

Determination of Molecular Orientation in Protein Films and Fibres by Raman Microspectroscopy

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Abstract

Raman microspectroscopy can provide high-quality spectra of single filaments having diameters smaller than 10 μm , allowing the determination of molecular orientation of very small samples. For systems with uniaxial symmetry, orientation can be quantitated by two order parameters, P_2 and P_4 , that are calculated from intensity ratios of polarized spectra. We have used the amide I band to determine the orientation of β -sheets in two protein-based systems for which quantitative data about orientation are needed. The first one concerns biosynthetic films made with a globular protein that can find applications as edible biodegradable food wrappings. The second example is spider silk, a natural material composed of fibrous proteins that arouses a lot of interest due to its outstanding mechanical properties. Both examples show that orientation measurements made with Raman microspectroscopy can be helpful in providing insights into the structure of protein-based systems and to discriminate between different molecular assemblies. The orientation of β -sheets in globular protein films seems to be perpendicular to the long axis of the filamentous aggregates that form the network as in amyloid fibrils. On the other hand, for the dragline silk of the spider *Nephila clavipes*, the β -sheets are aligned along the fiber axis, in agreement with the literature.

Keywords: Raman microspectroscopy, quantitative orientation measurements, film, fibre, protein aggregation, β -sheets

Résumé

La microspectroscopie Raman peut fournir des spectres d'excellente qualité d'un simple filament d'un diamètre inférieur à 10 μm , permettant ainsi de déterminer l'orientation de très petits échantillons. Pour des systèmes à symétrie uniaxe, l'orientation peut être

quantifiée à l'aide de deux paramètres d'ordre, P_2 et P_4 , calculés à partir de rapports d'intensité de spectres en lumière polarisée. Nous avons utilisé la bande amide I pour déterminer l'orientation de feuillettes β dans deux systèmes à base de protéines pour lesquels des données sur l'orientation sont nécessaires. Le premier porte sur des films bio-synthétiques de protéines globulaires pouvant avoir des applications comme emballage alimentaire biodégradable. Le second système est la soie d'araignée, un matériau naturel composé de protéines fibreuses qui suscite un grand intérêt en raison de ses propriétés mécaniques exceptionnelles. Ces deux exemples montrent que les mesures d'orientation par microspectroscopie Raman apportent de nouvelles informations sur la structure de systèmes à base de protéines, et permettent de discriminer différentes organisations moléculaires. L'orientation des feuillettes β dans les films de protéines globulaires semble être perpendiculaire à l'axe long des agrégats filamenteux qui forment le réseau, comme ce que l'on retrouve dans les fibres amyloïdes. Pour la soie d'araignée, les feuillettes β sont majoritairement alignés dans l'axe de la fibre, en accord avec la littérature.

Introduction

Raman microspectroscopy is an attractive technique for the molecular characterization of films and fibres since it can provide high-quality spectral and imaging data of very small samples. In addition, Raman spectroscopy allows the investigation of both molecular conformation and orientation. Indeed, it is a method a choice to gain detailed information about the distribution of molecular orientation. Since a precise description of orientation is required in order to understand the relationship between the mechanical properties of films and fibres and their molecular organizations, it is important to characterize such materials with Raman microspectroscopy.

For conventional Raman spectroscopy, the theory (1-3) and experimental methods (4-6) to determine orientation are well established for uniaxial systems,

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i.e. systems in which the molecular orientation is given by a single angle θ from a reference axis. In this case, orientation is described by a distribution function, $N(\theta)$, that can be expanded on a basis composed of Legendre polynomials. The two first coefficients of the expansion, the so-called order parameters P_2 and P_4 , can be calculated by conventional Raman spectroscopy from the intensity of linearly polarized spectra. The method has been used to determine the order parameters P_2 and P_4 in a limited number of systems including highly oriented samples of high density polyethylene (4-5) and poly(propylene terephthalate) (6) as well as nematic and smectic liquid-crystals (3).

