Phosphate ions alter the binding of daptomycin to living cell membranes

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Abstract

Advancements in antibiotic drug design are often hindered by missing information in how these small molecules interact with living cells. The antibiotic, daptomycin, has found clinical success, and emerging resistance, but a comprehensive picture of its mechanism of action has remained elusive. Using the surface-specific spectroscopy, second harmonic generation, we are able to quantitatively assess the binding of daptomycin to living cell membranes without the addition of exogenous labels. Our results reveal similar binding affinities for both gram-positive and gram-negative bacteria studied, including *E. coli*. More importantly, we show that phosphate ions influence the binding of daptomycin to the gram-positive bacterium *E. faecalis*. The role of environmental phosphate has not previously been considered in any proposed mechanism and its implications are expected to be important *in vivo*.

Introduction

The World Health Organization has listed multi-antibiotic resistance as one of the biggest global health threats in 2020.¹ In 2019, the Centers for Disease Control and Prevention

(CDC) reported that over 2.8 million new antibiotic-resistant infections are being documented every year, resulting in more than 35,000 yearly deaths in the United States alone.² The greatest threats arise from the growing number of multidrug-resistant bacterial strains.²⁻⁶ Of these strains, two of the gram-positive bacterial species that the CDC ranked as serious threats are vancomycin-resistant *Enterococcus faecalis* and methicillin-resistant *Staphylococcus aureus* (MRSA).^{2,3} When first discovered over 30 years ago, the antibiotic daptomycin yielded successful results for effectively treating these gram-positive bacterial infections.⁷⁻¹⁰ Considered one of the first antibiotics of its kind, daptomycin is a calcium-dependent cyclical lipopeptide drug that is believed to cause cell death through depolarization upon inserting into gram-positive bacterial membranes.^{9,11} The formation of the nascent drug complex and its interaction with living bacterial membranes is still poorly understood.⁹⁻¹³ The need for this information has become more imperative as daptomycin-resistant gram-positive strains are emerging quickly with minimal treatment options remaining.^{10,12-22} Considering the impact of various environmental factors on daptomycin's affinity for living bacterial membranes could provide new, crucial information for improved drug development. The work presented examines the initial native daptomycin-membrane adsorption affinities in living bacteria under different environmental conditions by measuring concentration-dependent isotherms using second harmonic generation (SHG), a surface-specific spectroscopy technique.

Daptomycin activity has been shown to require the presence of calcium ions and negatively charged phospholipids such as phosphatidylglycerol (PG, net charge = -1) and cardiolipin (CL, net charge = -2), the latter of which are found abundantly in gram-positive bacterial lipid-membrane compositions.^{11,23,24} When these two criteria are met, daptomycin is thought to insert its nonpolar tail into the bacterial membrane. However, calcium's role for facilitating daptomycin-membrane activity is still highly debated.¹² For example, while many have proposed that calcium ions predominately bind to daptomycin to produce an overall positively-charged complex to form, the composition of this complex is still not agreed

upon.^{11,23,25–32} An alternative theory suggests that Ca^{2+} ions interact with the bacterial membrane surface to help shield the negative charges of the phospholipids so that daptomycin can insert into the lipid-bilayer.³⁰ More so, the daptomycin:calcium (Dap- Ca^{2+}) interaction could be altered by other seemingly innocuous factors/species lurking within the surrounding environment. Recently, a new mechanism has been proposed in which lipid II, a cell biosynthesis precursor, has been implicated in daptomycin binding.³³ The roles of other ions found within various aqueous media environments, however, have not been fully explored possibly explaining the lack of consensus regarding this drug’s action.

