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Polarized Raman Spectroscopy of Aligned Insulin Fibrils

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Abstract

Amyloid fibrils are associated with many neurodegenerative diseases. The application of conventional techniques of structural biology, X-ray crystallography and solution NMR, for fibril characterization is limited because of the non-crystalline and insoluble nature of the fibrils. Here, polarized Raman spectroscopy was used to determine the orientation of selected chemical groups in aligned insulin fibrils, specifically of peptide carbonyls. The methodology is solely based on the measurement of the change in Raman scattered intensity as a function of the angle between the incident laser polarization and the aligned fibrils. The order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$ of the orientation distribution function were obtained, and the most probable distribution of C=O group orientation was calculated. The results indicate that the peptides' carbonyl groups are oriented at an angle of $13 \pm 5^\circ$ from the fibril axis, which is in consistent with previously reported qualitative descriptions of an almost parallel orientation of the C=O groups relative to the main fibril axis.

Introduction

Amyloid aggregation is a specific form of protein misfolding and self-assembly, which plays an important role in a wide variety of human diseases, including Parkinson's disease, Alzheimer's disease, and type II diabetes [1-3]. This association with deadly diseases has made amyloid fibrils the focus of intensive research over many decades. Amyloid fibrils are typically long, unbranched and morphologically diverse entities on the order of 5–40 nm in width [4-6]. Despite considerable diversity in the primary sequence of the constituent proteins, all amyloid fibrils share a number of common structural features. They adopt the classic 'cross- β ' structure in which individual strands in the β -sheets are aligned perpendicular to the long axis of the fibril [7, 8]. It is of fundamental importance to be able to determine the structural organization and biochemical properties of amyloid fibrils. This is a challenging problem because amyloid fibrils are non-crystalline and insoluble. Thus, techniques that are routinely used to analyze soluble proteins, such as X-ray crystallography and solution NMR, are not directly applicable to amyloid fibrils. The majority of structural information about amyloid fibrils, to date, has come from solid-state NMR [9], electron paramagnetic resonance [10], X-ray and electron fiber diffraction, scanning probe microscopy [1, 6], deep UV resonance Raman spectroscopy [11], infrared spectroscopy [12-14] and vibrational circular dichroism [5, 15].

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Raman spectroscopy is a powerful nondestructive technique for structural characterization of proteins and protein aggregates [16]. Its advantages include high sensitivity to changes in conformation and chemical bonding, as well as minimal sample preparation requirements. Moreover, measurements can be carried out on samples in different physical states, including powders, single crystals, homogeneous solutions or even dispersions. Raman scattering results from the interaction between the incoming light and the electronic structure of the molecule and is determined by the change in molecular polarizability. The polarization characteristics of Raman scattering are related to the polarizability tensor and carry symmetry information about chemical groups. Raman band anisotropy measurements allow for retrieval of this information, providing that the Raman tensor is known [17-26].

Additional structural information can be obtained if the polarized Raman measurements are performed on an anisotropic assembly of aligned species. There are a number of alignment techniques that have been used for molecular orientation, including methods using stretched polyethylene films as the matrix [25], shear flow orientation of macromolecules [27, 28], molecular combing [13] and preparation of dried stalk samples [29, 30]. Drop coating deposition Raman (DCDR) is a simple method that has been utilized in spectroscopic studies of proteins [31-33]. Briefly, DCDR involves depositing a micro-volume of protein solution on a suitable substrate, followed by solvent evaporation. As described in the literature, a drop of dilute protein solution forms a so-called “coffee ring” while drying [32, 34]. Liquid evaporation causes a net liquid flow outward from the center, producing a shear force that carries radially oriented fibrils toward the perimeter of the droplet. Owing to geometrical constraints, and the “edge effect”, fibrils moving close to the drop periphery have to change their orientation, which leads to self-organization along the perimeter of the droplet, parallel to the outer edge of the ring [35]. The DCDR method produces protein deposits that are in a solid-like state, which appear to remain substantially hydrated with considerable preservation of secondary structure [32]. Among the alignment methods reported, molecular combing has been used with polarized infrared spectroscopy to obtain structural information on oriented amyloid fibrils [13]. In the case of fibrils prepared as dried stalks, the orientation of specific groups, relative to the fibril axis, has also been investigated. To determine orientational information, two different polarized Raman spectra are typically acquired. Incident and scattered electric vectors are parallel to the aligned species in the case of the first spectrum, and both electric vectors are perpendicular to the alignment direction for the second spectrum [29, 30].