The method used for orientation measurements by micro-Raman spectroscopy has been recently reviewed (7). In this case, only four polarized spectra can be recorded and depolarization effects due to the microscope objective have to be considered in orientation calculations. Following the pioneering work on the analysis of polarization measurements in Raman microspectroscopy done by Turrell (8, 9), Sourrisseau and coworkers (10, 11) have further developed the method to determine quantitatively the orientation distribution. To date, this method has been applied to study photo-induced orientation of polymers containing azobenzene groups (10, 12). The equations that allow the calculation of P_2 and P_4 using Raman microspectroscopy for a vibrational mode having a cylindrical Raman tensor have recently been derived (13). These equations are required in order to determine the orientation of protein-based systems because 80% of the amide I mode is due to the C=O stretching vibration (14), a vibration for which the Raman tensor should be cylindrical. This mode is often used for protein studies since it gives rise to a strong band and is sensitive to secondary structures and hydrogen bonding. In this paper, the calculation method is applied on two protein systems, i.e. films of globular protein and spider silk fibres.

Self-supported protein films are interesting from the fundamental and applied point of views. As an application, edible films made of globular food proteins are envisaged as biodegradable food wrappings and coatings (15). In some cases, they can represent advantageous alternatives to synthetic polymer films. Biofilms made with commercial whey protein isolates (WPI) are the subject of a large number of studies because whey is a waste and a pollutant of the cheese industry that has to be valorized. The properties of these films are dominated by β -lactoglobulin (β -lg), the major protein constituent of whey. Edible films are commonly formed in two main steps: a heat-induced denaturation of a protein is first

carried out in aqueous solution, followed by a dehydration step. A plasticizer such as a sorbitol, glycerol or another polyol is usually added before water evaporation to provide elasticity to the final film. It is admitted that the native proteins unfold during the first heating step which allows the development of intermolecular cross-linkings during the second step, resulting in the formation of the film network (15). The structure of edible films has not been extensively studied and a better understanding of the molecular organization is required in order to optimize their mechanical and permeability properties. By drawing WPI-based films and by analyzing the subsequent molecular orientation, insights into film structure and properties should be gained.

Silk is a well known example of *in vivo* processed fibres. In particular, the dragline silk of spiders combines excellent rigidity and extensibility giving rise to a very resistant material that could actually not be reproduced by artificial means. The mechanical properties of such materials are highly dependent on the microstructure and orientation of the polypeptide chains. Silk is formed by fibrous proteins that are self-assembled into β -sheet crystallites embedded in amorphous domains (16-18). This combination of hard and soft domains is believed to be responsible for the outstanding mechanical properties of silk (16-18). However, the detailed organization of silk at the microscopic and molecular levels is still not fully understood. Tools are thus needed to elucidate the molecular conformation and to efficiently quantify the molecular orientation in silk in order to be able to spin regenerated fibres with comparable mechanical properties than natural ones. In this work, the potential of Raman microspectroscopy to provide quantitative data about molecular orientation in these protein-based systems is evaluated.

Theory

The theory of orientation measurements by Raman microspectroscopy for a cylindrical Raman tensor has been given in detail elsewhere (13). Therefore, only a brief survey is given here. For a system of uniaxial symmetry, the orientation distribution function, $N(\theta)$, can be expanded in a series of even Legendre polynomials in $\cos\theta$ (1):

$$N(\theta) = \sum_{\ell}^{\text{even}} \left(\ell + \frac{1}{2} \right) \cdot P_{\ell} \cdot P_{\ell}(\cos\theta) \quad (1)$$

where $P_{\ell}(\cos\theta)$ are the Legendre polynomials and P_{ℓ}

are the order parameters that can be determined experimentally. The first three order parameters are given by:

$$P_0 = 1, \quad P_2 = \frac{1}{2} \left(3 \langle \cos^2 \theta \rangle - 1 \right), \quad \text{and}$$

$$P_4 = \frac{1}{8} \left(35 \langle \cos^4 \theta \rangle - 30 \langle \cos^2 \theta \rangle + 3 \right) \quad (2)$$

For conventional Raman spectroscopy, the method to determine P_2 and P_4 has been developed by Bower (2) and Jen *et al.* (3). In the case of Raman microscopy, Turrell (8,9) has shown that the depolarization of the incident electric field in the focal plane of the objective and the solid angle of collection of the scattered light have to be taken into account when using high numerical aperture (NA) objectives.

