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Diffusion as a Probe of Peptide-Induced Membrane Domain Formation

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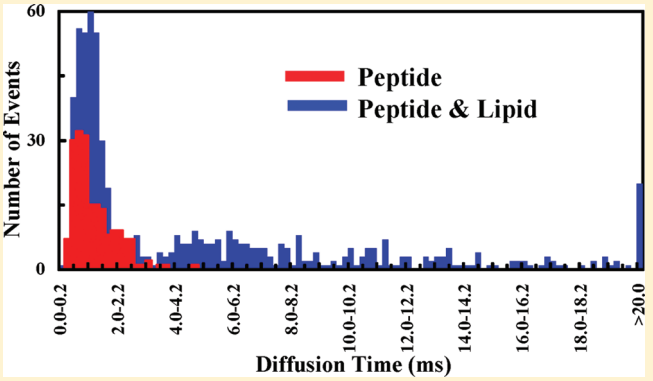
ABSTRACT: Recently, we have shown that association with an antimicrobial peptide (AMP) can drastically alter the diffusion behavior of the constituent lipids in model membranes (*Biochemistry* 49, 4672−4678). In particular, we found that the diffusion time of a tracer fluorescent lipid through a confocal volume measured via fluorescence correlation spectroscopy (FCS) is distributed over a wide range of time scales, indicating the formation of stable and/or transient membrane species that have different mobilities. A simple estimate, however, suggested that the slow diffusing species are too large to be attributed to AMP oligomers or pores that are tightly bound to a small number of lipids. Thus, we tentatively ascribed them to membrane domains and/or clusters that possess distinctively different diffusion properties. In order to further substantiate our previous conjecture, herein we study the diffusion behavior of the membrane-bound peptide molecules using the same AMPs and model membranes. Our results show, in contrast to our previous findings, that the diffusion times of the membrane-bound peptides exhibit a much narrower distribution that is more similar to that of the lipids in peptide-free membranes. Thus, taken together, these results indicate that while AMP molecules prompt domain formation in membranes, they are not tightly associated with the lipid domains thus formed. Instead, they are likely located at the boundary regions separating various domains and acting as mobile fences.



The mechanism of action of antimicrobial peptides (AMPs) has been the subject of extensive studies.[1−7] Findings from these studies have prompted the formulation of several models of membrane disruption by AMPs. For example, the ability of AMPs to disrupt the structural integrity of the targeted cell membranes has been attributed to (a) barrel-stave or/and toroidal pore formation,[8,9] (b) membrane dissolution in a detergent-like manner,[10,11] (c) formation of lipid−peptide domains,[12−25] (d) segregation of anionic lipids and zwitterionic lipids,[23−27] or (e) formation of nonlamellar phases.[28−30] Recently, we have shown that the diffusivity of individual lipids in an AMP-bound membrane, probed via fluorescence correlation spectroscopy (FCS), provides a sensitive means to monitor how AMP binding affects the membrane's structure and dynamics,[31] even at very low peptide/lipid ratios. As shown (Figure 1), results obtained from two well-studied AMPs, magainin 2 (mag2) and mastoparan X (mpX), showed that AMP binding can drastically alter the lipid diffusion behavior and that at relatively high peptide/lipid ratios the lipid diffusion times through a well-defined confocal volume, acquired by repeating measurements, are found to distribute over a wide range of time scales (the resultant distribution is hereafter referred to as τ_D distribution). While in our previous study we tentatively attributed the observed heterogeneity in lipid diffusion to AMP-induced domain formation,[31] which is consistent with a recent molecular simulation[26] as well as several experimental studies using supported lipid bilayers[17,18] and vesicles,[20−25,32,33] we have not ruled out other possibilities. For example, it is not clear to what extent the putative peptide oligomers and/or pores contribute to the observed distribution of lipid diffusion times.

