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Description	

Sum frequency generation spectroscopy of the attachment disc of a spider

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Abstract

The pyriform silk of the attachment disc of a spider was studied using infrared-visible vibrational sum frequency generation (SFG) spectroscopy. The spider can attach dragline and radial lines to many kinds of substrates in nature (concrete, alloy, metal, glass, plant branches, leaves, etc.) with the attachment disc. The adhesion can bear the spider's own weight, and resist the wind on its orb web. From our SFG spectroscopy study, the NH group of arginine side chain and/or NH₂ group of arginine and glutamine side chain in the amino acid sequence of the attachment silk proteins are suggested to be oriented in the disc. It was inferred from the observed doublet SFG peaks at around 3300 cm⁻¹ that the oriented peptide contains two kinds of structures.

Keywords: spider silk; orb web; pyriform silk; attachment disc; SFG;

1. Introduction

The orb-weaving spider can secrete seven types of silk for various uses by its seven types of secretory glands. The spider's dragline for vertical movement and the radial lines for orb-web construction are secreted from the large ampullate gland. On the other hand, the pyriform gland secretion is used to glue a dragline or a radial line onto surfaces [1–4]. Pyriform silk and glue are extruded from pyriform gland [5]. The pyriform gland is composed of two kinds of secretory cells [5]. One is located in the distal half of the glands, and produces pyriform spidroins with finely fibrillar proteinic granules through an extensive rough endoplasmic reticulum; another one is located in the proximal half of the gland, and secretes a glue-like component containing protein with a highly organized structure and unidentified carbohydrate [5]. In the proximal half of the gland, the carbohydrate component may be added to the protein [5].

The glue from the pyriform gland is an analogous liquid [6]. A stable cement-like attachment disc is formed in less than a second in a natural environment [7], and sticks the dragline and radial lines to the surface of materials [8]. The structure made of pyriform silk and cement-like glue for fixing the dragline and the radial line to the materials is called an attachment disc. Hundreds of spinnerets in the abdomen of the spider are brought into direct contact with the material's surface, and a large amount of pyriform silk is discharged from the spinnerets while being rubbed against the surface to form an attachment disc [9–12]. When a spider secretes attachment disc silk, its spinnerets are said to rub back-and-forth on parallel straight lines [12]. As shown in Figs. 1(b) and (c), the attachment disc of pyriform silk looks like a set of staple-pins and it fixes the dragline to the substrate.

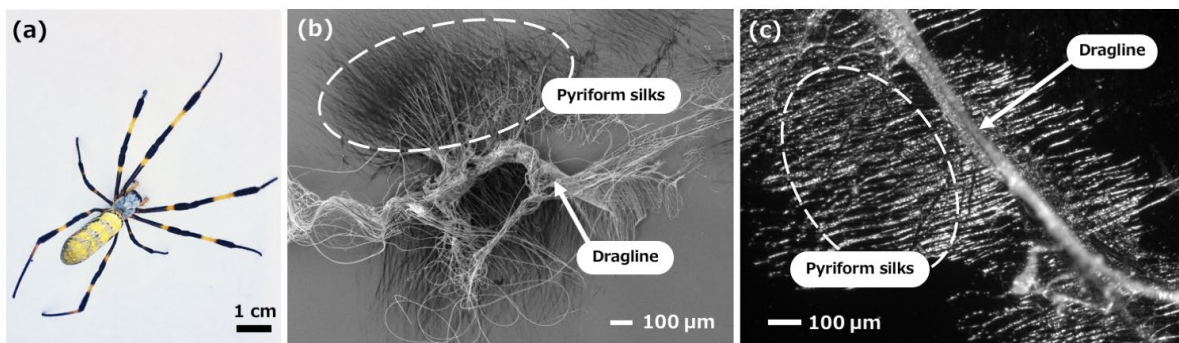


Figure 1. (a) Photograph of the spider (*N. clavata*) featured in this study. (b) Scanning electron microscopy (SEM, HITACHI TM3030Plus, acceleration voltage: 15 kV) image of an attachment disc of the spider. (c) Optical microscopy image of an attachment disc of the spider.

The attachment disc can apply a strong adhesive force to the surface of almost any material in nature. On the other hand, it has been reported that its adhesion strength or weakness depends greatly on the material [13,14]. However, the attachment disc adhesion strength to polytetrafluoroethylene (PTFE) is high in spite of its very low surface free energy [13]. On the other hand, the attachment disc cannot adhere to highly hydrophobic substrate surfaces, such as a

glass treated with dichlorodimethylsilane [7]. Besides this example, the mechanism of its adhesion remains unclear in many aspects.

