Rupture of Lipid Vesicles by a Broad-Spectrum Antiviral Peptide: Influence of Vesicle Size

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ABSTRACT: An amphipathic α-helical (AH) peptide was recently discovered that can rupture the lipid envelope of many viruses including HIV, hepatitis C, dengue, and herpes simplex. Despite its broad-spectrum activity, the AH peptide specifically targets small viruses only and does not affect large viruses. Indirect observations of virus size-specific targeting have been confirmed in a model system comprised of intact lipid vesicles on a gold substrate. Depending on vesicle size, AH peptide can promote vesicle rupture, but the mechanism by which vesicle size influences the rupture process remains to be elucidated. Herein, using the dynamic light scattering and quartz crystal microbalance with dissipation techniques, we have combined experiment and theory to understand the effects of vesicle size on the interaction between the AH peptide and vesicles. We identified that the AH peptide-binding interaction can induce a structural rearrangement of the vesicle’s lipid bilayer, which occurs independently of vesicle size. Kinetic analysis also revealed that AH peptide-binding occurs cooperatively for small vesicles only. Binding cooperativity is consistent with pore formation leading to vesicle rupture. By contrast, for large vesicles, AH peptide-binding is noncooperative and does not cause vesicle rupture, suggesting that the binding interaction occurs via a different mechanism. Compared to previous estimates that AH peptide is most effective against viruses with a diameter of less than 70 nm, our evidence validates that AH peptide may target a wider size range of enveloped viruses up to 160 nm in diameter. Taken together, our findings provide a quantitative rationale to understand the targeting specificity of AH peptide as a broad-spectrum antiviral drug candidate.

INTRODUCTION

With the rise in drug-resistant virus strains,1 the development of new antiviral medicines is a priority.2 While small-molecule drugs represent the majority of clinically approved antiviral medicines,3,4 new antiviral medicines is a priority.2 While small-molecule receptor-mediated attachment of virus particles to host cell enveloped viruses by interfering with the envelope rule. An emerging class of antiviral peptides directly targets viruses only and does not affect large viruses. Indirect observations of virus size-specific targeting have been confirmed in a model system comprised of intact lipid vesicles on a gold substrate. Depending on vesicle size, AH peptide can promote vesicle rupture, but the mechanism by which vesicle size influences the rupture process remains to be elucidated. Herein, using the dynamic light scattering and quartz crystal microbalance with dissipation techniques, we have combined experiment and theory to understand the effects of vesicle size on the interaction between the AH peptide and vesicles. We identified that the AH peptide-binding interaction can induce a structural rearrangement of the vesicle’s lipid bilayer, which occurs independently of vesicle size. Kinetic analysis also revealed that AH peptide-binding occurs cooperatively for small vesicles only. Binding cooperativity is consistent with pore formation leading to vesicle rupture. By contrast, for large vesicles, AH peptide-binding is noncooperative and does not cause vesicle rupture, suggesting that the binding interaction occurs via a different mechanism. Compared to previous estimates that AH peptide is most effective against viruses with a diameter of less than 70 nm, our evidence validates that AH peptide may target a wider size range of enveloped viruses up to 160 nm in diameter. Taken together, our findings provide a quantitative rationale to understand the targeting specificity of AH peptide as a broad-spectrum antiviral drug candidate.

corresponds to the functionally active state of the peptide.15 By contrast, antiviral peptides that interfere with the envelope membrane may show activity against a broad spectrum of viruses, in some cases targeting viruses that are smaller than a certain critical size.16,17 Antiviral peptides that target small viruses only are particularly advantageous from a therapeutic perspective because these peptides have potentially less membranotropic-induced cytotoxicity in relation to appreciably larger cell membranes.18 While there are relatively few candidates in the class of antiviral peptides at present, understanding of the design principles behind the engineering of membrane-targeting antiviral peptides is quickly developing, with one prominent example.

In the course of studying virus interactions with lipid membranes, an amphipathic α-helical peptide of a particular amino acid sequence (referred to hereafter as AH peptide) was discovered19 that can rupture virus particles of many enveloped viruses, including HIV,20 hepatitis C,16,18 dengue,18 and herpes simplex.21 The broad-spectrum antiviral activity of AH peptide...
has led to exciting insights into its mechanism of action, and several functionally related peptides have been identified in additional studies through either discovery or rational engineering. It has been demonstrated that AH peptide is a membrane-active agent that can promote rupture of the virus envelope. Since the lipids that form the envelope are derived from the infected host cell and are not encoded for in the virus genome, AH peptide’s mechanism of action hinders its inhibition via the evolution of drug-resistant virus strains. While AH peptide can rupture the lipid envelope of virus particles, it does not rupture cell membranes, thereby offering the peptide significant promise as an antiviral drug.

AH peptide has also been explored in the context of synthetic lipid membranes. In particular, the unique properties of AH peptide have enabled a novel method to fabricate two-dimensional planar lipid bilayers on solid supports such as gold and titanium oxide, which are intractable to planar bilayer formation by conventional methods. Using a combination of surface-sensitive measurement techniques and viscoelastic film modeling, it was demonstrated that AH peptide can bind to surface-adsorbed vesicles on either of these two substrates, eventually leading to vesicle rupture. AH peptide-mediated vesicle rupture causes a structural transformation that leads to the formation of a planar bilayer. To further understand the AH peptide-vesicle interaction, the process has also been monitored simultaneously by two surface-sensitive techniques, quartz crystal microbalance with dissipation (QCM-D) and ellipsometry, which are based on different physical principles. Simultaneous measurement allowed the acoustic and optical mass signals to be analyzed individually during the same process and also provided collective insight into the role of hydration. The key finding was that the initial binding of AH peptide to vesicles causes a large uptake in the amount of hydrodynamically coupled solvent in the vesicle adlayer, which is indicative of vesicle swelling as an intermediate step before vesicle rupture.

The effect of vesicle size on the interaction between AH peptide and intact vesicle layers on gold has also been investigated in relation to the efficiency of planar bilayer formation. The most efficient formation of planar bilayers was achieved with vesicles with less than a 70 nm average diameter. For vesicles with average diameter between 80 and 200 nm, incomplete bilayer formation was observed, while larger vesicles did not rupture but remained intact. Importantly, the vesicle size-dependence of the bilayer formation process also correlates relatively well with the known antiviral spectrum of AH peptide. A wide range of enveloped viruses, especially viruses with less than a 70 nm diameter, is susceptible to treatment by AH peptide via envelope rupture, while larger viruses are typically refractory to treatment. The correlation between studies involving vesicles and enveloped viruses supports the notion that vesicles are a reasonable model system to mimic enveloped viruses.

