Supporting Information: Azithromycin-Induced Changes to Bacterial Membrane Properties Monitored *In Vitro* by Second-Harmonic Light Scattering

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CONTENTS

■ MINIMUM INHIBITORY CONCENTRATIONS (MIC)

Minimum inhibitory concentration (MIC) was deduced using the broth dilution method[1], and quantified with flow cytometry. As shown in **Figure S1a**, for the standard protocol cell density of 10^5 ml^{-1}, our *E. coli* exhibit a MIC of ca. 0.5 μg/ml. It must be noted, however, that our SHS experiments require higher cell densities (ca. 10^8 ml^{-1}) to yield sufficient signal. Consequently, a MIC assessment was also performed for higher cell density (**Figure S1b**). In general, as cell density increases, it is reasonable that the MIC should also increase[1]. As expected, for the elevated cell density, the MIC increased to ca. 128 μg/ml. Nevertheless, for both high and low cell density conditions, the ratios of the MIC to the cell density were observed to be roughly equal, ca. 10^9 AZM molecules per bacteria cell. In other words, the same number of molecules per cell are required in order to induce the antibiotic effect. This strongly suggests that the same mechanism is at play for both conditions. Therefore, any information garnered in the high cell density conditions of the SHS experiment is equally representative of the low density case.

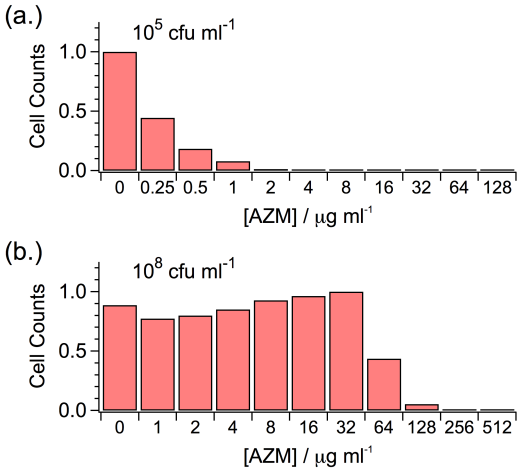

Figure S1. MIC determination for AZM with different cell densities of *E. coli* (mc4100) (a) Typical cell density for MIC determination, 10^5 ml^{-1}. (b) Typical cell density for SHS, 10^8 ml^{-1}.

In addition to AZM, MG is known to act as an antibiotic, but only at relatively high concentrations. Subsequently, in order to be certain that observed antibiotic effects stem solely from AZM, an identical MIC analysis was repeated for MG. For the elevated cell densities (ca. 10^8 ml^{-1}) required for our SHS experiments, the MG MIC for our strain of *E. coli* was deduced to be roughly 100 μM. Consequently, in order to avoid potential MG-induced antibiotic effects, the maximum MG concentration of our experiments was set to 25 μM.

■ NON-NORMALIZED DATA

Figure S2 highlights the effects of increasing concentrations of AZM (0 to 150 μM) on bacterial membrane properties. AZM was exposed to the cells for either 0 (**Figure S2a**) or 1 hour (**Figure S2b**) prior to initiating the SHS experiments. For all measurements, the concentration of MG was maintained at 25 μM. **Figure S2** contains the same information conveyed in **Figure 2**, but the measured SHS intensity

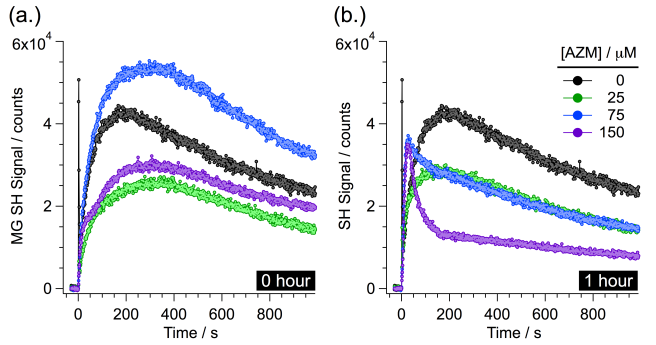

Figure S2. Time-resolved SHS signal collected during bacterial uptake of MG following (a) 0 hour or (b) 1 hour exposure to 0 (black), 25 (green), 75 (blue), or 150 (purple) μM AZM.

