Langmuir
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Duramycin-Induced Destabilization of a Phosphatidylethanolamine Monolayer at the Air−Water Interface Observed by Vibrational Sum-Frequency Generation Spectroscopy

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Received July 23, 2010. Revised Manuscript Received September 14, 2010

Duramycin is a small tetracyclic peptide which binds specifically to ethanolamine phospholipids (PE). In this study, we used lipid monolayers consisting of 1-palmitoyl-2-oleoyl phosphatidylethanolamine (POPE) and various phosphatidylcholines (PC) to investigate the effect of duramycin on the organization of lipids and its influence on surrounding water molecules, using vibrational sum-frequency generation spectroscopy in conjunction with surface pressure measurements and fluorescence microscopy. The results show that while duramycin has no effect on the PC lipid monolayers, it induces significant disorder of PE molecules and causes an increase of the PE monolayer surface pressure. Duramycin adopts a β-sheet conformation and is well-ordered at the air−water interface as well as after binding to PE. Our results are consistent with duramycin inserting into the PE monolayer via its hydrophobic end, exposing phenylalanine residues to the lipid. Binding of duramycin to PE broadens the hydrogen-bond distribution of lipid-bound water molecules, notably increasing the fraction of the less strongly hydrogen-bonded, possibly undercoordinated, water molecules. Fluorescence microscopy reveals that the interaction of duramycin with PE causes a change in the shape of the liquid-condensed domains of the PE monolayer from circular to horseshoe-like, indicating a reduction of line tension at the boundary of the two lipid phases. These results reveal that the first steps in the disruption of membrane integrity by duramycin consist of a reduction of the line tension, a decrease in the lipid order, and a weakening of the hydrogen bonding network of water around PE.

Introduction

Duramycin and cinnamycin are small, tetracyclic peptides, produced by Gram-positive bacteria.[1] Both peptides belong to the lantibiotic group of toxins and are known to disrupt bacterial and mammalian cell membranes.[2,3] One of the remarkable properties of these peptides is their ability to specifically bind to lipids containing phosphoethanolamine (PE)[4,5] in the lipid headgroup part (Figure 1A), separating it from other antimicrobial peptides for which the interaction with membranes is generally of electrostatic nature and therefore nonspecific.[6] The recent interest in duramycin/cinnamycin membrane interaction is motivated, on the one hand, by the possibility it offers to study the distribution and dynamics of specifically PE in cell membranes.[7−9] On the other hand, interest is motivated by the potential application of duramycin as a next generation antibiotic.[10] Although the specific interaction between duramycin and PE has been demonstrated,[2] it has remained challenging to determine the details of the interaction between duramycin and PE at the molecular level. Specific questions have remained unanswered, in particular regarding the degree to which the duramycin organization is collective, the effect of duramycin on the organization of lipids, and the role of water in the peptide−lipid interaction.

Figure 1B shows the amino acid sequence and approximate structure of cinnamycin and duramycin derived from nuclear magnetic resonance (NMR) studies.[11,12] A total of 19 amino acids assemble to form a hydrophobic end, involving three phenylalanine residues, along one side, and a hydrophilic part on the opposite side. The difference between these two peptides is the presence of arginine in cinnamycin where there is lysine in duramycin.

Cinnamycin is known to form a tight equimolar complex with PE. The proposed structure of the complex, based on the NMR analysis,[13] is depicted in Figure 2. There is a pocketlike region formed by residues Phe-7 through Ala-14 at the hydrophobic end, which specifically binds the ethanolamine end of PE. It is proposed that the interaction with the ethanolamine is stabilized

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(1) Oscáriz, J. C.; Pisabarro, A. G. *Int. Microbiol.* **2001**, *4*, 13–19.
(2) Iwamoto, K.; Hayakawa, T.; Murate, M.; Makino, A.; Ito, K.; Fujisawa, T.; Kobayashi, T. *Biophys. J.* **2007**, *93*(5), 1608–1619.
(3) Makino, A.; Baba, T.; Fujimoto, K.; Iwamoto, K.; Yano, Y.; Terada, N.; Ohno, S.; Sato, S. B.; Ohta, A.; Umeda, M.; Matsuzaki, K.; Kobayashi, T. *J. Biol. Chem.* **2003**, *278*(5), 3204–3209.
(4) Choung, S. Y.; Kobayashi, T.; Takemoto, K.; Ishitsuka, H.; Inoue, K. *Biochim. Biophys. Acta* **1988**, *940*, 180–187.
(5) Machaidze, G.; Ziegler, A.; Seelig, J. *Biochemistry* **2002**, *41*(6), 1965–1971.
(6) Brogden, K. A. *Nat. Rev. Microbiol.* **2005**, *3*(3), 238–250.
(7) Zhao, M.; Li, Z.; Bugenhagen, S. *J. Nucl. Med.* **2008**, *49*(8), 1345–1352.
(8) Zhao, M. *Amino Acids* **2009**, DOI 10.1007/s00726-009-0386-9.
(9) Emoto, K.; Kobayashi, T.; Yamaji, A.; Aizawa, H.; Yahara, I.; Inoue, K.; Umeda, M. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*(23), 12867–12872.
(10) Marshall, S. H.; Arenas, G. *Electron. J. Biotechnol.* **2003**, *6*(3), 271–284. ISSN 0717-3458. Available from: http://www.ejbiotechnology.info/content/vol6/issue3/full/1/reprint.html .
(11) Zimmermann, N.; Freund, S.; Fredenhagen, A.; Jung, G. *Eur. J. Biochem.* **1993**, *216*(2), 419–428.
(12) Kessler, H.; Mierke, D. F.; Saulitis, J.; Seip, S.; Steuernagel, S.; Wein, T.; Will, M. *Biopolymers* **1992**, *32*(4), 427–433.
(13) Hosoda, K.; Ohya, M.; Kohno, T.; Maeda, T.; Endo, S.; Wakamatsu, K. *J. Biochem.* **1996**, *119*(2), 226–230.

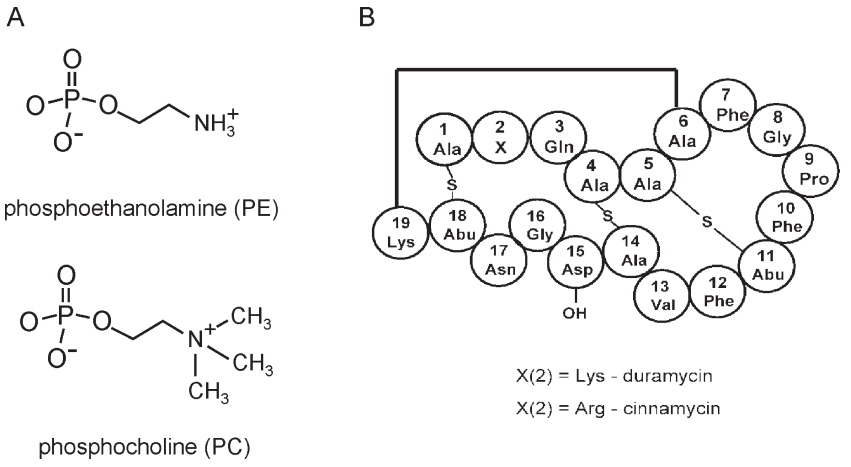

Figure 1. Schematic showing (A) phosphoethanolamine and phosphocholine of the lipid headgroup part, (B) the structure of duramycin and cinnamycin (Ro09-0198). Abbreviations: Ala, alanine; X_2, lysine (duramycin) or arginine (cinnamycin); Gln, glutamine; Phe, phenylalanine; Pro, proline; Abu, α-aminobutyric acid; Val, valine; Asp, aspartic acid; Gly, glycine; Asn, asparagine; Lys, lysine. Ala_6 is linked to Lys_{19} as lysinoalanine. The picture was drawn according to ref 11.

