Advanced raman spectroscopic methods for structural characterization of amyloid fibrils and bionanotubes

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Advanced Raman Spectroscopic Methods for Structural
Characterization of Amyloid Fibrils and Bionanotubes

by
Valentin Sereda

A Dissertation
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To my wife and my family
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ABSTRACT

Amyloid aggregation is a specific form of protein misfolding and self-assembly related to several degenerative pathologies like Alzheimer's and Parkinson's diseases, type-II diabetes, etc. This association with deadly diseases has made amyloid fibrils the focus of intensive research for many decades. Most recently, a significant interest to protein fibrils was also due to the application of the remarkable self-assembling capabilities to form well-structured nano-biomaterials. Self-assembly of short peptide sequences into well-organized structures is another important strategy for bottom-up fabrications of nano-biomaterials. One of the most commonly used building blocks is diphenylalanine, a short dipeptide that has been identified as playing a key role in the core-recognition motif of the Alzheimer’s disease, the β-amyloid protein. X-ray crystallography and solution NMR are two major tools of structural biology that have significant limitations when applied to such structures due to their non-crystalline and insoluble nature as well as large molecular weight.

This dissertation describes application of polarized Raman spectroscopy to advance the understanding of organization of amyloid fibrils and peptide nanotubes. In particular, it reports on the orientation of selected chemical groups and secondary structure elements in aligned insulin fibrils, including β-sheets, which are highly oriented in the cross-β core, and α-helices. Strong orientation of disulfide bonds in amyloid fibrils was revealed, indicating their association with the fibril core. The orientation of selected chemical groups relative to the main axis of the nanotube was determined. The obtained data indicate that there is only one orientation of di-D-phenylalanine with respect to the nanotube’s main axis.

Peptide microcrystals were used to identify spectroscopic signatures of amyloid-related conformations. The microcrystals were utilized for probing the core structures of fibrils prepared
from full-length proteins. We demonstrated that Raman spectroscopy in combination with advanced chemometrical methods can discriminate between parallel and anti-parallel β-sheet structures in peptide microcrystals and amyloid fibrils.

This dissertation also presents tip-enhanced Raman (TER) spectra of an individual insulin fibril, a protein cast film and a short peptide microcrystal mimicking the fibril core. Two different types of TER spectra were acquired depending on the “roughness” of the probed surface at the molecular level. Two tip enhancement mechanisms of Raman scattering, long- and short-range, were proposed based on the analogy with physical and chemical enhancement mechanisms, respectively, previously known in surface-enhanced Raman spectroscopy.
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CHAPTER 1. Structural characterization of protein aggregates by Raman spectroscopy

1.1 Introduction

Amyloid aggregation is a specific form of protein misfolding and self-assembly, which plays an important role in a number of human diseases, including Parkinson’s disease, Alzheimer’s disease, and type II diabetes.\textsuperscript{1-3} The disease-related amyloid fibrils are typically long, unbranched and morphologically diverse entities on the order of 5–40 nm in width.\textsuperscript{4-6} Meanwhile, short peptides also have the ability to self-assemble into nanostructures, including fibrils.\textsuperscript{7} While studying the formation of amyloid fibrils from aromatic peptides in solution, Gazit et al., has discovered that the core recognition motif of the β-amyloid polypeptide, the diphenylalanine polypeptide, forms discrete and hollow nanotubes.\textsuperscript{8,9}

It is of fundamental importance to be able to determine the structural organization and biochemical properties of such elongated species. However, this is a challenging problem because of the non-crystalline and insoluble nature of amyloid fibrils and nanotubes. Because of these limitations the experimental techniques like X-ray crystallography and solution NMR are not very effective.

It should be mentioned that solid state NMR (ssNMR) offers the most direct information about it at the moment. However, a site-specific isotope substitution is required for ssNMR which might be challenging for full-length proteins. To the best of our knowledge, Aβ1-40 is the longest peptide, which was used for the fibril structural studies by ssNMR so far. Even for a well-organized Aβ1-40 fibrils, the conformation of only 19 peptide bonds from the total of 40 were determined (Y10, V12, K16-A21, K28, A30-I32, L34, M35, G9, D23, and V36).\textsuperscript{10} Unordered segments of the fibrils were either absent from ssNMR spectra or yielded broad
Moreover, according to Tycko,\textsuperscript{14} multiple structural solutions are possible based on ssNMR data:

“The main problem is that, although one may be able to find a plausible set of resonance assignments that seems fully consistent with one’s multidimensional spectra, there is no guarantee that this set of assignments is unique.”

Raman spectroscopy is a powerful nondestructive technique for structural characterization of proteins and protein aggregates.\textsuperscript{15} 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.

1.2 Raman spectroscopy for determining the conformation and organization of polypeptide backbone

The conformation of polypeptide backbones is determined by dihedral angles. The Ramachandran dihedral psi (Ψ) angle of the Ramachandran plot, specifically determines the geometry of the secondary structure of proteins and peptides. By considering the Ψ and Φ angle combinations, one can relate secondary structure elements and the sites in a Ramachandran plot. More specifically, depending on dihedral angles, β-sheets can be organized in a parallel or anti-parallel fashion. It has been shown that Deep UV Raman spectroscopy provides unique opportunity to quantitatively determine the distribution of Ψ angles.\textsuperscript{15} In fact, amide III is one of the most structurally informative Raman bands due to its sensitively on the peptide bond Ramachandran Ψ dihedral angle that, in part, determines the secondary structure conformation.\textsuperscript{16} Asher et al,\textsuperscript{17} have established the dependence of the amide III band position on the Ramachandran Ψ dihedral angle with modest dependence on the Φ dihedral angle. This semi-
empirical approach has been developed initially for ultraviolet resonance Raman spectroscopy\textsuperscript{17} and later adopted for non-resonance Raman spectroscopy of peptides and proteins.\textsuperscript{18}

1.3 Structure and composition of the surface of protein aggregates

Structural characterization of protein aggregates is one of the major challenges in molecular biology. Vast majority of modern methods and techniques utilized for characterizing protein aggregates, amyloid fibrils in particular, probe the bulk properties. There are few current techniques that can probe the structure and composition of biological species on the nanometer scale. It is even more challenging to probe the surface of nanometer-size species, such as amyloid fibrils and other protein aggregates, although these surfaces play decisive roles in the biological activity and associated toxicity of these aggregates in vivo.

Tip-enhanced Raman spectroscopy (TERS) combines the advantages of vibrational spectroscopy and scanning probe microscopy and provides chemical information with nanometer spatial resolution.\textsuperscript{19-21} Due to the intense electromagnetic field localized near the apex of the tip “hot-spot”, the Raman signal of molecules near the tip can be enhanced by a factor of $10^4$-$10^8$.\textsuperscript{22,23} A high spatial resolution and sensitivity combined with an ability to provide molecular structural information has resulted in widespread application of TERS in probing various complex biological samples\textsuperscript{24} ranging from small biomolecules, such as adenine,\textsuperscript{25,26} amino acids,\textsuperscript{27,28} and cytochrome c,\textsuperscript{29} to collagen,\textsuperscript{30} biopolymers\textsuperscript{31,32} and lipid monolayers.\textsuperscript{33} This technique has been applied to nucleobases,\textsuperscript{34} RNA\textsuperscript{35} and DNA,\textsuperscript{36} with the potential for a direct label-free sequencing of a single DNA strand. TERS has also been used for virus detection\textsuperscript{37} and bacterial surface characterization.\textsuperscript{38,39} The label-free nature of TERS also appears to be capable of investigating protein–ligand binding.\textsuperscript{40,41} The chemical complexity of isolated mitochondria from yeast cells,\textsuperscript{42} membranes\textsuperscript{43,44} and human cells\textsuperscript{45,46} has been demonstrated by this technique.
1.4 Polarization Raman characteristic of anisotropic species

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, which is as a symmetric second-rank tensor, the so-called polarizability tensor. 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. The theoretical background of orientation measurements by Raman spectroscopy has been extensively described in the literature.

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. 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°. 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. Most often, the orientation of the polarization of the incident, as well as scattered, light is controlled in polarized Raman spectroscopic experiments.

There are a number of alignment techniques that have been used for molecular orientation, including methods using stretched polyethylene films as the matrix, shear flow orientation of macromolecules, molecular combing and preparation of dried stalk samples. Drop coating deposition Raman (DCDR) is a simple method that has been utilized
in spectroscopic studies of proteins. 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. 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. 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.

Among the alignment methods reported, molecular combing has been used with polarized infrared spectroscopy to obtain structural information on oriented amyloid fibrils. In the case of fibrils prepared as dried stalks, the orientation of specific groups, relative to the fibril axis, has also been investigated. It should be mentioned that structural information for a number of amyloidogenic peptides has also been obtained by means of infrared linear dichroism spectroscopy. 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 β₂-microglobulin.

1.4.1. Most probable orientation distribution function

The orientation of a molecule is a description of its spatial position with respect to a macroscopic XYZ coordinate system as described by three Euler’s angles (θ, φ, ψ). In general, the orientation information on scattering units can be described by means of the orientation distribution function $N(θ)$. 
Equation 1

\[ N(\theta) = \sum_{l}^{even} \left( l + \frac{1}{2} \right) \langle P_l \rangle P_l(cos\theta) \]

By assuming a uniaxial system with cylindrical symmetry, the orientation distribution function only depends on the angle \( \theta \) and can be expanded in a series of even Legendre polynomials \( \langle P_l \rangle \) of degree \( l \), whose average value is defined as the orientation order parameter.\(^{52}\)

Equation 2

\[ \langle P_l \rangle = \int_{0}^{\pi} P_l(cos\theta)N(\theta)sin(\theta)d\theta \]

For a system with cylindrical symmetry, the molecular orientation can be described by an orientation distribution function, \( N(\theta) \), for which \( N(\theta)sin(\theta)d\theta \) represents the fraction of chromophores oriented within the solid angle \( sin(\theta)d\theta \) at an angle \( \theta \) to the laboratory axis of the system (Figure 1.1).\(^{75}\)
Figure 1.1 (A) Schematic representation of the orientation of a polarizability ellipsoid in terms of Euler’s angles (θ, φ, ψ) and (B) an example of ODF dependent on θ angle only. XYZ represents the laboratory coordinate system (sample is oriented along the Z direction).

