Electronic Supporting Information for
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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1. Methods of BslA Expression and Purification

DNA encoding $BslA_{29\text{-}176}$ was synthesized by GenScript and cloned into the pGEX-6P-1 vector using the *Bam*HI and *Xho*I restriction sites. The resulting plasmid was then transformed into *E.coli* strain BL21(DE3). The cells were cultured at 37°C in 500 mL of Lysogeny broth (LB) containing 100 μg/ml of ampicillin, and induced with 250 μM of isopropyl β-thiogalactoside (IPTG) at OD_{600} of 0.6~0.8. The cells were then grown at 25°C for overnight expression, spun down and the pellets were stored at -80°C. For purification, the cells were resuspended in the lysis buffer (25 mM Tris-HCl, pH 7.4 and 250 mM NaCl) in the presence of EDTA-free Complete Protease Inhibitor (Roche). The cells were lysed by sonication and the cell debris were removed by centrifugation at 13,000 rpm at 4°C. 1 ml of Glutathione Sepharose 4B beads (GE Healthcare) were added to the clear lysate and the sample was nutated for 2-3 hrs at 4°C to allow for sufficient binding. The protein-bound beads were then collected by centrifugation, followed by first wash with the lysis buffer and second wash with the cleavage buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol). Next, 20 μL of PreScission protease in 3 mL of cleavage buffer were added to the beads and the sample was kept on a nutator for overnight cleavage at 4°C. The solution was collected and the remaining impurities were removed by gel filtration using Superdex 75. The purity of the protein was confirmed by SDS-PAGE. For all other experimental characterization, purified BslA was concentrated using centricons and buffer-exchanged into the storage buffer (10 mM phosphate buffer, pH 7.4 and 100 mM NaCl). The final concentration of BslA stock solution is 40 μM.

2. SFG Setup

Laser Setup: The broad-bandwidth SFG spectrometer used in the study was described in detail in *Ma et al*.[1] It has a 6-W regenerative amplifier seeded by a 120-fs 1.9-W Ti:sapphire oscillator (Mai Tai, Spectra-Physics) and is pumped by two Nd:YLF pump lasers (16 W, Empower, Spectra-Physics). A half of the amplifier output (3 W) pumped an OPA (TOPAS, Spectra-Physics) to generate a 120-fs pulsed IR beam in the range of 3800–900 cm^{-1}. The

other half (3W) of the amplifier 800-nm output entered a pulse shaper to yield ~2 ps pulses to a narrow bandwidth < 10 cm^{-1}. The pulse shaper comprises a grating, a planoconvex cylindrical lens, and a homemade slit. The reflected SFG signal was first filtered, then focused at the slit of the monochromator (SP-2558, Princeton Instruments), and finally detected by a CCD (Spec-10:400BR/LN, Princeton Instruments). The 800 nm beam has an incident angle of 56° and the IR beam has an incident angle of 69°. The spot size of the 800-nm beam is measured as ~60 μm and that of the IR beam is ~70 μm in diameter. To prevent heating effects from laser irradiation, a spectrometer with relatively long pulse duration (120 fs) and high repetition rate (5 kHz) is used. Therefore, relatively low IR pulse energy and power (~2 μJ, 10 mW, amide I) can be achieved. Also, the IR and visible beams are focused slightly below the interface to further minimize the heat effects. To determine the IR profile, we used the method described in Ma *et al*.[1] Specifically, we placed a nonlinear GaAs crystal on the sample stage and took a nonresonant SFG spectrum. The spectrum of GaAs provided the energy profile of the IR beam, which was used to normalize the SFG spectra. A box purged with dry nitrogen is used to house the optical path of the IR beam to minimize the effect of water vapor on the IR profile in the amide I vibrational region.

Polarization Settings in SFG Experiments: Figure S1 shows the two polarization settings used in our experiments. As shown in the figure, when the laser beams propagate in the *x*-*z* plane, *p* polarization indicates that the oscillation direction of the electric field is in the *x*-*z* plane, while *s* polarization indicates the oscillation direction along the *y*-axis. The polarization setting is described in the order of SFG-visible-IR beams. Namely, *ssp* denotes *s*-polarized SFG, *s*-polarized visible, and *p*-polarized IR, and *psp* denotes *p*-polarized SFG, *s*-polarized visible, and *p*-polarized IR.