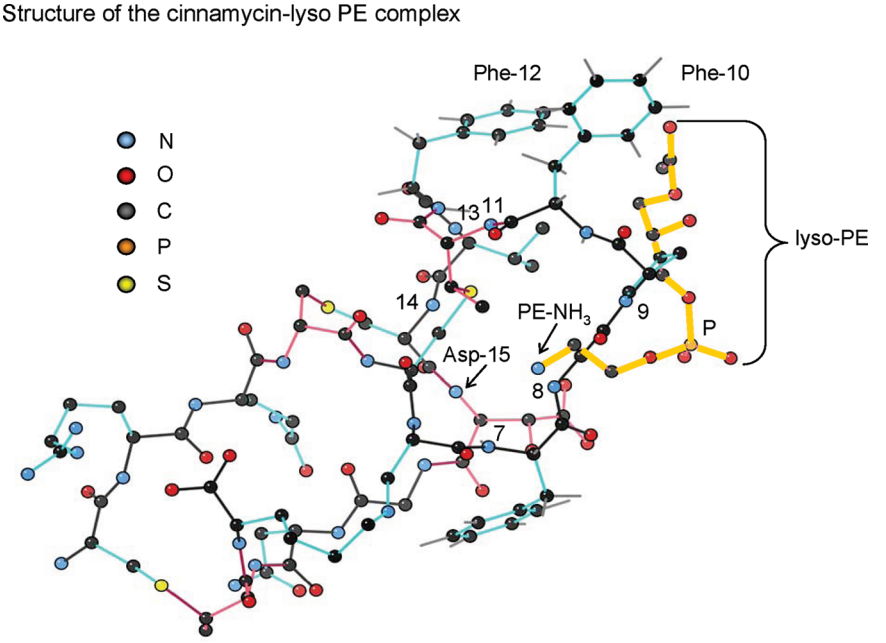

Figure 2. Three-dimensional structure of cinnamycin lyso−PE complex. The figure was prepared using the program King (http://kinemage.biochem.duke.edu) with the structure coordinates (PDB ID: 2dde) file downloaded from the Protein Data Bank (http://www.rcsb.org.pdb).[13] Numbers 7−14 denote residues forming the hydrophobic pocket.

by an ionic interaction between the ammonium group of phosphoethanolamine and the carboxylate group of Asp15 residue. Additional stability is provided by hydrophobic interactions involving Gly-8, Pro-9, Val-13, and two Phe-10 and 12 residues, with the glycerol moiety of the lipid.

Electron microscopy and small-angle X-ray scattering (SAXS) of PE containing liposomes have shown that the morphology of the lipid membrane drastically changes upon duramycin binding to PE within the bilayer.[2] Scanning probe microscopy studies of supported lipid bilayers also showed the shape change of PE-containing lipid bilayer by duramycin.[14] Specifically, duramycin destabilizes small (~10 nm diameter) lipid vesicles, causing surface-adsorbed vesicles to fuse on the surface, giving rise to lipid bilayers and multilayers. Moreover, increased membrane permeability has been observed after treatment with duramycin of cellular membranes containing PE.[2]

Here, we report our study of duramycin interaction with phospholipid monolayers using vibrational sum-frequency generation spectroscopy (VSFG). Surface sum-frequency generation (SFG) is a laser-based nonlinear vibrational spectroscopy and has recently been demonstrated to be a powerful technique to study the interaction between peptides and model membranes.[15,16] The strength of SFG lies in its ability to monitor (through their molecular vibrations) details of the organization and conformation of the three key players in the interaction: duramycin, lipids, and water.

(14) Matsunaga, S.; Matsunaga, T.; Iwamoto, K.; Yamada, T.; Shibayama, M.; Kawai, M.; Kobayashi, T. *Langmuir* **2009**, *25*(14), 8200–8207.

(15) Chen, X.; Wang, J.; Boughton, A. P.; Kristalyn, C. B.; Chen, Z. *J. Am. Chem. Soc.* **2007**, *129*(5), 1420–1427.

(16) Nguyen, K. T.; Le Clair, S. V.; Ye, S.; Chen, Z. *J. Phys. Chem. B* **2009**, *113*(36), 12358–12363.

Duramycin is studied through its amide vibrations as well as its C−H stretch vibration of the unsaturated =C−H groups of the aromatic phenylalanine amino acid residue. Lipids are studied using their C−H stretch vibrations, providing information about order in their tails.[17] Finally, changes in the lipid-bound water structure are obtained from the O−H (O−D) stretch vibration of (heavy) water. The use of a monolayer (rather than bilayers) allows for detailed control over the lipid properties (surface pressure and composition) and seems warranted, as we are interested in the initial steps of the duramycin−lipid interaction which may occur on one leaflet of a bilayer membrane.

We present data for pure lipid monolayers, pure duramycin monolayer, and lipid monolayers in the presence of duramycin. Our results confirm the specific duramycin−PE interaction. Novel observations include (1) the highly ordered and uniform orientation of duramycin molecules at the water−lipid interface; (2) duramycin-induced disorder of lipid molecules in the monolayer, despite the increase in the surface pressure and; (3) a weakening of the water hydrogen-bonding structure upon binding of duramycin. The latter two observations are tentatively attributed to the onset of membrane destabilization upon binding of duramycin.

Experimental Section

Materials. The following materials were purchased from Avanti Polar Lipids Inc. (Alabama, AL) and used without further purification: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE; 16:0/18:1), 1-palmitoyl(d31)-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (dPOPE; 16:0 D31/18:1), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC; 18:1/18:1), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC; 16:0/16:0), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC; 14:0/14:0), 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC; 18:1−12:0), and 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphoethanolamine (NBD-PE; 18:1−12:0), used as a fluorescent marker for microscopy measurements. All lipids were dissolved in chloroform (Sigma-Aldrich) to a final concentration of 0.5 μg/μL for nonlabeled lipids and 0.2 μg/μL for lipids labeled with a fluorescent tag.