For systems showing uniaxial symmetry, the polarized Raman spectroscopy technique allows for obtaining the second and fourth order parameters, ⟨P₂⟩ and ⟨P₄⟩, which are the first two coefficients of the Legendre polynomial expansion of the orientation distribution function.⁵³,⁵⁴,⁷⁶,⁷⁷

For example, in contrast to IR spectroscopy, higher the order coefficient, ⟨P₄⟩, can be obtained by means of polarized Raman spectroscopy. Consequently, a more detailed study about the molecular orientation distribution can be performed using polarized Raman spectroscopy. Furthermore, due to the fact that this technique is compatible with optical microscopy, polarized Raman spectroscopy is suitable for the study the material at the micron size scale or in a micron
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. From $\langle P_2 \rangle$ and $\langle P_4 \rangle$, the most probable orientation function, $N_{mp}(\theta)$ of the considered chemical group can be estimated using the information theory ($\theta$ is the angle around the fibril axis) by maximizing the information entropy of the distribution:\textsuperscript{52,63,78,79}

**Equation 3**

$$S[N_{mp}(\theta)] = -\int_0^\pi N(\theta) \ln(N(\theta)) \sin(\theta) \, d\theta$$

The most probable distribution function is then given by:

**Equation 4**

$$N_{mp}(\theta) = \frac{\exp[\lambda_2 \langle P_2 \rangle + \lambda_4 \langle P_4 \rangle]}{\int_{-1}^{+1} \exp[\lambda_2 \langle P_2 \rangle + \lambda_4 \langle P_4 \rangle]d(\cos \theta)}$$

where $\lambda_2$ and $\lambda_4$ are Lagrangian multipliers, are introduced to take care of the constraints while defining the maximum.

This is subject to the probability constraint:

**Equation 5**

$$\int_0^\pi N_{mp}(\theta) \sin(\theta) \, d\theta = 1$$

Once $\langle P_2 \rangle$ and $\langle P_4 \rangle$ are determined experimentally, $\lambda_2$ and $\lambda_4$ are numerically calculated using the constraints given by Eq 3.
Equation 6

\[ \langle P_2 \rangle = \int_{-1}^{+1} P_2(\cos \theta)N_{mp}(\theta)d(\cos \theta) \]

Equation 7

\[ \langle P_4 \rangle = \int_{-1}^{+1} P_4(\cos \theta)N_{mp}(\theta)d(\cos \theta) \]

It should be mentioned that the shape of the ODF significantly varies depending on the particular \( \langle P_2 \rangle \) and \( \langle P_4 \rangle \) couples. It also has been shown that parameters \( \langle P_2 \rangle \) and \( \langle P_4 \rangle \) are not totally independent (Figure 1.2), and values of \( \langle P_4 \rangle \) associated with a given \( \langle P_2 \rangle \) value are limited by:\textsuperscript{52}

Equation 8

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

Figure 1.2. Diagram and a schematic representation of different possible ODFs associated with \( \langle P_2 \rangle \) and \( \langle P_4 \rangle \).
The maximum value of \( \langle P_4 \rangle \) results in a bimodal distribution of the Raman tensor centered at 0° and 90°. If \( \langle P_4 \rangle = \langle P_4 \rangle_{\text{min}} \) the orientation distribution function is unimodal and is given by the delta function centered at an angle \( \theta_0 \).^{52,78,80}

**Equation 9**

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

This angle also corresponds to the mean value of the orientation distribution and can be calculated when only \( \langle P_2 \rangle \) is known.\(^{54,62}\)

### 1.4.2. Order parameters \( \langle P_2 \rangle \) and \( \langle P_4 \rangle \). Experimental approach

The following method can be successfully applied for polarized spectroscopic experiments 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.

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. (14, 15) or Eq. (20, 21), assuming the polarizability tensor is diagonal.\(^{76,81,82}\) Raman tensor and the principal axis is defined as the orientation of the largest polarizability oscillation.\(^{47,83}\)

**Equation 10**

\[
I^{VV}(\theta) \propto \langle P_4 \rangle [\cos^4 \theta - 6/7 \cos^2 \theta + 3/35] + \langle P_2 \rangle [6/7 \cos^2 \theta - 2/7] + 1/5
\]
Equation 11

\[ I_{VV}(0) \propto \langle P_4 \rangle 8/35 + \langle P_2 \rangle 4/7 + 1/5 \]

Equation 12

\[ I_{VH}(\theta) \propto \langle P_4 \rangle [-\cos^4 \theta + \cos^2 \theta - 4/35] + \langle P_2 \rangle /21 + 1/5 \]

Equation 13

\[ I_{VH}(0) \propto -\langle P_4 \rangle 4/35 + \langle P_2 \rangle /21 + 1/5 \]

Equation 14

\[ \frac{I_{VV}}{I_{VV}(0)} \propto \frac{\langle P_4 \rangle [\cos^4 \theta - 6/7 \cos^2 \theta + 3/35] + \langle P_2 \rangle [6/7 \cos^2 \theta - 2/7] + 1/5}{\langle P_4 \rangle 8/35 + \langle P_2 \rangle 4/7 + 1/5} \]

Equation 15

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

Here \( I_{VV} \) represents the Raman intensity when the polarization direction of both the polarizer and the analyzer is parallel to the alignment direction. The term \( I_{VH} \) defines the Raman intensity when the excitation polarizer is parallel to, and the analyzer polarizer is perpendicular to, the alignment direction; \( \theta \) is an angle between the polarization plane of the excitation laser and aligned species; and \( I_{VV}(0) \) refers to the Raman intensity when \( \theta = 0 \).

Regarding the case when polarizabilities \( \alpha_{xx} \), \( \alpha_{yy} \) and \( \alpha_{zz} \) are not equal and mutually perpendicular to each other, we also have incorporated a previously described approach.\[^{81,82}\]
Equation 16

\[ I^{VV}(\theta) \propto a + b(P_2) + 3c(P_4) - 3b(P_2)\cos^2\theta - 30c(P_4)\cos^2\theta + 35c(P_4)\cos^2\theta \]

Equation 17

\[ I^{VH}(\theta) \propto d - e(P_2) - 4c(P_4) + 35c(P_4)\cos^2\theta\sin^2\theta \]

Equation 18

\[ I^{VV}(0) \propto a - 2b(P_2) + 8c(P_4) \]

Equation 19

\[ I^{VH}(0) \propto d - e(P_2) - 4c(P_4) \]

Equation 20

\[ \frac{I^{VV}(\theta)}{I^{VV}(0)} = \frac{a + b(P_2) + 3c(P_4) - 3b(P_2)\cos^2\theta - 30c(P_4)\cos^2\theta + 35c(P_4)\cos^2\theta}{a - 2b(P_2) + 8c(P_4)} \]

Equation 21

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

where

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

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

\[ 12 \]
Equation 22

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

It should be emphasized that for the experimental setup without polarizer before the entrance slit of the monochromator all scattered light, with all polarizations \( I_{total}^{exp}(\theta) \), is collected. Assuming \( I_{total}^{exp} \propto I_{VV}^{exp} + I_{VH}^{exp} \), the Eq. (14, 15 and 20, 21) can be modified:

Equation 23

\[ I_{total}^{exp} \propto I_{VV}^{exp} + I_{VH}^{exp} \]

Equation 24

\[ I_{total}^{norm}(\theta) = \frac{I_{total}^{exp}(\theta)}{I_{total}^{exp}(0)} \]

Equation 25

\[ I_{total}^{exp}(\theta) \propto I_{VV}^{exp}(\theta) + I_{VH}^{exp}(\theta) \]

Equation 26

\[ I_{total}^{exp}(0) \propto I_{VV}^{exp}(0) + I_{VH}^{exp}(0) \]

Equation 27

\[ I_{total}^{norm}(\theta) \propto \frac{I_{VV}^{exp}(\theta) + I_{VH}^{exp}(\theta)}{I_{VV}^{exp}(0) + I_{VH}^{exp}(0)} = \frac{I_{VV}^{exp}(\theta)}{I_{VV}^{exp}(0) + I_{VH}^{exp}(0)} + \frac{I_{VH}^{exp}(\theta)}{I_{VV}^{exp}(0) + I_{VH}^{exp}(0)} \]

For the case when the orientation of the polarization of the incident and scattered light is controlled, the calculation procedure is based on the measurement of four polarized Raman...
spectra in the backscattering configuration. This method avoids the displacement or rotation of the sample, thus ensuring that the same point is irradiated. The former parameters allow for obtaining the most probable orientation distribution \( N_{mp}(\theta) \) using methodology described elsewhere.\(^{52,54,62,63,79,84}\)

Raman spectra are acquired using four polarization geometries (XX, ZZ, XZ, and ZX), where the first and second letters correspond to the incident and scattered light polarizations, respectively. Laboratory coordinate system (XYZ) is defined in such way that the Z-axis corresponds to direction of alignment and the Y-axis corresponds to the direction of both the incident and scattered light. The intensity ratios \( R_Z \) and \( R_X \) for individual Raman bands \( (I_{ZZ}, I_{ZX}, I_{XX}, \text{and } I_{XZ}) \) are related to parameters \( A \) and \( B \), which take into account the depolarization of the incident and scattered beams (see below for more details).