Figure 1 shows a scheme of the Raman apparatus and the definition of the laboratory frame. The stretching or fibre axis is along the z axis while the propagation direction of the incident and scattered radiation is along the y direction. I_{kl} ($k, l = x$ or z) is the measured intensity when the incident electric field is polarized in the k direction and the scattered light is polarized in the l direction. In the backscattering configuration, four linearly polarized spectra can be measured, providing two independent intensity ratios. Taking into consideration the microscope objective, the considered ratios are given by (8-11, 13):

$$R_1 = \frac{I_{zx}}{I_{zz}} = \frac{A \langle (\alpha_{zx})^2 \rangle + B \langle (\alpha_{zy})^2 \rangle}{A \langle (\alpha_{zz})^2 \rangle + B \langle (\alpha_{zy})^2 \rangle} \quad (3)$$

$$R_2 = \frac{I_{xz}}{I_{xx}} = \frac{A \langle (\alpha_{xz})^2 \rangle + B \langle (\alpha_{xy})^2 \rangle}{A \langle (\alpha_{xx})^2 \rangle + B \langle (\alpha_{xy})^2 \rangle} \quad (4)$$

where the constants A and B depend on the numerical aperture and the refractive index of the sample. A and B can be calculated numerically (8, 9, 13). Typically, for $NA=0.9$, $A=3.58$ and $B=0.74$.

For a Raman tensor with a cylindrical symmetry,

$$\alpha = \begin{pmatrix} \alpha_1 & & \\ & \alpha_1 & \\ & & \alpha_3 \end{pmatrix} = \alpha_3 \begin{pmatrix} a & & \\ & a & \\ & & 1 \end{pmatrix} \quad (5)$$

with $a = \alpha_1 / \alpha_3$

and the $\langle \alpha_{ij} \rangle$ averages in Equations 3 and 4 can be expressed as a functions of the principal components

of the Raman tensors, α_1 and α_3 , and the order parameters P_2 and P_4 as follows:

$$\langle (\alpha_{xx})^2 \rangle = \frac{1}{15} c - \frac{2}{21} d P_2 + \frac{3}{35} b P_4 \quad (6)$$

$$\langle (\alpha_{xz})^2 \rangle = \langle (\alpha_{zy})^2 \rangle = b \left(\frac{1}{15} + \frac{1}{21} P_2 - \frac{4}{35} P_4 \right) \quad (7)$$

$$\langle (\alpha_{xy})^2 \rangle = \langle (\alpha_{yx})^2 \rangle = b \left(\frac{1}{15} + \frac{1}{21} P_2 - \frac{4}{35} P_4 \right) \quad (8)$$

$$\langle (\alpha_{yy})^2 \rangle = b \left(\frac{1}{15} - \frac{2}{21} P_2 + \frac{1}{35} P_4 \right) \quad (9)$$

where

$$b = \alpha_3^2 (1-a)^2, \quad c = \alpha_3^2 (3+4a+8a^2)$$

$$\text{and } d = \alpha_3^2 (3+a-4a^2) \quad (10)$$

At this stage, there are 3 unknowns in Equations 3 and 4, namely P_2 , P_4 , and a .

The parameter a of the Raman tensor for a given vibration can be determined directly from an isotropic sample (i.e., $P_2 = P_4 = 0$) (13). In this case, the depolarization ratio, R_{iso} , only depends on a and is given by (see Equations 3 and 4):

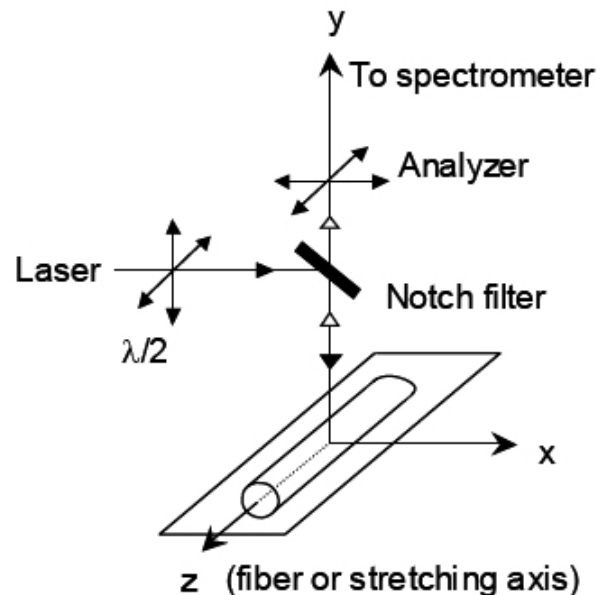


Figure 1. Scheme of the Raman apparatus and definition of the laboratory frame.