Pyriiform silk is mainly composed of proteins called pyriiform spidroin 1 (PySp1) and pyriiform spidroin 2 (PySp2) [6,8,15]. PySp1 contains block modules with a high degree of polarity and charge [6]. On the molecular scale, the amino acid sequences of pyriiform silks may interact strongly with the cement-like glue due to a high content of polar side chains. Pyriiform silks are not directly involved in the interaction with the substrate [7], but must be firmly attached to the cement-like glue. We have found in the past that an anisotropic secondary structure in the highly oriented spidroin (e.g., dragline or radial line) exhibits a second-order nonlinear optical response [16,17]. In this study, in order to get a further hint of the structure of the pyriiform silk, vibrational sum frequency generation (SFG) spectroscopy was used to investigate the physically anisotropic structure and the chemical species of the attachment disc. Since SFG occurs only in non-centrosymmetric structures, it can selectively observe oriented molecular components. Hence orientation of asymmetric molecular species can be analyzed by SFG. Since the SFG response is very weak, one must have as much sample material as possible in the probe laser spot, in order to get good S/N data. From this point of view also, attachment discs are easier to study than the other types of spider silk, since they are secreted in disc shapes and not in thin line shapes.

With the development of optical engineering, SFG vibrational spectroscopy has been widely used for the study of protein structure and molecular dynamics at various *in situ* surfaces and interfaces for the last 20 years [18–42]. The secondary structure of proteins can be analyzed by examining chiral NH stretching vibration and chiral amide I using chiral SFG [23,41]. On the other hand, our study uses the ‘achiral’ SFG method and it cannot analyze the secondary structures. While it is important to investigate the secondary structure of proteins in spider attachments, there

is no SFG study on attachment in the past. Hence this study is focused just on the second-order nonlinear response in molecular species. Here, the 'achiral' SFG method not only lets us assign the molecular species but also selectively detects non-centrosymmetric bulk structures including the secondary structure of proteins.

The cement-like glue has been reported to be protein rich in acidic groups and associated with a carbohydrate component [5]. A beads-on-a-string (BOAS) structure similar to the sticky aggregate glue droplets of orb web for catching preys is formed occasionally when the pyriform gland secretions are not in contact with the substrate [7]. Singla *et al.* [43] reported the SFG spectrum of the aggregate glue droplets of a spider orb web. Aggregate glue droplet is composed of glycoproteins (ASG-1 and ASG-2) [44–49], low-molecular-mass compounds (LMMCs) [50–55], inorganic salts [51,53,54], and bound water [43,56]. The attachment disc and the aggregate glue droplet have two similarities: the BOAS structure and the protein associated with a carbohydrate component. The attachment discs observed in our study should contain components in common with the aggregate glue droplet sample of Singla *et al.* [43]. On the other hand, our study also revealed different components from that in aggregate glue droplets.

2. Experimental Section

The samples consisted of attachment discs of *N. clavata*, or a spider shown in Fig. 1(a). The samples were collected 3-5 days before observation. The attachment discs were adhered to silicon wafer substrates by the spider. When we collected the samples, several silicon wafers (2 cm x 2 cm) were stuck on a sufficiently large sheet of paper. Spiders were captured on the JAIST campus (coordinates (WGS 84): E 136.5955, N 36.4451) in Ishikawa Prefecture. When a spider walked on the paper, it sometimes secreted an attachment disc under its belly. While secreting the pyriform

disc, the spider stopped and shook its belly part to left and right. We gently encouraged the spider to walk on the Si wafers and to secrete attachment discs on to the wafers. The detail of the sample collection is shown in the *Supporting data* (see Video S1).