The phosphate buffer (10 mM, pH = 7.5) was prepared with D_2O (Cambridge Isotope Laboratories, Inc., 99.93% purity) to avoid interference of O−H stretch vibrations with the C−H stretch vibrations. Duramycin from *Streptoverticillium cinnamoneus* was from Sigma (St. Louis, MO). It was dissolved in water to a final concentration of a stock solution 0.7 mM.

Lipid Monolayers. All experiments on lipid monolayers were performed at room temperature (22 °C) in a commercial microtrough (Delta Pi, Kibron, Finland, surface area of 12 cm^2). Droplets of lipid solutions in chloroform were spread onto a phosphate-buffered solution using a Hamilton microsyringe equipped with a repeating dispenser. The surface pressure was measured with a Dynaprobe instrument (Kibron, Finland), which consisted of a thin metal wire. The initial monolayer surface pressure (before injection of duramycin) was set in the range of 30−40 mN/m, similar to typical pressures of lipids in biological cell membranes,[18,19] and corresponding to a molecular density of lipid of about 2.7×10^{14} lipid molecules/cm^2.

The surface pressure was adjusted by changing the amount of lipid solution that was spread at the air−water interface. After equilibration, fluorescence images and SFG spectra were recorded on pure lipid monolayers and after injection of duramycin into the aqueous subphase. The duramycin solution (in water) was injected *beneath* the lipid monolayer with a microsyringe.

Surface Activity of Duramycin. Duramycin is an amphiphatic molecule and is therefore expected to be surface-active (i.e., adsorb at the air−water interface).[11] In order to study the insertion of duramycin into lipid monolayers, we ensured that the surface pressure due to the lipids was significantly larger than that attainable by the peptide. This allowed us to distinguish the surface activity of the peptide from the lipid−peptide interaction. An experiment recording the surface pressure at the air−water (phosphate buffer) interface of a duramycin solution indicated a maximum surface pressure of 15 mN/m, significantly lower than the surface pressure of lipid monolayers.

Fluorescence Microscopy. Fluorescence microscopy images were taken with an Olympus BX51 M microscope fitted with a 10× objective lens, NA 0.25, and a digital camera system (Olympus DP71). The optical resolution was 1.3 μm. A mixture of nonlabeled (99 mol %) and NBD-labeled lipids (1 mol %) was used to prepare lipid monolayers. The NBD dye is covalently attached to a single end of the lipid hydrocarbon chain. The addition of the fluorescent probe in the range of 0.1−2 mol % has no significant effect on the monolayer transition pressure.[20] A mercury lamp and band-pass filter (420−480 nm) were used for NBD excitation (NBD λ_{exc} = 465 nm λ_{emiss} = 534 nm). Lipids with fluorescent probes are known to partition into the liquid expanded phase (LE), causing bright fluorescent images.[21]

Vibrational Sum-Frequency Generation (VSFG). VSFG spectroscopy is a type of coherent second-order nonlinear optical spectroscopy, in which an infrared (IR) beam and a visible (VIS) beam are spatially and temporally combined at a surface or interface, generating a signal whose frequency is the sum of the infrared and visible frequencies.[22] The intensity of the emitted VSFG field is strongly enhanced when the IR frequency is resonant with a vibrational transition of molecule at the surface. The intensity of the signal for particular molecular vibration depends on the coverage of molecules at the interface (generally the dependence of the signal strength is quadratical with coverage) and on the orientation of their dipoles (increasing as more dipoles have the same orientation). Our VSFG setup was previously described in detail.[23] All VSFG spectra were recorded under *ssp* (SFG, VIS, IR) polarization conditions, in the range of 1500−3200 cm^{-1}, while scanning wavelength with the IR laser. The incident angles for the VIS and IR beams were 35° and 40° with respect to the surface normal, respectively. The spectra were normalized using a reference signal from z-cut quartz.

The SFG intensity is proportional to the square of the second-order nonlinear susceptibility $\chi^{(2)}$ of the sample and the intensities of the visible and infrared beams:

$$I_{SFG} \propto |\chi^{(2)}|^2 I_{VIS} I_{IR} \quad (1)$$

The susceptibility $\chi^{(2)}$ consists of a nonresonant term and a resonant term. Assuming that the resonant contributions can be approximated by Lorentzian functions, the overall susceptibility is

$$\chi^{(2)} = \chi^{(2)}{}_{NR} + \chi^{(2)}{}_{R} = A_0 \, e^{i\phi} + \sum_n \frac{A_n}{\omega_n - \omega_{IR} - i\Gamma_n} \quad (2)$$

where A_0 represents the amplitude of the nonresonant susceptibility, ϕ is the phase, and A_n is the amplitude of the nth vibrational mode, with resonant frequency ω_n and line width Γ_n. This equation shows that when the frequency of the incident infrared beam is in resonance with a vibrational mode (n), the SFG signal is

(17) Watry, M. R.; Tarbuck, T. L.; Richmond, G. L. *J. Phys.Chem. B* **2003**, *107*(2), 512–518.
(18) Demel, R. A.; Geurts van Kessel, W. S. M.; Zwaal, R. F. A.; Roelofsen, B.; van Deenen, L. L. M. *Biochim. Biophys. Acta* **1975**, *406*(1), 97–107.
(19) Blume, A. *Biochim. Biophys. Acta* **1979**, *557*, 32–44.
(20) Neville, F.; Cahuzac, M.; Konovalov, O.; Ishitsuka, Y.; Lee, K. Y. C.; Kuzmenko, I.; Kale, G. M.; Gidalevitz, D. *Biophys. J.* **2006**, *90*(4), 1275–1287.
(21) Knobler, C. M. *Science* **1990**, *249*(4971), 870–874.
(22) Shen, Y. R. *Nature* **1989**, *337*(6207), 519–525.
(23) Smits, M.; Sovago, M.; Wurpel, G. W. H.; Kim, D.; Müller, M.; Bonn, M. *J. Phys. Chem. C* **2007**, *111*(25), 8878–8883.

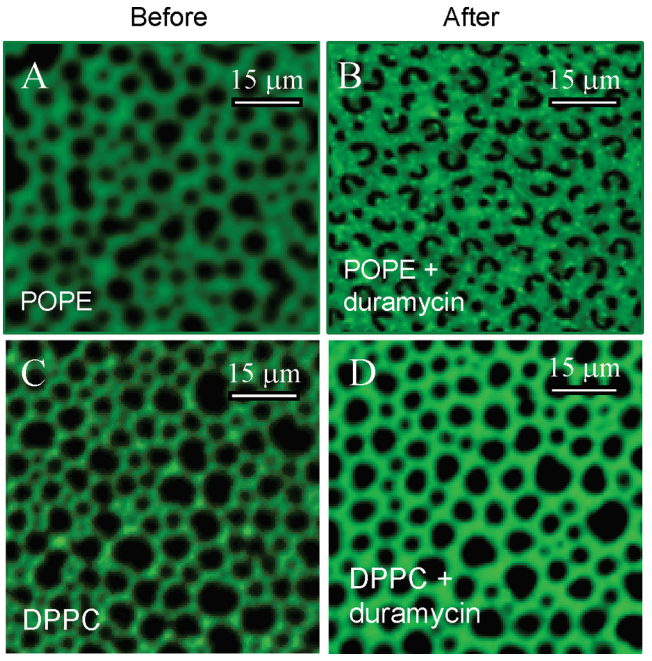

Figure 3. Fluorescence microscopy images for (A) a pure POPE monolayer, (B) the same POPE monolayer in the presence of duramycin, (C) a pure DPPC monolayer, and (D) the same DPPC monolayer in the presence of duramycin. To obtain fluorescent contrast, lipids without fluorescent label were mixed with NBD-tail labeled POPE or POPC (1 mol % NBD-PE or PC).

