Probing the absolute configuration of chiral molecules at aqueous interfaces

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We demonstrate that the enantiomers of chiral macromolecules at an aqueous interface can be distinguished with monolayer sensitivity using heterodyne-detected vibrational sum-frequency generation (VSFG). We perform VSFG spectroscopy with a polarization combination that selectively probes chiral molecular structures. By using frequencies far detuned from electronic resonances we probe the chiral macromolecular structures with high surface specificity. The phase of the sum-frequency light generated by the chiral molecules is determined using heterodyne detection. With this approach we can distinguish right-handed and left-handed helical peptides at a water-air interface. We thus show that heterodyne-detected VSFG is sensitive to the absolute configuration of complex, interfacial macromolecules and has the potential to determine the absolute configuration of enantiomers at interfaces.

Chirality is of importance in many areas of (bio)chemistry, ranging from enantioselective catalysis to supramolecular chemistry and protein biochemistry. In addition to point chirality associated with an asymmetric (stereogenic) carbon atom, macromolecular chirality such as the handedness of a helical polymer arises if chiral or even achiral building blocks are arranged in such a way that the macromolecular assembly is non-superimposable with its mirror image.

While chiroptical methods such as optical rotation, allow for the differentiation between enantiomers, the determination of the absolute configuration of a chiral molecule, i.e. the unambiguous identification of a right- or left-handed enantiomers without *a priori* knowledge, remains challenging and at present only few measurement techniques exist that offer this capability. Anomalous X-ray scattering dispersion has been the first technique to achieve this identification [1] and is now routinely applied to crystalline samples [2]. Recently, new tools for the determination of the absolute configuration of small molecules in the gas-phase have emerged. Chirality-sensitive three-wave-mixing in the microwave regime [3] allows to determine the absolute configuration when augmented by quantum chemical calculations [4]. Furthermore, it has been demonstrated that the imaging of fragmentation products resulting from laser-induced Coulomb explosions of chiral molecules allows for the determination of the absolute configuration [5–7].

In the liquid phase, the combination of circular dichroism (CD) and computational approaches is capable of identifying the absolute configuration. In particular vibrational circular dichroism (VCD) in the mid-infrared has matured into a powerful tool for the structural and conformational elucidation of small molecules in solution, owing to its sensitivity to distinct vibrational marker bands [8–14]. While CD-spectroscopy is a linear optical technique, the enantiomer-specificity of which results from inherently weak magnetic dipole or electric quadrupole interactions, the potential of non-linear optical techniques such as second harmonic generation (SHG) and sum-frequency generation (SFG) to study chiral molecules has been increasingly recognized over the past years [15–25]. Vibrational SFG-spectroscopy (VSFG) is a second-order non-linear optical technique, in which an infrared and a visible pulse are combined to generate light at their sum-frequency. This generation is enhanced in case the infrared light is in resonance with specific molecular vibrations. Within the electric dipole approximation, SFG is only allowed in media that lack inversion symmetry such as chiral media [27] or interfaces [28], where the inversion symmetry is inherently broken. Vibrational SFG therefore offers the structural resolution of vibrational spectroscopy with the specificity to non-centrosymmetric media. As a direct consequence, VSFG-signals can be generated from the bulk of an isotropic chiral medium and from interfaces with other media. The origin of a chiral VSFG-signal (bulk vs. interface) can be unambiguously determined by comparing the phase of the signal with a reference signal of known phase (see below).

Recently, chiral VSFG-spectra have been successfully measured for protein monolayers in the spectral region of