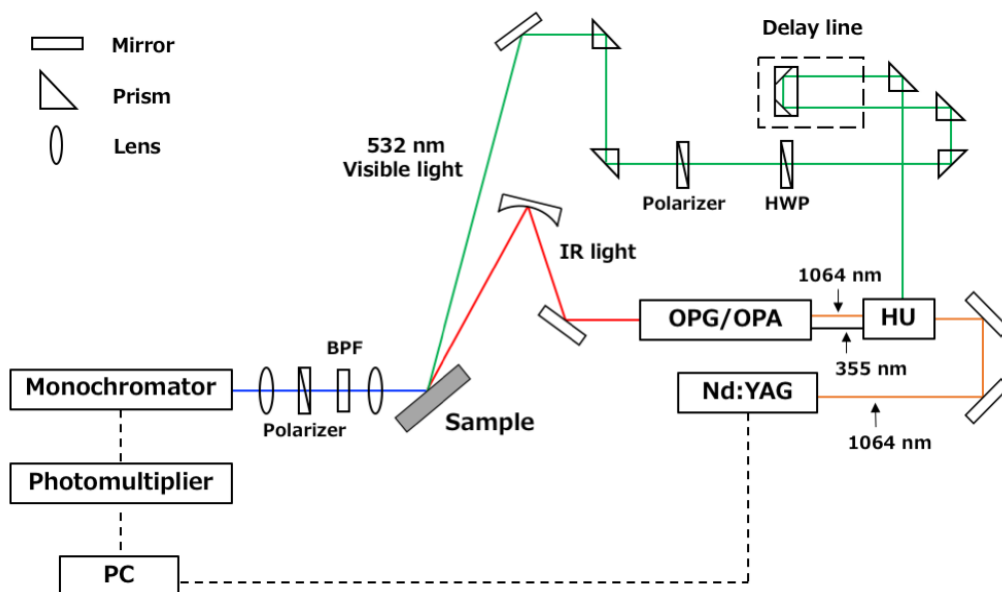


Figure 2. The optical setup of our SFG spectroscopy system. BPF: Band pass filter. HU: Harmonic unit. OPG/OPA: Optical parametric generation/amplifier. HWP: Half wave plate.

Figure 2 shows the optical setup of our SFG measurement system. In this study, we used doubled-frequency light pulses at a visible wavelength of 532 nm with the pulse time width set to 30 ps and the repetition rate of 10 Hz generated by a harmonic unit (EKSPHA HMPL/SH/TH/FH 183) excited by a mode-locked Nd:YAG laser (EKSPHA PL2143) system. Output from an optical parametric generator and amplifier system (OPG/OPA) (EKSPHA P6401DFG2-18) driven by the same Nd:YAG laser was used as the wavelength-tunable infrared (IR) light. The SFG signal was generated in the reflection direction from the sample by the IR and visible light pulses. It was then introduced into a monochromator (Nihon Koken Kogyo Co., Ltd., SG-100) and finally detected and recorded by a photomultiplier (HAMAMATSU R585) and a gated integrator system. The

resolution of the monochromator was set as 13.6 nm in the SFG measurement. The wavenumber resolution was determined by the spectral width of the IR output from our OPG/OPA and it was 3 cm^{-1} . Both visible and IR light beam spots were focused on the same area of 2 mm^2 on the sample surface. The energies of the visible and IR light were 35 $\mu\text{J}/\text{pulse}$ and 25-115 $\mu\text{J}/\text{pulse}$, respectively. The corresponding flux of the visible and IR pulses were 1.75 $\text{mJ}/\text{cm}^2\cdot\text{pulse}$ and 1.25-5.75 $\text{mJ}/\text{cm}^2\cdot\text{pulse}$, and the peak power densities on the samples were 58.3 TW/cm^2 and 41.7-192 TW/cm^2 , respectively. The polarization configurations PPP and SSP were used. Here the three capital letters denote the polarizations of the sum frequency output, visible input, and IR input pulses in that order. The maximum output energy of the IR light depended on the output wavelength of our OPG/OPA. The measured SFG signal was normalized by that from a GaAs(001) wafer under the same conditions. After the SFG measurement no damage of either nanometer or micrometer scale was detected on the sample using an optical microscope or an SEM. Additionally, since there is no time dependency of SFG, the damage was considered to be of negligible impact.

The measurement step of the Fourier-transform Infrared Spectrometer (FT-IR) (Perkin Elmer, Spectrum 100) was 1 cm^{-1} and the spectral resolution was 4 cm^{-1} . The attachment disc samples were separated from the Si substrates and measured on their own by attenuated total reflection (ATR).

3. Results

Figure 3 shows an FT-IR absorption spectrum of an attachment disc in the wavenumber region of 900-1800 cm^{-1} . Three absorption bands can be seen at 1200-1260 cm^{-1} , 1510-1580 cm^{-1} , and 1600-1700 cm^{-1} . These are the characteristic bands of the peptide group named amide III, amide II, and amide I, as indicated in the figure. Two absorption peaks are seen at 1167 cm^{-1} and 1454

cm^{-1} and are supposed to be the bands of β -poly-*L*-alanine (β -PAla) [57]. These results are consistent with the IR absorption spectrum of the dragline of the spider already reported [57]. Two more absorption peaks are seen at 1050 cm^{-1} and 1400 cm^{-1} . They are ascribed to serine [58] and/or lipids [59,60].

