

Miltefosine Impacts Small Molecule Transport in Gram-Positive Bacteria

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Abstract

Miltefosine (MLT) is an alkylphosphocholine with clinical success as an anticancer and antiparasitic drug. Although the mechanism of action of MLT is highly debated, the interaction of MLT with the membrane, specifically lipid rafts of eukaryotes, is well-documented. Recent reports suggest MLT impacts the functional membrane microdomains (FMMs) in bacteria - regions of the membrane structurally and functionally similar to lipid rafts. There have been conflicting reports, however, as to whether MLT impacts the overall fluidity of cellular plasma membranes. Here, we apply steady-state fluorescence techniques, generalized polarization of laurdan and anisotropy of diphenylhexatriene, to discern how MLT impacts the global fluidity of *Staphylococcus aureus* membranes. Additionally, we investigate how the transport of a range of small molecules is impacted by MLT for *S. aureus* and *Bacillus subtilis* by employing time-resolved second harmonic scattering. Overall, we observe MLT does not have an influence on the overall fluidity of *S. aureus* membranes. Additionally, we show that the transport of small molecules across the membrane can be significantly altered by MLT - although this is not the case for all molecules studied. The results presented here illustrate the potential use of MLT as an adjuvant to assist in the delivery of drug molecules in bacteria.

Introduction

Hexadecylphosphocholine, or Miltefosine (MLT), is a synthetic alkylphospholipid (APL) that was originally developed as an anticancer agent¹ and is currently in clinical use as an antiparasitic drug.² More recently, the antibacterial and antifungal activity of MLT has received attention.^{3–7} While only modest antibacterial activity has been observed, it has been suggested that MLT, and APLs more generally, could serve as a new framework for developing novel antibiotics or adjuvants.³ For the latter, it is imperative to investigate how the presence of MLT impacts small molecule transport through the membrane.

Despite decades of research, the mechanism of action of MLT remains a point of debate and discussion. While there is agreement that the plasma membrane is compromised with MLT treatment, it is not clear if this is achieved via lipid or protein targets. It has been shown that MLT specifically targets membranes composed of lipid raft constituents of eukaryotic and fungal species, such as cholesterol and ergosterol, respectively.^{4,8–12} As such, some have reported that this lipid raft association results in MLT impacting the cell membrane fluidity,^{13–16} while others report this is not the case.^{10,11} For instances when membrane fluidity was altered, there is evidence that supports the presence of proteins is required. Specifically, Alonso *et al.* demonstrated that stratum corneum membranes had a significant increase in membrane fluidity due to MLT but vesicles formed from the lipid extract of stratum corneum membranes had no change in fluidity.¹³ Additional mechanisms that have been proposed include miltefosine affecting the enzyme, cytochrome *c* oxidase,^{17,18} in eukaryotes and halting the cell's ability to synthesize phosphatidylcholine,^{8,19,20} both cases leading to cell death. Most MLT research, however, has focused on eukaryotic systems or model membranes containing lipid species that are less common or nonexistent in bacteria.

In bacteria, MLT has been shown to interrupt aggregation of flotillin in *Staphylococcus aureus* cell membranes.⁶ Flotillin are scaffold proteins that have been proposed as crucial components for the formation of functional membrane microdomains^{6,21} although inconsistent results remain.²² It is not clear, however, if such a change in protein behavior would

arise from direct MLT-protein interactions or, if instead, MLT alters the lipid environment preferred by the protein. In a recent paper, molecular dynamics simulations suggest that the lipids themselves are a major determinant in MLT passive transport into the membrane and may play a large role in its mechanism of action.¹⁰ When extending such questions to the potential use of MLT as an adjuvant species, it is necessary to consider how MLT's impact on the membrane alters small molecule transport through this important lipid boundary. In order to monitor transport dynamics, here, we implement time-resolved second harmonic scattering (trSHS).

SHS is a surface-specific spectroscopic technique based on second harmonic generation (SHG) originating from molecules adsorbed to surfaces in colloidal environments, such as that of living cell membranes.²³ SHG occurs when two photons of equal energy interact with a group of asymmetric molecules to produce light at the second harmonic, or at twice the energy of the incident photons. The emitted photons originating from an isotropic distribution of molecules, such as those in an aqueous solution, are incoherent leading to a weak, often insignificant signal. In contrast, once these molecules adsorb to a surface, the individual second harmonic signals originating from these molecules constructively interfere, producing appreciable SHS. The evolution of this signal over time can provide information on transport events of molecules within the membrane space. For instance, a decrease in SHS results from molecules becoming aligned in an anti-parallel fashion which is indicative of translocation from one leaflet into the other.^{24,25} Conversely, a rise in signal can be attributed to an overall greater alignment of the molecules to one another in the membrane,^{24,26} aggregation,^{27,28} or a change in the local chemical environment.^{29,30} Previous studies have demonstrated SHS and SHG can be used to quantify molecule-membrane interactions within model systems,^{31–40} eukaryotic species,^{16,41–44} and bacterial cells.^{24,25,45–56} Specifically, we have implemented this technique to elucidate how structural moieties of small molecules facilitate its transport through the Gram-positive membranes of living *S. aureus* and *Bacillus subtilis*.⁴⁷

After employing the steady-state fluorescent assays, generalized polarization (GP) of

laurdan and anisotropy of diphenylhexatriene (DPH), to determine the impact of MLT on global fluidity of *S. aureus* membranes, we monitor the trSHS of small molecules (malachite green,^{25,48,49,57–59} FM 2-10,^{46,47} FM 1-43,⁴⁷ and 4-Di-2-ASP⁴⁷) with previously documented transport behavior to assess the impact of MLT. We also consider the role of membrane composition by extending trSHS studies to another Gram-positive bacterial species, *B. subtilis*. Although we do not observe a change in the overall fluidity of *S. aureus* membrane, we do observe significant alterations in the interactions of multiple small molecule species with the lipid bilayer. These results are not universal, however, leading to a discussion of the localized impact of MLT on the membrane and the potential relationship to the small molecule structures that are affected.

Materials and Methods

Chemicals and Cell Treatment

Stock solutions of dye molecules FM 2-10 (Biotium), 4-Di-2-ASP (Sigma-Aldrich) and FM 1-43 (Biotium) were prepared in filter sterilized 80:20 Milli-Q water to DMSO to maintain a final DMSO concentration of 0.02% when introduced to cell solutions. Malachite green oxalate (Sigma-Aldrich) was prepared in sterile Milli-Q water. Stock solutions of laurdan (ThermoFisher Scientific), 1,6-Diphenyl-1,3,5-hexatriene (diphenylhexatriene, Sigma-Aldrich), and miltefosine (Sigma-Aldrich) were prepared in DMSO.

Single colonies of *Staphylococcus aureus* (ATCC27217) and *Bacillus subtilis* 168 grown on brain heart infusion (BHI) and Luria Bertani (LB) broth agar, respectively, were inoculated into liquid BHI and LB media, respectively. Cultures were grown anaerobically for *S. aureus* and aerobically at 250 RPM for *B. subtilis* overnight at 37°C. Aliquots were then resuspended in fresh liquid media to an optical density at 600 nm (OD_{600}) of 0.01 and allowed to grow to an OD_{600} of 0.2 at the same conditions. Cell samples were then filtered by centrifugation or vacuum filtration and washed twice with phosphate buffered saline (PBS). For trSHS

experiments, cells were resuspended in their respective media supplemented with 0.2% EC-OxyRase. For steady state fluorescence experiments, cells were resuspended in PBS. Final cell suspensions were measured to be at an OD_{600} of 0.2. MLT was incubated with cells at room temperature for a minimum of 10 minutes before experimentally probed.