Several Raman polarization schemes can be utilized for anisotropic samples. One of the approaches is based on varying the polarization direction of the excitation laser, with respect to the sample orientation, stepwise from 0° to 360° [36-38]. Another method is based on measurements of four polarized spectra, followed by the calculation of two depolarization ratios for two different orientations of the sample with respect to the incident beam [24, 39]. Most often, the orientation of the polarization of the incident, as well as scattered, light is controlled in polarized Raman spectroscopic experiments [25, 26, 30, 40].

The theoretical background of orientation measurements by Raman spectroscopy has been extensively described in the literature for *standard* polarized Raman spectroscopic measurements, involving independent control of the polarization of both the excitation beam

and the scattered light coupled to the spectrograph [20, 21]. For systems showing uniaxial symmetry, the Raman spectroscopy technique allows for obtaining the second and fourth order parameters, $\langle P_2 \rangle$ and $\langle P_4 \rangle$, respectively. These are the first two coefficients of the Legendre polynomial expansion of the orientation distribution function [23, 24, 41, 42]. The general procedure for determining the order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$ from polarized Raman measurements has been developed in detail by Bower [20]. By using polarized Raman spectroscopy, only the second, $\langle P_2 \rangle$, and the fourth, $\langle P_4 \rangle$, coefficients of the orientation function can be determined, and the orientation distribution can be estimated from these parameters by calculating the most probable orientation function, $N_{mp}(\theta)$ [22, 40, 43, 44].

Here, we describe a simple method for polarized Raman measurements on an anisotropic sample, which does not require controlling the polarization of the scattered light. Only the angle of incident laser polarization, with respect to the sample orientation, is controlled. This simplified method could be successfully applied for polarized spectroscopic experiments on an anisotropic sample using a Raman spectrograph, which is not equipped with light polarization accessories. We utilize this simplified approach for Raman spectroscopic polarization studies of aligned insulin fibrils. A drop coating deposition Raman method was utilized for fibril alignment. Although the DCDR method has not been used for the alignment of amyloid fibrils before, we found this method extremely efficient, resulting in more than an 80% degree of orientation. The high orientation order of the sample allowed for more accurate evaluation of chemical group orientation by the polarized Raman spectroscopic experiment. In this study, we determined the first two order parameters, $\langle P_2 \rangle$ and $\langle P_4 \rangle$, for the amide I vibrational mode, based on the change in Raman peak intensity as a function of the polarization rotation angle. The calculation of the most probable distribution revealed that peptide C=O groups in the insulin fibrils are well ordered and almost parallel to the fibril axis, diverging only $13 \pm 5^\circ$ relative to the main axis of the fibrils.

Materials and methods

Insulin fibril preparation and orientation

Both, human recombinant insulin and bovine insulin (I2643, I5500) were purchased from Sigma Aldrich (St Louis, MO) and used without further purification. Solutions of proteins, prepared immediately before fibrillation, were made by dissolving protein powder in 1 mL of H₂O, to achieve a final concentration of 10 g/L. The pH of the solution was adjusted to 2.5 (2.4 in the case of bovine insulin) by adding HCl. The solution was incubated at 65 °C for 24 hours without agitation. Prepared fibrils were washed with an acidic solution (HCl, pH 2.5 (2.4)) and centrifuged for 30 min at 12,000 g at 25 °C. This washing-spinning-resuspension procedure was repeated three times. Fibrils were sonicated for 5 minutes and then resuspended in pure water (dilution factor 1:100, v/v). Aliquots (5-20 μ L) of the fibril suspensions were dropped onto aluminum foil and air-dried.

The morphology of the prepared fibrils was characterized using atomic force microscopy (AFM). A drop of fibril solution was placed onto freshly cleaved mica and incubated for 2 minutes. The excess solution was then removed. The surface was dried under a slow stream of nitrogen before imaging. AFM scanning was performed in AC tapping mode using a

Smart SPM™-1000 fully motorized scanning probe microscope (AIST-NT Inc.) and Olympus AC160 tips.

