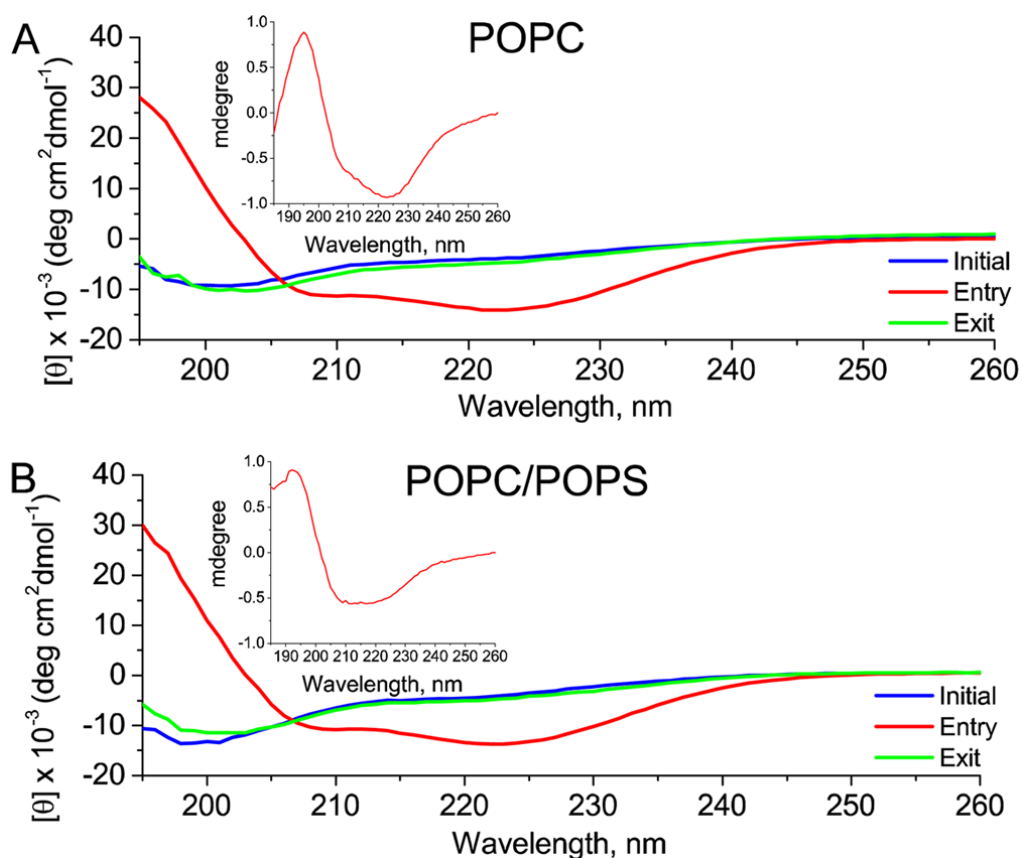


Figure 21. Reversible formation of the TM helix.

Circular dichroism (CD) for POPC (A) and POPC/POPS 90/10 (B). Peptide was incubated with vesicles at pH 8 (blue lines), and then acidified to pH 4 to induce membrane entry (red lines). Finally, the pH was raised back to 8 to attain exit (green lines). Insets are oriented CD experiments at pH 4.

fluorescence center of mass at neutral pH is different between POPC (349 nm) and POPC/POPS (354 nm). This suggests changes in the membrane depth of pHLIP as the tryptophan residues insert deeper into the POPC bilayer than in POPC/POPS (see Scheme 1). We propose that the observed decrease in the pK of entry results from the shallower position in the surface of negatively charged membranes. As a consequence, the hydration of key aspartate residues would increase, causing a decrease in their pK, and then the pK of membrane entry.

The entry pK and center of mass of the peripheral state are both altered in the presence of POPS. Since both pHLIP and POPS carry negative charge at neutral and basic pH, it seems reasonable that the observed differences could be due to electrostatic repulsions occurring between the negative charges found in pHLIP and POPS. To explore this possibility, NaCl was used to screen any electrostatic repulsion. Interestingly, addition of NaCl caused both the entry pK and the center of mass to return to values similar to those obtained in POPC (Figure 19B and Figure 20). These data indicate that electrostatic repulsion, despite not influencing significantly the affinity of pHLIP for vesicles, does play an important role for the insertion of pHLIP into negatively charged vesicles.

When pHLIP leaves the bloodstream and successfully localizes into the membrane of a tumor cell, it is in a position to be employed to diagnose and/or treat the tumor. However, pHLIP's journey could not end there, since the peptide does not irreversibly insert in the cancer cell membrane. Instead, thermodynamic and kinetic considerations are believed to determine the tendency of pHLIP to exit the membrane after the initial insertion and return to the surface (126). In our experimental system, membrane exit of pHLIP occurs when the acidic pH is raised back to neutrality (Figure 19, open symbols). Intrigued by the possibility of PS also playing a role on this process, we finally investigated the effect of the negative charge and its role on membrane exit of pHLIP. pH-titration experiments were again conducted to understand if the exit of pHLIP is

different between POPC and POPC/POPS. For POPC, the results demonstrated an exit pK of 6.2 ± 0.1 , in agreement with previous observations (Karabadzhak *et al.*, submitted). However, the addition of POPS led to an increase in the exit pK to 6.7 ± 0.2 (Figure 20), demonstrating that POPS alters the exit of pHLIP as well. This suggests that in the presence of POPS higher pH levels are required to cause the deprotonation of the aspartate residues leading to the exit. This can be rationalized in terms of PS discouraging the TM state of bearing charge *via* electrostatic repulsion. Consequently, this would shift up the pKa of the key aspartates.

Circular dichroism data showed that the membrane entry of pHLIP was not affected by the presence of POPS. However, it might occur that POPS could make the exit of pHLIP to be only partially reversible, hindering the return of pHLIP to a random coil from the TM helix. Circular dichroism was used to determine if the exit of pHLIP was as reversible in POPC/POPS as in POPC (Figure 21). Our CD results demonstrated high levels of reversibility regardless of the lipid headgroups (Figure 22).

Figure 23 depicts a conceptual model summarizing most of our results. For the membrane entry, we propose that electrostatic repulsion between negative charges in PS and peptide causes pHLIP to localize more shallowly on the membrane surface. Surprisingly, this charge-charge repulsion does not reduce the affinity for POPS vesicles compared to POPC. To rationalize these two observations we propose that in the membrane-bound state of pHLIP, most if not all of the negative charges in pHLIP point away from the membrane plane. This would not be surprising, since charged residues would be more stable in a hydrated environment than in the vicinity of the hydrophobic methylene groups of the acyl chains (138). Figure 19B shows the existence of

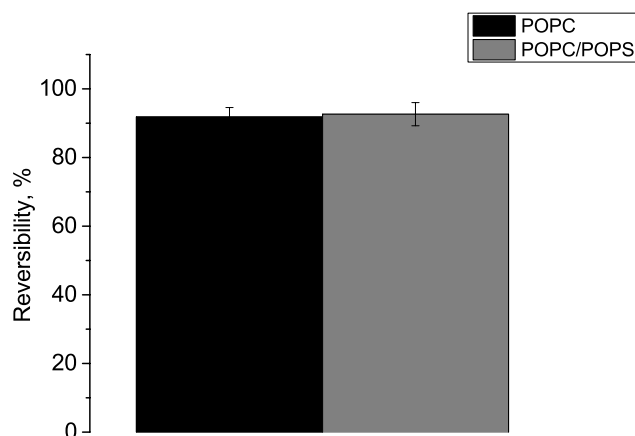


Figure 22. Reversibility of pHLIP.

Comparison of the reversibility of pHLIP in the presence of POPC and a mixture of POPC/POPS using CD. As represented in Figure 21, there was no significant difference in the amount of reversibility observed between POPC and POPC/POPS 90/10. Reversibility values were calculated by using the equation:

$$\left(\frac{\text{exit} - \text{entry}}{\text{initial} - \text{entry}} \right) \times 100$$

where exit is the ellipticity value at 222 nm for the exit state, initial is the ellipticity value at 222 nm for surface bound state, and entry is the ellipticity value at 222 nm for the inserted state. This was used to calculate the percent of reversibility for each lipid group tested.

hysteresis between the entry and exit transitions, with a pK difference close to 1 pH unit (Figure 20). Hysteresis is believed to occur when there is a large activation barrier to either folding or unfolding or both (117, 139). In the presence of PS, we observed differences between the peripheral state prior to and after insertion. Figure 19B shows that the CM value at pH 8 is 354 nm prior to formation of the TM and 349 nm after the membrane exit. Similarly, differences were also observed for the surface bound state in the fluorescence intensity at 335 nm (Figure 24) and monolayer experiments (Figure 25). We propose that the electrostatic repulsion imposes an energy barrier for the entry, resulting in the observed differences in the environment of the tryptophans. Interestingly, the difference in CM at pH 8 between POPC and POPC/POPS found prior to the entry (5 nm) vanishes after the exit. This suggests that the final location of pHLIP in the membrane (after exit) is not dependent of the membrane charge (Scheme 1). The differences in the location on the membrane surface before and after exit agree with the presence of hysteresis. An important membrane parameter that can impact the folding/insertion of membrane proteins into membranes is the membrane electrical potential (140, 141). The presence of negatively charged lipids affects the membrane surface potential. Consequently, we cannot rule out that the changes observed here in the presence of PS are not influenced by changes in the membrane electrical potential.

In this paper we report that the presence of a small percentage of negatively charged lipids severely impacts how pHLIP enters and exits membranes. Our CD data shows that the mechanism behind these changes is not a variation in the conformation of the peptide at the surface or inside the membrane. Instead, we propose that the observed pK differences result from subtle alterations in the environment of the acidic residues that control the entry and exit of pHLIP, with an important electrostatic repulsion component.

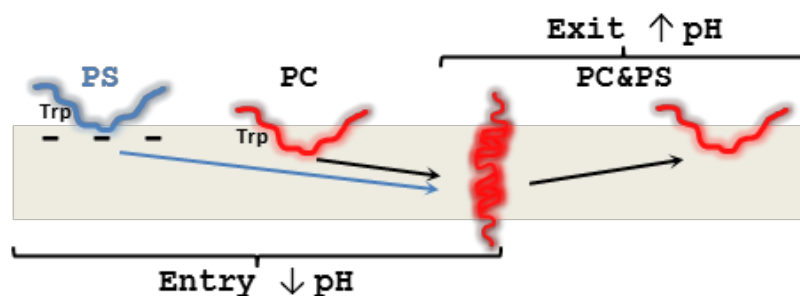


Figure 23. Model of membrane entry and exit of pHLIP.

The average location of the two Trp residues in pHLIP (W9 and W15) is shown. The position of the different acidic groups can be found in Table 1.

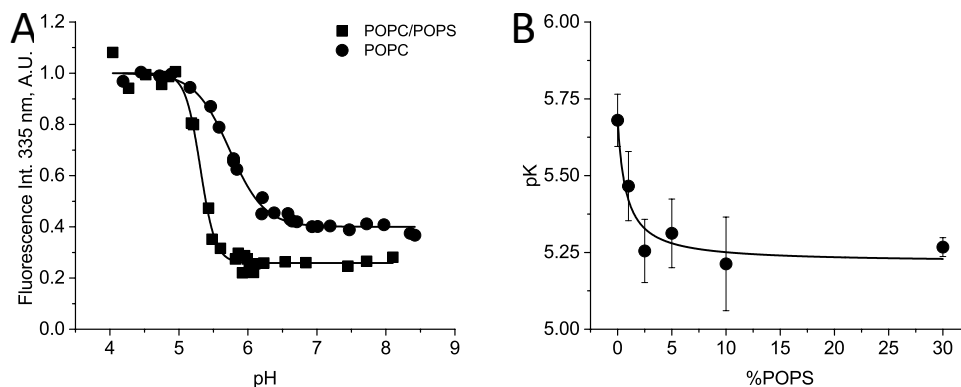


Figure 24. Fluorescence intensity characterization of the effect of PS on the membrane entry pK of pHLIP.

(A) Overlay of pH titrations demonstrating the change in pK for 10% PS (squares) compared to 0% PS (circles) in POPC. Data were normalized to average value for the acidic baseline (F_a). Curves were fitted to Eq. 1 (black line). pHLIP was incubated with lipid for one hour. pHLIP and lipid were added to a range of buffers (pH 4.1 to 8.5) to create the titration. Measurements were performed after the addition of pHLIP and lipid to the buffer range. Final peptide concentration was 1 μ M, and final lipid concentration was 200 μ M. (B) pK curve obtained from various pH titrations with differing percentages of PS showing that as PS percentage increased, pK decreased. Curve was fitted to Eq. 2, yielding a midpoint of PS as 0.8 ± 0.5 %.

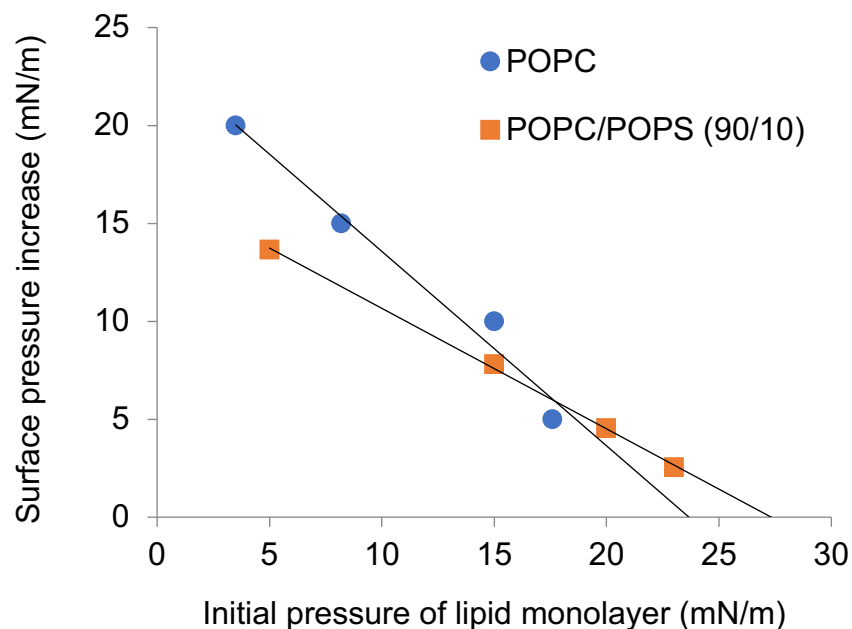


Figure 25. Effect of the binding of pHLIP to lipid monolayers.

The maximum increase in the surface pressure was determined for different initial pressures. The exclusion pressure was calculated by extrapolation of the fitting straight lines. The exclusion pressure was only 23.7 mN/m for POPC, and 27.3 mN/m for POPC/POPS 90/10.

Our results suggest that the presence of negatively charged lipids in the outer monolayer of the plasma membrane of cancer cells might affect how pHLIP targets tumors. By altering the protonation of the key aspartates in pHLIP, PS might hamper how pHLIP “senses” the extracellular acidity. Consequently, a higher proton concentration would be required for pHLIP to insert into tumors, discouraging peptide accumulation. Most tumors are only slightly acidic, while only a subset have extracellular pHs lower than 6.7 (125). Accordingly, the presence of PS at the outer surface of the membrane of cancer cells might hinder pHLIP targeting the more abundant, slightly acidic tumors. Still, this would not limit the targeting of the more acidic tumors. Understanding the effect of PS on the membrane insertion of pHLIP is a first step needed to refine the sequence of pHLIP, yielding pHLIP variants with improved tumor targeting.

3.6 Acknowledgements

We acknowledge Kristen Booth and Hannah McDonald for performing experiments.

Chapter IV: PS membrane asymmetry influences the folding and insertion of a transmembrane helix

4.1 Abstract

The plasma membrane (PM) has an asymmetric distribution of lipids between the inner and outer leaflets of its bilayer. A lipid of special interest in eukaryotic cells is the negatively charged phosphatidylserine (PS). In healthy cells, PS is actively sequestered to the inner leaflet of the PM but can redistribute to the outer leaflet when the cell is damaged or at the onset of apoptosis. The influence of this asymmetry and its loss on membrane protein structure and organization have not been widely addressed. Marginally hydrophobic membrane proteins contain acidic residues in their transmembrane sequence, which can enable topological transitions after membrane association. The pH low insertion peptide (pHLIP), which undergoes a topological reorientation and inserts into the membrane at low pH – as its name implies, is a useful and well-characterized model for studying these transitions. Although it is known that the inclusion of PS in symmetric vesicles affects the membrane insertion process of pHLIP by lowering the pH midpoint of insertion, it is unclear how PS asymmetry affects these topological transitions. Here, we studied pHLIP's topology using freely-floating asymmetric phosphatidylcholine (PC)/PS vesicles with PS enriched in the inner leaflet. We developed a modified protocol to create asymmetric vesicles containing PS and employed Annexin V labeled with an Alexa 568 fluorophore as a new probe to quantifying PS asymmetry. For pHLIP, membrane insertion was affected by the surface charge difference between bilayer leaflets as a result of the asymmetric distribution of charged lipids between the leaflets. We thus conclude that lipid asymmetry can have consequences for the behavior of membrane-associated proteins. A corollary is that model studies using symmetric bilayers to mimic the PM may fail to capture important aspects of protein-membrane interactions.

4.2 Introduction

The mammalian plasma membrane (PM) is a highly complex structure composed of hundreds of different lipids. Moreover, it has long been established that these lipids are not randomly arranged in the bilayer, but instead are asymmetrically distributed between the two bilayer leaflets (56). Specifically, the outer leaflet is enriched in phosphatidylcholines (PCs) and sphingomyelins, while the amino-containing glycerophospholipids such as phosphatidylserine (PS) and phosphatidylethanolamine (PE) are primarily located in the inner leaflet (56, 77-79). Lipid asymmetry is entropically unfavorable and must be actively maintained by the cell. Two classes of ATP-dependent transporters, with the ability to move phospholipids unidirectionally to a given leaflet (68, 83-85) have evolved for this purpose. These enzymes, termed flippases and floppases, counteract the movement of lipids down their concentration gradients by active or passive translocation, however, the scrambling of the lipids occurs through the non-ATP dependent activation of the scramblase transporter (68, 90, 142). Although there is still considerable uncertainty regarding the physiological role of PM asymmetry, its importance for proper cellular function is not in doubt (90).

The maintenance of proper PS asymmetry is important for cellular viability (90). The presence of the negatively-charged PS in the inner leaflet enhances the binding of many cytosolic proteins, including key signaling proteins such as phospholipase C or KRAS (143, 144). Unsurprisingly, the loss of PS asymmetry can lead to cellular malfunction and eventually death. For example, cell damage can result in the activation of scramblases, promiscuous lipid transporters that rapidly destroy membrane asymmetry (145). The resulting exposure of PS on the outer leaflet is a recognition signal for apoptosis to proceed via binding of annexins on the surface of macrophages (90).

Roughly half the mass of the membrane is proteins suggesting that membrane proteins are important constituents (146, 147). Transmembrane (TM) proteins contain sequences consisting largely of hydrophobic amino acids, driving protein insertion into the hydrophobic core of the membrane (148). Folding occurs through the formation of secondary structure that happens before membrane insertion (149, 150). It has also been proposed that membrane proteins fold independently of the lipid environment (151). A recent study investigated the importance of the lipid environment using the bacterial protein LacY (152) using a recently developed method to create lipid asymmetry in freely floating model membranes (153). The authors found that PE asymmetry led to topological reorientations of LacY. However, the experimental system was far from ideal as LacY dissipated bilayer asymmetry by strongly increasing the rate of lipid flip-flop (152). That membrane asymmetry can influence the topological orientation of membrane proteins is worthy of further consideration. PM proteins are synthesized in the symmetric membrane of the endoplasmic reticulum (ER), yet their final destination is an asymmetric membrane (79, 154). It is unknown if the dramatically different ER and lipid environment from the ER to the PM affects membrane proteins that have the potential to change their topology after membrane insertion.

Here, we use the pH-low insertion peptide (pHLIP) as a model system to study how PM asymmetry affects the folding and insertion of membrane proteins. pHLIP assumes two different membrane topologies depending on the pH, as a result of changes in protonation in its seven charged residues. This characteristic enables experimental control of pHLIP's topologies termed States II (membrane associated at neutral pH) and III (inserted as a TM helix at acidic pH) (4). Unlike membrane active peptides such as GALA, pHLIP does not cause membrane leakage or disruption, a requisite for maintaining membrane asymmetry (4, 155). The peptide has been studied in different membrane compositions and its behavior is well characterized (4, 5, 18, 95). Using symmetric PS vesicles, our lab previously showed that both the insertion pK and the

insertion depth in State II decreased with increasing PS concentration (95). We proposed that both observations can be explained by an unfavorable interaction between the negatively charged PS head group and the seven negative charges present on pHLIP at neutral pH (95). However, it is unknown how a more biologically faithful model system, one in which PS is enriched in the inner leaflet, would influence pHLIP insertion. To test this, we modified a technique for producing freely-floating vesicles in order to mimic the asymmetric distribution of PS in the PM (156). We found that PS asymmetry caused an increase in the midpoint of pHLIP insertion, suggesting that for a physiologically relevant transbilayer charge distribution— i.e., less negative charge in the outer compared to the inner leaflet— lowers the energetic barrier for insertion. This finding proposes a general role for PS asymmetry in promoting the folding of TM proteins.

