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# Understanding How Sterols Regulate Membrane Remodeling in **Supported Lipid Bilayers**

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ABSTRACT: The addition of single-chain lipid amphiphiles such as antimicrobial fatty acids and monoglycerides to confined, two-dimensional phospholipid bilayers can trigger the formation of three-dimensional membrane morphologies as a passive means to regulate stress. To date, relevant experimental studies have been conducted using pure phospholipid compositions, and extending such insights to more complex, biologically relevant lipid compositions that include phospholipids and sterols is warranted because sterols are important biological mediators of membrane stress relaxation. Herein, using the quartz crystal microbalancedissipation (QCM-D) technique, we investigated membrane remodeling behaviors triggered by the addition of sodium



dodecyl sulfate (SDS), lauric acid (LA), and glycerol monolaurate (GML) to supported lipid bilayers (SLBs) composed of phospholipid and cholesterol mixtures. The SLB platforms were prepared by the solvent-assisted lipid bilayer method in order to form cholesterol-rich SLBs with tunable cholesterol fractions (0-52 mol %). The addition of SDS or LA to fabricated SLBs induced tubule formation, and the extent of membrane remodeling was greater in SLBs with higher cholesterol fractions. In marked contrast, GML addition led to bud formation, and the extent of membrane remodeling was lower in SLBs with higher cholesterol fractions. To explain these empirical observations, we discuss how cholesterol influences the elastic (stiffness) and viscous (stress relaxation) properties of phospholipid/cholesterol lipid bilayers as well as how the membrane translocation properties of single-chain lipid amphiphiles affect the corresponding membrane morphological responses. Collectively, our findings demonstrate that single-chain lipid amphiphiles induce highly specific membrane morphological responses across both simplified and complex model membranes, and cholesterol can promote or inhibit membrane remodeling by a variety of molecular mechanisms.

# INTRODUCTION

Single-chain lipid amphiphiles such as free fatty acids and monoglycerides play important roles in a wide range of biological and biotechnology applications ranging from molecular evolution to biofuels.<sup>1-3</sup> One of the most promising directions involves understanding how single-chain lipid amphiphiles interact with phospholipid membranes, giving rise to the targeted inactivation of pathogenic microbes such as viruses and bacteria. Previous studies have demonstrated that certain fatty acids and monoglycerides can lyse enveloped viruses,<sup>4,5</sup> including human immunodeficiency virus,<sup>6</sup> herpes simplex virus,7 and respiratory syncytial virus.8 Furthermore, some compounds within this class can disrupt the cellular membranes surrounding Gram-positive and Gram-negative bacteria, resulting in either bacterial cell death or growth inhibition.<sup>2,9</sup> Within this scope, single-chain lipid amphiphiles

that exhibit broad-spectrum antimicrobial activity against bacteria, viruses, and other microorganisms are termed "antimicrobial lipids" and have been widely explored for several decades.<sup>10–14</sup> In the 1970s and 1980s, Kabara and colleagues conducted pioneering studies that investigated how the potency and scope of antimicrobial activity depend on the hydrocarbon chain length and number of degrees of unsaturation in antimicrobial lipids.<sup>15,16</sup> Importantly, these efforts led to the discovery that medium-chain, saturated fatty acids and their monoglyceride derivatives-i.e., lauric acid (LA) and glycerol monolaurate (GML)-exert particularly strong antimicrobial activity,<sup>17,18</sup> and these molecules have also been classified as

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Generally Recognized as Safe (GRAS) by the US Food and Drug Administration.<sup>2</sup>

Expanding on these biological insights, there has been renewed interest in developing experimental strategies to characterize the interactions between single-chain lipid amphiphiles and phospholipid membranes. Such efforts are helping to provide a physicochemical explanation of why fatty acids and monoglycerides exhibit different spectra of antimicrobial activity. While direct observation of the interactions between antimicrobial lipids and biological membranes typically requires a fixed treatment period and sample fixation before imaging (e.g., via scanning electron microscopy or atomic force microscopy), real-time monitoring of these interactions is possible using model membrane platforms. To date, dynamic light scattering and electron microscopy techniques have been utilized to study how fatty acids can induce the aggregation of small vesicles, including subsequent fission and growth of individual vesicles.<sup>19,20</sup> The shape transformation of giant unilamellar vesicles induced by oleic acid as well as the formation of new daughter vesicles has also been reported.<sup>21</sup> At the same time, while solution-based experimental systems provide insight into gross morphological changes, it is difficult to track molecular-level interactions at the nanoscale with such approaches, and surface-sensitive measurement platforms represent an alternative solution to address this measurement need.