Tabaei et al. recently employed a single tethered vesicle assay to further probe the binding interaction of AH peptide with small vesicles (average diameters ranging from 70 to 200 nm) at low peptide concentrations. Using this approach, it was concluded that AH peptide induces pore formation at a peptide-to-lipid ratio which is significantly lower than those of antimicrobial peptides. It was proposed that AH peptide prefers to rupture small vesicles due to membrane strain-dependent pore formation. Of note, in the study, the rate of peptide binding did not depend on vesicle size. However, the AH peptide concentration was limited to the sub-micromolar range, highlighting the challenge of characterizing the binding interaction of membrane-active agents at therapeutically relevant concentrations.

Despite the potential clinical significance of AH peptide’s targeting of small viruses, the mechanism of its targeting specificity, especially with regards to the rupture process, remains to be elucidated. Compared to previous QCM-D studies that focused on the formation of planar bilayers rather than on the kinetics of the vesicle rupture process, herein, we have experimentally scrutinized the rupture kinetics that are mediated by AH peptide. These experimental studies were performed with the QCM-D monitoring technique by using the intact vesicle platform on gold as a model system and yielded several novel findings, including (i) AH peptide binding was observed to induce a structural rearrangement of the lipid membrane independent of vesicle size; (ii) kinetic analysis demonstrated that AH peptide binding occurs cooperatively for small vesicles; (iii) AH peptide binding was noncooperative for large vesicles; and (iv) the size range of vesicles that can be ruptured by AH peptide is larger than was previously estimated (viruses less than 70 nm in diameter) and may encompass viruses up to 160 nm diameter. The effects of binding cooperativity are in agreement with the rupture kinetics and offer a quantitative rationale to understand the targeting specificity of AH peptide in its potential to be a broad-spectrum antiviral drug that works against a wide range of clinically important viruses.

### THEORETICAL ASPECTS

The AH peptide-induced structural transformation of adsorbed vesicles into a planar bilayer is a complex process. Interpretation of the vesicle-rupture kinetics, which are experimentally observed by tracking the characteristic physical properties of an ensemble of adsorbed vesicles, is complicated by the stochastic character of each individual vesicle rupture and also by the vesicle size distribution. For a single vesicle, the rupture process occurs at a certain time, and accordingly vesicle rupture itself can be considered to be instantaneous. However, for different vesicles, this time is different, even if the vesicles are of equivalent size. Thus, on the level of an ensemble of vesicles, the apparent duration of vesicle rupture may be long, especially for large vesicles with a relatively broad size distribution.

In our previous studies, we proposed a mean-field kinetic model that describes peptide binding to vesicles and peptide-induced pore formation and used the model to illustrate stochastic effects. The coalescence of pores, vesicle rupture, and lipid relaxation after rupture can, in general, hardly be described in detail on the mean-field level. Nevertheless, fitting and interpretation of the experimentally observed QCM-D kinetics related to the structural transformation can provide insight into the complex process, particularly on a comparative basis. Our present work, as already noted, is focused on the influence of vesicle size on the AH peptide-induced vesicle-rupture process. To facilitate understanding of the physics behind the fitting of the corresponding kinetics, we outline here a few general aspects of the theory of rate processes occurring on the surface of vesicles, with emphasis on the two factors directly related to our present experiments. Specifically, we briefly discuss the role of the peptide- peptide interaction and membrane curvature in the kinetics of peptide attachment to
the lipid bilayer and the role of the membrane curvature in the kinetics of pore formation.

Each step or substep on the pathway to vesicle rupture represents an activated process occurring, with the rate dependent on the coverage of a vesicle by AH peptide and the activation energy. As usual for rate processes, the activation energy is the difference between the reactant energies in the activated (transition) state and the ground state,

\[ E_a = E^0_a - E_0 \]  

(1)

Because of the direct or membrane-mediated peptide–peptide lateral interaction, the energies \( E_a \) and \( E_0 \) depend on the peptide coverage \( \theta \). On the simplest mean-field level, this dependence is considered to be linear, and accordingly the activation energy is represented as

\[ E_a = E^0_a + A\theta \]  

(2)

where \( A \) is a constant, and \( E^0_a \) is the value corresponding to \( \theta \to 0 \). Physically, the membrane-mediated peptide–peptide lateral interaction is related to peptide-induced strain of the membrane.

Besides peptide-induced strain, there is also strain related to vesicle size or, more specifically, to membrane curvature. In particular, the external part of each lipid layer in a vesicle is slightly stretched, while the internal part is slightly compressed. The linear tensile strain of the external part is \( \approx L/2R \), where \( L \) is the layer thickness and \( R \) is the vesicle radius. The corresponding contribution to the activation energy is proportional to this strain (as was earlier discussed in the context of enzymatic reactions at membrane interfaces); that is, the activation energy can be represented as

\[ E_a = E^0_a + B/R \]  

(3)

where \( B \) is a constant, and \( E^0_a \) is the value corresponding to \( R \to \infty \).

In general, the constants \( A \) and \( B \) may be positive or negative; that is, the peptide–peptide interaction and membrane curvature may suppress or facilitate a rate process. For example, the binding of peptides to a vesicle is usually accompanied by stretching of the lipid membrane. This effect indicates that the linear tensile strain of the external part of the bilayer is favorable for peptide binding, and accordingly the constant \( B \) is expected to be negative. As such, this effect may be important provided that the attachment is kinetically controlled (in the diffusion-controlled regime, the activation energy, if any, does not play a role). With increasing membrane curvature, the peptide binding energy increases (see, e.g., molecular dynamics simulations), and according to conventional Brensted–Evans–Polanyi arguments, the activation energy for peptide attachment is expected to decrease; that is, \( B \) is expected to be negative. In the context of our study, this effect is also important.

