A narrow amide I vibrational band observed by sum frequency generation spectroscopy reveals highly ordered structures of a biofilm protein at the air/water interface†

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We characterized BslA, a bacterial biofilm protein, at the air/water interface using vibrational sum frequency generation spectroscopy and observed one of the sharpest amide I bands ever reported. Combining methods of surface pressure measurements, thin film X-ray reflectivity, and atomic force microscopy, we showed extremely ordered BslA at the interface.

Biofilms are communities of microorganisms where cells are densely packed and attached to surfaces in extracellular matrices that consist mainly of exopolysaccharides, nucleic acids, and proteins.1 Biofilms constitute a protective mode of microbes to survive under hostile conditions.2 Biofilms facilitate gene transfer, quorum sensing, predation, and competition among colonies.3 Bacteria are shielded in biofilms from antimicrobial agents, making chronic infections a major challenge for the implantation of medical devices and bone replacement.4,5 Biofilms are characteristic of their amphiphilicity and stability, providing insights into molecular design of biomimetic materials, such as those used in biocontrol agents, inhibitors against metal corrosion, and bioreactors.5 Moreover, BslA is among the proteins that carry extreme properties, e.g., proteins expressed in thermophile with extremely low temperature,6 extremely high thermal stability,7 rhodopsin having extreme sensitivity for light detection for dim-light vision, antifreeze proteins lowering the freezing point to help animals survive at extremely low temperature, etc. Understanding the properties of these proteins not only is significant in protein science but also provides insight into molecular design of biomaterials. Since BslA is a newly identified protein shown to have extreme surface activity, characterization of its surface properties is therefore urgent and important.

Biofilms contain proteins as a major component. Some of these proteins are highly amphiphilic, belonging to the class of hydrophobins that facilitate formation of biofilms. Here, we focus on BslA (or YuaB) found in the biofilm of gram-positive bacteria, Bacillus subtilis that can grow on plants.6–8 BslA is known to facilitate the assembly of the extracellular matrix.6,7 Its crystal structure has been recently solved by Hobley et al. (Fig. 1A).9 The structure shows that 11 hydrophobic residues form a hydrophobic “cap” (green, Fig. 1A) and other residues form mainly hydrophilic β-strands (purple, Fig. 1A), exhibiting strong amphiphilicity.9 BslA shares little sequence homology and structural similarity with other hydrophobins.10 Uniquely, BslA does not use disulphide-bonded networks to stabilize surface structures. Instead, as Bromley et al. proposed,10 it changes conformations in the “cap” region from disordered loops to β-sheets (green, Fig. 1A) upon surface adsorption, exposing the hydrophobic residues for stabilization at interfaces.10 However, the molecular packing, structure, and orientation of BslA at the interfaces have not been fully understood, which require surface-specific methods for comprehensive characterizations.

Here, we combined surface-specific vibrational sum frequency generation spectroscopy (SFG) with surface pressure measurements, atomic force microscopy, and thin-film X-ray reflectivity to characterize BslA at the air/water interface, which is a model system for hydrophobic/hydrophilic interfaces. We expressed and purified a truncated version of BslA (see ESI† for procedures) with amino acids 29–176, the same construct as the one in the crystal structure.9 This truncated version was shown to be fully functional and observed one of the sharpest amide I bands ever reported. Combining methods of surface pressure measurements, thin film X-ray reflectivity, and atomic force microscopy, we showed extremely ordered BslA at the interface.

† Electronic supplementary information (ESI) available: Methods of BslA expression and purification; SFG setup; experimental procedure, data acquisition, and data analyses; supplementary data. See DOI: 10.1039/c5cc05743d
We first studied the adsorption and self-assembly process of BslA at the air/water interface. We obtained the adsorption isotherm at pH 7.4 (buffer: 10 mM phosphate and 100 mM NaCl) and 23 °C by monitoring the surface pressure at increasing concentrations of BslA. Fig. 1B shows that the surface pressure increases drastically at bulk concentrations lower than 1 μM, indicating strong adsorption of BslA at the interface. When the bulk concentration exceeds 1 μM, the interface is gradually saturated with BslA until reaching a maximum surface pressure of ~23 mN m⁻¹ at ~6 μM. The adsorption isotherm is fitted with the Langmuir model (see ESI†) as the red curve in Fig. 1B. The fitting yields an adsorption free energy (ΔG°) of ~8.65 kcal mol⁻¹. This large negative ΔG° reveals the strong surface activity of BslA at the air/water interface, even stronger than the amphiphilic long-chain alcohol, CH₃(CH₂)₉OH, with ΔG° of ~6.64 kcal mol⁻¹. ¹²

We then used vibrational SFG spectroscopy to characterize BslA at the air/water interface at the saturated level with a bulk concentration of 6 μM. Vibrational SFG spectroscopy is a non-invasive, label-free, and surface-selective vibrational spectroscopic method,¹³ which has been used to study biomolecules at interfaces.¹⁴,¹⁵ Since amide I bands are characteristic of protein secondary structures,¹⁶ we first acquired the conventional (achiral) SFG spectrum of BslA in the amide I vibrational region (1600–1700 cm⁻¹) using the ssp polarization setting (s-polarized SFG, s-polarized visible, and p-polarized infrared) (setup shown in ESI†).