In particular, supported lipid bilayers (SLBs) are simplified, two-dimensional phospholipid membranes that are commonly formed on silica-based surfaces and can be probed using surface-sensitive measurement techniques to monitor compound binding, membrane morphological responses, and changes in membrane fluidity. Using a single-component, zwitterionic phospholipid SLB platform, it was observed that the addition of docosahexaenoic acid (DHA) promoted the formation of elongated lipid protrusions as part of the membrane destabilization process.22 Similar morphological responses were observed when DHA was added to SLBs of different phospholipid compositions and under different ionic conditions.<sup>23</sup> Importantly, it has also been possible to distinguish the membrane morphological responses induced by medium-chain saturated fatty acids and monoglycerides, as exemplified by studies involving LA and GML.<sup>24</sup> Like DHA, LA as well as sodium dodecyl sulfate (SDS)-an anionic detergent with a saturated 12-carbon-long hydrocarbon chain-induced the formation of elongated lipid protrusions, whereas neutral GML promoted the formation of membrane buds; the difference in morphological responses was attributed in part to how molecular charge influences membrane translocation and corresponding release of membrane strain.<sup>24</sup> Additional medium-chain saturated fatty acids and monoglycerides exhibited similar behavior against single-component SLBs, and a correlation between the molecular structure and micellar aggregation properties of the compounds and their differential effects on membrane morphology and fluidity was established.<sup>25</sup> On the other hand, biological membranes are more complex and composed of a milieu of different phospholipids and sterols, and therefore the morphological responses observed in simplified models should be extended to more complex compositions. To this end, the formation of SLBs from E. coli and S. cerevisiae membrane extracts has been attempted, and the interactions of these SLB platforms with a monoglyceride were investigated.<sup>26</sup> However, it is challenging to prepare well-controlled SLBs with complex compositions,

and a bottom-up approach to systematically investigate how specific membrane components affect membrane morphological responses would be advantageous for deciphering mechanistic details.

Aside from phospholipids, sterols and sterol-like compounds are ringed molecules that are found in biological membranes and help to regulate membrane fluidity, permeability, and rigidity.<sup>27,28</sup> They also play important roles in membrane remodeling as part of biological processes such as endocytosis.<sup>29,30</sup> In mammalian cells, cholesterol is the most prominent sterol, and mammalian cell membranes contain as much as ~60 mol % cholesterol.<sup>31</sup> Unlike phospholipids, cholesterol molecules can rapidly translocate across bilayer leaflets and influence membrane properties.<sup>32,33</sup> Likewise, bacterial cell membranes contain hopanoids that act as functional analogues of cholesterol.<sup>34</sup> While sterols and sterollike hopanoids are prominent components in biological membranes, it is difficult to fabricate sterol-rich SLBs,<sup>3</sup> and solution-phase vesicles have been the main experimental system for studying the interactions between surfactants and phospholipid/cholesterol membrane compositions. For example, it has been observed that increasing amounts of cholesterol in vesicles impede membrane leakage and solubilization caused by SDS.<sup>37</sup> Extending these insights to uncover the influence of cholesterol on molecular-level interactions and corresponding membrane morphological responses is desirable, especially in the context of studying a broader range of membrane-active compounds like fatty acids and monoglycerides. Toward this goal, establishing cholesterol-rich SLB platforms would greatly advance measurement capabilities. While conventional fabrication methods are limited to preparing SLBs with no more than  $\sim$ 20 mol % cholesterol,<sup>36,38,39</sup> it was recently reported that cholesterol-rich SLBs with up to  $\sim$ 57 mol % cholesterol can be formed using the solvent-assisted lipid bilayer (SALB) method.<sup>39,40</sup> Such capabilities provide the basis for exploring how cholesterol, as a model sterol, influences membrane morphological responses induced by fatty acids and monoglycerides.

The goal of the present study was to investigate how cholesterol affects the membrane morphological responses induced by representative fatty acids and monoglycerides, namely LA and GML; SDS was also tested. Zwitterionic 1,2dioleoyl-sn-glycero-3-phosphocholine (DOPC) SLB platforms were prepared with varying fractions of cholesterol (0-52 mol)%) by the SALB formation method, and the addition of SDS, LA, and GML to prefabricated SLB platforms was tracked by quartz crystal microbalance-dissipation (OCM-D) measurements. The QCM-D technique offers a label-free measurement approach to monitor the changes in hydrodynamically coupled mass and viscoelastic properties of an adsorbate and therefore provides multiple signatures to characterize both SLB formation and subsequent membrane morphological responses, as previously established in multiple works.<sup>22,24,25</sup> Importantly, the QCM-D measurement signatures have been shown to correlate with the membrane morphological responses observed by fluorescence microscopy,<sup>24</sup> and hence the QCM-D measurements provide insight into membrane remodeling behaviors, including the type and extent of membrane morphological response patterns (i.e., tubule or bud formation). Following this measurement approach, we examined how cholesterol influences membrane morphological responses induced by single-chain lipid amphiphiles and discovered that the differential effects of cholesterol on



**Figure 1.** QCM-D evaluation of cholesterol-rich SLB formation. QCM-D measurements were performed to characterize the SLB formation process. The final (A)  $\Delta f$  shifts and (B)  $\Delta D$  shifts are reported after SLB formation was completed. The measurement shifts are relative to baseline recordings in equivalent aqueous buffer solution. The molar percentage (mol %) of cholesterol refers to the DOPC phospholipid/cholesterol mixture that was used in the SALB protocol. (C) QCM-D  $\Delta f$  shifts are reported as a function of time for cholesterol extraction from cholesterol-rich SLBs upon M $\beta$ CD treatment. After SLB formation, the  $\Delta f$  shifts were normalized to baseline values at t = 0 min, and 1 mM M $\beta$ CD was then added starting at t = 5 min. Positive  $\Delta f$  shifts indicate cholesterol extraction from the SLB platform. (D) Calculated molar percentage of cholesterol in SLB platform as a function of the input cholesterol fraction in the lipid mixture used in the SALB protocol. Mean and standard deviation (error bars) are reported from five or more technical replicates.

regulating membrane remodeling depend on the elastic (stiffness) and viscous (stress relaxation) properties of phospholipid/cholesterol lipid bilayers.