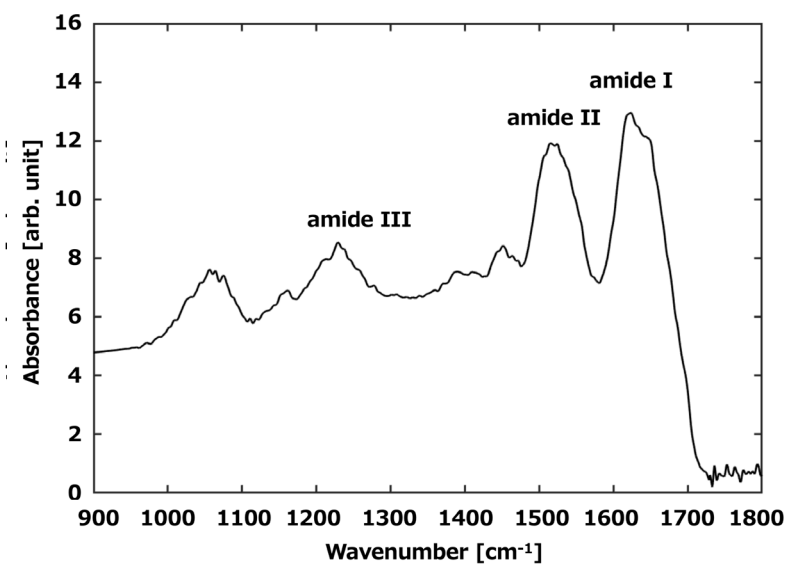


Figure 3. FT-IR spectrum of the $900\text{-}1800 \text{ cm}^{-1}$ region of an attachment disc of a spider (*N. clavata*). Bands attributed to amide I, II and III are marked.

Figure 4 shows an FT-IR absorption spectrum and SFG spectra of an attachment disc in the wavenumber region of $2800\text{-}3500 \text{ cm}^{-1}$. The wavenumber resolution of the SFG spectrum is about 10 cm^{-1} after a Savitzky-Golay smoothing treatment [61] with three smoothing points. The incident plane of the two excitation light fields is parallel to the pyriform silk fiber axis of the attachment disc. The polarization configuration are PPP and SSP for the middle and bottom spectra, respectively. We obtained SFG spectra at seven different positions on four different attachment disc samples with good reproducibility. The IR absorption spectrum shows two wide vibrational bands at $2850\text{-}3000 \text{ cm}^{-1}$ and $3100\text{-}3500 \text{ cm}^{-1}$. The IR spectrum at $2800\text{-}3500 \text{ cm}^{-1}$ in Fig. 4(a) is consistent with the IR spectrum of the spider dragline reported by Papadopoulos *et al.* [57].

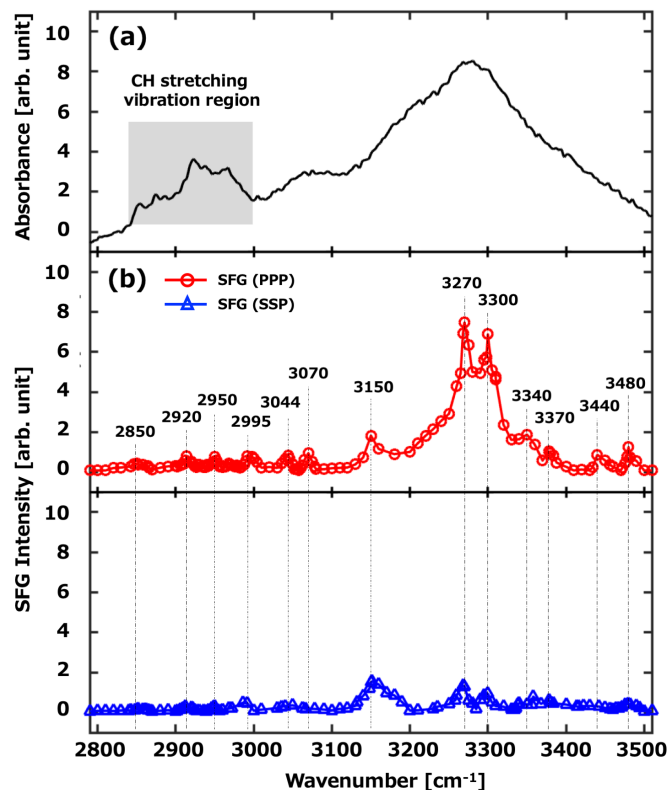


Figure 4. (a) FT-IR and (b) SFG spectra of the 2800-3500 cm^{-1} region of an attachment disc of a spider (*N. clavata*). The solid lines of the SFG spectra are the result of Savitzky-Golay smoothing. The wavenumber resolution is $\sim 10 \text{ cm}^{-1}$ after the smoothing.

The wavenumbers of the 13 detected peaks in the SFG spectra are shown in Fig. 4(b). The bands below 3000 cm^{-1} are assigned to CH_n vibrations. 3044 cm^{-1} and 3070 cm^{-1} peaks are assigned to the CH stretching of the aromatic ring. 3270 cm^{-1} and 3300 cm^{-1} are assigned to NH of β -sheet [62–64] and α -helix [65–69], respectively. The assignment of the SFG bands in Fig. 4(b) based on the literature is shown in Table 1. In the polarization configuration of SSP, the relative intensity of the peak at 3150 cm^{-1} is slightly stronger than that in the PPP configuration.