Time-Resolved Second Harmonic Scattering

TrSHS experiments were performed using home-built instruments previously described in detail elsewhere.⁴⁶ Briefly, the output of a Ti:sapphire oscillator (80 MHz) was compressed to <100 fs and focused into a 2 mm quartz flow cell (Starna) that housed the cell samples. The resulting signals were then collected with a series of lenses and directed towards a photomultiplier tube (PMT) for detection. Filters were implemented to block any second harmonic (400 nm) produced by optics before the sample housing and fundamental light (800 nm) after the sample. In addition to SHS, two-photon fluorescence (TPF) was detected simultaneously by using a 450 longpass dichroic mirror to direct the TPF signals to second PMT. The second harmonic responses were selected using a 400/10 bandpass filter before the PMT. TPF responses were collected using a 625/50 bandpass filter. The power sent to the sample was controlled using a half-waveplate and polarizer. For FM 2-10 and 4-Di-2-ASP, the power was set to 35 mW and for experiments with MG and FM 1-43, the power was set to 100 mW. These experiments were accomplished using a gravity flow peristaltic pump at a rate of <7 mL per minute.

Immediately prior to trSHS experiments, the quartz flow cell was passivated to ensure there was no cell adsorption to the surface during the course of the trials. First, the sample chamber was sterilized with 1 M hydrochloric acid, then rinsed with a copious amount of sterilized Milli-Q water. Then, we used an established bovine serum albumin (BSA)/glutaraldehyde cross-linking procedure, previously described.⁶⁰ In short, 50 mM BSA (Sigma-Aldrich) was incubated for 10 minutes, rinsed with 10 mM TRIS buffer (corrected to pH 7.4) (Sigma-Aldrich), and then replaced with another 10 minute incubation of a 1% glu-

taraldehyde (Thermo-Fisher Scientific) solution. Finally, the flow cell was rinsed with the TRIS buffer. All solutions were sterilized before performing the cross-linking procedure.

Generalized Polarization Measurements

Membrane fluidity was assessed using generalized polarization (GP) measurements of laurdan. Laurdan is a fluorescent molecule that shifts its emission spectra due to the polarity of the surrounding environments. For instance, for environments with low polarity such as that of rigid regions of the membrane, the emission maximum of laurdan is 440 nm. When laurdan is in an environment of high polarity (e.g. a fluid membrane), laurdan has an emission maximum shifted to 490 nm. The ratio of the emission intensities at 440 nm and 490 nm, or GP, can be calculated to quantify changes in membrane fluidity according to Equation 1,

$$GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}} \quad (1)$$

where I_{440} and I_{490} are the fluorescence intensities at 440 nm and 490 nm, respectively. For GP measurements, after cells were treated with MLT as outlined above, samples were washed by centrifugation, resuspended in PBS, and then incubated with 0.5 μ M laurdan at 37°C for $\sim$ 30 minutes in the dark. Measurements were then taken with a Perkin Elmer LS 55 fluorometer with 350 nm excitation at room temperature.

Steady State Fluorescence Anisotropy

Another measure of fluidity used here is the steady state fluorescence anisotropy of diphenyl-hexatriene (DPH). DPH preferentially localizes in the hydrophobic region of the membrane. As such, the orientation and rotational freedom of the DPH molecules in this region directly reflects the fluidity of the environment it is residing in. This can be quantified by calculating the anisotropy, $\langle r \rangle$, of DPH:

$$\langle r \rangle = \frac{I_{VV} - I_{VH}G}{I_{VV} + 2I_{VH}G} \quad (2)$$

$$G = \frac{I_{HV}}{I_{HH}} \quad (3)$$

where I is the fluorescence intensity at different excitation and emission polarizer combinations, denoted by the subscripts, respectively, where V is vertical and H is horizontal, and G is a correction factor to account for detector sensitivity.

Anisotropy measurements were recorded with an Agilent Cary Eclipse fluorometer. After cells were treated with MLT, cells were resuspended in PBS and incubated with 10 μ M DPH for $\sim$ 45 minutes at 37°C. Samples were excited at 350 nm and emission was collected at 430 nm. Measurements were taken at room temperature.

Results and Discussion

MLT Impact on Global Membrane Fluidity

While there has been debate in the literature regarding the impact MLT has on the fluidity of the membranes it interacts with,^{10,11,13–16} no such studies have been performed with bacteria. Therefore, we first sought to investigate whether the presence of MLT results in a more fluid membrane environment in *S. aureus*. In order to do so, we employ two steady state fluorescence techniques: GP of laurdan and steady-state anisotropy of DPH. Both laurdan and DPH are fluorescent molecules commonly used as probes to assess membrane fluidity.^{61,62} As the laurdan chromophore is polar, it reports on the presence of water near the junction between the lipid headgroup and tail with a higher GP value associated with a decrease in the membrane fluidity.^{61,63} The nonpolar DPH molecule is expected to embed more deeply within the hydrophobic portion of the membrane and a lower DPH anisotropy value indicates higher membrane fluidity.^{63,64} While many studies find similar trends between these different

probe assays,⁶² occasional differences are noted.⁶⁴

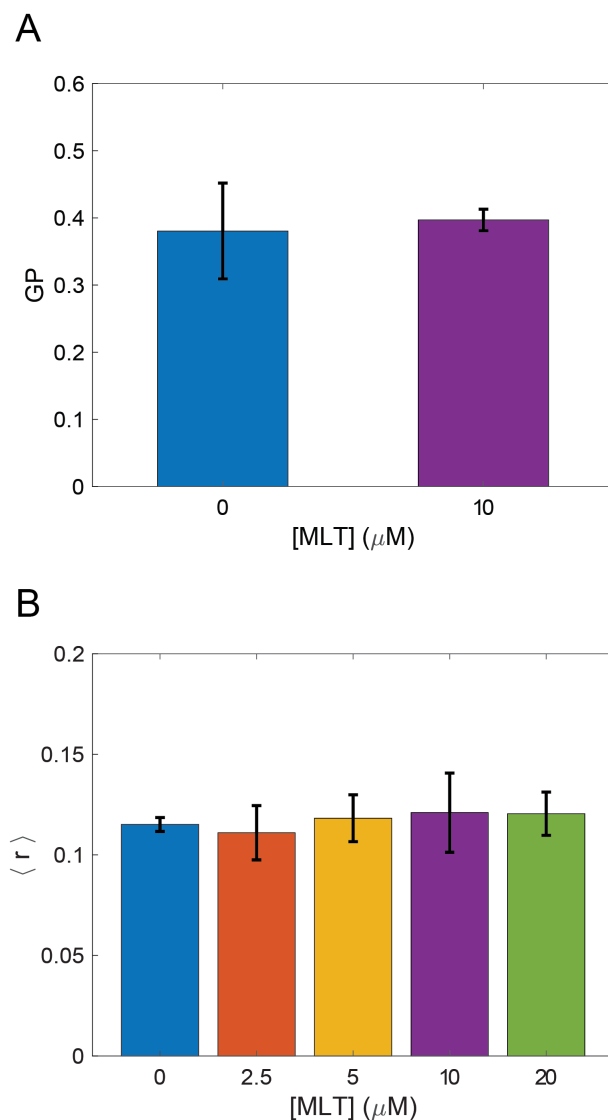


Figure 1: Steady state fluorescence measurements of fluidity with (A) laurdan and (B) DPH. (A) Average GP values of laurdan for *S. aureus* with 0 μ M MLT (blue) and 10 μ M MLT (purple). (B) Average anisotropy ($\langle r \rangle$) of DPH with *S. aureus* is shown as a function of MLT concentration. For each concentration of MLT, $n = 3$.