4.3 Materials and Methods

Materials. The lipids 1-palmitoyl-d31-2-oleoyl-*sn*-glycero-3-phosphocholine (POPCd31), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) were purchased from Avanti Polar Lipids (Alabaster, AL) and used as is. Annexin V, Alexa Fluor 568 conjugate (Annexin V-568) was purchased from ThermoFisher Scientific (Waltham, MA) and assayed for concentration with an Agilent Cary 100 UV-Vis spectrophotometer (Santa Clara, CA) using an extinction coefficient of $23,380 \text{ M}^{-1}\text{cm}^{-1}$. pHLIP (sequence: N_t-AAEQNPYIYWARYADWLFTTPLLALLVDADEGTCG-C_t), synthesized using standard solid phase protocols and purified by reverse-phase high performance liquid chromatography (HPLC) to greater than 95% purity, was purchased from P3 Biosystems (Louisville, KY). A lyophilized pHLIP stock was dissolved in buffer (10 mM NaPi pH 8.0) and assayed for concentration by UV-Vis using an extinction coefficient of $13,940 \text{ M}^{-1} \text{ cm}^{-1}$. Methyl- β -cyclodextrin (M β CD), sucrose, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2-(*N*-morpholino) ethanesulfonic acid (MES), calcium chloride (CaCl₂), sodium acetate (NaOAc) buffer, and sodium phosphate buffer (NaPi) were all purchased from Sigma-Aldrich (St. Louis, MO). Buffers were prepared by weighing the powder and adding ultrapure water to obtain the desired concentration. HEPES, MES, NaOAc buffers were adjusted to the correct pH using hydrochloric acid or sodium hydroxide. Ultrapure water was obtained from a Millipore Milli-Q Academic (MilliPore Sigma, Burlington, MA) source.

Asymmetric vesicle preparation and quantification. Asymmetric vesicles were prepared following the protocol from (156) with minor modifications due to the inclusion of POPS. Briefly, PS asymmetry was generated by using M β CD to catalyze the exchange of POPCd31 from multilamellar vesicles (MLVs) to the outer leaflet of large unilamellar vesicles (LUVs) composed of POPCd31 and POPS in a molar ratio of 93/7. Lipid films for donor MLVs were hydrated with

0.75 mL of 20% w/w sucrose, and films for acceptor vesicles were hydrated with 0.5 mL ultrapure water; the concentrations of aqueous donor and acceptor were 25.3 mM and 12.6 mM, respectively. Donors were subjected to 4x freeze/thaw cycles while acceptors went through 5x cycles. Donor MLVs were diluted to 4.38 mM and incubated with 4.33 mL of 35 mM M β CD for two hours at room temperature in an 8:1 molar ratio. The acceptors were extruded through a 100 nm pore size polycarbonate membrane (31 passes) using a Mini Extruder (Avanti Polar Lipids, Inc., Alabaster, AL) to form LUVs. Acceptor LUVs were then added to the donor/M β CD mixture and incubated for one hour at 30°C, (although the original protocol calls for incubation at room temperature, we found that a slightly increased temperature allowed for greater PS exchange). The donor:acceptor molar ratio during exchange was 3:1. After the exchange step, residual MLVs were removed by centrifugation at 20,000 x g for one hour, and the supernatant containing the asymmetric vesicles was washed 4 times with buffer (1 mM HEPES/1 mM CaCl₂/pH 7.4) and concentrated using a 100,000 MWCO centrifugal filter device (Amicon Ultra-15, EMD Millipore, Billerica MA). Gas chromatography coupled to mass spectrometry (GC/MS) (Agilent Technologies 7890A, Santa Clara, CA) was used to quantify the initial and final POPS concentrations in the vesicles after derivatization of the lipids to fatty acid methyl esters (FAMES), as in (156). The FAMES derived from protiated and deuterated palmitic acid were separately resolved by GC (157), allowing for quantitation of the mole fractions of POPCd31 and POPS (156). Three replicate samples of the initial acceptor vesicles and of the final asymmetric vesicles were measured to determine error bars. The mol % of POPS in the outer leaflet was calculated using the equation:

$$V_T C_T = C_A V_A + C_B V_B, \quad (\text{Eq. 1})$$

where V_T is the total fractional vesicle volume (1), C_T is the total concentration of POPS, C_A and C_B are the concentrations of POPS in the outer and inner leaflet, and V_A and V_B are the volume

fractions of the outer and inner leaflet (0.51 and 0.49, respectively for 100 nm diameter vesicles). Vesicle concentration was determined by an inorganic phosphate assay (158). Dynamic light scattering (DLS) was used to determine the size and polydispersity of both the acceptors and asymmetric vesicles using either a Brookhaven Instruments BI-200SM (Brookhaven Instruments, Holtsville, NY) or a Wyatt DynaPro NanoStar (Wyatt Technology, Santa Barbara, CA) instrument. For the Wyatt instrument, the MW-R model was set for globular proteins and the Rg model was set for spheres. For the Brookhaven Instrument, 90-degree light scattering was measured using a 633 nm HeNe laser light source operated at 30 mW and a detector aperture of 200 μ m. Data collection time was 4 minutes and the obtained autocorrelation curve was fit with both CONTIN and cumulant analyses. DLS measurements were taken at 25°C.

Annexin V assay and lipid flip flop. Symmetric POPC vesicles with 0, 1, 2.5, 3.5, 5, 8, 10, 15, 20, and 50 mol % POPS were prepared in buffer (1 mM HEPES/1 mM CaCl₂/pH 7.4) as described above. Annexin V-568 (stock concentration determined via UV/Vis) and buffer (25 mM NaOAc/1 mM CaCl₂/pH 5.4) were added to the vesicles and incubated in the dark for 1 hour; the final lipid and Annexin V-568 concentrations were 50 μ M and 0.52 μ M, respectively, and the final sample pH was 5.5. Fluorescence measurements were made at room temperature with a Photon Technology International (Edison, NJ) Quanta Master fluorometer using the following instrument settings: excitation wavelength 579 nm, emission wavelength 601 nm, 15 s integration, 90° excitation polarization, 0° emission polarization, and 4.8 nm excitation and emission slit widths. Appropriate lipid blanks were subtracted in all cases, and changes in intensity were normalized to the POPC control. Symmetric samples of varying POPS concentration were used to determine a calibration curve that was fitted with the equation:

$$Signal = F_0 + \Delta F(K_p x)/(55.3 + K_p x), \quad (\text{Eq. 2})$$

where F_0 is the initial fluorescence intensity, ΔF is the change in fluorescence intensity, x is the mol % of POPS, and 55.3 is the molar concentration of water (12, 149). This equation was used to determine the molar partition coefficient, K_p , of Annexin V binding to the membrane in the presence of different levels of PS. Using this assay, we determined the molar % of POPS exposed at the outer leaflet of the asymmetric samples via a decrease in Annexin V-568 intensity as it bound PS. Using Annexin V-568 intensity values in the presence of asymmetric samples, we normalized the values to the PC control and inputted them into Eq. 2 to determine the mol % POPS in the outer leaflet. Measurements on asymmetric samples were taken for multiple days to assess the level of asymmetry and lipid flip flop.

Lipid flip flop in the presence of pHLIP was determined by performing the Annexin assay with asymmetric vesicles. pHLIP was incubated with POPC (control) and asymmetric vesicles for 1 hour at pH 7.4. Annexin V-568 was then added and incubated as described earlier. The final pH was 5.5, and the final pHLIP concentration was 0.25 μ M. Intensity changes in the presence of pHLIP were analyzed as described earlier.

Calculation of leaflet surface potentials. The surface potential of each leaflet in the asymmetric bilayer was calculated using the Grahame equation:

$$\sigma = \sqrt{8c_0\epsilon\epsilon_0k_BT} \sinh\left(\frac{e\psi_0}{2k_BT}\right), \quad (\text{Eq. 3})$$

where σ is the surface charge density, c_0 is the ion concentration, ϵ is the dielectric constant (here, 78.3), ϵ_0 is the vacuum permittivity, k_B is the Boltzmann constant, T is the absolute temperature (here, 298.2 K), and ψ_0 is the surface potential (159). The surface charge density was calculated using the Guoy-Chapman Theory (159) separately for each leaflet using the measured POPS concentrations, and assuming an average area per lipid of 62.7 $\text{\AA}^2$ (160). Solving Eq. 3 for ψ_0 gives the surface potential.

Intrinsic tryptophan fluorescence spectroscopy. Symmetric POPCd31 vesicles with 0, 3, 7, and 30 mol % POPS were prepared *via* extrusion using a 100-nm pore size membrane (Whatman, UK) using a Mini Extruder (Avanti Polar Lipids, Inc., Alabaster, AL) in 10 mM NaP_i pH 8 to form LUVs. Asymmetric vesicles were in 1 mM HEPES/1 mM CaCl₂/pH 7.4. Both symmetric and asymmetric vesicles were incubated with pHLIP for 1 hour at room temperature, for a final lipid:peptide molar ratio of 200:1. Final peptide concentration was 1 μ M. It was previously shown that inclusion of the C-terminal cysteine in pHLIP does not cause disulfide-mediated dimerization (14). A pH titration was performed by adjusting the pH of the different samples with 100 mM stocks of NaOAc, MES, or HEPES buffers (25 μ L), to obtain the desired final pH values. The final sample volume was 140 μ L for symmetric samples, and 125 μ L for asymmetric samples. A 2.5 mm-bulb pH-electrode (Microelectrodes, Inc., Bedford, NH) was employed to measure the final pH of each sample. Emission spectra were recorded using a Photon Technology International (Edison, NJ) Quanta Master fluorometer at room temperature with the following settings: excitation wavelength 280 nm, emission wavelength range 310-400 nm, and excitation and emission slits 3 nm. Appropriate lipid blanks were subtracted in all cases. Data were analyzed by calculating the spectral center of mass (CM) with the following equation:

$$CM = \sum_i I_i \lambda_i / \sum_i I_i, \quad (\text{Eq. 4})$$

where I_i is the fluorescence intensity at wavelength λ_i . CM uses the entire spectral range of the data to inform on the local environment of the two Trp residues (99, 100). The data were also analyzed by monitoring changes in the fluorescence emission intensity FI at 335 nm, which is directly proportional to the population of molecular species present (101). CM and FI pH-titrations were then fitted to determine the pK using the equation:

$$Signal = (F_a + F_b 10^{m(pH-pK)}) / (1 + 10^{m(pH-pK)}), \quad (\text{Eq. 5})$$

where F_a is the acidic baseline, F_b is the basic baseline, m is the slope of the transition, pK is the midpoint of the curve, and *Signal* refers to the changes in the fluorescence or circular dichroism signals as a function of pH.

Circular Dichroism. CD measurements were performed using a Jasco (Easton, MD) J-815 spectropolarimeter at 25°C. pHLIP was incubated with POPC, symmetric POPC/POPS 97/3, or asymmetric vesicles (prepared as described earlier) in 10 mM NaP_i buffer (pH 8.0) for 1 hour. The pH was then adjusted with 100 mM NaOAc or NaP_i to a range of desired final pH values. The final sample volume, in each case, was 250 μ L. For POPC samples, the lipid:peptide molar ratio was 200:1 with a final peptide concentration of 7 μ M and a final lipid concentration of 1.4 mM. For the POPC/POPS 97/3 and asymmetric samples, the final peptide concentration was 3 μ M and a final lipid concentration of 600 μ M. Helical content changes of pHLIP were determined by measuring the ellipticity at 222 nm as a function of pH as in (15). Molar ellipticity was determined with the following equation:

$$[\theta] = \Theta / (10lc(N - 1)), \text{ (Eq. 6)}$$

where Θ is the measured ellipticity, l is the cell path length, c is the protein concentration, and N is the number of amino acids (here, 38) (161). Calculated molar ellipticity at 222 nm was plotted against measured pH, and the resulting sigmoidal transition was fitted using Eq. 2 to obtain the pK_{CD} . Spectra were collected from 260-195 nm for samples at pH 8 and 4 using the same temperature and scan rate as earlier, but with a 1 nm step size. Spectra were collected to check for secondary structure other than at 222 nm, which allowed for a detailed comparison of pHLIP in symmetric and asymmetric membranes.

Statistical analysis. Statistical analysis was performed on the pK values and samples from the annexin assay to determine if the observed changes were significant. The analysis was performed

using SPSSv25 software (IBM Analytics, Armonk, NY). One-way ANOVA and two different post hoc multiple comparisons tests were used based on the homoscedasticity (2-sided Dunnett t-test) or heteroscedasticity (Dunnett T3) of the data. Both the 2-sided Dunnett t-test and the Dunnett T3 set one variable as the control to compare to all other treatment groups. A 2-sided Dunnett t-test was used to determine the statistical significance of pK values determined via Trp fluorescence and CD by comparing all samples to the asymmetric vesicles sample. A one-way ANOVA was used to determine the statistical significance of the annexin assay results (Fig. S1). A Dunnett T3 test was used to determine the statistical significance of the stability of POPS asymmetry by comparing day 1 after the exchange to all subsequent days. A 2-sided Dunnett t-test was used to determine the statistical significance of lipid flip flop in the presence of pHLIP by comparing samples in the presence of pHLIP to samples without pHLIP. $P \leq 0.05$ was considered significant for all tests.

4.4 Results

Preparation and quantification of asymmetric PS vesicles. The goal of this study was to understand how PS asymmetry influences the folding and insertion of a TM helix in a well-controlled and characterized model membrane system. A wide variety of techniques have been developed for the preparation of asymmetric bilayers, each with strengths and weaknesses (162, 163). For our study, it was important to use freely-floating vesicles, rather than solid supported bilayers, and to avoid the use of osmolytes that can potentially interact with the bilayer and/or create membrane tension (153, 164). To this end, we used the technique of methyl- β -cyclodextrin-mediated lipid exchange pioneered by the London group (153) with modifications that eliminate the requirement for concentrated sucrose in the vesicle core (Figure 26) (156). Our strategy was to prepare symmetric POPC/POPS acceptor vesicles with PS as a minor component (~ 7 mol%), and then replace the POPS in the outer leaflet with POPC from a donor vesicle pool to generate asymmetric vesicles (aLUVs) with PS enriched in the inner leaflet.

aLUVs, once prepared, will gradually equilibrate to a symmetric state via passive lipid flip-flop. Although some studies have reported fast flip-flop (half times of seconds to minutes) in supported bilayers (165), there is general agreement that lipid flip-flop is much slower (half times of hours to days) in vesicles (68, 70, 142). To monitor PS asymmetry, we first used GC/MS to determine the total PS concentration in the aLUVs (156). Because GC/MS cannot distinguish between inner and outer leaflet lipid populations (156, 166), we used externally added Annexin V conjugated with the Alexa-568 dye (AnnexinV-568) to determine the amount of PS exposed in the outer leaflet. Annexin V binds specifically to PS headgroups in the presence of calcium (167) and is routinely used in cell biology to determine the onset of apoptosis, which is marked by the exposure of PS to the extracellular environment (168). In symmetric vesicles, we observed a monotonic decrease in the fluorescence intensity of Annexin V-568 as PS concentration was

increased (Figure 27). Using this data as a calibration curve, we then assayed the concentration of exposed PS in aLUVs. Prior to outer leaflet exchange, the acceptor LUVs had an average exposed PS concentration of ~ 7 mol% ($N = 5$), which decreased to ~ 3 mol% in the aLUVs (Figure 28). Statistical analysis revealed no significant differences in exposed PS concentration among the aLUV exchanges ($P > 0.05$), an indication that the preparation of asymmetric PS-containing vesicles was robust and reproducible.

Next, we investigated the stability of aLUVs by monitoring PS asymmetry over time. Figure 29A shows that no significant loss of asymmetry in four-day old aLUVs occurred ($P > 0.05$). We also examined the influence of pHLIP on the stability of PS asymmetry. It has been previously reported that the presence of transmembrane peptides can accelerate lipid flip-flop and lead to a rapid loss of membrane asymmetry (169). However, we found that pHLIP in either State II or III did not lead to a significant loss of PS asymmetry over 3 hours via the annexin assay, a period of time greater than the time needed to complete our fluorescence spectroscopic assay ($P > 0.05$) (Figure 29B). We also observed no changes in lipid flip-flop at room temperature over a period of 108 hours (data not shown). These results indicate that pHLIP does not influence the rate of POPS flip-flop on our experimental timescales.

PS membrane asymmetry influences the insertion pK of pHLIP. The pK of insertion (midpoint of insertion) (4, 14) is a key parameter describing pHLIP's interaction with membranes. pHLIP insertion occurs in multiple steps as pH is lowered (20, 21), and we recently reported that the insertion process is characterized by at least three macroscopic pK values that require different analyses and/or techniques for understanding (15, 17). Specifically, when using the fluorescence emission spectra of pHLIP's two Trp residues to monitor insertion into symmetric POPC vesicles,

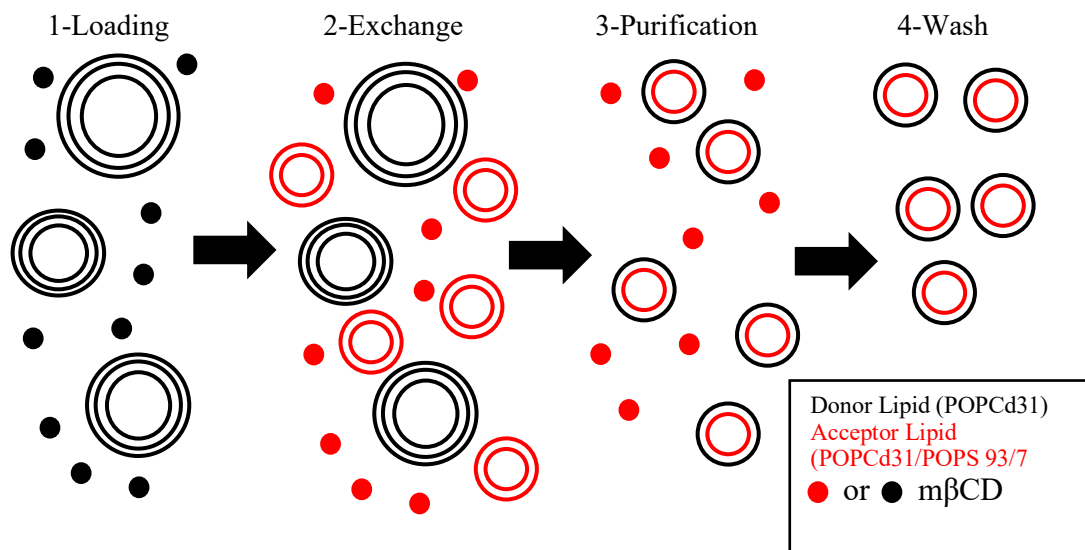


Figure 26. Cyclodextrin mediated exchange used to generate asymmetric vesicles.

Steps were as follows: 1- mβCD is incubated with sucrose-loaded donor multilamellar vesicles to load donor lipid into mβCD. 2- Acceptor unilamellar vesicles are added for exchange to proceed with mβCD delivering donor lipid to the acceptors. 3- Donors are removed by centrifugation. 4- Asymmetric vesicles are washed in buffer of interest to remove mβCD. Black mβCD corresponds to the POPCd31-loaded state, and red mβCD is the loaded form containing POPS. Adapted from (156, 157).

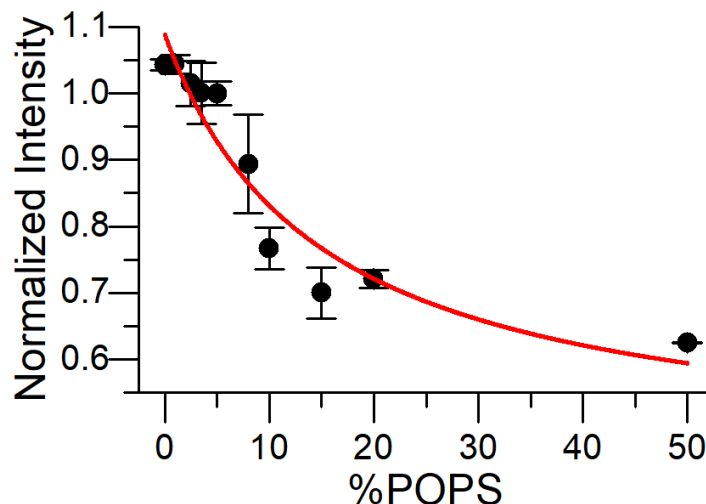


Figure 27. Annexin V-568 used to quantify the amount of PS in the outer bilayer leaflet.

