Creation of Lipid Partitions by Deposition of Amphipathic Viral Peptides

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Phospholipid vesicles exhibit a natural characteristic to fuse and reform into a continuous single bilayer membrane on hydrophilic solid substrates such as glass, mica, and silica. The resulting solid-supported bilayer mimics physiological tendencies such as lipid flip—flop and lateral mobility. The lateral mobility of fluorescently labeled lipids fused into solid-supported bilayers is found to change upon deposition on the membrane surface of an amphipathic α-helical peptide (AH) derived from the hepatitis C virus (HCV) NS5A protein. The binding of the AH peptide to a phospholipid bilayer, with the helical axis parallel to the bilayer, leads to immobilization of the bilayer. We used AFM to better understand the mechanistic details of this specific interaction, and determined that the diminished fluidity of the bilayer is due to membrane thinning. Utilizing this specific interaction between AH peptides and lipid molecules, we demonstrate a novel process for the creation of lipid partitions by employing AH peptides as agents to immobilize lipid molecules, thus creating a patterned solid support with partition-defined areas of freely mobile lipid bilayers. This architecture could have a wide range of applications in novel sensing, biotechnology, high-throughput screening, and biomimetic strategies.

Introduction

Phospholipid assemblies exhibit natural tendencies to fuse and assemble into a single bilayer membrane on silica1−3 and on several other hydrophilic solid supports.4−6 The resulting supported membrane maintains many of the biological and physiological characteristics of free membranes, including lipid flip−flop7 between proximal and distal leaflets and lateral fluidity.3 In particular, the lateral mobility of supported membranes is a unique feature that distinguishes them from other adlayers. A variety of techniques have been pioneered by Boxer and co-workers8−12 to fabricate supported membranes that are partitioned into isolated compartments. These include "photolithographic patterning",9,13 "microfluidic flow patterning",14 and "microprinting"9,15 methods, each of which is designed to control the lateral fluidity of the supported membranes. One of the common ways to create diffusion barriers for fluid membranes involves the use of substrates on which patterns of foreign barrier materials have been deposited prior to the assembly of the supported lipid bilayer. Several foreign metals (Au, Al, Cr, and Ti) and metal oxides (Al2O3 and TiO2) resit formation of fluid membranes on solid supports.8,10,11,15,16 However, only one example of a biomaterial (fibronectin12) has been employed to create effective barriers.

In this article, we demonstrate a new method to create a lipid partition, which is derived from the molecular interactions between an amphipathic α-helical (AH) peptide and a supported lipid bilayer. In the course of our studies of the membrane-binding dynamics of a peptide derived from the hepatitis C virus (HCV) nonstructural protein 5A (NS5A), we have determined that an amphipathic α-helical peptide (AH) containing the N-terminal 31 amino acids of NS5A possesses the structural characteristics required for binding to a membrane.17 Particularly intriguing is that the interaction between this AH peptide and a lipid bilayer was found to alter the mobility of the bilayer on a solid support and that the resulting bilayer can also be partitioned by patterned arrays of the AH peptide.

To better understand the mechanistic details of AH peptide−lipid interaction, the present study utilizes various techniques including quartz crystal microbalance-dissipation (QCM-D), fluorescence recovery after photobleaching (FRAP), imaging ellipsometry, and atomic force microscopy (AFM).

First, we use 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) containing a fluorescent lipid probe (N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt-Texas Red-DHPE) in order to form a complete planar bilayer on SiOx quartz crystal supports, as described previously.1 FRAP measurements are then used to monitor the lateral mobility of the supported membranes

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Conventional inorganic barriers impose a nonbiological material within the surface system, resulting in a partition with physical and chemical properties that are different from the lipid bilayer. We propose a new partitioning method to generate barriers to lipid bilayer mobility while providing a surface that retains the same physical and chemical properties of the lipid bilayer. This is made possible by the architecture of the barrier as formed by the AH peptide. Specifically, the peptide becomes fixed to the substrate with the hydrophobic residues of the peptide being oriented away from the substrate such that the tails of lipids are bound to the peptide and the lipid heads are oriented outward. The resulting barrier is thus effectively composed of a fixed lipid monolayer, providing a surface that is overall more bio-mimetic than with conventional methods.