# EXPERIMENTAL SECTION

**Reagents.** 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Lauric acid, sodium dodecyl sulfate, and methyl- $\beta$ -cyclodextrin were obtained from Sigma-Aldrich (St. Louis, MO). Glycerol monolaurate was purchased from Abcam (Cambridge, UK). The aqueous buffer solution was 10 mM Tris buffer solution [pH 7.5] with 150 mM NaCl. Phosphate-buffered saline (PBS) was purchased from Gibco (Carlsbad, CA). All other reagents were obtained from Sigma-Aldrich. All solutions were prepared with Milli-Q-treated water (>18 M $\Omega$ ·cm) (Millipore, Billerica, MA). The dry compounds were stored in a dark cabinet.

Sample Preparation. DOPC lipid powder and cholesterol powder were dissolved in isopropanol at 10 mg/mL lipid concentration, mixed to the desired molar ratio, and then diluted to a 0.3 mg/mL total lipid concentration. Dissolved DOPC lipid powder and cholesterol powder were stored in -20 °C fridge. Stock solutions of LA and GML were first prepared by dissolving the weighed compounds in ethanol to a final concentration of 200 mM. An aliquot from the ethanol solutions was diluted 100-fold with PBS to make 2 mM stock solutions and then further diluted in PBS as appropriate. The SDS test samples were prepared by dissolving the weighed compound in PBS (with 1% ethanol) to a 2 mM stock concentration and then further diluted in PBS as appropriate. To increase the solubility of all test samples, the solutions were heated in a 70 °C water bath for 30 min. After heating, the solutions were cooled and then further diluted to the appropriate test concentration. All solutions were prepared on the same day as the experiments.

Quartz Crystal Microbalance-Dissipation (QCM-D). QCM-D experiments with a Q-Sense E4 instrument (Q-Sense AB, Gothenburg, Sweden) were conducted in order to characterize the interactions between the test compounds and supported lipid bilayers with varying DOPC/cholesterol molar ratios. The QCM-D technique monitors changes in resonance frequency ( $\Delta f$ ) and energy dissipation ( $\Delta D$ ) of an oscillating, piezoelectric quartz crystal sensor chip as a function of time, which reflect the acoustic mass and viscoelastic properties, respectively, of an adsorbate on the surface.<sup>41</sup> The sensor chip had a fundamental frequency of 5 MHz and a sputter-coated, 50 nm thick layer of silicon dioxide (model no. QSX 303, Q-Sense AB) The experimental data were collected at the third (n = 3), fifth (n = 5), seventh (n = 7), ninth (n = 9), and 11th (n = 11) odd overtones using the QSoft software program (Q-Sense AB), and the data were normalized according to the overtone number. Data processing was performed in the QTools (Q-Sense AB) and OriginPro 8.5 (OriginLab, Northampton, MA) software packages. All presented data were collected at the seventh overtone. Before the experiment, the sensor chips were sequentially rinsed with water and ethanol, dried with nitrogen gas, and subjected to oxygen plasma treatment for 1 min with an Expanded Plasma Cleaner (model no. PDC-002, Harrick Plasma, Ithaca, NY). Initially, cholesterol-rich SLB on silicon dioxide surface was made by using the SALB technique.<sup>42</sup> During the experiments, the temperature in the measurement cell was maintained at 25.0 ± 0.5 °C. A peristaltic pump (Reglo Digital, Ismatec, Glattbrugg, Switzerland) was used to inject liquid samples into the measurement chamber at a flow rate of 50  $\mu$ L/min.

#### RESULTS AND DISCUSSION

Formation of Cholesterol-Rich Supported Lipid Bilayers. Supported lipid bilayers on silicon dioxide substrates were prepared from mixtures of DOPC phospholipid and cholesterol by employing the solvent-assisted lipid bilayer (SALB) technique.<sup>39</sup> The QCM-D technique was employed to track the SLB formation process on silicon dioxide-coated sensor chips and measure changes in resonance frequency ( $\Delta f$ )



**Figure 2.** Effect of SDS treatment on mass and viscoelastic properties of cholesterol-rich SLBs. QCM-D measurements were performed, and representative  $\Delta f$  (blue squares) and  $\Delta D$  (red triangles) shifts are reported as a function of time for DOPC phospholipid/cholesterol SLBs with (A) 0, (B) 17, (C) 35, and (D) 52 mol % cholesterol. The initial measurement values correspond to fabricated SLB platforms on the sensor surface. 1 mM SDS was added starting at  $t = 5 \min (\operatorname{arrow 1})$ , and a buffer washing step was then performed after the measurement signals stabilized (arrow 2).