Equation 3 describes the effect of vesicle size on an elementary rate process. Physically and mathematically, this effect is simple because it includes only membrane curvature. However, in the case of pore formation, there are also more integral effects. For spontaneous formation of relatively large pores with radius \( r \), for example, the change in the free energy can be represented as

\[ \Delta F = -\pi \kappa r^2 / R^2 + 2\pi \gamma r \]  

(4)

where the first term corresponds to the energy gain due to the decrease in the bending energy with a pore formation-related increase of \( R \) (\( \kappa \) is the bending rigidity, and \( \kappa / R^2 \) is the membrane bending energy per unit area), and the second term is the energy loss due to the line tension (\( \gamma \) is the corresponding coefficient). Minimization of this expression yields the critical pore radius and energy,

\[ r_n = \gamma R^2 / \kappa \]  

(5)

and

\[ \Delta E_n = \pi \gamma R^2 / \kappa \]  

(6)

This critical energy can be identified with the activation energy for pore formation. Thus, eq 6 indicates that pores can form easily in small vesicles, while with increasing vesicle size, pore formation can be kinetically hindered. Quantitatively, this effect is weak if one uses the conventional value of the bending rigidity \( \kappa = 25k_B T \), where \( k_B \) is the Boltzmann constant, and \( T \) is room temperature. Here, we note that \( \kappa = 25k_B T \) corresponds to large vesicles. For small vesicles (with \( R \leq 50 \) nm), the bending rigidity can be appreciably larger.

In the case of peptide-induced pore formation, membrane bending can contribute to the apparent activation energy of pore formation in analogy with eq 6. The rate of pore formation and, accordingly, the rate of vesicle rupture may also dramatically depend on the peptide coverage directly or via peptide-induced membrane strain. Because of the dependence of the peptide binding energy and activation energies of elementary steps on \( R \) (eq 3), the coverage depends on \( R \), and accordingly the pore formation and vesicle rupture rates may strongly depend on \( R \) as well. Specifically, as noted below eq 3, the activation energy for peptide attachment is expected to increase with decreasing \( R \), and accordingly the peptide adsorption rate and coverage are expected to increase. The rate of peptide-induced pore formation dramatically increases with increasing peptide coverage (because this process occurs via nucleation involving a few peptides) and may also increase due to the bending energy (eq 6). In addition, the cooperative effects on peptide adsorption may facilitate the nucleation of pores. Collectively, these effects explain why vesicle size or, more specifically, membrane curvature affects the interaction between AH peptide and a membrane.

**MATERIALS AND METHODS**

**Vesicle Preparation.** Small unilamellar vesicles composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) (Avanti Polar Lipids, Alabaster, AL) were prepared by the extrusion method, as previously described. As-supplied lipids were dissolved in chloroform, which was evaporated under nitrogen gas, forming a dried lipid film. After it was stored overnight in a vacuum desiccator to remove chloroform, the lipid film was hydrated in water at a nominal lipid concentration of 5 mg·mL⁻¹. The suspension was then vortexed for 5 min to form large multilamellar vesicles. To reduce vesicle lamellarity, seven cycles of freeze–thaw treatment were performed. In each cycle, the vesicle suspension was quickly frozen in liquid nitrogen before being thawed in a 60 °C water bath and then vortexed. After the freeze–thaw treatment was completed, the vesicles were passed through track-etched polycarbonate membranes of either 1000, 800, 400, 200, 100, 50, or 30 nm pore diameter a minimum of 19 times by using a miniextruder (Avanti Polar Lipids). The resulting small
ultramembrane vesicles were diluted to 0.1 mg·mL⁻¹ before experiment. A tris(hydroxymethyl)aminomethane (Tris) buffer (10 mM Tris and 150 mM NaCl, pH 7.5) was used to dilute the vesicle solutions. All buffer solutions were prepared in 18.2 MΩ·cm Milli-Q water (Millipore, Billerica, MA).

**Dynamic Light Scattering.** A 90Plus particle size analyzer (Brookhaven Instruments, Holtsville, NY) with a 658.0 nm monochromatic laser was used to measure the intensity-weighted size distribution of vesicle populations after extrusion. To minimize the reflection effect, all measurements were taken at the scattering angle of 90°, and the intensity autocorrelation function was measured, which can be fit directly to yield the intensity-weighted size distribution of vesicles in solution, as previously described. In this work, the intensity-weighted Gaussian and histogram profiles of extruded vesicles in solution are reported.

**Quartz Crystal Microbalance-Dissipation.** A Q-Sense E4 instrument (Q-Sense AB, Gothenburg, Sweden) was used to monitor in situ adsorption of vesicles onto silicon oxide, as described elsewhere. Briefly, the crystal was initially driven at its resonance frequency, and then the drive circuit was short-circuited. The exponential decay of the crystal oscillation was recorded and analyzed, yielding the changes in frequency and dissipation at 5, 15, 25, 35, 45, and 55 MHz. The measurement data presented in the main text were recorded at the third overtone (15 MHz) and normalized based on the overtone number (n = 3). The temperature of the measurement cell was 25.0 °C, with an in-cell Peltier element to control thermal fluctuations to within ±0.5 °C. To account for the additional contribution of peptide-related mass to the vesicle adlayer, we calculated the acoustic mass of the adlayer as a function of time by using the Voigt–Voinova model. In the context of the AH peptide-mediated structural transformation, we present this calculation in the form of effective Voight thickness as a function of time by assuming a constant effective film density, as previously described in related work on the subject, and discuss it further below.

The model was based on QCM-D measurement data that were collected at the third, fifth, seventh, ninth, and eleventh overtones. For large vesicles, only the third, fifth, and seventh overtones were used. As previously noted by Larsson et al., the Voigt–Voinova model is a simplistic representation of the biological system under study, and the parameters obtained from the fitting should be considered as effective parameters. In this case, we assumed the vesicles and peptides form one layer having a uniform effective density that was constrained to be 1100 kg·m⁻³, and the viscosity of the bulk aqueous solution was fixed at 0.001 Pa·s⁻¹. The effective Voight mass (i.e., the product of effective density and thickness) was independent of the chosen effective density (within a certain range), suggesting that the model is reasonable to describe the structural transformation.