has not been normalized. The observed variation in the measured SH signal could reasonably stem from a variety of sources, including: variations in cell density or cell size, as well as changes in the orientation of the surface adsorbed MG. Changes in cell density would result in a linear variation in the magnitude of the measured SHS signal. Similarly, variations in cell size could result in quadratic changes in the measured SHS signal. Specifically, changes in cell size would be accompanied by changes in the surface area of the cell, and therefore (potentially) change the maximum surface adsorption concentration (i.e., SHS scales as the square of the surface concentration). Furthermore, as more AZM competes for available surface adsorption sites, this would likely result in changes to the ensemble MG orientation at the membrane

surface, and hence a significant variation in the resulting SH signal[2]. Investigation of these effects would require polarization-dependent measurements, which are beyond the scope of the current study. Nevertheless, while these effects would cause notable changes in the magnitude of the measured SHS signal, they would have no effect on the kinetics (i.e., signal rise and decay) of the measured signal.

■ EXPERIMENTAL METHODS

Materials

A 1 mM stock solution of malachite green (MG) oxalate (Cat. No.: M9015, Sigma Aldrich) was prepared in distilled deionized water (Millipore, 18.2 MΩ.cm). A 1024 µg/ml stock solution of azithromycin, AZM (Cat. No.: 75199, Sigma-Aldrich) was prepared in a solution composed of 1:1 v/v of ethanol : distilled deionized water and stored at 4°C.

Bacteria Strain

Escherichia coli (*E. coli*, mc4100 strain, ATCC 35695) was cultivated on Lauria Broth agar (LB Broth with agar Lennox, Cat. No.: L2897, Sigma-Aldrich) medium plates at 37°C for ca. 24 hours and then stored at 4°C for future use.

Broth Dilution Experiments for MIC Measurements

To obtain statistical results for MIC analysis, four separate single-colonies of *E. coli* were grown aerobically at 37°C in 4 mL of Terrific Broth (TB) culture media in a shaking flask at 150 rpm for ca. 6 hours. The TB media was composed of 9.52 g TB (Cat. No.: T0918, Sigma-Aldrich) and enriched with 1.6 mL glycerin (Cat. No.: G31-1, Fisher Scientific, USA) in 200 mL distilled deionized water (Millipore, 18.2 MΩ.cm), then autoclaved at 121°C for 20 min. For each sample, 1 ml of the cultured bacteria was transferred to a UV-Vis spectrometer to measure the optical density of the bacteria suspensions at 600 nm. Two bacteria stock samples, with cell densities of ca. 5×10^5 and 5×10^8 cfu/ml, were prepared (i.e., $OD_{600} = 0.1$ is equivalent to 10^8 cfu/ml) from each cultivated colony to be used for MIC assessment. To measure the MIC for AZM, a series of AZM solutions with final concentrations ranging from 0 to 512 µg/ml (i.e., 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 µg/ml) were prepared in a 96-well microtiter plate by combining 50 µl AZM (of various concentrations) and 50 µl of the bacteria stock suspensions. An identical preparation was also performed to deduce the MIC for MG. The last well in each series contained 100 µl of pure TB media (i.e., no bacteria, AZM, or MG), and was used as a control experiment. The 96-well plate was then placed on the shaker to allow the bacteria to grow aerobically at 37°C at 150 rpm for ca. 18 hours. To prevent solvent evaporation in the wells, a distilled deionized water bath was also placed in the shaker. To estimate the number of cultivated bacteria in each well, we performed flow cytometry (FC) measurements by using a BD Accuri C6 Flow Cytometer. In order to reduce the effect of debris in the FC analysis, thresholds for forward scattering (FSC) and side scattering (SSC) signals were set to 10,000 for FSC-H and 100 for SSC-H. FC experiments were carried out with a medium flow rate and a limited total volume of 10 µl of sample. The FSC signals describe the relative size of the particles (i.e., bacteria or debris) passing through the laser beam. In order to do single-cell analysis (i.e., remove doublets from our calculations), we exploited an FSC-A versus FSC-H counter plot (**Figure S3a**) in which doublets appear as a separate population toward higher FSC-A values. **Figure S3b** depicts the FSC-H plots of the singlet region for untreated *E. coli* (black) and *E. coli* treated with 64 µg/ml AZM (low cell density = 10^5 ml^{-1}, blue signal) and 256 µg/ml AZM (high cell density = 10^8 ml^{-1}, red signal). The healthy untreated control sample of bacteria showed a well-resolved high population (centered around ca. 10^5) in the FSC-H histogram. Conversely, in the above MIC AZM-treated samples, there is no apparent population of bacteria, only a disperse population at very small values of FSC-H signal, which corresponds to debris. **Figure S3c** depicts the same analysis but for MG. As shown, in the *E. coli* sample treated with 32 µg/ml MG (high cell density = 10^8 ml^{-1}, red signal), there is no significant population of bacteria, only a disperse population of debris.