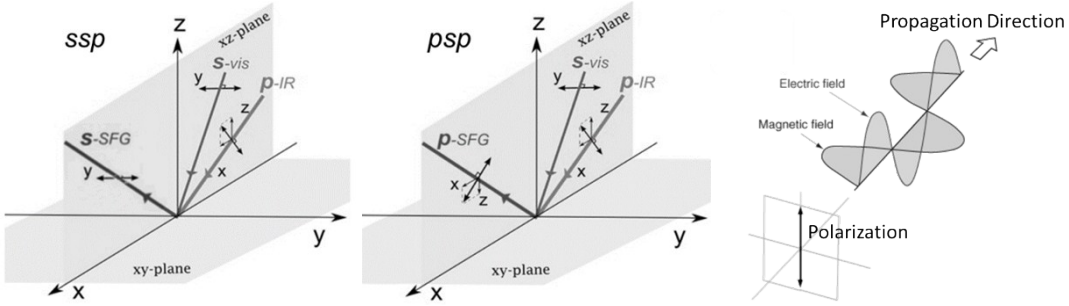
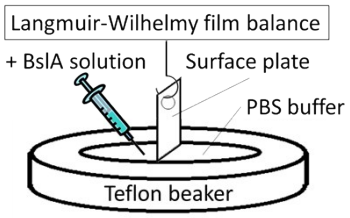


Figure S1. The *ssp* and *psp* polarization settings in SFG experiments.

3. Experimental Procedure, Data Acquisition, and Data Analyses

Surface Adsorption Isotherm: Scheme S1 illustrates the method to obtain surface adsorption isotherms. The surface pressures of BslA solutions of different concentrations were measured in a Teflon beaker (diameter = 4 cm) using a Langmuir-Wilhelmy film balance (KSV, Finland) with a piece of filter paper as a surface plate. Before the addition of BslA stock solution, the surface pressure of the phosphate buffer was set to zero. The stock solution was then added in increments into the bulk of the PBS buffer with a Hamilton

microsyringe. The surface pressure was monitored in real time during the process. For each increment, 20 min was allowed to reach adsorption equilibrium before the reading on the balance was taken.



Scheme S1. An experiment to obtain the surface adsorption isotherm of BslA.

Derivation of the Langmuir Model: The Langmuir model is based on the equilibrium between the adsorption of bulk molecules (BM) onto the empty sites (ES) and the desorption of molecules from the filled sites (FS) at the interface:

$$\mathrm{BM} + \mathrm{ES} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{FS} \qquad \text{(S1)}.$$

Suppose *C* is the number of molecules per unit volume before adsorption (equivalent to molar bulk concentration), *N* is the number of molecules per unit volume adsorbed at the interface, of which the maximum N_{max} is a constant determined by the number of empty sites at the interface. At equilibrium,

$$\frac{dN}{dt} = k_1(C - N)(N_{\max} - N) - k_{-1}N = 0 \qquad \text{(S2)}.$$

For adsorption to a flat surface, N_{max} is usually much smaller than the bulk concentration *C*.[2] Therefore, Equation S2 can be simplified as:

$$\frac{dN}{dt} = k_1 C(N_{\max} - N) - k_{-1}N = 0 \qquad \text{(S3)}.$$

By solving Equation S3, the expression for Langmuir model can be expressed as

$$\frac{N}{N_{\max}} = \frac{1}{1 + (KC)^{-1}} \qquad \text{(S4)},$$

where $K = k_1/k_{-1}$. By fitting N/N_{max} (proportional to surface pressure) versus bulk concentration (*C*) of BslA, the adsorption equilibrium constant *K* can be obtained. The adsorption free energy (ΔG) can then be calculated:

$$\Delta G = -RT \ln K \qquad \text{(S5)},$$

where *R* is the gas constant and *T* is the temperature.

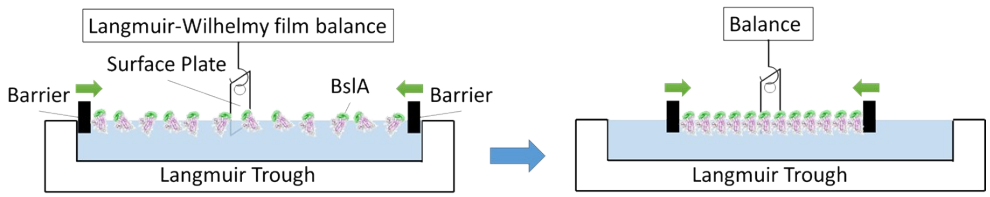
SFG Experiments: The setup of SFG spectrometer was described in Section 2. BslA was added using a Hamilton microsyringe into a Teflon beaker (Diameter = 4 cm) that contained 5 mL PBS buffer (10 mM phosphate, 100 mM NaCl, pH = 7.4). The *ssp* and *psp* polarization settings were used for the characterization. During spectral acquisition, the visible and the