The affinity of daptomycin for the membranes of living bacteria is challenging to determine as the drug’s native, and relatively weak, fluorescence in the visible overlaps within the autofluorescence spectral region for bacterial cells. This is often remedied by studies using either model lipid vesicle systems to avoid cellular autofluorescence signals altogether or by using fluorescently-labeled daptomycin with bacterial cells to cause a spectral shift outside of the autofluorescence region and increase the overall quantum yield of the detected molecule. However, the majority of model membrane spectroscopy studies utilized large unilamellar vesicles (LUVs) containing phosphatidylcholine (PC) phospholipids to indicate both fluorescently-labeled and native daptomycin interaction.^{23,24,34–40} The diameter range of an LUV is defined as 100-1,000 nm whereas the diameter of a gram-positive bacterial cell ranges between 1.5 - 2 microns. The size discrepancies between the LUV and a gram-positive bacterial cell could lead to inaccurate representations of the bacterial membrane curvature and possibly alter the drug’s true mechanism for how it interacts with living cell membranes.⁴¹ PC phospholipids are also not native to the lipid compositions of gram-positive bacteria, such as *E. faecalis*, and could lead to potentially false mechanisms of action for daptomycin. In addition, model membrane studies lose the ability to account for the complex diversity of lipid and protein species found in biological membranes which vary between bacterial strains. This was demonstrated in our previous work where the variance between the lipid-membrane compositions for *E. faecalis* and *S. aureus* was observed to yield different dynamics for the

probe molecule FM 4-64 as it interacted with the membranes over time.⁴²

While the fluorescent-labeling approach is considered a standard technique for optical studies, dye molecules can alter molecular interactions which may lead to inaccurate assumptions regarding the native drug mechanisms.^{43,44} One study noted this discrepancy by showing fluorescently-labeled daptomycin rapidly diffusing into *Staphylococcus epidermis* within minutes as compared to the native daptomycin case which required several hours for diffusion.⁴⁴ As an alternative, SHG is a nonlinear optical phenomena that only generates signal from chromophores in a noncentrosymmetric environment, such as those in a biological membrane. This removes background from autofluorescent sources in cells, such as the metabolites like NADH,^{45,46} and provides a new means to probe the binding of small molecules, such as drugs, to the membranes of living bacterial cells. Over the past few decades, SHG has been utilized to monitor numerous small molecule-membrane interactions in model systems^{47–59} as well as in living cell systems.^{42,60–68}

SHG occurs when two photons of frequency ω are upconverted to a single photon of frequency 2ω when they interact with a noncentrosymmetric sample. A more detailed explanation of SHG is provided in the SI, but in our sample the molecule being probed generates coherent SHG signal when it satisfies the noncentrosymmetric criteria by embedding into the lipid bilayer.⁶⁹ A significant increase in the generated SHG signal arises when an electronic transition in the molecule of interest is resonant with either the fundamental or second harmonic frequency. In daptomycin, this resonance condition is met by the kynurenine residue. This chromophore has a weak absorption at 380 nm with a tail that overlaps with the 400 nm second harmonic of our laser source. Fluorescence studies have shown an increase in fluorescence signal from the kynurenine residue as native daptomycin inserted into model membranes indicating that the kynurenine residue embedded into the membrane where it became sterically restricted.^{25,35}

Langmuir adsorption isotherms can be measured using SHG spectroscopy without the addition of exogenous labels to determine and compare daptomycin’s binding affinities for

cell membranes while in various environmental conditions, essentially revealing daptomycin's unaltered initial interactions with living bacterial systems. Before now, past daptomycin isotherm studies required the use of model membrane systems or fluorescent tags.^{23,37,40,70–73} SHG Langmuir adsorption isotherms have been previously employed to assess the binding affinities for different small molecule-membrane systems such as in model membranes^{74–82} and in living cell membranes^{42,64,65} The cartoon schematic in Fig. 1 represents the adsorption

Figure 1: Cartoon schematic of the daptomycin adsorption onto the membrane. Not shown are the Ca^{2+} ions that are known to be necessary but whose exact role is still debated.

of free daptomycin molecules in solution, D_{free} , onto the empty membrane sites, M_{empty} , and producing daptomycin-bound membrane sites MD_{bound} that generate the measured SHG signal.