$$R_{iso} = R_1 = R_2 = \frac{b(A+B)}{Ac+Bb} = \frac{(1-a^2)(A+B)}{(8a^2+4a+3)A+(1-a^2)B} \quad (11)$$

Knowing a , P_2 and P_4 can be calculated from the experimental values of R_1 and R_2 .

As expressed by Equations 3 and 4, the polarized intensities depend on the orientation of the molecules. In addition, as demonstrated by Frisk *et al.* (10), the simple parameter defined by $p=1-(I_{xx}/I_{zz})$ can also be used to qualitatively characterize molecular orientation. This parameter is simple to obtain and can replace the more complex parameters P_2 and P_4 , especially when the Raman tensor is unknown.

Experimental

Protein films were made with a WPI obtained from Davisco Foods International Inc. (Le Sueur, MN). They were prepared according to the procedure of McHugh *et al.* (19). WPI was first dissolved during one hour in distilled water at a concentration of 10% w/v. Then, the protein was denatured by heat at 80°C for 30 min. and rapidly cooled to room temperature in a water bath. Then, the plasticizer (diethylene glycol, DEG) was added and the resulting filmogene solution was mixed for at least 15 min. (WPI/DEG weight ratio = 1). This solution was deposited for three days on a glass Petri dish in a 50% relative humidity (RH) environment, allowing evaporation which resulted in the formation of a transparent film that was peeled off from the glass surface. The dimensions of the films were 2 × 15 mm with a thickness of 0.45 mm. They were drawn manually with a stretcher from Manning Applied Technologies (Troy, ID) at 20 0.5°C and 100% RH. The draw ratio λ was calculated as $\lambda=(I-I_0)/I_0$, where I_0 is the initial length of the sample and I the length upon stretching. Spectra were recorded 20 min. after elongation by steps of 0.5 mm.

The golden orb-weaving spider used in this work belongs to the species *Nephila clavipes* (*N. clavipes*) and comes from the south of the United States. Fresh dragline silk samples were reeled directly from the fully awake spider at a drawing speed of 2 cm.s⁻¹. Silk monofilaments were mounted on glass microscope slides with double-sided tape. Measurements were made at 20 ± 0.5°C and 20% RH on three collected fibres and at three different points on each fibre.

Raman measurements were made using a LabRam 800HR spectrometer (Jobin Yvon Horiba, Villeneuve d'Ascq, France) coupled to a Olympus BX30 microscope. Silk and film samples were irradiated with the 632.8 nm line of a He-Ne laser (Melles Griot, Carlsbad, CA) and with the 514.5 nm line of an argon ion laser (Spectra-Physics, Model 2020, Mountain View, CA), respectively. The beam was focussed down with a 10× (films) or 100× (fibres) Olympus objective (0.25 and 0.9 NA, respectively) generating an intensity of ~5-10 mW at the sample. The confocal hole and the entrance slit of the monochromator were fixed at 200 and 100 μm, respectively. A half-wave plate (Melles Griot, Carlsbad, CA) was used to orient the polarization of the incident laser beam either parallel (z) or perpendicular (x) to the fiber axis. A polarizer was placed before the entrance slit of the monochromator to allow the detection of the polarized scattered light along the z and x directions. A broad-band quarter-wave plate was placed after the polarizer to eliminate the polarization dependence of the grating. The measurement time of a single spectrum was typically around one min.