Figure 1A shows the GP values for laurdan in *S. aureus* membranes either with or without a MLT incubation prior to the measurement. For the cells not exposed to MLT, the GP value obtained is 0.381 ± 0.07 which is consistent with values others have reported for exponential

phase *S. aureus* at room temperature.^{65,66} In comparison to the cells exposed to MLT, there is no significant difference. This suggests that there is no overall increase in the fluidity of the *S. aureus* membrane due to the presence of MLT. The anisotropy of DPH adds further support to this conclusion. As can be seen in Fig. 1B, the DPH anisotropy values are found to be 0.115 ± 0.003 which is also consistent with previous literature values⁶⁷ and remains unchanged over multiple MLT concentrations.

Small Molecule Organization and Transport

For consideration of MLT as an adjuvant to treat bacterial infections, understanding how MLT affects the interaction of small molecules with the membrane, and moreover their transport through it, is crucial. Four dye molecules with different established membrane interactions are studied here: MG, FM 2-10, 4-Di-2-ASP, and FM 1-43. MG is a small, positively charged molecule that has been extensively characterized with model liposomes^{33,37,38,68} as well as *Escherichia coli*^{25,48,57–59} and eukaryotic cells.⁴³ In all previous work, MG has exhibited an SHS response consistent with a relatively rapid passive diffusion through lipid bilayers. Further, we have previously reported on the bacterial membrane interactions of the three styryl molecules listed where slight variances in their structures result in differences in their affinity for and translocation within the membranes of *S. aureus*.^{46,47} For instance, we have proposed that for molecules with short hydrophobic tails and small headgroups (FM 2-10 and 4-Di-2-ASP, respectively), the molecules have a greater propensity to remain in the outer leaflet of the membrane. After ~ 1 hour, however, the molecules produce a rise in SHS signal indicative of further organization within the membrane.^{46,47} Further, we demonstrated that the introduction of molecules with larger branched tails (FM 1-43) result in a rapid decrease in SHS. This response is due to the molecules moving from the outer leaflet to the inner leaflet of the membrane.⁴⁷

Immediate alterations to small molecule transport induced by MLT can be seen by examining the behavior of MG. Figure 2 shows that at 0 μ M MLT, the molecules rapidly diffuse

through the hydrophobic portion of the membrane in agreement with MG SHS studies with both liposomal membranes^{33,37,38,68} as well as *E. coli* cells.^{25,48,57–59} After *S. aureus* cells are treated with 12 μM MLT (Fig. 2), the trSHS decreases more rapidly compared to untreated cells. These results indicate that MG may be experiencing a more fluid membrane environment to mediate quicker passive diffusion across the lipid bilayer.⁶⁸

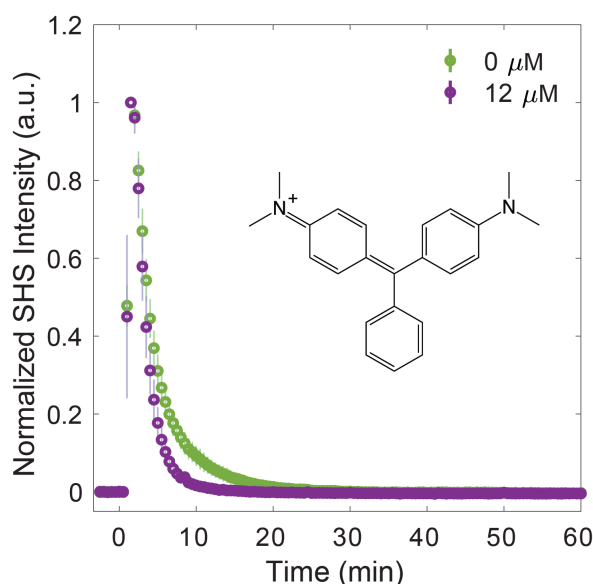


Figure 2: Normalized trSHS trials of *S. aureus* with 25 μM MG with 0 μM MLT (green) and 12 μM MLT (purple). The structure of MG is shown in the plot. For each condition, $n = 3$. Averaged data is shown as circles and the shaded error bars represent the standard deviation between the individual normalized trials.

More complex dynamics are observed when moving to the styryl dye molecules which have a stronger association with the membrane environment. Past studies with FM 2-10 and 4-Di-2-ASP hypothesized that the molecule undergoes organization in the membrane of *S. aureus*. This hypothesis arises from the fact that the SHS signal in these samples rises over time and can be understood by examining the factors that contribute to the intensity of the SHS signal (I_{SHS}). Specifically, $I_{SHS} = |\chi_{eff}^{(2)} E_{\omega} E_{\omega}|^2$ where E_{ω} is the incoming electric

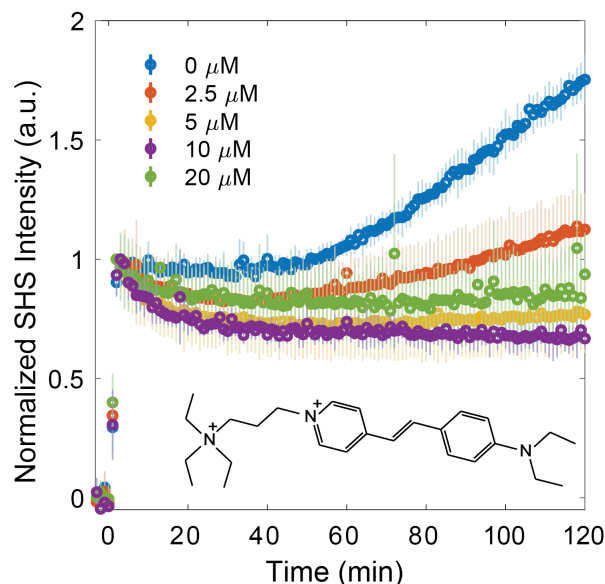


Figure 3: Normalized trSHS trials of 16 μM FM 2-10 with *S. aureus* with increasing concentrations of MLT. The structure of FM 2-10 is shown in the plot. For each concentration of MLT, $n = 3$. Averaged data is shown as circles and the shaded error bars represent the standard deviation between the individual normalized trials.

field and $\chi_{eff}^{(2)}$ is the second order effective susceptibility which can be further decomposed to $\chi_{eff}^{(2)} \propto N_s \langle \beta \rangle$. Here, N_s is the population of probe molecules in the membrane while $\langle \beta \rangle$ is the orientational average of the hyperpolarizability. As we have previously determined, the population of cells throughout the duration of our measurements is static and subsequently there is no increase in membrane probe population,⁴⁶ therefore the SHS rise is attributed to $\langle \beta \rangle$. This term can increase through a change in the probe molecule's environment either due to differences in solvation or an overall increase in molecular alignment due to environmental rigidity.

Since MLT has been shown to disrupt membrane regions of increased rigidity,⁸ the membrane interactions of FM 2-10 and 4-Di-2-ASP were studied here. As can be seen in Figure 3, when no MLT is present, the SHS signal is relatively static over the first 40 minutes but then

nearly doubles after two hours. This behavior, and especially the rise, are clearly altered by MLT. As the concentration of MLT increases from 0 to 20 μM , the SHS rise is diminished and eventually lost. A reduced SHS rise is also observed for 4-Di-2-ASP after cells are treated with 10 μM MLT (Figure 4).