IR beams were focused slightly below the air/water interface. The raw spectra were normalized by the IR profile, which was provided by the nonresonant spectrum from the surface of a nonlinear GaAs crystal. The normalized spectra were fitted into the following equation:

$$I_{SFG} \propto \left| \chi_{NR}^{(2)} + \sum_q \frac{A_q}{\omega_{IR} - \omega_q + i\Gamma} \right|^2 \quad \text{(S6)},$$

where I_{SFG} is the sum frequency intensity, $\chi_{NR}^{(2)}$ is the nonresonant second-order susceptibility, ω_{IR} is the input IR frequency, and A_q, ω_q, and Γ_q are the amplitude, resonant frequency, and damping factor of the qth vibration mode, respectively. In our experiments, $\chi_{NR}^{(2)}$ is the susceptibility for the air/water interface.

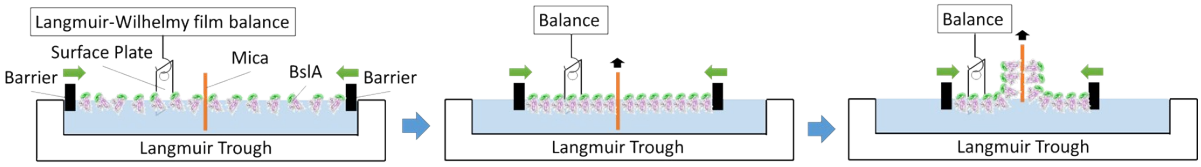
Surface Compression Isotherm: Scheme S2 describes the experimental setting to obtain a surface compression isotherm. A Langmuir trough (KN2002, KSV Instruments Ltd, Finland) was used for compression of monolayers, which operates via KSV Nima Software to control two symmetric barriers. BslA stock solution was added onto the surface of the PBS buffer in the trough with a Hamilton microsyringe. Then, the monolayer was compressed by the two barriers at the constant speed of 20 mm/min, while the surface pressure was monitored using a piece of filter paper as a surface plate attached to the Langmuir-Wilhelmy film balance (KSV, Finland). The surface pressure-molecular area (π-A) isotherm was obtained by plotting the surface pressure against molecular area, where the molecular area was calculated by dividing the surface area of the trough by the total number of BslA molecules.



Scheme S2. Experimental procedure to obtain the surface pressure isotherm for BslA.

Langmuir-Blodgett Film Preparation: Scheme S3 describes the experimental procedure for the preparation of Langmuir-Blodgett Film. A piece of freshly cleaved V-1 grade mica sheet (Electron Microscopy Sciences, Hatfield, PA) was submerged into a mixture of 98% sulfuric acid and 30% hydrogen peroxide (1:1, v/v) for 10 min. After the mica sheet was washed with water and dried, it was attached to a KSV dipper and placed in the well in the center of the Langmuir trough. PBS buffer solution was then added into the trough until the mica sheet was completely submerged. After BslA was added onto the PBS buffer subphase and reached adsorption equilibrium at the air/water interface, the two barriers on the trough started the compression until the monolayer reached the target surface pressure (23 mN/m). Langmuir-Blodgett (LB) films of BslA were obtained by vertically pulling the mica

sheet from the subphase at a constant speed of 1 mm/min while the surface pressure was kept constant by the barriers. The deposited films were dried in the air for 24 hours before characterizations.



Scheme S3. Experimental procedure for the preparation of Langmuir-Blodgett film of BslA.

X-ray Thin Film Reflectivity: Specular X-ray reflectivity (XRR) experiments in parallel beam geometry were performed with a Rigaku SmartLab x-ray diffractometer using Cu Kα radiation and a Graphite monochromator coupled with a scintillation counter on receiving side, operated at 45 kV and 200 mA.

AFM Imaging: AFM images were obtained with an Agilent 5420 AFM instrument (Agilent, Santa Clara, CA). All the images were taken under taping mode in air with an uncoated silicon probe which has a typical force constant of 40 N/m. All images were taken at a resolution of 512 × 512 points and processed using PicoView software.

4. Supplementary Data

Compression-Decompression Isotherm of BslA Monolayer.