Raman spectroscopy

A Renishaw inVia confocal Raman spectrometer equipped with a research-grade Leica microscope with a 50x objective (NA = 0.50, Olympus), which produced a laser spot size of approximately 2 μm, was used to collect spectra. A 785 nm laser was utilized for excitation with the power adjusted to approximately 5 mW. Each spectrum was an average of twenty 40-second acquisitions over a range of 550–1800 cm⁻¹. No damage or spectral modifications were observed in samples under these conditions. The spectrometer was calibrated before Raman spectra collection using a silicon reference standard (520 cm⁻¹). For polarization measurements, the excitation laser was focused on the edge of a dried droplet. We utilized the fact that the laser beam is linearly polarized and an anisotropic sample of oriented fibrils could be specifically oriented relative to the beam polarization. No polarizer was placed in front of the camera, and all collected scattered light was coupled to the spectrometer. An optical scrambler was installed before the spectrometer entrance slit to eliminate the polarization dependence of the grating. Local landmarks on the samples were employed to ensure that the same area of a fibril sample was analyzed for polarization measurements.

Data acquisition was performed using WiRE 3.2 software; the GRAMS v7.01 (ThermoGalactic, Salem, NH) software package was used to remove cosmic rays, and for baseline subtraction over the 550–1800 cm⁻¹ spectral range, using a polynomial baseline followed by 7–11 points smoothing. The spectra were normalized by total area. Raman polarization-dependent data were fitted using SigmaPlot 12 software (Systat Software Inc). The order parameters were calculated from the peak-height intensity of the amide I band in the polarized spectra.

Analysis of polarized Raman spectroscopic data

For obtaining information about molecular orientation, the average values of the Legendre polynomial $\langle P_2 \rangle$ and $\langle P_4 \rangle$ can be determined by fitting the experimentally determined Raman scattering intensity using either Eq. (1, 2) or Eq. (3, 4), assuming the polarizability tensor is diagonal [41, 45, 46].

$$\begin{aligned}
 I^{VV}(\theta) &\propto \langle P_4 \rangle \left[\cos^4\theta - 6/7 \cos^2\theta + 3/35 \right] + \langle P_2 \rangle \left[6/7 \cos^2\theta - 2/7 \right] + 1/5 \\
 I^{VV}(0) &\propto \langle P_4 \rangle 8/35 + \langle P_2 \rangle 4/7 + 1/5 \\
 I^{VH}(\theta) &\propto \langle P_4 \rangle \left[-\cos^4\theta + \cos^2\theta - 4/35 \right] + \langle P_2 \rangle / 21 + 1/5 \\
 I^{VH}(0) &\propto -\langle P_4 \rangle 4/35 + \langle P_2 \rangle / 21 + 1/5 \\
 \frac{I^{VV}}{I^{VV}(0)} &\propto \frac{\langle P_4 \rangle [\cos^4\theta - 6/7 \cos^2\theta + 3/35] + \langle P_2 \rangle [6/7 \cos^2\theta - 2/7] + 1/5}{\langle P_4 \rangle 8/35 + \langle P_2 \rangle 4/7 + 1/5}
 \end{aligned} \tag{1}$$

$$\frac{I^{VH}}{I^{VV}(0)} \propto \frac{\langle P_4 \rangle \left[-\cos^4\theta + \cos^2\theta - 4/35 \right] + \langle P_2 \rangle / 21 + 1/5}{\langle P_4 \rangle 8/35 + \langle P_2 \rangle 4/7 + 1/5} \tag{2}$$

Here I^{VV} represents the Raman intensity when the polarization direction of both the polarizer and the analyzer is parallel to the fibril's main axis. The term I^{VH} defines the Raman intensity when the excitation polarizer is parallel to, and the analyzer polarizer is perpendicular to, the fibril's main axis; θ is an angle between the polarization plane of the excitation laser and aligned fibrils; and $I^{VV}(0)$ refers to the Raman intensity when $\theta = 0$.