**Experimental Section**

**AH and NH Peptides.** Amphipathic α-helical peptides (AH) and nonamphipathic nonhelical peptides (NH) were synthesized by Anaspec Corp. (San Jose, CA). NH peptides were designed to substitute three charged amino acids into the AH peptide, spaced at intervals along the predicted amphipathic helix such that a sustained hydrophobic patch remained. The sequences of each peptide are as follows: AH peptide: H-Ser-Gly-Ser-Trp-Leu-Arg-Asp-Val-Trp-Asp-Trp-Ile-Cys-Thr-Val-Leu-Thr-Asp-Phe-Lys-Thr-Trp-Leu-Gln-Ser-Lys-Leu-Asp-Tyr-Lys-Asp-NH$_2$ and NH peptide: H-Ser-Gly-Ser-Trp-Leu-Arg-Asp-Val-Trp-Asp-Trp-Trp-Ile-Cys-Thr-Val-Leu-Thr-Asp-Lys-Thr-Leu-Gln-Ser-Lys-Leu-Asp-Tyr-Lys-Asp-NH$_2$ (introduced charged amino acids are indicated in bold and are underlined). For the fluorescent labeled AH peptide, 5/6-carboxy-tetramethylrhodamine- Ser-Gly-Ser-Trp-Leu-Arg-Asp-Val-Trp-Asp-Trp-Ile-Cys-Thr-Val-Leu-Thr-Asp-Phe-Lys-Thr-Trp-Leu-Gln-Ser-Lys-Leu-Asp-Tyr-Lys-Asp-NH$_2$ (biotin)-NH$_2$ was used (R-AH). In order to ensure the solubility of R-AH peptide, 5% dimethyl sulfoxide (DMSO) was included in the Tris buffer.

**Small Unilamellar Vesicle Preparation.** Small unilamellar vesicles of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) (Avanti Polar Lipids, Alabaster, USA) were prepared by the extrusion method. N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (Texas Red- DHPE) was purchased from Molecular Probes (Eugene, OR). Lipids were first mixed in chloroform, and the solvent was evaporated to form a lipid film on the wall of a round-bottomed flask. For FRAP measurements, a molar ratio of 99.5:0.5 for POPC to Texas Red-DHPE was prepared. For QCM-D measurement, we used a Tris buffer: 10 mM Tris [pH 7.5] and 150 mM NaCl solution with 1 mM EDTA in 18.2 MΩ cm MilliQ water (MilliPore, Oregon). Extruded unilamellar vesicles (referred to simply as vesicles) were prepared in the following manner. Lipid films were prepared by drying the lipids dissolved in chloroform under a gentle stream of nitrogen at room temperature. The resulting lipid film was then placed under vacuum for at least 5 h in order to eliminate any chloroform residue. Multilamellar vesicles were prepared by first swelling the lipid film in aqueous solution, then vortexing periodically for 5 min. The resulting multilamellar vesicles were subsequently sized by a mini extruder (Avanti Polar Lipids, Alabaster, USA) through 30 nm track-etched polycarbonate membranes. Vesicles were generally prepared at a nominal lipid concentration of ~5 mg/mL for QCM-D and FRAP and then subsequently diluted before experiments. Vesicles were generally used within 1 h of preparation.

**Fabrication of PDMS Stamps.** A polydimethylsiloxane (PDMS) elastomer kit (Sylgard 184) and curing agent (Dow Corning) were used for the fabrication of PDMS stamps, as described in Whitesides et al. Briefly, each stamp of a micropatterned master was replicated using Sylgard 184 (Dow Corning) with a 10-to-1 weight ratio of PDMS-to-curing agent. PDMS was first thoroughly mixed with the curing agent and allowed to cure for 30 min at room temperature. Then the mixed elastomer was poured into a master mold and left in vacuum overnight. The PDMS stamp is inherently very hydrophobic, but its surface can be temporarily converted to a hydrophilic

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Figure 1. Schematic overview of experimental strategy and microcontact printing schemes used to create AH peptide barriers for compartmentalization of lipid bilayer. Cross sectional (middle panels) and top-down views (right panels) are presented. A. Schematic outline of microcontact printing process to create AH peptide pattern. B. FRAP process to confirm that AH barriers function to restrict the mobility of the lipid bilayers.