and energy dissipation ( $\Delta D$ ) that are related to an adsorbate's mass and viscoelastic properties, respectively.<sup>42</sup> For the QCM-D measurements, a baseline recording was first made in aqueous buffer solution, and then the solution was exchanged to water-miscible isopropanol solution. Afterward, a 0.3 mg/mL mixture of DOPC and cholesterol in isopropanol—premixed to the desired molar ratio (0–52 mol % cholesterol input)—was deposited on the silicon dioxide surface, followed by solvent exchange to aqueous buffer solution, and this process resulted in SLB formation, as determined by the final  $\Delta f$  and  $\Delta D$  shifts relative to the baseline recording.

The final QCM-D measurement values are presented in Figure 1A,B and show a dependence on the molar fraction of cholesterol in the lipid mixture. For the DOPC phospholipid composition, the final  $\Delta f$  and  $\Delta D$  shifts were  $-26 \pm 1.5$  Hz and  $(0.3 \pm 0.2) \times 10^{-6}$ , respectively, which agree well with literature values for successful SLB formation.<sup>36,43</sup> Similar values were obtained for DOPC/cholesterol SLBs with 17 mol % cholesterol, in which case the  $\Delta f$  and  $\Delta D$  shifts were  $-26 \pm$ 1.3 Hz and  $(0.6 \pm 0.2) \times 10^{-6}$ , respectively. On the other hand, DOPC/cholesterol SLBs with 35 and 52 mol % cholesterol fractions had appreciably larger measurement responses, and the final  $\Delta f$  and  $\Delta D$  shifts were around -32 Hz and  $1.3 \times 10^{-6}$ , respectively. These variations in the QCM-D measurement responses as a function of cholesterol fraction are indicative of differences in membrane organization, prompting us to quantify the amount of cholesterol in the DOPC/cholesterol SLB platforms.

To determine the amount of cholesterol in the SLB platforms, the fabricated SLBs were treated with 1 mM methyl- $\beta$ -cyclodextrin (M $\beta$ CD), which can extract cholesterol from lipid membranes.<sup>44</sup> Figure 1C shows the normalized  $\Delta f$  shifts for fabricated SLBs after M $\beta$ CD injection at t = 5 min. The normalized values at the initial time point correspond to successful SLB formation, and M $\beta$ CD extraction of cholesterol

from the SLBs led to positive  $\Delta f$  shifts, which indicates successful removal of cholesterol. Indeed, the corresponding kinetics exhibited monotonic behavior,<sup>44</sup> and there was negligible measurement response when M $\beta$ CD treatment was performed on DOPC SLBs. The magnitude of the  $\Delta f$  shifts increased according to the molar fraction of cholesterol used in the initial mixture. By applying the Sauerbrey model to quantify the mass of cholesterol removed and the mass of DOPC phospholipids remaining, the molar fraction of cholesterol in the SLB was calculated (Figure 1D).<sup>39</sup> For 17 mol % cholesterol in the initial mixture, the resulting SLB had 12  $\pm$ 2 mol % cholesterol, and the SLBs formed from mixtures with 35 and 52 mol % cholesterol had  $32 \pm 2$  and  $48 \pm 2$  mol % cholesterol, respectively. Hence, the cholesterol fraction in the SLB platform could be tuned from ~10 to ~50 mol % cholesterol and supports that the trend in QCM-D measurement responses relates to the effects of cholesterol fraction on membrane organization, as discussed below.

At relatively low molar fractions of cholesterol, mixtures of unsaturated phospholipids and cholesterol form a coexistence of liquid-disordered, fluid L<sub>d</sub> phases and liquid-ordered L<sub>o</sub> phases, the latter of which exhibits short-range orientational order and long-range translational disorder.<sup>45,46</sup> Above a certain molar fraction of cholesterol ( $\sim 20-30$  mol %), phase coexistence no longer occurs, and the mixture assumes the L<sub>o</sub> phase state.<sup>47</sup> This phase transition coincides with the condensing effect of cholesterol whereby the molecular density of the lipid mixture becomes greater as phospholipid and cholesterol form a stoichiometric complex at higher cholesterol fractions.44 Based on the amount of cholesterol in the SLB platforms as determined by M $\beta$ CD extraction, the molecular area per lipid slightly decreases from 67 Å<sup>2</sup> for a DOPC lipid membrane (0 mol % cholesterol input) to 60  $Å^2$  for DOPC/ cholesterol lipid membranes prepared from 17 mol % cholesterol input.<sup>48</sup> This small difference in molecular packing