**RESULTS**

**Influence of Vesicle Extrusion Process on Vesicle Size.** In previous works, interpreting the effects of vesicle size on the interaction between AH peptide and vesicles was based on the average vesicle diameter. While the average vesicle diameter can provide information about trends related to AH peptide’s interaction with lipid vesicles, it is obviously not sufficient to represent the size distribution of vesicles in the population depending on the case. As a result, conclusions may be limited to observation of trends without more quantitative insight into the mechanism behind the observed behavior. This issue is particularly relevant for extruded vesicles, which were used in the two former studies as well as in this study. Depending on the extrusion filter pore size, extruded vesicles may have a single, monodisperse population or exhibit a multimodal size distribution, corresponding to two or more subpopulations of vesicles. To characterize the vesicle size distribution, we performed dynamic light scattering (DLS) experiments on vesicles that were extruded through pore diameters ranging from 30 to 1000 nm. Experimentally, the extruded vesicles exhibited a wide range of sizes, with average intensity-weighted diameters ranging from 58 ± 1 to 925 ± 46 nm (Table 1). The intensity-weighted Gaussian and histogram profiles for each vesicle size are presented in the Supporting Information (Figure S1). In general, the average size of extruded vesicles increased as a function of extrusion pore diameter. Additionally, there was a greater tendency for vesicles extruded through larger pore sizes to exhibit a bimodal size distribution. In such cases, the intensity-weighted diameters corresponding to the peak of each subpopulation are reported as well. N/A indicates Not Applicable.

<table>
<thead>
<tr>
<th>extrusion pore (nm)</th>
<th>average vesicle diameter (nm)</th>
<th>first peak (nm)</th>
<th>second peak (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>58 ± 1</td>
<td>59</td>
<td>N/A</td>
</tr>
<tr>
<td>50</td>
<td>80 ± 1</td>
<td>87</td>
<td>N/A</td>
</tr>
<tr>
<td>80</td>
<td>94 ± 1</td>
<td>97</td>
<td>N/A</td>
</tr>
<tr>
<td>100</td>
<td>122 ± 1</td>
<td>85</td>
<td>165</td>
</tr>
<tr>
<td>200</td>
<td>161 ± 2</td>
<td>161</td>
<td>N/A</td>
</tr>
<tr>
<td>400</td>
<td>241 ± 10</td>
<td>156</td>
<td>631</td>
</tr>
<tr>
<td>800</td>
<td>791 ± 17</td>
<td>217</td>
<td>1538</td>
</tr>
<tr>
<td>1000</td>
<td>925 ± 46</td>
<td>432</td>
<td>2375</td>
</tr>
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</table>

"Dynamic light scattering measurements were performed to measure the average intensity-weighted diameter of extruded vesicles that were passed through extrusion pore filter, with vesicle diameters ranging between 30 and 1000 nm. Overall, the average size of extruded vesicles increased as a function of extrusion pore diameter. Additionally, there was a greater tendency for vesicles extruded through larger pore sizes to exhibit a bimodal size distribution. In such cases, the intensity-weighted diameters corresponding to the peak of each subpopulation are reported as well. N/A indicates Not Applicable."

Here, we discuss the main trends in vesicle size distribution, as presented in Table 1. For small vesicles extruded through 30, 50, or 80 nm diameter pores, there was only one peak, corresponding to a single population of vesicles. In each case, the average vesicle diameter corresponded to a value larger than the corresponding pore diameter, and this finding is in agreement with past results. By contrast, for larger vesicles, there was a trend toward bimodal distributions. Vesicles extruded through 100 nm pores had a bimodal distribution, with peaks around 85 and 165 nm in diameter. Vesicles extruded through 200 and 400 nm diameter pores also had subpopulations with peaks around 160 nm. For vesicles extruded through 200 nm diameter pores, there was only one population, as reflected by an average vesicle diameter of 161 ± 2 nm. By contrast, vesicles extruded through 400 nm diameter pores had a bimodal distribution, with one subpopulation around 156 nm in diameter and another subpopulation around 631 nm in diameter. Similar bimodal distributions were also observed for larger vesicles in this study. The smaller of the two subpopulations was around 217 or 432 nm in diameter for vesicles extruded through 800 or 1000 nm diameter pores, respectively. In both of these two cases, the second subpopulation corresponded to an average diameter greater than the first.
than 1000 nm.\textsuperscript{46} In the following sections, the vesicle sizes are reported as the average intensity-weighted diameter. Where applicable, we also discuss the implications of the bimodal distribution observed for vesicles extruded through large pore sizes. For general guidance, we note here that, in the case of bimodal vesicle size distributions in solution, the vesicles in the subpopulation with smaller size are expected to be more represented in the intact vesicle adlayer on the substrate, because their rate of diffusion to the surface is higher, and accordingly these vesicles play a larger role in the adsorption process.

**Interaction between AH Peptide and Surface-Adsorbed Vesicles.** We next studied the interaction between AH peptide and lipid vesicles of varying size by measuring the adsorption kinetics associated with this process. QCM-D monitoring was employed to follow corresponding changes in resonance frequency and energy dissipation. Before explaining particular events related to different-size vesicles, we provide general commentary about how the experiments were performed, as presented in Figure 1. After a baseline measurement signal was established with a gold substrate immersed in aqueous buffer solution, vesicles were added onto the gold substrate (Figure 1, arrow 1). Vesicle adsorption led to a decrease in resonance frequency and to an increase in energy dissipation. After saturation, coverage of vesicles was reached, and the measurement signals again stabilized. A 13 \(\mu\)M AH peptide solution was then added (Figure 1, arrow 2), and the interaction between AH peptide and vesicles was monitored in real time. As described in the Introduction, the adsorption kinetics showed different behaviors depending on vesicle size. In all cases, a decrease in resonance frequency and an increase in energy dissipation initially occurred. This behavior corresponds to AH peptide binding and an associated increase in hydration. Among generally small vesicles, a maximum change in resonance frequency occurred and was followed by vesicle rupture, as indicated by a subsequent increase in resonance frequency and a decrease in energy dissipation. In some cases, vesicle rupture led to the formation of a planar bilayer, whereas in other cases, vesicle rupture did not promote formation of a complete bilayer. Among larger vesicles, vesicle rupture was minimal. As described below, the experimental results of the QCM-D measurements can be classified into three groups based on the changes in resonance frequency and energy dissipation. Within each group, we present experimental data related to one particular vesicle size (Figures 1−3), and the rest of the experimental data is provided in the Supporting Information.