In order to substantiate our previous conclusions and to further reveal the identity of the slowly diffusing species, herein we use FCS to measure the diffusion times of membrane-bound mag2 and mpX at various peptide/lipid ratios. The underlying premise is that if stable or transient (i.e., stable on the time scale of diffusion through the confocal volume) peptide−lipid clusters of different sizes are formed, distribution of peptide diffusion times should be, to a large extent, similar to the distribution of lipid diffusion times. On the other hand, different distribution scenarios would arise if those slowly diffusing species were composed mostly of lipids. Our results show that for both mag2 and mpX the resultant τ_D distributions are markedly different from those obtained with lipids, suggesting that AMPs prompt but do not directly participate in the formation of lipid domains.

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MATERIALS AND METHODS

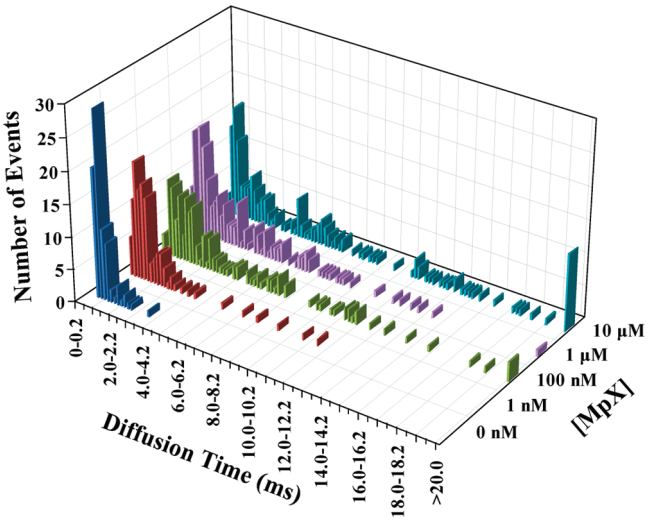

Figure 1. τ_D distributions of fluorescent labeled lipids in POPC GUVs obtained at different bulk mpX concentrations, as indicated (reproduced with permission from ref 31).

Peptides. Mag2 (sequence: GIGKFLHSAKKWGKAFVG EIMNS) and mpX (sequence: INWKGIAAMAKKLL) were synthesized using the standard fluoren-9-ylmethoxycarbonyl- (Fmoc-) based solid-phase method on a PS3 peptide synthesizer (Protein Technologies, MA). The dye molecule, 5- (and 6-) carboxytetramethylrhodamine (5,6-TAMRA), was manually coupled to the N-terminus of the peptide (for mag2 one additional glycine residue was added to the N-terminus). The coupling reaction was performed prior to any cleavage reactions to minimize any undesirable couplings to side chains or the C-terminus, according to the method of Bilgiçer and Kumar.[34] Prior to manual cleavage of the peptide from the resin, which was performed using a cocktail of 95% trifluoroacetic acid, 2.5% water, and 2.5% triisopropylsaline, the unreacted dye was washed off using *N*,*N*-dimethylformamide (DMF). The dye-labeled peptide product was further purified by reverse-phase chromatography and verified by matrix-assisted laser desorption ionization (MALDI) mass spectroscopy. The fluorescently labeled mpX and mag2 are hereafter referred to as TMR-mpX and TMR-mag2, respectively.

Giant Unilamellar Vesicles. Giant unilamellar vesicles were prepared according to an electroswelling method,[35] and the details have been described previously.[31]

FCS Experiment. The FCS setup, sample preparation, data acquisition, and analysis protocols are same as those used in our previous study.[31] Briefly, all of the FCS measurements were performed by placing the focus of the excitation laser beam near the center of the apical regions of giant unilamellar vesicles (GUVs) of approximately 10−50 μm in diameter, which remained static and intact on the time scale of the FCS measurement. Each FCS curve was obtained by correlating the fluorescence signal for a duration of 40 s and fit to a two-dimension diffusion equation to yield a characteristic diffusion time (τ_D). On average, for each experimental condition (i.e., a specific bulk peptide concentration) more than 250 FCS traces were collected from approximately 25 GUVs. In addition, the final (bulk) concentration of the labeled peptide in all of the peptide−GUV samples was kept at 1 nM, and the reported peptide concentration corresponds to the sum of the labeled and unlabeled peptide concentrations. In order to present the data in a histogram format (i.e., τ_D distribution), the experimentally determined diffusion times were binned with a bin size of 200 μs.