Langmuir monolayer of BslA was first compressed from 0 mN/m to 35 mN/m and then decompressed to 0 mN/m at the constant speed of 20 mm/min. This procedure was repeated for three times to yield the compression-decompression isotherm shown below. The isotherm from the three cycles overlaps well with one another, indicating negligible desorption of BslA from the interface.

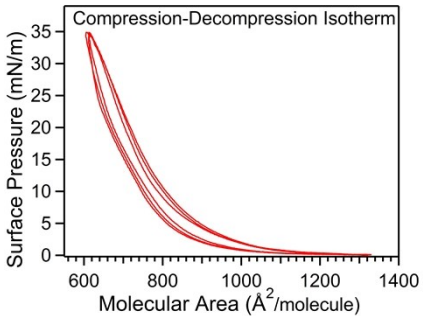


Figure S2. Compression-decompression isotherm (3 cycles) for BslA monolayer at the air/water interface.

Fitting Parameters for the Linear Portion of BslA Compression Isotherm (Figure 2A).

Fitted portion: (Molecular Area, Surface Pressure) = (572.69, 31.76) to (648.98, 20.70).

Fitting function: Surface Pressure = y-intercept + slope x Molecular Area.

Fitting parameters: y-intercept = 116.32 ± 0.32, slope = -0.148 ± 0.001.

AFM Images in Different Probing Areas at 23 mN/m

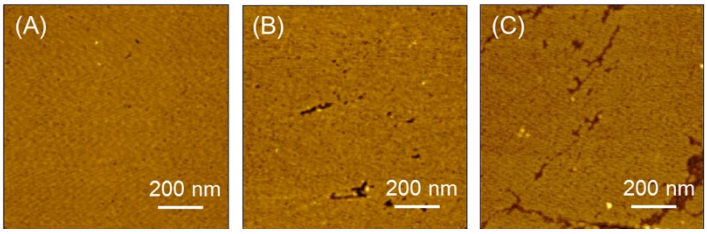


Figure S3. AFM images of Langmuir-Blodgett (LB) films of BslA on mica: (A) a perfect smooth coverage, (B) a large area of smooth coverage with minor defects (presented as Figure 2B in the main text), and (C) a large area of smooth coverage with relatively more defects.

Measured XRR Patterns of Mica.

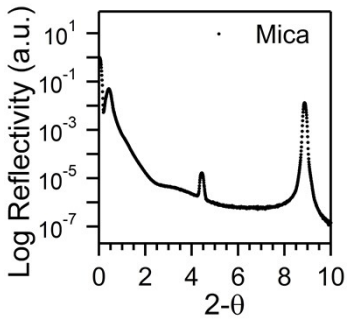


Figure S4. X-ray reflectivity curve of mica.

Table S1. Parameters obtained from the XRR fitting for BslA on mica prepared at the surface pressure of 23 mN/m

Material	Thickness (Å)	Density (gr/cm^3)	Roughness (Å)
Hydrophobic cap	9.5 ± 0.1	1.25 ± 0.02	0.04
Hydrophilic body	22.0 ± 0.2	1.14 ± 0.01	0.36
Hydrophilic tail	4.2 ± 0.1	0.18 ± 0.02	0.06
Mica grade V1	--	3.2	0.00

Peak Width as function of BslA concentration.