**Figure 3.** Effect of LA treatment on mass and viscoelastic properties of cholesterol-rich SLBs. QCM-D measurements were performed, and representative  $\Delta f$  (blue squares) and  $\Delta D$  (red triangles) shifts are reported as a function of time for DOPC phospholipid/cholesterol SLBs with (A) 0, (B) 17, (C) 35, and (D) 52 mol % cholesterol. The initial measurement values correspond to fabricated SLB platforms on the sensor surface. 2 mM LA was added starting at t = 5 min (arrow 1), and a buffer washing step was then performed after the measured signals stabilized (arrow 2).

is consistent with the similar QCM-D measurement results obtained for these two cases. On the other hand, the molecular area per lipid decreases more appreciably to 45 and 42 Å<sup>2</sup> for DOPC/cholesterol lipid membranes prepared from 35 and 52 mol % cholesterol inputs, respectively. Compared to the DOPC phospholipid case, the expected increase in molecular packing is around 33-37% for the higher cholesterol fraction cases, and this trend agrees well with the experimentally measured decrease in the QCM-D frequency shifts of around 19%. Indeed, as the magnitude of the QCM-D frequency shift is proportional to the adsorbed mass for rigid adlayers, the experimental findings support that the molecular density of SLBs with high cholesterol fractions is higher than conventional DOPC SLBs and SLBs with lower cholesterol fractions. Taken together, the data support that cholesterol-rich SLBs with <20 mol % cholesterol likely coexist in the  $L_d$  and  $L_o$  phases, while cholesterol-rich SLBs with >30 mol % cholesterol are in the L<sub>o</sub> phase state.

Evaluation of Membrane Morphological Responses. After confirming the successful formation of DOCP/cholesterol SLBs, we next investigated how cholesterol fraction influences membrane morphological responses induced by SDS, LA, and GML. The test compounds were added to prefabricated SLBs under continuous flow conditions, and QCM-D measurements were performed in order to track the corresponding membrane morphological responses, as established in previous studies.<sup>24,25</sup> After SLB formation was complete, the QCM-D measurements were continued, and the measurement times were normalized such that t = 0 min corresponds to the time after SLB formation followed by a stabilization period and  $t = 5 \min$ indicates when compound addition to the SLB platform began under continuous flow conditions. Above their corresponding critical micelle concentration (CMC) values, the test compounds are known to induce membrane morphological responses, and we therefore selected appropriate concentrations of each compound for evaluation: 1 mM SDS, 2 mM LA, and 125  $\mu$ M GML. The QCM-D measurement responses are presented in Figures 2–4. Of note, at t = 0 min, the  $\Delta f$  and  $\Delta D$  shifts correspond to SLB formation ( $\Delta f_{\rm bilayer}$  and  $\Delta D_{\rm bilayer}$ ), and the corresponding net  $\Delta f$  and  $\Delta D$  shifts reported below are the  $\Delta f_{\rm measured} - \Delta f_{\rm bilayer}$  and  $\Delta D_{\rm measured} - \Delta D_{\rm bilayer}$  shifts, respectively. By reporting the net measurement shifts, it is possible to compare responses obtained on SLB platforms with different cholesterol fractions.

Sodium Dodecyl Sulfate. Figure 2 presents the effect of 1 mM SDS on the  $\Delta f$  and  $\Delta D$  shifts as a function of cholesterol fraction in the SLB platform. SDS addition to 100 mol % DOPC SLBs exhibited two-step interaction kinetics, and there was an initially rapid decrease in the  $\Delta f$  shift to around -35 Hz and an increase in the  $\Delta D$  shift to around  $6 \times 10^{-6}$ . These values corresponded to an inflection point that was followed by an increase in the  $\Delta f$  and  $\Delta D$  shifts to -2 Hz and  $0 \times 10^{-6}$ respectively (Figure 2A). After a buffer washing step, the final  $\Delta f$  and  $\Delta D$  shifts were -1 Hz and  $0 \times 10^{-6}$ , respectively, and these final measurement responses are equivalent to a bare silicon dioxide sensor surface in buffer solution, indicating essentially complete removal of adsorbed lipid molecules. The interaction kinetics and final measurement result are consistent with SDS intercalation causing membrane strain that leads to tubule formation,<sup>24</sup> resulting in membrane solubilization and SLB removal.

Interestingly, while cholesterol is known to increase membrane resistance to SDS-induced solubilization in suspended vesicle systems, similar two-step interaction kinetics were also observed for DOPC/cholesterol SLBs across all tested cases. In all cases, there was nearly complete membrane solubilization while the magnitude of the QCM-D measurement responses at the inflection point depended on the cholesterol fraction. For SLBs with 17 mol % cholesterol, the  $\Delta f$  and  $\Delta D$  shifts reached their inflection points around



**Figure 4.** Effect of GML treatment on mass and viscoelastic properties of cholesterol-rich SLBs. QCM-D measurements were performed, and representative  $\Delta f$  (blue squares) and  $\Delta D$  (red triangles) shifts are reported as a function of time for DOPC phospholipid/cholesterol SLBs with (A) 0, (B) 17, (C) 35, and (D) 52 mol % cholesterol. The initial measurement values correspond to fabricated SLB platforms on the sensor surface. 125  $\mu$ M GML was added starting at t = 5 min (arrow 1), and a buffer washing step was then performed after the measurement signals stabilized (arrow 2).