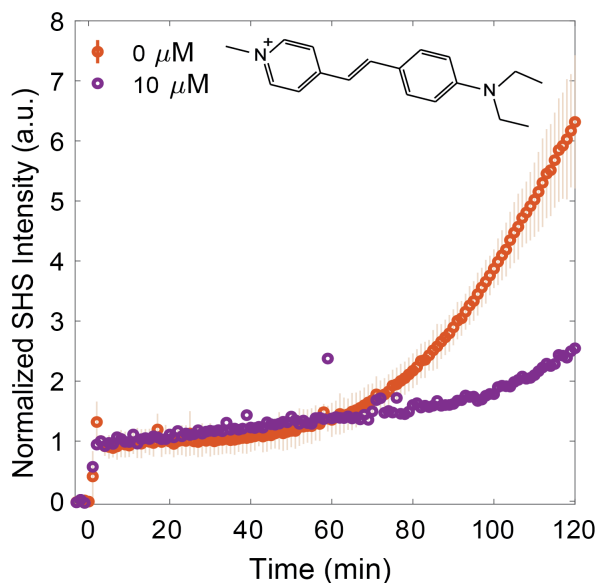


Figure 4: Normalized trSHS trials of *S. aureus* with 16 μM 4-Di-2-ASP with 0 μM MLT (orange) and 10 μM MLT (purple). The structure of 4-Di-2-ASP is shown in the plot. For 0 μM MLT, $n = 3$ and the shaded error bars represent the standard deviation between the individual normalized trials; for 10 μM MLT, $n=1$.

Another change induced by MLT is also observed within the first 20 minutes. Whereas the SHS signal of FM 2-10 with no MLT present displays a static signal, cells treated with MLT exhibit an SHS decay after the initial adsorption of FM 2-10. Such a decrease is most often interpreted as a movement of some population of the probe molecule to the inner leaflet.^{24,25,48} Alternatively, the same way a more rigid environment would induce a rising SHS signal, a more fluid environment can cause a reduction as the alignment of the molecules is decreased. More support for this argument can be seen by examining the trends

in SHS intensity values. While Fig. 3 shows the time-resolved SHS signals normalized to the initial absorption peak to better differentiate the dynamic changes, Fig. 5 compares the initial SHS intensity after adsorption to that of the two-photon fluorescence signal taken at the same time. As FM 2-10 only exhibits significant fluorescence when embedded in the membrane environment, monitoring the fluorescence signal allows us to separately monitor the population contribution to the SHS signal. As can be seen in Fig. 5, the fluorescence intensity for different MLT concentrations remains unchanged, suggesting that the same number of probe molecules are adsorbed onto the membrane. In contrast, the SHS intensity shows an overall decreasing trend with increasing MLT concentration suggesting that $\langle\beta\rangle$ is decreasing over these conditions. Taken together, these data suggest that FM 2-10 does not experience a more rigid environment over time and that, in fact, it's initial lipid environment may be more fluid, potentially promoting translocation to the inner leaflet.

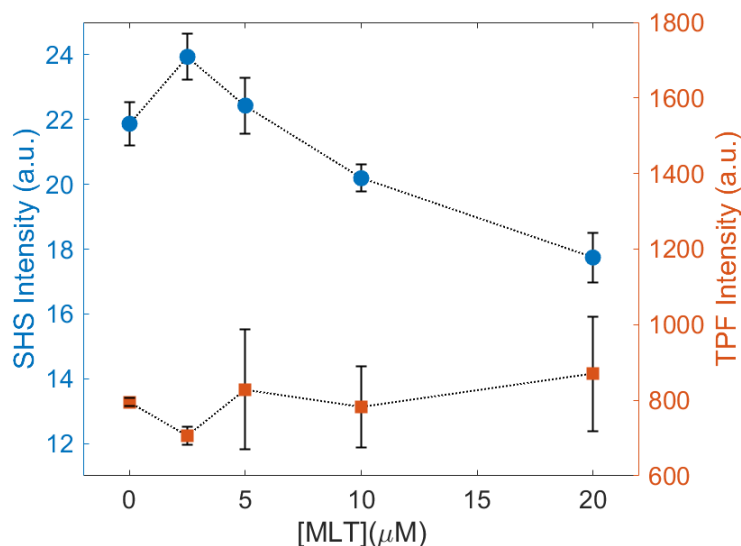


Figure 5: Initial intensities of trSHS (circles, left axis) and TPF (squares, right axis) signals of *S. aureus* with 16 μ M FM 2-10.

We have previously shown that the membrane composition itself plays a large role in how small molecules trespass the bacterial surface.⁴⁷ Specifically, we observed that for FM 2-10 and 4-Di-2-ASP, where a rise over the two hour time period is seen with *S. aureus*, a slight rise is followed with a decrease in SHS intensity over time for *B. subtilis*. This discrepancy was

proposed to be attributed to the generally more fluid membrane of *B. subtilis*, which in turn facilitated translocation events, whereas *S. aureus* mediated molecule organization in the outer leaflet over the same time period.⁴⁷ The fact these membranes facilitate starkly different transport properties of FM 2-10 allows an opportunity to determine how translatable MLT's impact is for different species. Figure 6 shows that when MLT is introduced to *B. subtilis*, FM 2-10 produces an immediate decrease in SHS intensity after initial adsorption, similar, albeit more rapid, to what was witnessed with *S. aureus* (Fig. 3). Following this first translocation event, the SHS intensity displays a slight increase over time and then remains relatively stagnant for the duration of the experiment.

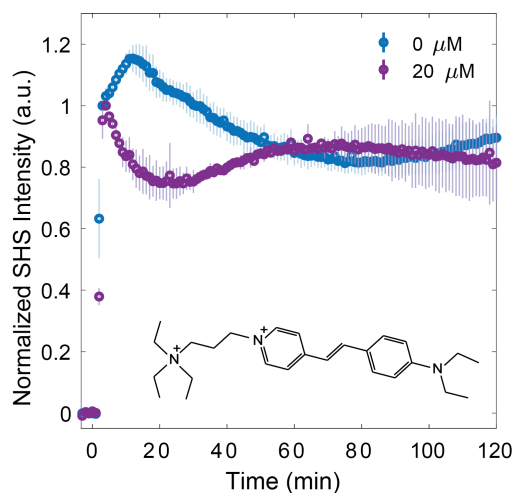


Figure 6: Normalized trSHS trials of *B. subtilis* with 16 μM FM 2-10 with 0 μM MLT (blue) and 20 μM MLT (purple). The structure of FM 2-10 is shown in the plot. For each condition, $n = 3$. Averaged data is shown as circles and the shaded error bars represent the standard deviation between the individual normalized trials.

Although the two cell species have different FM 2-10 transport in the absence of MLT, the final result after MLT incubation is very similar. This could be due to MLT having the same mechanism of action for both cell types. While *S. aureus* and *B. subtilis* have differences in their overall phospholipid membrane composition,⁶⁹ they do have similarities that could

explain the analogous MLT results. For instance, both membranes have been shown to be equipped with flotillin scaffold proteins^{6,21,70} - proteins that are largely conserved across different domains of life and hypothesized to play a role in MLT's mechanism of action for bacteria.^{6,71} Additionally, both membrane species are known to moderate their membrane fluidity via isoprenoid-derived minor lipid species.⁷²⁻⁷⁴ For instance, *S. aureus* produces carotenoids, such as staphyloxanthin, and *B. subtilis* synthesizes hopanoids.^{75,76} Hopanoids in particular are structurally and functionally similar to that of ergosterol and cholesterol in eukaryotes.⁷⁷⁻⁷⁹ Subsequently, both of these sterols in eukaryotes have been shown to be important constituents for MLT activity.^{4,8-12} Therefore, flotillin proteins and/or isoprenoid-derived molecules could be potential targets for MLT in the bacterial species studied here.