**Equation 28**

\[
R_Z = \frac{I_{ZX}}{I_{ZZ}} = \frac{A\langle (\alpha_{ZX})^2 \rangle + B\langle (\alpha_{ZY})^2 \rangle}{A\langle (\alpha_{ZZ})^2 \rangle + B\langle (\alpha_{ZY})^2 \rangle}
\]

**Equation 29**

\[
R_X = \frac{I_{XZ}}{I_{XX}} = \frac{A\langle (\alpha_{XZ})^2 \rangle + B\langle (\alpha_{XY})^2 \rangle}{A\langle (\alpha_{XX})^2 \rangle + B\langle (\alpha_{XY})^2 \rangle}
\]

Components of the tensor, \( \langle (\alpha_{ij})^2 \rangle \), can be related to the components in the molecular frame and the orientation parameters \( \langle P_2 \rangle \) and \( \langle P_4 \rangle \).\(^{50,54,62}\)

**Equation 30**

\[
\langle (\alpha_{XX})^2 \rangle = \frac{1}{15} c - \frac{2}{21} d \langle P_2 \rangle + \frac{3}{35} b \langle P_4 \rangle
\]
Equation 31

\[ \langle (\alpha_{ZX})^2 \rangle = \langle (\alpha_{XZ})^2 \rangle = \langle (\alpha_{ZY})^2 \rangle = b \left( \frac{1}{15} + \frac{1}{21} \langle P_2 \rangle - \frac{4}{35} \langle P_4 \rangle \right) \]

Equation 32

\[ \langle (\alpha_{ZZ})^2 \rangle = \frac{1}{15} c + \frac{4}{21} d \langle P_2 \rangle + \frac{8}{35} b \langle P_4 \rangle \]

Equation 33

\[ \langle (\alpha_{XY})^2 \rangle = b \left( \frac{1}{15} - \frac{2}{21} \langle P_2 \rangle + \frac{1}{35} \langle P_4 \rangle \right) \]

where

Equation 34

\[ b = \alpha_3^2 (1-a)^2 \]

Equation 35

\[ c = \alpha_3^2 (3 + 4a + 8a^2) \]

Equation 36

\[ d = \alpha_3^2 (3 + a - 3a^2) \]

In general, the evaluation of molecular orientation does not require absolute solutions for the tensor components but only involves the ratios of the diagonal components of the tensor. For a Raman tensor with cylindrical symmetry:
Equation 37

\[ \alpha = \begin{pmatrix} \alpha_1 \\ \alpha_2 \\ \alpha_3 \end{pmatrix} = \alpha_3 \begin{pmatrix} a \\ a \\ 1 \end{pmatrix} \]

where \( a = \alpha_1/\alpha_3 = \alpha_2/\alpha_3 \).

The parameter \( a \) of the Raman tensor can be determined from the depolarization ratio, \( R_{iso} \), acquired for an isotropic sample from Eqs. (28, 29) (for which \( \langle P_2 \rangle = \langle P_4 \rangle = 0 \)).

Equation 38

\[ R_{iso} = R_Z = R_X = \frac{(A + B)(1 - a)^2}{A(8a^2 + 4a + 3) + B(1 - a)^2} \]

The calculations (Eqs. 28, 29 and 38) also require the use of additional correction parameters,\(^{85,86} \) \( A \) and \( B \), which come from integrations of the squares of the electric vector components (Eq. 39) over the solid angle of light collection, \( \Omega \)

Equation 39

\[ I = \int_V \int_\Omega |E_e(\alpha)E_s|^2 \, d\Omega \, dV \]

where \( V \) is the total volume, \( E_e \) and \( E_s \) are the electric vectors of the excitation and scattered radiation respectively, and \( \alpha \) is the polarizability tensor.\(^{54,86} \)

The constants \( A \) and \( B \) take into account the depolarization of the incident and scattered beams in the focal plane associated with the use of a high numerical aperture objective (\( NA \)), objective semi-angular aperture (\( \theta_m \)), and a sample refractive index \( n \) according to Eqs. (40-42).
Equation 40

\[ A = \pi^2 \int_0^{\theta_m} (\cos^2 \beta + 1) \sin \beta \, d\beta = \pi^2 \left( \frac{4}{3} \cos \theta_m - \frac{1}{3} \cos^3 \theta_m \right) \]

Equation 41

\[ B = 2\pi^2 \int_0^{\theta_m} \sin^3 \beta \, d\beta \, 2\pi^2 \left( \frac{2}{3} - \cos \theta_m + \frac{1}{3} \cos^3 \theta_m \right) \]

Equation 42

\[ \theta_m = \sin^{-1} \left( \frac{NA}{n} \right) \]

In addition, using known elements of the Raman tensors, \( r_1 = \alpha_1/\alpha_3 \) and \( r_2 = \alpha_2/\alpha_3 \), the parameters \( \langle P_2 \rangle \) and \( \langle P_4 \rangle \) can be also determined from the intensity ratios \( R_Z \) and \( R_X \). It should be emphasized, that this approach does not require experimental determination of the depolarization ratio, \( R_{iso} \) that is assumed to be constant.

The elements \( \langle (\alpha_{ij})^2 \rangle \) can be related the orientation parameters \( \langle P_2 \rangle \) and \( \langle P_4 \rangle \) by using following equations:\textsuperscript{62}

Equation 43

\[ \langle (\alpha_{ZX})^2 \rangle = \langle (\alpha_{XZ})^2 \rangle = \langle (\alpha_{ZY})^2 \rangle = N_0 d - 2N_2 e \langle P_2 \rangle - 8N_4 c \langle P_4 \rangle \]

Equation 44

\[ \langle (\alpha_{ZZ})^2 \rangle = N_0 a - 8N_2 b \langle P_2 \rangle + 16N_4 c \langle P_4 \rangle \]

Equation 45

\[ \langle (\alpha_{XX})^2 \rangle = N_0 a + 4N_2 b \langle P_2 \rangle + 6N_4 c \langle P_4 \rangle \]
\begin{equation}
\langle (\alpha_{XY})^2 \rangle = N_0 d + 4 N_2 e(P_2) + 2 N_4 c(P_4)
\end{equation}

\(N_l\) are the factors for \(l = 0, 2, 4\) given by

\begin{equation}
N_0 = \frac{1}{\sqrt{2}}, N_2 = \frac{\sqrt{5}}{\sqrt{2}}, N_4 = \frac{3}{\sqrt{2}}
\end{equation}

\(a, b, c, d, e\) parameters, which contain the components of the tensor in the molecular frame

\begin{equation}
a = \alpha_3^2 \frac{\sqrt{2}}{15} \left[ 3(r_1^2 + r_2^2 + 1) + 2(r_1 r_2 + r_1 + r_2) \right]
\end{equation}

\begin{equation}
b = \alpha_3^2 \frac{1}{210} \sqrt{\frac{5}{2}} \left[ 3r_1^2 + 3r_2^2 + 2r_1 r_2 - r_1 - r_2 - 6 \right]
\end{equation}

\begin{equation}
c = \alpha_3^2 \frac{1}{840\sqrt{2}} \left[ 3r_1^2 + 3r_2^2 + 2r_1 r_2 - 8r_1 - 8r_2 + 8 \right]
\end{equation}

\begin{equation}
d = \alpha_3^2 \frac{\sqrt{2}}{15} \left[ (r_1^2 + r_2^2 + 1) - (r_1 r_2 + r_1 + r_2) \right]
\end{equation}

\begin{equation}
e = \alpha_3^2 \frac{1}{210} \sqrt{\frac{5}{2}} \left[ r_1^2 + r_2^2 - 4r_1 r_2 + 2r_1 + 2r_2 - 2 \right]
\end{equation}
CHAPTER 2. Experimental section

2.1 Materials

Human recombinant insulin, bovine insulin (I2643, I5500) and hen egg white lysozyme (L-6876) were purchased from Sigma Aldrich (St Louis, MO) and used without further purification. Solutions of proteins were prepared immediately before fibrillation. Insulin fibrils 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. Hen egg white lysozyme (HEWL) (60 mg/mL) has been incubated at 65 °C and pH 2.1, for 3 days to prepare fibrils.

Aβ thirty-four to forty-two (LMVGGVVIA) and Aβ sixteen to twenty-two (KLVWFAE) peptides were obtained in purified form from the GenScript Corp., Piscataway, NJ. Aβ thirty-four to forty-two fibrils were prepared by dissolving 1 mg of Aβ thirty-four to forty-two peptide powder in 50 μL of 88% formic acid followed by dilution of the resulting solution with water to obtain a 10% formic acid solution. This final mixture was slowly evaporated for 24 h at 24 °C.¹⁰ In case of Aβ sixteen to twenty-two peptide (KLVWFAE), fibrillized samples were prepared by incubation of aqueous solutions at a peptide concentration of approximately 200 μM, at a temperature of 24 °C, and with 1.0 mM phosphate buffer at pH 7.0.¹¹

Prepared fibrils were washed with the corresponding solution 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). For a sample alignment, aliquots (5-20 μL) of the fibril suspensions were dropped onto aluminum foil and air-dried.

Short peptide (LVEALYL, TFQINS, IFQINS, VAEALYL and YTIAAL) microcrystals were kindly provided by Prof. David Eisenberg (Howard Hughes Medical Institute, UCLA-DOE
Institute for Genomics and Proteomics, Los Angeles CA). The di-D-phenylalanine nanotubes were provided by Dr. Milana C. Vasudev (Department of Bioengineering, University of Massachusetts Dartmouth).