Regarding the case when polarizabilities α_{xx} , α_{yy} and α_{zz} , are not equal and mutually perpendicular to each other, we also have incorporated a previously described approach [45, 46]:

$$\begin{aligned} I^{VV}(\theta) &\propto a + b \langle P_2 \rangle + 3c \langle P_2 \rangle - 3b \langle P_2 \rangle \cos^2 \theta - 30c \langle P_4 \rangle \cos^2 \theta + 35c \langle P_4 \rangle \cos^2 \theta \\ I^{VV}(\theta) &\propto d - e \langle P_2 \rangle - 4c \langle P_4 \rangle + 35c \langle P_4 \rangle \cos^2 \theta \sin^2 \theta \\ I^{VV}(0) &\propto a - 2b \langle P_2 \rangle + 8c \langle P_4 \rangle \\ I^{VH}(0) &\propto d - e \langle P_2 \rangle - 4c \langle P_4 \rangle \\ \frac{I^{VV}(\theta)}{I^{VV}(0)} &= \frac{a + b \langle P_2 \rangle + 3c \langle P_2 \rangle - 3b \langle P_2 \rangle \cos^2 \theta - 30c \langle P_4 \rangle \cos^2 \theta + 35c \langle P_4 \rangle \cos^2 \theta}{a - 2b \langle P_2 \rangle + 8c \langle P_4 \rangle} \end{aligned} \quad (3)$$

$$\frac{I^{VH}(\theta)}{I^{VV}(0)} = \frac{d - e \langle P_2 \rangle - 4c \langle P_4 \rangle + 35c \langle P_4 \rangle \cos^2 \theta \sin^2 \theta}{a - 2b \langle P_2 \rangle + 8c \langle P_4 \rangle} \quad (4)$$

where

$$\begin{aligned} a &= (8r^2 + 4r + 3), b \\ &= (8r^2 - 2r - 6) \\ &/21, c \\ &= (r - 1)^2 / 35, d \\ &= (r - 1)^2 / 15, e \\ &= - (r - 1)^2 / 21 \quad \text{and} \\ &/r = \alpha'_{xx} / \alpha'_{zz}, \alpha'_{zz} \\ &= \delta \alpha_{zz} / \delta q, \alpha'_{xx} \\ &= \delta \alpha_{xx} / \delta q. \end{aligned}$$

The differential polarizability ratio r is connected with the depolarization ratio R_{iso} by the expression:

$$R_{iso} = \frac{(r - 1)^2}{(3 + 4r + 8r^2)}$$

It should be emphasized that in our experimental setup *no polarizer was placed before the entrance slit* of the monochromator. As a result, all scattered light, with all polarizations ($I_{total}^{exp}(\theta)$), was collected. Assuming $I_{total}^{exp} \propto I^{VV} + I^{VH}$, we modified Eq. (1-2 and 3-4):

$$\begin{aligned}
\frac{exp}{total} I &\propto I^{VV} + I^{VH} \\
\frac{exp}{total} I(\theta) &= \frac{\frac{exp}{total} I(\theta)}{\frac{exp}{total} I(0)} \\
\frac{exp}{total} I(\theta) &\propto I^{VV}(\theta) + I^{VH}(\theta) \\
\frac{exp}{total} I(0) &\propto I^{VV}(0) + I^{VH}(0) \\
\frac{exp}{total} I(\theta) &\propto \frac{I^{VV}(\theta) + I^{VH}(\theta)}{I^{VV}(0) + I^{VH}(0)} = \frac{I^{VV}(\theta)}{I^{VV}(0) + I^{VH}(0)} + \frac{I^{VH}(\theta)}{I^{VV}(0) + I^{VH}(0)}
\end{aligned} \tag{5}$$

It has been shown that the tensor of the amide I vibrational mode is not cylindrical, but a_{xx}/a_{zz} is much larger than a_{yy}/a_{zz} , where a_{xx} , a_{yy} and a_{zz} are zero (principal axis) components of the Raman tensor, and the principal axis is defined as the orientation of the largest polarizability oscillation. [17, 47]. The amide I normal mode is affected by the nature of the side chain and depends on the secondary structure of the backbone. Krim et al. have shown that vibrations of adjacent amide chromophores are coupled and delocalized along the polypeptide backbone [48, 49]. Asher and coworkers [50, 51] have experimentally demonstrated that a negligible vibrational coupling occurs between adjacent peptide bonds for amide I modes in the polyproline II (PPII) conformation. For α -helix conformation, they found that the amide I vibrational mode exhibited noticeable interamide coupling. The amide I Raman tensor of isolated peptide group has been determined [52] and shown to be transferable to peptides in β -sheet and α -helical conformations [17]. The study of the amide I vibrational mode (which is mainly attributed to the C=O stretching vibration) of *N. edulis dragline* has shown that obtaining $\langle P_2 \rangle$ using a cylindrical Raman tensor or using a noncylindrical Raman tensor gives the same value within experimental error [44]. Therefore, the error associated with the cylindrical tensor approximation is expected to be small for $\langle P_2 \rangle$ and $\langle P_4 \rangle$ measurements in the case of the amide I vibrational mode, and should not affect the overall conclusions of this work.