Linearly polarized Raman intensities, identified as I_{xx} , I_{xz} , I_{zx} and I_{zz} (see Theory), were obtained by measuring the maximum of the amide I peak considering a linear baseline in the 1750-1500 cm⁻¹ region. The spectra were 7-points smoothed before measuring intensities and the intensities were corrected to account for the polarization dependence of the optical elements of the Raman microscope. The correction factor was determined experimentally from the intensity ratio of the spectra obtained for isotropic films made of WPI or regenerated *Bombyx mori* (*B. mori*) silk. All spectral manipulations were performed using GRAMS/AI 7.0 (ThermoGalactic, Salem, NH). The isotropic refractive index of silk used for the orientation calculations was 1.5, which is very close to what was previously determined for *B. mori* silk and to our own measurements on *N. clavipes* (data not shown).

Results and Discussion

Globular protein films

Figure 2 shows the linearly polarized Raman spectra in the amide I region of an unstretched and stretched WPI film at $\lambda=1.2$, which is the maximum draw ratio that could be achieved before film breaking. To our knowledge, this is the first report about edible globular protein films studied by Raman spectroscopy. The profile of the I_{xx} and I_{zz} amide I bands as well as the position of the peak

maximum at 1667 cm^{-1} both clearly indicate that β -sheet is the dominant secondary structure. Since these films are composed of protein aggregates, the Raman spectra demonstrate that β -sheet formation is involved in the aggregation process, and it confirms the potential role that β -sheets may play in the film mechanical properties (20, 21). For the unstretched film, the I_{xx} and I_{zz} spectra on one hand, and the I_{xz} and I_{zx} spectra on the other hand, are equal. This is expected for an isotropic sample for which $P_2 = P_4 = 0$. The measured depolarization ratio is $R_{iso} = 0.2$, which corresponds to a value of $a = \alpha_1/\alpha_3$ of 0.16. The stretched film reveals different I_{xx} and I_{zz} intensities, showing that the film is no longer isotropic.

To investigate the molecular orientation upon drawing, the qualitative parameter $p=1-(I_{xx}/I_{zz})$ (see Theory) is plotted as a function of λ in Figure 3. As one can see, p increases progressively with the draw ratio showing that the proteins orient themselves with stretching. The fact that p increases indicates that the C=O amide groups tend to align along the stretching axis. At maximum drawing ($\lambda=1.2$), it is found from the intensity of the four polarized spectra that $R_1 = 0.17$ and $R_2 = 0.21$, which gives $P_2 = 0.06$ and $P_4 = 0.005$. The theoretical limit values of P_2 are $-0.5 \leq P_2 \leq 1$ and correspond to a perfect orientation at 0° ($P_2 = 1$) and 90° ($P_2 = -0.5$) from the z axis. The P_2 value for the film is very small, indicating that the orientation is not extensive and that Raman microspectroscopy is a sensitive technique for reorientation measurements. The fact that P_2 is positive also indicates that the C=O groups tend to align along the z axis.

Globular protein films are thought to be composed of a network of cross-linked proteins with intercalated plasticizer molecules that provide elasticity to the film (15). However, the structure of the protein aggregates is not known. It has been shown by infrared spectroscopy that the first heating step in film formation induces protein self-aggregation via the formation of intermolecular antiparallel β -sheets (20,21). It is known that in this structure, the polypeptide chains are parallel and connected by H-bonds, with the C=O groups being perpendicular to the sheet. The heat-induced aggregation and the formation of protein gels is a well-documented phenomenon (22, 23). Indeed, it is well known that many globular proteins, and especially β -lg, form intermolecular β -sheets upon heating, giving rise to aggregation (24,25). Ultimately, a self-supported gel can be obtained if the temperature and concentration are high enough and if the heating time is long enough. Since the thermal behaviour of WPI is dominated by that of β -lg, the first denaturation step consists of a heat-induced aggregation of the proteins,