Aligned samples of nanotubes, were prepared by adding dry nanotubes to deionized water. The prepared suspension was sonicated for 5 min and then aliquots (5-20 μL) of the nanotube suspensions were dropped onto aluminum foil and air-dried.

For TERS experiments on native insulin, bovine insulin was dissolved (0.5 g/L) in distilled water. Aliquots (20-100 μL) of the fibril suspensions or protein solution were dropped onto a substrate with gold nanoplates. After the surface was exposed to the solution for 2–3 min, the solution excess was gently removed, and the surface was rinsed with water and dried under an argon flow. The gold nanoplates were synthesized and deposited on glass slides as previously described.90

AppNano ACCESS™-NC-A probes were used. First layer - SiO2 (50Å); second Layer - Ag (250Å) followed by the protection layer of Al (50Å). The deposition rate for all materials - 0.5 – 1Å per sec. Vacuum in evaporation chamber - 5 × 10⁻⁷ Torr or lower.

2.2 Instrumentation

Polarized Raman spectra were recorded by rotating anisotropic samples relative to the fixed direction of the laser beam polarization. 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. 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.

Polarized Raman spectra were also recorded in the backscattering geometry with a LabRam HR Evolution Raman microscope (Horiba Jobin Yvon). A 785 nm diode laser was focused on the sample with a 100x objective (0.9 NA-Olympus) with the power adjusted to approximately 10 mW. Raman spectra were acquired using four polarization geometries (XX, ZZ, XZ, and ZX), where the first and second letters correspond to the incident and scattered light polarizations, respectively. A half wave plate was used to select the polarization of the incident laser beam and a polarizer was used to select the X or Z component of the scattered beam. An optical scrambler was installed before the spectrometer entrance slit to eliminate the polarization dependence of the grating. The depolarization ratio was determined with polarized measurements on isotropic human insulin films/powders.

Tip-enhanced Raman spectra and AFM images were acquired on a Nanofinder ® 30 - CombiScope ™ 1000 (AIST-NT, USA). The following conditions were used: oil immersion objective, 60×, NA = 1.45 (Olympus, Japan); Continuous wave Diode Pumped Solid State laser (KLASTECH®, wavelength 532nm, Germany) as the excitation source.

2.3 Data analysis

Data acquisition and processing were performed using WiRE 3.2 software; the GRAMS v7.01 (ThermoGalactic, Salem, NH) software package and LabSpec6 software (Horiba Jobin Yvon). The spectra were baseline corrected over the 300–1800 cm⁻¹ spectral range using a
polynomial baseline, followed by 7–11 points smoothing. Raman polarization-dependent data were fitted using SigmaPlot 12 software (Systat Software Inc). All calculations were performed in MATLAB (version R2015a) and Maxima, a computer algebraic system.91
CHAPTER 3. Structural organization of insulin fibrils

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3.1 Introduction

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. 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.92,93 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.

Polarized Raman spectroscopy is uniquely suitable for probing of molecular conformation. Moreover, the orientation of individual structural units in the sample can be evaluated based on polarized Raman measurements conducted on an anisotropic sample of aligned species. Polarization characteristics of Raman scattering are related to the polarizability tensor and contain information regarding the symmetry of chemical groups. Raman band anisotropy measurements allow for retrieval of this information if the Raman tensor is known.47-56
3.2 Orientation of chemical groups

Structural information can be obtained if the polarized Raman measurements are performed on an anisotropic assembly of aligned species. Oriented samples of insulin fibrils were prepared using the drop coating deposition Raman method,\textsuperscript{67-69} in which the insulin fibril suspension was deposited onto aluminum foil, followed by solvent evaporation. The drop coating technique resulted in the formation of a visible ring at the very edge of the dry droplet, allowing for easy focusing of the excitation laser beam. The “coffee ring” diameter (droplet size) varied from 5 to 10 mm.

At first, polarized Raman spectroscopy was used to determine the orientation of the principal axis of the amide I Raman tensor, with respect to the fibril axis in aligned insulin fibrils. 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. No polarizer was used for the collection of scattered light.

Raman spectra were measured while the angle between the fibril sample and the excitation beam polarization was changed gradually from 0° to 124°. 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.
**Figure 3.1.** 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:** Schematic representation of the experimental design.

It is evident from spectra presented in Figure 3.1 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 assignment of the major Raman bands marked in Figure 3.1 is shown in Table 3.1, based on literature data. 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.
**Figure 3.2.** The normalized Raman scattering intensities (black dots) for $I_{\text{norm}}^{\exp}(\theta)$ vs. rotational angle ($\theta$) for human insulin fibrils. The black line is calculated using Eq. 27. The red and blue lines represent 95% confidence and prediction intervals, respectively.

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 ($0^\circ$) and perpendicular ($90^\circ$) orientations (Figure 3.1). Second, from the polar plot (Figure 3.2), by evaluating the polarization angle at which a maximum Raman intensity is observed for the amide I vibration.

The most prominent Raman peak exhibiting an orientation-dependent intensity is the amide I peak at 1674 cm$^{-1}$ (Figure 3.1) that is in agreement with previously published data for insulin fibrils.
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. The amide I Raman band is mainly associated with C=O stretching mode mixed with contributions from C-N stretching and Cα-C-N deformation. Also, 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.

**Table 3.1** Peak assignments for the Raman spectrum of insulin fibrils

<table>
<thead>
<tr>
<th>Raman shift, cm⁻¹</th>
<th>Peak assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1674</td>
<td>Amide I, mainly C=O in β-sheets</td>
</tr>
<tr>
<td>1616</td>
<td>Tyr, C₂-C₃, C₆-C₅ in-phase stretching</td>
</tr>
<tr>
<td>1448</td>
<td>(CH₂) deformation, scissoring mode</td>
</tr>
<tr>
<td>1200-1300</td>
<td>Amide III</td>
</tr>
<tr>
<td>1003</td>
<td>Phe, Tyr ring breathing</td>
</tr>
<tr>
<td>853</td>
<td>Tyr, Fermi resonance</td>
</tr>
<tr>
<td>830</td>
<td>Tyr, ring breathing</td>
</tr>
<tr>
<td>644</td>
<td>Tyr, ring deformation</td>
</tr>
<tr>
<td>621</td>
<td>Phe, in-plane ring deformation</td>
</tr>
</tbody>
</table>

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 (Figure 3.1). 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.

\[
\frac{(I_{\max} - I_{\min})}{I_{\max}} \text{ is equal to approximately 80% for an aligned insulin fibril sample (Figure 3.1), where } I_{\max} \text{ and } I_{\min} \text{ are amide I Raman intensities obtained at } 0^0 \text{ and } 90^0, \text{ respectively. This expression gives us an estimation of a minimum value for the degree of orientation or alignment in the sample. Considering absolutely perfect alignment, it can be shown that any deviation in the angle between the Raman tensor (even with only one non-zero component) and the main axis of a fibril will result in a decrease in the value obtained by the expression } \frac{(I_{\max} - I_{\min})}{I_{\max}}.
\]

As described in Chapter 1, 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 spectroscopy, for a Raman tensor showing cylindrical symmetry. We used the intensity of the amide I band to determine the orientation of the principal axis of the amide I Raman tensor, with respect to the fibril axis. The normalized Raman scattering intensity for amide I (1674 cm\(^{-1}\)) is plotted in Figure 3.2. The black solid line corresponds to the theoretical Raman intensity, as computed from Eq. 27 (section 1.6.1). 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 3.2, and the corresponding most probable orientation distribution function \(N_{mp}(\theta)\) is plotted in Figure 3.3. 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 3.2). It has been shown that 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.

**Table 3.2** Orientation order parameters for oriented human and bovine insulin fibrils

<table>
<thead>
<tr>
<th></th>
<th>( \langle P_2 \rangle^a )</th>
<th>( \langle P_4 \rangle^a )</th>
<th>( \langle P_2 \rangle^b )</th>
<th>( \langle P_4 \rangle^b )</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human insulin fibrils</td>
<td>0.48</td>
<td>0.17</td>
<td>0.49</td>
<td>0.29</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>0.51*</td>
<td>-0.16*</td>
<td>0.54*</td>
<td>-0.12*</td>
<td></td>
</tr>
<tr>
<td>Bovine insulin fibrils</td>
<td>0.47</td>
<td>0.1</td>
<td>0.49</td>
<td>0.09</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>0.51*</td>
<td>-0.16*</td>
<td>0.52*</td>
<td>-0.15*</td>
<td></td>
</tr>
</tbody>
</table>

Experimental data were fitted with Eqs. 27, 14 – 15 (\( a \)) and 20 - 21 (\( b \)).

\[ \langle P_4 \rangle = \langle P_4 \rangle_{\text{min}} = \frac{1}{18}(35\langle P_2 \rangle^2 - 10\langle P_2 \rangle - 7). \]

For comparative purposes, the order parameter \( \langle P_2 \rangle \) was also calculated (Table 3.2) while keeping the parameter \( \langle P_4 \rangle \) fixed during the fitting procedure: \( \langle P_4 \rangle = \langle P_4 \rangle_{\text{min}}. \)
Figure 3.3. (A) The most probable orientation distribution function \( N_{mp}(\theta) \) determined for human insulin fibrils, (B) orientation distribution function, \( N_{mp}(\theta) \times \sin(\theta) \) of the amide I Raman tensor for insulin fibrils, where \( \langle P_2 \rangle = 0.48 \) and \( \langle P_4 \rangle = 0.17 \).

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 (Figure 1.2, Eq.8), and thus a unimodal distribution is expected. Based on the Eq. 9, the angle \( \theta_0 \) for the principal axis of the amide I Raman tensor has a value \( 34 \pm 2^\circ \). 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 3.3, 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.\(^{106,107}\) The \( N_{mp}(\theta) \times \sin(\theta) \) plot (Figure 3.3B) shows the maximum of the
distribution at $\theta = 23 \pm 5^\circ$, and the mean of the distribution yields *average* orientation angles of $34 \pm 4^\circ$.