The interaction of AH peptide with vesicles of small size (<80 nm diameter) led to complete rupture and formation of a planar bilayer (Figure 1A). AH peptide first binds to the vesicles until there is a critical coverage of pores in the vesicles. At this point, membrane destabilization causes swelling of the vesicle adlayer, and then vesicle rupture and lipid relaxation occur finally. The unique feature of this case is that the final changes in resonance frequency and energy dissipation correspond to the values of a planar bilayer, that is, approximately \(-26 \text{ Hz}\) and less than \(0.5 \times 10^{-6}\), respectively. The interaction of AH peptide with vesicles between 94 and 161 nm diameter exhibited similar kinetic behavior (Figure 1B). However, in these cases, the final changes in resonance frequency and energy dissipation do not correspond to the values of a planar bilayer. In particular, the QCM-D measurement results suggest that the adlayer maintains viscoelastic properties characteristic of intact vesicles. This result is likely due to the relatively broad size distribution of vesicles and appears to indicate that vesicles below a critical size are ruptured completely, while larger vesicles remain intact.

By contrast, the interaction between AH peptide and large vesicles of 241 nm diameter or greater showed different kinetic behavior (Figure 1C). AH peptide binding to vesicles was observed predominately, and there was evidence of only minor, if any, vesicle rupture, on the basis of the change in resonance frequency. While a maximum change in resonance frequency was still observed, the magnitude of change corresponding to the initial binding stage of AH peptide was significantly smaller, and the overall shape of the rupture profile was shallower. Additionally, there was no maximum change in the energy dissipation, which is observed only if there is a structural transformation involving vesicle rupture. Collectively, this evidence indicates that the majority of large vesicles remain intact upon interaction with AH peptide.
Lag Time between QCM-D Frequency and Dissipation Signals. In cases of vesicle rupture, we observed a lag time between the maximum changes in resonance frequency and energy dissipation. To further analyze the structural transformation process, we removed the dependence on time and plotted time-independent curves that show the relationship between changes in resonance frequency and energy dissipation (Figure 2 and, in the Supporting Information, Figure S3). A similar approach has been utilized in previous works\textsuperscript{49−52} to analyze the interaction between antimicrobial peptides and solid-supported model membranes. Generally, such analysis has been applied to compare the mechanisms of how different antimicrobial peptides disrupt planar bilayers. In the present case, we instead focus exclusively on AH peptide and investigate how time-independent curves may provide additional information about the influence of vesicle size on the structural transformation process.

On the basis of the QCM-D measurement results, we identified three stages of the interaction between AH peptide and vesicles, including (i) AH peptide binding interaction caused an increase in bound mass, corresponding to a maximum change in the resonance frequency; (ii) the mass load of the adlayer decreased while the energy dissipation continued to increase until reaching a maximum (in some cases); and (iii) if a maximum change in energy dissipation was observed, then there was an additional stage corresponding to vesicle rupture and lipid relaxation. Furthermore, we analyzed the particular influence of vesicle size on the AH peptide-
mediated structural transformation resulting in complete vesicle rupture to form a planar bilayer, incomplete vesicle rupture, or minimal to no vesicle rupture.

If there was complete vesicle rupture (e.g., small vesicles below 80 nm diameter), then all three stages were identified, and the behavior across regimes was generally similar for all vesicle sizes (Figure 2A). Of note, the observed changes in and the behavior across regimes was generally similar for all vesicle swelling occurs, presumably until reaching a threshold size range. Since pore formation is membrane strain-dependent, this relationship suggests that AH peptide may induce the formation of pores in smaller vesicles to a greater extent, that is, more kinetically favorable due to vesicle size-dependence of pore formation as described in the Theoretical Aspects section, and/or starting at a lower peptide-to-lipid ratio. In stage (ii), vesicle swelling occurs, presumably until reaching a threshold level at which the structural transformation occurs. Despite some differences in stage (i) related to vesicle size, the behavior in stage (ii) is nearly identical for vesicles in this size range, suggesting that the structural transformation, including the vesicle swelling process, follows a general pathway.

If there was incomplete rupture (e.g., vesicles between 94 and 161 nm diameter), then all three stages were again identified (Figure 2B). Compared to smaller vesicles, the changes in physical properties in each stage from (i) to (iii) were less significant. In particular, the vesicle swelling process observed in stage (ii) had only a minor effect on the adlayer properties. Additionally, stage (iii) did not continue until a planar bilayer was formed. Rather, the QCM-D measurement signals indicated the presence of remaining viscoelastic elements, presumably unruptured vesicles ($\Delta f < \pm 50$ Hz and $\Delta D < 5 \times 10^{-6}$, as compared to baseline values). The interaction of AH peptide with 161 nm diameter vesicles showed evidence of vesicle rupture and lipid relaxation, but the rupture process was only intermediate in progression. The remaining adsorbate had physical properties that are characteristic of an intact vesicle adlayer.

If there was minimal to no vesicle rupture (e.g., 241 nm diameter or larger vesicles), then there was not a complete structural transformation, but rather stages (i) and (ii) occurred only (Figure 2C). There was a maximum change in energy dissipation—indicating the completion of stage (i)—but vesicle rupture and lipid relaxation did not occur thereafter. For larger vesicles, only a maximum change in resonance frequency was observed; no such change in energy dissipation occurred, suggesting that the vesicle swelling process never reached the threshold level required to induce vesicle rupture. Nevertheless, there were appreciably larger increases in energy dissipation that occurred during stage (ii) (up to $\Delta D > 25 \times 10^{-6}$ in this regime alone, and $\Delta D > 60 \times 10^{-6}$, as compared to baseline values), while the mass load of the adlayer decreased, and there was limited evidence of vesicle rupture to any extent. Collectively, the findings suggest that the interaction of AH peptide with large vesicles may follow a different pathway than the rupture process observed for small vesicles.