The amide I spectrum (Fig. 1C) gives surprisingly sharp spectral features. Fitting the spectrum (Table 1), we identified two vibrational bands and assigned them to β-turn (1669 cm⁻¹) and the B₁ mode of antiparallel β-sheets (1685 cm⁻¹). These assignments are consistent with the standard amide I frequencies for the secondary structures and the crystal structure of BslA, with extensive antiparallel β-sheet strands connected by β-turns (Fig. 1A). ³ Notably, the damping coefficient for the 1685 cm⁻¹ band is only 5.25 cm⁻¹, corresponding to a full-width-at-half-maximum (FWHM) of 10.50 cm⁻¹, approaching the spectral resolution of our broad bandwidth SFG spectrometer (~8 cm⁻¹). This amide I band is broader at lower BslA bulk concentration (Table S2, ESI†). It is much narrower than that for common proteins, which typically have FWHM greater than 20 cm⁻¹ for single secondary structures observed using conventional vibrational methods, such as infrared and SFG (Table S3, ESI†).⁶,¹⁷ Our observed sharp amide I band is unusual because inhomogeneity of the amide groups in protein structures and various extents of hydrogen-bonding interactions with solvents often broaden the amide I band. Since SFG signals depend highly on molecular ordering and orientation, the sharp amide I band (Fig. 1C) led to the hypothesis that BslA self-assembles into extremely ordered and compact structures at the air/water interface.

We further acquired the chiral SFG spectrum using the psp polarization setting (p-polarized SFG, s-polarized visible, and p-polarized IR) under the same conditions (Fig. 1D). Chiral SFG has the advantages of being free of spectral background from achiral molecules, including water.¹⁸,²⁰ Due to the difference in selection rules,²⁰ the chiral SFG spectrum (Fig. 1D) of BslA at the air/water interface is different from the achiral SFG spectrum (Fig. 1C). It exhibits only one amide I band at 1639 cm⁻¹, which is at the high-frequency end of the antiparallel B₁ vibrational mode and the low-frequency end of disordered structures.¹⁶ We ruled out the assignment of disordered structures because they should not exhibit chiral amide I SFG signals.¹⁸,²⁰ Thus, we assigned the 1639 cm⁻¹ band to the B₁ vibrational mode of antiparallel β-sheets, consistent with the assignment of the 1685 cm⁻¹ band in the achiral spectrum (Fig. 1C) to the B₁ mode of antiparallel β-sheets.¹⁶

We then examined the narrow 1685 cm⁻¹ band (Fig. 1C) under acid-denaturing conditions. We added HCl solution to decrease the pH from 7.4 to 1.2 to denature BslA, and then obtained the achiral and chiral SFG spectra. The achiral SFG

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![Fig. 1](https://example.com/f1.png)

**Table 1** Fitting parameters of achiral and chiral SFG spectra for BslA

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Amide I (achiral-ssp) Fig. 1C (pH 7.4)</th>
<th>Amide I (achiral-ssp) Fig. 1E (pH 1.2)</th>
</tr>
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<tbody>
<tr>
<td>Zₐ (a.u.)</td>
<td>0.020 ± 0.005</td>
<td>-0.005 ± 0.003</td>
</tr>
<tr>
<td>ω (cm⁻¹)</td>
<td>1668.5 ± 0.7</td>
<td>1668.6 ± 0.8</td>
</tr>
<tr>
<td>A (a.u.)</td>
<td>1.99 ± 0.24</td>
<td>3.97 ± 0.10</td>
</tr>
<tr>
<td>Γ (a.u.)</td>
<td>11.75 ± 1.29</td>
<td>20.15 ± 0.62</td>
</tr>
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</table>
The spectrum shows that the sharp band at 1685 cm⁻¹ vanishes, leaving a broad band at 1669 cm⁻¹ (Fig. 1E), while the chiral SFG spectrum shows that the 1639 cm⁻¹ band disappears (Fig. 1F). The vanishing vibrational bands at 1685 cm⁻¹ in the achiral spectrum (Fig. 1C) and 1639 cm⁻¹ in the chiral spectrum (Fig. 1D) indicate that the acidic condition denatures the antiparallel β-sheets at the interface. The remaining band at 1669 cm⁻¹ in the achiral spectrum (Fig. 1E) can originate from β-turns and/or and ordered structures commonly found in denatured proteins. In addition, the damping coefficient of the 1669 cm⁻¹ band in the achiral spectra increases from 11.75 cm⁻¹ (Fig. 1C) to 16.65 cm⁻¹ (Fig. 1E), suggesting that the denatured protein is more disordered and/or more inhomogeneous. The disappearance of the achiral 1685 cm⁻¹ narrow band and the chiral 1639 cm⁻¹ band is due to the structural changes, rather than desorption of BslA from the interface as the intensity of the achiral amide I band (1669 cm⁻¹) is comparable to that before denaturation. These results show that the unusually narrow amide I vibrational band at the neutral pH (Fig. 1C) comes from properly assembled, folded BslA at the air/water interface.

