Identification of the response of protein N-H vibrations in vibrational sum-frequency generation spectroscopy of aqueous protein films

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Abstract:

The N-H stretching vibration is an important probe for investigating structural and functional properties of proteins but is often difficult to analyze as it overlaps with the O-H stretching vibration of water molecules. In this work we investigate the N-H signals of hydrophobins using conventional (VSFG) and heterodyne-detected vibrational sum-frequency generation spectroscopy (HD-VSDG). Hydrophobins represent a group of surface active proteins that form highly-ordered protein films at the water-air interface and that give rise to prominent vibrational modes. We find that in conventional VSFG spectra N-H specific signals show significant changes in shape and intensity upon altering the pH values. These changes can easily be misinterpreted for conformational changes of the protein. Using HD-VSFG experiments, we demonstrate, that for hydrophobin films the change of the N-H response with pH can be well explained from the interference of the N-H response with the broad interfacial water O-H stretch band.

Introduction:

Protein adsorption to interfaces is of great significance in several fields of research and applications. An accurate determination of the secondary structure and molecular orientation of proteins at interfaces is important to understand and ultimately to control their functions. In the past protein interfaces have been studied using techniques such as ellipsometry, neutron reflection, X-ray reflectivity and surface tension measurements[1-3]. In recent years, vibrational sum-frequency generation spectroscopy (VSFG) has become an increasingly used technique to probe the structure, molecular composition and orientation of interfacial protein films. In VSFG an infrared light pulse and a visible pulse are combined at an interface to generate light at their sum-frequency. The generation is enhanced in case the infrared light is resonant with a molecular vibration. For the bulk the generation of sum-frequency light is symmetry forbidden, making the technique highly surface-specific.

VSFG has been successfully applied to study the structure, kinetics and interactions of proteins at various interfaces[4-7]. At the air-water interface most reported studies focused on analyzing the amide I and C-H stretching regions. A few research groups have studied the protein N-H stretching modes using VSFG, but the measurement of these signals are complicated due to the spectral overlap with the O-H stretching vibration of water molecules[8, 9]. As a result little information is currently available on the N-H response of proteins at the interface, and possible changes in this response following a modification of the temperature or the pH.

Hydrophobins are a group of very surface active proteins that are produced by filamentous fungi and are known for their unique functions related to the interaction and control of interfaces[10, 11]. They are known to be extremely stable and can withstand a broad range of pH values and most common denaturants[2, 12]. At the water-air interface hydrophobins assemble into very stable, highly-ordered protein films that form an ideal system for VSFG studies. Here we investigate the class II hydrophobin HFBII from *Trichoderma reesei* using both conventional (VSFG) and heterodyne-detected vibrational sum-frequency generation spectroscopy (HD-VSFG).

Results and Discussion:

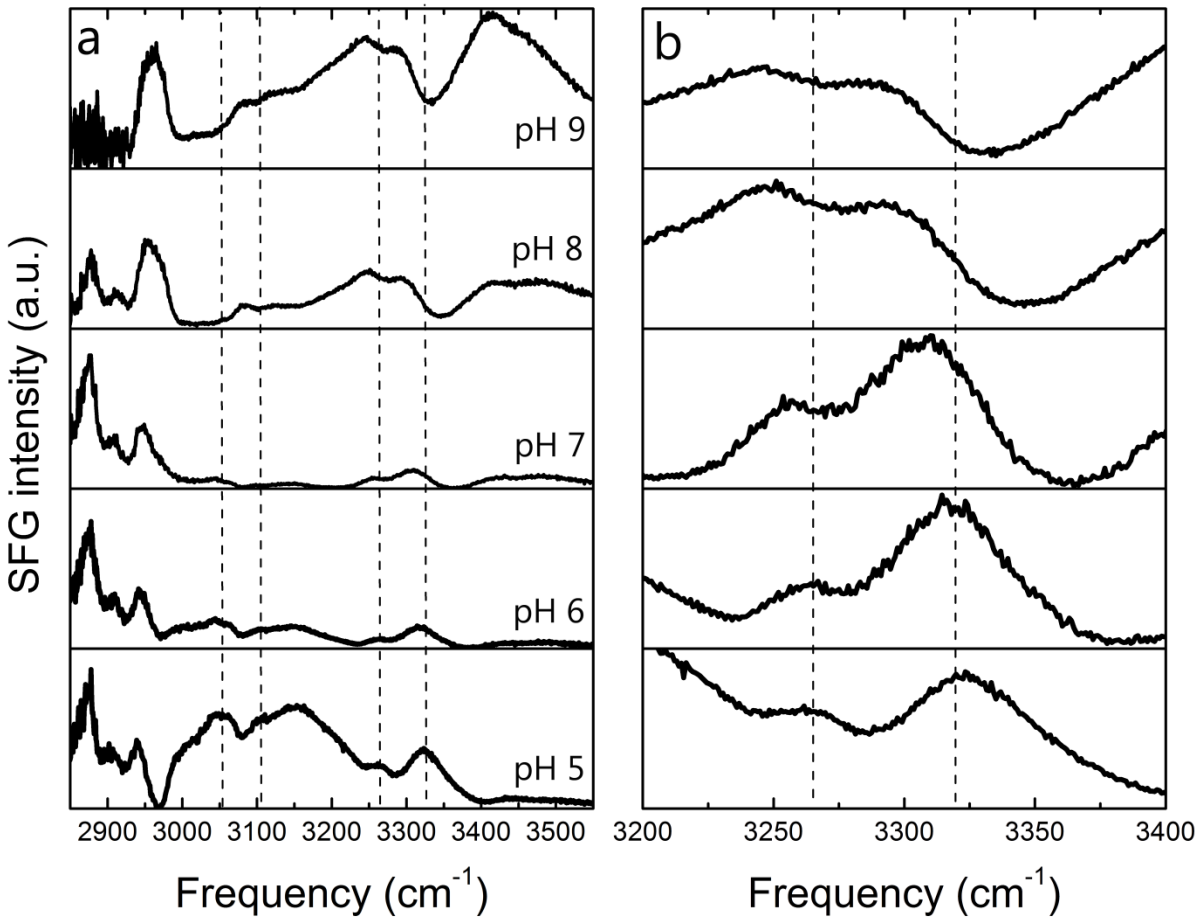

Figure 1: (a) Vibrational SFG spectra of a class II hydrophobin HFBII film adsorbed at the water-air interface, measured with ssp polarization configuration (SFG=s, vis=s, IR=p-polarized). The spectra were taken at different pH values as indicated. The dashed lines indicate the vibrational modes of interest at ~3050 cm^{-1}, 3105 cm^{-1}, 3270 cm^{-1} and 3340 cm^{-1}. (b) Magnification of the response of the N-H stretching modes at ~3270 cm^{-1} and 3340 cm^{-1}.

In Figure 1 we present pH-dependent VSFG spectra of the class II hydrophobin HFBII adsorbed to the air-water interface. In the frequency region of 2800-3100 cm^{-1} the vibrational SFG spectra show strong signals of C-H stretching vibrations. We assign these bands to the methyl symmetric stretch (~2880 cm^{-1}), the methine stretch (~2905 cm^{-1}), a Fermi resonance (~2940 cm^{-1}) and

aromatic C-H (~3050 cm^{-1}) vibrations[13]. At frequencies >3100 cm^{-1} the spectrum of HBFII shows the broad response of the O-H stretching band of interfacial water molecules. We further identify resonances at ~3270 cm^{-1} and ~3340 cm^{-1} which we assign to the N-H stretch vibrations of the β-sheet and β-turn structural elements present in hydrophobin (Supporting Figure 1). These structural elements also lead to clear signals in the amide I region[14]. The weak signal at ~3100 cm^{-1} is assigned to the amide B band of the N-H vibration[15].

Hydrophobins are known to be stable in a wide range of pH values, and it has been shown that the adsorption of hydrophobins at the water-air interface is independent of the solution pH over a wide range of pH values[2, 12]. Changing the pH of the solution has a significant impact on the shape and intensity of the C-H, N-H and particularly the O-H vibrational bands of the conventional intensity VSFG spectra. We find that at pH levels close to the isoelectric point (IEP) of HFBII (~ 6.7) the VSFG intensity of the water signals is negligible. At pH values below (positive net charge) and above the IEP (negative net charge), the intensity of the O-H vibrational bands increase markedly, agreeing with previous VSFG studies of proteins at the water-air interface[16-18]. We further observe that the signals at 3100, 3270 cm^{-1} and 3340 cm^{-1} show an identical pH-dependence as the signal at 3050 cm^{-1}. For pH values >7 all these signals appear as negative features in the intensity VSFG spectra, and for pH values <7 they appear as positive features. It is well-known that the band at 3050 cm^{-1} originates from the C-H stretch vibration of aromatic rings and that the origin of the apparent phase reversal does not lie in a change in the orientation of the C-H groups, but rather in the change of the orientation of the interfacial water molecules that can be easily oriented[16, 18]. At pH<7 the 3050 cm^{-1} band interferes constructively with the broad O-H stretching band (pH<7) leading to a positive feature in the intensity VSFG spectrum, while at pH>7 destructive interference is observed. Given the similar pH dependence of the signals at 3100, 3270 cm^{-1} and 3340 cm^{-1}, we conclude that these signals arise from vibrations of the protein that show a similar interference effect with the broad O-H stretching band as the band at 3050 cm^{-1}. The pH dependence of the interference makes it highly unlikely that the signals at 3100, 3270 cm^{-1} and 3340 cm^{-1} would be due to water O-H bands as these obviously are not expected to change their interference with other water O-H modes as a function of pH.