In the presence of PS, fluorescent Annexin V-568 exhibits decreased intensity with increasing concentration of exposed (outer leaflet) PS. Symmetric PC vesicles containing various levels of PS were used to generate a calibration curve. Red line is a fit to the data using a binding model (Eq. 2).

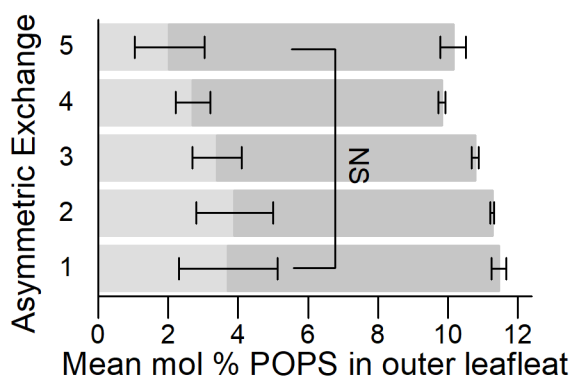


Figure 28. Comparison of PS levels in the outer bilayer leaflet before (dark gray) and after (light gray) exchange.

The calibration curve in A is used to determine the level of membrane asymmetry for days post exchange. The mean level of PS asymmetry for five different asymmetric exchanges is shown to verify the reproducibility of the exchange protocol. Statistical analysis (one-way ANOVA) concluded there were no statistical differences between the five different exchanges. NS; no significance. Error bars are standard deviation.

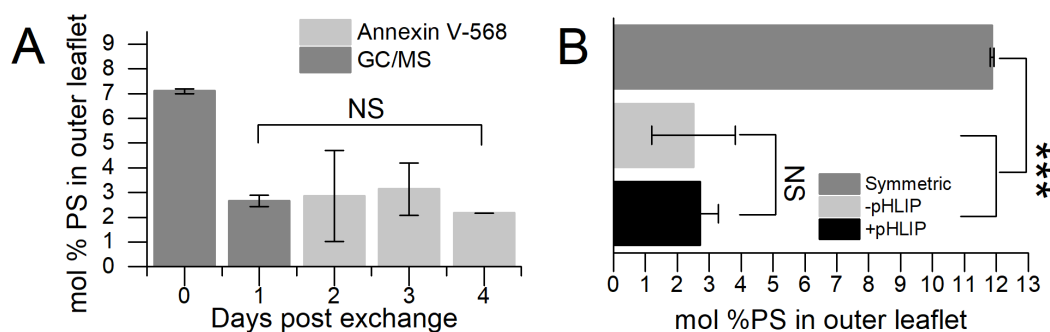


Figure 29. PS asymmetry is stable for days post exchange and in the presence of pHLIP.

(A) Stability of asymmetric vesicles monitored for a period of four days post exchange. Day zero indicates the level of PS in the outer leaflet prior to exchange. Days 1-4 display the level of asymmetry post exchange. Both GC/MS and Annexin V-568 were used to determine the level of membrane asymmetry independently. Error bars represent the measurement uncertainty for each day obtained from replicates. (B) Comparison of PS levels in the outer leaflet of asymmetric vesicles incubated in the presence and absence of pHLIP. Here, pHLIP was incubated with asymmetric vesicles in both State II and III within the same sample by changing the pH after a given period of time. The symmetric vesicle PS level is prior to exchange and the asymmetric vesicle level is post exchange. Here the symmetric PS level was ~12%. The inclusion of pHLIP in the asymmetric vesicles does not lead to a loss of membrane asymmetry over a three-hour period post addition of pHLIP. ***, $P \leq 0.005$; NS; no significance. Error bars are standard deviation.

we found different insertion pK values depending on whether the response metric was the spectral center-of-mass (CM) or the fluorescence intensity at fixed wavelength (FI) (15). We refer to these insertion pH midpoints as pK_{CM} and pK_{FI} , respectively.

We first used both native Trp and nitrobenzoxadiazole (NBD) conjugated to a C-terminal Cys residue fluorescence, as described previously (15), to determine pHLIP insertion pK s in symmetric POPCd31/POPS vesicles with increasing POPS concentration. As previously reported (130), decreasing the solution pH caused a shift in the Trp emission maximum to shorter wavelengths and an increase in intensity (Figure 30A). These changes result from alterations in the local environment of the Trp residues, consistent with pHLIP insertion into the membrane. As NBD is also an environmentally sensitive dye, similar changes were observed compared to Trp (105-109). We then analyzed the emission spectra to determine the insertion pK_{CM} , pK_{FI} , and pK_{NBD} (Figure 30B-E and Figure 31). We found that pK_{FI} was ~ 0.5 units lower than pK_{CM} at each PS concentration and that both pK_{CM} (Figure 30D) and pK_{FI} (Figure 30E) decreased by ~ 0.4 units as POPS concentration increased from 0 to 7 mol %. Similarly, we observed a pK_{NBD} that was comparable to previous results, as well as a decrease in the presence of symmetric PS to a concentration of 7% (15). The comparable influence of PS suggests that deuteration does not influence the insertion of pHLIP. However, symmetric membranes do not recapitulate the PM, promoting the study of PS asymmetry.

We next examined aLUVs that typically contained ~ 3 mol % PS in the outer leaflet and ~ 7 mol % PS in the inner leaflet. We observed a statistically significant increase in both pK_{CM} and pK_{FI} compared to symmetric membranes with similar PS concentration ($P < 0.05$). Specifically, pK_{CM} of the asymmetric vesicles was 6.26 ± 0.14 compared to 5.91 ± 0.15 and 5.79 ± 0.07 for

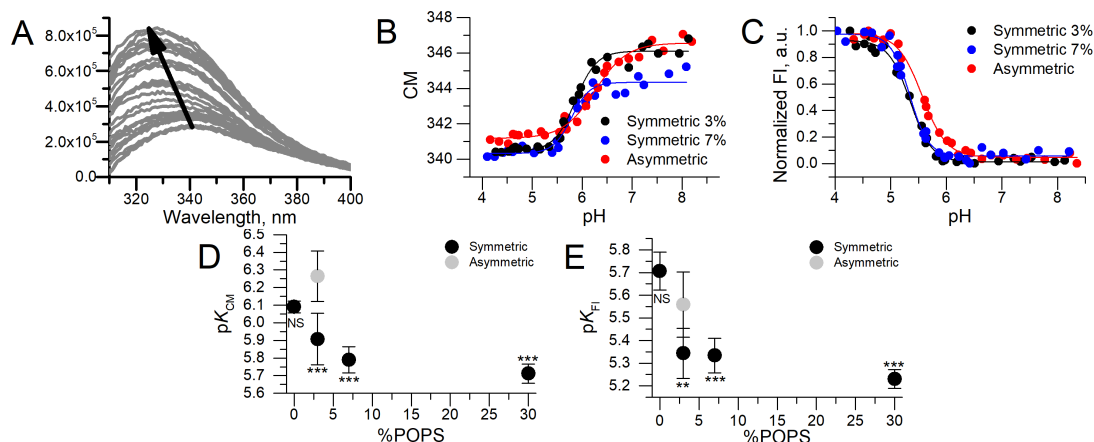


Figure 30. PS membrane asymmetry alters pK_{CM} and pK_{FI} .

Intrinsic Trp fluorescence indicates PS membrane asymmetry affects the pK of insertion of pHLIP causing a basic shift in the pK values compared to symmetric PS. (A) Representative Trp spectra displaying changes in Trp fluorescence as pH is decreased (arrow) in asymmetric vesicles. (B) and (C) Representative pK titrations for CM and FI Trp fluorescence analysis comparing asymmetric samples with symmetric samples with 3% and 7% PS. Titrations were fit to Eq. 3 to yield pK_{CM} and pK_{FI} . (D) and (E) shows a comparison of pK of insertion between asymmetric and symmetric vesicles with varying levels of PS for both a CM (D) and FI (E) analysis. Statistical analysis was conducted comparing asymmetric to symmetric samples (one-way ANOVA, 2-sided Dunnett t-test). **, $P \leq 0.01$; ***, $P \leq 0.005$; NS; no significance. Error bars are standard deviation.

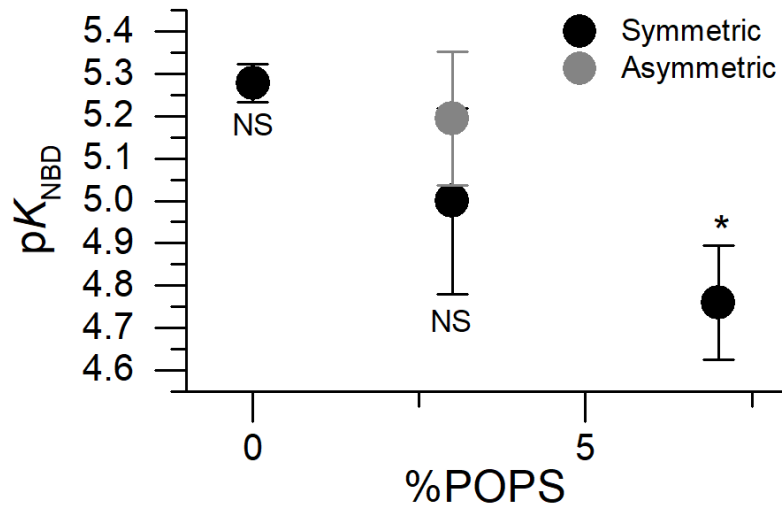


Figure 31. PS membrane asymmetry has no influence on pK_{NBD} .

NBD conjugated to a C-terminal Cys residue fluorescence was used to determine the pK of translocation. We found that PS membrane asymmetry does not influence the pK of translocation of pHLIP, compared to symmetric 3% PS. Statistical analysis was conducted comparing asymmetric to symmetric samples (one-way ANOVA, 2-sided Dunnett t-test). *, $P \leq 0.05$; NS; no significance. Error bars are standard deviation.

symmetric vesicles containing 3 and 7 mol % PS, respectively (Figure 30D). Similar changes were observed in pK_{FI} – the aLUV value was 5.56 ± 0.14 , while it was 5.34 ± 0.11 and 5.33 ± 0.08 for symmetric samples containing 3 and 7 mol % PS, respectively (Figure 30E). Remarkably, pK_{CM} for the aLUVs was even higher than that of symmetric vesicles lacking PS, with the difference in the two pK values (i.e., $pK_{CM} - pK_{FI}$) increasing to nearly 0.7 units, compared to 0.4-0.55 units for symmetric membranes. For pK_{NBD} , we observed no statistically significant difference in the aLUV samples when compared to symmetric samples containing 3% PS. The increase in pK_{CM} and pK_{FI} suggests that PS asymmetry affects the membrane insertion of pHLIP in a manner that cannot be simply predicted from the outer leaflet composition.

Secondary structure formation is not altered by PS membrane asymmetry. pHLIP must adopt a stable secondary structure prior to membrane insertion (149, 170). Using circular dichroism (CD), we previously observed that PS has no influence on the secondary structure of pHLIP in State II and III in symmetric vesicles composed of POPC/POPS 90/10 mol% (95). Figure 32A shows CD spectra of pHLIP in symmetric (3 mol % PS) and asymmetric vesicles at pH values representing State II (pH 8) and State III (pH 4), revealing that pHLIP exhibits comparable helical content whether the membrane contains an asymmetric or symmetric PS distribution. This result suggests that PS asymmetry has little effect on pHLIP secondary structure at the initial and final states of the insertion process.

As mentioned in the previous section, we recently proposed that different analysis methods for determining the insertion pK report independently on the protonation of different acidic residues in pHLIP (15). Specifically, we found that pK_{CD} informs on the formation of helical content on the membrane surface. Since pK_{CM} and pK_{FI} showed a significant increase in

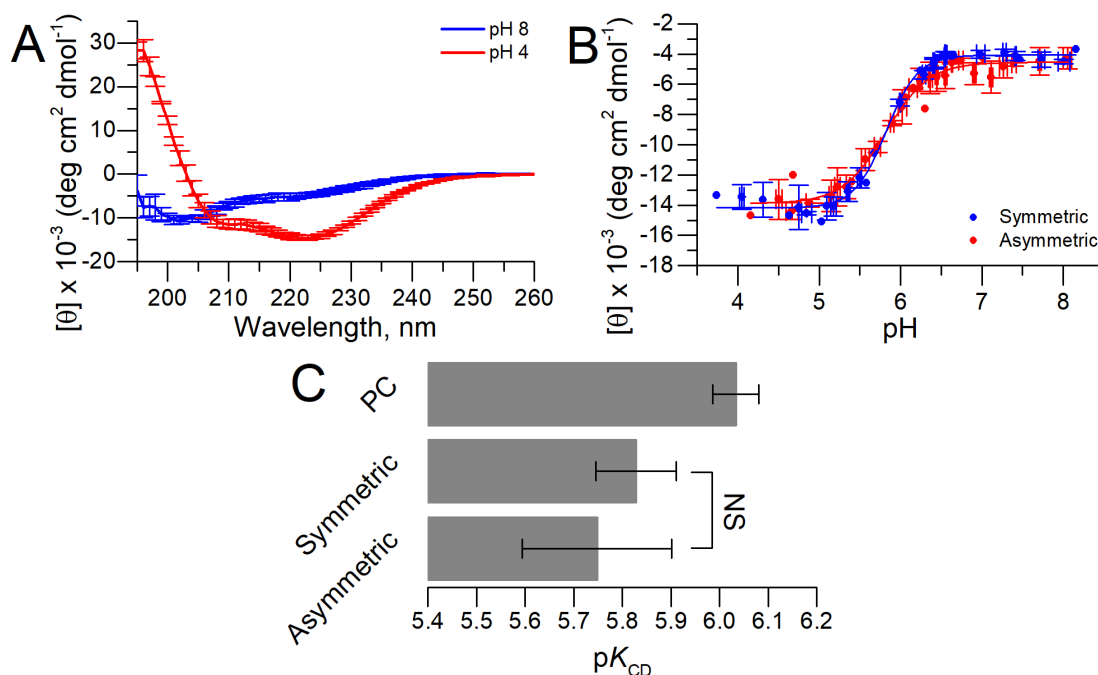


Figure 32. PS membrane asymmetry has no substantial influence on the helical formation of pHLIP.

(A) Average CD spectra of pHLIP in the presence of PS asymmetry. pH 8 and 4 represent the membrane absorbed and transmembrane states of pHLIP, respectively. (B) Average CD titrations comparing symmetric and asymmetric POPC/POPS vesicles with 3% PS in the outer bilayer leaflets for both cases. Lines indicate fits to the data using equation 3. (C) pK_{CD} obtained from titrations of PC and symmetric PC/PS with 3% PS, and with asymmetric PS samples. No statistical significance (one-way ANOVA) is observed between symmetric and asymmetric PS, suggesting that asymmetric PS does not influence the insertion process of pHLIP as monitored by CD. NS; no significance.

asymmetric PS vesicles, we investigated if pK_{CD} was similarly affected. Figure 32B shows a comparison of average pH titrations of symmetric (3 mol% PS) LUVs and aLUVs containing ~ 3 mol% PS in the outer leaflet. Figure 32C shows no significant effect of PS membrane asymmetry on the pK_{CD} compared to symmetric samples ($P > 0.05$), suggesting that PS asymmetry affects only some steps in the membrane insertion of pHLIP.

4.5 Discussion

PS asymmetric vesicles can be prepared and are stable over multiple days. Membrane lipid asymmetry is a key property of cellular membranes (56). Membrane asymmetry is maintained by ATP-dependent enzymes that translocate lipids to their intended leaflet with high head group specificity, while subgroups of the ATP binding cassette proteins flop lipids with low head group specificity (68, 83-85). Loss of these mechanisms of controlled lipid localization is a property of apoptosis and cell death (90). For example, Scott's syndrome, a bleeding disorder, is associated with a problem in the regulation of membrane asymmetry, and is the only disease known to be with linked membrane asymmetry (171).

Here, we modified a recently developed technique that used cyclodextrin to create tensionless PC vesicles with an asymmetric chain distribution (70, 156). We aimed instead to create vesicles with an asymmetric PS distribution mimicking that found in the mammalian PM. We found that minor variations had to be made to the original technique to accommodate the movement of PS, possibly due to the slight differences in how m β CD solubilizes PS compared to PC (172). Once the vesicles were prepared, we needed to measure the level of PS asymmetry. A variety of techniques have been described for measuring the asymmetry of different lipid species and we first settled on nuclear magnetic resonance (NMR) as used by Heberle *et al.* for determining PS asymmetry (156). However, this approach failed, possibly due to a strong interaction between the positively charged chemical shift reagent Pr^{3+} and the negatively charged PS head group (data not shown). We also explored using trinitrobenzenesulfonic acid, a molecule known to interact with

PE and PS (173). Unfortunately, TNBS showed little sensitivity to PS and failed to accurately detect PS levels in symmetric vesicles (data not shown).

After exhausting the available options from the literature, we took advantage of the well-known PS binding properties of Annexin V (167, 168), turning to a fluorescent version of this protein. We discovered that the fluorescence intensity of the Annexin V-568 conjugate is sensitive to the concentration of PS in the outer leaflet, which allowed us to assay for PS concentration in asymmetric vesicles using a calibration curve prepared from symmetric vesicles. We speculate the decrease in Alexa 568 fluorescent intensity is due to quenching caused natural amino acids like Trp (174). Using this assay, together with GC/MS, we were able to consistently quantify the level of PS asymmetry in our aLUVs. The annexin assay showed that PS asymmetry could be generated and was stable for multiple days (Figs. 27 and 29). Using this same assay, we also determined that pHLIP did not disrupt asymmetry (Figure 29B). This finding is of fundamental importance, since proteins and peptides often cause a loss of lipid bilayer asymmetry (152, 169), impeding studies looking into the effect of lipid asymmetry.

PS asymmetry may be particularly stable in part due to the unfavorable free energy barrier for transporting a negatively charged head group across the apolar membrane core. Moreover, pHLIP, with its many negative charges in State II, interacts adversely with PS (95). In State III, pHLIP does not disrupt the membrane and only interacts with a few lipid shells in close proximity to it (4, 13). This could explain our observation that pHLIP does not increase the rate of PS flip flop.

PS asymmetry influences the insertion of pHLIP. The interaction of pHLIP with bilayers also depends on the specific lipid composition. However, all previous studies were carried out with symmetric bilayers (5, 18, 95, 130, 175). We have reported that symmetric PS vesicles decreased the insertion pK compared to PC vesicles, with a saturation at ~ 5 mol% PS (95). This was proposed

to result from the unfavorable interaction between the negative charge on the PS headgroup and the seven negative charges on pHLIP at neutral pH (95). In a similar report, symmetric vesicles containing an assortment of lipid head groups, including PS, were studied to examine how their presence influenced pHLIP (175). This study also concluded that PS affects the insertion of pHLIP in symmetric membranes. However, an asymmetric distribution of PS, mimicking the plasma membrane, was missing from all these studies.