In this part of our study, 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. 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. 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 preferred orientation of the principal axis of the Raman tensor with respect to the fibril axis was calculated by multiplying $N_{mp}(\theta)$ 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. In order to obtain this type of information we utilized an experimental setup which allows controlling excitation and scattered light independently (Chapter 1, section 1.6).

A drop coating deposition Raman method was utilized for human insulin fibrils alignment. An optical microscopic image of a portion of the edge of a dried drop of an insulin fibril solution is shown in Figure 3.4. The Z-axis was selected as the direction along the edge of the dried droplet.
**Figure 3.4.** Aligned insulin fibrils prepared by drop coating deposition. Bright-field microscopy image of a portion of the ring formed by drying the fibril solution on aluminum foil. The assigned coordinate system for the orientation of laser polarization relatively to the sample is shown schematically.

In order to characterize the orientation of insulin fibrils in the sample, and sample homogeneity, an additional set of measurements were made. First, polarized Raman spectra (ZZ) were collected by varying the sample orientation (around the Y-axis), with respect to the polarization direction of the excitation laser beam, stepwise from $0^\circ$ to $160^\circ$ (Figure 3.5). As expected, the maximum and minimum Raman intensities of the amide I band were observed at $0^\circ$ and $90^\circ$, respectively.
Figure 3.5. Orientation-dependent Raman spectra (amide I region) as a function of the excitation polarization angle. A series of polarized Raman spectra (in the ZZ polarization geometry) were recorded over the range 0-160° by rotating the sample around the Y-axis.

Secondly, a series of polarized Raman spectra (ZZ polarization geometry) were collected along the line (schematically shown on Figure 3.4), which is perpendicular to the edge of the droplet with 1 μm steps. Figure 3.6A demonstrates that all spectra showed remarkable reproducibility, indicating that the sample was highly homogeneous in that area, which is much larger than the size of the focused laser spot. It is well known that spectral data preprocessing is an important step. Removal of any background, i.e. fluorescence background could potentially alter the precision in the determination of \( \langle P_2 \rangle \) and \( \langle P_4 \rangle \). Polarized Raman spectra before any preprocessing steps are shown on Figure 3.6B.
Figure 3.6. Representative polarized Raman spectra (ZZ polarization geometry) of aligned insulin fibrils. (A) Spectra were collected along the line schematically represented in Figure 3.4 with 1 μm steps. (B) Raw polarized spectra of aligned insulin fibrils (before background correction).
Figure 3.7 shows polarized Raman spectra of aligned human insulin fibrils obtained for four polarization geometries. Significant differences in intensity were observed for several vibrational modes in the Raman spectra acquired using the $XX$ and $ZZ$ polarization geometries. In particular, the bands in the 1575–1720 cm$^{-1}$ and 480–550 cm$^{-1}$ regions, corresponding to the amide I$^{96,108}$ and disulfide stretching modes,$^{109}$ respectively, exhibited major variations.

![Normalized polarized Raman spectra of oriented human insulin fibrils.](image)

Figure 3.7. Normalized polarized Raman spectra of oriented human insulin fibrils.

To obtain qualitative information regarding the degree of alignment, we calculated the orientation parameter $f$, also termed the pseudo-order parameter, using the following formula:$^{75}$

$$f = 1 -(I_{XX}/I_{ZZ})$$
where $I_{XX}$ and $I_{ZZ}$ are the Raman intensities of the peak of interest in the XX and ZZ spectra, respectively. For an isotropic sample, $f$ equals zero and increases as the molecular orientation increases along the Z direction until it reaches 1 for a perfect parallel orientation. We obtained $f$ values of 0.88 and 0.91 for the 1672 cm$^{-1}$ amide I band and 513 cm$^{-1}$ S-S stretching vibration band, respectively.

It has been shown previously$^{96-98}$ that Raman spectra of amyloid fibrils in general, and the amide I band in particular, are dominated by the contribution from the fibril core, which has a well-ordered β-sheet structure with β-strands arranged parallel or anti-parallel to each other and perpendicular to the long axis of the fibril.$^{66,110}$ Consequently, because of the cross-β structure of the fibril core, peptide carbonyl groups are oriented nearly parallel to the main axis of the fibril.$^{57,66,73}$

It is currently well accepted that the fibril cross-β core is surrounded by randomly oriented protein regions with various secondary structures, including α-helices, β-turns, and disordered conformations.$^{111-114}$ Using tip-enhanced Raman spectroscopy, we have recently found that the secondary structure configuration and the amino acid residue composition are different on the surface of two insulin fibril polymorphs.$^{115}$
Figure 3.8. Band decomposition of the amide I region of the polarized Raman spectra of insulin fibrils for various polarization geometries.

The amide I normal vibrational mode depends on the C═O hydrogen bonding, on the vibrational coupling between adjacent amide units, and, consequently, on the protein secondary structure. Therefore, the decomposition of the amide I Raman band on individual components allows for the determination of the protein secondary structure composition. The amide I region (1575-1720 cm$^{-1}$) of the polarized Raman spectra was fitted using Gaussian and Lorentzian functions, including three components representing the main secondary structural elements. Figure 3.8 shows the band decomposition for the four polarized spectra with different polarization geometries. The component at 1672 cm$^{-1}$ is assigned to the well-ordered β-sheet structure, whereas the band at ca. 1655 cm$^{-1}$ is attributed to the α-helical conformation. The
component at approximately 1685 cm\(^{-1}\) could reflect a composite contribution from PPII, a disordered structure. The bands at 1589, 1604, and 1616 cm\(^{-1}\), which could be attributed to ring modes of Phe and Tyr residues\(^{94,117}\) were also included in the band-fitting protocol.

Protein Raman bands at 510 ± 5, 525 ± 5, and 540 ± 5 cm\(^{-1}\) could be assigned to S-S stretching vibrations, \(v(\text{SS})\), in gauche–gauche–gauche (ggg), gauche–gauche–trans (ggt) and trans–gauche–trans (tgt) conformations of the C-C-S-S-C-C segment, respectively.\(^{118-121}\) Gaussian deconvolution of the Raman spectra of naive insulin and isotropic insulin fibril powders in the 470–580 cm\(^{-1}\) range revealed that the ratio between the peak areas assigned to the ggg and ggt conformations was approximately 2:1. Therefore, two out of the three disulfides most likely contribute to the 513 cm\(^{-1}\) Raman band, whereas the remaining S-S bond is responsible for the 530 cm\(^{-1}\) band. The positions of the disulfide bands in the Raman spectrum of native insulin powder were in agreement with the literature data.\(^{67,94}\) The Raman peaks at approximately 490 cm\(^{-1}\) found in the spectra of native insulin and isotropic insulin fibril powders can be assigned to skeletal bending.\(^{94}\) Based on the crystal structure of human insulin,\(^{122}\) we assigned the most intense peak at 513 cm\(^{-1}\) (S-S) to two disulfides ([B] Cys7-[A] Cys7 and [B] Cys19-[A] Cys20) with a ggg conformation, whereas the 530 cm\(^{-1}\) peak was attributed to the disulfide bond ([A] Cys11-[A] Cys6) with a ggt conformation.
Figure 3.9. Band decomposition of the S-S stretching vibrational mode region of the polarized Raman spectra of oriented insulin fibrils for various polarization geometries.

It is known that S–S stretching Raman bands have a very low depolarization coefficient, and the Raman tensor for the totally symmetric stretching vibration of the disulfide bridge is oriented parallel to the S-S bond.\textsuperscript{123,124} As shown in Figure 3.9, the intensity of the band at 513 cm\textsuperscript{-1} is very strong in the ZZ orientation and weak in the XX orientation, indicating a high level of orientation of the disulfide bonds with a ggg conformation along the main axis of the insulin fibrils. To the best of our knowledge, this is the first experimental observation of a preferential orientation of disulfide bonds in amyloid fibrils.
Quantitative interpretation of polarized Raman spectra (Figure 3.7) requires the Raman tensor of a particular band. In general, a Raman tensor relates the polarization direction of the exciting light to the polarization direction of the scattered light. A Raman tensor corresponding to a particular vibrational mode can be described in terms of three non-zero components (principal axes) that can be determined using polarized Raman spectroscopic measurements.

The polarized Raman spectra of oriented fibrils are depicted in Figure 3.7. Significant intensity variations are evident for several bands in the ZZ and XX spectra. The fact that the crossed-polarized spectra (ZX and XZ) overlap within the experimental error confirms that no displacements and rotations of the sample occurred during the measurements. Most importantly, this result satisfies the conditions for the application of the uniaxial model, which allows for quantitative determination of the order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$ from the polarized Raman spectra.