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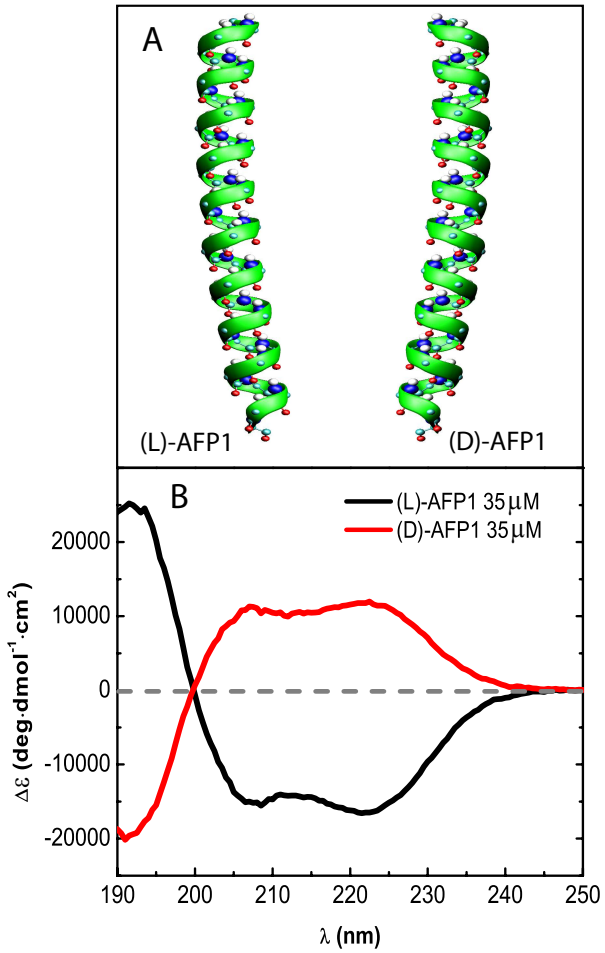

FIG. 1. (A) Structure of AFP synthesized from L- and D-amino acids; the structure of (D)-AFP1 has been generated by a mirror-image transformation of the structure of (L)-AFP1 published in [26](Protein Data Bank identifier 1WFA). (B) Near UV-circular dichroism spectra obtained from solutions of (L)- and (D)-AFP1 at 15 °, respectively. The CD-spectra exhibit the characteristic features of an α-helix, showing extrema in the molar ellipticity at 207 and 222 nm. The opposite sign of the CD-spectra confirms that (L)- and (D)-AFP1 indeed form helices with opposite handedness and are enantiomeric to each other.

the amide I vibration (C=O-stretch) and the NH-stretch vibration [19, 21–23], which allowed e.g. to obtain unprecedented insights into the aggregation mechanism of amyloidic peptides at water-lipid interfaces [22]. While these studies have focussed on the intensity of the generated SFG-light $I_{sfg} \propto |\chi^{(2)}|^2$, the sign of the non-linear susceptibility $\chi^{(2)}_{chiral}$ of a chiral molecule changes between enantiomers, and thus information on the absolute configuration of chiral molecules can be obtained if the real and imaginary part of $\chi^{(2)}_{chiral}$ are determined [15–18, 24, 25]. This has been demonstrated for bulk solutions of limonene [24] and BINOL (1,1'-bi-2-naphtol)[25]. Recently, the first phase-resolved chiral VSFG-spectra have been reported for a few proteins at the air-water interface [29]. Here, we show how heterodyne-detected chiral VSFG spectroscopy can be used to distinguish between enantiomers of interfacial (bio)macromolecules with monolayer sensitivity. We show that this method allows for an unambiguous distinction between left- and right-handed α-helical peptides at the water-air interface.

We use VSFG to study the biologically relevant type I antifreeze peptide (AFP1) [30] at the water-air interface. AFP1 is naturally found in the body fluids of the cold-adapted winter flounder fish, forming a natural protection against freeze damage from ice-crystals by adsorbing to the ice-crystal surfaces and inhibiting further growth [31]. AFP1 is 37 amino acids in length and fully α-helical. The handedness of an α-helix is defined by the chirality of the amino acids (L- or D-enantiomers) and constitutes a form of supramolecular chirality. We have synthesized two AFP1 peptides with either (L)- or (D)-amino acid configuration, which allows us to selectively control the handedness of the helix and hence the absolute configuration of the molecule. The helicity of (L)- and (D)-AFP1 in the bulk of the solution was studied by circular dichroism. The CD-spectra in Fig. 1B show the characteristics of an α-helix, but with opposite sign for (L)-AFP1 and (D)-AFP1, confirming that the two peptides form indeed helices with opposite handedness. The temperature-dependence of the CD-spectra shows that the thermal stability of (L)- and (D)-AFP1 is highly similar and agree with literature values (see Supporting Information [32]).