-45 Hz and 7 × 10<sup>-6</sup>, respectively (Figure 2B). Similarly, for SLBs with 35 and 52 mol % cholesterol, the  $\Delta f$  shifts at the inflection point were -50 and -54 Hz, respectively (Figure 2C,D). The corresponding  $\Delta D$  shifts were 10 × 10<sup>-6</sup> and 12 × 10<sup>-6</sup>, respectively.

Because the initial  $\Delta f_{\rm bilayer}$  and  $\Delta D_{\rm bilayer}$  shifts depended on the cholesterol fraction, we also calculated the net  $\Delta f$  and  $\Delta D$ shifts at the inflection point for direct comparison across the different SLB platforms. The net  $\Delta f$  shifts were -9, -15, -19, and -21 Hz for 0, 17, 35, and 52 mol % cholesterol SLBs, respectively. The corresponding net  $\Delta D$  shifts were  $6 \times 10^{-6}$ ,  $7 \times 10^{-6}$ ,  $9 \times 10^{-6}$ , and  $11 \times 10^{-6}$ , respectively. These values indicate that the responses associated with tubule formation become larger with increasing cholesterol fraction in the SLB platform.

Lauric Acid. Figure 3 presents the effect of 2 mM LA on the  $\Delta f$  and  $\Delta D$  shifts as a function of cholesterol fraction in the SLB platform. As with SDS, LA addition to 100 mol % DOPC SLBs exhibited more subtle two-step interaction kinetics that also indicate tubule formation.<sup>24</sup> Upon LA addition, the  $\Delta f$  shift initially decreased to around -40 Hz with a concomitant increase in the  $\Delta D$  shift to around  $4 \times 10^{-6}$  (Figure 3A). Then, the  $\Delta f$  shift began to increase until reaching around -35 Hz while the  $\Delta D$  shift further increased to  $6 \times 10^{-6}$ . After a buffer washing step, the final  $\Delta f$  and  $\Delta D$  shifts were around -19 Hz and  $2 \times 10^{-6}$ , respectively. These values indicate that adsorbed lipid mass remained on the sensor surface, supporting that LA induces membrane morphological responses without solubilizing the DOPC lipid bilayer.

With increasing cholesterol fraction, the interaction process exhibited more complex behavior and greater membrane solubilization was observed. For SLBs with 17 mol % cholesterol, the  $\Delta f$  and  $\Delta D$  shifts reached their inflection points around -42 Hz, and the corresponding  $\Delta D$  shift was

around  $5 \times 10^{-6}$  before there was a suddenly sharp increase in the  $\Delta D$  shift to around 8 × 10<sup>-6</sup> (Figure 3B). After a buffer washing step, the final  $\Delta f$  and  $\Delta D$  shifts stabilized around -22 Hz and 3  $\times$  10<sup>-6</sup>, respectively. For SLBs with 35 mol % cholesterol, similar interaction kinetics were observed although the magnitude of the  $\Delta D$  shift was more pronounced, reaching a maximum  $\Delta D$  shift of  $11 \times 10^{-6}$  (Figure 3C). On the other hand, for SLBs with 52 mol % cholesterol, the  $\Delta f$  shift reached its inflection point around -49 Hz before increasing up to around -22 Hz while the  $\Delta D$  shift quickly rose and stabilized at around  $14 \times 10^{-6}$  throughout the interaction process (Figure 3D). Interestingly, upon buffer washing, the  $\Delta f$  shift rose to around -8 Hz and the  $\Delta D$  shift decreased to around  $3 \times 10^{-6}$ . These measurement values indicate that LA induced the greatest extent of membrane solubilization for highly cholesterol-rich SLBs, suggesting that the stiffer membrane structures are more prone to destabilization.

For direct comparison, we also report the net  $\Delta f$  and  $\Delta D$  shifts at the inflection point for the different SLB platforms. The net  $\Delta f$  shifts were -14, -14, -16, and -19 Hz for 0, 17, 35, and 52 mol % cholesterol SLBs, respectively. The corresponding net  $\Delta D$  shifts were 6, 8, 10, and  $13 \times 10^{-6}$ , respectively. These values indicate that the measurement responses associated with tubule formation become larger with increasing cholesterol fraction in the SLB platform. This trend is particularly pronounced in the  $\Delta D$  shift response and mirrors the trends observed in the SDS case. As both SDS and LA are anionic molecules that induce tubule formation, the results suggest that the cholesterol fraction influences the morphological response to heightened membrane strain, likely stemming from how cholesterol affects the viscoelastic properties of lipid membranes.