before and after the addition of specific peptides. Next, in order to create patterns with the AH peptide, we use microcontact printing (µCP) to pattern fluorescently labeled AH peptide on a SiO$_x$ quartz crystal, as described in the experimental sections and Figure 1. We then utilize imaging ellipsometry to confirm the uniformity and height of the AH-patterned regions, and the QCM-D technique to follow vesicle fusion kinetics on the AH-patterned SiO$_x$ quartz crystal. FRAP measurements were then again used to monitor micropatterns of lipid bilayers established by deposition of AH peptides. Finally, we wanted to obtain evidence of the mechanistic details of the viral peptide interaction with lipid molecules. In order to do that, we employed AFM to capture the peptide-induced changes in lipid membranes observed during the interactions between the AH peptide and the lipid bilayer on a mica surface. This leads to an important clue about the diminished fluidity of the bilayers.

As anticipated, we were able to distinguish the formation of bilayers that retained mobility on the bare unpatterned SiO$_x$ regions, whereas the AH-patterned regions successfully acted as barriers to mobility, thus establishing the desired partitioned supported membrane.
form by brief treatment with oxygen plasma. The PDMS stamps used for patterning the AH peptide where plasma oxidized. It is worth noting that Zou et al. has shown that the curing temperature of plasma treatment can affect the material properties of PDMS (Mater. Res. Soc. Symp. Proc. Vol. 782). Therefore, in certain experimental settings where this can occur, researchers should take precaution to extract the oligomers from PDMS by swelling and deswelling in organic solvents (see Whitesides et al. Anal. Chem. 2003, 75, 6544–6554).

Quartz Crystal Microbalance-Dissipation (QCM-D). In order to study in situ adsorption and microscopic studies of the adsorbed layer, we utilized a Q-Sense D300 (Q-Sense AB, Gothenburg, Sweden) with the window chamber QWIC 301. AT-cut crystals (Q-Sense) of 14 mm in diameter with 50 nm thermally evaporated SiOx were used for all vesicle interaction and adsorption studies. The crystal was initially driven at its resonance frequency. To capture the characteristic dissipation, the drive circuit was short circuited and the exponential decay of the crystal oscillation was recorded and analyzed, yielding the frequency and dissipation changes at 5, 15, 25, and 35 MHz. The temperature of the Q-Sense cell was set at 25.0 °C and accurately controlled by a Peltier element in the cell with fluctuation smaller than ±0.5 °C. Each QCM crystal was treated with oxygen plasma at ∼80 W for ∼5 min prior to use (March Plasmad Plasma Etcher, March Instruments, California).

Epifluorescence Microscopy and Fluorescence Recovery after Photobleaching (FRAP). A Nikon Eclipse E800 upright microscope with an epifluorescence package was used to observe the peptide pattern and membranes on a quartz solid surface. 10× and 40× water-immersion objectives were used to acquire images of the patterned membrane submerged in a Tris buffer. Bleaching was achieved with a 100 W high-pressure mercury light source using a contracted field diaphragm and 40× objective lens. A high-resolution air-cooled CCD camera (Photometrics CoolSNAP, Roper Scientific) was used to capture the images, and MetaMorph software (Universal Imaging, CA) was used to collect image stacks to analyze the digitized fluorescence counts of the specified regions. Openlab 4.0 (Improvision Inc. England) was used to render 3-D profiling and provide snapshots of the images. FRAP analysis was performed to extract diffusion coefficients from the temporal fluorescence intensity profiles of selected regions during recovery. The diffusion coefficients were calculated based on the Axelrod method16 by the following equation:

\[ D = \frac{0.224}{w^2} \left(1 - \frac{t}{t_{1/2}}\right) \]

where \( D \) is the fluorophore diffusion constant, \( w \) is the radius of the bleached area, and \( t_{1/2} \) is the half recovery time defined by the recovery fraction, \( f(t_{1/2}) = (f(0) - F(0))/[F(t \rightarrow \infty) - F(0)] \approx 0.5. \)

Fluorescence recovery of the bleached region recovered to 76% (Figure 2F). This suggests that NH does not significantly interact with the supported bilayers to alter the fluidity of the supported bilayer (Figure 2C).