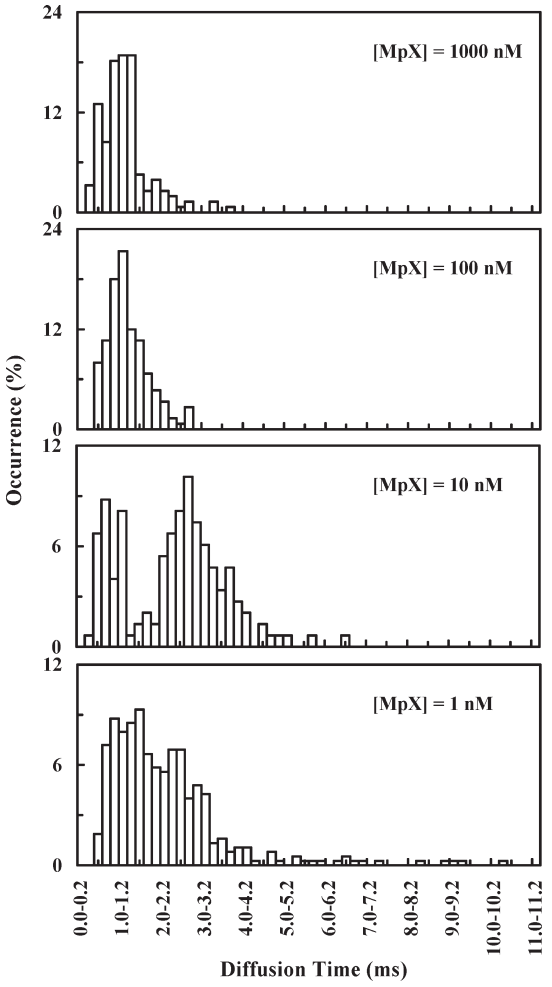

Figure 2. τ_D distributions of TMR-mpX in the membranes of POPC GUVs obtained at different bulk mpX concentrations, as indicated.

RESULTS

Mastoparan X. As shown (Figure 2), the diffusion of mpX in the membrane of POPC GUVs is heterogeneous, especially at low peptide concentrations. For example, at 1 nM mpX the diffusion time of the peptide shows a broad distribution ranging from 600 μs to more than 10 ms with an average value of 2.2 ms. Since the peptide diffusion time in buffer (i.e., in the absence of membranes) is 82 ± 12 μs, these data indicate that the diffusivity of membrane-bound peptides is significantly smaller than that of the free peptide. What is more interesting is that the τ_D distribution of the membrane-bound peptide becomes narrower with increasing peptide concentration, which is in stark contrast with that obtained with the lipid diffusion (Figure 1). For example, at 1 μM peptide concentration the diffusion times are distributed between 400 μs and 3 ms, whereas under the same conditions the lipid diffusion times sample a much wider time range. In addition, the average diffusion time of the membrane-bound peptide at 1 μM is approximately 1.0 ms, which is similar