enhanced. For most systems, including the one studied here, $\chi^{(2)}$ can only be nonzero at an interface, where the symmetry is broken. Thus, the probe depth of VSFG is strictly defined by the region that lacks inversion symmetry. The ability of VSFG to provide structural information about surfaces makes it particularly well-suited to the study of lipid interfaces and membrane physics, without the need to attach fluorescent labels.[23−25] For phospholipids monolayers, characteristic vibrations of lipid tails and heads can be observed, as well as vibrations of water molecules around the lipid headgroups and water molecules present close to the lipid hydrocarbon chains.[24,26] Equation 2 was used to fit the VSFG spectra, as described in the literature.[24,27]

Results and Discussion

Reduction of Line Tension at the Phase Boundary of Lipid Domains: Fluorescence Microscopy. POPE shows gel to liquid crystalline phase transition temperature (T_c) at 25 °C.[28] In Figure 3, we examined fluorescence images before and after injection of duramycin below a POPE monolayer at 22 °C, which is below the T_c of POPE. We also studied the effect of duramycin on DPPC (T_c = 41 °C).[29] In all images, two coexisting phases are seen: (i) a liquid-condensed (LC) phase appearing as black (nonfluorescent) domains and (ii) a liquid expanded (LE) phase appearing as bright (fluorescent) domains.[21] The existence of two phases is consistent with the presence of a plateau in the pressure−area isotherm of a pure POPE and DPPC monolayer.[30,31] For pure monolayers, the condensed domains have a circular shape with an average diameter of 6 μm. The circular morphology is due to the finite line tension between the two lipid phases.[32]

When duramycin is injected into the subphase beneath the POPE monolayer (Figure 3B), the LC domains developed a horseshoe-like shape. According to ref 33, the area/perimeter (A/P) ratio of a domain is directly proportional to the line tension.[33] The A/P ratio, calculated from the number of pixels in the domain and on its perimeter, is 3.7 for the circular and 1.8 for the horseshoe-like domains. This decrease in the A/P ratio indicates that duramycin reduces the line tension at the boundary between the LC and LE phases. The morphology of the POPE monolayer changed quickly after addition of duramycin to the subphase. The sharper contrast in the fluorescence images with duramycin present indicates that the lateral diffusional mobility of the domains was greatly reduced. There was no change in the DPPC domain structure when duramycin was added beneath the DPPC monolayer (Figure 3D). The same experiment for a DOPC (liquid crystalline at 22 °C)[34] monolayer also revealed no changes in the monolayer morphology by duramycin, where it should be noted that for DOPC there is no coexistence of different phases at room temperature.[31]

A theory developed by the McConnell group[35−37] interprets the size and shape of lipid domains at the air−water interface as a competition between line tension and dipole densities. In particular, this theory states that line tension favors large circular domains, whereas long-range dipolar and electrostatic forces favor small and/or extended and irregular domain sizes and shapes. Thus, it is likely that the irregular domain shapes that we observed for the POPE monolayer after injection of duramycin (Figure 3B) were due to an increase of electrostatic dipole densities that occurred within the lipid domains after duramycin binding. More specifically, our POPE/duramycin system has an ionic electrostatic dipole, leading to a predominance of long-range dipolar forces.[35] Indeed ion pair formation between the positively charged amino group of PE and the negatively charged aspartic acid (Asp-15) residue of cinnamycin has been suggested based on the NMR studies.[13] According to the theory, an increased contribution of electrostatic interaction must be associated with a reduction in line tension which is observed as a change of the domains shape in the fluorescence images.

Vibrational Sum-Frequency Generation Spectroscopy. Here, we used VSFG to study the arrangement of duramycin and lipid molecules at the air−water and lipid−water interfaces. For this, we recorded vibrational fingerprints in the amide I band, the lipid C−H stretch vibrations, and the water O−D stretch vibrations. The amide I band arises primarily from in-plane peptide C═O stretch vibrations.[38] In our experiments, we used D_2O, since our setup works better at O−D than O−H stretch frequencies. The use of D_2O also allowed us to avoid the overlap between the amide I band and the H_2O bending mode.

Conformation of Duramycin at Interfaces: SFG in the Amide I Region. The peptide conformation at the lipid−water

(24) Ma, G.; Allen, H. C. *Langmuir* **2006**, *22*(12), 5341–5349.
(25) Liu, J.; Conboy, J. C. *J. Am. Chem. Soc.* **2004**, *126*(29), 8894–8895.
(26) Sovago, M.; Vartiainen, E.; Bonn, M. *J. Chem. Phys.* **2009**, *131*(16), 161107-1–161107-4.
(27) Zhuang, X.; Miranda, P. B.; Kim, D.; Shen, Y. R. *Phys. Rev. B* **1999**, *59*(19), 12632–12640.
(28) Epand, R. M.; Bottega, R. *Biochim. Biophys. Acta* **1988**, *944*(2), 144–154.
(29) Jacobson, K.; Papahadjopoulos, D. *Biochemistry* **1975**, *14*(1), 152–161.
(30) Domènech, Ò.; Torrent-Burgués, J.; Merino, S.; Sanz, F.; Montero, M. T.; Hernández-Borrell, J. *Colloids Surf., B* **2005**, *41*(4), 233–238.
(31) Tamm, L. K.; McConnell, H. M. *Biophys. J.* **1985**, *47*(1), 105–113.
(32) Akimov, S. A.; Kuzmin, P. I.; Zimmerberg, J.; Cohen, F. S.; Chizmadzhev, Y. A. *J. Electroanal. Chem.* **2004**, *564*, 13–18.
(33) Lin, W.-C.; Blanchette, C. D.; Longo, M. L. *Biophys. J.* **2007**, *92*(8), 2831–2841.
(34) Ulrich, A. S.; Sami, M.; Watts, A. *Biochim. Biophys. Acta* **1994**, *1191*(1), 225–230.
(35) Benvegnu, D. J.; McConnell, H. M. *J. Phys. Chem.* **1993**, *97*(25), 6686–6691.
(36) Benvegnu, D. J.; McConnell, H. M. *J. Phys. Chem.* **1992**, *96*(16), 6820–6824.
(37) McConnell, H. M. *Annu. Rev. Phys. Chem.* **1991**, *42*(1), 171–195.
(38) Chen, X.; Wang, J.; Sniadecki, J. J.; Even, M. A.; Chen, Z. *Langmuir* **2005**, *21*(7), 2662–2664.