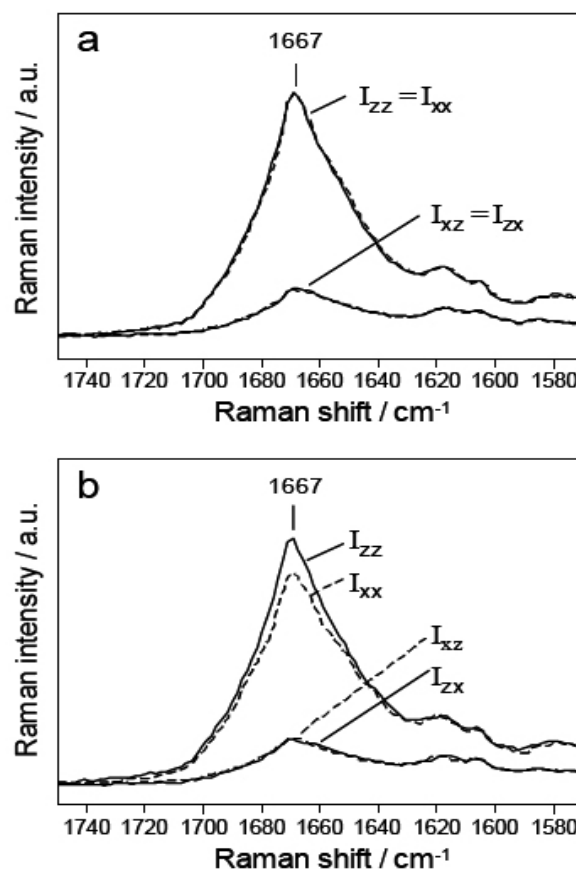


Figure 2. Polarized Raman spectra in the amide I region of an (a) unstretched and (b) stretched ($\lambda = 1.2$) WPI film.

without formation of a network. Since the heating time and temperature are not too extensive, the solution is in fact composed of soluble aggregates. This condition is required to allow the casting of films by dehydration. In addition, it is well known that, in our conditions, WPI and β -lg can form the so-called fine-stranded gels (26, 27). These gels are transparent and composed of strands (filamentous aggregates) that result from an ordered aggregation of the proteins. These filaments, that are made up by β -sheets, are also formed during the first step of the film formation process (data not shown). They may interconnect and/or grow upon dehydration to form the film network. But how are the β -sheets organized within these filamentous aggregates?

The present data reveal that p increases with λ , confirming that the C=O groups reorient in the drawing direction. This result is in contradiction with the intuitive hypothesis inferring that the polypeptide chains (i.e., the β -sheets) would be aligned along the filamentous aggregates. Indeed, if that was the case, the C=O groups would reorient perpendicular to the drawing direction causing p to decrease since the aggregates would align along

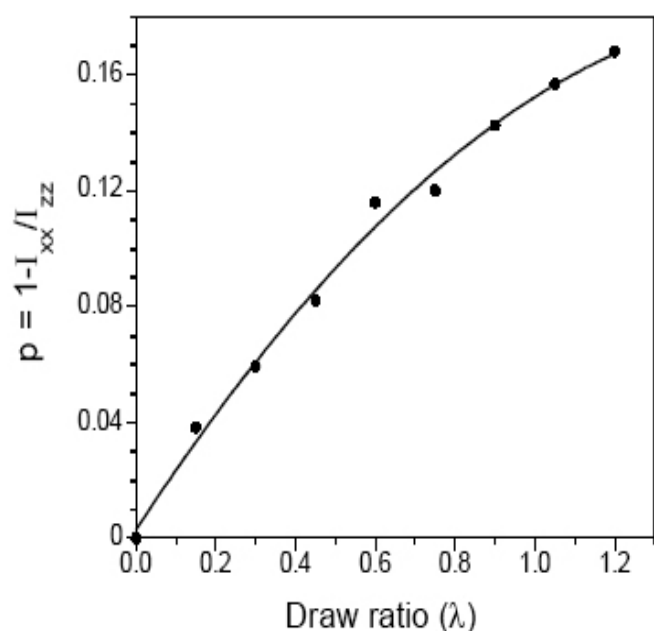


Figure 3. Plot of the qualitative orientation parameter $p = 1 - I_{xx} / I_{zz}$ as a function of the draw ratio (λ) for a WPI film. The full line is a fit of the experimental data that is only a guide for the eye.

the drawing direction (we infer that the Raman tensor is aligned along the C=O direction, i.e. perpendicular to the β -sheet). The Raman data rather suggest that the β -sheets are perpendicular to the filament axis. In this case, the carbonyl groups reoriented in the z direction (and the β -sheets in the x direction) when the aggregates were aligned in the z direction upon drawing. The orientation of the β -sheets in the film is represented in Figure 4a. Such β -sheets that are perpendicular to the filament direction are reminiscent of the structural organization of cross β -sheets that are found in protein fibrils and that have pathological effects in amyloid diseases (28). This new finding may provide some clues to understand the previously proposed relations between fine-stranded aggregates formed by globular proteins and amyloid fibrils (29).