Glycerol Monolaurate. Figure 4 presents the effect of 125  $\mu$ M GML on the  $\Delta f$  and  $\Delta D$  shifts as a function of



Figure 5. Trend in QCM-D measurement shifts for membrane remodeling behavior induced by different test compounds. Maximum  $\Delta f$  and  $\Delta D$  shifts upon SDS, LA, or GML treatment are presented as a function of cholesterol fraction in the DOPC phospholipid/cholesterol SLB platforms. The net  $\Delta f$  and  $\Delta D$  shifts are reported as  $\Delta f_{\text{measured}} - \Delta f_{\text{bilayer}}$  and  $\Delta D_{\text{measured}} - \Delta D_{\text{bilayer}}$  shifts, respectively, in order to normalize the magnitude of the measurement shifts across the different SLB platforms. Mean and standard deviation (error bars) are reported from three or more technical replicates.

cholesterol fraction in the SLB platform. GML addition to 100 mol % DOPC SLBs led to a gradual decrease in the  $\Delta f$  shift down to -112 Hz with a corresponding increase in the  $\Delta D$ shift to  $20 \times 10^{-6}$  (Figure 4A). These values indicate that GML induces bud formation<sup>24</sup> and relates to the nonionic character of GML and its increased rate of membrane translocation across the two bilayer leaflets. After a buffer washing step, the final  $\Delta f$  and  $\Delta D$  shifts were around -25 Hz and 1  $\times$  10<sup>-6</sup>, respectively, further indicating that GML does not cause membrane solubilization. Similar measurement trends were observed for cholesterol-rich SLBs, with marked decreases in the magnitudes of the  $\Delta f$  and  $\Delta D$  shifts decreased at higher cholesterol fractions. For SLBs with 17 and 35 mol % cholesterol, the maximum  $\Delta f$  shifts were around -98 and -90 Hz, respectively, and the corresponding  $\Delta D$  shifts were around 14 and  $11 \times 10^{-6}$ , respectively (Figure 4B,C). On the other hand, for SLBs with 52 mol % cholesterol, the maximum  $\Delta f$  shift was similar, but the  $\Delta D$  shift was only  $8 \times 10^{-6}$  (Figure 4D). In all cases, the final responses after the buffer washing step were roughly equivalent to the values obtained for SLBs, indicating that GML causes less membrane disruption to cholesterol-rich SLBs than SDS and LA.

As such, the net  $\Delta f$  shifts were -87, -69, -61, and -60 Hz for 0, 17, 35, and 52 mol % cholesterol SLBs, respectively. Likewise, the corresponding net  $\Delta D$  shifts were 20, 14, 11, and  $7 \times 10^{-6}$  for 0, 17, 35, and 52 mol % cholesterol SLBs, respectively. This trend is opposite to that observed upon SDS and LA treatment in which cases the maximum  $\Delta D$  shift increased for SLBs with greater cholesterol fractions. Hence, SDS and LA vs GML had distinct effects on the membrane morphological response, including how cholesterol fraction influences the measured extent of remodeling behavior as well as the corresponding effects on membrane disruption as assessed post-treatment.

# Influence of Cholesterol on Membrane Remodeling.

The aforementioned results establish that the specific membrane morphological changes triggered by addition of SDS, LA, or GML to a SLB platform occur largely independent of the cholesterol fraction. This finding supports the general importance for lipid membranes to regulate stress passively through membrane remodeling, and in the case of confined, two-dimensional SLBs, such conditions give rise to membrane protrusions with distinct three-dimensional geometries.<sup>49,50</sup> Indeed, the insertion of single-chain lipid amphiphiles and related compounds induces membrane strain (deformation) in SLBs, leading to nucleation events that promote membrane budding (via caps) or formation of elongated tubule structures.<sup>24,25</sup> Nonionic compounds such as GML induce relatively low membrane strain leading to bud formation, while anionic compounds such as SDS and LA induce larger membrane strain that causes tubule formation.<sup>24</sup> A key difference between these two cases is that nonionic GML molecules can quickly translocate across the two leaflets and equilibrate, whereas anionic molecules such as SDS and LA have slower rates of translocation, and hence there is a concentration disequilibrium across the two leaflets, which contributes to larger membrane strains.<sup>9</sup> For all tested cholesterol fractions, the QCM-D data are consistent with these expected behaviors, with buds causing large shifts in the frequency and energy dissipation responses and tubules causing relatively small shifts in the frequency response and a large shift in the energy dissipation response. In both scenarios, the measurement responses arise from the formation of threedimensional structures (buds or tubes) protruding from the surface, and larger measurement responses correlate with more significant morphological changes and membrane remodeling.

What is striking about the experimental data is that the effect of cholesterol fraction on the membrane morphological responses appears to depend on which particular single-chain lipid amphiphile was added. As presented in Figure 5, the maximum QCM-D measurement responses increased as the cholesterol fraction became greater for the SDS and LA cases, while the maximum QCM-D measurement responses decreased as the cholesterol fraction became greater for the GML case. In turn, this finding indicates that the extent of tubule formation was greater for SLBs with higher cholesterol fractions while the extent of membrane budding due to GML insertion decreased accordingly. In other words, the extent of membrane remodeling depends not only on the cholesterol fraction but also which compound was added to the SLB platform.