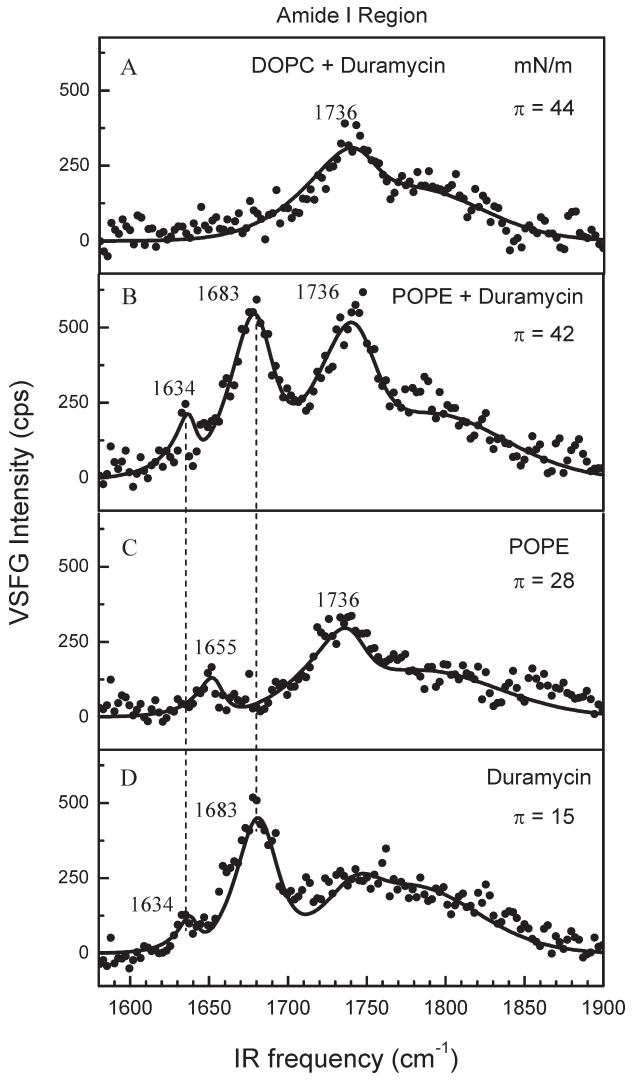

Figure 4. VSFG spectra in the C=O stretch region for (A) a DOPC monolayer, (B) a POPE monolayer in the presence of duramycin at a solution concentration of 0.7 μM, (C) a POPE monolayer in the absence of duramycin, and (D) a pure duramycin monolayer. The solid lines represent fits to the data using a Lorentzian model.

interface influences the biological activity.[39] It is however difficult to observe the structure of the peptide at the lipid−water interface. Only a few spectroscopic methods, such as SFG, attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR), and circular dichroism (CD) can provide such information. Some studies have shown that the conformation of the peptide in solution is different from its conformation after binding to a lipid matrix.[40] In particular, most of the α-helical peptides tend to change structure upon adsorption.[41]

Figure 4 shows VSFG spectra in the amide I region for a pure monolayer of duramycin (Figure 4D), a pure monolayer of POPE (Figure 4C), and for the POPE and DOPC monolayers after addition of duramycin (Figure 4B and A, respectively). The spectrum for duramycin (Figure 4D) has two sharp peaks, one at 1634 cm^{-1} and the other one at ~1683 cm^{-1}. According to the literature, the band at 1634 cm^{-1} can be assigned to the B_2 mode and the band at 1683 cm^{-1} to the B_1/B_3 modes, both associated with antiparallel β-sheets.[42,43] The observation of strong amide bands in the SFG spectrum implies that duramycin molecules are highly oriented at the air−water interface. The uniform orientation of duramycin at the interface is a prerequisite for its spectroscopic detection using SFG; for a disordered monolayer, the signals from oppositely oriented C=O dipoles would cancel out. The 1736 cm^{-1} peak observed for both PE and PC monolayers (Figure 4C, B, and A) is due to the C=O stretch vibration of the lipid ester group. The spectrum for the pure POPE monolayer (Figure 4C) contains an additional peak at 1655 cm^{-1} which we tentatively assigned to the N−D bending motion of the ethanolamine group, where the D atom bridges N with O in the PO_4 group. It is highly probable that hydrogen atoms on the NH_3 group are replaced with deuterium atoms, since D_2O was a medium in the buffer solution. A comparison of Figure 4B and D implies that the structure of duramycin at the lipid−water interface (Figure 4B) is the same as it is at the air−water interface (Figure 4D). This is in agreement with a recent molecular dynamics simulation study on the structural stability of β-peptides at an interface.[44]

No bands associated with the peptide vibrational modes were observed when duramycin was present in the subphase beneath the DOPC monolayer (Figure 4A). It appears that duramycin does not form an ordered interfacial structure near the DOPC monolayer. This observation is consistent with the previously observed lack of interaction between duramycin and PC lipids in vesicle studies.[2]

Destabilization of Lipids in the Presence of Duramycin: SFG in the C−H Stretch Region. The unique selection rules of SFG that inversion symmetry must be broken allows one to investigate the order of lipids at the air−water interface by monitoring the intensity ratio of the methyl and the methylene groups in the lipid alkyl chains.[17] Figure 5 shows VSFG spectra in the C−H stretch region for monolayers of DMPC (T_c = 24 °C)[45] and POPE spread at the air−water interface (Figure 5A and C). The solid lines in the figures were obtained by fitting using eq 2. Three intense peaks are seen in both spectra, which are assigned, according to previous studies, to the symmetric CH_2 stretch mode at 2850 cm^{-1} (CH_2−SS), the symmetric stretch mode of the terminal CH_3 group at 2875 cm^{-1} (CH_3−SS), and a third peak at around 2950 cm^{-1} is ascribed to the asymmetric CH_3 stretch mode, in combination with contributions from Fermi resonances.[27] When lipid molecules are ordered, the SFG intensity of the methylene relative to methyl stretch is low, due to, respectively, the inversion symmetry of an all-trans alkyl chain and collective orientation of the methyl groups. On the other hand, when gauche defects are formed, the inversion symmetry within the alkyl chain is broken and the relative intensity of the methylene symmetric stretch mode increases; the disorder reduces the methyl intensity.[46] In the spectrum for pure POPE (Figure 5C) monolayer, the CH_2 symmetric stretch relative to the CH_3 symmetric stretch is much more pronounced than that for DMPC (Figure 5A), suggesting that tails of the lipids are less ordered. This disorder in POPE is due to kinks in the lipid tails formed by the presence of an unsaturated C=C bond in the POPE molecules.