To further evaluate the surface activity of BslA, we obtained its surface compression isotherm. We monitored the surface pressure while compressing the BslA monolayer at the air/water interface using two barriers in a Langmuir trough (Fig. 2A), a common method used for studying stability and packing of interfaces using two barriers in a Langmuir trough (Fig. 2A). We speculate that the difference between the occupied area (1.14 g cm⁻³) of the crystal structure oriented with the hydrophobic “cap” facing up, two lines are drawn to depict the approximate height of BslA at the mica surface and the large smooth coverage in the macroscopic length scale of µm is due to densely packed, highly ordered BslA molecules with the cap facing up. We further examined this proposed molecular picture using thin film X-ray reflectivity.

We performed thin film X-ray reflectivity (XRR) experiments to further characterize the LB film of BslA prepared at the same surface pressure of 23 mN m⁻¹. The log reflectivity curve (Fig. 3A) exhibits a clear fringe pattern as a result of the interference between the partially reflected X-rays from the air/film and film/substrate interfaces. We also observed peaks associated with Bragg reflections (peaks at ~4.5° and ~9.0°), which are characteristic of the mica crystalline substrate. The XRR result for mica without the Bragg peaks exhibits a clear fringe pattern as a result of the interference between the partially reflected X-rays from the air/film and film/substrate interfaces (Fig. 3A). The log reflectivity curve (Fig. 3A) exhibits a clear fringe pattern as a result of the interference between the partially reflected X-rays from the air/film and film/substrate interfaces (Fig. 3A). The log reflectivity curve (Fig. 3A) exhibits a clear fringe pattern as a result of the interference between the partially reflected X-rays from the air/film and film/substrate interfaces (Fig. 3A). The log reflectivity curve (Fig. 3A) exhibits a clear fringe pattern as a result of the interference between the partially reflected X-rays from the air/film and film/substrate interfaces (Fig. 3A).
B-factor in this region (33) is higher than that in the regions of β-sheets (25), indicating higher flexibility. In vivo studies showed that bacteria expressing BslA with mutations in this region do not exhibit significant changes in hydrophobicity of the biofilms. However, we cannot exclude the possibility that this region may still undergo conformational changes to facilitate intermolecular interactions. The total thickness and values of the density in the fitting model are consistent with the crystal structure (Fig. 1A) at a full surface coverage that orients vertically with the higher density hydrophobic cap facing up (Fig. 3B), confirming the formation of a densely packed monolayer of BslA at the air/water interface.

In summary, we observed an unusually narrow amide I vibrational band in the SFG spectrum of BslA at the air/water interface. We further characterized BslA at the air/water interface by performing surface pressure measurements, AFM imaging, and thin film X-ray reflectivity experiments. On the basis of these characterization, we attributed the narrow amide I band to the extremely ordered, neatly oriented, and highly compact structures of BslA at the interface, consistent with the recent report of highly ordered 2D lattices formed by BslA. Our results also support that BslA can likely form a stable and robust outmost layer at the air/water interface or hydrophobic/water interfaces as a protective shield for materials. Finally, our work reinforces the proposal that BslA likely forms the outermost surface structures of biofilms to protect them from antibiotics for treating infectious diseases.

This work is supported by the National Science Foundation (NSF) Grant CHE 1213362 and National Institutes of Health (NIH) Grant 1R56DK105381-01 to E. C. Y. Y., CRISP MRSEC NSF DMR 1119826 to C. H. A., and CBET 1355317 to R. M. L. M. A. is partially supported by a Postdoctoral Fellowship of Mexico's National Council of Science and Technology.

**Notes and references**