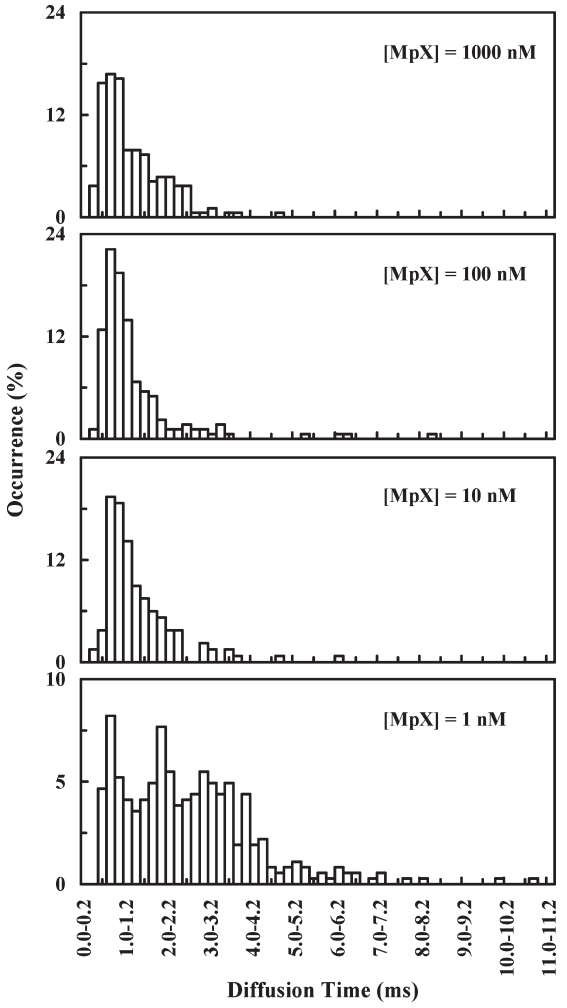

Figure 3. τ_D distributions of TMR-mpX in the membranes of POPC/POPG (3/1) GUVs obtained at different bulk mpX concentrations, as indicated.

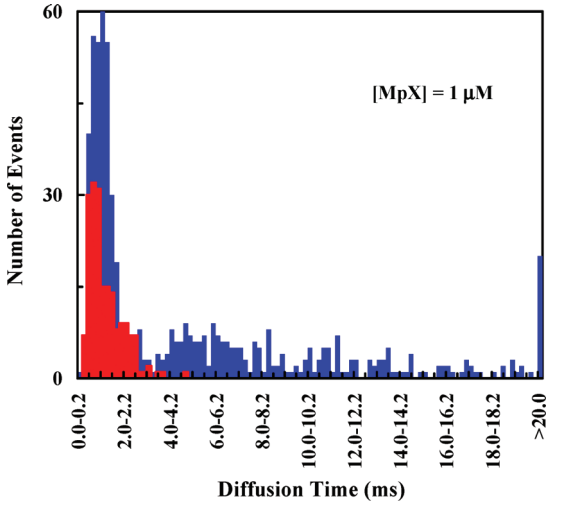

Figure 4. Comparison of the τ_D distributions of TMR-mpX (red) and TMR-mpX and TR-DHPE (blue) in the membranes of POPC/POPG (3/1) GUVs. In both cases the peptide concentration was 1 μM.