To understand the physicochemical basis for this difference, let us consider the ways in which cholesterol affects lipid membrane properties. First, the addition of cholesterol is known to increase membrane stiffness in line with classical theories of elastic membranes.<sup>51,52</sup> The two main contributing factors to the elastic energy of a curved lipid bilayer are the packing of individual lipid molecules (denoted by the spontaneous curvature) and the density disequilibrium of lipid molecules in the two leaflets arising from compression and expansion in the negatively and positively curved leaflets, respectively.<sup>53,54</sup> Of note, equilibration of phospholipid densities across the two leaflets is slow because the energy barrier for phospholipid flip-flop is high. Treating a lipid bilayer as a purely elastic material, it would therefore be predicted that a cholesterol-rich bilayer would resist deformation more than a cholesterol-free bilayer. However, experimentally, it has been observed that cholesterol-rich membranes undergo greater deformation than pure phospholipid membranes.<sup>55,56</sup> Such behavior has been explained by Szostak and colleagues by noting the propensity of cholesterol to flip-flop across the bilayer leaflets and aid stress relaxation by helping to equilibrate membrane density across the two leaflets.<sup>57</sup> As a result, cholesterol-rich membranes can deform to a greater extent than pure phospholipid membranes in response to stress, with transbilayer redistribution of cholesterol molecules helping to relax tension. This flip-flop behavior introduces a viscous component to phospholipid/cholesterol membranes, and deformation of complex, cholesterol-rich membranes exhibits a viscoelastic response<sup>58</sup> that is influenced by the interplay of elastic (membrane stiffness) and viscous (cholesterol flip-flop) properties.

As mentioned above, the extent of membrane remodeling as a function of cholesterol fraction in the SLB depended on the particular compound triggering the membrane morphological response, and these differential trends can be understood by taking into account the physicochemical properties of each compound and how they relate to the elastic and viscous properties of the phospholipid/cholesterol SLB platform. For the GML case, the extent of membrane remodeling decreased with increasing cholesterol fraction, and this trend agrees with classical theories of membrane elasticity whereby deformation would decrease with increasing membrane stiffness (greater cholesterol fraction). This trend is consistent with the fact that GML can rapidly translocate across the bilayer leaflets, and hence cholesterol flip-flop is likely a less important factor for stress relaxation. As such, the membrane morphological responses to GML addition appear to depend more strongly on the elastic properties of the SLB platform. By contrast, for the SDS and LA cases, the extent of membrane remodeling increased with increasing cholesterol fraction, and this observation supports that the viscous properties of the SLB

platform are the main contributing factor to influence the membrane morphological responses in these two cases. Indeed, there is a high energy barrier for anionic SDS and LA molecules to translocate across bilayer leaflets, giving rise to asymmetric strain, and cholesterol flip-flop would therefore be an important factor in relaxing membrane tension. Taken together, the manner in which cholesterol affects membrane remodeling processes arising from SDS, LA, and GML addition to SLBs can be understood by taking into account how cholesterol influences the material properties of complex lipid bilayers as well as the physicochemical properties of the added compounds, especially molecular charge.

Looking forward, it would also be interesting to explore the molecular composition of buds and tubules in order to determine if cholesterol or phospholipid molecules preferentially self-assemble together with single-chain lipid amphiphiles in these morphological structures or rather a similar membrane composition to the precursor SLB platform is maintained. Such questions are especially pertinent to SLB platforms with intermediate cholesterol fractions ( $\sim$ 15–20 mol %), in which case phase coexistence occurs, and the pattern of membrane remodeling may vary across the SLB surface depending on the membrane phase state in a local region.

### CONCLUSIONS AND PROSPECTS

In this work, we have explored how cholesterol influences the membrane morphological responses that occur when singlechain lipid amphiphiles are added to SLB platforms. By employing the SALB method, it was possible to fabricate SLB platforms with defined molar fractions of cholesterol across the fluid, liquid-disordered, and liquid-ordered phase states. It was discovered that the three tested compounds-SDS, LA, and GML-each induce specific and distinct membrane morphological responses in both pure phospholipid and phospholipid/ cholesterol SLBs, indicating that the formation of threedimensional protrusions is a generic mechanism to regulate stress in confined lipid bilayer systems. Interestingly, the presence of cholesterol strongly influenced the extent of membrane remodeling in a manner that depended on which compound was added. When SDS or LA was added to SLB platforms, elongated tubules formed and the extent of membrane remodeling was greater in SLBs with higher cholesterol fractions. In marked contrast, GML addition caused bud formation, and the extent of membrane remodeling in this case was lower in SLBs with higher cholesterol fractions. As such, cholesterol either promoted or inhibited membrane remodeling, and this multifaceted behavior could be understood by taking into account how cholesterol influences the elastic (stiffness) and viscous (stress relaxation) properties of phospholipid/cholesterol lipid bilayers as well as how the membrane translocation properties of different single-chain lipid amphiphiles vary. Looking forward, such insights advance our understanding of how cholesterol influences the viscoelastic properties of biological membranes and functions as a mediator of stress relaxation in lipid bilayers. In addition, our findings demonstrate that single-chain lipid amphiphiles such as free fatty acids and monoglycerides induce highly specific membrane morphological responses across both simplified and complex model membranes, motivating their continued exploration as antimicrobial candidates.

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#### Notes

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