Fig. 2A shows the intensity VSFG-spectrum of (L)-AFP1 obtained under ssp-polarization conditions (s-polarized SFG, s-polarized VIS, p-polarized IR). The spectrum consists of a broad band around ~ 3200 cm^{-1} originating from the OH-stretch vibration of interfacial water molecules, and two additional narrow resonances at 2890 cm^{-1} and 2950 cm^{-1} which originate from the symmetric and antisymmetric CH_3-stretching vibrations [33] of methyl-groups in the amino acid side chains of AFPI. Additionally, a peak on the high frequency side of the broad OH-band is observed at 3350 cm^{-1}. Based on its resonance position and its narrow line-width, we assign this band to an NH-stretch vibration. While the signal could in principle also originate from NH-groups in the side-chains of amino acids (lysine and arginine) or the N-terminus/amidated C-terminus of the peptide, the overwhelming prevalence of backbone-NH-groups (36) over side-chain (2) or N/C-terminal NH-groups (2) supports an assignment to backbone NH-vibrations.

In Fig. 2B we show chiral-specific VSFG-spectra of (L)- and (D)-AFP1 measured under psp-polarization conditions. The broad signal from the OH-stretch vibration of water molecules observed in Fig. 2B is completely absent from the psp SFG spectrum, and only a single, sharp feature with a width of ~ 100 cm^{-1} is observed in the spectrum, which we assign to the NH-stretch vibration of the peptide backbone [22]. Both the resonance frequency and the width of the peak are

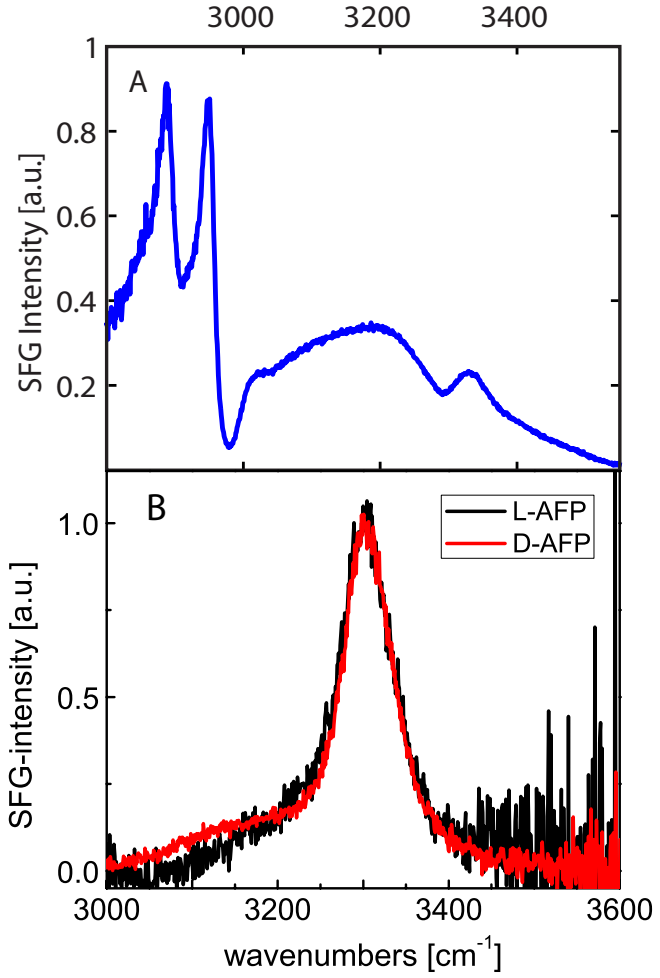

FIG. 2. (A) Intensity SFG-spectrum obtained from an aqueous solutions of (L)-AFP1 under ssp (achiral) polarization conditions. The sharp resonances below 3000 cm^{-1} originate from CH-stretch vibrations of the peptide and the broad resonance centered around $\sim$ 3200 cm^{-1} originates from the OH-stretch vibration of interfacial water molecules. A peak on the high frequency side of the broad OH-band can be observed around 3350 cm^{-1}. (B) Comparison of intensity SFG-spectrum obtained from an aqueous solutions of (L)-AFP1 and (D)-AFP1 under psp polarization conditions. In the chiral SFG-spectrum of panel (B), only one spectrally narrow feature, originating from the NH-stretch vibration of the peptide backbone [22], is observed.

in good agreement with values recently reported for the NH-stretch vibration of a number of other α-helical peptides and proteins [22]. The intensity-spectra in Fig. 2B only demonstrate the presence of chiral molecules at the water-air interface, but do not provide information on their absolute configuration, i.e. the handedness of the helices. The nature of the enantiomer can be determined by measuring the phase of $\chi^{(2)}$, which requires heterodyne detection of the SFG light. The phase-resolved $\chi^{(2)}$-spectrum is obtained by spectral interferometry using a Fourier filtering approach similar to the one described in Ref. [34].