The intensity ratios, $R_Z = I_{ZX}/I_{ZZ}$ and $R_X = I_{XZ}/I_{XX}$, of the amide I band components and $\nu(SS)$, together with the corresponding values of the order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$, are summarized in Table 3.3. Parameters $A$ and $B$ were calculated to be respectively, and $n$ was set to 1.55.
Table 3.3 Calculated intensity ratios $R_z$ and $R_x$, order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$, and Lagrange multipliers $\lambda_2$ and $\lambda_4$ for amide I and $\nu(S-S)$ Raman tensors in human insulin fibrils

<table>
<thead>
<tr>
<th>Raman shift, cm$^{-1}$</th>
<th>$R_z$</th>
<th>$R_x$</th>
<th>$\langle P_2 \rangle$</th>
<th>$\langle P_4 \rangle$</th>
<th>$\lambda_2$</th>
<th>$\lambda_4$</th>
<th>$R_{iso}$</th>
<th>$a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1672 (β-sheet)</td>
<td>0.11</td>
<td>1.12</td>
<td>0.65</td>
<td>0.43</td>
<td>2.1</td>
<td>1.52</td>
<td>0.47</td>
<td>-0.115</td>
</tr>
<tr>
<td>1655 (α-helix)</td>
<td>0.19</td>
<td>1.23</td>
<td>0.51</td>
<td>0.22</td>
<td>2.09</td>
<td>0.43</td>
<td>0.49</td>
<td>-0.131</td>
</tr>
<tr>
<td>1685 (PPII, disordered</td>
<td>0.22</td>
<td>1.38</td>
<td>0.5</td>
<td>0.2</td>
<td>2.11</td>
<td>0.31</td>
<td>0.45</td>
<td>-0.172</td>
</tr>
<tr>
<td>structure)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>513 $\nu(S-S)$</td>
<td>0.1</td>
<td>1.23</td>
<td>0.69</td>
<td>0.46</td>
<td>2.3</td>
<td>1.6</td>
<td>0.47</td>
<td>-0.115</td>
</tr>
</tbody>
</table>

We calculated the most probable orientation distribution function, $N_{mp}(\theta)$, based on experimentally determined $\langle P_2 \rangle$ and $\langle P_4 \rangle$ values and numerically calculated Lagrangian multipliers ($\lambda_2$ and $\lambda_4$) using a well-developed approach for quantitative characterization of Raman tensor orientation,\textsuperscript{52,63,78,79} where $\theta$ is the angle between the fibril axis and the principal axis of the polarizability tensor. Because a uniaxial cylindrical symmetry was assumed, multiplication of the most probable orientation distribution function by $sin(\theta)$ allows for the preferred orientation with respect to the fibril axis to be obtained, which corresponds to the maximum $N_{mp}(\theta)sin(\theta)$ function (Figure 3.10). The mean of this distribution function characterizes the average orientation angle.\textsuperscript{106,107}

Figure 3.10A shows $N_{mp}(\theta)$ associated with various secondary structure components of the amide I band. In each case, $N_{mp}(\theta)$ is Gaussian, unimodal, and centered at $\theta = 0^\circ$. Thus, it can be concluded that different structural components are not randomly oriented but exhibit a certain level of orientation. As expected, the β-sheet component (1672 cm$^{-1}$) showed the highest level of
orientation among all the secondary structure elements. The distribution of orientation was broader for the α-helix (1655 cm\(^{-1}\)) and PPII disordered structure (1685 cm\(^{-1}\)) than for the β-sheet. The \(N_{mp}(\theta)\sin(\theta)\) function of the 1672 cm\(^{-1}\) band reached a maximum at \(\theta = 12^\circ\) with an average orientation angle of approximately 26° (Figure 3.10B). For the other two components of the amide I region, representing α-helices (ca. 1655 cm\(^{-1}\)) and composite contributions from PPII and disordered regions (near 1685 cm\(^{-1}\)), the average orientation angles of PART were 33° and 35°, respectively.

**Figure 3.10.** (A) The most probable orientation distribution function \(N_{mp}(\theta)\) and (B) the calculated orientation distribution function \(N_{mp}(\theta)\sin(\theta)\) of Raman tensors obtained for amide I Raman spectroscopic components and a disulfide Raman band of human insulin fibrils.

The ultimate goal of the polarized Raman spectroscopic study is to determine the orientation of chemical groups in an amyloid fibril based on the orientation distribution functions obtained for the polarizability tensors. The knowledge of the orientation of the polarizability tensor relative to the corresponding chemical group is required for this determination. The largest polarizability oscillation for the amide I vibration of the isolated amide chromophore occurs along the line that is in the plane of the peptide group and at an angle of 34° with respect to the
peptide C═O bond.\textsuperscript{83,126} However, Krim et al. has shown that vibrations of adjacent amide chromophores are coupled and delocalized along the polypeptide backbone.\textsuperscript{105,108} Furthermore, Asher and coworkers have experimentally demonstrated that the amide I vibrational mode exhibited noticeable inter-amide coupling in the α-helix conformation.\textsuperscript{128,129} For this reason, it is more appropriate to consider the amide I Raman tensor for β-sheet and α-helix structures rather than that for the isolated peptide group. The largest principal axis for the β-sheet amide I Raman tensor is in the plane of the peptide bond and nearly parallel to the peptide carbonyls.\textsuperscript{126,130} It has also been shown for the α-helix that the Raman tensor axis with the largest magnitude is parallel to the helix main axis, whereas the other two tensor axes are equivalent and perpendicular to the main axis.\textsuperscript{126} Thus, the average and preferred angle distribution for β-sheet and α-helix amide I Raman tensors obtained from experimental results using the $N_{\text{mp}}(\theta)\sin(\theta)$ function represent the C═O orientation relative to the fibril axis.

The width of the orientation distribution function is used typically to estimate the uncertainty in determining the most probable orientation angle of a chemical group based on the polarized Raman spectroscopic measurements.\textsuperscript{106} The half-height half-width of the orientation distribution function is about 15° for β-sheet and disulfide bonds in insulin fibrils (Figure 3.10). A higher orientation variability of C═O groups in α-helix, disordered and PPII structures results in a broader distribution (about 25°). In the case of a perfect alignment of fibrils in an anisotropic sample and a single orientation of chemical groups at a certain angle, the orientation distribution function is expected to be unimodal and given by the delta function centered at that angle. Several factors could result in broadening the orientation distribution function including an imperfect fibril alignment and multiple orientations of chemical groups with respect to the axis of the fibril. For example, the Eisenberg model predicts several different orientations of C═O
groups in the insulin fibril core.\textsuperscript{4} We used this prediction data to build a corresponding orientation distribution function assuming a perfect fibril alignment. The fact that there is an excellent agreement between this modeled distribution function and the function obtained experimentally (Figure 3.12A) indicates that the obtained broadening of the most probable orientation distribution function is indeed due to various orientations of carbonyl groups. In other words, the width of the orientation distribution function of C=O groups in the insulin fibril core is determined by the inhomogeneous broadening. The latter could be potentially tested by the site-specific isotope substitution of C=O groups and obtaining the orientation distribution functions for uniquely oriented labeled groups.

To evaluate the orientation of C═O groups for disordered portions of fibrils, we used the Raman tensor for the amide I mode proposed by Tsuboi and colleagues by investigating the Raman spectra of a uniaxial tetragonal aspartame.\textsuperscript{131} As mentioned previously, the angle of 34° has been reported between this tensor and the peptide C═O bond for the isolated amide chromophore.\textsuperscript{83,126} Once the angle between the carbonyl group and the PART is known, the orientation order parameters for the C═O bonds, \( \langle P_2 \rangle^{\text{CO}} \) and \( \langle P_4 \rangle^{\text{CO}} \), can be calculated from the experimentally determined \( \langle P_2 \rangle \) and \( \langle P_4 \rangle \) values using the Legendre addition theorem.\textsuperscript{132}

Figure 3.12B shows the most probable orientation distribution function of C=O groups in the disordered parts of an insulin fibril (Figure 3.12, blue curve) calculated based on \( \langle P_2 \rangle^{\text{CO}} \) and \( \langle P_4 \rangle^{\text{CO}} \). The function shows a binormal distribution for the C=O group orientation with maxima at approximately 10° and 90°. This behavior can be explained assuming that at least two populations of disordered elements might co-occur within the fibril. These two populations consist of a similar number of groups and exhibit very different orientations, namely parallel and perpendicular to the fibril axis. As discussed in detail below, this behavior of carbonyl groups in
the disordered portion of an insulin fibril qualitatively agrees with Eisenberg’s model, but quantitative comparisons result in several possible limitations. First, the $34^\circ$ angle between the C═O group and the PART reported for an isolated peptide group might not be a good approximation for disordered peptide chains because of a possible vibrational coupling between adjacent peptide groups. Additionally, it has been shown that for a specific $\langle P_2 \rangle$, there is a single most probable value of the order parameter $\langle P_4 \rangle_{mp}$. Therefore, the most probable value of the orientation order parameter $\langle P_4 \rangle_{mp}^{CO}$ was approximated from the associated $\langle P_2 \rangle^{CO}$. In this case, the resultant most probable distribution function for the C═O had a single maximum at approximately $34^\circ$ and the average orientation angle of C═O was approximately $45^\circ$. Consequently, the calculated most probable orientation distribution function of the C═O groups in disordered parts of the insulin fibril should be considered as tentative.

The $R_Z$ and $R_X$ ratios as well as the order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$ for disulfide bonds (513 cm$^{-1}$ Raman band) are presented in Table 3.3. The $N_{mp}(\theta)$ function is centered at $\theta = 0^\circ$ (Figure 3.10A) and the maximum of the $N_{mp}(\theta)\sin(\theta)$ function is at $\theta = 12^\circ$ (Figure 3.10B). The mean distribution yielded an average orientation angle of $24^\circ$. It is worth noting that these values are very close to those obtained for the $\beta$-sheet component of the amide I band. It has been shown previously that disulfide bonds remain intact and preserve their conformation during insulin fibrillation. Assuming that the Raman cross-section of a disulfide bond is independent of the conformation ($\sigma_{ggg} = \sigma_{ggt}$), we conclude that the two disulfide bonds ([B] Cys7-[A] Cys7 and [B] Cys19-[A] Cys20) with $ggg$ conformation are preferably orientated at approximately $12 \pm 8^\circ$ with respect to the fibril axis. It should be noted that the 530 cm$^{-1}$ Raman band assigned to the [A] Cys11-[A] Cys6 disulfide bond with $ggt$ conformation did not show significant
polarization dependence that suggests that these S-S groups do not have a preferred orientation relative to the main axis of the fibril.