Here we study the effect of PS asymmetry on the membrane insertion of pHLIP. However, pHLIP's membrane insertion is not fully described by one insertion pK and have been reported using different analysis methods (15). Specifically, in symmetric PC bilayers, pK_{CM} and pK_{CD} reported on the same pHLIP protonation event, whereas pK_{FI} reported on different ones (15). We found that the insertion pK in asymmetric PS vesicles determined from Trp fluorescence using both a CM and FI analysis is significantly increased ($P < 0.05$) compared to a similar symmetric distribution of PS (Figure 30). However, pK_{CD} , which describes the midpoint of helical formation (15), was unaffected ($P > 0.05$) (Figure 32). Furthermore, we also determined that the pK_{NBD} (pK of translocation) was not influenced by PS membrane asymmetry compared to symmetric membranes with 3% PS. No changes in pK_{CD} or pK_{NBD} may indicate that PS asymmetry promotes the integration of Trp residues into the membrane before helical formation is complete, and that it does not influence the translocation of the C-terminus across the membrane (Figure 33). As seen in Figure 30 and 32, the titration reported by CM is largely complete before a large change occurs in the titration reported by CD, which indicates that in asymmetric membranes CM and CD are decoupled in reporting the protonation of acidic residues (Figure 33) (15). The decoupling of CM

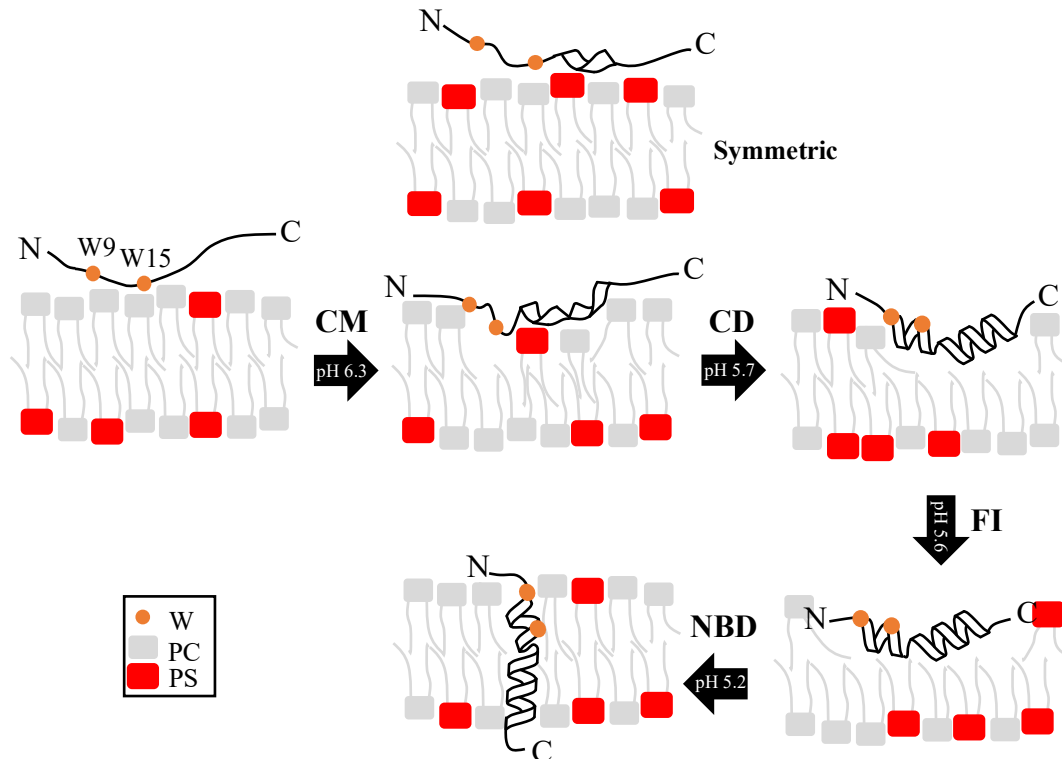


Figure 33. Influence of PS membrane asymmetry on the insertion of pHLIP.

The black arrows represent the transitions between each step in the membrane insertion process, with the corresponding pH midpoints. Three transitions are represented by their insertion pK . The first transition represents the pK determined via CM. This transition is where the observed influences of membrane asymmetry are most prominent. Comparing to symmetric PS (directly above), the N-terminal region including the two Trp residues is more closely associated with the membrane in the presence of membrane asymmetry.

and CD suggests that PS asymmetry might change the pK_a of Asp25 and Asp33 of pHLIP. Decoupling is not observed in samples containing symmetric PC or PS meaning that early protonations reported by CM are occurring before pHLIP begins to adopt its secondary structure in aLUVs. Equally important, the titration reported by FI only started at the point where the CM titration ends (Figure 30) suggesting that CM and FI, as in symmetric POPC membranes, are reporting on different protonation steps in the insertion process (15). Together, the data indicates that PS asymmetry affects the protonation of Asp residues 14, 25, 31, and 33, as protonations of these residues are reported by CM, FI, and CD (15).

Changes in membrane electrostatics promote an altered insertion of pHLIP. An asymmetric distribution of PS across the two leaflets is expected to change the electrostatics of the membrane (87, 176, 177). Lipid bilayers have three distinct potentials, namely the surface, transmembrane, and the dipole potentials (176). The surface potential is created by a buildup of charge on the membrane surface which propagates out from the membrane and into the aqueous solution (176). A concentration gradient of ions between the two aqueous solutions bathing the bilayer causes the transmembrane potential (176). Finally, the dipole potential is a positive potential centered in the hydrophobic core of the membrane, which arises from permanent dipoles in the lipid molecules (176). The observed increases in pK_{CM} and pK_{FI} could be caused by a change in one of these potentials (87, 176, 177). For example, an asymmetric distribution of negatively charged lipids should create a surface potential difference across the membrane (87, 177, 178). As a result, an additional transmembrane potential could potentially arise, not resulting in this case from an ion gradient across the membrane, but due to the asymmetric charge distribution from the PS head group. The membrane surface potential for each leaflet can in principle be calculated using the Guoy-Chapman model and the Grahame equation (Eq. 3) (159, 179). Although our protocol for creating PS asymmetry yielded only a small PS difference of ~ 4 mol%, this translates into an $\sim$

+30 mV difference in surface potential across the membrane, with a less negative exterior. We propose that this surface potential might attract the negatively charged Asp and Glu side chains in pHLIP, leading to the peptide residing deeper in the interfacial region (87). Due to the higher dielectric constant and reduced hydration at this deeper location, the pK_a values of these residues would be expected to increase (123). In contrast, pK_{CD} was not affected by PS asymmetry (Figure 33), suggesting that asymmetry-induced changes in the surface potential do not affect the helical content of pHLIP (149) despite influencing its location at the membrane surface. Some caution with this interpretation is warranted, as surface potential differences may not be the only factor driving the observed pK shift. We cannot rule out that lipid asymmetry affects the lipid dipoles in a manner that alters the membrane dipole potential and thus influences the insertion pK of pHLIP (180, 181).

pHLIP as a model can elucidate the influence of asymmetry on marginally hydrophobic TM domains. TM domains are primarily composed of hydrophobic amino acids, which anchor the domain into the bilayer. However, polar amino acids are commonly found in TM domains and indeed, Asp and Glu represent $\sim 5\%$ of the residues in TM sequences (148, 182). Typically, these polar residues are functionally important, such as in bacteriorhodopsin, where Asp 85 and 96 (corresponding to Asp 14 and Asp 25 in pHLIP) mediate proton transfer across the membrane (183). A second example is the mammalian sodium/hydrogen exchanger NHE1, where conserved Asp residues located within the membrane environment allow this exchanger to control the internal pH of the cell (184). A more accurate amino acid sequence predictor of TM propensity discovered overlap between sequences that were both non-TM and TM (185). These sequences are termed marginally hydrophobic TM domains (mTMD) (186). mTMDs occur in membrane proteins and are unique in that they can assume two different topologies. These involve either movement of helices in and out of the bilayer, or reorientation of helices within the bilayer after insertion (187-

189). This orientation change is a direct consequence of the presence of polar residues, including Asp and Glu, which reduce their hydrophobicity compared to standard TMD (187-189). Some examples of proteins that have shown dual topology and mTMDs are the human aquaporin water channel APQ1, the hepadnaviral large envelope protein, and the ATP-gated ion channel subunit P2X2 (along with the related ASIC protein) (190-192).

Like mTMDs, pHLIP can adopt two different membrane topologies because of the content of its polar residues. In the case of pHLIP, the topological change can be easily measured in reconstituted systems, as TM insertion is triggered by a mere drop in pH. We propose that pHLIP might be used as a model system to gain mechanistic insights into topological transitions in membranes and speculate that membrane asymmetry might influence the membrane location of other peptides and proteins. Specifically, mTMD could experience topological transitions when exposed to membrane asymmetry changes, particularly those containing amino acids with charged side chains.

TM proteins are synthesized at the membrane of the endoplasmic reticulum and are transported to the PM *via* the Golgi apparatus. Membrane proteins experience environmental changes during this transit. Specifically, there is a difference in environmental pH, as the lumen of the Golgi is acidic, while the ER lumen and the cytoplasm have a neutral pH (193). However, it is poorly understood if such pH changes alter mTMD's structure *via* changes to the protonation state of Asp, Glu or His residues. A second important consideration is that the ER and PM are different both in terms of lipid composition and asymmetry. Thus, the ER is delineated by a symmetric membrane, while the PM exhibits asymmetry of multiple lipid species, including PS (79). We have shown recently that the topology of pHLIP is affected by changes in symmetric lipid composition (95). Here, we show that not only the presence, but also their distribution in the bilayer impacts the location of pHLIP in the membrane. In the future work, we are interested in determining if

asymmetry changes to the ER and PM can trigger mTMD topological changes, which could affect the folding and activity of membrane proteins in different cellular membranes.

4.6 Acknowledgments

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Chapter V: A transmembrane helix reduces the dynamics of membrane thickness fluctuations

5.1 Abstract

Lipid membranes are highly dynamic assemblies that exhibit a hierarchy of motions ranging from rotation of individual lipid molecules to collective membrane fluctuations. The latter, particularly thickness fluctuations, have recently become experimentally accessible with the development of neutron spin echo (NSE) techniques. Cellular membranes contain membrane-absorbed and transmembrane proteins that carry out myriads of biological functions. However, how these proteins affect membrane dynamics, including thickness fluctuations, remains an open question. Here, we use the pH-low insertion peptide (pHLIP) to address this question. The location of pHLIP in the membrane is controlled by pH, as it transitions from a membrane-absorbed to a transmembrane state below a critical pH value. The use of pHLIP thereby allows studying the impact both classes of membrane interactions with an identical amino acid sequence. In this study, we measured membrane thickness fluctuations with NSE, and the static membrane thickness with small-angle neutron and x-ray scattering (SANS and SAXS, respectively). NSE detected differences between the two membrane states in a complex lipid membrane composed of dioleoylphosphatidylcholine/dioleoylphosphatidylserine/cholesterol (DOPC/DOPS/Chol). Specifically, pHLIP in the membrane-absorbed orientation showed no influence on the membrane's fluctuations. However, as a transmembrane helix, pHLIP strongly reduced the rate of membrane thickness fluctuations. Both SANS and SAXS showed that neither of pHLIP's conformations affected the average bilayer thickness. We suggest that the suppression of thickness fluctuations may result changes in membrane viscosity induced by the presence a transmembrane helix.

5.2 Introduction

Lipids in membranes display a variety of different motions, including both individual and collective bilayer fluctuations (64). The latter of which includes dynamic motions such as membrane bending and thickness fluctuations. Thickness fluctuations are described as in- and out-of-plane collective motions of lipids that result in local deviation from the average bilayer thickness. They have been proposed to be an important component of pore formation in membranes, suggesting a role in biological function (194, 195). Although thickness fluctuations have been investigated from a theoretical standpoint (74), they have only recently become accessible to experimental studies with the development of neutron spin echo (NSE) techniques (64, 74-76, 196). NSE is an inelastic neutron scattering technique that can access the appropriate length- (several to 100s of angstroms) and time-scales (picoseconds to 100s of nanoseconds) in which thickness fluctuations occur (194, 197). Previous studies used NSE to investigate single-component saturated lipid bilayers with varying acyl chain length and lipid phase states, finding that these membranes exhibit thickness fluctuations that are consistent with theoretical predictions (64, 75, 76, 196). It was discovered that only fluid phase bilayers exhibit detectable thickness fluctuations, suggesting that lipid physicochemical properties can significantly influence such membrane dynamics (64, 75). While these findings are important, biological membranes contain a substantial amount of protein in addition to lipid. However, a potential influence of membrane proteins on membrane thickness fluctuations remains an open question. The timescale of membrane thickness fluctuations coincides with that of membrane protein folding and some conformational changes (75, 198), suggesting a potential reciprocal influence.

Membrane proteins account for approximately half the mass of the mammalian plasma membrane (146, 147). These proteins can be grouped into two broad categories, membrane-absorbed and transmembrane proteins, both of which are crucial to the cell's viability. It is necessary to study both categories to understand the effects of proteins on membrane thickness

fluctuations. Although there is no perfect model system, the pH-low insertion peptide (pHLIP) can explore both membrane positions. pHLIP is a soluble peptide in aqueous solution (State I), that adsorbs to the membrane surface at neutral pH (State II), and finally forms a transmembrane (TM) α -helix when the pH of the environment acidifies (State III) (4). Within the sequence of pHLIP are seven acidic groups whose protonation triggers membrane insertion, allowing for controllable conformational changes (15). Since the conformation can be tuned by adjusting the acidity of the solution, pHLIP can represent both membrane-adsorbed and transmembrane proteins without the need to use two different model systems. The use of pHLIP also allows for easy NSE sample preparation, at neutral pH as the peptide is soluble in water (10).

In this study, we utilize pHLIP in a new capacity as a model peptide to probe the effects of a polypeptide chain on thickness fluctuations. We used a simplified model membrane system composed of dioleoylphosphatidylcholine (DOPC), cholesterol (Chol), and the negatively charged lipid dioleoylphosphatidylserine (DOPS) added to promote unilamellarity. The primary reason for choosing these lipids is that pHLIP/membrane interaction is reasonably well understood from previous studies of a similar system composed of DOPC and various levels of Chol (18) as well as compositions that include negatively charged lipids (95). A secondary motivation is that NSE thickness fluctuation measurements have not previously been conducted in unsaturated membranes. We found that pHLIP in either the membrane-adsorbed or transmembrane conformation did not largely affect the average membrane thickness as determined by small-angle neutron and X-ray scattering (SANS and SAXS, respectively). However, the TM helix of pHLIP reduced the rate at which the membrane thickness fluctuates compared to lipid only, yet the amplitude of the fluctuations was unaffected. A recent refinement of the NSE analysis suggested that TM pHLIP caused a large increase in the viscosity of the membrane. We propose that the

suppression of thickness fluctuations may result from an increase in membrane viscosity, therefore slowing down the motions of lipids.

5.3 Materials and Methods

Materials. 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (sodium salt) (DOPS), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), and cholesterol from ovine wool (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL) and used as is. D₂O (99.96% D) was purchased from Cambridge Isotope Laboratories (Andover, MA). pHLIP (sequence: N₁-AAEQNPYIYWARYADWLFTTPLLDDALLVDADEGT-C₁) was synthesized using standard solid phase protocols (P3 Biosystems, Louisville, KY) and purified by reverse-phase high performance liquid chromatography (HPLC) to greater than 95% purity. 9-(2,2-dicyanovinyl)julolidine (DCVJ) was purchased from Sigma-Aldrich (St. Louis, MO). 1,6-diphenyl-1,3,5-hexatriene (DPH) was purchased from Fisher Scientific (Hampton, NH). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Hampton, NH). Sodium phosphate dibasic (>98.5%) and sodium phosphate monobasic monohydrate (98%) were purchased from Sigma-Aldrich (St. Louis, MO) and were used to prepare sodium phosphate buffer. Sodium deuterioxide (NaOD) and deuterium chloride (DCl) were purchased from Sigma-Aldrich (St. Louis, MO) and were used to adjust the final pH of the small angle neutron and x-ray samples as well as the neutron spin echo samples.

Synthesis of deuterated lipids. DOPC-d₆₆ was synthesized from *sn*-glycero-3-phosphocholine CdCl₂ complex (GPC•CdCl₂, Santa Cruz Biotechnology, Dallas, TX) and oleic acid-d₃₄ (98% D, Sigma-Aldrich, St. Louis, MO) by the method of Mena & Djerassi (1985) (199). Dry toluene and dry, ethanol-free chloroform for reactions were obtained from Sigma-Aldrich. In a representative procedure, GPC•CdCl₂ (490 mg, 1.11 mmol) was dried by rotary evaporation from toluene (3×5 mL) followed by storage *in vacuo* for 2 h, then suspended in 5 mL of chloroform (8 mL) with ultrasonication. Oleic acid-d₃₄ (725 μ L, 722 mg, 2.28 mmol, 2.05 equiv) was added via syringe, followed by a solution of *N,N'*-dicyclohexylcarbodiimide (1.41 g, 6.84 mmol, 6.15 equiv) in

chloroform (2 mL) was added in portions with swirling. *N,N*-Dimethylaminopyridine (279 mg, 2.28 mmol, 2.05 equiv) was added as a solid and dissolved by swirling. The solution was sonicated for ~18 h, at which point TLC indicated substantial completion of the reaction. TLC analysis was performed on silica gel plates using 65:25:4 CHCl₃:MeOH:H₂O (v:v:v) as the mobile phase and iodine for visualization.

The product was purified by chromatography on silica gel using sequential elution with 65:25:0, 65:25:2 and 65:25:3 (v/v/v) CHCl₃:MeOH:H₂O. Fractions containing DOPC-d₆₆ (as judged by TLC) were pooled and rotary evaporated. To remove trace metal ions and acidic impurities, the residue was taken up in a mixture of 10 mL of chloroform, 20 mL of methanol and 8 mL of aqueous buffer containing Tris (30 mM) and EDTA (20 mM) at pH 8.0. The mixture was shaken for 2 min in a separatory funnel, after which additional chloroform (10 mL) and water (10 mL) were added to induce phase separation. The chloroform (lower) phase was washed 3× with 10 mM NaCl, dried over Na₂SO₄ and rotary evaporated to dryness. The combined yield from two preparations using 1.97 g of oleic acid-d₃₄ was 1.20 g (45%) of DOPC-d₆₆.

The synthesis of perdeuterated cholesterol (Chol-d₄₀) was adapted from a protocol provided by Prof. Howard Riezman (University of Geneva) by using *Saccharomyces cerevisiae* RH6829 that was genetically modified to produce cholesterol rather than ergosterol. *S. cerevisiae* RH682941 was cultured in a medium containing yeast nitrogen base (7 g/L), yeast extract (5 g/L), d-glucose (12.5 g/L), uracil (30 mg/L) and l-leucine (30 mg/L) in D₂O. 1-L cultures were each inoculated with 0.5 mL of an overnight culture grown in yeast extract peptone dextrose (YPD)–H₂O medium. The cultures were shaken at 30 °C for 4 days during growth, after which the cells were harvested by centrifugation (5,000 x g, 10 min) and washed once with water. The wet cell pellet (~8 g) was resuspended in alcoholic potassium hydroxide⁴² (45.2 g of potassium hydroxide and 0.3 g of pyrogallol dissolved in 17 mL of water, then diluted with 100 mL of absolute ethanol)

and refluxed for 2 h. An additional 25 mL of absolute ethanol was added, and the suspension was refluxed for an additional 1 h. After cooling to room temperature, the mixture was diluted with water (40 mL) and extracted with 3×35 mL of n-heptane. The combined heptane extracts were dried using a rotary evaporator, re-dissolved in a small amount of chloroform, and chromatographed on a column of silica gel packed with and eluted with 4:1 hexanes:ethyl acetate. Fractions containing substantially pure cholesterol, as judged by thin layer chromatography on silica gel (4:1 hexanes:ethyl acetate eluent, phosphomolybdic acid stain), were combined and evaporated to dryness. The cholesterol was further purified by recrystallization twice from methanol. Gas chromatography/mass spectrometry (GC/MS) was performed after conversion of the cholesterol to its trimethylsilyl derivative using N,O-bis(trimethylsilyl)trifluoroacetamide containing 1% chlorotrimethylsilane (60 °C, 30 min). The GC/MS analysis showed 87% substitution of D for H, which corresponds to an average of 40 deuterium atoms per cholesterol molecule.

Intrinsic tryptophan fluorescence spectroscopy. A lyophilized pHLIP stock was dissolved in 10 mM sodium phosphate (NaPi) pH 8.0 buffer. DOPC/DOPS/Chol 76/4/20 vesicles were prepared by extrusion through a 100 nm pore size polycarbonate membrane using a Mini Extruder (Avanti Polar Lipids, Inc., Alabaster, AL) in the same buffer to form large unilamellar vesicles (LUV). pHLIP was added externally and incubated for 1 h at room temperature (~ 23°C) for a final lipid:peptide molar ratio of 150:1. The final peptide concentration was 1 μ M. To perform a pH insertion titration, sample pH was adjusted by mixing with 100 mM stocks of sodium acetate (NaOAc), MES [2-(*N*-morpholino) ethanesulfonic acid], or HEPES [(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)] buffers (25 μ L), to obtain the desired pH values. The final sample volume was 140 μ L. The final pH of each sample was measured using a 2.5 mm bulb pH-electrode (Microelectrodes, Inc., Bedford, NH). Tryptophan fluorescence emission spectra were recorded

using a Photon Technology International (Edison, NJ) Quanta Master fluorometer at room temperature with the excitation wavelength set to 280 nm and an emission wavelength range of 310-400 nm, with excitation and emission slits set to 3 nm. Lipid blanks were subtracted in all cases. Data were analyzed by monitoring changes in the fluorescence intensity (FI) at 335 nm, which is directly proportional to the population of molecular species present (101). FI pH-titrations were then fitted to determine the pK_{FI} using:

$$Signal = (F_a + F_b 10^{m(pH-pK_{FI})}) / (1 + 10^{m(pH-pK_{FI})}), (Eq. 1)$$

where F_a is the acidic baseline, F_b is the basic baseline, m is the slope of the transition, and pK_{FI} is the FI midpoint of the curve, and signal is fluorescence changes.