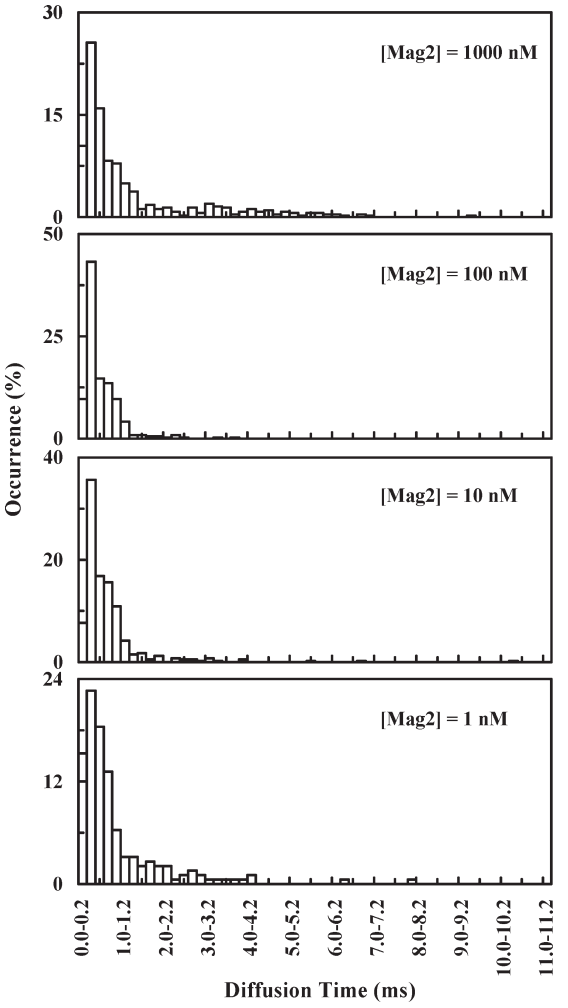

Figure 5. τ_D distributions of TMR-mag2 in the membranes of POPC GUVs obtained at different bulk mag2 concentrations, as indicated.

to the average diffusion time (~1.2 ms) of the lipids in peptide-free POPC GUVs.[31]

As shown (Figure 3), the τ_D distributions of mpX obtained with POPC/POPG (3/1) GUVs qualitatively show the same behavior. In this case, the most noticeable difference is that the τ_D distribution appears to be less sensitive to the peptide concentration; namely, at peptide concentrations of $\geq$10 nM, the distributions are more or less the same. This presumably arises from the stronger binding affinity of cationic AMPs toward membranes containing anionic lipids (e.g., POPG). Similar to that observed in POPC GUVs, the average diffusion time of the peptide at 1 μM is about 1.2 ms.

As a control experiment, we have also measured the τ_D distribution of a mpX-GUV sample that contains a trace amount of fluorescently labeled mpX (1 nM) and lipid (i.e., 0.002% TR-DHPE).[31] As shown (Figure 4), simultaneous excitation of the fluorescent peptide and lipid results in a τ_D distribution that is drastically different from that resulting from only the diffusion of the peptide. Taken together, these results decisively indicate that the lateral diffusion of a significant fraction of the membrane-bound peptides is independent of the diffusion of the lipid or lipid species.

Magainin 2. As shown (Figures 5 and 6), the τ_D distributions of mag2 obtained with both POPC and POPC/POPG (3/1)

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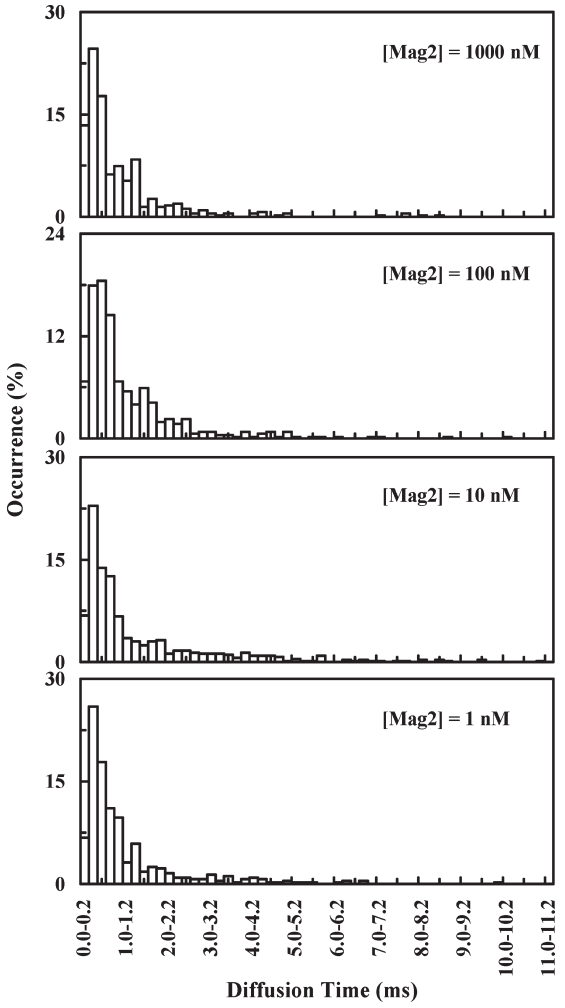

Figure 6. τ_D distributions of TMR-mag2 in the membranes of POPC/POPG (3/1) GUVs obtained at different bulk mag2 concentrations, as indicated.

