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The Negative Charge of the Membrane Has Opposite Effects on the Membrane Entry and Exit of pH-Low Insertion Peptide

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Supporting Information

ABSTRACT: The pH-low insertion peptide (pHLIP) targets acidic diseases such as cancer. The acidity of the environment causes key aspartic acids in pHLIP to become protonated, 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 p*K* values.

The full extent of the complexity of tumors as organs has only recently been recognized. Tumors not only contain cancer cells, but a number of seemingly healthy, specialized cells are also found in the tumor microenvironment.[1] The composition of the tumor microenvironment is severely influenced by metabolites pumped out of tumor cells. Malignant cells have a metabolism altered compared to that of 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.[2] Because acidity is a general property of solid tumors, it holds great promise as a targeting principle in the design of technologies to fight cancer. A leading technology for targeting tumor acidity is the pHLIP (pH-low insertion peptide).[3] The pHLIP is soluble in aqueous solution, but when membranes are made available, it binds to them in a disordered conformation.[4] 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.[5,6] The pH sensing properties of pHLIP lead to its accumulation in multiple types of murine tumors, including melanoma, prostate carcinoma, and lymphoma,[3,7] as well as in human tumor biopsies.[8]

The physical properties of the lipid bilayer impact the interaction of pHLIP with lipid membranes. For example, the structure that pHLIP adopts at the membrane surface has been proposed to be influenced by the bilayer elastic bending modulus.[9] 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 with different compositions. Cell studies suggested that this is due to differences in the fluidity of the membrane.[9] Several differences exist between the membranes of healthy and tumor cells, one being their charge.[10] 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.[11] Conversely, healthy mammalian cells lack PS at their surface.[12] 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.[3] 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 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 with different compositions.

We first studied the acquisition of the surface-bound state by comparing the binding affinity of pHLIP for zwitterionic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) vesicles and negatively charged POPC/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) (70/30 molar ratio) vesicles. We hypothesized that binding of pHLIP to POPS-containing vesicles would be hindered, because of the negative charge that phosphatidylserine carries on its headgroup. The fluorescence intensity of a pHLIP−NBD complex was monitored to assess the binding affinity for the different lipid headgroups (detailed Experimental Procedures can be found in the Supporting Information). Strikingly, we found that pHLIP displayed a similar affinity for both lipid headgroups (Figure S1 of the Supporting Information). The obtained partition coefficients, $(2.1 \pm 0.4) \times 10^5$ and $(1.6 \pm 0.5) \times 10^5$ for

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POPC and POPC/POPS vesicles, respectively, result in very similar binding ΔG values: -7.3 ± 0.2 and -7.1 ± 0.3 kcal/mol, respectively. Unexpectedly, this indicates that the negative charge of POPS has no effect on pHLIP binding, suggesting that the affinity for the negatively charged vesicles is not reduced because of 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.[13] 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.[14] Our results (Figures 1 and 2) demonstrate that the

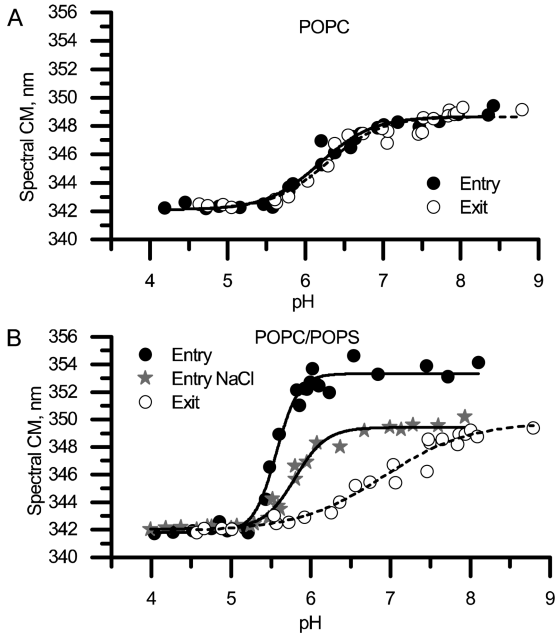

Figure 1. Membrane entry and exit transitions. Acidification leads pHLIP to insert into the membrane (entry). After a subsequent increase in pH, pHLIP leaves the membrane core (exit). (A) POPC and (B) 90/10 POPC/POPS vesicles. Stars show how 0.4 M NaCl affects entry in 95/5 POPC/POPS vesicles.

presence of POPS causes a sharp decrease in the entry pK, until saturation occurs at ~5% POPS with a pK of ≈5.6 (Figure 2). The overall content of PS in the plasma membrane of mammals is around 10%.[12] Consequently, the observed pK decrease was obtained for physiological levels of PS.

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 3). At pH 8, pHLIP binds to the periphery of either vesicle type in a largely disordered 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

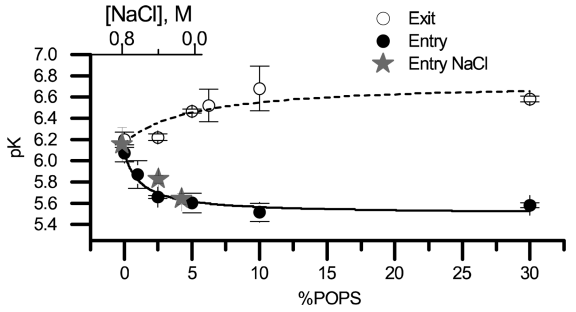

Figure 2. Impact of POPS on pK values for pHLIP membrane entry (filled symbols) and exit (empty symbols). The role of electrostatic interactions is assayed with varying NaCl concentrations in the entry experiments with 95/5 POPC/POPS vesicles.