Circular dichroism (CD). pHLIP was incubated with DOPC/DOPS/Chol vesicles (prepared as described earlier) in 10 mM NaPi pH 8.0 buffer for 1 h. Afterwards, the pH was adjusted with 100 mM NaOAc or NaPi (62.5 μ L) to the desired final pH values. The final sample volume was 250 μ L. The lipid:peptide molar ratio was 150:1 with a final peptide concentration of 5 μ M. The final pH of the samples was measured after spectra were recorded. CD spectra were acquired using a Jasco (Easton, MD) J-815 spectropolarimeter interfaced with a Peltier system. Spectra were recorded at 25°C using a 2 mm cuvette with a scan rate of 100 nm/min, and 20-40 accumulations were performed. Raw data were converted into mean residue ellipticity according to:

$$[\theta] = \frac{\theta}{10lc(N-1)}, (Eq. 2)$$

where Θ is the measured ellipticity, l is the path length of the cell in cm, c is the protein concentration in M, and N is the number of amino acids. Appropriate blanks were subtracted in all cases.

Viscosity and fluidity measurements. DCVJ was prepared in a chloroform stock.

DOPC/DOPS/Chol vesicles (prepared as described earlier) were incubated with pHLIP (prepared as described earlier) and DCVJ by adding pHLIP to the vesicles and then adding the vesicles/pHLIP solution to a dried film of DCVJ. The solution was vortexed for 10 min in the presence of the DCVJ film and then incubated for 1 h in the dark. Afterwards, the pH of the samples was adjusted using either with 100 mM NaOAc or NaP_i to the desired final pH values. Final pHLIP concentration was 6.7 μ M, final DCVJ concentration was 4.5 μ M, and final lipid concentration was 1 mM with a lipid:peptide ratio of 150:1. DCVJ protocol was modeled after (200). DCVJ emission spectra were recorded using a Photon Technology International Quanta Master fluorometer at 25°C with the excitation wavelength set to 433 nm and an emission wavelength range of 450-600 nm, with excitation and emission slits set to 3 nm. Lipid blanks were subtracted in all cases. The final pH of each sample was measured using a 2.5 mm-bulb pH-electrode. Data was analyzed by taking the fluorescence intensity peak wavelength (486 nm) and normalizing to the lipid only sample.

A DPH stock was prepared in DMSO. DOPC/DOPS/Chol vesicles (prepared as described earlier) were incubated with DPH by adding DPH to the vesicles. The solution was incubated for 1 h in the dark. Final DMSO percentage in the sample was ~4%. After 1 h, pHLIP was added and incubated for an additional hour in the dark. Afterwards, the pH of the samples was adjusted using either 100 mM NaOAc or NaP_i to the desired final pH values. The final pHLIP concentration was 10 μ M, final DPH concentration was 3 μ M, and final lipid concentration was 1.5 mM with a lipid:peptide ratio of 150:1. The final pH of each sample was measured using a 2.5 mm-bulb pH-electrode. Data was collected on a Horiba Fluorolog-3 spectrofluorometer (Kyoto, Japan) at room temperature with the excitation wavelength set to 360 nm and an emission wavelength set to 430 nm, with excitation and emission slits set to 4 nm. Polarizers were used in the vertical (V) and horizontal (H) positions in the VV, VH, HV, and HH settings. 10 measurements at each polarizer

combination were averaged for the final value. Lipid blanks were subtracted in all cases. Anisotropy was determined using:

$$\langle r \rangle = \frac{I_{VV}I_{HH} - I_{HV}I_{VH}}{I_{VV}I_{HH} + 2I_{HV}I_{VH}}, (Eq. 3)$$

where I is the measured fluorescence intensity, and the first and second subscript positions denote the positions of the excitation and emission polarizers, respectively (201).

Statistical analysis. Statistical analysis was performed on the viscosity and fluidity values to determine if the observed changes were significant. The analysis was performed using SPSSv25 software (IBM Analytics, Armonk, NY). For the viscosity data, a Kruskal-Wallis Test was used due to the normalized data violating the assumption of normally distributed data. For the fluidity data, a one-way ANOVA was used. $P \leq 0.05$ was considered significant for all tests.

Small-angle neutron and X-ray scattering (SANS and SAXS). D₂O suspensions of 100 nm extruded vesicles composed of DOPC, DOPS and Chol (as well as the perdeuterated variants DOPC-d₆₆ and Chol-d₄₀) (~20 mg/mL) were prepared as described above. To access the two states of pHLIP, measurements were performed at two pH values, pH 8 for State II and pH 4 for State III, obtained by using either NaOD or DCl, respectively. Lyophilized pHLIP was weighed and added to vesicle suspensions (pH 8) at a lipid:peptide ratio of 150:1.

Small-angle neutron scattering (SANS) experiments were performed at the NGB-30-meter SANS instrument at the National Institute of Standards and Technology (NIST) NIST Center for Neutron Research (NCNR) and BL-6 (EQ-SANS) at the Spallation Neutron Source (SNS) at Oak Ridge National Laboratory (ORNL). LUV suspensions were loaded into 1 mm path-length quartz banjo cells (Hellma USA, Plainview, NY) and mounted in a temperature-controlled cell holder with ~ 1°C accuracy. EQ-SANS data were taken at a 4.0 m sample-to-detector distance (SDD)

with a 10.0–13.5 Å wavelength band and a 2.5 m SDD with a 2.5–6.0 Å wavelength band for a total usable scattering vector range of $0.005 \text{ Å}^{-1} < q < 0.5 \text{ Å}^{-1}$. Scattered neutrons were collected with a two-dimensional (2D) ($1 \times 1 \text{ m}$) ^3He position-sensitive detector (ORDELA, Inc., Oak Ridge, TN) with 256×192 pixels. The 2D data from the two instrument configurations were reduced and stitched together using the software package Mantid from ORNL. During reduction, data were corrected for detector pixel sensitivity, dark current, and sample transmission, and background scattering from water. SANS experiments on similarly prepared samples were also performed on the CHRNS 30m SANS beamline at NCNR. Measurements were done using the standard SANS configuration (neutron wavelength of 6.0 Å and SDD of 1m, 4 m and 13 m) combined with the lens configuration (wavelength of 8.4 Å and SDD of 13 m), accessing an overall q -range between $\sim 0.001 \text{ Å}^{-1}$ and 0.5 Å^{-1} . The 2D scattering signals, collected on a $640 \text{ mm} \times 640 \text{ mm}$ ^3He position-sensitive detector (pixel size: $5.08 \text{ mm} \times 5.08 \text{ mm}$), were normalized and corrected for all instrumental effects, such as empty cell scattering and background, using the NCNR Igor data reduction software (202). The same reduction software was used to obtain the 1D scattering patterns presented here by circularly averaging the normalized 2D data and subsequently stitching data sets over adjacent q -ranges obtained using the different instrument configurations described above. Small angle x-ray scattering (SAXS) were performed on the same samples used in the SANS experiments using a Rigaku BioSAXS-2000 home source system with a Pilatus 100K 2D detector and a HF007 copper rotating anode (Rigaku Americas, The Woodlands, TX). SAXS data were collected at a fixed SDD using a silver behenate calibration standard, with a typical data collection time of 3 h. For all scattering data, the one-dimensional scattering intensity $I(q)$ [$q = 4\pi \sin(\theta)/\lambda$, where λ is the neutron or X-ray wavelength, and 2θ is the scattering angle relative to the incident beam] was obtained by radial averaging of the corrected 2D data.

Scattering data were analyzed following Doktorova et al. (203). Briefly, differently contrasted scattering data (i.e., SANS data for different deuterated lipid variants, and SAXS data) were jointly refined with a model that accounts for coherent scattering contributions arising from transverse (out-of-plane) scattering length density (SLD) variation within the bilayer. Transverse SLD variation for SANS and SAXS arises primarily from the different nuclear and electronic composition, respectively, of the lipid headgroup and hydrocarbon layers. In principle, the SLD profile is influenced by the presence of membrane-absorbed or inserted protein, which has a different neutron SLD and electron density than lipid or water. However, because of the low mass fraction of protein used in these experiments ($< 1\%$), we neglected the protein contribution to the SLD profile. Any detectable changes in the scattering curves are due to the influence of the protein on bilayer structure. The transverse SLD profiles were derived from the underlying lipid volume probability distributions within the bilayer, modeled as the sum of separate distributions for the lipid headgroups and hydrocarbon chains. The total unit cell volume was calculated as a mole-fraction weighted sum of lipid component volumes obtained from literature and constrained in the fit, leaving adjustable parameters for the area per lipid A_L and headgroup thickness D_H . Additional structural parameters, including the total bilayer thickness D_B and the hydrocarbon thickness $2D_C$, were derived from relationships between the adjustable parameters and the lipid headgroup and hydrocarbon volumes. From the unit cell volume probability profile, NSLD and ED profiles were then obtained as a sum of the separate headgroup and hydrocarbon volume probability distributions multiplied by their respective total scattering lengths.

For each nominal sample composition (e.g., DOPC/DOPS/Chol 74/4/20 mol%), separate SANS samples with different contrast were prepared using protiated and perdeuterated variants of DOPC (i.e., DOPC or DOPC- d_{66}) and Chol (i.e., Chol or Chol- d_{40}). A joint analysis of all available SANS and SAXS data for a given composition, temperature and pH was implemented in

Mathematica 11.0 (Wolfram Research, Champaign, IL). A complete list of the structural parameters is found in Table S2.

Complementary SANS data analysis was performed using the built-in core-multi-shell model in the SasView software by employing a three-shell representation of the head-tail-head groups of the lipid vesicle (204). The model assumes a Gaussian distribution of the vesicle radius (which is fitted over the low- q range of the data) but does not take into account gradients in the layer interfaces (due to the Gaussian distribution of different lipid subgroups) – it is rather based on a crude SLD slab model of the three layers.

Neutron spin echo (NSE). Suspensions of 100 nm extruded vesicles were prepared in D₂O buffer as described above at a concentration of 50 mg/mL. Fully protiated lipids and cholesterol were used for measurements of bending fluctuations. On the other hand, perdeuterated forms of DOPC and cholesterol (synthesized at ORNL as described above) were used for measurements of thickness fluctuation signals. Protiated and perdeuterated samples were loaded in 2 mm and 4 mm thick quartz cells, respectively, for optimal scattering signal while maintaining reasonable thresholds on multiple scattering and incoherent background effects. To access the different states of pHLIP, measurements were run at two pH values, pH 8 for State II and pH 4 for State III of the peptide, as described above using DCl or NaOD. Lyophilized pHLIP was weighed and added to samples at pH 8 at a lipid:peptide ratio of 150:1.

NSE experiments were conducted on the NSE spectrometers at NCNR and SNS. NSE measurements at NCNR were performed using the following conditions: 1) Protiated samples: $\lambda = 8 \text{ \AA}$ and $11 \text{ \AA}$, $0.04 \text{ \AA}^{-1} < q < 0.1 \text{ \AA}^{-1}$; and 2) Perdeuterated samples: $\lambda = 6 \text{ \AA}$, $8 \text{ \AA}$ and $11 \text{ \AA}$, $0.04 \text{ \AA}^{-1} < q < 0.18 \text{ \AA}^{-1}$. Analogous measurements were run on D₂O buffer and carbon black (resolution sample) for proper data normalization and background subtraction. Reduction and processing of

the raw data was performed using the Data Analysis and Visualization Environment (DAVE) software developed at NIST (205). The data processing yields the normalized intermediate scattering function $S(q, t)/S(q, 0)$ as a function of Fourier time, t , for discrete q -values within the accessed q -range. For lipid membranes, the decay of the intermediate scattering function is fitted to a stretched exponential function with a stretching exponent of $2/3$ such that:

$$S(q, t)/S(q, 0) = \exp [(-\Gamma(q)t)^{2/3}], \text{ (Eq. 4)}$$

where $\Gamma(q)$ represents the decay rate at individual q -values. Analysis of the decay rates to extract the bending and thickness fluctuations parameters was performed using a Mathematica code that was developed for NSE data fitting. Experiments performed on the NSE BL-15 at ORNL were performed over different neutron wavelength bands (due to the broad wavelength distribution of pulsed neutron sources), and the intensity was grouped over different scattering rings to obtain the decay functions described earlier at discrete q -values. The measurements were planned with sets of target neutron wavelengths and mean q -values to access an overall q -range of $0.05 \text{ \AA}^{-1}$ to $0.15 \text{ \AA}^{-1}$ for both protiated and perdeuterated samples (albeit with different λ - q configurations). The instrument resolution and the D_2O buffer were measured under the same sample configurations for proper data reduction and normalization. The reduction of the SNS-NSE data was performed using a Python script that reads, groups, plots and fits the collected data and generates a list of values for $S(q, t)/S(q, 0)$ as a function of the Fourier time and the mean q -value of grouped scattering rings. The subsequent fitting of the data to a stretched exponential function, the extraction of the decay rates, and the analysis of the $\Gamma(q)$ patterns were done following the same protocols described above for the NCNR-NSE data.

5.4 Results

Determination of insertion pH of pHLIP. We used an array of complementary biophysical techniques to investigate the membrane association of pHLIP and its influence on membrane structure and dynamics. Experiments were carried out in unilamellar lipid vesicles composed of DOPC, Chol, and DOPS, in a 76/20/4 molar ratio.

A previous study probed how pHLIP responded to pH in the presence of DOPC membranes with varying levels of Chol. Specifically, it was determined that pHLIP inserted with a pK_{SM} of ~ 5.6 in the presence of DOPC membranes containing 20 mol% Chol (18). However, the effect of negatively charged lipids in membranes containing cholesterol has not been studied. Here, we incorporated in our vesicles 4% of DOPS, which was suited for our experimental needs. Specifically, in the early stages of the current study we found that our DOPC/Chol suspensions extruded through 100 nm pores were not completely unilamellar, but instead had some contamination with multilamellar vesicles (Figure 34A), as revealed by the appearance of Bragg peaks. Consistent with previous reports (206), we could eliminate this contamination by doping the bilayers with 4 mol% of the negatively charged lipid DOPS (Figure 34B). The lipid mixture we use here, while simple, recapitulates several properties of the plasma membrane, as it is fluid, contains cholesterol, and negatively charged lipids.

To understand the pH responsive properties of pHLIP in the presence of the charged dopant, we performed pH titrations. Using native Trp fluorescence, we determined the pK_{FI} to be 4.90 ± 0.08 (Figure 35A) (15). A decrease in the pK_{FI} in the presence of a negatively charged lipid falls in line with a previous study that observed a decrease in the pK_{FI} in the presence of palmitoyloleoylphosphatidylserine (POPS) doped vesicles (95). With this data, we also determined

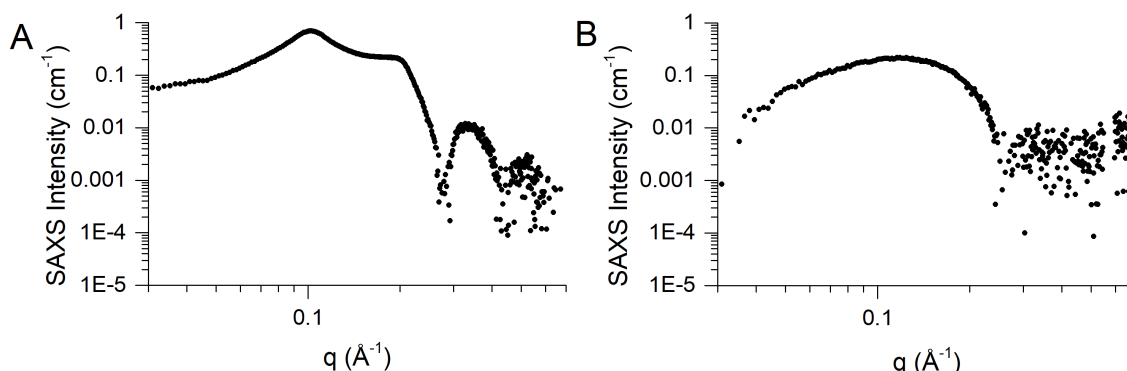


Figure 34. Inclusion of the charged lipid DOPS ensures unilamellar liposomes.

Comparison of DOPC only liposomes (A) to DOPC liposomes that include DOPS at 4 mol% (B). Bragg peaks in the scattering curve indicate the presence of pauci-lamellar populations of vesicles (A) whereas with the inclusion of DOPS, the Bragg peaks are no longer observed (B). Loss of Bragg peaks indicates a unilamellar population.

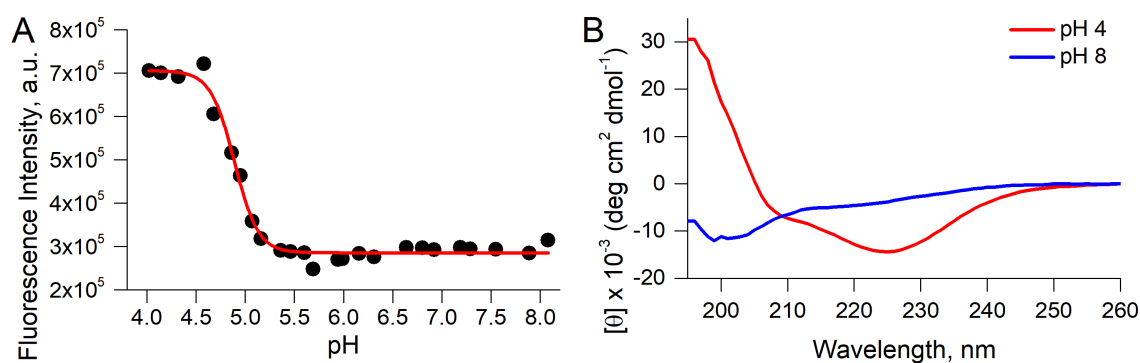


Figure 35. pHLIP responds in a pH dependent fashion in DOPC/DOPS/Chol vesicles.

(A) The increase in Trp fluorescence intensity at low pH indicates pHLIP insertion into the bilayer. Also shown is a fit of the data (red line) using Eq. 1 to obtain the midpoint of insertion (pK_{FI}). (B) Representative circular dichroism spectra showing that pHLIP adopts a mostly unstructured conformation in State II at pH 8 (blue) and folds into an alpha helix in State III at pH 4 (red).

the pH values at which pHLIP is stably in State II or State III, allowing us to decide on pH values used in the later scattering experiments. This is observed in the baselines found in Figure 35A.

Upon transition from State II to State III, pHLIP undergoes a large change in secondary structure (4, 10). Formation of an alpha helix is a hallmark of pHLIP's pH responsive property. Using circular dichroism (CD), we found that pHLIP was mostly unstructured in State II, but formed a TM α -helix in State III in the lipid composition used here, agreeing with previous reports in other lipid compositions (Figure 35B) (4, 10). Data from both fluorescence and CD illustrates how pHLIP responds to pH in the lipid composition studied here, but with a lower pK than observed previously for a similar composition. However, this is not surprising since both Chol and PS have been observed to decrease the pK (18, 95).

The rate of membrane thickness fluctuations is reduced in the presence of pHLIP in the transmembrane state, but not in the membrane absorbed state. After characterizing pHLIP's pH interaction with the membrane composition of interest, we turned to NSE to investigate pHLIP's influence on membrane dynamics. Membrane bilayers display different modes of collective motion that involve large membrane portions, including bending and thickness fluctuations. While the effect of proteins on membrane bending fluctuations has been studied (207), it is not known if polypeptides have an effect on membrane thickness fluctuations. Specifically, membrane thickness fluctuations are described as in- and out-of-plane motions of lipids in collective groups that result in local aberration from the average bilayer thickness (73, 74). NSE has the distinct ability to measure these fluctuations, as this technique probes the appropriate length- and time-scales in which they occur (194, 197). Using tail contrast matched vesicles composed of perdeuterated DOPC and deuterated cholesterol, we investigated the influence of pHLIP in both State II and III on membrane thickness fluctuations.