In order to investigate the effect of duramycin on the lipid organization, we first recorded VSFG spectra in the C−H region

(39) Powers, J.-P. S.; Hancock, R. E. W. *Peptides* **2003**, *24*(11), 1681–1691.
(40) Matsuzaki, K.; Horikiri, C. *Biochemistry* **1999**, *38*(13), 4137–4142.
(41) Shai, Y. *Biochim. Biophys. Acta* **1999**, *1462*(1−2), 55–70.
(42) Hilario, J.; Kubelka, J.; Keiderling, T. A. *J. Am. Chem. Soc.* **2003**, *125*(25), 7562–7574.
(43) Thundimadathil, J.; Roeske, R. W.; Guo, L. *Biophys. J.* **2006**, *90*(3), 947–955.
(44) Miller, C. A.; Abbott, N. L.; de Pablo, J. J. *Langmuir* **2009**, *25*(5), 2811–2823.
(45) Caffrey, M.; Hogan, J. *Chem. Phys. Lipids* **1992**, *61*(1), 1–109.
(46) Sovago, M.; Wurpel, G. W. H.; Smits, M.; Müller, M.; Bonn, M. *J. Am. Chem. Soc.* **2007**, *129*(36), 11079–11084.

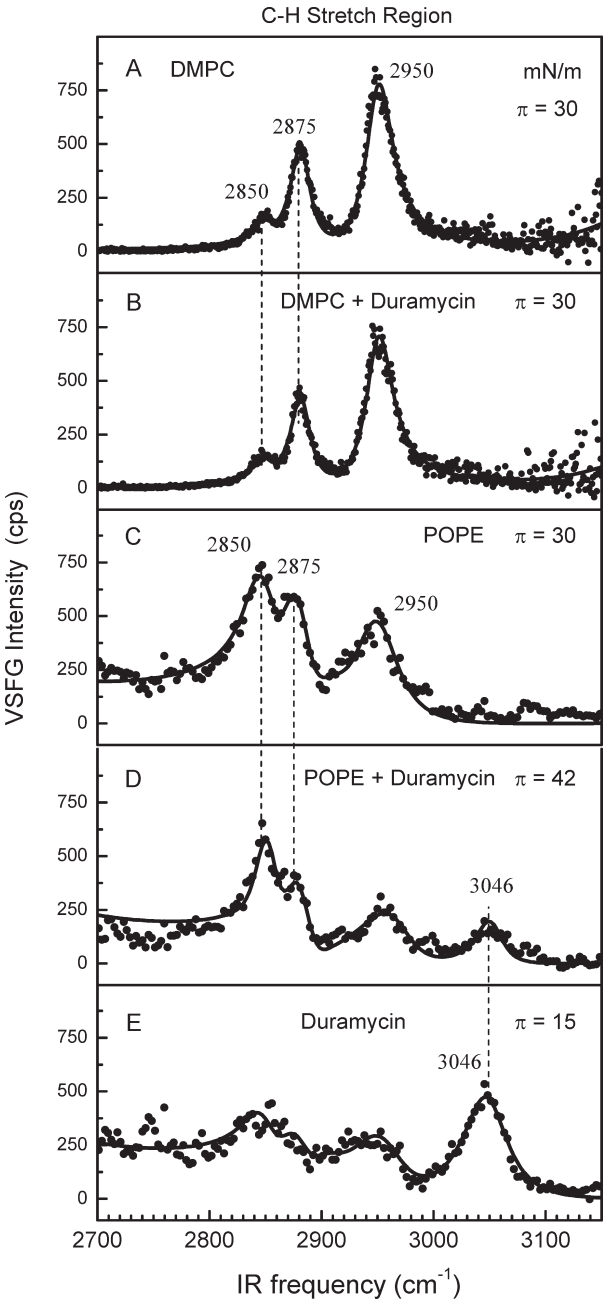

Figure 5. VSFG spectra in the C−H stretch region for (A) a pure DMPC monolayer (deposited on H_2O), (B) the same DMPC monolayer in the presence of duramycin, (C) a pure POPE monolayer, (D) the same POPE monolayer in the presence of duramycin, and (E) a pure duramycin monolayer. The solid lines represent fits to the data using a Lorentzian model. The monolayer surface pressure is given in the right corner of each panel. The final duramycin concentration is below 0.7 μM.

for duramycin present at the air−water interface (Figure 5E). In this spectrum, an intense peak at 3046 cm^{-1} is observed in addition to symmetric and asymmetric CH_3 and CH_2 stretches (2800−3000 cm^{-1}). A peak at 3046 cm^{-1} is typical for the C−H stretch mode of aromatic compounds and originates from the only aromatic amino acid contained in duramycin, the phenylanine (Phe) residues.[47,48] The marked intensity of the 3046 cm^{-1} peak indicates that duramycin molecules are highly oriented at the air−water interface, which was also concluded from the SFG spectra in the amide I region.

Figure 5B and D shows the effect of duramycin on the lipid tails of the DMPC and POPE monolayer. The major difference is that there is no effect of duramycin on the DMPC signal (and DOPC, data not shown), whereas for the POPE monolayer several changes are evident: the appearance of the 3046 cm^{-1} peak and changes in the remainder of the spectrum. The monolayer surface pressure also increased from 30 to 42 mN/m, indicating incorporation of duramycin into the POPE containing monolayer,[20] whereas the surface pressure for DMPC remained at 30 mN/m. The observation that the Phe peak at 3046 cm^{-1} is comparable in strength to the lipid C−H stretch bands is somewhat remarkable; given the fact that duramycin contains only three phenylalanine amino acids, with one benzene moiety each. The Raman and IR cross sections of the aromatic =C−H stretch are comparable to that of the C−H stretches of the methyl and methylene groups of the lipid. This indicates that the same degree of ordering is present in the duramycin and in the lipid tails. Hence, we can conclude not only that there is very little variation in peptide structure, but that for the ensemble of peptides their orientation is quite uniform.

The addition of duramycin into the subphase below POPE (Figure 5D) monolayer causes a signal intensity decrease over the entire frequency range, which may reflect an overall increase in disorder of the conformation for lipid molecules at the interface. It also results in spectral changes, most notably an increase in the CH_2 symmetric stretch at 2850 cm^{-1} relative to the CH_3 symmetric stretch at 2875 cm^{-1} (comparison of Figure 5C and D). This can be understood from symmetry arguments: when the methylene groups in an alkyl chain change from ordered trans to disordered cis conformation, a local center of inversion disappears which renders the CH_2 modes SFG active. The CH_3 intensities on the other hand will simultaneously decrease, due to a broadening of the angular distributions of chain tilt angles. Given the preceding arguments, the intensity of the CH_2−SS relative to that of the CH_3−SS constitutes an appropriate semiquantitative measure of lipid disorder in pure lipid monolayers.[49] However, since duramycin has oscillator strength in the same spectral region, it is difficult to separate the contributions of the lipids from that of the peptide. To confirm that duramycin indeed induces disorder in the POPE lipid monolayer, we performed experiments on a monolayer containing partially deuterated POPE, having one deuterated alkyl tail. The resultant SFG spectra together with the peak assignment[24] are shown in Figure 6. It is apparent that the intensity of the CD_2 symmetric stretch peak increases upon injection of duramycin, pointing to an increased average number of gauche defects within the alkyl chains, which make an increased number of CD_2 groups SFG-active.