The SFG peaks were detected only when the incident plane and the fiber axes of the

attachment disc silk were parallel to one other. When the fiber axes of the attachment disc silk were perpendicular to the incident plane, the incident light was scattered strongly by the attachment disc and did not enter the detector in the regular reflection direction. The linear optical reflectance of the attachment disc on the silicon substrate increased linearly as a function of the angle between the silk fiber axis and the incident plane. The reflectance is at the maximum when the fiber axis of the silk is parallel to the incident surface (see Figure S1). We tried to measure the chiral SFG component by using a combination of SPP and PSP polarization [70], but the signal was below the noise level.

4. Discussion

4.1. Assignment of the observed peaks

In this study, our main concern is the peaks at 3150 cm^{-1} , 3270 cm^{-1} and 3300 cm^{-1} . Here, before going to these peaks, we check the observed peaks in the FT-IR and SFG spectra. Figures 3 and 4(a) show IR vibration bands in the $900\text{-}1800\text{ cm}^{-1}$ and $2800\text{-}3600\text{ cm}^{-1}$ regions. FT-IR data of $1800\text{-}2800\text{ cm}^{-1}$ is shown in Figure S2 in the *Supporting data* file. The infrared absorption spectrum of the attachment disc silk in Fig. 3 including the IR vibration bands of amides I, II and III, is quite similar to that of the spider's dragline already reported [57]. This is because both of them consist of proteins.

Figure 4(b) shows SFG bands in the $2800\text{-}3600\text{ cm}^{-1}$ region. The non-zero second-order nonlinear optical response of the attachment disc silk was confirmed by a second harmonic generation (SHG) microscopy (see Figure S3 in the *Supporting data*). Figure 4 shows several peaks in the SFG and IR spectra. In our experimental process, bare silicon substrates give SFG signals at the noise level in the CH, NH and OH stretching region at $2800\text{-}3600\text{ cm}^{-1}$. Si is famous for

having a strong SHG response from its surface dangling bonds, but SHG and SFG become very weak if there is a native oxide layer on the surface [71]. The fact that the SFG from bare Si is negligible in our experimental condition further means that SFG contribution from environmental contamination is also at our noise level. Additionally, our previous paper [16] reported the existence of “SHG” signals from the radial lines and draglines of spider silk but not from the spiral lines. If the contamination with non-centrosymmetric structures in our sample preparation process contributed to the second order optical nonlinearity, there should be SHG signals from all the types of spider silk. Hence, we can say that the effects of contamination with non-centrosymmetric structures can be excluded from our sample preparation conditions in our later discussion.

Table 1. Tentative assignment of the 2800-3500 cm^{-1} region of the vibrational SFG peaks of the attachment disc of the spider (*N. clavata*).

attachment disc SFG (cm^{-1})	Possible assignment
2850	$\nu_{\text{ss}}(\text{CH}_2)$ [72–75]
2920	$\nu_{\text{as}}(\text{CH}_2)$ [75]
2950	Fermi-resonance of $\nu_{\text{as}}(\text{CH}_3)$ [76] or OCH_3 [77–79]
2995	$\nu_{\text{as}}(\text{CH})$ [80]
3044	$\nu(\text{CH})$ aromatic ring [81–84]
3070	$\nu(\text{CH})$ aromatic ring [81,84–86]
3150	NH of Arg side chain [87], Fermi-resonance of $\nu_{\text{ss}}(\text{NH}_2)$ [88]
3270	β -sheet [62–64]
3300	α -helix [65–69]
3340	NH [67]
3370	OH [89,90], $\nu_{\text{ss}}(\text{NH})$ [91]
3440	OH of hydroxyl group [92–94], NH [66,67,95,96]
3480	OH [97], NH [66]

ν : stretching vibration, ν_{ss} : symmetric stretching, ν_{as} : asymmetric stretching

As shown in Table 1, four peaks of the CH stretching vibration region (2850-3000 cm^{-1}) in the FT-IR spectrum in Fig. 4(a) are assigned to: CH_2 symmetric stretching [72–75] at 2850 cm^{-1} , CH_2 asymmetric stretching [75] at 2916-2924 cm^{-1} , Fermi-resonance of $\nu_{\text{as}}(\text{CH}_3)$ [76] or OCH_3 [77–79] at 2950 cm^{-1} , and CH asymmetric stretching [80] at 2995 cm^{-1} . As the origin of the aromatic ring signal, phenylalanine and tyrosine are considered among the amino acids constituting pyriform spidroin [8,98].