To further check the assignment of the signals at 3100, 3270 cm^{-1} and 3340 cm^{-1} to protein N-H vibrations, we also measured VSFG experiments of HFBII dissolved in D_2O. Clearly, if these signals would be due to water O-H vibrations they should not be observed due to the H/D-exchange. Figure 2 shows VSFG spectra of the HFBII in D_2O with different solution pH. We observe a persistence of all three resonances at 3100, 3270 cm^{-1} and 3340 cm^{-1}, which constitutes conclusive evidence that these signals arise from N-H vibrations. The results of the HFBII in D_2O spectra further show that the N-H vibrations belong to the protein backbone, as N-H groups in the side-chains are known to show rapid isotope exchange[15]. The slow and incomplete H/D-exchange also illustrates that the HFBII films at the interface possess a very rigid structure.

We observe that the intensity of the N-H signals varies at different pH-values. At low pH we observe all three signals while at pH values >IEP of HFBII only one N-H signal is observed. We explain these changes from a pH dependence of the overall orientation (tilt) of the hydrophobin molecules at the interface. Similar orientation-induced changes of the spectral response are observed in the amide I region of the VSFG spectrum[19].

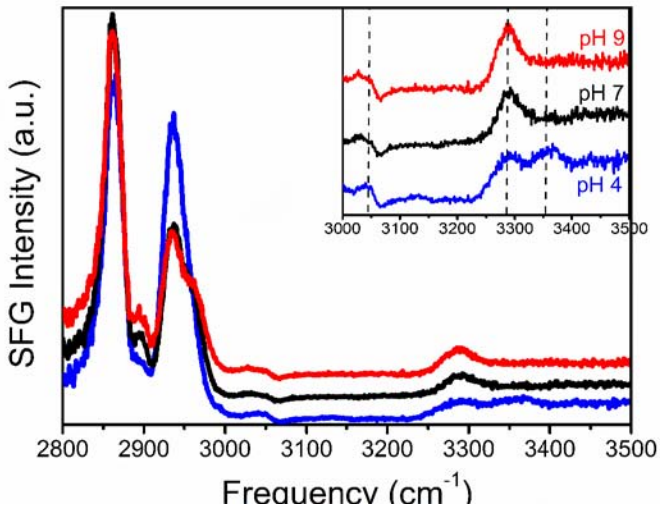

Figure 2: (a) Vibrational SFG spectra of a class II hydrophobin HFBII film adsorbed at the D_2O-air interface, measured with ssp polarization configuration (SFG=s, vis=s, IR=p-polarized). The spectra were taken at different pH values as indicated. The dashed lines indicate the vibrational modes of interest at ~3050 cm^{-1}, 3270 cm^{-1} and 3350 cm^{-1}. (b) Magnification of the response of the N-H stretching modes at ~3270 cm^{-1} and 3350 cm^{-1}.