NSE measures the decay rate, Γ , of membrane dynamics at length scales which are determined by the wavevector transfer, q . If the q -dependence of Γ shows a simple q^3 dependence, as predicted by Zilman and Granek (208), then the measurements are describing changes in bending fluctuations (Figure 36, blue data points). However, a deviation from the observed q^3 dependence of Γ suggests that excess dynamics in the form of thickness fluctuations are occurring (Figure 36, red data points) (75). Previous studies using tail-contrasted vesicles have observed that both single component and multi-component lipid bilayers display thickness fluctuations (64, 75, 76). To access the effect of pHLIP on bending fluctuations we first measured fully protiated vesicles in the absence and presence of pHLIP in either State II or III (Figure 37 and Table 6). Analysis of the data was conducted using:

$$\Gamma_{bend} = 0.0058 \frac{k_B T}{\eta_{D2O}} \sqrt{\frac{k_B T}{\kappa}} q^3, (Eq. 5)$$

to extract the membrane bending modulus, κ , of the samples where $k_B T$ is the thermal energy, Γ_{bend} is the decay corresponding to bending fluctuations, and η_{D2O} is the viscosity of D₂O. Our results showed that κ did not change in the presence of pHLIP at either neutral or acidic pH (Figure 37 and Table 6).

Next, tail contrasted vesicles (where only DOPS was fully protiated) were used to determine if pHLIP influenced thickness fluctuations. We analyzed that data using the analysis model proposed by Nagao *et al.* (64), where thickness fluctuations manifest as excess dynamics on top of bending fluctuation modes, and are well described by a Lorentzian in Γ - q space such that:

$$\frac{\Gamma}{q^3} = \frac{\Gamma_{bend}}{q^3} + \frac{\Gamma_{TF}}{q_0^3} \frac{1}{1 + (q - q_0)^2 \zeta^2}, (Eq. 6)$$

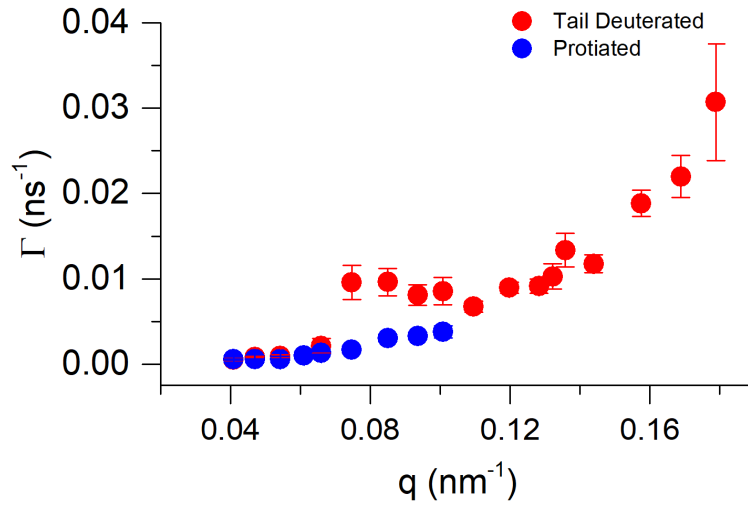


Figure 36. q dependence of the decay rate, Γ , for protiated and tail deuterated samples.

The q^3 dependence of the bending data (blue symbols) is represented by the first term in Eq. 5. Tail deuterated samples (red symbols) show an enhancement in the decay rates indicating the presence of thickness fluctuations occurring on top of bending fluctuations.

Table 6. Dynamic parameters obtained from the analysis of the neutron spin echo data from DOPC/DOPS/Chol bilayers in the presence and absence of pHLIP.

Dynamic parameters for the model include the bending modulus κ , the timescale of the thickness fluctuation Γ_{TF} , the amplitude of the thickness fluctuation Δd_m , the area compressibility modulus K_a , and the membrane viscosity μ .

		Bending Modulus	Thickness Fluctuations		Area Compressibility	Viscosity
pH	pHLIP	κ ($k_B T$)	Γ_{TF} ($ns^{-1} \cdot 10^{-3}$)	Δd_m (nm)	K_a (N/m)	μ (nPa·s·m)
8	-	30.34 ± 2.47	7.3 ± 0.9	0.374	0.262 ± 0.021	31.92 ± 3.49
4	-	29.22 ± 1.33	7.3 ± 1.7	0.355	0.261 ± 0.021	32.02 ± 4.14
8	+	26.88 ± 1.81	6.5 ± 2.3	0.295	0.232 ± 0.018	30.51 ± 3.52
4	+	32.90 ± 2.75	1.7 ± 0.6	0.288	0.284 ± 0.031	108.14 ± 9.76

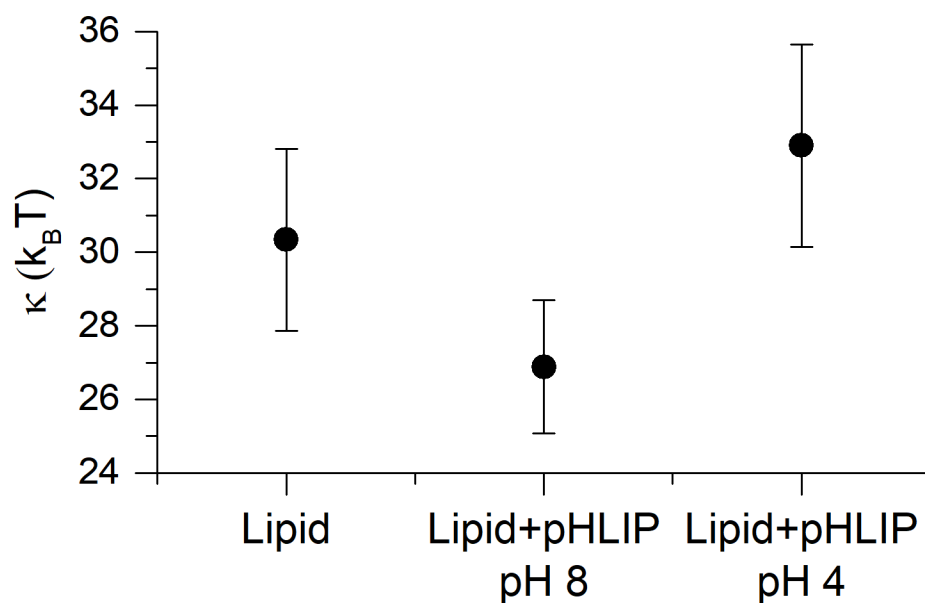


Figure 37. Bending fluctuations are not influenced by the presence of pHLIP.

Bending modulus values determined from Eq. 5 comparing lipid only to samples containing pHLIP in State II or State III. No change greater than the error of the measurement is observed for the bending modulus in any condition. Error bars are fitting errors.

where Γ_{TF} is the rate of relaxation of thickness fluctuations, and q_0 is the peak position of Γ/q^3 . $1/\zeta$ is the width of the Lorentzian which determines the fluctuation amplitude, $\Delta d_m \approx \pi/\zeta q_0^2$. Using these expressions, data were analyzed in the presence and absence of pHLIP to determine both Γ_{TF} and Δd_m (Figure 38A and Table 6). Experiments in the presence of peptide yielded thickness fluctuations with Γ_{TF} and Δd_m values comparable to previous reports with different lipids (Table 6) (64, 75, 76). Upon the inclusion of pHLIP at pH 8, we observed no Γ_{TF} changes. However, once pHLIP assumed the TM configuration, it caused a large Γ_{TF} suppression (Figure 38B and Table 6). Interestingly, no large change on Δd_m was caused by pHLIP in either State (Figure 39 and Table 6). We performed control experiments that showed that pH had no effect on the NSE data (Figure 40), indicating that the experimental Γ_{TF} changes result from the presence of the TM helix.

Recently, a new expression has been devised to extract additional information pertaining to membrane physical properties from NSE data (76). The new expression extracts additional elastic and viscous parameters:

$$\frac{\Gamma}{q^3} = \frac{\Gamma_b}{q^3} + \frac{K_A k_B T}{\mu q_0^3 k_B T + 4\mu q_0 K_A A_0 (q - q_0)^2}, (Eq. 7)$$

where q_0 is the peak position of Γ/q^3 , K_A is the area compressibility modulus determined from NSE experiments using protiated lipids, $k_B T$ is the thermal energy, Γ_b is the decay corresponding to bending fluctuations, μ is the membrane viscosity, and A_0 is the area per lipid. Fitting to this equation allowed to extract data the effect of pHLIP on both K_A and μ . We observed that the presence of pHLIP at the membrane surface or as a TM helix did not change K_A (Figure 41). Membrane adsorbed pHLIP did not have an effect on membrane viscosity (μ). However, as observed in when Eq. 6 was used for the analysis, the TM state of pHLIP caused a large μ increase (Figure 38C and Table 6).

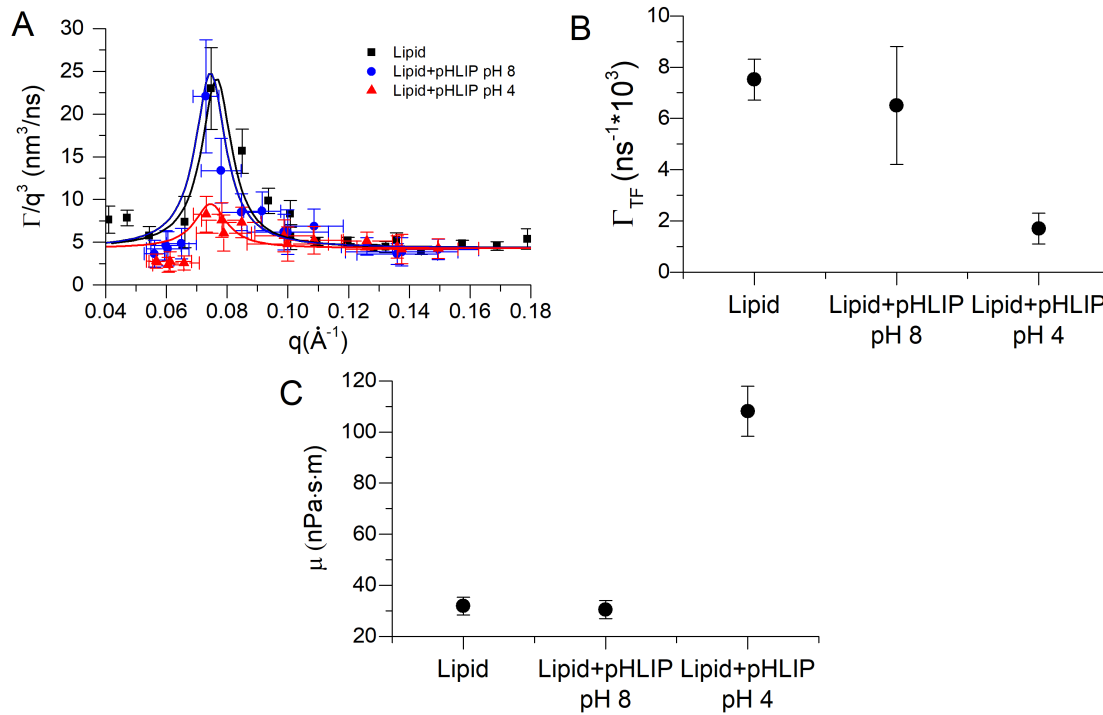


Figure 38. State III pHLIP slows down the thickness fluctuation rate along with an increase in membrane viscosity.

(A) The normalized relaxation rate Γ/q^3 as a function of q for DOPC/DOPS/Chol vesicles that include tail deuterated DOPC and Chol lipids. The height of the peak describes the rate of the fluctuations, and the width of the peak describes the amplitude. To obtain both the rate and the amplitude of the thickness fluctuations, each curve in A is fit with Eq. 6 (lines are the fit). (B) The rate of thickness fluctuations is suppressed only in the presence of State III pHLIP. (C) Correlated with the decrease in thickness fluctuations, membrane viscosity is drastically enhanced by State III pHLIP. Error bars are fitting errors.

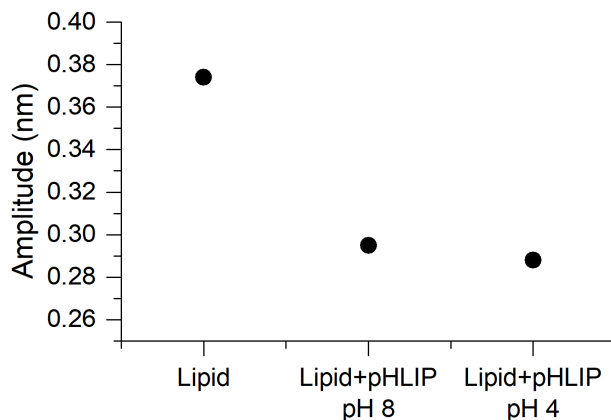


Figure 39. pHLIP does not change the amplitude of the thickness fluctuations.

Amplitude values for the thickness fluctuations determined from Eq. 6 comparing lipid only to samples containing pHLIP in State II or State III. No large change is observed for the thickness fluctuation amplitude in any condition.

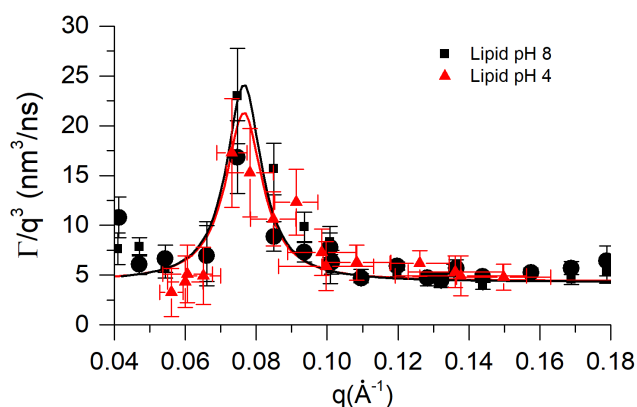


Figure 40. pH does not influence the measured thickness fluctuations.

The normalized relaxation rate Γ/q^3 as a function of q for DOPC/DOPS/Chol vesicles that include tail deuterated DOPC and Chol lipids. The height of the peak describes the rate of the fluctuations and the width are not affected by changing the pH. Error bars are fitting errors.

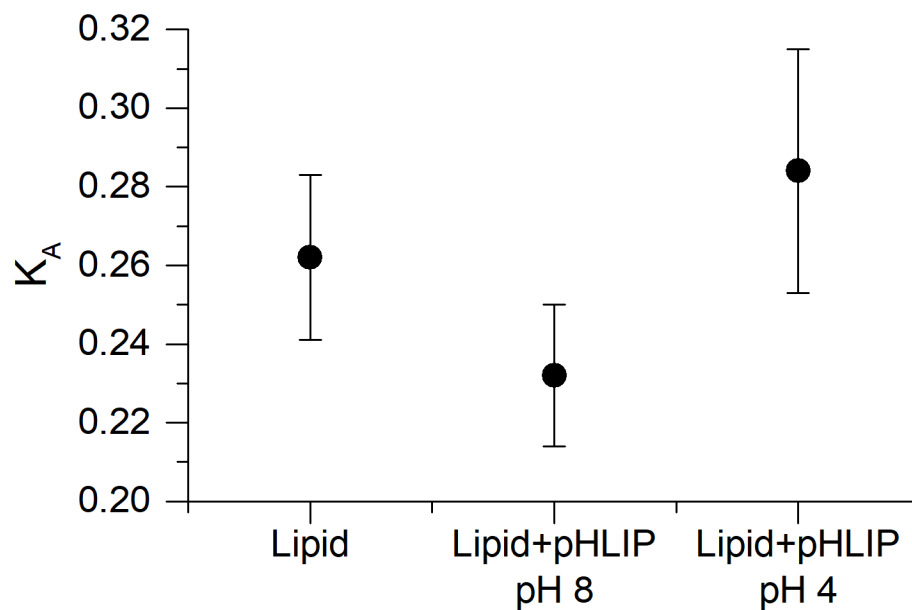


Figure 41. The area compressibility modulus (K_A) is not altered by pHLP.

K_A determined from Eq. 7 comparing lipid only to samples containing pHLP in State II or State III. No large change is observed for the K_A in any condition. Error bars are fitting errors.

The average bilayer thickness is unaffected by pHLIP in any orientation. The interaction of membrane proteins and their lipidic environment is driven by the sequestering of hydrophobic components present in each (209). To understand how the hydrophobic thickness of pHLIP compares to the membrane composition of interest here, we determined the bilayer structural properties in the absence and presence of pHLIP. To gain a deeper insight into this question, we employed both SANS and SAXS. With SANS, we used contrast variation within our samples to take advantage of the large neutron scattering length density difference between hydrogen and deuterium (210). To gain the highest resolution possible in our structural parameters, we jointly analyzed the SANS and SAXS data with a modified slab model, and also employed a core-multi-shell model to analyze the SANS data (210).

To obtain structural information, vesicles were measured in the presence and absence of externally applied pHLIP at pH 8 and 4. We measured SANS and SAXS on both protiated and deuterated samples (i.e., prepared using chain perdeuterated DOPC and a highly deuterated cholesterol) in D₂O buffer to obtain three differently contrasted datasets. We carried out both a joint and core-multi-shell model analysis of the data to extract the following structural parameters: the area per lipid (A_L), total bilayer thickness (Luzzati thickness) (D_B), hydrophobic thickness ($2D_c$), and phosphate-to-phosphate distance (D_{HH}) (Figures 42-44, and Table 7). We found quite good agreement between the two models, suggesting our obtained structural parameters are robust. Of particular interest here is the bilayer's hydrophobic thickness, which might change if hydrophobic mismatch were occurring between the lipid membrane and TM pHLIP. Instead, we found that addition of pHLIP did not have an effect on the bilayer structure (including hydrophobic thickness) in either its membrane-absorbed or inserted state (Table 7). Figures 45 and 46 show the

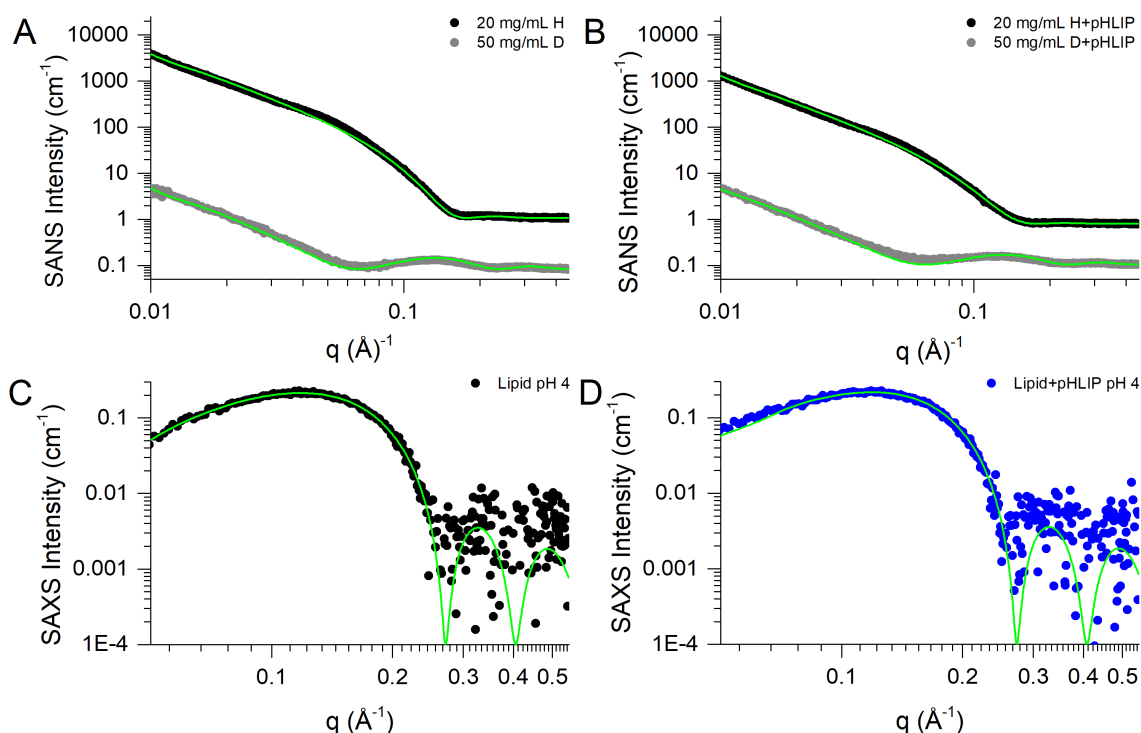


Figure 42. Average bilayer thickness of DOPC/DOPS/Chol vesicles is not significantly different in the presence of transmembrane pHLIP as determined by SANS and SAXS.

SANS (A, B) and SAXS (C, D) measurements of DOPC/DOPS/Chol vesicles in the absence (A,C) or presence of transmembrane pHLIP (B,D). Irrespective of pHLIP's inclusion, scattering curves display similarities suggesting pHLIP has little influence on static structural properties of the membrane such as bilayer thickness. Green line indicates the use of the joint analysis model to fit the data in all cases. Data is offset for clarity. SANS data includes protiated and tail deuterated vesicles in 100% D_2O .