GUVs also show that the translational diffusion property of the AMP is distinctively different from that of the lipid.[31] In particular, these and the data obtained with mpX all indicate that a large percentage of the membrane-bound AMP molecules have a diffusion coefficient (e.g., for the current setup a τ_D of 1 ms gives rise to a $D = 1.3 \times 10^{-7}$ cm^2/s) that is comparable to that measured for other peptides and proteins,[36] especially at relatively high peptide concentrations (e.g., 1 μM) where the AMP molecules are expected to form oligomers and induce membrane domain formation. In conjunction with our previous results,[31] this finding is interesting as it suggests that the role of the AMPs is to prompt the formation of stable and/or transient lipid clusters or domains that are distinguishable by their diffusion times. Additionally, at the same bulk peptide concentration the τ_D distribution of mag2 is measurably different from that of mpX, which is expected since the peptide sequence is known to play a key role in AMP−membrane interactions.

DISCUSSION

Recently, we demonstrated that FCS is a sensitive method to probe AMP−membrane interactions.[31] In particular, we showed that binding of an AMP can cause the diffusion times of the constituent lipids of two model membranes to significantly deviate from their intrinsic values (i.e., those measured in the absence of any peptides). In addition, we found in many cases that the diffusion times spread over a wide range of time and are too long to be ascribed to the diffusions of peptide oligomers. Thus, we tentatively attributed the observed heterogeneity in lipid diffusion to AMP-induced membrane domain formation.[31] While this interpretation is consistent with several studies,[16−27] our previous data in themselves do not elucidate the peptide's role. In other words, we were unable to determine whether these slow diffusing species, monitored via a fluorescent lipid, correspond to tightly bound peptide−lipid clusters or lipid domains. In addition, a recent molecular dynamics simulation by Niemelä et al.[37] indicated that, in a membrane containing a transmembrane protein channel, the diffusion of the lipids could be intrinsically heterogeneous, as the lipids close to the protein are found to diffuse much slower compared to those far from the protein. Therefore, we seek to better understand our previous results by measuring the diffusion time of the AMP of interest. If the observed diffusing species consist of both AMP molecules and lipids, which are tightly bound to each other, it can be assumed that measurements of either the lipid diffusion or peptide diffusion would result in similar τ_D distributions. On the other hand, if the diffusion of peptide species is not slaved to the diffusion of the lipid species, a different set of results would emerge.

As shown (Figures 2 and 3), at relatively low peptide concentrations (e.g., 1 and 10 nM) the diffusion of the membrane-bound mpX is rather heterogeneous, and in some cases (e.g., at 10 nM) two distinctive distributions are observed. In particular, the corresponding τ_D distribution is similar to that obtained with a pH low insertion peptide (pHLIP) that is bound to membrane surface.[38] Because the diffusivity of a membrane-bound object is expected to depend on its size and orientation, as well as the strength of interaction with the surrounding lipid molecules, these results seem to be consistent with the notion that at low peptide/lipid ratios the AMPs occupy primarily the surface-bound or S state [39] wherein the peptide may sample an ensemble of conformations and/or orientations.[40,41] An alternative interpretation is that this heterogeneity in peptide diffusion reflects the formation of an ensemble of transiently populated and dynamic peptide−lipid clusters, as observed in the simulation of Niemelä et al.,[37] that have different sizes and hence different diffusion times.