Spider silk

Silk is interesting to compare with the protein films since they also contain β -sheets, but the proteins are fibrous rather than globular. Figure 5 presents the polarized spectra in the amide I region of a dragline silk thread of the *N. clavipes* spider. As seen in the I_{xx} spectrum, the amide I band is narrow and located at 1666 cm^{-1} , showing that the β -sheet is the most abundant structure. The fact that the I_{xx} amide I band is narrower than the WPI film indicates that the amount of β -sheets and that the level of orientation are higher in silk. A larger intensity difference

is observed between the I_{xx} and I_{zz} spectra than for the stretched globular protein film at maximum elongation before breaking, showing that the spidroin molecules are much more oriented in silk. It is also noted that, in this case, I_{xx} is stronger than I_{zz} , which clearly indicates that for silk the carbonyl groups of the polypeptide chains are mainly aligned perpendicular to the fibre axis. Consequently, the polypeptide chains are mainly oriented along the fibre axis.

The isotropic sample used to measure the depolarization ratio is a regenerated film made with the silk of the silkworm *B. mori*. The preparation of such films has been described elsewhere (13). It consists in casting a solubilized silk aqueous solution onto polyethylene films, drying for 48 hours in dry air, and immersing the resulting film in methanol which induces a conversion of disordered proteins into β -sheets. The depolarization for this film is $R_{iso} = 0.21$, which is close to the value found above for WPI film. This suggests that the parameter a for the β -sheet is rather independent of the nature of the protein. The calculation gives the following order parameters: $P_2 = -0.27 \pm 0.002$ and $P_4 = -0.12 \pm 0.002$. The absolute value of P_2 is large, indicating a high level of orientation. The fact that P_2 is negative shows that the carbonyl groups are significantly oriented in the x direction, as noted above. This is in good agreement with the proposed models for the structural organization of silk (16-18) for which the crystallites are formed with extended β -sheets that are mainly aligned along the fibre direction (see Figure 4b).

If one only considers the parameter P_2 , a “mean” orientation angle of the β -sheets can be calculated. One should, however, keep in mind that this angle corresponds to an orientation average of the molecules, and that a more precise view of the orientation distribution is provided by considering both P_2 and P_4 . From the value of P_2 , the C=O groups are found to be oriented at 67° from the fibre axis. Since the carbonyl groups are perpendicular to the β -sheet axis, the average orientation angle of the β -sheets can be calculated using the Legendre addition theorem (30). For *N. clavipes*, the β -sheets are oriented at 34° in average from the fibre axis, which is in good agreement with that obtained by NMR spectroscopy for silk of the same species (17).

The P_2 and P_4 values obtained for *N. clavipes* are close to those recently reported for the spider *Nephila edulis* ($P_2 = -0.32 \pm 0.002$ and $P_4 = 0.13 \pm 0.002$) (13). At this time it is difficult to assign such variations to differences between the silk of different species. Other factors such as the drawing conditions of silk can also