The NH stretching vibration usually serves as a probe to investigate the structural and functional properties of proteins [66,68]. Fermi resonance by the NH stretching vibration and the amide II harmonic leads to bands [66] at 3100-3500 cm^{-1} . The peak of 3150 cm^{-1} is discussed in detail in section 4.4. The SFG peaks at 3270 cm^{-1} and in the 3300-3360 cm^{-1} region in Fig. 4(b) are typical modes in NH stretching vibration [62–64,66–69]. At circa 1630 cm^{-1} in the FT-IR spectrum in Fig. 3, we see a typical amide I peak [99]. For the SFG peaks between 3270 and 3300 cm^{-1} , the intensity for polarization combination of PPP is significantly stronger than that of SSP. Since there is non-zero second order nonlinearity arising from NH bonds, it is suggested that the second order nonlinearity was caused by oriented amino acid residues. The doublet peaks of 3270 and 3300 cm^{-1} are discussed in detail in section 4.3. The SFG peak at 3440 cm^{-1} is near the frequency 3480 cm^{-1} in the hydroxyl vibration region of protein [92–94,100,101]. However, the assignment of the peaks between 3370 cm^{-1} and 3480 cm^{-1} is difficult.

4.2. Comparison of the SFG spectral data in this study with those of the aggregate glue droplet on an orb web reported by Singla et al. [43]

Here we compare the SFG spectra of the attachment disc in this study with those of the aggregate glue droplet of the orb web and its LMMCs extract by Singla *et al.* [43]. The common

features of the spectra shared by the attachment disc, the aggregate glue droplet and the LMMCs are as below.

- 1) The peaks of CH_n at $2800\text{-}3000\text{ cm}^{-1}$ are seen.
- 2) The peak centered at 3270 cm^{-1} are seen for the attachment disc and the glycoproteins in aggregate glue droplet.
- 3) The SFG spectra of LMMCs [43] show a peak near 3150 cm^{-1} for the SSP polarization combination. The enhancement of the SFG peak intensity at 3150 cm^{-1} for SSP polarization combination is also seen for the attachment disc in Fig. 4(b).

Differences are in the following.

- i) No peak was observed around 3270 cm^{-1} for LMMCs by Singla *et al.* [29].
- ii) At 3270 cm^{-1} the SFG spectra of aggregate glue droplet by Singla *et al.* shows one broad peak [29], while the attachment disc in this study shows doublet peaks at 3270 cm^{-1} and 3300 cm^{-1} .
- iii) The peaks at 3150 cm^{-1} in the SFG spectra of the attachment disc in this study are prominent, but not in that of aggregate glue droplet.

From the common features of 1) and 2), we guess that both the attachment disc and the aggregate glue droplet contain hydrocarbons and peptide chains. As it is well known, the early secretions of the component associated with the carbohydrate of the cement-like glue of the attachment disc appear in cells with the Golgi apparatus in the proximal part of the pyriform gland [5]. Since most glycosylation reactions occur in the Golgi apparatus [102], the component associated with the carbohydrate in the cement-like glue of the attachment disc may be a glycoprotein.

From the difference point i), the SFG spectrum of the LMMCs extract by Singla *et al.* shows no NH peak [43] and so there is no oriented peptide bond in LMMCs. In fact, the LMMCs in aggregate

glue droplet consist of γ -aminobutyramide, *N*-acetyltaurine, choline, betaine, isethionic acid and pyrrolidone [50–55], and so there can be no peptide bonds in LMMCs. On the other hand, the common feature 3) suggests the existence of LMMCs in the cement-like glue of the attachment like in aggregate glue droplet. Concerning the difference point ii), the splitting of the peak is discussed in detail in section 4.3. The peak at 3150 cm^{-1} mentioned in the difference point iii) can be assigned either as LMMCs, glutamine, or arginine in spidroin, as it will be discussed in section 4.4.

Summarizing this section, the SFG spectra show that glycoproteins may exist both in the attachment disc and aggregate glue droplet. On the other hand, since there are differences in the SFG spectra, the attachment disc contains different peptides aggregation from that in the aggregate glue droplet.

4.3. Discussion on the origin of the splitting of 3270 and 3300 cm^{-1} peaks

The infrared absorption frequency of secondary amide in the solid state depends on the type of hydrogen bonding. In some cases of dipeptides, polypeptides, and proteins, two or multiple absorption peaks are found [66]. Since the main NH absorption of secondary amides occurs near 3270 cm^{-1} in the solid state [66,103–105], the 3270 cm^{-1} peak in Fig. 4(b) can be assigned to the secondary amides. The splitting of the 3270 and 3300 cm^{-1} peaks is considered to be due to two kinds of structures in (1) α -helical and/or (2) β -sheet structures. For discussing candidate (1), we refer to the results of Lee *et al.* [65]. Experimentally, oriented and unoriented α -helical poly (*L*-alanine) (β -PLA) show doublet structures at amide A peak in IR [65] and polarized Raman spectra [106]. This amide A doublet was suggested to arise from two different structures, and the higher frequency component was assigned to the standard α -helical structure in the peptide chains [65].