In our experiments, the concentration of daptomycin is increased while monitoring the SHG response to assess the availability of empty membrane sites, M_{empty} . The results can be fit by the equation:

$$\theta = \frac{\frac{[c]}{K_d}}{1 + \frac{[c]}{K_d}} \quad (1)$$

where c is the concentration of daptomycin and θ represents the fraction of MD_{bound} to M_{total} , such that:

$$\theta = \frac{MD_{bound}}{M_{total}} \quad (2)$$

K_d in Eq. 1 is the dissociation constant ($K_d = \frac{k_{-1}}{k_1}$) and represents the concentration at which half of the membrane sites are occupied.⁶⁹ A smaller K_d value corresponds to a stronger

association with the membrane.

Daptomycin’s activity against bacteria has been shown to be impacted by local environment factors including: (1) the ions and molecules in the solvent environment^{35,83–85} and (2) the lipid-membrane composition of the bacterial species.^{18,20,86–89} Here we consider how these factors influence the initial step of daptomycin activity by examining its membrane adsorption/binding. In our experiments detailed below, the daptomycin membrane affinities for the gram-positive bacterial species *E. faecalis* were determined for solutions with different ionic composition. In order to study the effects arising from the natural membrane composition, we also compared the SHG isotherms for daptomycin with different bacterial species.

Results and discussion

Ion-Mediated Effects: The Role of Phosphate Ions

Components found in the surrounding local environment can modify small-molecular interactions at the membrane surface.^{64,90,91} Most biological salines, such as 0.1 M phosphate buffered saline (PBS) and 0.9% sodium chloride (NaCl), are composed of multiple ionic species with well-defined concentrations. In contrast, bacterial growth in complex media are less defined and contain a wide variety of nutrients such as proteins, lipids, free ions and vitamins. Although, these biological solutions generally have similar pH values around 7.4, the varying range of ionic and molecular species can dramatically affect how small molecules, such as daptomycin, interact with bacterial membranes.^{35,83–85} Specific ion effects on membrane processes due to the presence of free ionic species in aqueous environments have been previously investigated using SHG.^{53,64}

Figure 2 shows isotherms for daptomycin adsorption onto the membranes of living *E. faecalis* cells in environments with different ionic compositions. The K_d values extracted from the fits of the data to the Langmuir equation (Eq. 1) are provided in Table 1. In Fig. 2A, the

Figure 2: SHG isotherms of daptomycin adsorption studies on *E. faecalis* cells with a constant 1:8 Dap: Ca^{2+} ratio (—), *E. faecalis* cells with a constant 1:8 Dap: Mg^{2+} ratio (—), and blank controls with a constant 1:8 Dap: Ca^{2+} ratio and no cells present. (—) Cells were suspended in solutions of (A) 0.9% NaCl saline and (B) 0.1 M PBS. The individual trials are shown as $\times$. The averages for the curve fits are shown as $\circ$.

cells are suspended in 0.9% NaCl saline and the SHG signal is recorded as the concentration of daptomycin is increased. The blue data points and fit (—) show that the largest signal is observed in NaCl when calcium ions are also introduced. Here a 1:8 daptomycin:calcium concentration ratio is maintained throughout the experiment. In contrast, significantly less signal is observed if either the cells are absent (—, blank control) or if the calcium ions

are replaced with magnesium ions (—). For the blank control, the linear signal observed with increasing concentration is consistent with hyper-Rayleigh scattering (HRS). HRS is incoherent second harmonic scattering that scales linearly with concentration and arises in this sample from the daptomycin aggregates forming in solution.^{23,27–32} In contrast, the SHG from a single cell is coherent with a forward propagating signal based on the size of the cells.⁶⁹ The SHG signals observed when calcium is replaced by magnesium in the presence of cells is similar to the blank control suggesting that daptomycin is unable to bind to cell membranes under these conditions without calcium present. This is in agreement with other studies that have shown that daptomycin can only bind to membranes in the presence of Mg^{2+} with significantly higher concentrations.²⁷ Overall, our isotherms not only confirm daptomycin’s well-known calcium dependency^{11,23,23,25–30,30–32} but also that our measured SHG signals are specific to daptomycin adsorption.

Table 1: Average K_d values for the daptomycin binding affinity when using a 1:8 Dap: Ca^{2+} constant ratio with *E. faecalis* cells for n trials.