**Kinetics of AH Peptide Binding to Vesicles.** To further understand the effects of vesicle size on AH peptide binding to vesicles, we performed kinetic analysis by using a phenomenological model. In this model, the dependence of the number of bound peptides on time is described as

$$N = C t^\alpha$$

(7)

where $C$ is a constant, and $\alpha$ is the corresponding exponent. If one assumes the vesicle surface is uniform and that peptide association can occur at any site, then the number of bound peptides would initially increase linearly as a function of time before the peptide coverage becomes appreciable. The kinetics may occur such that $\alpha = 1$ or $1/2$ almost up to saturation if, for example, peptide binding were diffusion-limited under flow or no flow conditions, respectively. Likewise, if $\alpha < 1$ or $\alpha > 1$, then peptide binding is kinetically limited and noncooperative or cooperative, respectively. The latter two adsorption regimes occurred such that the adsorption activation energy increases or decreases with increasing coverage due to lateral adsorbate–adsorbate interactions (eq 2). Following this line, we fitted the time-dependence of the change in effective Voigt thickness of the entire adlayer as

$$d = d_0 + D(t - t_1)^\alpha$$

(8)

where $d_0$ is the initial thickness, and $D(t - t_1)^\alpha$ is the thickness corresponding to peptide binding ($D$, $d_0$, and $\alpha$ are parameters in the fitting, and $t_1$ is fixed as the initial time at which peptide binding occurs; note that eq 7 implies that $t_1 = 0$.) The fitting was performed from $t = t_1$ up to the time at which the maximum in effective Voigt thickness is reached (Figure S4 of the Supporting Information). The latter is interpreted as the rupture time, and vesicle rupture is assumed to occur before saturation of bound peptide. The fitting parameters $d_0$, $D$, and $\alpha$ form the basis of our analysis (Table 2).

**Table 2. Kinetic Analysis of AH Peptide Binding to Vesicles of Different Size**

<table>
<thead>
<tr>
<th>average vesicle diameter (nm)</th>
<th>$d_0$ (nm)</th>
<th>$D$ (nm·min$^{-1}$)</th>
<th>$\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>58</td>
<td>25.6 ± 0.2</td>
<td>3.05 ± 0.14</td>
<td>1.41 ± 0.03</td>
</tr>
<tr>
<td>80</td>
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<td>2.74 ± 0.15</td>
<td>1.28 ± 0.03</td>
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<tr>
<td>94</td>
<td>25.0 ± 0.0</td>
<td>1.98 ± 0.06</td>
<td>1.14 ± 0.02</td>
</tr>
<tr>
<td>122</td>
<td>34.7 (fixed)</td>
<td>2.96 ± 0.06</td>
<td>0.60 ± 0.01</td>
</tr>
<tr>
<td>161</td>
<td>61.4 (fixed)</td>
<td>2.91 ± 0.13</td>
<td>0.77 ± 0.02</td>
</tr>
<tr>
<td>241</td>
<td>66.3 (fixed)</td>
<td>4.33 ± 0.12</td>
<td>0.60 ± 0.01</td>
</tr>
<tr>
<td>791</td>
<td>73.7 (fixed)</td>
<td>10.5 ± 0.1</td>
<td>0.36 ± 0.00</td>
</tr>
<tr>
<td>925</td>
<td>90.8 (fixed)</td>
<td>15.2 ± 0.0</td>
<td>0.32 ± 0.00</td>
</tr>
</tbody>
</table>

* A phenomenological model was applied to quantitatively describe the effects of vesicle size (reported as intensity-weighted, average diameter) on AH peptide binding to vesicles. An equation corresponding to the model was developed to fit the QCM-D measurement data in the form of effective Voigt thickness as a function of time. The fitting range was defined as stage (i) in the structural transformation, which corresponds to initial peptide binding to the intact vesicle layer up until the maximum in effective Voigt thickness is reached. The parameters are defined as follows: $d$ is the thickness of the vesicle adlayer at time $t$; $d_0$ is the initial thickness; $D$, $b$, and $\alpha$ are fitting parameters; and $c$ is fixed as the initial time at which peptide binding occurs. For larger vesicles, $d_0$ was fixed in the fitting procedure in order to improve the fit during the initial time before peptide binding occurred.

Concerning $d_0$, we repeat that in our experiments the intact vesicle adlayer is close to saturation. Under such conditions, the surface concentration of vesicles is proportional to $1/R^2$. The vesicle area is proportional to $R^2$. Thus, the lipid mass is nearly independent of $R$. The first guess might be that $d_0$ depends primarily on the lipid mass and accordingly is expected to be independent of $R$. Our calculations indicate, however, that $d_0$ increases with increasing $R$, especially trend-wise when
comparing small and large vesicles. This effect may be related to more effective trapping of water by larger vesicles or to differences in the degree of vesicle deformation as a function of vesicle size.

Concerning $\alpha$, we note the general tendency of this parameter to decrease with increasing $R$. For 94 nm diameter or smaller vesicles, $\alpha$ was always greater than 1, and the values increased with decreasing vesicle size, up to $\alpha = 1.41 \pm 0.03$ for 58 nm diameter vesicles. During the structural transformation from intact vesicles to a planar bilayer, the binding of AH peptide was therefore cooperative, suggesting that binding of additional peptides is promoted by already bound peptides that are involved in processes related to vesicle fusion/swelling. Of note, vesicles and virus particles in this size range are susceptible to efficient lysis by AH peptide. Outside this size range, AH peptide is less effective, and we similarly observed a change in binding cooperativity.

Binding of AH peptide to vesicles between 122 and 241 nm diameter was noncooperative ($\alpha$ between 0.6 and 0.8). Although 122 nm diameter vesicles still ruptured appreciably, there is likely a thermodynamic balance that must be considered between the free energy of pore formation and the membrane strain induced by peptide binding, along with vesicle deformation. This example is notable because the physical properties of the adlayers were identical for 122 nm diameter or smaller vesicles, indicating that there was greater deformation of larger vesicles in this size range. For appreciably larger vesicles, $\alpha$ became as low as 0.32 ± 0.00. Hence, the obtained values of $\alpha$ not only showed a dependence on vesicle size, but there was also a transition between cooperative and noncooperative binding of AH peptide to vesicles.