Another important approximation of the proposed approach, discussed below, relates to the assumption that fibrils are perfectly oriented in the sample.

Results and discussion

Polarized Raman spectra of aligned fibrils

Human and bovine insulin fibrils were prepared and washed as described in the experimental section. The fibril morphology was determined to be consistent with the literature data [5] using Atomic Force Microscopy (AFM) (data not shown). Oriented samples of insulin fibrils were prepared using the drop coating deposition Raman method [31-33], in which the insulin fibril suspension was deposited onto aluminum foil, followed by solvent evaporation (Fig. 1).

A series of polarized Raman spectra were recorded over the range 1-124° by rotating samples relative to the fixed direction of the laser beam polarization. For each spectrum obtained, normalization by total area was applied to take into account possible changes in the volume of the sample exposed as a result of rotation.

It is evident from spectra presented in Fig. 1 that the rotation of aligned insulin fibrils with respect to the incident laser polarization causes significant variation in the intensity of

several Raman bands. The anisotropy of these Raman bands originates from the preferential orientation of certain chemical moieties and their associated vibrational modes, with respect to the fibril axis.

For a sample of randomly oriented fibrils, the Raman intensity as a function of polarization angle is a constant, and systematic deviation from this indicates the degree of fibril alignment. The orientation of the main axis of a fibril can be deduced in two ways. First, by analyzing the difference in the Raman intensity of the amide I band between parallel (0°) and perpendicular (90°) orientations (Fig. 1). Second, from the polar plot (Fig. 2), by evaluating the polarization angle at which a maximum Raman intensity is observed for the amide I vibration.

It has been shown previously that Raman spectra of amyloid fibrils, in particular the amide I band, are dominated by the contribution from the fibril core, which has a well-ordered β -sheet structure [53-55]. The assignment of the major Raman bands marked in Fig. 1 is shown in Table 1, based on literature data [24, 29, 56, 57]. The amide I Raman band is mainly associated with C=O stretching mode mixed with contributions from C-N stretching and C α -C-N deformation [58-61]. The amide I band is a sensitive marker of protein secondary structure because its wavenumber depends on C=O hydrogen bonding and the interaction between adjacent amide units, which are influenced by the three-dimensional structure of the polypeptide backbone [48, 62, 63].

The most prominent Raman peak exhibiting an orientation-dependent intensity is the amide I peak at 1674 cm^{-1} (Fig. 1) that is in an agreement with previously published data for insulin fibrils [53]. Measey and Schweitzer-Stenner have shown [64, 65] that the anisotropic Raman amide I profile is a good tool for discriminating between parallel and antiparallel β -sheets. It is expected that the amide I position for antiparallel β -sheets might be upshifted in contrast to parallel β -sheets because of weaker hydrogen bonding in the former case. However the amide I position does not necessarily report on the type of β -sheet because other factors affect the peak position also. For example, it has been shown [65] that simulated anisotropic Raman amide I profile of antiparallel and parallel β -sheets depends on the number of strands. Specifically, the decrease in the number of strands from 12 to 1 causes a large upshift of amide I peak for the parallel β -sheet and a negligible peak shift in the case of antiparallel β -sheet. Also, even 3° twist and/or 2° bend per strand causes detectible upshift of amide I band of the parallel β -sheet.

The largest intensity of the amide I band occurred when the main axes of the fibrils were parallel to the direction of the electric field component of the polarized laser radiation. The intensity of this peak decreased more than five-fold when the sample was rotated from 0° to 90° , retaining only 18% of its maximum intensity (Fig. 1). This significant change in the intensity indicates both an excellent alignment of fibrils in the sample and a strong orientation of the Raman tensor of the associated amide I vibrational mode along the main axis of an insulin fibril.