The most interesting feature of these experiments was revealed when the mobility of the Texas Red–DHPE fluorophore-doped lipid bilayer was monitored after addition of the AH peptide. Observations of labeled-lipid mobility indicate that the binding of AH peptide on supported lipid bilayers diminished the mobility of lipid molecules. To further investigate the binding affinity of AH peptide, we varied the peptide concentration between 0.820 μM and up to 76% (Figure 2F). This suggests that NH does not significantly interact with the supported bilayers to alter the fluidity of the supported bilayer (Figure 2C).

Results

The AH Peptide Alters Mobility of Solid Supported Bilayers. Results characterizing molecular mobility in the supported membranes upon addition of the amphipathic helix (AH) peptide are summarized in Figure 2. First a lipid bilayer containing Texas Red–DHPE was fused onto a SiOx quartz crystal. Following the formation of the bilayer, we performed FRAP to check the mobility via recovery of fluorescence in the photobleached area of the bilayer. In the absence of added peptide (Figure 2A), the diffusion coefficient in the photobleached target area was 1.97 ± 0.39 um² s⁻¹, indicating normal recovery of fluorescence due to the fluidity of the lipid bilayers. The line integrated and normalized changes in recovery, \( \Delta_f \), indicate that the intensity of the bleached region recovered to 89% of the original planar bilayer, as shown in Figure 2D. In marked contrast, when we added the AH peptide (AH concentration ∼ 13 μM) on the supported membrane, the bilayer failed to demonstrate recovery of the fluorescence into the bleached area (Figure 2, panels B and E). As a control, addition of a peptide termed NH (containing the same sequence as AH except for three charged amino acid substitutions in the hydrophobic face that disrupt its amphipathic and helical nature)17,21 resulted in normal fluidity of the bilayer (1.96 ± 0.25 um² s⁻¹) with relatively normal recovery of \( \Delta_f \) to 76% (Figure 2F). This suggests that NH does not significantly interact with the supported bilayers to alter the fluidity of the supported bilayer (Figure 2C).

Figure 2. AH peptides immobilize lipid molecules on SiO₂ quartz crystals. The images represent FRAP experiments on sequential fluorescence images of membrane consisting of POPC (99.5 mol %) and Texas Red–DHPE (0.5 mol %) with and without added AH or NH peptides (13 µM). The graphs represent the corresponding traces of normalized fluorescence intensity across the bleaching spots from $t \sim 0$ to 500 s for a series of FRAP experiments probing the changes in recovery. The parameter, $\Delta f$, stands for the linearly integrated and normalized difference between $t \sim 0$ and 500 s intensity traces. A value of 0 indicates fixation due to lipid molecules being immobilized by AH, and a value of 1 indicates complete recovery of the bleached spot on the lipid bilayer. A. FRAP experiments for POPC mixed with DHPE indicate that normal recovery takes place at $t \sim 475$ s with 99% recovery of bleached spot with diffusion coefficient of 1.97 ± 0.39 nm² s⁻¹. B. FRAP experiments for POPC mixed with DHPE with added AH peptide indicate that no recovery can be detected at $t \sim 513$ s with 1.1% recovery of bleached spot with diffusion coefficient of 0.00 nm² s⁻¹. C. FRAP experiments for POPC mixed with DHPE with added NH peptide indicate that relatively complete recovery takes place at $t \sim 510$ s with 79% recovery of bleached spot with 1.96 ± 0.25 nm² s⁻¹. D. Corresponding recovery trace for Figure 2A. E. Corresponding recovery trace for Figure 2B. F. Corresponding recovery trace for Figure 2C.

Microcontact Printing (µCP) to Pattern AH Peptides on a SiO₂ Quartz Crystal. AH peptide binding into the lipid bilayer may act as a barrier to lateral diffusion of lipid molecules within the supported bilayer, as a result of immobilization of lipid molecules. We hypothesized that this biomolecular-binding phenomenon could be exploited to allow the creation of biocompartments within two-dimensional fluid environments of supported lipid bilayers. To test this hypothesis, we used microcontact printing (µCP) to pattern fluorescent labeled AH peptides (R-AH) on a SiO₂ quartz crystal. Due to its inherently high hydrophobicity, a polydimethylsiloxane (PDMS) stamp was found to be an excellent material to pattern the amphiphatic peptide. Figure 4A displays AH peptide patterns created by physisorption binding using variously shaped PDMS stamps onto SiO₂ quartz crystals in Tris buffer. The driving force to retain the AH peptides on silica is presumably due to interactions of charged amino acids within the hydrophilic face of the AH peptide and the hydroxyl (–OH) groups of SiO₂. Imaging ellipsometry was used to reveal that the surface-adsorbed peptides appeared to form a single monolayer (height ~ 2 nm), as displayed in Figure 4B.