To study the effect of the pH on the N-H vibrations in more detail, we measure heterodyne detected VSFG spectra of the protein films. HD-VSFG provides the imaginary (Im) and real parts (Re) of $\chi^{(2)}$ as a function of frequency, and thus provides important additional information in comparison to conventional intensity VSFG that only measures the absolute square of the second-order nonlinear susceptibility $\chi^{(2)}$. The sign of the Im[$\chi^{(2)}$] spectrum is directly connected to the absolute orientation of the molecules at the interface, and therefore provide direct information on the molecular-scale structure[20]. In Figure 3 we show HD-VSFG spectra of HFBII adsorbed to the air-water interface measured in ssp polarization (s-SFG, s-VIS, p-IR). We recorded spectra at pH values 4.5, 7 and 8 which correspond to a negatively, positively and neutral protein layer at the interface[12]. In the C-H stretching region negative bands appear at ~2880 cm^{-1} and ~2940 cm^{-1} and are attributed to the methyl symmetric stretch ($\nu CH_{3,SS}$) and a methyl Fermi resonance ($\nu CH_{3,FR}$), respectively. The positive band at ~2975 cm^{-1} can be assigned to the methyl anti-symmetric stretching ($\nu CH_{3,AS}$) vibration. The negative sign of $\nu CH_{3,SS}$ and $\nu CH_{3,FR}$ bands and the positive sign of $\nu CH_{3,AS}$ together indicate that the terminal methyl CH molecules are pointing towards the air[21, 22]. The hydrophobic patch of HFBII consists entirely of aliphatic amino acid residues, possessing terminal methyl groups (Valine, Leucine, Isoleucine, Alanine)[23], thus agreeing with a molecular picture in which HFBII absorbs at the water-air interface with its hydrophobic patch closest to the air. Changing the pH of the solution does not have a significant effect on the intensity of the C-H signals, indicating that the net orientation of the C-H groups of the protein does not change when varying the pH. We observe that the overall signal of the Im $\chi(2)$ spectrum changes sign because the signal is dominated by the response of the O-H vibrations of the interfacial water molecules. The O-H groups change their net orientation, following the pH dependent overall charge state of the protein. The N-H bands observed at 3270 cm^{-1} and 3310 cm^{-1} constitute relatively small and narrow additional features that add to the broad response of the interfacial water O-H band. At all three pH values, these additional features have a negative sign, meaning that the corresponding protein N-H groups do not change their orientation when the pH is varied. The amplitudes of the N-H signals do change as a function of pH, which can be well explained from a small change of the overall tilt of the proteins at the interface. The results obtained with HD-VSFG are highly consistent with the conventional intensity VSFG spectra as demonstrated by the comparison of the constructed $|\chi^{(2)}|^2$ with the magnitude spectra (SFig. 2).

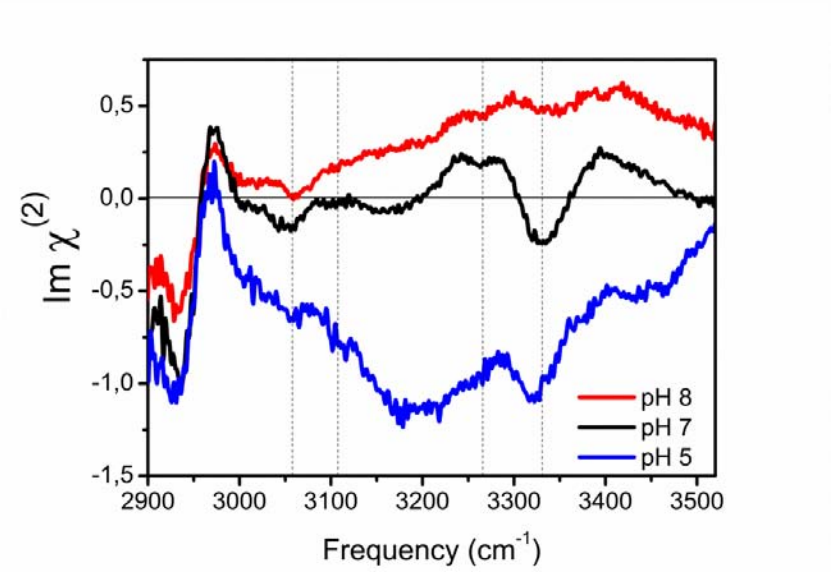

Figure 3: Imaginary χ(2) spectra of a HFBII film at the water-air interface. Spectra were recorded in ssp polarization (s-SFG, s-VIS, p-IR) and at pH values above (red) below (blue) and close to the isoelectric point (black). The dashed lines indicate the vibrational modes at ~3050 cm^{-1}, ~3105 cm^{-1}, ~3270 cm^{-1} and ~3340 cm^{-1}.