$(I_{\max} - I_{\min})/I_{\max}$ is equal to approximately 80% for an aligned insulin fibril sample (Fig. 1), where $I_{\max}$ and $I_{\min}$ are amide I Raman intensities obtained at 0° and 90° , respectively. This

expression gives us an estimation of a minimum value for the degree of orientation or alignment in the sample. Considering absolutely perfect alignment, it can be shown that any deviation in the angle between the Raman tensor (even with only one non-zero component) and the main axis of a fibril will result in a decrease in the value obtained by the expression $(I_{\max} - I_{\min})/I_{\max}$.

Determination of the molecular orientation distribution coefficients

As described in the experimental section, it is possible, for systems showing uniaxial symmetry, to quantitatively determine the order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$ by polarized Raman microspectroscopy, for a Raman tensor showing cylindrical symmetry. We used the intensity of the amide I band to determine the orientation of the carbonyl groups in a β -sheet, with respect to the fibril axis. The normalized Raman scattering intensity for amide I (1674 cm^{-1}) is plotted in Fig. 2. The black solid line corresponds to the theoretical Raman intensity, as computed from Eq. 5. Consideration should be given to possible errors associated with intensity measurements and fitting procedures, imperfect fibril orientation within the sample, and contribution from the less oriented parts of the fibrils.

Values obtained for coefficients $\langle P_2 \rangle$ and $\langle P_4 \rangle$ from the experimental scattered Raman intensity are listed in Table 2, and the corresponding most probable orientation distribution function $N_{\text{mp}}(\theta)$ is plotted in Figure 3.

For aligned insulin fibrils, the value of $\langle P_2 \rangle$ obtained for the peak at 1674 cm^{-1} is approximately 0.48 ± 0.01 . The $\langle P_2 \rangle$ order parameter defines the molecular orientation; values -0.5 and 1 define perfect orientation at 90° and 0° , respectively, from the fibril axis. A positive $\langle P_2 \rangle$ value corresponding to the 1674 cm^{-1} band indicates that the principal axis of the Raman tensor of the amide I vibration is preferentially oriented parallel to the fibril axis. It is apparent that the values of orientation parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$ are similar for human and bovine insulin fibrils (Table 2). As has been shown previously, the segment of the insulin B-chain with the sequence LVEALYL is central to the cross- β spine of the insulin fibril [4]. Both bovine and human insulin possess the LVEALYL sequence in their amino acid chains. In addition, the only difference in the fibrillation conditions between human and bovine insulin is a mere 0.1 pH unit. Thus, taking into account a predominant contribution from the cross- β core in the amide I band, it is expected that bovine and human insulin fibrils have the same structural organization.

For comparative purposes, the order parameter $\langle P_2 \rangle$ was also calculated (Table 2) while keeping the parameter $\langle P_4 \rangle$ fixed during the fitting procedure: $\langle P_4 = \langle P_4 \rangle_{\min}$. It has been shown that parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$ are not totally independent [22], and values of $\langle P_4 \rangle$ associated with a given $\langle P_2 \rangle$ value are limited by:

$$\langle P_4 \rangle_{\min} = \frac{1}{18} (35\langle P_2 \rangle^2 - 10\langle P_2 \rangle - 7) \leq \langle P_4 \rangle \leq \frac{1}{12} (5\langle P_2 \rangle + 7) = \langle P_4 \rangle_{\max}$$

The maximum value of $\langle P_4 \rangle$ results in a bimodal distribution centered at 0° and 90° . The values of $\langle P_4 \rangle$ determined from the experimental data are closer to the lower limit of allowed $\langle P_4 \rangle$ values, and thus a unimodal distribution is expected.

If $\langle P_4 \rangle = \langle P_4 \rangle$, the orientation distribution function is unimodal and is given by the delta function centered at an angle θ_0 [22, 43, 66]. This angle also corresponds to the mean value of the orientation distribution and can be calculated when only $\langle P_2 \rangle$ is known [24, 39].

$$\theta_0 = \arccos\left(\frac{2\langle P_2 \rangle + 1}{3}\right)^{1/2}$$

Based on the above equation, the angle θ_0 for the principal axis of the amide I Raman tensor has a value $34^\circ \pm 2$. The combined values of $\langle P_2 \rangle$ and $\langle P_4 \rangle$ provide valuable information on the shape of the orientation distribution function. As shown in Figure 3, the distribution of orientations, described by the function $N_{mp}(\theta)$, is Gaussian and centered at $\theta=0^\circ$. The shape of the calculated distribution function indicates that the principal axis of the amide I Raman tensor has a preferred orientation along the longitudinal (main) axis of the fibril.