Vesicle Fusion Kinetics on AH-Patterned Surfaces using QCM-D. To further investigate the effects of kinetics due to the AH peptide on SiO₂, we used one of the AH-patterned SiO₂ quartz crystals described above (Figure 4A-2), and we monitored the kinetics of POPC vesicle fusion into lipid bilayers on SiO₂ quartz crystal by quartz crystal microbalance-dissipation (QCM-D) with the window chamber (QWiC 301). Following the injection of POPC vesicle solution (0.12 mg mL⁻¹), the mass uptake is fast, as exhibited by the huge frequency and dissipation changes. The kinetics show the measured total viscoelastic load coupled to the oscillations of the quartz crystal. Upon the saturation of critical coverage of the intact vesicles, they then continuously ruptured and formed bilayers, which is evident from the loss of large amounts of trapped water. Figure 5 demonstrates the vesicle rupturing and the formation of bilayers on two distinct surfaces (nearly complete and the AH peptide-patterned SiO₂ surfaces). Since the containment of the AH peptide pattern reduced the active area of the intact vesicles, the average critical coverage decreased down to 30% (critical coverage of the transformation is 42 Hz on a bare SiO₂ surface and decreases down to 30 Hz on an AH-patterned SiO₂ surface). Formation of a complete

of the monolayer exhibit defects. Linescans demonstrate the lipid layer formation on the AH-patterned region, as measured by the intensity of the fluorescence-doped lipid molecule (Texas Red—DHPE). The linescans of three different parts of the images provide further support for the different formations of lipid assemblies on the two distinct surfaces; linescan 1 displays the complete bilayer (high intensity) on the bare SiO$_2$, on both sides of uniformly formed (lower intensity) monolayer regions overlaying the surface printed with AH peptide. The boundaries of the AH peptide and bare SiO$_2$ regions are identified as pixel positions 45 and 90. Linescan 2 demonstrates that the lipid assembly layer on AH peptide is formed in a nonuniform fashion. This nonuniform layer is attributed to the lipid interaction with the AH peptide and the nonuniform surface properties generated by AH peptide adsorption. Linescan 3 distinctly reveals the disruption of lipid assembly layers on the AH-patterned region.

**Recovery Test in an AH-Patterned Region using FRAP.**

We also performed recovery tests on the AH barrier substrate, as shown in Figure 5D. The fluidity of lipids in the various regions of patterned SiO$_2$ solid supports was monitored using the FRAP technique. As shown in Figure 6A, the early stages of recovery of the bleached spot confirmed that the bilayers retain fluidity within the bare SiO$_2$ surface, whereas the lack of recovery on the AH-patterned surface indicated that the AH peptide successfully immobilized a lipid monolayer while it acted as a barrier to the supported lipid bilayer. Figure 6B corresponds to the linescans of the series of fluorescence images. The AH-patterned region (up to pixel position ~400) fails to recover fluorescence, suggesting that the AH peptide immobilized the lipid molecules in the AH-patterned layer.