Additionally, the value of $D$ depended on vesicle size. In principle, $D$ is related to the association rate constant of the membrane–peptide interaction. For all vesicles between 58 and 161 nm diameter, the value of $D$ consistently ranged between approximately 2 to 3 nm-min$^{-1}$. However, for larger vesicles, $D$ increased and was equal to 10.5 ± 0.1 and 15.2 ± 0.0 nm-min$^{-1}$ for 791 and 925 nm diameter vesicles, respectively. These values suggest that the association rate constant or some parameter related to this constant may be influenced by vesicle size, at least to some degree. Because of possible effects of penetration depth on the QCM-D measurement signal in the case of large vesicles, the values of $D$ measured for small and large vesicles cannot be directly compared. Overall, analysis of the kinetic parameters related to AH peptide binding show that vesicle size can influence binding cooperativity and/or the association rate constant, and the range of these effects are consistent with the experimental data on vesicle rupture.

**Kinetics of Vesicle Rupturing Induced by AH Peptide.**

Using a related phenomenological model, we also performed kinetic analysis on the effect of vesicle size on vesicle rupturing induced by AH peptide. The coalescence of vesicle rupture and lipid relaxation was cumulatively described by

$$d = d_a + G \exp\left[-(t - t_s)/\tau\right]$$

(9)

where $d$ is the thickness of the adlayer, $d_a$, $G$, and $\tau$ are fitting parameters, and $t_s$ is fixed as the starting time of the rupture process. The fitting range was chosen to be associated with stages (ii) and (iii) in the structural transformation, occurring after reaching the maximum in effective Voigt thickness (Figure S4 of the Supporting Information). For each vesicle size, the parameters $G$ and $\tau$ were varied, while $d_a$ was fixed to describe adlayer thickness after the structural transformation is complete (Table 3). No rupture process was observed for 791 or 925 nm diameter vesicles, so fitting was not performed in these cases.

**Table 3. Kinetic Analysis of Vesicle Rupture Induced by AH Peptide**

<table>
<thead>
<tr>
<th>average vesicle diameter (nm)</th>
<th>$d$ (nm)</th>
<th>$G$ (nm)</th>
<th>$\tau$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>58</td>
<td>5.67 ± 0.02</td>
<td>53.6 ± 0.2</td>
<td>5.21 ± 0.02</td>
</tr>
<tr>
<td>80</td>
<td>5.18 ± 0.00</td>
<td>42.5 ± 0.1</td>
<td>4.46 ± 0.01</td>
</tr>
<tr>
<td>94</td>
<td>7.62 ± 0.02</td>
<td>27.2 ± 0.1</td>
<td>22.6 ± 0.1</td>
</tr>
<tr>
<td>122</td>
<td>21.1 ± 0.2</td>
<td>21.1 ± 0.2</td>
<td>79.2 ± 1.6</td>
</tr>
<tr>
<td>161</td>
<td>58.5 ± 0.0</td>
<td>22.2 ± 0.0</td>
<td>33.6 ± 0.4</td>
</tr>
<tr>
<td>241</td>
<td>68.2 ± 0.3</td>
<td>26.5 ± 0.3</td>
<td>53.6 ± 1.3</td>
</tr>
<tr>
<td>791</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>925</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*A phenomenological model was applied to quantitatively describe the effects of vesicle size (reported as DLS intensity-weighted, average diameter) on vesicle rupture induced by AH peptide, specifically the coalescence of vesicle rupture and lipid relaxation. An equation corresponding to the model was developed to fit the QCM-D measurement data in the form of effective Voigt thickness as a function of time. The fitting range was defined as stages (ii) and (iii) in the structural transformation, which corresponds to the time after reaching the maximum in effective Voigt thickness. The parameters are defined as follows: $d$ is the thickness of the adlayer, $d_a$, $G$, and $\tau$ are fitting parameters, and $t_s$ is fixed as the starting time of the rupture process. No rupture process was observed for 800 and 1000 nm vesicles, so fitting was not performed in these cases. N/A indicates Not Applicable.*

The parameters $G$ and $d_a$ are related because $G$ represents the magnitude of the change in adlayer thickness during the rupture process (i.e., from maximum in effective Voigt thickness to final effective Voigt thickness), and $d_a$ represents the value of the adlayer’s final effective Voigt thickness. The fitting identified that the greatest values for $G$ were observed for 80 nm diameter or smaller vesicles; the values ranged from 42.5 ± 0.1 to 53.6 ± 0.2 nm. For vesicles between 122 and 241 nm diameter, the value of $G$ was always between 21.1 ± 0.2 and 27.2 ± 0.1 nm. Compared to $G$ obtained for smaller vesicles, these values suggest that the structural transformation is incomplete. Note that the magnitude of $G$ is roughly equivalent for incompletely ruptured vesicle adlayers irrespective of vesicle size or initial adlayer thickness, the latter of which is related to the deformation of adsorbed vesicles.

In terms of the final adlayer thicknesses, the values of $d_a$ indicate that 80 nm diameter vesicles or smaller ruptured completely. In these cases, $d_a$ was approximately 5 nm, which is indicative of a planar bilayer on the gold substrate. Nearly complete vesicle rupture was also observed for 94 nm diameter vesicles, and $d_a$ was 7.62 ± 0.02 nm. Larger vesicles were ruptured less efficiently, and $d_a$ was correspondingly greater. Large vesicles between 161 and 241 nm diameter had $d_a$ values that ranged from 58.5 ± 0.0 to 68.2 ± 0.3 nm, suggesting limited vesicle rupture. An interesting case was observed for 122 nm diameter vesicles, for which $d_a$ was 21.1 ± 0.2 nm. This intermediate value suggests that a sizable fraction of vesicles were ruptured while another sizable fraction of vesicles remained intact.

Additionally, our model also describes the duration of the vesicle rupture process by fitting a decay parameter, $\tau$. The collective rupture of 80 nm diameter or smaller vesicles occurred quickly, with $\tau$ on the order of 5 min. Although 94 nm diameter vesicles were almost completely ruptured, $\tau$ was
membrane expansion that arises from asymmetric peptide rearrangements of the membrane may be unbalanced and/or a structural transformation. Specifically, membrane destabilization may occur without vesicle rupture: (i) vesicles with 122 nm average diameter, which had only one population; and (ii) vesicles with 161 nm average diameter, which had one subpopulation around 165 nm and another subpopulation around 156 nm. As previously noted, the average vesicle diameter may not be a suitable predictor to estimate the vesicle size range that is targeted by AH peptide because the average diameter may not accurately reflect the size distribution of vesicles. In light of this consideration, we analyze cases of inefficient vesicle rupture observed in this study to more clearly define the targeting range of AH peptide.