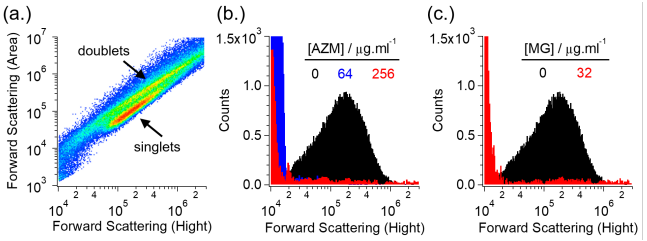

Figure S3. Representative flow cytometry signals collected after broth dilution experiments. (a.) Contour plot profiles of untreated *E. coli* depicting two distinct populations corresponding to doublet and singlet bacteria populations. (b.) The FSC histograms of samples of low (10^5 ml^{-1}, blue signal) and high (10^8 ml^{-1}, red signal) cell densities, treated with 64 and 256 µg/ml AZM, respectively. The histogram of untreated *E. coli* is shown in black. (c.) The FSC histograms of samples of high cell density (10^8 ml^{-1}, red signal) treated with 32 µg/ml MG (100 µM).

Bacteria Sample Preparation for SHS Experiments

A single colony of the bacteria was grown aerobically at 37°C in 50 mL of TB culture media in a shaking flask at 150 rpm for ca. 8 hours (i.e., mid-to-late exponential phase). The harvested bacteria were centrifuged (i.e., 1500xg, 5 min, room temperature) and then washed twice with enough phosphate buffer saline (i.e., 1xPBS; pH=7.3) to remove waste and residual TB. For each washing step, a Rotamix (10101-RKVSD, ATR Inc.) was used at 20 rpm to re-suspend the cells in 1xPBS, while applying minimal biomechanical forces to the cells. After each washing step, the supernatant was removed and the resulting cellular pellets were collected for preparation of the *E. coli* stock samples in 1xPBS.

Time-Resolved Second-Harmonic Laser Scattering

For each SHS experiment, an appropriate amount of each stock was used to achieve the final concentration of 25 µM MG, and either 0, 18.7, 56.2, or 112.4 µg/ml AZM (i.e., 0, 25, 75, and 150 µM AZM) in the liquid flow jet. All solutions were allowed to equilibrate to room temperature prior to initiating the experiments. Our SHS experimental setup has been described previously in detail[3]. The setup consists of a mode-locked Ti:Sapphire laser (Coherent, Micra V, oscillator only) tuned to 800±10 nm and delivering pulses of ca. 150 fs at a repetition rate of 76 MHz with ~ 4 nJ pulse energy. The aver-

age output power of the laser is ca. 300 mW. A long-pass filter (Schott, RG695) was placed before the objective lens to remove any residual light generated at the second harmonic frequency prior reaching the sample.

SHS was measured while the sample circulated in a liquid flow system (i.e., to minimize laser absorption losses and multiple scattering effects), which was formed by pumping the sample through a circular stainless-steel nozzle (1/16" inner diameter). Nalgene tubing (Nalge Nunc, Inc.) was used both to connect the sample reservoir with the inlet of a motorized liquid pump (Micropump, Inc.), as well as to recollect the sample back into the reservoir.

For instantaneous (0 hour) exposure experiments, an aliquot of the bacteria stock suspension (with a final cell density of ca. 10^8 ml^{-1}) was added into the flowing MG or MG+AZM solutions (at t=0 s) to achieve the appropriate final concentration of 25 μM of MG and 0 to 150 μM of AZM. For the 1 hour exposure experiments, we first treated the bacteria with 0 to 150 μM of AZM for 1 hour, then added sufficient volume of the treated bacteria stock suspension (with a final density of ca. 10^8 ml^{-1}) into the flowing MG or MG+AZM solutions (at t=0 s) to achieve the appropriate final concentration of 25 μM of MG and 0 to 150 μM of AZM. The second harmonic light (ca. 400 nm) was collected in the forward scattering direction after passing through a collective lens, a BG39 band-pass filter, and finally a monochromator. A photomultiplier tube (Hamamatsu, R585) was used to detect the second-harmonic photons. The second-harmonic signal passed through a preamplifier (Stanford Research System, SR440), and was processed through a correlated photon counting system (Stanford Research System, SRS SR400). Signal was collected in 1 second intervals, with a gate time of 0.5 seconds. The second-harmonic signal was recorded at 400 nm.

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