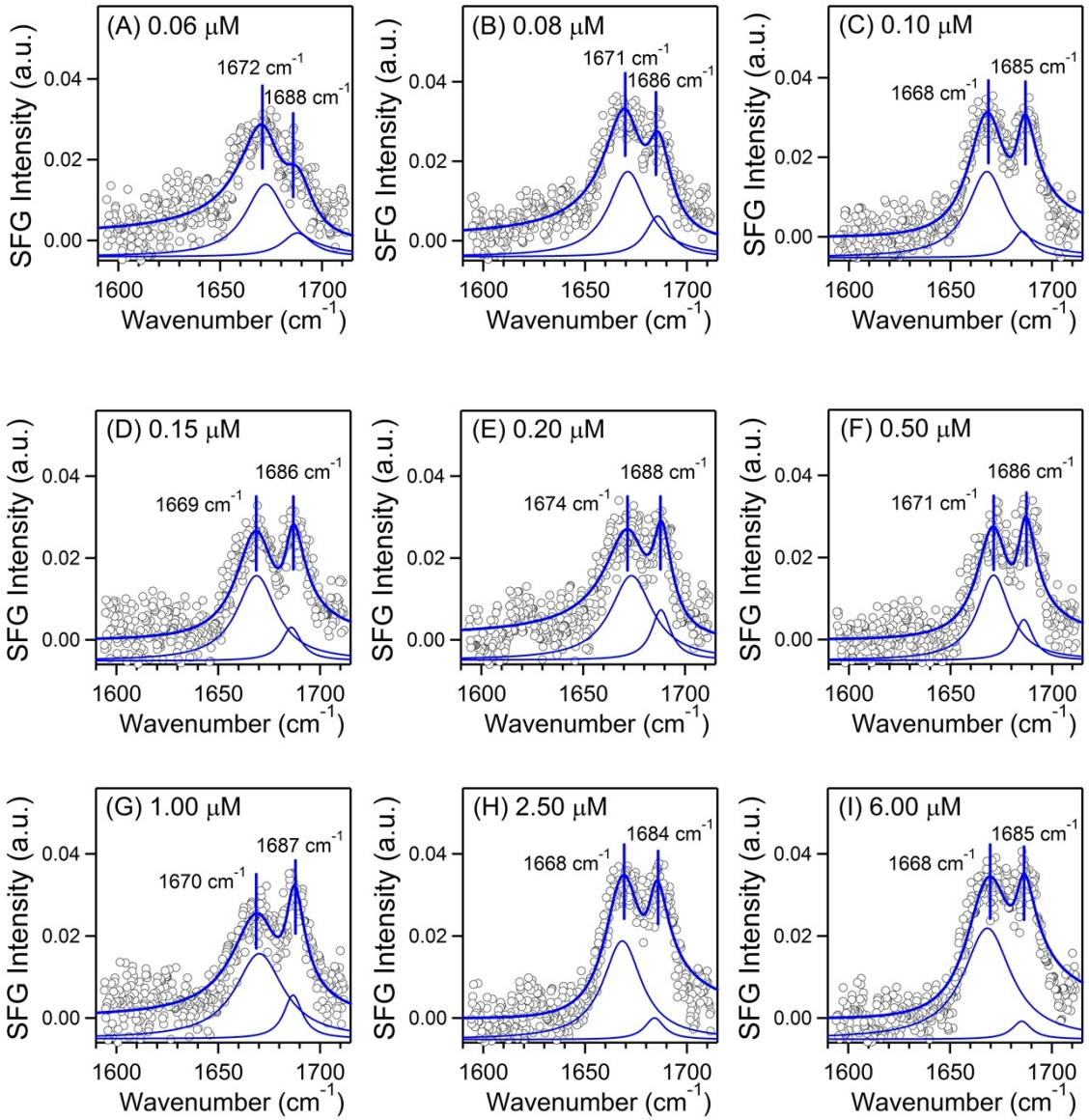


Figure S5. Concentration-dependent *ssp* spectra of BslA at the air/water interface at pH 7.4. Acquisition time: 10 min. Parameters for spectral fitting are shown in Table S2 on the next page.

Table S2. Spectral fitting parameters

Concentration	(A) 0.06 μM	(B) 0.08 μM	(C) 0.10 μM
χ_{NR} (a.u.)	-0.029 ± 0.006	-0.023± 0.004	0.020 ± 0.009
A (a.u)	1.67 ± 0.47	1.71 ± 0.27	1.92 ± 0.20
ω (cm^{-1})	1672.4 ± 0.8	1671.0 ± 0.5	1667.9 ± 0.8
Γ (cm^{-1})	12.30 ± 1.81	11.49 ± 1.08	11.50 ± 0.95
A (a.u)	0.70 ± 0.38	0.76 ± 0.20	0.55 ± 0.16
ω (cm^{-1})	1687.9 ± 0.8	1685.7 ± 0.4	1685.4 ± 0.4
Γ (cm^{-1})	9.01 ± 2.72	7.38 ± 1.11	5.92 ± 0.94
Concentration	(D) 0.15 μM	(E) 0.20 μM	(F) 0.50 μM
χ_{NR} (a.u.)	0.011 ± 0.004	-0.023 ± 0.008	0.012 ± 0.006
A (a.u)	1.79 ± 0.17	1.72 ± 0.34	1.47 ± 0.19
ω (cm^{-1})	1668.8 ± 0.5	1673.7 ± 0.7	1671.2 ± 0.5
Γ (cm^{-1})	11.76 ± 0.93	12.50 ± 1.51	9.71 ± 0.97
A (a.u)	0.55 ± 0.12	0.61 ± 0.19	0.58 ± 0.15
ω (cm^{-1})	1685.9 ± 0.3	1688.2 ± 0.5	1686.1 ± 0.4
Γ (cm^{-1})	5.81 ± 0.81	5.71 ± 1.01	5.52 ± 0.87
Concentration	(G) 1.00 μM	(H) 2.50 μM	(I) 6.00 μM
χ_{NR} (a.u.)	-0.005 ± 0.007	0.026 ± 0.006	0.029 ± 0.013
A (a.u)	2.08 ± 0.27	2.02 ± 0.19	2.52 ± 0.29
ω (cm^{-1})	1670.2 ± 0.7	1668.2 ± 0.5	1668.3 ± 1.1
Γ (cm^{-1})	14.34 ± 1.41	11.47 ± 0.92	14.21 ± 1.42
A (a.u)	0.57 ± 0.14	0.48 ± 0.15	0.41 ± 0.20
ω (cm^{-1})	1686.7 ± 0.4	1684.1 ± 0.3	1685.2 ± 0.5
Γ (cm^{-1})	5.52 ± 0.77	5.81 ± 1.01	5.78 ± 1.46