Thus, in our result, the peak at 3300 cm^{-1} in Fig. 4(b) can be assigned to the α -helical structure. In Lee *et al.*'s paper [65], the lower frequency peak was not assigned, but was suggested to originate from another peptide structure. Thus, the peptides detected by SFG in this study may consist of two structures.

Since 3270 cm^{-1} peak in the FT-IR spectrum is frequently assigned to a hydrogen bond in the β -sheet [62–64], the β -sheet can be the candidate origin (2) of 3270 cm^{-1} peak. The peak splitting may originate from two kinds of structures in the peptide chain of β -sheet. The band between 3270 and 3310 cm^{-1} is normally modified by two factors: transition dipole coupling (TDC) and Fermi resonance [107]. The resonance frequency in NH mode depends on the strength of the $\text{NH}\cdots\text{O}=\text{C}$ hydrogen bond, and the farther the distance of $\text{H}\cdots\text{O}$, the higher the resonance frequency [107]. Therefore, the $\text{NH}\cdots\text{O}=\text{C}$ hydrogen bond strength related to the SFG peak at 3300 cm^{-1} should be weaker than that at 3270 cm^{-1} .

Here, we note that the peak at 3270 cm^{-1} was observed in a broad peak in FT-IR, but was observed in a doublet by SFG. In FT-IR vibrations of all the molecular bonds are observed regardless of the molecular orientation. In SFG non-centrosymmetric structures or oriented asymmetric molecules are selectively observed. Namely, in Fig. 4(b) two types of oriented peptides were selectively observed by SFG from differently oriented peptides with various types of structures. On the other hand, only one peptide structure was observed in aggregate glue droplets by Singla *et al.* [43]

4.4. Discussion on the origin of the 3150 cm^{-1} peak and its polarization dependence in the SFG spectra

Figure 4(b) shows peaks in the SFG spectra at 3150 cm^{-1} . There are five candidate origins of

this peak: 1) NH group of arginine side chains; 2) NH₂ group of glutamine and/or arginine side chains; 3) LMMCs; 4) reorganized molecular bonding between the cement-like glue and pyriform spidroin and the 5) cyclic lactam with ring sizes of seven or less.

First, the 3150 cm⁻¹ peak may arise from the NH groups or NH₂ groups in the side chains of amino acid. NH group is contained in the side chain of arginine, histidine, and tryptophan among the 20 kinds of amino acids constituting natural protein. Pyriform spidroin forming the spider silk contains ~2.6% arginine (R), ~0.05% histidine (H), and does not contain tryptophan (W) [8,98]. One histidine is present in the amino acid sequences of pyriform silk proteins of the *A. trifasciata* spider, but not in *N. clavate* [98] studied in this work. The 3150 cm⁻¹ peak is characteristic of NH stretch in the side chain of arginine [87,108]. So here, as candidate 1), the SFG peak of 3150 cm⁻¹ can be assigned as NH group of arginine side chains.

On the other hand, the 3150 cm⁻¹ peak is frequently assigned as the Fermi-resonance splitting of $\nu_{ss}(\text{NH}_2)$ [88]. NH₂ group is contained in the side chain of aspartic acid, glutamine, arginine, asparagine, and lysine among the 20 kinds of amino acids constituting natural protein. Pyriform spidroin forming the spider silk contains ~15.5% glutamine (Q), ~2.6% arginine (R), ~2.3% asparagine (N), and ~0.5% lysine (K) [8,98]. Furthermore, cDNA analysis has identified repetitive motifs (e.g., QQASVSQS and QQSSLAQS) made of 8 amino acids in the pyriform spidroin, and these motifs contain a large amount of glutamine [98]. Among these amino acid molecules only glutamine and arginine have an vibrational resonance peak near 3150 cm⁻¹ [109], while asparagine [110], and lysine [111] does not. So here, as candidate 2), the SFG peak of 3150 cm⁻¹ can be assigned as NH₂ group of glutamine and arginine side chains.

As explained in the common feature 3) in section 4.2, the SFG peaks at 3150 cm⁻¹ of the attachment disc and LMMCs of aggregate glue droplet shared similar polarization dependence.