Bacterial Species	Saline Environment	n	K_d (μM)
<i>E. faecalis</i>	0.9% NaCl	4	73 ± 10
<i>E. faecalis</i>	PBS	5	104 ± 9
<i>E. coli</i>	PBS	2	92 ± 24
<i>S. aureus</i>	PBS	3	147 ± 33

Turning to Fig. 2B, analogous behavior is seen when the experiments are repeated with the *E. faecalis* cells suspended in PBS. Again, the highest signal is observed when calcium ions are present to facilitate the binding of daptomycin with the cell membranes (—). Looking at the PBS data in more detail, however, reveals multiple key differences. First, the SHG intensities are significantly larger than those from similar concentrations of daptomycin in NaCl. This suggests that in PBS there are either a larger number of surface sites available for daptomycin binding, or that when bound, the daptomycin molecules generate more signal from, for example, more uniform alignment.⁹² A second difference observed for the PBS data is that the blank control (—) is not linear but instead exhibits a Langmuir shape. The Langmuir shape arises from a sharp increase in the initial signal as SHG scales quadratically

with concentration that begins to level off at higher concentrations as surface sites are filled, preventing additional drug adsorption. In the blank control, there are no cells and thus no membrane surface sites. As such, the plateau observed in the blank control must arise from another limiting reactant.

Calcium and phosphate ions are known to form a precipitate, and a control study was performed in which only calcium was titrated into PBS solution to determine the maximum calcium concentration that could be added to PBS before scatter from these precipitates was significant (Supporting Information, Fig. S1). This control study did not yield a linear nor a Langmuir trend as the calcium concentration was increased again confirming that our SHG isotherm measurements are reporting on daptomycin. The calcium and phosphate control study, however, did exhibit significant scatter for calcium concentrations at and above 10 mM. To stay well under this limit, the maximum calcium concentration was limited to be 8 mM for all of the SHG isotherm trials to ensure minimal contribution from calcium-phosphate aggregates.

Since daptomycin has been suggested to form aggregate complexes with ionic species,^{10,31,32,44,52,93} dynamic light scattering (DLS) was employed to determine the size of daptomycin aggregates forming within three different solutions. Figure 3 shows the size of the aggregates formed in NaCl, PBS and water when 1:0.5 (*top*) and 1:8 (*bottom*) concentration ratios of Dap:Ca²⁺ are present. A 1 mM final daptomycin concentration was used throughout the DLS measurements in order to match the highest daptomycin concentration used during the isotherm experiments. When the Dap:Ca²⁺ is 1:0.5 (Fig. 3 *top*) the only solution that produces an aggregate species is NaCl (—) with this complex having a diameter <10 nm. This species is also present when the relative amount of calcium is increased to a 1:8 Dap:Ca²⁺ ratio (Fig. 3 *bottom*) and a species with a similar size also appears in water solution (—). The most prominent feature in the 1:8 data, however, is a significantly larger aggregate species, approximately $\sim 1 \mu\text{m}$ in diameter, which is only present in the PBS sample (Fig. 3A, —). The fact that this species only forms in the PBS solution with a relatively large concentra-

tion of calcium ions suggests that a unique daptomycin complex is formed with calcium that includes an ionic component of PBS.

Figure 3: (A) DLS diameter size measurements of Dap:Ca²⁺ aggregates when in different solutions: 0.9% NaCl saline (—), PBS (—) and H₂O (—). Aggregate sizes are shown for 1:0.5 (top) and 1:8 (bottom) Dap:Ca²⁺ ratios. Daptomycin concentration was held constant at 1 mM for all DLS trials. (B) Environmental ion effects on 1:8 Dap:Ca²⁺ ratio daptomycin concentration-dependent SHG isotherms in sterile saline blank controls containing various ion species, such as 0.9% NaCl (—), 0.9% NaCl + 1.35 mM KCl (—), 0.9% NaCl + 5 mM Na₂HPO₄ (—) and 0.1 M PBS (—).