**AFM Measurement of Time Lapse of Membrane Thinning due to AH Peptide.**

To understand the nature of the AH peptide and lipid bilayer interaction, we performed an AFM experiment. Figure 7 shows a typical noncontact mode AFM image of a lipid bilayer patch. The dark regions represent the atomically flat mica surface, whereas the white light areas display the lipid bilayer patch. The red arrowheads (panels f, j, r, and v) indicate the location of the scan line. In order to confirm the height of the bilayer patch, we scratched the center portion using a fast scan (10 Hz) of 200 nm size for 4 min. The height of the bilayer patch was found to be 4.5 nm, the normal thickness of a physiological membrane (see the Supporting Information). In order to identify changes in the conformation of the lipid bilayer due to AH peptide interactions, we performed time lapse AFM experiments with increasing AH peptide concentrations added to the lipid bilayer, as shown in Figure 7, panels a–v. We injected different concentrations as described in the caption of Figure 7. Briefly, we first added 0.82 uM AH peptide (panel c) after imaging several bare lipid bilayers (panels a and b). The height of the lipid bilayers were maintained, and no dramatic changes occurred up to 1.63 uM AH peptide concentration (panel j). In marked contrast, the bilayer started to thin, spreading out on the mica surface at a concentration of 3.25 uM AH peptide, when the height of lipid bilayers decreased by about 1 nm (panel r). The thinning process continued with increased amounts of AH peptide concentrations. At a concentration of 13 uM AH peptide (panel v), the mica surface became saturated with lipid molecules that had completely spread out on the mica surface. Therefore, we no longer see the bare mica surface. The height difference between the lipid layer patch and spread regions is ~0.1 nm. It is noteworthy that the AH peptide concentration is a more crucial factor in the bilayer interaction than is the time period. AH peptide concentrations up to 1.63 uM did not make any conformation or height changes to the bilayer patch over a 2-h period. For higher AH peptide concentrations, the bilayer started to thin, spreading out on the mica surface.
concentrations (26 µM), the bilayer patches immediately became disrupted (data not shown).

Discussion

Peptide molecules that are known to affect membrane translocations have been extensively studied in the past.23,24 These peptides are often referred to as cell-permeating peptides and are thought to play an important role in the processes of fusion, disruption, and pore formation.23–25 The importance of these peptides for drug delivery is recognized in their ability to uniformly transport large, biologically active molecules into mammalian cells. Antimicrobial peptides help destroy bacteria by binding or disturbing lipid plasma membranes. The positively charged faces of antimicrobial peptides interact toward the negatively charged bacterial membranes. Previous studies with model membranes have demonstrated that amphipathic α-helical peptides penetrate the lipid membrane. The penetration leads to the expansion26 of the outer leaflet of the bilayer and the thinning of the bilayer during the early stages of antimicrobial peptide–membrane interactions.27

The binding and membrane-permeating process of the AH peptide is not clearly understood. Our previous study demonstrated that the AH peptide acts as an agent to disrupt lipid vesicle adsorption to a solid support and triggers the transformation of vesicles into a bilayer. For possible interpretations of the AH binding results, we turn to literature on the action of antimicrobial peptides as a basis. The mechanism of peptide-induced membrane interaction and disruption provides useful information for drug

![Figure 5. Comparing the QCM-D responses for two different substrates; Changes in resonant frequency and dissipation as a function of time with and without AH patterning (Figure 4A-2, cup-holder shape) on a SiOx surface. A. To distinguish the vesicle fusion kinetics between the AH-patterned SiOx surface (Figure 4A-2) and bare SiOx (nonpatterned) surfaces, we measured the frequency changes as a function of time. After 3 min of stabilizing the frequency signal, the POPC vesicle solution was injected into the liquid cell. Following the injection of the POPC vesicle solution, vesicles formed bilayers on the two distinct surfaces. It is worth noting that the difference in frequency kinetics is due mainly to the difference in the active area caused by the containment of the bilayer (see main text for details) B. The corresponding energy dissipation associated with vesicle fusion kinetics is shown. A dissipation value of less than ~0.25 confirmed that both surfaces have formed rigid bilayers. C. Both panels represent the resulting bilayer formation (left panel: bare SiOx, quartz crystal surface; right panel: AH-patterned SiOx, quartz crystal surface). The white line represents a 200 µm scale bar. D. The image is a more detailed representation of Figure 5C (right panel). Although the region between the circles is a lipid monolayer on top of the AH peptide, the circular regions are defect-free lipid bilayers on a bare SiOx surface. The complete bilayer formed on a bare SiOx, quartz crystal. In marked contrast, the lipid layer formed on an AH-patterned SiOx, quartz crystal. The three linescan graphs are represented in the image to confirm the uniformity of the lipid layers.