The increase in CD_2 stretch intensity thus testifies to increased molecular disorder of lipids in the POPE monolayer, despite the surface pressure increase from 31 to 39 mN/m. It is uncommon to observe a reduced lipid order when the monolayer surface pressure increases. The observation that the symmetric CD_3 stretch intensity remains unchanged suggests that binding of duramycin to PE lipids does not affect the average orientation (or orientational distribution) of lipid molecules.

Destabilization of Lipid-Bound Water in the Presence of Duramycin: SFG in the O−D Stretch Region. The specific binding of duramycin to PE lipids must be associated with a significant restructuring of water molecules associated with lipids

(47) Kim, G.; Gurau, M. C.; Lim, S.-M.; Cremer, P. S. *J. Phys. Chem. B* **2003**, *107*(6), 1403–1409.
(48) Kim, G.; Gurau, M.; Kim, J.; Cremer, P. S. *Langmuir* **2002**, *18*(7), 2807–2811.
(49) Gurau, M. C.; Lim, S.-M.; Castellana, E. T.; Albertorio, F.; Kataoka, S.; Cremer, P. S. *J. Am. Chem. Soc.* **2004**, *126*(34), 10522–10523.

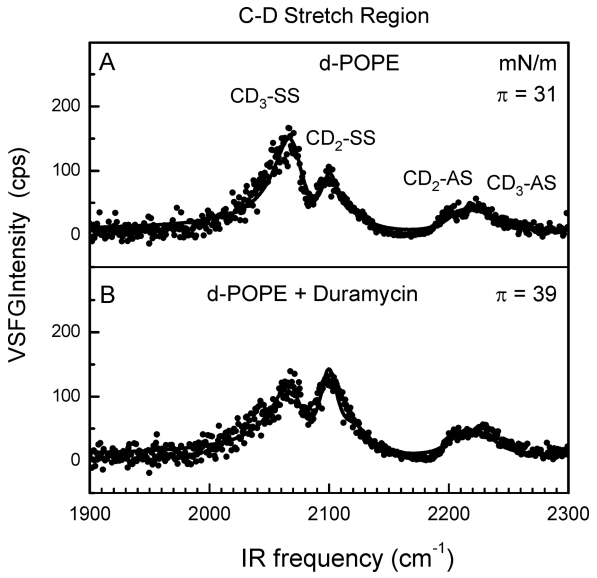

Figure 6. VSFG spectra in the C−D stretch region for (A) a pure dPOPE monolayer (POPE with the saturated lipid alkyl chain deuterated) and (B) the same dPOPE monolayer (deposited on H_2O) in the presence of duramycin at a solution concentration of 0.7 μM.

and duramycin. Although water is anticipated to play an important role in interactions of lipids with peptides, there is little experimental evidence on water involvement in these biologically important interactions because it is difficult to access information on the small number of water molecules located in hydration shells of lipids and peptides. Small quantities of water molecules are detectable using SFG under the condition that their dipoles are uniformly oriented and that symmetry of the environment is broken.

Figure 7 shows VSFG spectra in the O−D stretch region for a pure monolayer of DOPC (Figure 7A) and POPE (Figure 7C) spread at the air−water interface. The spectra for both lipid monolayers are characterized by the presence of a broad peak at 2350 cm^{-1} with a shoulder at ~2500 cm^{-1}. It has been estimated that approximately 10−20 water molecules are associated with each phospholipid molecule.[50,51] These water molecules form a complex network of hydrogen bonds with other water molecules, but also with hydrogen bond-donating and -accepting moieties within the lipid headgroup, including the glycerol group and the carbonyl group of the lipid. The strength of these hydrogen bonds is affected by the local membrane environment and is reflected in the frequency of the O−D stretch mode, with lower frequencies indicating stronger hydrogen bonding. The stretch frequency of an isolated D_2O molecule is at ~2730 cm^{-1}.[52] Accordingly, when the measured O−D frequency is closer to 2730 cm^{-1}, the D_2O molecule is less constrained by the hydrogen-bond environment. The spectra shown in Figure 7A and C indicate fairly strong hydrogen bonding of water molecules associated with DOPC and POPE lipid molecules. The main peak at 2350 cm^{-1} and the shoulder at higher frequency[53] can be attributed to the O−D stretch mode of water molecules in the extended hydrogen-bonding network, in which there are up to four hydrogen bonds per water molecule. This is because water in crystalline ice films

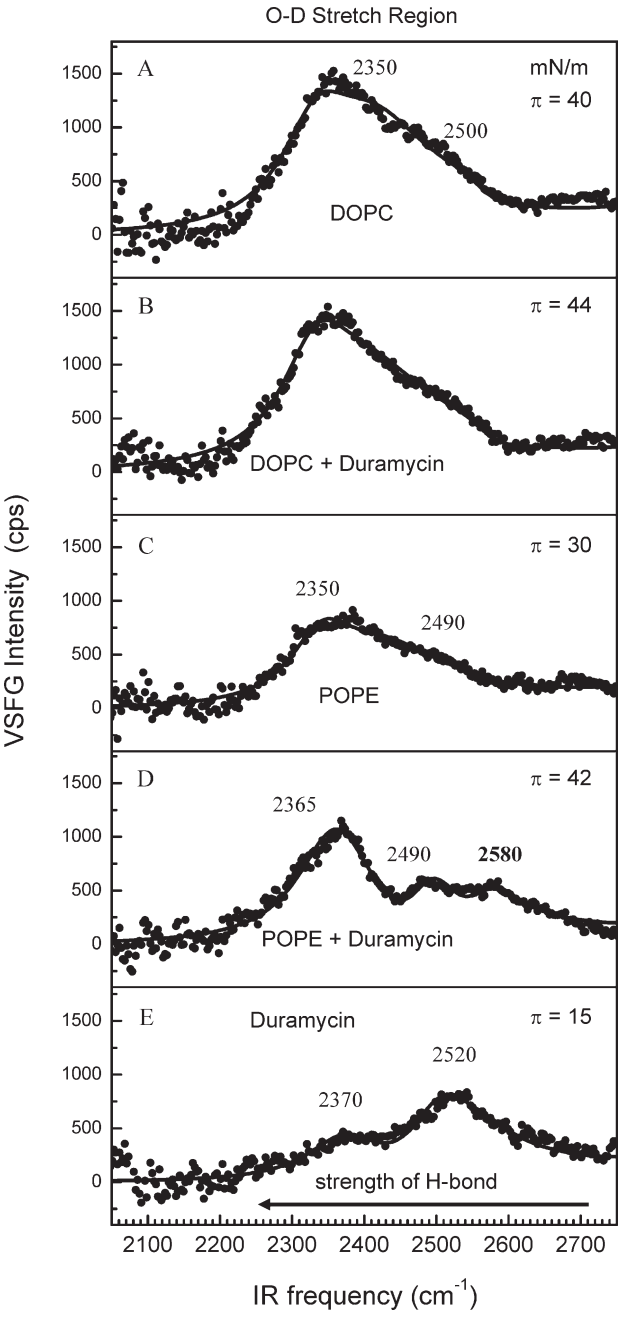

Figure 7. VSFG spectra in the O−D stretch region for (A) a pure DOPC monolayer, (B) the same DOPC monolayer in the presence of duramycin, (C) a pure POPE monolayer, (D) the same POPE monolayer in the presence of duramycin, and (E) a pure duramycin monolayer. The solid lines represent fits to the data using a Lorentzian model. The surface pressure is given in the right corner of each panel. The final duramycin concentration is below 0.7 μM.