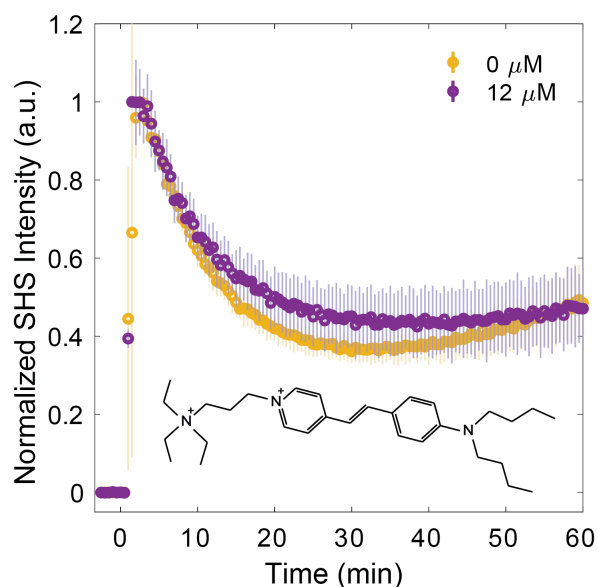


Figure 7: Normalized trSHS trials of *S. aureus* with 16 μM FM 1-43 with 0 μM MLT (yellow) and 12 μM MLT (purple). The structure of FM 1-43 is shown in the plot. For each condition, $n = 3$. Averaged data is shown as circles and the shaded error bars represent the standard deviation between the individual normalized trials.

When attempting to understand how MLT does not affect laurdan and DPH behavior

but does affect the behavior of MG, FM 2-10 and 4-Di-2-ASP, one may be quick to note the neutral forms of the former group of molecules vs. the ionic forms of the latter. This justification, however, is refuted by examining FM 1-43. Unlike the other three dyes measured with trSHS, FM 1-43 transport is unaffected by the presence of MLT. As can be seen in Fig. 7, after initial adsorption, the SHS signal from FM 1-43 decreases over 30 minutes before stabilizing. The signal from the MLT-affected cells exhibits the same behavior within the error of the measurement.

We propose two possible explanations for the differences in MLT's impact for these five different probe molecules. First, MLT may induce a more local effect to specific regions, *i. e.* domains, within the membrane that is limiting in its impact to only those molecules that are strongly associated with the same domains. This local impact is what has been proposed before for its behavior.^{6,8} The novelty of our measurements is then the suggestion that MG, FM 2-10 and 4-Di-2-ASP have a preferential association with such domains. Previous work has shown that both laurdan^{63,80} and DPH⁶⁴ can partition into multiple different lipid phases promoting a more homogeneous distribution within the membrane. In contrast, styryl dyes have been seen to heterogeneously localize within bacterial membranes⁸¹ although this is not always the case.⁸² The heterogeneous distribution has been attributed to the cationic charge on the styryl dyes interacting with the negatively charged phosphatidylglycerol (PG) lipid headgroup.⁸¹ It is important to note, however, that such heterogeneous behavior was seen explicitly for FM 1-43 which we find to be unaffected by MLT.

As a second, not necessarily unrelated, explanation for the differing behavior, we consider the hydrophobic character of the probe molecules. As noted previously, both laurdan and DPH are neutral molecules predominantly interacting with lipids below the headgroup. Beyond those two molecules, the only other one not impacted by MLT was FM 1-43. In comparison to MG, FM 2-10 and 4-Di-2-ASP, FM 1-43 contains the largest hydrophilic moiety. As such it may be that those molecules with stronger interactions with the lipid tails and hydrophobic core of the membrane are less susceptible to MLT alterations.

Conclusions

The results of our trSHS experiments show that MLT does hold promise as an adjuvant species for small molecule antibiotics targeting Gram-positive bacteria. Our steady-state fluorescence measurements with laurdan and DPH show no evidence of a large-scale increase in the membranes of *S. aureus* cells induced by MLT. This result supports previous work by others^{10,11} but extends this finding to bacteria. In contrast, multiple small molecules exhibit altered interactions with the membranes after exposure to MLT. These alterations led to an overall higher propensity for the molecules to more efficiently traverse the lipid bilayer. While the lack of impact on FM 1-43 dynamics may seem limiting for MLT's use, it could alternatively be viewed as promising for future selectivity. Work remains, however, to better understand MLT's relationship with different small molecules and the mechanism by which MLT impacts bacterial membranes overall.

References

- (1) Eibl, H.; Unger, C. Hexadecylphosphocholine: a new and selective antitumor drug. *Cancer Treatment Reviews* **1990**, *17*, 233–242.
- (2) Dorlo, T. P. C.; Balasegaram, M.; Beijnen, J. H.; de Vries, P. J. Miltefosine: a review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis. *Journal of Antimicrobial Chemotherapy* **2012**, *67*, 2576–2597.
- (3) Obando, D.; Widmer, F.; Wright, L. C.; Sorrell, T. C.; Jolliffe, K. A. Synthesis, antifungal and antimicrobial activity of alkylphospholipids. *Bioorganic & Medicinal Chemistry* **2007**, *15*, 5158–5165.
- (4) Wu, Y.; Wu, M.; Gao, J.; Ying, C. Antifungal Activity and Mode of Action of Miltefosine Against Clinical Isolates of *Candida krusei*. *Frontiers in Microbiology* **2020**, *11*, 854.

- (5) Margaritova Zaharieva, M.; Dimitrov Kroumov, A.; Dimitrova, L.; Tsvetkova, I.; Trochopoulos, A.; Mihaylov Konstantinov, S.; Reinhold Berger, M.; Momchilova, M.; Yoncheva, K.; Miladinov Najdenski, H. Micellar curcumin improves the antibacterial activity of the alkylphosphocholines erufosine and miltefosine against pathogenic *Staphylococcus aureus* strains. *Biotechnology & Biotechnological Equipment* **2019**, *33*, 38–53, Publisher: Taylor & Francis eprint: <https://doi.org/10.1080/13102818.2018.1533792>.
- (6) Koch, G.; Wermser, C.; Acosta, I. C.; Kricks, L.; Stengel, S. T.; Yepes, A.; Lopez, D. Attenuating *Staphylococcus aureus* Virulence by Targeting Flotillin Protein Scaffold Activity. *Cell Chemical Biology* **2017**, *24*, 845–857.e6.
- (7) Llull, D.; Rivas, L.; García, E. In Vitro Bactericidal Activity of the Antiprotozoal Drug Miltefosine against *Streptococcus pneumoniae* and Other Pathogenic Streptococci. *Antimicrobial Agents and Chemotherapy* **2007**, *51*, 1844–1848, Publisher: American Society for Microbiology.
- (8) Luit, A. H. v. d.; Budde, M.; Ruurs, P.; Verheij, M.; Blitterswijk, W. J. v. Alkyllysophospholipid Accumulates in Lipid Rafts and Induces Apoptosis via Raft-dependent Endocytosis and Inhibition of Phosphatidylcholine Synthesis *. *Journal of Biological Chemistry* **2002**, *277*, 39541–39547, Publisher: Elsevier.
- (9) Zulueta Díaz, Y. d. l. M.; Fanani, M. L. Crossregulation between the insertion of Hexadecylphosphocholine (miltefosine) into lipid membranes and their rheology and lateral structure. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **2017**, *1859*, 1891–1899.
- (10) Malta de Sá, M.; Sresht, V.; Rangel-Yagui, C. O.; Blankschtein, D. Understanding Miltefosine–Membrane Interactions Using Molecular Dynamics Simulations. *Langmuir* **2015**, *31*, 4503–4512, Publisher: American Chemical Society.
- (11) Castro, B. M.; Fedorov, A.; Hornillos, V.; Delgado, J.; Acuña, A. U.; Mollinedo, F.;