In altering the fluidity of the lipid bilayer.30 Sequential processes of binding to the bilayer membrane interface Rotation around the AH peptide’s longitudinal axis leads to a face to the target bilayer’s phospholipid headgroups by arranging amphipathic R of vesicles, creating microvilli structures on the outer leaflet of that, just as in the early stages of antimicrobial mode of action, 27–29 the AH peptide, when added to the vesicles, leads to the expansion of vesicles, creating microvilli structures on the outer leaflet of the vesicles and causing the vesicles to rupture.4

Figure 6. Sequential fluorescence images of an AH-patterned SiO₂ quartz crystal surface resulting from FRAP experiments. A. The series of fluorescence images demonstrates FRAP experiments for fluidity of the bilayer that is deposited in the same pattern as Figure 5D. Background lipid layers containing POPC (99.5 mol %) and TR-DHPE (0.5 mol %) are displayed on bare SiO₂. B. The depicted graph shows the line scans that correspond to the sequential fluorescence images. The series of fluorescence images of recovery on the bleached spot is shown with corresponding line scan results presented in panel A. At 12 min of recovery, the bleached spot recovered ~40% of fluorescence intensity (panel A4). At 26 min of recovery, the round boundary can be observed (panel A6). At 69 min of recovery, the round boundary is completely visible (panel A8). The visibility of the round boundary confirms that the AH peptide acts as the barrier.

development and has received increasing attention. We speculate that, just as in the early stages of antimicrobial mode of action,27–29 the AH peptide, when added to the vesicles, leads to the expansion of vesicles, creating microvilli structures on the outer leaflet of the vesicles and causing the vesicles to rupture.4 One model suggests that the secondary structure of the amphipathic α-helical peptide is the essential factor in the sequential processes of binding to the bilayer membrane interface and in altering the fluidity of the lipid bilayer.30–35 These processes are envisaged to occur as follows: the AH peptide’s hydrophilic face contains conserved charged residues, whereas its hydrophobic face contains hydrophobic residues, with well-organized Trp residues located at the interface of the two sides of the helix.24,36 These structural features promote the binding of the hydrophilic face to the target bilayer’s phospholipid headgroups by arranging the AHs hydrophilic surface such that it faces the headgroups. Rotation around the AH peptide’s longitudinal axis leads to a reorientation with the hydrophobic residues toward the hydrophilic core of the membrane, resulting in the binding of the AH peptide within the bilayer.

In order to understand how the interaction of lipid bilayers and AH peptide impair the mobility of bilayers at the molecular level, we performed the AFM experiments on mica surfaces. In particular, during AFM experiments, peptide-induced changes in lipid membranes were observed, and the time-lapse series of images provided information about the dynamics of the process.

The Huang group has suggested that membrane thinning is an intermediate process that is critical in the overall activity of antimicrobial peptides.27,29,37–39 Huang et al.40 have demonstrated that amphipathic helical peptides initially bind electrostatically with the helical axes parallel to the bilayer surface at low concentrations. They used a simple geometric model for the elasticity of the membrane to describe peptide-induced changes in the lipid head group region and the resulting membrane thinning effect. Mecke et al.29 employed Huang’s simple geometric model to interpret their AFM results on the interaction of antimicrobial peptides with lipid bilayers. According to Huang’s geometric model, as depicted in Figure 8, we speculate that changes occur in the thickness of the bilayer.27,29,33 In the complete planar bilayer, as shown in Figure 8A, the area occupied by each lipid molecule is well defined. Upon AH peptide binding on the bilayer, the AH peptide inserts itself into the lipid headgroups, triggering the disruption of the monolayer. As a result, the membrane thins and spreads out. Using the conservation of volume, we can calculate the expected height changes Δh as follows:

\[
\Delta h = \frac{\Delta A + \pi h}{A}
\]

where A is the average area available in the plane of the bilayer per lipid molecule, R is the peptide/lipid ratio, ΔA is the area of the cross-section along the peptide helical axis, and h is the length of the hydrocarbon tail for the lipid molecule. For example, we calculated Δh from eq 1, given the following values: length and area of a POPC lipid molecule are 1.4 nm and 68.3 Å², respectively. Length, diameter, and ΔA of AH peptide33 (31 residues) are 4.7 nm, 2.0 nm, and 940 Å², respectively (obtained from Hyperchem software) and give a change in membrane thickness of Δh ≤ 1.3 nm with assumption of R = 1.

The above analysis assumes that the AH peptide acts only on the outer leaflet of the bilayer41 and only for the case of a defect-free planar bilayer. Our data, however, suggests that the defective regions serve as available areas for displaced lipid molecules to form a layer. Moreover, at the saturated AH peptide concentration (52 μM), immediate disruption of the bilayer patch is observed. This data indicates that the interaction of the AH peptide does not exclusively occur at the outer leaflet of the bilayer. We suggest that these phenomena occur via two different processes, as described below.