Discussion

At pH values close to the IEP the protein contains no net charge and consequently no electric field induced ordering of the dipoles of the water molecules. At pH values above the isoelectric point of hydrophobin (IEP~ 6.7) the protein contains a negative net charge and aligns water molecules with their O-H groups pointing towards the air phase, while at lower pH values the positive net charge leads to the O-H-bonds pointing to the water phase[5, 24]. It is well-established that pH-induced changes in the C-H signals of proteins in conventional VSFG spectra are solely due to the change of the relative phase of the C-H and O-H signals, rather than changes in the response of the C-H stretch vibrations[18]. In several VSFG studies of proteins the aromatic C-H stretching band at ~3050 cm^{-1} has thus been used to deduce the absolute water orientation at the protein/water interface[6, 16]. While the influence on the C-H vibration is well known, no information currently exist on a possible interference of the water O-H response with the protein N-H vibrational bands. The HD-VSFG results of Figure 2 clearly show that the pure Imχ(2) responses of the N-H vibrations show very little change when the pH is varied. We thus conclude that the changes observed at ~3100 cm^{-1}, ~3270 cm^{-1} and ~3340 cm^{-1} in the intensity VSFG

spectra with varying pH result from a similar interference with the water O-H response as is observed for the aromatic C-H vibration at ~3050 cm^{-1}. Due to this interference the N-H bands appear as positive peaks at pH values below the IEP, and as negative feature for pH values above the IEP. The strong changes in frequency, amplitude and sign of the N-H signals in the conventional intensity VSFG spectra can thus be mostly attributed to interference effects, the responses of the N-H modes themselves are quite pH independent. Here it should be realized that hydrophobins are structurally rigid and form a highly-ordered interfacial layers[6]. Most other proteins do undergo conformational changes and even partially unfold when they adsorb at the water/air interface. For these proteins the responses of the N-H signals may in fact change when the pH is varied. The present results demonstrate that HD-VSFG measurements are superior to intensity VSFG measurements in revealing conformational changes from the response of the N-H vibrations.

Conclusion

We performed conventional and heterodyne-detected vibrational sum-frequency generation (HD-VSFG) measurements to study the dependence of the N-H signals of proteins on pH. In our conventional VSFG spectra we find that the N-H related responses show significant changes in shape and intensity following a modification of the pH. These changes are not due to conformational changes of the protein, but rather result from the interference of the N-H response with the broad interfacial water band, as we illustrate with HD-VSFG experiments. We conclude that the use of HD-VSFG is essential to distinguish pH-induced conformational changes of proteins from changes in the interference with the background signal of water.

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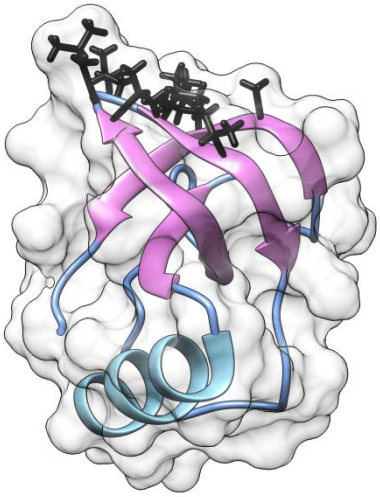
Supporting Information:

Methods:

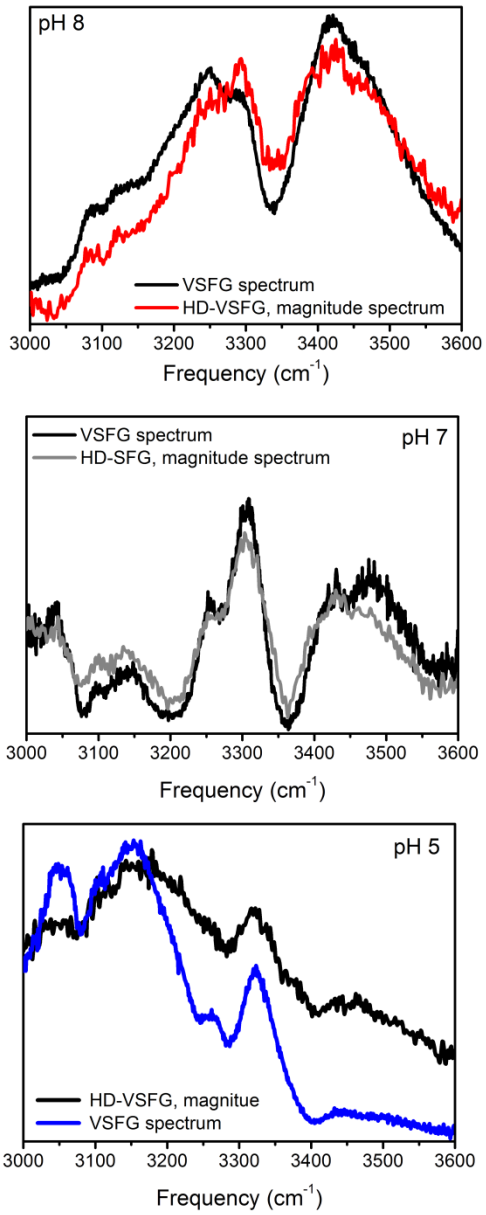
The laser source for the vibrational sum-frequency generation experiments is a regenerative Ti:Sapphire amplifier (Coherent) producing 800 nm pulses at a 1 kHz repetition rate with a pulse duration of 35 fs and a pulse energy of 3.5 mJ. Approximately one third of the laser output is used to pump a home-built optical parametric amplifier and a difference-frequency mixing stage. This nonlinear frequency conversion produces broadband mid-IR pulses (tuneable from 2-10 μm, with a bandwidth of 600 cm^{-1} (FWHM), and a pulse energy of 10-20 μJ). The IR pulses have a sufficiently large bandwidth to measure the complete SFG spectrum of the OH stretch vibrations of H_2O. Another part of the 800 nm pulse is sent through an etalon to narrow down its bandwidth to ~15 cm^{-1}. The resulting narrow-band 800 nm pulse (VIS) and the broadband IR pulse are directed to the sample surface at angles of ~50° and ~55°, respectively, to generate light at the sum frequency. The VIS and IR beams are focused in spatial and temporal overlap on the sample surface with 200 mm and 100 mm focal length lenses, respectively. The SFG light generated at the surface is sent to a monochromator and detected with an Electron-Multiplied Charge Coupled Device (EMCCD, Andor Technologies). The conventional VSFG spectra are background subtracted (blocked IR) and normalized to a reference SFG spectrum measured from z-cut quartz. The acquisition time of a typical VSFG measurement is 600s.

We use heterodyne-detected VSFG to determine the imaginary and real parts of $\chi(2)$. In HD-VSFG the SFG electric field generated from the sample is combined with that of a local oscillator (LO) at the same frequency. The LO sum-frequency (LO-SFG) light is generated by first focusing the IR and VIS beams on a metal surface to generate a strong non-resonant $\chi(2)$ SFG signal. This signal is delayed with respect to the IR and VIS beams by passing it through a silica plate (~ 1 mm). The LO-SFG and the IR and VIS beams are refocused on the sample surface using a spherical mirror. The IR and VIS beam generate the SFG signal of the sample. Subsequently, the LO-SFG and sample SFG beams are collimated, sent into a monochromator and detected with a CCD. The detected interference pattern contains cross terms from which we extract the real and imaginary $\chi(2)$ part using Fourier transformation. To obtain the Im $\chi(2)$ of the sample, we compare the signal with the HD-VSFG signal of a reference sample for which the phase of the SFG light is known. For this purpose we used a z-cut quartz crystal that was

oriented in such a way that the bulk electric dipole contribution of quartz was maximized. For HD-SFG measurements it is crucial that the HD-VSFG signal from the reference (z-cut quartz crystal) is generated at the same height as the sample. A mismatch in height would result in a phase shift of the recorded spectra and thus in the extracted real and imaginary $\chi(2)$. We control the height of the reference quartz crystal and the sample by monitoring the position of the VSFG signal on the CCD camera. This enables us to define the SFG signal on the camera with a precision of 1 pixel size (16 x 16 μm). Together with our setup geometry this leads to a phase uncertainty of $\sim\pi/10$ (~20 degrees). The phase uncertainty does not affect our experimental results nor their interpretation, as the possible flipping of vibrations as a function of pH would involve much larger phase changes $\sim\pi$ (180 degrees). The typical acquisition time of a HD-SFG spectrum is 120 s. All SFG measurements were performed in H_2O (Milipore) or D_2O (Cambridge Laboratories) and the pH was adjusted using hydrochloric acid or sodium hydroxide. The pH (Mettler Toledo FE20) of the samples was checked before and after each measurement. Class II hydrophobins were provided by VTT research and purified as described in Ref.[25].



Supporting Figure 1: Three-dimensional structure of T. reesei hydrophobin HFBII. Hydrophobins fold into compact structures with a β-barrel core and a distinguishable hydrophobic patch (colored in black).



Supplementary Figure 2: Comparison of the HD-VSFG magnitude (Imaginary χ(2))2 + (Real χ(2))2 and conventional VSFG spectrum of a HFBII film at the air-water interface. Spectra were obtained at different pH values as indicated in the figure.

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