Assuming a uniaxial cylindrical symmetry, multiplication of $N_{mp}(\theta)$ by $\sin(\theta)$ gives the probability that the principal axis of the amide I Raman tensor is oriented at an angle with respect to the fibril's main axis. The mean of this distribution characterizes the average orientation angle [67, 68]. The $N_{mp}(\theta) * \sin(\theta)$ plot (Fig. 4) shows the maximum of the distribution at $\theta=21^\circ \pm 5$, and the mean of the distribution yields *average* orientation angles of $34^\circ \pm 4$.

In this respect it is worth noting that the largest polarizability oscillation for the amide I band takes place along a line that is in the plane of the peptide group and at an angle of 34° to the peptide C=O bond [17, 69]. This means that the *average* orientation of carbonyl groups is nearly parallel to the fibril's main axis. This is in agreement with data previously reported for aligned amyloid fibrils, studied by polarized infrared and Raman spectroscopy [13, 29, 70]. At the same time, based on the maximum position of the distribution $N_{mp}(\theta) * \sin(\theta)$, the preferred orientation of C=O groups with respect to the fibril axis is approximately $13 \pm 5^\circ$. Supported by the fact that the C=O groups in a β -sheet are perpendicular to the β -strand, these results show unambiguously that the β -strands are nearly perpendicular to the fibril's main axis, as represented by a well-documented cross- β structure of amyloid fibrils. Thus, our results are in agreement with the report, which demonstrated that the fibrils possess a cross- β structure, with β -strands arranged parallel or antiparallel to each other and perpendicular to the long axis of the fibril [13, 71].

It should be mentioned that structural information for a number of amyloidogenic peptides has also been obtained by means of infrared linear dichroism spectroscopy [13, 70, 72]. In particular, inclination angles for specific C=O bonds have been reported with respect to the fibril axis of aligned amyloid fibrils, prepared from the core fragment (21-31 peptide, [21NFLNCYVSGFH³¹]) of β_2 -microglobulin [72, 73]. By applying isotope substitution and the amide I band decomposition procedure, followed by the estimation of the number of residues per secondary structure, Hiramatsu *et al.* have concluded that two C=O bonds in the β -sheet are oriented at 0° , three in the β -sheet structure at 27° , four in the random coil portion at 47° , and another two in the β -turn or β -bulge at 32° , with respect to the fibril axis. Based on these reported data, the average angle for the 21-31 peptide of β_2 -microglobulin

fibrils could be estimated as 32° . Taking into account only the angle values for C=O bonds which are part of the β -sheet core, an angle of $\sim 16^\circ$ with respect to the fibril axis is obtained, which is close to the value of preferred orientation of C=O groups obtained in this work for full-length protein fibrils.

Orientation of aromatic amino acid side chains—Several bands attributed to aromatic amino acid residues also show significant dichroism. Peaks at 643, 830 and 1616 cm^{-1} are assigned to various vibrational modes of the phenoxyl ring, which is the aromatic part of the tyrosine side chain. Raman tensors for these vibrational modes have been reported previously [69, 74]. Because each insulin monomer contains four tyrosine residues, obtaining quantitative information about the orientation of the individual side chains would be challenging unless all four have the same orientation. However, we did manage to obtain some qualitative information about the average orientation of tyrosine phenoxyl rings. Tyrosine Raman bands at 830 and 643 cm^{-1} have higher intensity with laser polarization perpendicular (90°) to the main axis of the insulin fibrils. Accordingly, the average orientation of the tyrosine phenoxyl ring planes is closer to being perpendicular than parallel to the fibril axis.