The relative intensity of the SFG peak at the 3150 cm^{-1} SSP polarization combination of the attachment disc is strong, but that of the LMMCs is weak. The possibility that the SFG peak of 3150 cm^{-1} on the attachment disc is derived from LMMCs cannot be ruled out as candidate 3). However, since 3150 cm^{-1} of LMMCs is very weak, the peak of 3150 cm^{-1} in Fig. 4(b) should be dominated by contributions from other molecules than LMMCs.

In a report of SFG spectra by Dreesen *et al.* [112] on the binding of avidin and biocytin, the interaction between the two substances created a specific order in the molecule. Two new $\nu(\text{CH})$ vibrations centered on 3050 cm^{-1} and $\nu(\text{NH})$ centered on 3150 cm^{-1} were obtained. They were not originally present in avidin or biotin when they were separate [112]. The reorganization of the biocytin conformation after bonding to avidin eliminated the vibrations in the original CH_2 chain of biocytin [112]. The NH vibration at 3150 cm^{-1} can be assigned to avidin, biocytin, or both [112]. This result of Dreesen *et al.* [112] can explain the SFG peak 3150 cm^{-1} for the attachment disc in Fig. 4. In the attachment, the pyriform spidroin silks are thought to be bound to the cement-like glue [7]. New vibration modes of 3044 cm^{-1} $\nu(\text{CH})$ and 3150 cm^{-1} $\nu(\text{NH})$ may have been created by the interaction between the carbohydrate component of cement-like glue and pyriform spidroin (candidate 4)). These two peaks were not in the SFG spectrum of aggregate glue droplet [43]. However, the reorganization between the cement-like glue and the pyriform spidroin occurs only near the boundary of these two materials, and it may not generate strong SFG.

For cyclic lactams (candidate 5)) with ring sizes of seven or less in a solid state, the band near 3280 cm^{-1} is replaced by a new one near 3175 cm^{-1} [66,104,113,114]. Darmon *et al.* [104] suggested that the absorption near 3280 cm^{-1} is due to hydrogen bonds between amides in the *trans*-configuration, whereas the absorption near 3160 cm^{-1} arises from ones in the *cis*-configurations. However, the cyclic lactam is unlikely to form an oriented cluster in pyriform silk,

and it may not be the origin of the 3150 cm^{-1} peak.

In Fig. 4(b), the relative SFG intensity at 3150 cm^{-1} is almost the same between SSP and PPP polarization combination. Since this mode has a quite different polarization dependence from those of the NH mode at 3270 cm^{-1} and 3300 cm^{-1} , the molecular vibration at 3150 cm^{-1} should be oriented statistically in a different direction from the average NH bonds in the whole attachment disc. The orientation of NH and/or NH_2 at the amino acid residue may have some effect on the interaction between the pyriform silk and the cement-like glue. However, since interfacial vibration between NH and/or NH_2 groups and cement-like glue was not directly observed, we cannot say anything concerning the molecular mechanism of the adhesion property.

5. Summary and Conclusions

SFG spectra of spider silk in the attachment disc have been obtained in the hydrogen stretching vibration region between 2800 and 3500 cm^{-1} . Doublet peaks of the NH band due to the peptide component of the attachment disc were observed in the SFG spectrum at 3250-3320 cm^{-1} . This doublet can be attributed to two different NH vibration modes. The doublet peaks can be assigned to the α -helix and/or β -sheet structure. By comparing χ_{ppp} and χ_{ssp} , we concluded that the molecular vibration at 3150 cm^{-1} should be oriented statistically in a different direction from the average NH bonds in the whole attachment disc. This vibration mode is assigned to the NH group of arginine side chain and/or NH_2 group of arginine and glutamine side chain. Although the SFG spectrum of the attachment disc and aggregate glue droplet contains common properties, the behavior of 3150 cm^{-1} and 3300 cm^{-1} of the attachment disc is clearly different from that of the aggregate glue droplet. An observation by SFG microscopy will give more information in the future, while the analysis should be upgraded to the one using a DFT method.

CRedit authorship contribution statement

Yue Zhao: Conceptualization, Visualization, Writing – original draft, Methodology. **Lin Liang:** Investigation, Data curation. **Yanrong Li:** Investigation, Data curation. **Khuat Thi Thu Hien:** Supervision. **Goro Mizutani:** Supervision, Validation. **Harvey N. Rutt:** Validation.

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Appendix A. Supplementary data

Video S1. The detail of sample collection. **Figure S1.** The angular dependence of the specular linear optical reflectivity of the attachment disc. **Figure S2.** The FT-IR spectrum of the 1800-2800 cm⁻¹ region of an attachment disc of a spider (*N. clavata*). **Figure S3.** The second harmonic generation microscopy of the attachment disc.

Supplementary data associated with this article can be found in the online version, at DOI:

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