- Prieto, M. Edelfosine and Miltefosine Effects on Lipid Raft Properties: Membrane Biophysics in Cell Death by Antitumor Lipids. *The Journal of Physical Chemistry B* **2013**, *117*, 7929–7940, Publisher: American Chemical Society.
- (12) Heczková, B.; Slotte, J. P. Effect of anti-tumor ether lipids on ordered domains in model membranes. *FEBS Letters* **2006**, *580*, 2471–2476.
- (13) Alonso, L.; Mendanha, S. A.; Marquezin, C. A.; Berardi, M.; Ito, A. S.; Acuña, A. U.; Alonso, A. Interaction of miltefosine with intercellular membranes of stratum corneum and biomimetic lipid vesicles. *International Journal of Pharmaceutics* **2012**, *434*, 391–398.
- (14) Fernandes, K. S.; de Souza, P. E. N.; Dorta, M. L.; Alonso, A. The cytotoxic activity of miltefosine against *Leishmania* and macrophages is associated with dynamic changes in plasma membrane proteins. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **2017**, *1859*, 1–9.
- (15) Petit, K.; Suwalsky, M.; Colina, J. R.; Aguilar, L. F.; Jemiola-Rzeminska, M.; Strzalka, K. *In vitro* effects of the antitumor drug miltefosine on human erythrocytes and molecular models of its membrane. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **2019**, *1861*, 17–25.
- (16) Moreira, R. A.; Mendanha, S. A.; Fernandes, K. S.; Matos, G. G.; Alonso, L.; Dorta, M. L.; Alonso, A. Miltefosine Increases Lipid and Protein Dynamics in *Leishmania amazonensis* Membranes at Concentrations Similar to Those Needed for Cytotoxicity Activity. *Antimicrobial Agents and Chemotherapy* **2014**, *58*, 3021–3028, Publisher: American Society for Microbiology.
- (17) Zuo, X.; Djordjevic, J. T.; Oei, J. B.; Desmarini, D.; Schibeci, S. D.; Jolliffe, K. A.; Sorrell, T. C. Miltefosine Induces Apoptosis-Like Cell Death in Yeast via Cox9p in Cy-

- tochrome c Oxidase. *Molecular Pharmacology* **2011**, *80*, 476–485, Publisher: American Society for Pharmacology and Experimental Therapeutics Section: Article.
- (18) Luque-Ortega, J. R.; Rivas, L. Miltefosine (Hexadecylphosphocholine) Inhibits Cytochrome c Oxidase in *Leishmania donovani* Promastigotes. *Antimicrobial Agents and Chemotherapy* **2007**, *51*, 1327–1332, Publisher: American Society for Microbiology.
 - (19) van Blitterswijk, W. J.; Verheij, M. Anticancer Alkylphospholipids: Mechanisms of Action, Cellular Sensitivity and Resistance, and Clinical Prospects. *Current Pharmaceutical Design* **2008**, *14*, 2061–2074.
 - (20) Rakotomanga, M.; Blanc, S.; Gaudin, K.; Chaminade, P.; Loiseau, P. M. Miltefosine Affects Lipid Metabolism in *Leishmania donovani* Promastigotes. *Antimicrobial Agents and Chemotherapy* **2007**, *51*, 1425–1430, Publisher: American Society for Microbiology.
 - (21) López, D.; Kolter, R. Functional microdomains in bacterial membranes. *Genes & Development* **2010**, *24*, 1893–1902, Company: Cold Spring Harbor Laboratory Press Distributor: Cold Spring Harbor Laboratory Press Institution: Cold Spring Harbor Laboratory Press Label: Cold Spring Harbor Laboratory Press Publisher: Cold Spring Harbor Lab.
 - (22) Strahl, H.; Errington, J. Bacterial Membranes: Structure, Domains, and Function. *Annual Review of Microbiology* **2017**, *71*, 519–538, eprint: <https://doi.org/10.1146/annurev-micro-102215-095630>.
 - (23) Gonella, G.; Dai, H.-L. Second Harmonic Light Scattering from the Surface of Colloidal Objects: Theory and Applications. *Langmuir* **2014**, *30*, 2588–2599, Publisher: American Chemical Society.
 - (24) Page, E. F.; Blake, M. J.; Foley, G. A.; Calhoun, T. R. Monitoring membranes: The exploration of biological bilayers with second harmonic generation. *Chemical Physics Reviews* **2022**, *3*, 041307.

- (25) Wilhelm, M. J.; Dai, H.-L. Molecule-Membrane Interactions in Biological Cells Studied with Second Harmonic Light Scattering. *Chemistry – An Asian Journal* **2020**, *15*, 200–213, eprint: <https://onlinelibrary.wiley.com/doi/pdf/10.1002/asia.201901406>.
- (26) Hou, Y.; Li, J.; Liu, X.; Ruan, Y.; Chen, S.-l.; Yuan, Q.; Gan, W. The effect of side group on the dynamic behavior of anthracyclines on DOPG lipid membranes revealed by second harmonic generation and fluorescence. *Chemical Physics* **2021**, *541*, 111036.
- (27) Steinhoff, R.; Chi, L. F.; Marowsky, G.; Möbius, D. Protonation and monolayer aggregation studied by second-harmonic generation. *JOSA B* **1989**, *6*, 843–847, Publisher: Optica Publishing Group.
- (28) Marowsky, G.; Steinhoff, R. Hemicyanine monolayer orientation studied by second-harmonic generation. *Optics Letters* **1988**, *13*, 707–709, Publisher: Optica Publishing Group.
- (29) Stähelin, M.; Burland, D. M.; Rice, J. E. Solvent dependence of the second order hyperpolarizability in p-nitroaniline. *Chemical Physics Letters* **1992**, *191*, 245–250.
- (30) Bartkowiak, W.; Zaleśny, R.; Niewodniczański, W.; Leszczynski, J. Quantum Chemical Calculations of the First- and Second-Order Hyperpolarizabilities of Molecules in Solutions. *The Journal of Physical Chemistry A* **2001**, *105*, 10702–10710, Publisher: American Chemical Society.
- (31) Moreaux, L.; Sandre, O.; Mertz, J. Membrane imaging by second-harmonic generation microscopy. *JOSA B* **2000**, *17*, 1685–1694, Publisher: Optica Publishing Group.
- (32) Nguyen, T. T.; Conboy, J. C. High-Throughput Screening of Drug–Lipid Membrane Interactions via Counter-Propagating Second Harmonic Generation Imaging. *Analytical Chemistry* **2011**, *83*, 5979–5988.