In contrast, the data obtained with mag2 are less pronounced in this regard. As shown (Figures 5 and 6), in both types of membranes mag2 exhibits a τ_D distribution that shows a high probability of occurrence within the time range of 100−600 μs. It is known that mpX binds to POPC membranes at least 10 times stronger than mag2[42] and that in the entire concentration range studied mag2 is much less effective than mpX at altering the diffusivity of the lipids in POPC membranes.[31] Thus, for POPC GUVs such fast diffusing species (i.e., those diffusing faster than the lipids) may correspond to peptides that are only weakly associated with the membrane surface. A recent single-particle tracking study has shown that when a cell-penetrating peptide "floats" on the headgroup region of a membrane, its diffusion coefficient is much larger than that of the lipid.[43] However, the results obtained with POPC/POPG GUVs are more difficult to explain. Interestingly, our previous study on the lipid diffusion in POPC/POPG GUVs indicated that mag2 causes a significant fraction of the fluorescently labeled lipids to diffuse faster than

(i.e., $\tau_D \approx 400\ \mu s$) the lipids in peptide-free membranes. Thus, these results provide strong evidence suggesting that a large number of mag2 molecules are located in loosely packed regions of the membrane, where the effective membrane viscosity is expected to be smaller than that of well-packed or more ordered lipid regions. This picture is consistent with the finding that transmembrane helices prefer the liquid-disordered phase in model membranes.[16−23,44]

What is more interesting is that at relatively high peptide concentrations the τ_D distribution of the membrane-bound peptide differs significantly from that of the lipid (e.g., Figure 4). Our previous study showed that the heterogeneity in lipid diffusion increases with increasing peptide concentration,[31] presumably due to an increased degree of membrane structural disintegration by the AMP, whereas our present study indicates that in comparison the peptide diffusion is remarkably less heterogeneous and on average much faster than the lipid diffusion. These results, taken together, provide compelling evidence suggesting that a large fraction of membrane-bound peptides, regardless of their oligomeric states, do not form stable clusters with the membrane lipids. In other words, the exceedingly wide distribution of diffusion times we previously observed using a tracer fluorescent lipid[31] in AMP-bound membranes is a manifestation of AMP-induced perturbations to the local lipid organization or peptide-induced membrane domain formation. Moreover, the wide τ_D distribution of the lipid suggests that the resulting membrane domains are dynamic and varied in size.[45]

AMP-induced membrane domain formation has also been observed or suggested in other studies.[16−27,46−48] For example, a molecular dynamics simulation suggested that for membranes composed of both zwitterionic (e.g., PE) and anionic (e.g., PG) lipids, binding of an AMP can induce ordering of PG-rich domains by reducing the electrostatic repulsion between the negatively charged PG lipids.[26] Similarly, an atomic force microscopy study[18] indicated that a cationic peptide, PFWRIRIRR-amide (PR-9), can induce domain formation by preferential interaction with the cardiolipins in supported bilayers consisting of POPE/TOCL (3/1) lipids at a peptide/lipid ratio of 1/1. While several views have been put forward to explain peptide-induced membrane domain formation, they are all based on the notion that domain formation is the consequence of the preferential interaction between the cationic residues in the peptide and the anionic lipid headgroups, which leads to lipid segregation in membranes composed of both anionic and zwitterionic lipids,[15,18,21,24−27,46−48] or the preferential binding of AMPs to lipid regions that have a lower phase transition temperature,[17−19,22,26] or the preferential binding of peptides to lipids that have a high intrinsic negative curvature.[16,45,49]

Regardless of the mechanism by which domains are formed, we can safely assume that the τ_D distribution of the peptide would be similar to that of the lipid domains if such domains were composed of tightly packed lipid and peptide molecules so that they diffuse together. The visible "tail" in the τ_D distributions of mag2 (Figure 6) seems to suggest that a small number of mag2 molecules may indeed diffuse together with various lipid domains as their diffusion times are too long to be accounted for as individual peptide species. Mag2 is longer than mpX and contains an additional charged residue as well as two polar residues. In mag2, these charged and polar residues are distributed rather evenly over the entire length of the peptide, whereas in mpX the charged residues are located toward the termini of the peptide. As a result, mag2 exhibits a stronger binding affinity toward anionic lipids. Thus, it is quite plausible that the slow diffusing peptide species observed in the case of mag2 correspond to a small population of peptides that are more tightly bound to and hence diffuse together with lipid domains that are rich in POPG. This notion is consistent with the computer simulation study of Niemelä et al.,[37] which showed that a membrane-bound protein can diffuse as a dynamic complex with the surrounding lipids. In addition, one might expect that for more potent lipid clustering agents their diffusion could become entirely slaved to that of the clusters thus formed.