Conclusions

The conversion of proteins or peptides from their soluble functional states into structures referred to as amyloid fibrils has been implicated in a large number of diseases. Atomic-level structural characterization of amyloid fibrils is challenging because of the limitations of solution NMR and X-ray crystallography when applied to insoluble, noncrystalline protein aggregates. Polarized Raman spectroscopy is uniquely suitable for probing amyloid fibrils, which can be aligned with high efficiency in an anisotropic sample. We used a simple approach, based on the so-called “coffee stain” phenomenon, for preparing well-oriented insulin fibrils. Raman spectra were measured while the angle between the fibril sample and the excitation beam polarization was changed gradually from 0° to 124° . No polarizer was used for the collection of scattered light. A dramatic change in the amide I band intensity was observed by varying the polarization orientation, suggesting a good alignment of fibrils in the sample. We developed a method for the quantitative evaluation of the orientation of selected chemical groups, relative to the main fibril axis, using polarized Raman spectroscopic data. This method involves calculating order parameters The $\langle P_2 \rangle$ and $\langle P_4 \rangle$ of the most probable orientation function ($N_{\text{mp}}(\theta)$) of the Raman tensor. The preferred orientation of the principal axis of the Raman tensor with respect to the fibril axis was calculated by multiplying $N_{\text{mp}}(\theta)$ by $\sin(\theta)$. Knowing the relationship between the principal axis of the Raman tensor and the corresponding chemical moiety, it is possible to obtain orientation information for the latter.

Here, bovine and human insulin fibrils, prepared using the same procedure, were investigated. Atomic force microscopy indicated a similar morphology of the two protein fibrils. We specifically focused on determination of the peptide carbonyl group orientation in this study. We obtained similar results for the *average* orientation of C=O groups in the two types of insulin fibrils, indicating a similar structural organization of the fibril core. Our results indicate that the preferred orientation distribution of the peptide carbonyl group is

centered at an angle approximately 13° from the fibril axis. The method proposed here is not limited to peptide carbonyl groups specifically, but can also be applied to other chemical groups as long as the Raman tensors are known.

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Figure 1.

Representative Raman spectra of aligned insulin fibrils as a function of the excitation polarization angle (θ). For convenience, parallel (0°) and perpendicular (90°) orientations are shown in blue and red, respectively. **Insert:** bright-field image of a portion of the ring formed by an evaporated solution of insulin fibrils, with a schematic representation of an angle between the polarization plane of the excitation laser (red arrow) and the aligned fibrils.

Figure 2.

The normalized Raman scattering intensities (black dots) for $^{exp}_{norm} I(\theta)$ vs. rotational angle (θ) for human insulin fibrils. The black line is calculated using Eq. 5. The blue and red lines represent 95% confidence and prediction intervals, respectively.

Figure 3. Polar representations of the most probable orientation distribution function ($N_{mp}(\theta)$) determined for human insulin fibrils, where $\langle P_2 \rangle = 0.48$ and $\langle P_4 \rangle = 0.17$.

Figure 4. Polar representations of the orientation distribution function, $N_{mp}(\theta) * \sin(\theta)$, of the amide I Raman tensor for insulin fibrils, where $\langle P_2 \rangle = 0.48$ and $\langle P_4 \rangle = 0.17$.

Table 1

Peak assignments for the Raman spectrum of insulin fibrils

Raman wavenumber/cm ⁻¹	Peak assignment
1674	Amide I, mainly C=O in β -sheets
1616	Tyr, C ₂ -C ₃ , C ₆ -C ₅ in-phase stretching
1448	(CH ₂) deformation, scissoring mode
1200-1300	Amide III
1003	Phe, Tyr ring breathing
853	Tyr, Fermi resonance
830	Tyr, ring breathing
644	Tyr, ring deformation
621	Phe, in-plane ring deformation

Table 2

Orientation order parameters for oriented human and bovine insulin fibrils

	Raman wavenumber/cm ⁻¹	$\langle P_2 \rangle^a$	$\langle P_4 \rangle^a$	$\langle P_2 \rangle^b$	$\langle P_4 \rangle^b$	R^2
Human insulin fibrils	1674	$\frac{0.48}{0.51}^*$	$\frac{0.17}{-0.16}^*$	$\frac{0.49}{0.54}^*$	$\frac{0.29}{-0.12}^*$	0.95
Bovine insulin fibrils		$\frac{0.47}{0.51}^*$	$\frac{0.1}{-0.16}^*$	$\frac{0.49}{0.52}^*$	$\frac{0.09}{-0.15}^*$	0.97

Experimental data were fitted with equation 5, 1 - 2(a) and 3 - 4 (b).

$$^*\langle P_4 \rangle = \langle P_4 \rangle_{min} = \frac{1}{18} \left(35 \langle P_2 \rangle^2 - 10 \langle P_2 \rangle - 7 \right).$$