First, as shown in the model scheme in Figure 8B, after the insertion of the AH peptide in the outer leaflet of the bilayer, the hydrophilic portion of the AH peptide can be attracted to the

headgroups of the bottom leaflet of the bilayer, as well as the hydroxyl (−OH) groups on the hydrophilic silicon surface. This process resembles the initial interactions between the outer leaflet of the bilayer and the hydrophilic portion of the AH peptide. Such a mechanism is well-corroborated by the FRAP analysis, which shows that the mobility of lipid molecules decreases as the AH peptide concentration increases.

In the case of an AH peptide-patterned surface, the converse interaction takes place first (i.e., the lipid acyl chains interact with the surface-exposed hydrophobic face of the AH). Lipid molecules fuse as nonuniform layers on the top of the AH peptide-patterned surface due to the interaction between the acyl chains of lipid molecules and the hydrophobic faces of the AH peptide. The hydrophobic portion is only 1/3 of the helical structure as

Figure 7. Time lapse images of AFM confirming membrane thinning due to AH peptide. Typical noncontact mode AFM image of a supported phospholipid bilayer is shown. Several lipid bilayer patches (4.2 nm higher than the mica, yellow white area) are formed on the mica surface (black brown area). Red arrows indicate the location of the scan line represented at the bottom of the panels. We found that the thickness of the bilayer decreased with the increase in AH peptide concentration. We used a closed liquid cell in order to wash out residual AH peptide between each dosage increase of AH peptide, as depicted in the figure. First, well-defined regions of bilayer patches on the mica surface from −18 to −6 min were imaged (panels a and b). At 0 min, 0.82 uM of AH peptide was added, and then images were recorded to 36 min (panels c–f). After washing with Tris buffer, 1.63 μM of AH peptide were added at 42 min and scans were taken until 77 min (panels g–j). Height images and profiles suggested that the bilayer patches are not altered at lower concentrations of AH peptide. The thinning process starts at concentrations above 3.25 μM at 82 to 106 min (panels k–n). After washing with additional buffer, 6.50 μM of AH peptide were added at 120 min (panel o) in order to further investigate any thinning effects. Further, the AH peptide concentration was increased to 13 μM until 210 min, and then the thinning effect was continuously observed (panels s–v), until the bilayer patches were completely thinned out. Corresponding scan profiles for the above height images are also presented.
depicted in the model picture in Figure 8C (calculated from the Hypercam program). We suggest that due to limited partial exposure of the hydrophilic portion of the AH peptide to lipids, unfavorable interaction between hydrophilic peptide residues and acyl chains of lipid molecules might represent the main driving force in disturbing the monolayer assembly, resulting in nonuniform lipid layer formation above a surface of AH peptides. This disruption drives the impairment of lipid mobility.

**Conclusion**

AH peptide binding to the lipid bilayer impairs the free lateral diffusion of lipid molecules within the supported bilayer. AFM allows direct visualization of the peptide adsorption process with high resolution. Upon AH peptide binding on the bilayer patch, we were able to detect simultaneously the thinning of the membrane and the disrupted free lipid molecules that filled up the diffused regions. The AH peptide—bilayer interaction alters the fluidity of the bilayers, as observed by FRAP. The early stages of recovery of a bleached spot confirmed that the bilayers on the bare portions of SiO$_2$ retained fluidity, whereas the lack of recovery on a AH peptide-patterned surface indicated that the AH-peptide regions successfully acted as a barrier to fluidity.

This strategy thus provides a direct method for preparing biocompatible membrane barriers that can be exploited to generate a convenient and highly defined model for a cell membrane. Moreover, this strategy should enable the fabrication of arrays of membrane domains that can be studied independently of each other on a single support. This, in turn, could have many exciting possible applications in bioanalytical devices.

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**Supporting Information Available:** Characterization and investigation of the bilayer patch formation by AFM. In addition, we also report control experiments using a “faux-treated” stamp with no peptide and a stamp coated with the NH (nonhelical) peptide, as shown in Figure 2. This information is available free of charge via the Internet at http://pubs.acs.org.

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