However, for both mag2 and mpX our data show that the majority of the peptide diffusion times are comparable to or faster than the mean diffusion time of the lipid in unperturbed membranes (Figures 2, 3, 5, and 6), which indicates that the lateral diffusion of most peptides is not hindered by the formation of more slowly diffusing lipid domains. In fact, the fast diffusion behavior of the peptides suggests that they are situated in a low-viscosity region of the membrane. For instance, the mean diffusion time of TMR-mpX in the membrane of POPC/POPG (3/1) GUVs is 1.2 ms at 1 μM peptide concentration (Figure 3), giving rise to a mean diffusion coefficient of 1.1×10^{-7} cm^2/s, which is significantly larger than that measured for various lipid domains diffusing in the membrane of GUVs.[45] Thus, our results are more consistent with a mechanism wherein the peptides stabilize domains by settling at the interface of the ordered domain and disordered region of the membrane or by partitioning within the more disordered regions of the membrane.[17,19,22,26,33,50−52] In other words, the peptide molecules in this case behave more like mobile fences or obstacles than nucleation sites for membrane domain formation. It has been observed in simulation studies[53,54] that fixed obstacles in membranes can lead to domain formation by reducing the line tension.[33,44] In light of the current findings, it would be very useful to carry out similar computational studies to further investigate the role of mobile obstacles in membrane domain formations. Moreover, several studies[33,55−57] have speculated that the size of domains formed due to peptide binding could be too small to detect by conventional optical microscopic methods. Thus, as demonstrated in the current and other studies,[31,58] FCS provides an easy and alternative method for "imaging" nano- and microdomains in membranes.

In addition, the observation that mpX can effectively induce domain formation in zwitterionic POPC membranes[31] further suggests that other factors, besides electrostatic interactions, are also important determinants of peptide-induced membrane domain formation. MpX has a large hydrophobic moment but is too short to fully span the membrane. Nevertheless, mpX can effectively induce membrane thinning, resulting in negative membrane curvature.[49,59] Therefore, hydrophobic mismatch between the AMP and membrane thickness may also be a major cause of domain formation,[16,60] since it is known that the disordered fluid lipid chains are effectively shorter than the ordered lipid chains[61] and that each lipid tends to be surrounded by lipids with similar chain lengths in order to protect the hydrophobic core from the surrounding water.

CONCLUSIONS

It is well-known that high concentrations of AMPs can cause lipid vesicles to leak or burst. However, at low concentrations the effect of an AMP to the structural integrity of lipid membranes of interest is more difficult to assess. Previously, we demonstrated

that the lateral diffusion of lipids is a sensitive probe of the underlying AMP−membrane interactions, even at very low peptide/lipid ratios, and that AMP binding leads to formation of a wide variety of lipid species with varying diffusion time. In order to provide a better understanding of the nature of these species, herein we measured the distribution of diffusion times of two membrane-bound AMPs through a well-defined confocal excitation volume using fluorescence correlation spectroscopy. We found that at AMP concentrations of 100 nM and higher the distribution of the characteristic diffusion times of the membrane-bound peptides is much narrower than that of the lipids and also with a faster mean. Thus, these findings indicate that while AMPs induce membrane domain formation, they do not do so by forming tightly bound peptide−lipid clusters. Instead, our results suggest a mechanism of domain formation wherein the AMP molecules (i.e., monomers and/or oligomers) reside within the transition region between domains and behave as mobile fences.

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Author Contributions

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ABBREVIATIONS

FCS, fluorescence correlation spectroscopy; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt); GUV, giant unilamellar vesicle; mag2, magainin 2-amide; mpX, mastoparan X; TMR, tetramethylrhodamine.

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