In particular, we discuss three cases of incomplete vesicle rupture: (i) vesicles with 122 nm average diameter, which had one subpopulation around 85 nm and another subpopulation around 165 nm; (ii) vesicles with 161 nm average diameter, which had only one population; and (iii) vesicles with 241 nm average diameter, which had one subpopulation around 156 nm and another subpopulation around 631 nm. Of note, the vesicle sizes were obtained by DLS measurements in bulk solution. As smaller vesicles diffuse more quickly than larger vesicles, according to diffusion-limited kinetics, we assume that, for bimodal size distributions, vesicles belonging to the sub-population with smaller size represent the major fraction of vesicles on the substrate.

The on this assumption, the adsorption of vesicles with 122, 161, and 241 nm DLS-measured average diameters would probably lead to an intact vesicle adlayer with vesicles possessing, on average, 85, 161, and 156 nm diameters, respectively. As vesicle rupture was observed in all three cases, we may conclude that AH peptide can rupture vesicles up to 160 nm diameter. Inefficient rupture, as defined previously, is not due to incomplete rupture of vesicles on a single vesicle level but because the QCM-D measurement captures interaction kinetics in relation to a collection of vesicles. Hence, inefficient rupture may be redefined in the case of...
adsorbed vesicles above or below the critical size such that rupture occurs for vesicles at or below the critical size, and no rupture occurs for vesicles above the critical size. Furthermore, in this study, no vesicle rupture was observed among vesicles in which the small-size subpopulation was greater than 200 nm diameter, which is outside the targeting size range of AH peptide.

Collectively, the experimental data support that AH peptide may target enveloped viruses up to 160 nm diameter, which is in agreement with previous findings related to HIV20 and herpes simplex virus.21 On the basis of this finding, we present an updated graph that correlates the target vesicle size range of AH peptide with the known antiviral spectrum of CSA, the AH peptide analogue (Figure 4). When other factors such as virus morphology, shape, and spike density are accounted for (Table S1 of the Supporting Information), AH peptide and analogues thereof are clearly potent mediators of virion rupture by membrane destabilization. Compared to vesicles of relatively simple composition, an additional consideration with enveloped viruses is that many types of virions contain an appreciable amount of cholesterol, which is also known to influence vesicle bending rigidity and must be considered alongside virus size. The broad spectrum of targeted viruses which bud from different parts of host cells with varying lipid composition further supports that AH peptide is a promising antiviral agent. Hence, these findings motivate the continued development of AH peptide-based therapeutics to target enveloped viruses across a wider range of virus sizes than previously suggested.

### CONCLUSION

The motivation of this work was to understand how AH peptide selectively deactivates small enveloped viruses by rupture of the envelope. To address this issue, we studied lipid vesicles of varying size, which provide a model system for enveloped viruses. A combination of experiment and theory was applied to analyze the system under consideration. The theory indicates that membrane curvature may facilitate vesicle rupture due to a higher rate of AH peptide adsorption and higher rate of pore formation. The interaction of AH peptide with vesicles of different sizes was studied experimentally by the QCM-D technique. The experimental results were consistent with the predictions. As previously observed, AH peptide ruptured small vesicles to form a planar bilayer on gold. No such rupture was observed with large vesicles. We also gained new insight into the rupture process based on the QCM-D measurement data, including the following two key observations:

(1) We identified three stages to describe changes in the physical properties in the adlayer, including: (i) peptide binding; (ii) structural rearrangement, including pore formation; and (iii) vesicle rupture. In particular, the structural rearrangement within stage (ii) was newly identified and is observed when the energy dissipation of the film increases, even as the mass load of the film decreases. This regime occurs for all vesicle sizes, irrespective of whether or not vesicle rupture occurs eventually.

(2) AH peptide binding to small vesicles occurs cooperatively. By contrast, for large vesicles, AH peptide binding was noncooperative. The findings from the kinetic analysis demonstrate the unique properties of AH peptide, as compared to antimicrobial peptides that can also disrupt the lipid membranes of infectious pathogens. AH peptide can target a broad spectrum of enveloped viruses within a certain size range, whereas antimicrobial peptides generally target specific bacterial pathogens on the basis of membrane charge distribution or lipid composition.

In summary, we have analyzed the rupture mechanism of AH peptide and identified factors related to its selective targeting of small, enveloped viruses. This work indicates that AH peptide may rupture a wider size range of enveloped viruses than previously estimated. On the basis of a combination of experiment and theory, we estimate that AH peptide is able to rupture enveloped viruses up to 160 nm diameter, which is in better agreement with virological studies than previous estimates. These findings have significant implications for developing new antiviral medicines, and future work may be directed toward translating the findings from small vesicles to biological systems of medical importance.

### ASSOCIATED CONTENT

More detailed information is provided about the full experimental data sets (Figures S1–S3), as well as the biophysical properties of several viruses (Table S1). This information is available free of charge via the Internet at http://pubs.acs.org/.

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**Figure 4.** Correlation of virus size range and antiviral activity spectrum of AH peptide. The antiviral activity of the shortened AH peptide analogue termed CSA was assessed previously by Cheng et al.18 On the basis of the QCM-D measurement results obtained in the present study along with the in vitro experimental data in the former study,18 the graph represents the correlation between virus diameter and susceptibility to AH peptide treatment. We assume the size range of vesicles susceptible to AH peptide treatment in our model system is equivalent to the size range of enveloped virus particles that are susceptible to AH peptide treatment. The size range of virus diameters by virus type is represented by the black bars. Viruses are reported as being either susceptible (blue) or refractory (red) to AH peptide treatment. On the basis of the experimental results presented in this study, the virus targeting range of AH peptide is reported to be enveloped viruses up to 160 nm diameter, as indicated by the vertical green dotted line. In general, enveloped viruses that are spherical and within the targeting range are susceptible to AH peptide treatment. In the graph, viruses with specific properties are denoted as follows: (a) Nonenveloped virions; (b) Pleomorphic virion morphology (including spherical subpopulation); and (c) Nonspherical, regular virion morphology. More information about virus sizes and morphologies is available in the Supporting Information.
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Notes
The authors declare no competing financial interest.

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