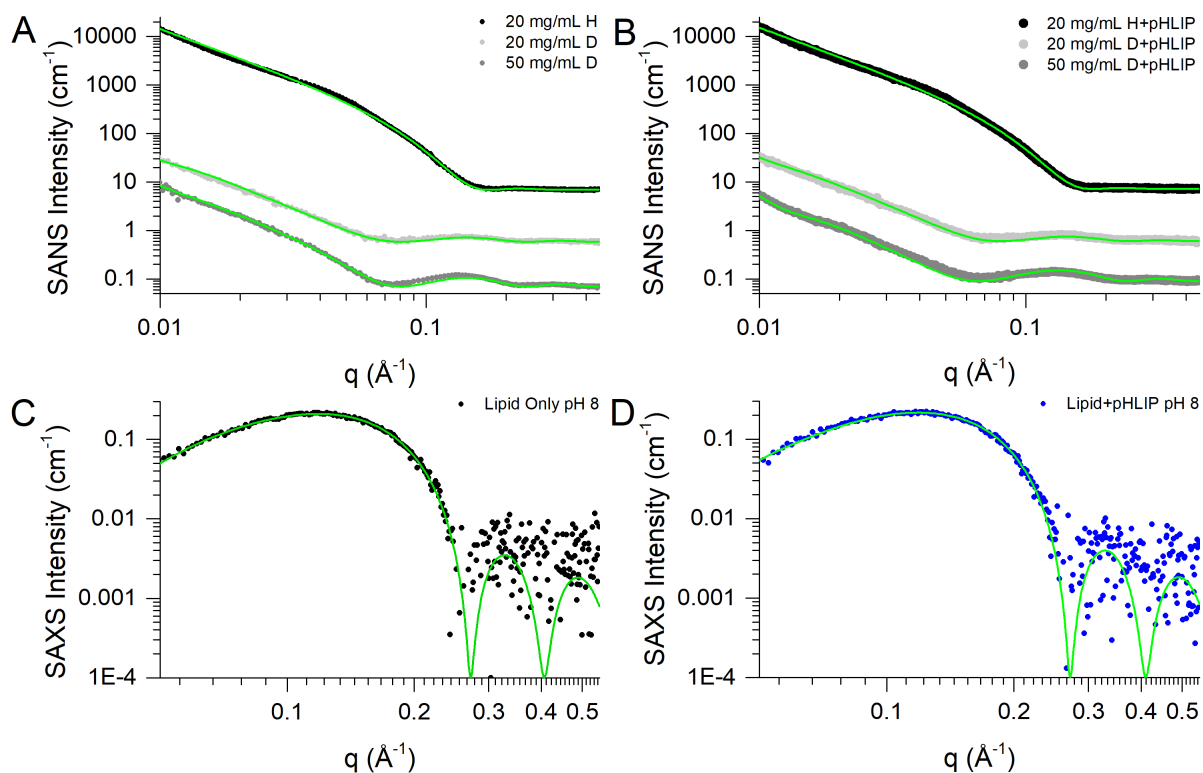


Figure 43. Joint analysis determines the average bilayer thickness of DOPC/DOPS/Chol vesicles is not significantly different in the presence of State II pHLIP as determined by SANS and SAXS.

SANS (A, B) and SAXS (C, D) measurements of DOPC/DOPS/Chol vesicles in the absence (A, C) or presence of State II pHLIP (B, D). Irrespective of pHLIP's inclusion, scattering curves display similarities suggesting pHLIP has little influence on static structural properties of the membrane such as bilayer thickness. Green line indicates the use of the joint analysis model to fit the data in all cases. Data is offset for clarity. SANS data includes protiated vesicles and two concentrations of tail deuterated vesicles in 100% D_2O .

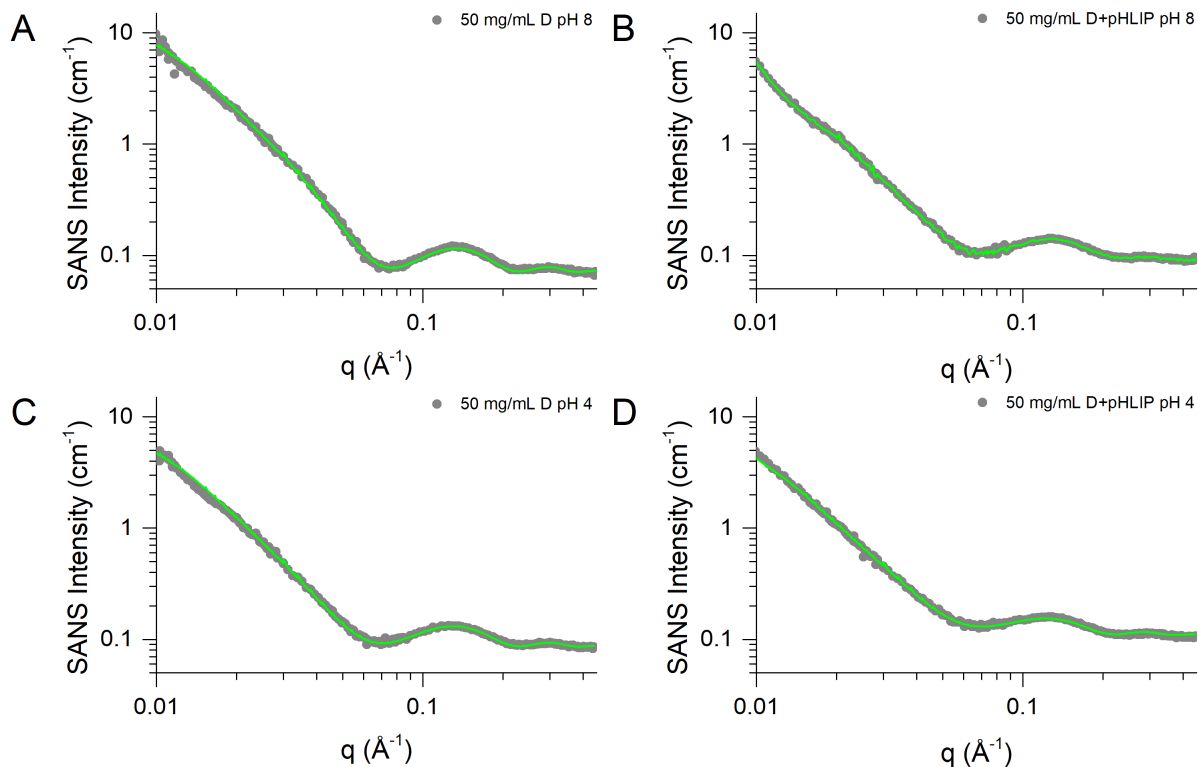


Figure 44. Core-multi-shell model analysis determines the average bilayer thickness of DOPC/DOPS/Chol vesicles is not largely different in the presence of pHLIP.

SANS measurements of DOPC/DOPS/Chol at 50 mg/mL vesicles in the absence (A,C) or presence of pHLIP (B,D). Scattering curves display similarities suggesting pHLIP has little structural influence on the membrane. Green line indicates a model fit to the data in all cases.

Table 7. Structural parameters obtained from both the joint and core-multi-shell model analysis of SANS/SAXS scattering data from DOPC/DOPS/Chol bilayers.

Structural parameters for the model include the area per lipid A_L , total bilayer thickness D_B , hydrophobic thickness $2D_C$, and phosphate-to-phosphate thickness D_{HH} . Errors were obtained using a bootstrap method described in the Supporting Information for the joint analysis. Joint analysis values are referred to as Joint, and core-multi-shell analysis values as Shell. Here, the core-multi-shell analysis was only used to analysis SANS data (Figure 44).

		Joint	Shell	Joint	Shell	Joint	Shell	Joint
pH	pHLIP	$A_L [\text{\AA}^2]$	$A_L [\text{\AA}^2]$	$D_B [\text{\AA}]$	$D_B [\text{\AA}]$	$2D_C [\text{\AA}]$	$2D_C [\text{\AA}]$	$D_{HH} [\text{\AA}]$
8	–	60.3 ± 0.8	59.17 ± 0.12	38.6 ± 0.5	39.29	29.7 ± 0.4	30.29	37.0 ± 0.1
8	+	60.2 ± 0.5	58.82 ± 0.16	38.6 ± 0.3	39.52	29.8 ± 0.2	30.47	37.0 ± 0.1
4	–	61.1 ± 0.6	59.65 ± 0.32	38.0 ± 0.4	39.64	29.3 ± 0.3	30.55	37.0 ± 0.1
4	+	60.4 ± 0.07	57.65 ± 0.16	38.5 ± 0.06	40.32	29.7 ± 0.05	31.08	37.2 ± 0.1

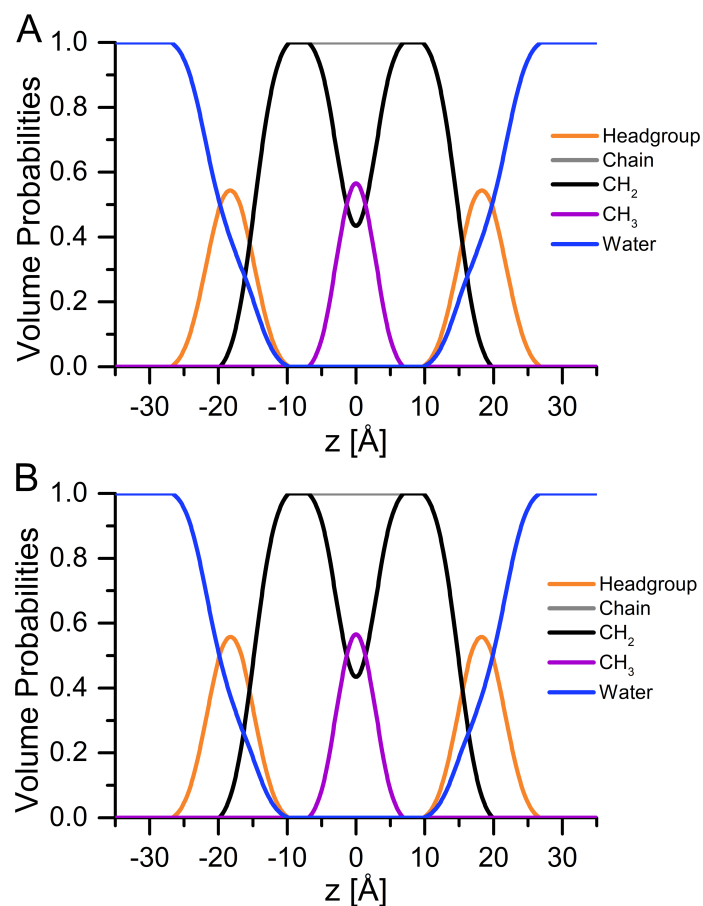


Figure 45. pHLIP does not change the bilayer structure.

Transbilayer volume probability profiles for DOPC/DOPS/Chol vesicles at pH 4 determined from the experimental SANS/SAXS scattering data. Comparison between the bilayer in the absence (A) and presence of transmembrane pHLIP (B) shows no significant effect of pHLIP on the distribution of lipid headgroup or hydrocarbon chain components.

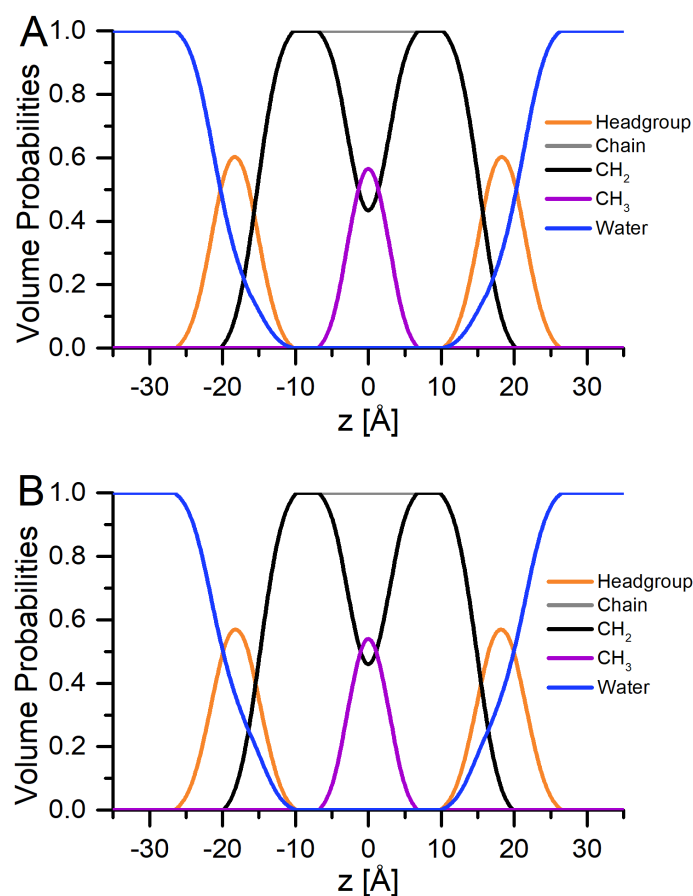


Figure 46. State II pHLIP does not change the bilayer structure.

Transbilayer volume probability profiles for DOPC/DOPS/Chol vesicles at pH 8 determined from the experimental SANS/SAXS scattering data. Comparison between the bilayer in the absence (A) and presence of pHLIP in State II (B) shows no significant effect of pHLIP on the distribution of lipid headgroup or hydrocarbon chain components.

volume probability profiles of lipid component groups determined from the joint scattering analysis. From these volume probability profiles, the position and distribution of the lipid headgroup and hydrocarbon chain components can be compared (Figure 45 and 46). The volume probability profiles are similar in the presence or absence of transmembrane pHLIP, suggesting that pHLIP has little to no influence on the position or distribution of the bilayer components under these conditions. This result suggests that a change in membrane thickness does not explain the changes the TM state helix of pHLIP causes on membrane thickness fluctuations.

Molecular probes do not detect effects of pHLIP on membrane viscous properties. Membrane lipids move both around their axes as well as laterally with the plane of the membrane. Changes in the phase state or inclusion of molecules such as cholesterol can influence both types of diffusive movements (211-213). Additional techniques can be employed to study lipid diffusion such as molecular probes (200, 201). To further probe the effect of pHLIP on membrane dynamics, we employed DCVJ and DPH dyes to probe changes in viscosity or fluidity, respectively.

DCVJ is a molecular rotor that measures the viscosity of a medium via changes in torsional relaxation (200). If the relaxation of the rotor is restricted by means such as increases in viscosity, the fluorescent signal increases (200). Using this probe, we assayed for viscosity changes induced by pHLIP (Figure 47A). We found that DCVJ did not detect a statistically significant change in membrane viscosity in the presence of TM or adsorbed pHLIP (Figure 47A) ($P > 0.05$). As a control for the sensitivity of DCVJ, we measured fluorescent intensity changes of dimyristolphosphatidylcholine (DMPC) as a function of phase state. DMPC has a gel to fluid phase transition (T_m) at $\sim 25^\circ\text{C}$. Assaying temperatures both well below and above this T_m we observed a large increase in the fluorescent intensity of DCVJ as temperature decreased (Figure 48). This

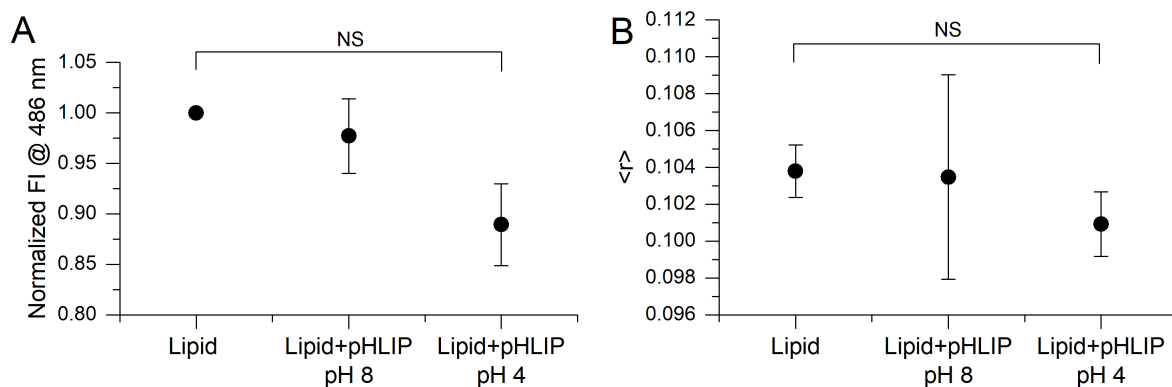


Figure 47. Membrane viscosity and fluidity investigated via fluorescent probes is not altered in the presence of pHLIP.

Membrane viscosity and fluidity were measured using the fluorescent probes DCVJ and DPH, respectively. (A) Changes at the peak fluorescence intensity of DCVJ (486 nm) were normalized to the lipid only sample. No statistical significance was observed in the presence of pHLIP in either state ($P > 0.05$). (B) Anisotropy ($\langle r \rangle$) of DPH was compared between the absence and presence of pHLIP in either State II or III. No statistical significance was observed with the inclusion of pHLIP in State II or III ($P > 0.05$). NS; no significance. Error bars are standard deviation. $N=3$.

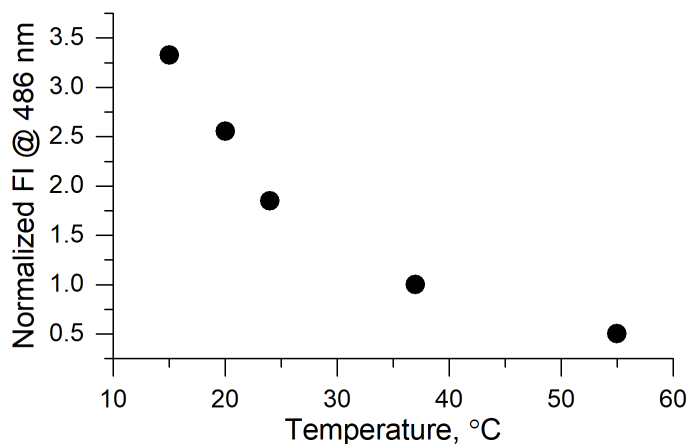


Figure 48. DCVJ responds to changes in the phase state of DMPC.

DCVJ measurements in DMPC vesicles as a function of temperature. The data shows that the intensity of DCVJ increases as DMPC transitions from the fluid to gel phase due to a decrease in temperature. Data was taken at the peak of the fluorescence spectra of DCVJ and is normalized to the 37°C data point.

control shows that DCVJ, in our experimental conditions, detects the membrane becoming more viscous as the lipid transitions to the gel state.

DPH anisotropy measurements were conducted to probe pHLIP effects on membrane fluidity. DPH absorbs light that is polarized on its long axis (201). The anisotropy of DPH can be determined based on how the molecule rotates and loses its initial polarization during its excited state, which is influenced by the fluidity of the environment (201). Employing this probe, we assessed if pHLIP changed the fluidity of the membrane (Figure 47B). In agreement with the DCVJ results, we observed that pHLIP did not cause a significant membrane fluidity change (Figure 47B) ($P > 0.05$).

5.5 Discussion

Cellular membranes are dynamic entities where lipids and proteins undergo a series of different motions. Some of these movements are local, occurring over small length-scales, while others involve collective motions of multiple molecules. One of such motions involves coordinated changes in the lipid acyl chains that result in the membrane thickness oscillating around an average value. It has only recently been possible to measure the minimal parameters that define membrane thickness fluctuations: the rate and amplitude (64, 75, 76). Here, we take advantage of such advances to determine if a TM helix influences thickness fluctuations.

We performed NSE experiments with vesicles composed of unsaturated lipids in the presence of pHLIP, a model membrane peptide. By using unsaturated lipids, we probe additional lipid species known to be essential to the plasma membrane. We chose pHLIP since a small pH change triggers its transition from a membrane surface to a TM configuration. Thus, the effect of the same polypeptide in the two fundamental types of membrane interactions can be compared. We in turn discovered a membrane composition that leads to the lowest pK of insertion observed for pHLIP.

The additive effect of DOPS and Chol lead to the lowest observed insertion pK of pHLIP.

Previous pHLIP studies have focused on understanding how membrane characteristics may influence pHLIP's efficacy for targeted cancer therapy (16, 39, 96). Here, we instead use pHLIP as a model polypeptide to understand protein influence on membrane structural and dynamical properties. pHLIP adsorbs to the surface of the membrane and, as external acidity increases, inserts into the membrane. Membrane insertion of pHLIP is typically studied *via* a pK of insertion assay (4, 10, 18). The presence of negatively charged lipids, specifically phosphatidylserine, decreases the pK (95), as does the presence of cholesterol in the bilayer (18). It was suggested that the negative charge of the PS headgroup interacts unfavorably with negatively charged pHLIP in membrane-adsorbed state, leading to increased solvation of pHLIP on the membrane surface (95).