In Fig. 3, we plot the real and imaginary $\chi^{(2)}$ of L-and D-type AFP1 in the spectral region of the NH-stretch vibration obtained under psp-polarization settings. The opposite phase of the backbone NH-vibration resulting from the different signs of $\chi^{(2)}_{chiral}$ for right-handed (L-AFP1) and left-handed (D-AFP1) α-helical peptides is clearly resolved. This is most clearly seen in the absorptive imaginary part of $\chi^{(2)}$ that shows a sign change between (L)-AFP1 and (D)-AFP1. The dispersive real parts of $\chi^{(2)}_{chiral}$ reflect the same trend, with $\mathrm{Re}(\chi^{(2)}_{chiral})$ of (L)-AFP1 being negative at $\omega < \omega_0$, exhibiting a zero-crossing at ω_0 and becoming positive $\omega > \omega_0$. For (D)-AFP1 the opposite trend is observed, which is fully consistent with the opposite sign of the imaginary parts of $\chi^{(2)}_{chiral}$.

A chiral VSFG signal can in principle originate both from the bulk and the surface of a solution, owing to the non-centrosymmetric nature of a chiral medium. The clarify the origin of the measured SFG-signal, the *absolute* phase of the chiral VSFG signal needs to be unambiguously determined. We achieve this by comparing the phase of the chiral VSFG signal generated from the AFP-sample (E_{sample}) with the phase of the SFG signal from a reference quartz crystal with a well-known phase (E_{quartz}). To this purpose, we replace the sample by a right-handed, z-cut quartz crystal with the optical axis (z-axis) oriented parallel to the surface normal. For quartz the sum-frequency generation process is non-resonant, meaning that $\chi^{(2)}$ is real and frequency independent. Hence, the ratio of E_{sample} obtained from the AFP-solution and E_{quartz} directly yields the values of the real and imaginary $\chi^{(2)}_{sample}$ of the AFP molecules at the water-air interface according to

$$\chi^{(2)}_{sample}(\omega) = \chi^{(2)}_{quartz}(\omega)\frac{E_{sample}(\omega)}{E_{quartz}(\omega)} \qquad (1)$$

where the sign of $\chi^{(2)}_{quartz}(\omega)$ is determined by the handedness and the orientation of the crystal.

Eq. 1 assumes that there is no phase-shift between $E_{sample}(\omega)$ and $E_{quartz}(\omega)$. This assumption is valid if E_{sample} and E_{quartz} originate either *both* from an interface or *both* from the bulk of a medium. If one of the two fields originates from an interface while the other is generated in the bulk of a medium, a relative phase-shift between $E_{sample}(\omega)$ and $E_{quartz}(\omega)$ of $\pi/2$ is expected [35, 36]. We orient the quartz-crystal in such a way that its bulk $\chi^{(2)}$ equals zero and thus a surface-specific $E_{quartz}(\omega)$ is observed (see the Supporting Information [32] for a detailed description of the phase determination). The frequency dependence of the real and imaginary parts of $\chi^{(2)}_{chiral}$ as obtained from Eq. 1 and shown in Fig. 3 unambiguously confirms that the psp SFG signal originates from AFP molecules located at the water-air interface: in case these signals would originate from bulk AFP molecules, the detected $\chi^{(2)}_{chiral}$ would be phase