- (33) Kumal, R. R.; Nguyenhuu, H.; Winter, J. E.; McCarley, R. L.; Haber, L. H. Impacts of Salt, Buffer, and Lipid Nature on Molecular Adsorption and Transport in Liposomes As Observed by Second Harmonic Generation. *The Journal of Physical Chemistry C* **2017**, *121*, 15851–15860, Publisher: American Chemical Society.
- (34) Dikkumbura, A. S.; Aucoin, A. V.; Ali, R. O.; Dalier, A.; Gilbert, D. W.; Schneider, G. J.; Haber, L. H. Influence of Acetaminophen on Molecular Adsorption and Transport Properties at Colloidal Liposome Surfaces Studied by Second Harmonic Generation Spectroscopy. *Langmuir* **2022**, *38*, 3852–3859, Publisher: American Chemical Society.
- (35) Hamal, P.; Subasinghe Don, V.; Nguyenhuu, H.; Ranasinghe, J. C.; Nauman, J. A.; McCarley, R. L.; Kumar, R.; Haber, L. H. Influence of Temperature on Molecular Adsorption and Transport at Liposome Surfaces Studied by Molecular Dynamics Simulations and Second Harmonic Generation Spectroscopy. *The Journal of Physical Chemistry B* **2021**, *125*, 10506–10513, Publisher: American Chemical Society.
- (36) Hamal, P.; Nguyenhuu, H.; Subasinghe Don, V.; Kumal, R. R.; Kumar, R.; McCarley, R. L.; Haber, L. H. Molecular Adsorption and Transport at Liposome Surfaces Studied by Molecular Dynamics Simulations and Second Harmonic Generation Spectroscopy. *The Journal of Physical Chemistry B* **2019**, *123*, 7722–7730, Publisher: American Chemical Society.
- (37) Yan, E. C. Y.; Eienthal, K. B. Effect of Cholesterol on Molecular Transport of Organic Cations across Liposome Bilayers Probed by Second Harmonic Generation. *Biophysical Journal* **2000**, *79*, 898–903.
- (38) Srivastava, A.; Eienthal, K. B. Kinetics of molecular transport across a liposome bilayer. *Chemical Physics Letters* **1998**, *292*, 345–351.
- (39) Ruan, Y.; Guha, P.; Chen, S.-L.; Yuan, Q.; Gan, W. Observing the structural varia-

- tions on binary complex vesicle surfaces and the influence on molecular transportation. *Chemical Physics* **2021**, *548*, 111250.
- (40) Chen, S. L.; Liang, Y. Z.; Hou, Y.; Wang, H.; Wu, X.; Gan, W.; Yuan, Q. Simple physics in and easy manipulating of the interfacial behavior of charged molecules on drug delivery vesicles. *Materials Today Physics* **2019**, *9*, 100092.
- (41) Li, B.; Li, J.; Gan, W.; Tan, Y.; Yuan, Q. Unveiling the Molecular Dynamics in a Living Cell to the Subcellular Organelle Level Using Second-Harmonic Generation Spectroscopy and Microscopy. *Analytical Chemistry* **2021**, *93*, 14146–14152, Publisher: American Chemical Society.
- (42) Campagnola, P. J.; Wei, M.-d.; Lewis, A.; Loew, L. M. High-Resolution Nonlinear Optical Imaging of Live Cells by Second Harmonic Generation. *Biophysical Journal* **1999**, *77*, 3341–3349.
- (43) Sharifian Gh., M.; Wilhelm, M. J.; Moore, M.; Dai, H.-L. Spatially Resolved Membrane Transport in a Single Cell Imaged by Second Harmonic Light Scattering. *Biochemistry* **2019**, *58*, 1841–1844, Publisher: American Chemical Society.
- (44) Zeng, J.; Eckenrode, H. M.; Dai, H.-L.; Wilhelm, M. J. Adsorption and transport of charged vs. neutral hydrophobic molecules at the membrane of murine erythroleukemia (MEL) cells. *Colloids and Surfaces B: Biointerfaces* **2015**, *127*, 122–129.
- (45) Miller, L. N.; Blake, M. J.; Page, E. F.; Castillo, H. B.; Calhoun, T. R. Phosphate Ions Alter the Binding of Daptomycin to Living Bacterial Cell Surfaces. *ACS Infectious Diseases* **2021**, Publisher: American Chemical Society.
- (46) Miller, L. N.; Brewer, W. T.; Williams, J. D.; Fozo, E. M.; Calhoun, T. R. Second Harmonic Generation Spectroscopy of Membrane Probe Dynamics in Gram-Positive Bacteria. *Biophysical Journal* **2019**, *117*, 1419–1428.

- (47) Blake, M. J.; Castillo, H. B.; Curtis, A. E.; Calhoun, T. R. Facilitating flip-flop: Structural tuning of molecule-membrane interactions in living bacteria. *Biophysical Journal* **2023**, *122*, 1735–1747.
- (48) Zeng, J.; Eckenrode, H. M.; Dounce, S. M.; Dai, H.-L. Time-Resolved Molecular Transport across Living Cell Membranes. *Biophysical Journal* **2013**, *104*, 139–145.
- (49) Wu, T.; Wilhelm, M. J.; Li, Y.; Ma, J.; Dai, H.-L. Indole Facilitates Antimicrobial Uptake in Bacteria. *ACS Infectious Diseases* **2022**, Publisher: American Chemical Society.
- (50) Wilhelm, M. J.; Sharifian Gh., M.; Dai, H.-L. Chemically Induced Changes to Membrane Permeability in Living Cells Probed with Nonlinear Light Scattering. *Biochemistry* **2015**, *54*, 4427–4430, Publisher: American Chemical Society.
- (51) Wilhelm, M. J.; Sharifian Gh., M.; Wu, T.; Li, Y.; Chang, C.-M.; Ma, J.; Dai, H.-L. Determination of bacterial surface charge density via saturation of adsorbed ions. *Biophysical Journal* **2021**, *120*, 2461–2470.
- (52) Wilhelm, M. J.; Sheffield, J. B.; Sharifian Gh., M.; Wu, Y.; Spahr, C.; Gonella, G.; Xu, B.; Dai, H.-L. Gram's Stain Does Not Cross the Bacterial Cytoplasmic Membrane. *ACS Chemical Biology* **2015**, *10*, 1711–1717, Publisher: American Chemical Society.
- (53) Wilhelm, M. J.; Sharifian Gh., M.; Dai, H.-L. Influence of molecular structure on passive membrane transport: A case study by second harmonic light scattering. *The Journal of Chemical Physics* **2019**, *150*, 104705, Publisher: American Institute of Physics.
- (54) Wilhelm, M. J.; Sheffield, J. B.; Gonella, G.; Wu, Y.; Spahr, C.; Zeng, J.; Xu, B.; Dai, H.-L. Real-time molecular uptake and membrane-specific transport in living cells by optical microscopy and nonlinear light scattering. *Chemical Physics Letters* **2014**, *605-606*, 158–163.