5. Comparison of Amide I Peak Width

1. The spectral parameters of vibrational studies of proteins at interfaces by surface-selective vibrational spectroscopy are summarized for several model peptides (IAPP, LKα14, bovine rhodopsin, pHLIP, alamethicin, tachyplesin I) and native proteins (fibrinogen, insulin, lysozyme, bovine serum albumin, human serum albumin).
2. The vibrational methods include sum frequency generation spectroscopy (SFG), attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) and infrared reflection-absorption spectroscopy (IRRAS)
3. All the SFG peaks are for the ssp polarization setting.

Table S3. Comparison of Peak Width of Amide I Bands of Proteins

Molecular System	Interface	Method	Secondary structure	Peak position (cm^{-1})	FWHM (cm^{-1})	Reference
Rat IAPP	Lipid/water	SFG	Disordered	1650	46	J. Am. Chem. Soc., 2011, 133, 8094–8097
Human IAPP	Lipid/water	SFG	Parallel β-sheet	1662	26	
$LK_{\alpha}14$	Air/water	SFG	α-helix	1651	34	
Bovine rhodopsin	Air/water	SFG	α-helix	1647	47	
pHLIP	Air/water	SFG	α-helix	1666	51	
Alamethicin	Lipid/water	SFG	3_{10}-helix	1635	~17	J. Phys. Chem. B 2010, 114, 3334–3340
			α-helix/3_{10}-helix	1670	~39	
Tachyplesin I	Water/polystyrene	SFG	Antiparallel β-sheet	1633	~22	Langmuir 2005, 21, 2662–2664
			β-turn	1664	~26	
			Antiparallel β-sheet	1688	~23	
		ATR-FTIR	Antiparallel β-sheet	1633	~38	
			Random structures	1658	~22	
			β-turn	1671	~31	
			Antiparallel β-sheet	1690	~25	
Fibrinogen	Water/polystyrene	SFG	β-sheet	1630	~30	PNAS 2005, 102, 14,

			α-helix	1650	~32	4978-4983
	316LVM stainless steel surface	IRRAS	β-sheet	~1636	~30	Phys. Chem. Chem. Phys., 2008, 10, 2502–2512
			α-helix	~1657	~28	
			β-turn	~1673	~20	
Insulin (1 mg/mL)	Air/water	SFG	α-helix	1654	29	Phys. Chem. Chem. Phys., 2014, 16, 26722--26724
Insulin (50 mg/mL)			α-helix	1653	26	
Lysozyme	Lipid/water	SFG	Protein aggregates	1660	48	Langmuir 2014, 30, 7736−7744
			Antiparallel β-sheet	1685	38	
Bovine serum albumin	316LVM stainless steel surface	IRRAS	β-sheet	~1628	~27	Biomacromolecules 2007, 8, 2836-2844
			α-helix	~1655	~33	
			β-turn	~1678	~30	
Human serum albumin	Calcium phosphates ceramics surface	ATR-FTIR	Random structures	1636	~42	J. Biomed. Mater. Res.-A 2015, article ASAP, DOI: 10.1002/jbm.a.35496
			α-helix	1656	~26	
			β-turn	1675	~22	

References:

1. G. Ma, J. Liu, L. Fu and E. C. Y. Yan, *Appl. Spectrosc.*, 2009, **63**, 528-537.
2. H. F. Wang, E. C. Y. Yan, Y. Liu and K. B. Eisenthal, *J. Phys. Chem. B*, 1998, **102**, 4446-4450.