PBS contains the following ionic species: potassium (K⁺), sodium (Na⁺), chloride (Cl⁻), dihydrogen phosphate (H₂PO₄⁻) and hydrogen phosphate (HPO₄²⁻). To determine which of the ionic species found in PBS is contributing to forming the large Dap-Ca²⁺ complex, 0.9% NaCl saline solutions were made with the addition of either KCl or Na₂HPO₄ containing 1/2 of the molar equivalents typically used to make 0.1 M PBS. Daptomycin concentration-dependent isotherm control studies were performed without the presence of cells for each of the saline solutions using a constant 1:8 Dap:Ca²⁺ ion ratio throughout the experiment (Fig. 3B).

Signal resulting from the addition of potassium ions to 0.9% NaCl saline (Fig. 3B,—) closely modeled the signal trend seen in the 0.9% NaCl saline only sample (Fig. 3B,—). Both of these signals rose linearly with concentration consistent with incoherent HRS. As an aside, the high intensities seen from HRS in the NaCl blank prevented higher concentrations

of daptomycin to be examined for the isotherms presented in Fig. 2A. An extended plot of this data can be found as Fig. S2 in the Supporting Information. Coming back to Fig. 3B, the addition of phosphate ions into 0.9% NaCl saline (—) does not display the same linear behavior as the NaCl + K⁺ (—) but instead closely resembles the signal from the PBS trial (—). These results suggest that free phosphate ions found in the aqueous environment contribute to forming the large and unique daptomycin-Ca²⁺ complexes. Furthermore, the formation of these clusters directly impacts the binding of daptomycin to the cell membrane as evidenced by the significantly higher SHG signal observed for these conditions (Fig. 2B, —). Environmental phosphate ions are not currently considered to play a role in any of the proposed mechanisms for daptomycin binding^{11,23,25–32} suggesting that these models are still incomplete.

Differences between Bacterial Membrane Compositions

Bacterial species have unique lipid-membrane compositions which aid in regulating small-molecule interactions at the membrane surface.^{94–98} Daptomycin activity is specific for gram-positive bacterial species and has been shown to have no effect on gram-negative species such as *E. coli*.^{7,8,99} As previously mentioned, daptomycin requires the presence of negatively charged lipid species and has a high affinity for PG phospholipids¹¹ which are commonly found as the major lipid-class in gram-positive bacteria, such as in *E. faecalis*.^{100,101} Studies have also shown that variations to bacterial lipid-membrane compositions can alter daptomycin behavior.^{86,89} To determine how bacterial phospholipid-membrane compositions affect the initial binding affinity for daptomycin, SHG adsorption isotherms were measured with *E. coli* and *S. aureus* while maintaining a constant 1:8 Dap:Ca²⁺ ion ratio as the daptomycin concentration was increased. The SHG adsorption isotherms for each bacteria as well as the previous data from *E. faecalis* are shown in Fig. 4 and the extracted K_d are reported in Table 1.

It is immediately apparent that despite being inactive against *E. coli*, daptomycin still

Figure 4: SHG isotherms of initial daptomycin adsorption onto the membranes of different bacterial species. *E. faecalis* cells (—), *E. coli* cells (—), *S. aureus* cells (—) and a comparison to the 1:8 Dap:Ca²⁺ PBS blank control (—). Trials were performed in a PBS environment using a 1:8 Dap:Ca²⁺ ratio. The individual trials are shown as x. The averages for the curve fits are shown as o.

binds to its outer membrane (Fig. 4,—). Prior studies have suggested that the lack of daptomycin activity could be due to the drug’s inability to either interact with or disrupt the cytoplasmic membrane.⁹⁹ Our results unequivocally show that the former suggestion is not the case. Further, our results are supported by a study by Faust *et al.* that revealed daptomycin was capable of permeating model spheroplast vesicles consisting of phospholipid species extracted from the cytoplasmic membrane of *E. coli* cells.¹⁰²