The bands at 643, 830 and 1209 cm$^{-1}$, which could be assigned to various vibrational modes of the phenoxyl ring, and the bands at 1003 and 1032 cm$^{-1}$, which arise from phenylalanine residues, also showed different Raman scattering intensity for XX and ZZ configurations (Figure 3.7). It is well known that aromatic residues play an important role in the stability of protein structures through the involvement of hydrogen bonds or in the stabilization of amyloid fibrils as a result of π-π stacking between aromatic residues.$^{136}$ In addition, the Raman bands at approximately 830–850 cm$^{-1}$ can be useful in determining the local environment of the Tyr side chains. It has been shown previously that the intensity ratio $I_{850}/I_{830}$ depends strongly on the hydrogen-bonding state of the phenoxyl group.$^{137}$

Raman tensors for several vibrational modes of the tyrosine residue have been previously reported.$^{117,126}$ Each insulin monomer contains four tyrosine and three phenylalanine residues, which do not necessarily have the same orientation relative to the main fibril axis. Obtaining information regarding the orientation of the individual side chains would require site-specific labeling,$^{58}$ which we plan to accomplish in the future. However, the approach described by Tsuboi$^{48,138}$ allows for information regarding the average orientation of the phenolic ring relative to the fibril axis to be obtained. Using the polarized Raman intensity ratios ($I_{ZZ}/I_{XX}$) of the bands at 643, 830 and 1209 cm$^{-1}$ and the known values of the corresponding Raman tensors,$^{117}$ the average orientation angle of the phenolic ring normal is $60 \pm 10^\circ$ relative to the fibril axis.

3.3 Evaluation of existing structural models

Dobson and colleagues$^{139}$ have proposed a hypothetical arrangement of the polypeptide chains in the insulin amyloid protofilament (Figure 3.11B) based on cryo-EM data. Their model
is qualitative, i.e., it does not provide structural information with atomic-level resolution, but it suggests that each insulin molecule occupies two β-strand layers with the strands parallel to each other. One can imagine that due to specific retentions two inter-chain disulfide bonds should be oriented rather parallel then perpendicular to the fibril axis, even though, Jimenez et al. specifically stated that “orientations of the termini and disulfide bonds within the curved structure are arbitrary”. We found a strong orientation of two inter-chain disulfide bonds ([B] Cys7-[A] Cys7 and [B] Cys19-[A] Cys20) along the main axis of the fibril that does not contradict to the model.

**Figure 3.11.** Structural models of the insulin fibril proposed by (A) Eisenberg and coworkers and (B) Dobson and coworkers.

Eisenberg and coworkers have determined a limited set of possible arrangements of peptides in the cross-β structure, depending on the organization of the β-strands and β-sheets with respect to one another. The insulin fibril model has been built based on the crystal structures of short amyloidogenic peptides LVEALYL and LYQLENY, which constitute the insulin fibril core. These peptide sequences are found in the B and A chains of insulin, respectively. The proposed model predicts the structural organization of the entire insulin fibril (Figure 3.11A). We utilized the model to determine the orientation of disulfide bonds and the
fibril core β-strands, α-helical and disordered parts, and compare the results to those of our polarized Raman spectroscopic study. Specifically, Figure 3.12 shows the comparison of the carbonyl group orientation in the fibril core (A) and disordered parts (B) of the fibril obtained from the polarized Raman spectroscopic data and that predicted by the model. The model-predicted angles are presented in the form of histograms and smooth Kernel functions (red curves). In the case of the fibril core (Figure 3.12A), the Kernel function showed a similar shape and close position (maximum at 14° and average at 18°) to those of the most probable distribution function (black curve, maximum at 12° and average at 24°) determined from the polarized Raman spectroscopic data. This result indicates an excellent agreement between the results of our polarized Raman spectroscopic study and Eisenberg’s model in the case of the fibril core.

Figure 3.12. The orientation of C=O groups relative to the main fibril axis in the β-sheet core (A) and disordered parts (B) of human insulin fibrils: comparison of the polarized Raman spectroscopic data with those predicted by Eisenberg’s model. The orientation distribution functions obtained from the polarized Raman spectroscopic data (black curves). Histograms and smooth Kernel functions (blue curves) represent the angle distribution predicted by the model. The data were normalized for comparison.
Based on Eisenberg’s model, the angle distribution (blue curve) of peptide carbonyls in the PPII and disordered regions with respect to the fibril axis has two maxima at approximately 25° and 72° according to the Kernel function shown in Figure 3.12B. The latter is in qualitative agreement with the most probable distribution function (black curve) for C=O groups calculated based on the polarized Raman measurements. A noticeable difference between the two distribution functions (blue and black curves in Figure 3.12B) might result from a potential inaccuracy in calculating the angular distribution function for C=O groups based on the distribution function obtained for the corresponding PART as discussed above. Taking into account the composite contribution from disordered regions and PPII, we conclude that there is a qualitative agreement between the estimated distribution of the C═O group orientation based on polarized Raman measurements and that obtained from Eisenberg’s model. The 1655-cm⁻¹ amide I sub-band assigned to α-helical structures showed strong polarization dependence with the ZZ component being four times the XX component. This result indicates that the α-helices are well aligned and their orientation is close to the main axis of the fibril. In addition to this qualitative estimation, we calculated the average orientation angle for α-helix carbonyls relative to the fibril axis to be 33° based on the most probable distribution function. In contrast to our conclusion, Eisenberg’s model predicts the average angle for α-helix C═O groups to be approximately 78°.

It is known that the frequency of the S–S stretching vibrational mode correlates with the internal rotation about the CS and CC bonds of the CCS-SCC moiety, which allows for differentiating the $ggg$, $ggt$, and $tgt$ conformations based on Raman spectra. The analysis of the S–S group orientation based on polarized Raman spectroscopy is simplified by the fact that the S–S stretching vibrational mode polarizability tensor is parallel to the S–S group. Our experimental data imply quite confidently that the two disulfide groups ([B] Cys7-[A] Cys7 and
[B] Cys19-[A] Cys20) are parallel to each other and nearly parallel to the main fibril axis. The most probable angle between these two S-S groups and the fibril axis was estimated to be 12°. This conclusion disagrees with Eisenberg’s model, which predicts that the two disulfide bonds, [B] Cys7-[A] Cys7 and [A] Cys6-[A] Cys11, are parallel to each other and oriented at approximately 72° with respect to the fibril axis. The third S-S bond, [B] Cys19-[A] Cys20, possesses an orientation at approximately 52°.

Our results clearly show that two inter-chain disulfide bonds are highly oriented relative to the fibril axis. Moreover, their orientation is parallel to the β-sheet component or, in other words, to the peptide carbonyl groups in the β-sheet (Figure 3.10). Such a strong and uniform orientation may indicate that these S-S groups are part of the fibril cross-β core. This observation is in agreement with our recent hydrogen-deuterium exchange data demonstrating that all of the disulfide bonds are located at the edge of the hydrophobic fibril core of insulin fibrils. Furthermore, the tendency of Cys residues to participate in the formation of β-sheet areas on the surface of insulin fibrils was demonstrated with tip-enhanced Raman spectroscopy.

Overall, the comparison of the orientation of carboxyl and disulfide groups evaluated based on the polarized Raman spectroscopy of aligned insulin fibrils and predicted by Eisenberg’s model showed excellent agreement for the fibril core carbonyls. At the same time, a significant difference was found for the orientation of α-helices and disulfide bonds. The model was built for the entire insulin fibril based mainly on the determined crystal structures of short amyloidogenic peptides LVEALYL and LYQLENY, which constitute the fibril core. As a result, the fibril core structure was modeled accurately, whereas the remainder of the fibril, including disordered portions and α-helices, were not.
CHAPTER 4. Orientation of di-D-phenylalanine molecules in a nanotube

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4.1 Introduction

The self-assembly of short peptide sequences into nanostructures has become an important strategy for bottom-up fabrications of nanostructures. One of the most commonly used building blocks is the diphenylalanine, a short dipeptide which has been identified as playing a key role in the core-recognition motif of the Alzheimer’s disease, the β-amyloid protein (Aβ). Such peptide-based molecular building blocks can used to design highly organized nanoscale structures and several groups have demonstrated the rich polymorphism of self-assembled diphenylalanine and their ability to into nanotubes, nanofibrils, nanospheres and hydrogels. Diphenylalanine nanotubes exhibit unique functional properties such as crystallinity, high aspect ratios and high thermal stability and their applications include drug delivery, sensing, and nanoelectronics.

The information about structural organization of short peptide nanotubes is limited to X-ray diffraction measurements carried out on crystals (not nanotubes) and theoretical calculations. Polarized Raman spectroscopy offers many advantages for the study of peptide nanotubes since it provides molecular level information about structural organization. For a uniaxially oriented system, the technique of polarized Raman scattering provides direct measurements of the second \( \langle P_2 \rangle \) and the forth \( \langle P_4 \rangle \), order parameters. Combined with an ODF analysis this method becomes a powerful and relatively easy tool to quantify orientation of individual structural units in nanotubes.
4.2 Orientation of chemical groups

In this study, polarized Raman spectroscopy was used for understanding the orientation of di-D-phenylalanine (D-FF) molecules within a nanotube. Polarized Raman spectra XX, ZZ, XZ, and ZX were measured for a bundle of well-aligned nanotubes.