has been reported to have vibrational response at approximately the same frequency.[54] These water molecules are located near the phospholipid headgroups.[26,55,56]

No change in the O−D vibrational spectrum of water molecules was observed when duramycin was injected into the subphase below the DOPC monolayer (Figure 7B). In contrast, the

(50) Nagle, J. F.; Wiener, M. C. *Biochim. Biophys. Acta* **1988**, *942*(1), 1–10.
(51) McIntosh, T. J.; Simon, S. A. *Biochemistry* **1986**, *25*, 4948–4952.
(52) Sovago, M.; Campen, R. K.; Bakker, H. J.; Bonn, M. *Chem. Phys. Lett.* **2009**, *470*(1−3), 7–12.
(53) Sovago, M.; Campen, R. K.; Wurpel, G. W. H.; Müller, M.; Bakker, H. J.; Bonn, M. *Phys. Rev. Lett.* **2008**, *100*(17), 173901-4.
(54) Kondo, T.; Kato, H. S.; Kawai, M.; Bonn, M. *Chem. Phys. Lett.* **2007**, *448* (1−3), 121–126.
(55) Mondal, J. A.; Nihonyanagi, S.; Yamaguchi, S.; Tahara, T. *J. Am. Chem. Soc.* **2010**, *132*(31), 10656–10657.
(56) Chen, X.; Hua, W.; Huang, Z.; Allen, H. C. *J. Am. Chem. Soc.* **2010**, *132*(32), 11336–11342.

SFG spectrum in the O−D stretch changes significantly when duramycin is added into the subphase below the POPE monolayer (Figure 7D). Most notably, a new peak at 2580 cm^{-1} appears. The peak position is significantly blue-shifted from the highest frequencies observed for pure POPE (2490 cm^{-1}) and pure duramycin (2520 cm^{-1}). It manifests the presence of water molecules in a weaker hydrogen-bonding environment, which must be related to association of water molecules with the duramycin−POPE complex. The water signal strength upon injection of duramycin does not change much compared to the pure POPE monolayer (Figure 7C), suggesting that there is no significant charge screening induced by duramycin. This in turn may indicate that there is little interaction between the ethanolamine group of the lipid and duramycin.

Little is known about how the binding of amphiphilic peptides to lipids affects the local water hydrogen-bonding environment at the lipid−water interface. Theoretical studies have shown that the number of water−water hydrogen bonds increases when an amphiphatic peptide diffuses from bulk solution to the interface.[44] This contrasts our finding of weakened hydrogen bonding observed here. On the other hand, time-resolved fluorescence spectroscopy studies of gramicidin showed that a peptide can shift water molecules deep into the lipid bilayer, pushing them closer to the lipid alkyl chains.[57] Penetration of water as far as the glycerol backbone of the lipid and between fatty acyl chain packing defects has also been reported.[58] Water in this hydrophobic environment would be expected to be more weakly hydrogen-bonded and display an increased vibrational frequency. Thus, we propose that the peak we observed at 2580 cm^{-1} in the duramycin + POPE system (Figure 7D) arises from water molecules that duramycin has forced into a hydrophobic region of the duramycin−lipid complex, for instance, in the vicinity of the lipid fatty acyl chains. In any case, it is apparent that binding of duramycin with POPE lipids is associated with a weakening of the hydrogen-bonded structure of water molecules in the hydration shells of the peptide and the lipid. To the best of our knowledge, this constitutes the first spectroscopic evidence for restructuring of interfacial water occurring upon peptide binding to lipids.

Conclusions

In this study, we used fluorescence microscopy and VSFG to investigate the interaction of duramycin with lipid monolayers composed of lipids containing phosphoethanolamine (PE) and phosphocholine (PC) in their headgroup parts. The results are consistent with previous reports that the interaction of duramycin with the POPE lipid monolayer system is headgroup-specific: there was no sign of duramycin−lipid interaction for any of the PC-headgroup containing lipids studied here. A lipid monolayer as a simple model of the cell membrane reflects the specific interaction observed previously in lipid vesicles or cells studies. Fluorescence microscopy shows that duramycin causes reduction of the line tension at the phase boundary between the LC and LE phases in the POPE monolayer. SFG data in the amide I region indicate that duramycin has a β-sheet conformation at the air−water interface and also after binding to POPE, and that duramycin is uniformly oriented, both at the air−water interface and after penetration into the hydrophobic core of the POPE lipid monolayer. This is further corroborated by a relatively intense aromatic =C−H band from the peptide phenylanaline amino acids, also consistent with both an ordered structure of the peptide and a collective orientation of the ensemble. Monitoring the C−H stretch vibrations of the lipids reveals that duramycin causes an increase of disorder of the lipid alkyl chains of the POPE monolayer. Water vibrations reveal an increase in the population of weakly hydrogen-bonded water molecules, giving rise to a characteristic feature in the O−D region at 2580 cm^{-1}.

These results illustrate the first steps in the action of the antimicrobial peptide duramycin to disrupt membrane integrity: upon adsorption of duramycin, the line tension between different lipid phases is reduced. Most notably, our results provide a glimpse of duramycin action at the molecular level; duramycin adsorption occurs in a highly ordered fashion; duramycin molecules are collectively aligned and highly ordered at the molecular level. Interaction of duramycin with the monolayer results in reduced lipid order and a weakening of the water hydrogen-bonding network around PE. The vibrational fingerprints of the specific interaction of the antibiotic peptide with lipids will allow for rapid screening of various peptides, with antimicrobial properties, using lipid monolayers and surface-sensitive vibrational techniques.

Acknowledgment. This study was financially supported in part by RIKEN President Discretionary Fund (2004−2006, 2010−2012), Lipid Dynamics Project of RIKEN, and Grants-in-Aid for Scientific Research on Promotion of Novel Interdisciplinary Fields Based on Nanotechnology and Materials from the Ministry of Education, Culture, Sports, Science and Technology of Japan. One of the authors (I.I.R.) thanks the Japan Society for the Promotion of Science (JSPS) for the financial support. M.B. acknowledges support from the Young Academy of the Royal Dutch Academy of Sciences. The authors thank R. Pool for experimental help.

(57) Ho, C.; Stubbs, C. D. *Biophys. J.* **1992**, *63*(4), 897–902.
(58) Zhou, F.; Schulten, K. *J. Phys. Chem.* **1995**, *99*(7), 2194–2207.