The daptomycin binding affinities appear to be similar for all of the bacterial species with the extracted K_d averages shown Table 1. Fig. 4 reveals that *E. faecalis* (—) yielded Langmuir-shaped curves with higher I_{SHG} in comparison to the gram-negative *E. coli* (—). The lower intensities for *E. coli* can be explained by a lower number of binding sites for daptomycin as this species contains approximately ~30% PG phospholipids in its phospholipid-membrane composition^{103–105} as opposed to the combined ~38% PG and ~42% CL for *E. faecalis*.¹⁰⁶

S. aureus cells produce the lowest intensity SHG for the daptomycin isotherm (Fig. 4,—). The intensity is still greater than that exhibited in the blank control sample demonstrating

clear daptomycin binding which is consistent with the fact that daptomycin is known to be active against this gram-positive species.^{7,8,29,107–109} In addition, the relative amount of negative phospholipids in the *S. aureus* membranes is similar to that of *E. faecalis*.¹⁵ Instead, we expect that the lower SHG signal arising from daptomycin binding to *S. aureus* membranes arises from larger fluctuations in the molecular alignment. The lipid composition of *S. aureus* has been shown to contain a higher percentage of branched fatty acids^{110,111} leading to an overall higher fluidity.¹¹² When the molecule embeds into such a membrane, the fluidity will allow for it to sample a larger range of angles reducing the overall average orientation and thus the SHG intensity.

Conclusions

The work presented here includes the first reports for native daptomycin binding affinity constants with living bacterial membranes under different environmental conditions. These efforts were realized through the use of SHG spectroscopy which showcases its capabilities for quantifying small molecule interactions in living biological systems label-free. In doing so, the presence of free phosphate ions in the PBS environment were revealed to participate in the formation of a large and unique daptomycin- Ca^{2+} complexes. This phosphate-induced daptomycin- Ca^{2+} complex also yielded adsorption signals with larger intensities upon binding to *E. faecalis* membranes in comparison to the phosphate-free environment. We can conclude then that the environmental phosphate ions alter the daptomycin binding and needs to be considered in future proposed mechanisms for this drug. The K_d values for multiple bacterial species were also measured which revealed that daptomycin has similar binding affinities for both the gram-positive and gram-negative bacteria studied. While daptomycin is known to not be effective against the gram-negative *E. coli*,^{7,8,99} the SHG daptomycin adsorption isotherms clearly show that daptomycin does bind to *E. coli* membranes. Overall, our SHG studies have provided new insight into the factors affecting the initial binding of

daptomycin to living cells.

Methods

Bacterial Cultures

For testing the gram-positive bacterial species, single colonies of *E. faecalis* (OG1RF) and *S. aureus* (ATCC 27217) were grown on brain heart infusion (BHI) agar plates and inoculated into BHI liquid medium with a 1:5 liquid-to-head space volume ratio. The liquid cultures were statically grown overnight at 37°C. For the gram-negative bacterial studies, single colonies of *E. coli* (K-12) were taken from Luria broth (LB) agar plates and inoculated into LB medium with a 1:5 liquid-to-head space volume ratio and grown overnight to stationary phase while shaking at 250 revolutions per minute at 37°C. Once the cultures reached stationary phase, the cells were diluted into either fresh BHI or LB media to an optical density at 600 nm (OD_{600}) of ~ 0.01 and grown under the same overnight growth parameters until reaching an OD_{600} of ~ 0.3 . The bacterial cultures were washed twice via vacuum filtration with either sterile PBS or sterile 0.9% NaCl, dependent on the experimental isotherm saline conditions being tested. The cells were then resuspended in either PBS or 0.9% NaCl saline, each containing 2% OxyRase, to a final $OD_{600} \sim 0.3$. The final cell densities were measured using the UV-Vis spectrometer before performing the isotherm trials.

Drug Solution Prep for Daptomycin-Dependent Isotherms

For the daptomycin concentration-dependent isotherm trials, the daptomycin stock solutions were prepared using either the Ca^{2+} or Mg^{2+} ions. The dicationic stocks were made by dissolving calcium chloride or magnesium chloride in an 80:20 ratio of MilliQ deionized water (H_2O):dimethylsulfoxide (DMSO) to a final concentration of 800 mM. The X^{2+} (where $X^{2+} = Ca^{2+}$ or Mg^{2+}) ionic stock solutions were each filter-sterilized using 0.2 micron syringe filters.