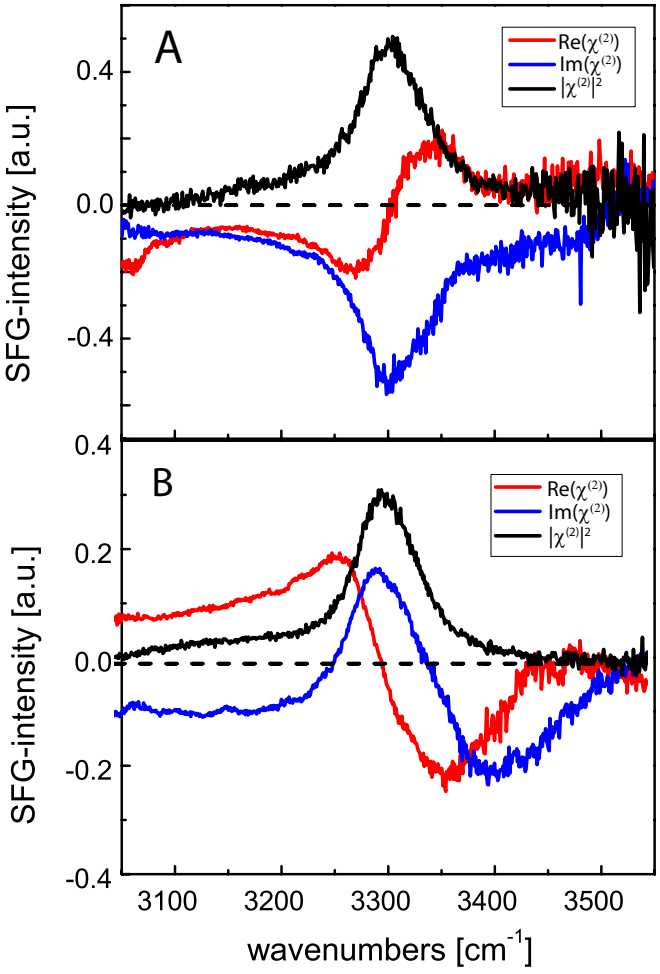

FIG. 3. (A) Real and imaginary $\chi^{(2)}$ measured for L-type AFP1. (B) As (A) for D-type AFP1. The intensity VSFG-spectra (proportional to $|\chi^{(2)}|^2$) of (L)- and (D)-AFP1 are shown in black for comparison.

shifted by $\pi/2$, which would result in an absorptive line shape for the real part of $\chi^{(2)}_{chiral}$ and a dispersive line shape, i.e. a sign change at the resonance frequency of 3350 cm^{-1}, for the imaginary part of $\chi^{(2)}_{chiral}$.

We thus find that heterodyne-detected chiral SFG allows for the determination of the amplitude and phase of $\chi^{(2)}_{chiral}$. This $\chi^{(2)}_{chiral}$ can be compared with a calculation of the molecular hyperpolarizabilities and thus of $\chi^{(2)}_{chiral}$ by means of a quantum-chemical computations, thereby allowing for a determination of the absolute configuration of interfacial molecules without *a priori* knowledge on the nature of the enantiomer. This type of calculations have already been demonstrated for molecular systems of comparable size [37]. This approach is similar to what has become possible for chiral molecules in the bulk phase by combining vibrational CD-spectroscopy with quantum-chemical methods. Due to the low sample amount that is required and short data integration times (<10 minutes), the experimental approach outlined here is perfectly suited to study (bio)chemical transformations at interfaces, offering unique insight in structural and conformational rearrangements of interfacial macromolecules.

In conclusion, we have demonstrated heterodyne-detected chiral vibrational sum-frequency generation (VSFG) of the left- and right-handed enantiomers of α-helical antifreeze peptides, and we have demonstrated that the signal originates from peptide molecules at the air-water interface. By interfering the SFG light generated at the sample surface with the light of a local oscillator, we determine the real and imaginary part of the second-order susceptibility $\chi^{(2)}_{chiral}$, which directly reflects the absolute configuration of the chiral molecule. This method may find many applications, especially in fields where stereoselective (bio)chemical processes at interfaces play an important role.

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ACKNOWLEDGMENTS

This work is part of the research program of the "Stichting voor Fundamenteel Onderzoek der Materie (FOM)", which is financially supported by the "Nederlandse organisatie voor Wetenschappelijk Onderzoek (NWO)". IKV gratefully acknowledges the Netherlands Organisation for Scientific Research (NWO - VENI Grant: 700.10.406) and the European Union through the Marie Curie Fellowship program FP7-PEOPLE-2011-CIG (Contract No. 293788) for funding.