- (55) Sharifian Gh., M.; Wilhelm, M. J.; Dai, H.-L. Azithromycin-Induced Changes to Bacterial Membrane Properties Monitored in Vitro by Second-Harmonic Light Scattering. *ACS Medicinal Chemistry Letters* **2018**, *9*, 569–574.
- (56) Sharifian Gh., M.; Wilhelm, M. J.; Dai, H.-L. Label-Free Optical Method for Quantifying Molecular Transport Across Cellular Membranes In Vitro. *The Journal of Physical Chemistry Letters* **2016**, *7*, 3406–3411, Publisher: American Chemical Society.
- (57) Sharifian Gh., M.; Wilhelm, M. J.; Dai, H.-L. Observing mechanosensitive channels in action in living bacteria. *Biophysical Reports* **2024**, *4*, Publisher: Elsevier.
- (58) Kumar, D.; Gayen, A.; Chandra, M. Hypo-osmotic Stress Increases Permeability of Individual Barriers in Escherichia coli Cell Envelope, Enabling Rapid Drug Transport. *ACS Infectious Diseases* **2023**, *9*, 2471–2481, Publisher: American Chemical Society.
- (59) Gayen, A.; Kumar, D.; Matheshwaran, S.; Chandra, M. Unveiling the Modulating Role of Extracellular pH in Permeation and Accumulation of Small Molecules in Subcellular Compartments of Gram-negative Escherichia coli using Nonlinear Spectroscopy. *Analytical Chemistry* **2019**, *91*, 7662–7671, Publisher: American Chemical Society.
- (60) Hyeon Park, J.; Naw Sut, T.; A. Jackman, J.; Rahim Ferhan, A.; Kyeong Yoon, B.; Cho, N.-J. Controlling adsorption and passivation properties of bovine serum albumin on silica surfaces by ionic strength modulation and cross-linking. *Physical Chemistry Chemical Physics* **2017**, *19*, 8854–8865, Publisher: Royal Society of Chemistry.
- (61) Jay, A. G.; Hamilton, J. A. Disorder Amidst Membrane Order: Standardizing Laurdan Generalized Polarization and Membrane Fluidity Terms. *Journal of Fluorescence* **2017**, *27*, 243–249.
- (62) Mykytczuk, N. C. S.; Trevors, J. T.; Leduc, L. G.; Ferroni, G. D. Fluorescence polarization in studies of bacterial cytoplasmic membrane fluidity under environmental stress. *Progress in Biophysics and Molecular Biology* **2007**, *95*, 60–82.

- (63) Vanounou, S.; Pines, D.; Pines, E.; Parola, A. H.; Fishov, I. Co-existence of Domains with Distinct Order and Polarity in Fluid Bacterial Membranes. *Photochemistry and Photobiology* **2002**, *76*, 1–11, eprint: [https://onlinelibrary.wiley.com/doi/pdf/10.1562/0031-8655\(2002\)076:0001:CODWDO2.0.CO;2](https://onlinelibrary.wiley.com/doi/pdf/10.1562/0031-8655(2002)076:0001:CODWDO2.0.CO;2).
- (64) López, C. S.; Garda, H. A.; Rivas, E. A. The effect of osmotic stress on the biophysical behavior of the *Bacillus subtilis* membrane studied by dynamic and steady-state fluorescence anisotropy. *Archives of Biochemistry and Biophysics* **2002**, *408*, 220–228.
- (65) Perez-Lopez, M. I.; Mendez-Reina, R.; Trier, S.; Herrfurth, C.; Feussner, I.; Bernal, A.; Forero-Shelton, M.; Leidy, C. Variations in carotenoid content and acyl chain composition in exponential, stationary and biofilm states of *Staphylococcus aureus*, and their influence on membrane biophysical properties. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **2019**, *1861*, 978–987.
- (66) Bessa, L. J.; Ferreira, M.; Gameiro, P. Data on Laurdan spectroscopic analyses to compare membrane fluidity between susceptible and multidrug-resistant bacteria. *Data in Brief* **2018**, *21*, 128–132.
- (67) Bessa, L. J.; Ferreira, M.; Gameiro, P. Evaluation of membrane fluidity of multidrug-resistant isolates of *Escherichia coli* and *Staphylococcus aureus* in presence and absence of antibiotics. *Journal of Photochemistry and Photobiology B: Biology* **2018**, *181*, 150–156.
- (68) Wu, T.; Wilhelm, M. J.; Ma, J.; Li, Y.; Wu, Y.; Dai, H.-L. Influence of Phase Transitions on Diffusive Molecular Transport Across Biological Membranes. *Angewandte Chemie International Edition* **2022**, *61*, e202205608, eprint: <https://onlinelibrary.wiley.com/doi/pdf/10.1002/anie.202205608>.

- (69) Malanovic, N.; Lohner, K. Antimicrobial Peptides Targeting Gram-Positive Bacteria. *Pharmaceuticals* **2016**, *9*, 59.
- (70) Lopez, D.; Koch, G. Exploring functional membrane microdomains in bacteria: an overview. *Current Opinion in Microbiology* **2017**, *36*, 76–84.
- (71) Morrow, I. C.; Parton, R. G. Flotillins and the PHB Domain Protein Family: Rafts, Worms and Anaesthetics. *Traffic* **2005**, *6*, 725–740, _eprint: <https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1600-0854.2005.00318.x>.
- (72) Willdigg, J. R.; Helmann, J. D. Mini Review: Bacterial Membrane Composition and Its Modulation in Response to Stress. *Frontiers in Molecular Biosciences* **2021**, *8*, Publisher: Frontiers.
- (73) Bramkamp, M. Fluidity is the way to life: lipid phase separation in bacterial membranes. *The EMBO Journal* **2022**, *41*, e110737, Publisher: John Wiley & Sons, Ltd.
- (74) Mitchison-Field, L. M.; Belin, B. J. Bacterial lipid biophysics and membrane organization. *Current Opinion in Microbiology* **2023**, *74*, 102315.
- (75) López, G.-D.; Suesca, E.; Álvarez Rivera, G.; Rosato, A. E.; Ibáñez, E.; Cifuentes, A.; Leidy, C.; Carazzone, C. Carotenogenesis of *Staphylococcus aureus*: New insights and impact on membrane biophysical properties. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **2021**, *1866*, 158941.
- (76) van Tilburg, A. Y.; Warmer, P.; van Heel, A. J.; Sauer, U.; Kuipers, O. P. Membrane composition and organization of *Bacillus subtilis* 168 and its genome-reduced derivative miniBacillus PG10. *Microbial Biotechnology* **2022**, *15*, 1633–1651, _eprint: <https://onlinelibrary.wiley.com/doi/pdf/10.1111/1751-7915.13978>.
- (77) Rohmer, M.; Bouvier, P.; Ourisson, G. Molecular evolution of biomembranes: structural equivalents and phylogenetic precursors of sterols. *Proceedings of the National Academy*

of Sciences **1979**, 76, 847–851, Publisher: Proceedings of the National Academy of Sciences.

- (78) Sáenz, J. P.; Sezgin, E.; Schwille, P.; Simons, K. Functional convergence of hopanoids and sterols in membrane ordering. *Proceedings of the National Academy of Sciences* **2012**, 109, 14236–14240, Publisher: Proceedings of the National Academy of Sciences.
- (79) Sáenz, J. P.; Grosser, D.; Bradley, A. S.; Lagny, T. J.; Lavrynenko, O.; Broda, M.; Simons, K. Hopanoids as functional analogues of cholesterol in bacterial membranes. *Proceedings of the National Academy of Sciences of the United States of America* **2015**, 112, 11971–11976.
- (80) Wenzel, M.; Vischer, N. O. E.; Strahl, H.; Hamoen, L. W. Assessing Membrane Fluidity and Visualizing Fluid Membrane Domains in Bacteria Using Fluorescent Membrane Dyes. *Bio-protocol* **2018**, 8, e3063.
- (81) Barák, I.; Muchová, K.; Wilkinson, A. J.; O'Toole, P. J.; Pavlendová, N. Lipid spirals in *Bacillus subtilis* and their role in cell division. *Molecular Microbiology* **2008**, 68, 1315–1327, eprint: <https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1365-2958.2008.06236.x>.
- (82) Johnson, A. S.; van Horck, S.; Lewis, P. J. Dynamic localization of membrane proteins in *Bacillus subtilis*. *Microbiology* **2004**, 150, 2815–2824, Publisher: Microbiology Society,.