Here, we find that the inclusion of both PS and Chol in a phosphatidylcholine vesicle further decreases the pK of insertion to the lowest observed for pHLIP (Figure 35A). This could suggest that membrane properties that influence pHLIP's insertion are additive in their effect on the insertion pK . Knowing this, we asked how does pHLIP influence membrane dynamics in the present lipid composition.

NSE detects pHLIP's influence on lipid viscosity causing a large reduction in the thickness fluctuation rate. Previously, thickness fluctuations have been studied in fully saturated lipid systems with the use of NSE (64, 75, 76). Recent advances allow for the extraction of additional membrane properties including membrane viscosity *via* translational diffusion of the lipids (76, 214, 215). A key membrane component missing from all previous studies was the inclusion of a membrane protein. Figure 38A shows that when pHLIP forms a TM helix, it strongly reduces the magnitude of relaxation rate (Γ/q^3) peak at $q=0.07 \text{ \AA}^{-1}$. Further analysis revealed that the observed differences did not result from a change in how much the thickness deviated from the average value (fluctuation amplitude, Δd_m), which is $\sim 10\%$ of the average bilayer thickness (75). Instead, changes resulted from a 3-4 fold decrease in Γ_{TF} , the rate of thickness fluctuations. Additionally, we determined that, in the presence of the TM helix, there was an increase in the viscosity parameter, μ . However, the membrane-absorbed state of pHLIP did not cause a change in either Γ_{TF} or μ (Figure 38 and Table 6). The data here would suggest that Γ_{TF} is suppressed by the increase in membrane viscosity. To further test this, we performed additional experiments to examine the influence of a TM helix on membrane thickness fluctuations. First, we performed small-angle scattering experiments to determine if pHLIP influenced the average membrane thickness.

Average membrane thickness is not influenced by pHLIP. Matching of the hydrophobic acyl chains and the TM region is energetically favorable due to the seclusion of hydrophobic components away from aqueous solvent otherwise a change in the system occurs (216-218). Such

changes can include membrane thickness, helix tilt, oligomerization, peptide backbone deformation, backbone kinking, and loss of a TM state (216-218). Changes in membrane thickness to accommodate either a longer or shorter TM helix have been observed previously (216, 217, 219-221). However, our data show no effect of pHLIP on the structural properties of the membrane (membrane thickness) in either state (Figures 41, 42, 44-46, and Table 7), comparable to the finding by Narayanan *et al* (222). However, we must note that the TM helix of pHLIP could tilt to alleviate hydrophobic mismatch. As a result, we propose that the observed effect of TM pHLIP on Γ_{TF} is not a consequence of hydrophobic mismatch. This result promoted us to further investigate changes in membrane viscosity *via* the use of DPH and DCVJ molecular probes.

pHLIP changes membrane viscosity by influencing the translational but not the rotational diffusion of lipids. Lipid movement within the bilayer occurs both rotationally around their axes, as well as translationally within the plane of the membrane. By using probes that are rotationally sensitive to their environment, one can measure changes in rotational diffusion. Here we used two different rotationally sensitive probes, DPH and DCVJ, that measure membrane fluidity and viscosity, respectively (200, 201). We found that neither membrane-absorbed pHLIP nor the TM helix of pHLIP affected membrane viscosity or fluidity as probed rotationally (Figure 47). However, this conclusion initially disagrees with the NSE data showing a large increase in membrane viscosity. To address this, a further discussion detailing the differences in the two techniques is required.

Here we use both neutron scattering and fluorescence spectroscopy to investigate viscous properties of the membrane. NSE measures the translational diffusion of lipids, whereas, the fluorescent probes DPH and DCVJ, measure rotational diffusion. Since rotational and translational diffusion measurements can be used to study membrane viscosity, both NSE and fluorescence can measure membrane viscosity changes. However, fundamental differences exist between rotational

(rotation about an axis) and translational (lateral movement within the plane of the membrane) diffusion (214, 215). A key difference is the range of energy propagation of their individual effects (214). In a 2D environment, the energy of rotational diffusion falls off as $1/r$, where r is the distance between two particles (214). However, the energy of translational diffusion decreases as $\log r$, suggesting that translational diffusion has farther reaching effects (214).

The influence of membrane inclusions, such as membrane proteins, and Chol on membrane viscosity has been determined using both translational and rotational diffusion as readouts. Sar1p, a vesicle trafficking protein, increased the level of membrane viscosity as additional Sar1p was added (212). Chol, a rigid molecular constituent of the plasma membrane, also increases membrane viscosity (213, 223, 224). Although both entities increased membrane viscosity, differences lie in the measurement techniques. DPH, a rotationally sensitive probe, was used to determine the influence Chol on viscosity, while Sar1p influence on viscosity was examined *via* translational diffusion (212, 224). Looking to Figure 49, we see that viscosity increases as a function of Chol concentration. Figure 49 also shows that NSE detects viscosity changes at lower Chol concentrations (9-1 lipid to Chol ratio) than those determined *via* DPH (3-1 lipid to Chol ratio). This would suggest that NSE, through examining translational diffusion, is more sensitive to low percentage inclusions in the membrane.

The contrast between NSE and the molecular probes suggest that the inclusion of pHLIP at low densities (150-1 lipid to peptide ratio) does not influence rotational diffusion in the same capacity as it does translational diffusion. Both the molecular probes and pHLIP are in relatively low membrane densities. The spatial separation between either DPH or DCVJ and pHLIP may inhibit the molecular probes from sensing pHLIP's influence on membrane viscosity due to the rapid loss of energy propagation. Thus, the molecular probes could be only sensing a localized viscosity that pHLIP may not disturb. With NSE, probe density is no longer a limitation as neutrons

are interacting with all lipids. This large-scale interaction allows NSE to detect the influence of pHLIP on translation diffusion and furthermore membrane viscosity.

We propose that pHLIP slows the rate of thickness fluctuations by increasing membrane viscosity (Figure 50). Although we observe that Chol also increases membrane viscosity (Figure 49), pHLIP affects viscosity to a higher extent. We suggest that pHLIP, being a TM helix interacting with both leaflets of the bilayer at once, leads to increased interleaflet coupling. Interleaflet coupling has been suggested to occur due to both interdigitation of acyl chains and by the rapid flip-flop of Chol (225-227). pHLIP forms a stable TM helix which could allow pHLIP to increase interleaflet coupling to a higher degree than acyl chain length or rapid Chol translocation. This agrees with pHLIP influencing viscosity greater than Chol even though pHLIP is at a lower density.

Conclusions. Here we have shown that a TM helix suppresses the rate of thickness fluctuations by increasing membrane viscosity, without altering the fluctuation amplitude. From SANS and SAXS, the effect on thickness fluctuations does not appear to be driven by hydrophobic mismatch between pHLIP and the membrane. However, we propose that pHLIP increases interleaflet coupling, driving increases in viscosity. A fast fluctuating membrane may inhibit protein motions known to occur in similar timescales (75, 198). Suppressing the rate of thickness fluctuations may serve as a mechanism for protein signaling, without the necessity of contact. Slower membrane fluctuations may allow protein motions, such as domain movements, to occur that might have been

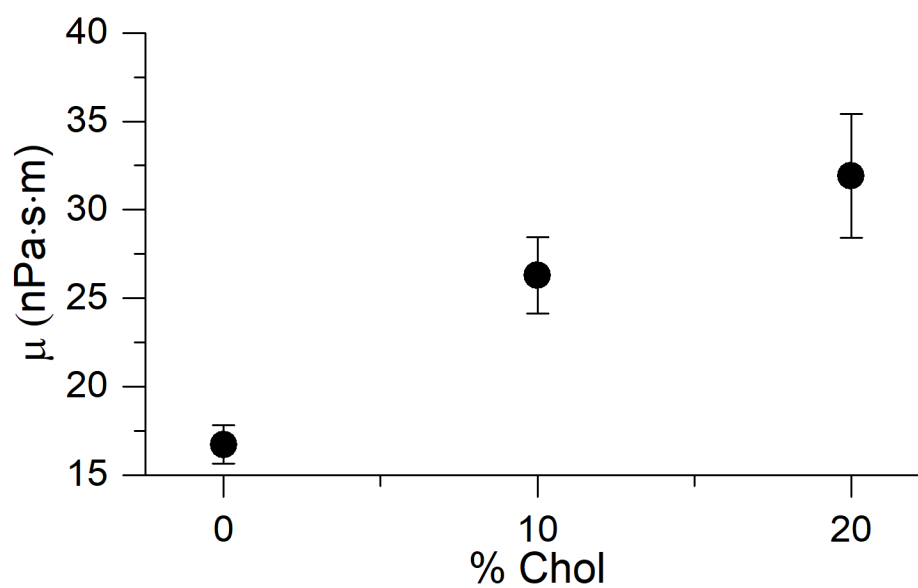


Figure 49. Membrane viscosity is influence by the concentration of Chol.

NSE data was analyzed with the use of Eq. 7 to determine the effect of various levels of Chol on membrane viscosity from NSE data. Increasing the molar % of Chol in the membrane lead to an increase in membrane viscosity.

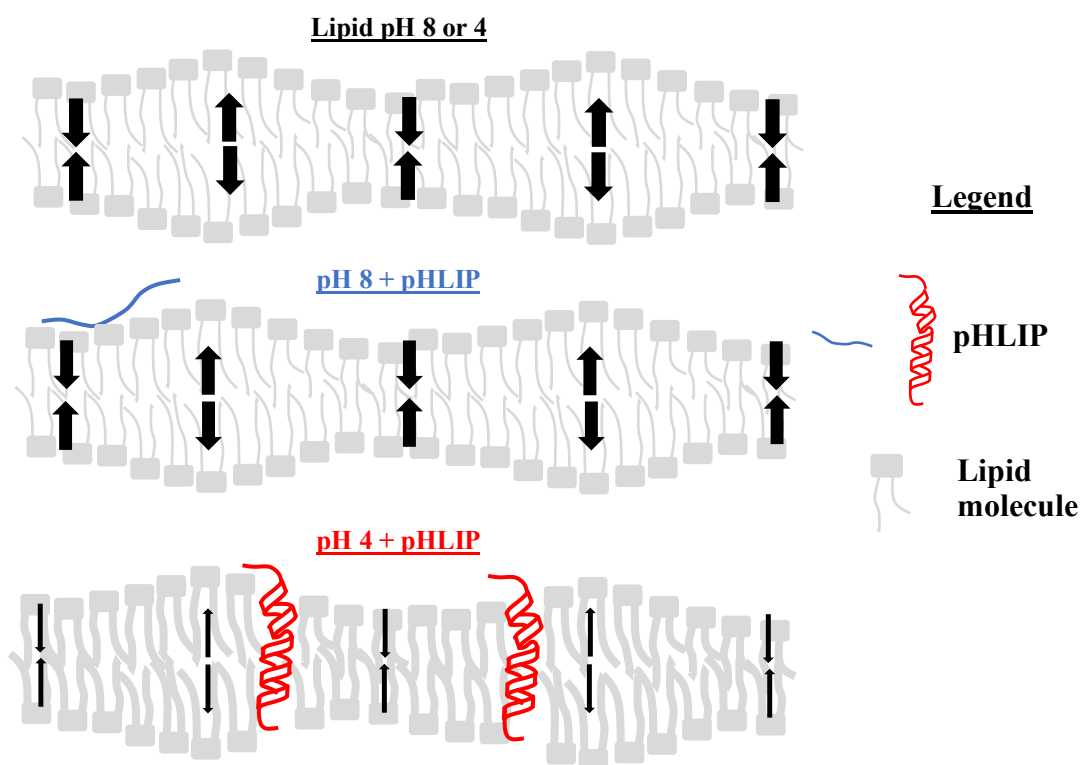


Figure 50. State III pHLIP leads to a suppression of membrane thickness fluctuations by way of increasing the membrane viscosity.

In DOPC/DOPS/Chol liposomes, thickness fluctuations occur irrespective of pH with a characteristic amplitude (represented by the height of the black arrows) and timescale (thickness of the black arrows). In the presence of State II pHLIP (pH 8+pHLIP), no change in thickness fluctuations are observed. Once pHLIP inserts into the membrane as State III (pH 4+pHLIP), the timescale of the thickness fluctuations decreases drastically along with an increase in membrane viscosity. The amplitude of the thickness fluctuations shows no variation in any conditions described. Black arrows are to scaled for amplitude and timescale changes. Thicker acyl chains indicate an increase in viscosity.

suppressed in the presence of a more rapidly fluctuating membrane. Additionally, suppressing thickness fluctuations may suggest that membrane proteins limit the occurrence of pore formation to allow the membrane to function as a semi-permeable membrane (195). Future studies will aim to investigate protein signaling *via* suppression of thickness fluctuations.

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Chapter VI: Conclusions and future directions

6.1 Conclusions

In this work, we used biophysical techniques to gain a deeper insight into the complex peptide-lipid interactions of pHLIP. Although pHLIP has primarily been studied in its relation to cancer applications, here we aimed to use pHLIP as a model system to study peptide-lipid interactions. Starting with the key parameter that describes pHLIP's insertion into the bilayer, the pK of insertion, we showed that different techniques determine distinctive values of the pK (15). We discovered that the gold standard SM analysis technique may not truly represent the pK of insertion. Instead, we suggested that by using a C-terminal NBD label, the pK of translocation can be obtained and more faithfully represents the insertion and transmembrane formation of pHLIP. These results advance the pHLIP field by allowing for a more precise determination of the pH at which pHLIP inserts a cargo into a cancerous cell. Additionally, knowledge of the different pK of insertion values can also be used to address which acidic groups in the sequence are influenced by changes in the membrane composition.

pHLIP has been extensively studied interacting with model membranes composed solely of PC phospholipids (4, 18). However, the plasma membrane is a chemically diverse entity containing a variety of additional phospholipid head groups other than just PC (57). The negatively charged lipid PS represents ~10% of the phospholipids in the plasma membrane and was chosen to be included in our model systems (57, 79). A healthy cell sequesters PS from the extracellular environment; however, PS asymmetry is lost in cancerous cells (91). In symmetric vesicles, we found that the inclusion of symmetric PS decreases the insertion pK to ~5.6 (~6.0 in PC) with a saturating point at ~5% PS (95). The presence of PS in the outer leaflet of cancerous cells would decrease the insertion pK of pHLIP. This result suggests that pHLIP may insert at a lower pH in cancerous tissues than previously considered. As cancerous tissues are typically only ~0.5 pH units

more acidic than their healthy counterparts, our study would propose that pHLIP needs to be modified to insert at a higher pH in the presence of PS exposure in cancerous cells (228).

Understanding how pHLIP interacts with symmetric PS systems prompted us to investigate how pHLIP may be influenced by an asymmetric distribution of PS. Using a recently developed technique to form stable asymmetric membranes, we investigated how membrane asymmetry affects the pK_{CM} of pHLIP (156, 157). We found that PS membrane asymmetry led to a basic shift in the pK of insertion compared to symmetric PS. We propose that this upshift in the pK of insertion was due to a change in one of the three membrane potentials, specifically the surface potential difference across the bilayer (176, 177). Since pHLIP shares similarities with mTMDs (inclusion of acidic residues and ability to reorient its topology), we hypothesized that this result suggests that membrane asymmetry influences the final topology of mTMDs in the plasma membrane (186).

As the previous studies describe how pHLIP is influenced by the membrane, we choose to investigate how pHLIP affects membrane physical properties. Membrane bilayers are associated with static thickness as well as dynamic properties, such as individual and collective motions of the lipids (63, 64). The use of small angle scattering opened the possibility of examining peptide-membrane interactions from the lipid stand point. With small angle scattering, we investigated how the membrane is affected by the presence of pHLIP. SANS and SAXS showed that pHLIP does not largely affect the average membrane thickness in either State II or State III. NSE suggested that in State II, pHLIP has no influence on membrane thickness fluctuations; however, in State III, TM pHLIP suppressed the rate membrane fluctuations. Thickness fluctuations occur at similar timescales such as protein folding and have been suggested to have implications in membrane pore formation (195, 229). pHLIP, as a model transmembrane protein, suppressing thickness fluctuations may suggest that membrane proteins limit the occurrence of pore formation

to allow the membrane to function as a semi-permeable membrane (195). Transmembrane inclusions (pHLIP) may act as barriers within the membrane to break up collective fluctuations.

The plasma membrane is an intricate constituent of the cell that contains a variety of lipids that are constantly in motion. Applying new techniques to incorporate the diversity of this lipid bilayer has allowed us to advance our understanding of its intimate relationship with pHLIP. A detailed understanding of the membrane insertion process along with the inclusion of more biologically relevant lipids has allowed us to ask additional biologically significant questions. We find that adding complexity to the membrane both influences pHLIP and is affected by pHLIP at each complexity level increase. Now that we have a clearer understanding of how pHLIP behaves in systems containing lipids with negatively charged head groups and systems that demonstrate collective motions, pHLIP can be employed as a model peptide to answer further questions involving peptide-lipid interactions. This work opens the doors for the use of pHLIP as a model peptide.

6.2 Future Directions

We have only begun to investigate the questions that can now be asked using pHLIP as a model peptide. Although many variants of pHLIP have been created and investigated, none have been assayed in model systems that contain PS (12, 25). Many of the current variants have been assayed to determine effectiveness against cancerous cells compared to pHLIP. pHLIP showed a decrease in the insertion pK in the presence of PS (95). This finding is not ideal as pHLIP will only target highly acidic tumors. There is potential that other variants such as pHLIP with the replacement of Asp 14 or 25 with Glu to display a pK of insertion similar to the extra-cellular pH of the tumor environment (25). Additional studies including pK of insertion assays should be conducted to determine if any of the pHLIP variants show great promise in the presence of PS.

Probing membrane asymmetry via the inclusion of PS asymmetry in model membranes is only the starting point. Additional lipids such as PE and SM should be included both individually and together in these studies to determine if other lipids influence the insertion of pHLIP. Not only should the lipid head groups be studied; investigation probing the influence of fluidity differences across the membrane should also be addressed. SM lipids typically possess a fully saturated N-acyl chain, whereas the glycerophospholipids commonly have a fully saturated acyl chain and an unsaturated acyl chain in the *sn*-1 and *sn*-2 positions, respectively (230). In asymmetric model membranes, SM presence in the outer leaflet reduces the fluidity relative to an inner leaflet composed of unsaturated glycerophospholipids (153) giving these vesicles leaflet fluidity asymmetry. Thus, the plasma membrane has an asymmetric distribution of lipid acyl chains present creating differences in fluidity between the two leaflets of the lipid bilayer, which might cause differences in packing and order across the bilayer. How leaflet fluidity influences pHLIP and in turn influences mTMDs is still unknown.

The influence of pHLIP on membrane physical properties was only investigated a single lipid system in State II and III. For future studies, it would be ideal to include additional lipids that contain a variety of acyl chain lengths and to also probe more than just State II and III. Looking at how pHLIP influences the static membrane thickness via thinning or thickening, SANS and SAXS will be employed. Future studies would use a range of acyl chains from 14:0 PC to 22:0 PC and additional pH points to probe affects near the *pK* of insertion. Preliminary data with 14:0 PC vesicles suggests that pHLIP can thicken the membrane in State III, as hydrophobic matching occurs due to the thin hydrophobic portion of 14:0 PC. Future NSE measurements should be used to investigate thickness fluctuations at the *pK* of insertion. A previous reports suggests that, at the *pK* of insertion, the membrane may be in a local non-bilayer state (17). Results from SANS, SAXS,

and NSE could help to explain how pHLIP is able to delivery large cargo across the membrane by creating membrane disruption at the pK of insertion.

Finally, differences in the level of saturation of the acyl chains in the outer leaflet and the inclusion of cholesterol in the plasma membrane has been speculated to lead to phase separation known as lipid rafts. Lipid rafts have been implicated in signal transduction and membrane trafficking (231). Rafts are thought to mediate key events in these processes when necessary for their function (231). Evidence of raft involvement in signaling has been observed in several different cellular signaling types (232-234). In times of inactive membrane trafficking or cellular signaling, raft components form clusters of nanoscale (~ 10 nm) domains that fluctuate in composition (235, 236). Once stimulation occurs, these nanoscale raft domains coalesce via lipid-lipid, protein-lipid, and protein-protein interactions, forming larger domains that bring the proteins in close proximity for cell signaling or membrane trafficking to proceed (235, 237). Targeting proteins to lipids rafts is thought to occur through lipid modifications such as GPI-anchors, palmitoylation, or myristylation; however, it is unknown how phase separation is affected by proteins outside of the raft phase (238-240). Using pHLIP as a model transmembrane protein, we can investigate how membrane proteins outside the raft phase influence lipid raft size, function, and existence.

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