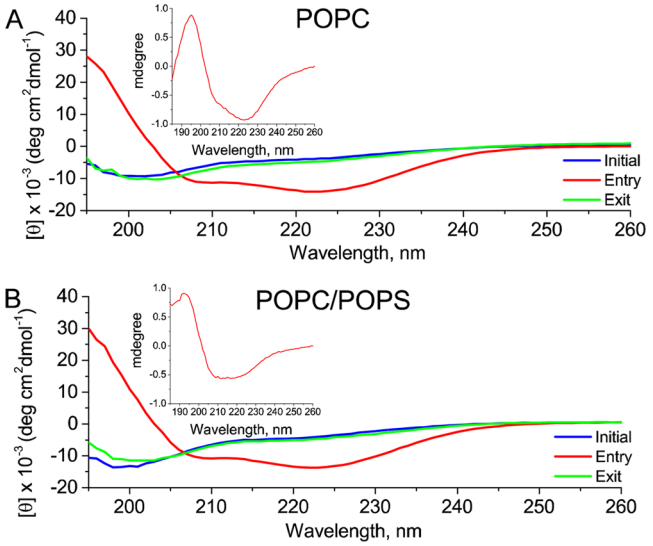

Figure 3. Reversible formation of the TM helix. Circular dichroism (CD) for (A) POPC and (B) 90/10 POPC/POPS vesicles. Peptide was incubated with vesicles at pH 8 (blue lines) and then acidified to pH 4 to induce membrane entry (red lines). Finally, the pH was increased back to 8 to attain exit (green lines). Insets show results of oriented CD experiments at pH 4.

POPS did not influence these spectral features, indicating that POPS does not affect 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 of Figure 3). Despite the small differences observed for both lipids, their OCD spectra share features (maximum at ~195 nm and broad minima between 210 and 225 nm), typically observed for a TM helix.[15] The combination of the two types of CD experiments demonstrated that pHLIP still forms a TM helix in the presence of negatively charged lipids.

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.[16] 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

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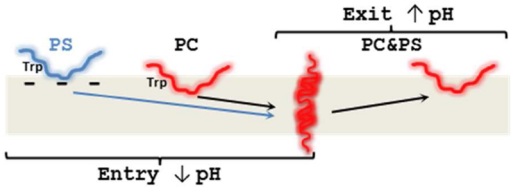
hydrated when the membrane contains POPS. Insights pertaining to the peripheral state of pHLIP can be gained by examining the degree of insertion in the bilayer of the two tryptophan residues of pHLIP (Figure S2 of the Supporting Information). This information can be used to assess if the negative charge of POPS affects the location of pHLIP on the membrane surface compared to POPC. Figure 1 shows that the fluorescence center of mass at neutral pH is different between POPC (349 nm) and POPC/POPS (354 nm) vesicles. This suggests changes in the membrane depth of pHLIP as the tryptophan residues insert deeper into the POPC bilayer than in POPC/POPS vesicles (see Scheme 1). We propose that the

Scheme 1. Model of Membrane Entry and Exit of pHLIP[a]



[a]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 Figure S2 of the Supporting Information.

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. Because 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 (Figures 1B and 2). These data indicate that electrostatic repulsion, despite not significantly influencing the affinity of pHLIP for vesicles, does play an important role in 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, because the peptide does not irreversibly insert into 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.[3] In our experimental system, membrane exit of pHLIP occurs when the acidic pH is raised back to neutrality (Figure 1, empty symbols). Intrigued by the possibility of PS also playing a role in 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 vesicles. For POPC, the results demonstrated an exit pK of 6.2 ± 0.1, in agreement with previous observations (Karabadzhak et al., manuscript submitted for publication). However, the addition of POPS led to an increase in the exit pK to 6.7 ± 0.2 (Figure 2), demonstrating that POPS alters the exit of pHLIP, as well. This suggests that in the presence of POPS higher pH values 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 increase the pK_a values of the key aspartates.

CD data showed that the membrane entry of pHLIP was not affected by POPS. However, it is possible that POPS could make the exit of pHLIP only partially reversible, hindering the return of pHLIP to a largely disordered conformation from the TM helix. CD was used to determine if the exit of pHLIP was as reversible in POPC/POPS vesicles as in POPC vesicles (Figure 3). Our CD results demonstrated high reversibility regardless of the lipid headgroups (Figure S7 of the Supporting Information).

Scheme 1 depicts a conceptual model summarizing most of our results. For 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 that for POPC vesicles. 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, because charged residues would be more stable in a hydrated environment than in the vicinity of the hydrophobic methylene groups of the acyl chains.[17] Figure 1B shows the existence of hysteresis between the entry and exit transitions, with a pK difference close to 1 pH unit (Figure 2). Hysteresis is believed to occur when there is a large activation barrier to either folding, unfolding, or both.[18,19] In the presence of PS, we observed differences between the peripheral state prior to and after insertion. Figure 1B 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 S4 of the Supporting Information) and monolayer experiments (Figure S8 of the Supporting Information). 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 vesicles 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 on 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 and/or insertion of membrane proteins into membranes is the membrane electrical potential.[20,21] The presence of negatively charged lipids affects the membrane surface potential. Consequently, we cannot rule out the possibility 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 show that the mechanism behind these changes is not a variation of 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

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of the acidic residues that control the entry and exit of pHLIP, with an important electrostatic repulsion component.

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.[22] 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.

■ ASSOCIATED CONTENT

Supporting Information

Detailed methods and supplementary figures and references. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

H.L.S., V.P.N., D.S.A., K.R.B., F.L.D., and J.B. performed the research. H.L.S., V.P.N., J.B., and F.N.B analyzed the data. H.L.S., V.P.N., J.B., and F.N.B. wrote the manuscript.

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Notes

The authors declare no competing financial interest.

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