For maintaining a constant 1:8 Dap:X²⁺ ion ratio for at each of the different daptomycin concentrations studied for the isotherms, a 100 mM daptomycin stock solution was prepared using the 800 mM X²⁺ ion solutions as the solvent. Additional 1:8 Dap:X²⁺ ion stock solutions were made by performing serial dilutions using sterile 80:20 H₂O:DMSO as the solvent to achieve daptomycin stock solutions with concentrations ranging from 0 mM to 100 mM. The daptomycin stock solutions were freshly prepared each day immediately before the isotherm experiments were conducted.

Daptomycin Concentration-Dependent Isotherms

Approximately 990 μ L of the prepared bacterial culture solutions from Section were aliquoted into sterile microcentrifuge vials and 10 μ L of the 1:8 Dap:X²⁺ solution corresponding to the appropriate drug concentration was added to the vials immediately before each drug concentration SHG measurement was conducted. The final DMSO percentage was held constant at 0.2% for each isotherm sample.

Sample Cell Preparation

A quartz flow cell, with a 2 mm path length, was passified using a bovine serum albumin (BSA) - 1% glutaraldehyde crosslinking protocol¹¹³ and flushed with Tris buffer (pH adjusted using HCl to $\sim 7.4 \pm 0.1$) prior to the experiments. Approximately 20-30 minutes before the bacterial cells reached the appropriate cell density, a solution of 50 mM BSA was prepared in cold (4°C) 10 mM Tris buffer and filter-sterilized using a 0.2 micron syringe filter. The BSA solution was introduced into the flow cell where it was allowed to incubate for approximately 10 minutes. Cold 10 mM Tris buffer containing 1% glutaraldehyde was introduced to the flow cell following the BSA incubation period and allowed to incubate for approximately 10 minutes. Finally, 10 mM Tris buffer was used to flush out the 1% glutaraldehyde solution from the flow cell. About three 1 mL aliquots of the prepped bacterial solutions were micropipetted into the flow cell and discarded to help remove the remaining Tris buffer in

the flow cell before the SHG measurements were taken.

SHG Spectroscopy Apparatus

SHG signals were collected using a home-built spectroscopy instrument previously reported elsewhere.⁴² Briefly, the 80 MHz output of a MaiTai titanium:sapphire oscillator (Spectra-Physics) with pulse widths compressed to ~ 80 fs and centered at 800 nm was used to excite the sample. The average power at the sample position was adjusted to be ~ 350 mW for all of the experiments. The SHG signals, centered at 400 nm were isolated with a series of optical filters before being detected with a photon-counting PMT. The SHG signals were collected every 250 ms and averaged over 3 s.

Given that previous SHG bacterial membrane studies showed that a molecule smaller than daptomycin with no significant membrane association took approximately ~ 28 seconds to cross the outer membrane of *E. coli*,⁶⁴ we do not expect significant internalization of the daptomycin within the 3 s time frame for the measurement of each data point. Total run time for each full SHG isotherm data set was approximately ~ 20 min. Each experimental condition was replicated with the number of trials reported as n in Table 1.

Data Processing

For a given set of experimental conditions, each SHG isotherm trial was scaled to the average value of 0 μM concentration SHG signal arising from HRS. The scaled intensities for each of the trial were averaged together, before fitting to the Langmuir equation (Eq. 1) using Matlab. The average K_d constants and standard deviations were extracted from the fit parameters.

Dynamic Light Scattering

Dynamic light scattering (DLS) measurements were taken with a Zetasizer Nano ZS to determine the daptomycin- Ca^{2+} aggregate diameter size of various Dap: Ca^{2+} ratios in sterile solutions consisting of DI H_2O , 0.9% NaCl saline and PBS. Daptomycin concentration was held constant at 1 mM for all of the trials.

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Supporting Information Available

Supporting Information is available free of charge:

- Expanded description of SHG
- Figures S1 and S2

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