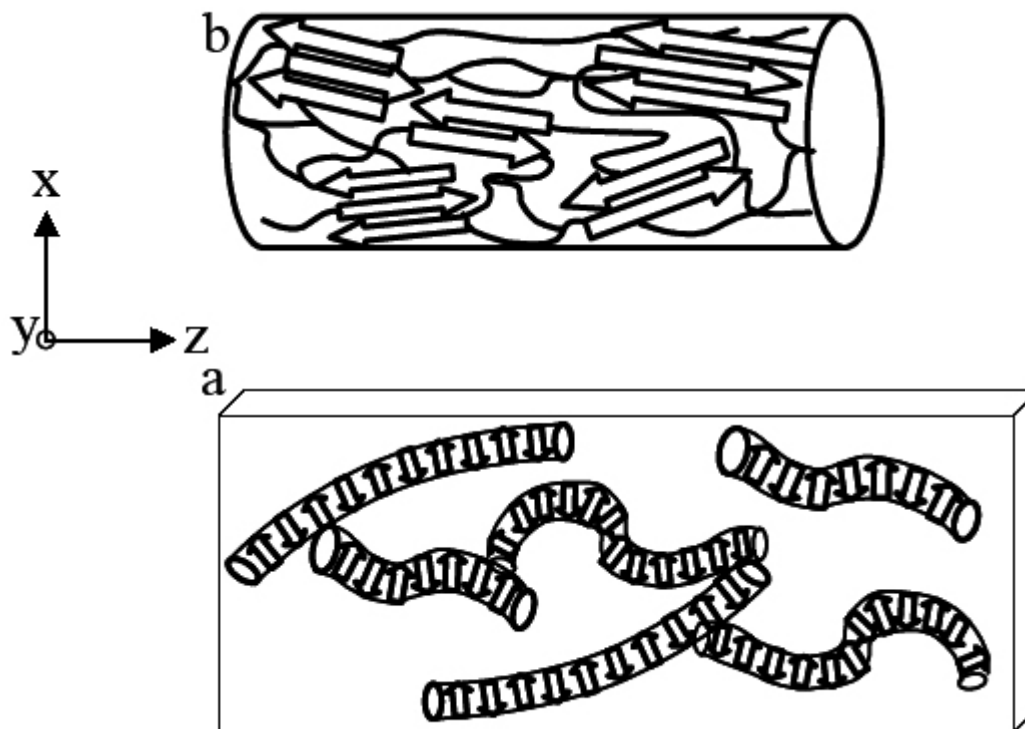


Figure 4. Comparison of the orientation of β -sheets in (a) globular protein-based film and (b) spider silk fibre. β -Sheets are represented by arrows, amorphous domains by lines.

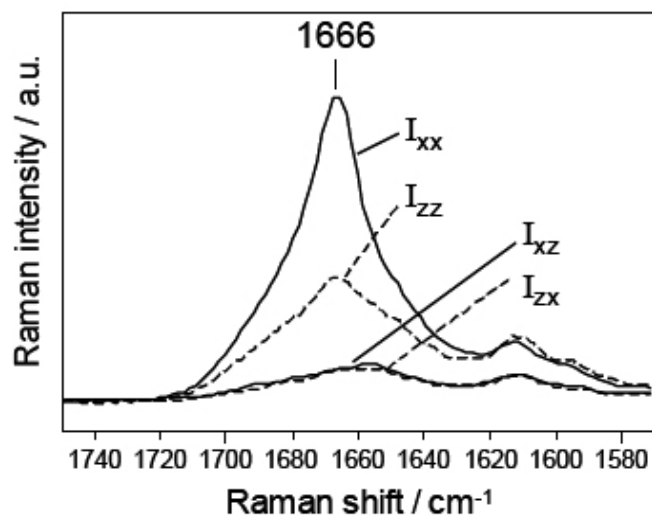


Figure 5. Polarized Raman spectra in the amide I region of a dragline silk of a *N. clavipes* spider.

have a significant impact on the level of orientation. More work is thus needed to precisely correlate quantitative orientation parameters with other variables.

Conclusion

A method to quantitatively determine the orientation of molecules using a vibrational mode for which the Raman tensor is cylindrical is presented. It has been applied to two oriented protein systems, stretched globular protein films and spider silk that exhibit different types of protein β -sheet assembly. In particular, globular protein films are formed by a network of protein aggregates in which the β -sheets are aligned perpendicular to filamentous aggregate long axis, similarly to what is found in amyloid fibrils. For spider silk, the β -sheets are principally oriented along the fibre direction. Orientation measurements made with Raman microspectroscopy are thus found to be successful in providing insights into the structure of protein-based systems and to discriminate different molecular organizations. This technique seems also to be sensitive to small levels of orientation and allows the comparison of samples that exhibit small differences in orientation. Future works will focus on the correlation between the order parameters on one hand, and the preparation conditions and mechanical properties of films and fibres on the other hand.

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