Significant differences in the intensities were found for many bands in ZZ and XX polarized Raman spectra (Figure 4.1). For example, the band at 1131 cm$^{-1}$, which can be assigned to the NH$_3^+$ rocking vibration mode,$^{146,147}$ $\rho$(NH$_3^+$), exhibited major variations. The position of this band in the Raman spectrum indicates that amine group in the D-FF nanotube is protonated. Moreover, the appearance of the band is in agreement with another band at about 495 cm$^{-1}$, which is assigned to the torsion vibration of NH$_3^+$ group, $\tau$(NH$_3^+$).$^{147-149}$

Figure 4.1. Normalized polarized Raman spectra of oriented di-D-phenylalanine nanotubes.
It is important to make sure that the nanotubes are well aligned in the sample. To estimate the degree of alignment, we calculated the orientation parameter $f$. We obtained a value for $f$ of 0.98 for the 1131 cm$^{-1}$ band, which indicates an excellent alignment.

The lower portion of Figure 4.1 shows the Raman spectra from the two cross-polarizations, XZ and ZX. The fact that these crossed-polarized spectra overlap within an experimental error confirms that no displacements or rotations of the sample occurred during the measurements. Most importantly, this result verifies experimentally the accuracy of our polarization Raman measurements and shows that the conditions for the application of the uniaxial model for the polarized Raman spectral data analysis are satisfied.

We used the intensity of the amide I band to determine the orientation of the carbonyl groups in D-FF nanotubes, with respect to the nanotube axis. For nanotubes, the amide I band is located at 1645 cm$^{-1}$, as seen in Figure 4.1. For the aligned D-FF nanotubes, calculated intensity ratios $R_Z = I_{ZX}/I_{ZZ}$ and $R_X = I_{XZ}/I_{XX}$, for this peak were 0.1±0.02 and 0.2±0.03. Additional correction parameters, $A$, $B$ were 4.088 and 1.0624 respectively, the refractive index ($n$) was equal to 1.4.

To evaluate the orientation of the principal axis of the Raman tensor (PART) with respect to the main axis of D-FF nanotubes we used the Raman tensor of the amide I and amide III modes reported by Lekprasert et al. Tensor elements of the amide I and the amide III vibrational modes are represented in the form of ratios $r_1 = a_1/a_3$ and $r_2 = a_2/a_3$. For the amide I, $r_1$ and $r_2$ were -0.016, 0.274 and for the amide III, $r_1$ and $r_2$ were -0.085, 0.356. Based on the above, values of $\langle P_2 \rangle$ and $\langle P_4 \rangle$ obtained for the amide I were approximately 0.18 and 0.31, respectively.
Figure 4.2. (A) The most probable orientation distribution function $N_{mp}(\theta)$ and (B) the calculated orientation distribution function $N_{mp}^{CO}(\theta)\sin(\theta)$ of C═O groups relative to the main axis of di-Diphenylalanine nanotubes.

Figure 4.2A shows that $N_{mp}(\theta)$ of the PART for the amide I band is bimodal with a main Gaussian peak centered at 0° with a width at half height of 15°, and a second much smaller contribution centered at 90°. In this case, it might indicate that the only a fraction of the PART for the amide I band were oriented perpendicularly to the nanotubes axis, while the remaining fraction were oriented parallel to the main axis. We obtained the information about orientation of the PART but we decided to calculate the orientation of carbonyl groups within the aligned nanotubes. To evaluate the orientation of C═O groups the angle (33°)$^{47,83}$ between the PART and carbonyl bond has to be taken into consideration. The orientation order parameters for the C═O bonds, $\langle P_2 \rangle^{CO}$ and $\langle P_4 \rangle^{CO}$, were then calculated from the experimentally determined $\langle P_2 \rangle$ and $\langle P_4 \rangle$ values for PART of the amide I Raman tensor using the Legendre addition theorem$^{132}$ followed by calculation of the most probable orientation distribution function $N_{mp}^{CO}(\theta)$. Figure 4.2B
clearly shows that in case of carbonyl groups the $N^{CO}_{mp} (\theta) \sin(\theta)$ function was much broader, however, it reached maximum at $\theta = 50^\circ$ with an average orientation angle of approximately $52^\circ$ relative to the main Z-axis.

The amide III band spans the range of 1220-1250 cm$^{-1}$ and is primarily due to the in-phase combination of N-H in-plane-bending and C-N stretching vibrations.$^{151}$ For D-FF, the amide III band was located at 1245 cm$^{-1}$. Applying the same approach, the order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$ for the amide III Raman tensor were 0.35 and -0.55. Surprisingly, the value of $\langle P_4 \rangle$ falls in the allowed region determined by the condition of $\langle P_4 \rangle = \langle P_4 \rangle_{\text{min}}$. As already mentioned, fulfilling the requirements where $\langle P_4 \rangle = \langle P_4 \rangle_{\text{min}}$, the orientation angle was obtained using Eq.16 showing that the amide III Raman tensor makes $41^\circ$ with the nanotube axis. Moreover, applying the Legendre addition theorem$^{132}$ the orientation of C═O groups at $56^\circ$ from the nanotube’s main axis was found. An agreement between these two estimations based on two independent vibrational modes indicates a high reliability of the method. It should be noted that in case of the amide I band, the value of $\langle P_4 \rangle$ may fall higher than the $\langle P_4 \rangle = \langle P_4 \rangle_{\text{min}}$ level due to possible limited applicability of the known localized polarizability tensors used for calculations, and/or experimental errors. Interestingly, Lekpraset at al. have shown that for nanotubes prepared from L-diphenylalanine the orientation of the PART corresponding to the amide I and amide III were $59 \pm 5^\circ$ and $41 \pm 4^\circ$. However, the predominant orientation of C═O bond was $0 \pm 5^\circ$ to the nanotube axis.$^{62}$

The band at 1418 cm$^{-1}$, which was assigned to the symmetric stretching vibration $\nu_s \text{COO}^-$, also showed a strong polarization effect (Figure 4.1). The intensity ratios $R_Z = I_{ZX}/I_{ZZ} = 1.1$ and $R_X = I_{XZ}/I_{XX} = 0.2$ were measured for this band. However, due to unavailability of the
isotropic sample of D-FF we used the calculated isotropic depolarization ratio of 0.52 which was previously reported for $v_s\text{COO}^-$ vibrational mode.\textsuperscript{83}

**Figure 4.3.** (A) The most probable orientation distribution function $N_{mp}(\theta)$ and (B) the calculated orientation distribution function $N_{mp}(\theta)\sin(\theta)$ of the major axis of the Raman tensor of the symmetric stretching vibration vsCOO- with respect to the oriented di-D-phenylalanine nanotubes.

Values of $\langle P_2 \rangle$ and $\langle P_4 \rangle$ obtained for the band at 1418 cm$^{-1}$ were approximately -0.4 and 0.2. The function $N_{mp}(\theta)$ is unimodal with a Gaussian peak centered at 90° with a width at half height of 15 (Figure 4.3). Interestingly, $N_{mp}(\theta)\sin(\theta)$ function peaks at 90° with the average orientation angle of 78°. It should be noted that the orientation of the local Raman tensor axes with respect to the COO$^-$ plane have shown to be slightly different. In particular, for the COO$^-$ stretching vibration, the major axis of the Raman tensor lies within the carboxylate plane\textsuperscript{147} (Figure 4.3, blue lines) and 12.7° out of the COO$^-$ plane.\textsuperscript{83} Parameters $\langle P_2 \rangle_{\text{COO}^-}$ and $\langle P_4 \rangle_{\text{COO}^-}$ and the most probable orientation distribution function for the case when the major axis of the
Raman tensor is 12.7° out of COO⁻ plane were calculated using the Legendre addition theorem (Figure 4.3, red lines). The functions represent the range of distributions present within the nanotubes. Obtained results clearly show that in both cases, the majority of COO⁻ groups were perpendicular to the nanotube axis although the distribution was broader when an angle at 12.7° out of COO⁻ plane was considered.

The intense band located at 1000 cm⁻¹, seen in Figure 4.1, corresponds to the in-plane breathing mode of the phenylalanine benzene rings. Due to the lack of the information on Raman tensors the quantitative information for this vibrational mode, the orientation cannot be obtained. However, qualitative information was still obtained due to the difference in the intensity of this band between the perpendicular and parallel polarizations, (Figure 4.1) and assuming that the largest polarizability change occurs within the plane of the phenyl ring. The band has higher intensity in the case of XX polarization, and thus, the plane normal to the phenylalanine ring planes is closer to being perpendicular than to being parallel to the axis of the nanotube. Therefore, the phenylalanine ring planes should be oriented parallel rather than perpendicular, relative to the nanotube axis.

As previously mentioned, the bands at 495 and 1131 cm⁻¹ were assigned to torsional and rocking vibrations of NH₃⁺ group, respectively. Despite the fact that Raman tensors corresponding to NH₃⁺ vibrational modes are not available, qualitative estimation of orientation can be made. First, the almost practically maximum possible polarization dependence observed for the band at 1131 cm⁻¹ indicates that the corresponding Raman tensor is anisotropic. Furthermore, the perfect orientation parallel to the nanotube axis unambiguously implies that the PART should be oriented parallel, or along the chemical bond. Secondly, our assumption is based on the reported polarized Raman data of L-valine crystals. L-valine crystallizes in a
monoclinic structure, space group P2₁, extending parallel to the (001) plane. Authors defined the y-axis as the axis along the longest dimension of the crystal; the z-axis and x-axis were mutually perpendicular. Raman spectra have been collected with three different scattering geometries, Z(yy)Z, Z(xx)Z and Z(yx)Z, where the first and the last letter represents the incident and scattered light propagation direction. Interestingly, it has been shown that the relative intensity of the ρ(NH₃⁺) vibration mode in case of Z(xx)Z polarization geometry was almost three times higher comparing to the intensity for Z(yy)Z geometry. Moreover, for the torsional vibration of NH₃⁺, the relative intensity was more the four times lower than the intensity for rocking vibrations of NH₃⁺ group for Z(yy)Z geometry. Based on these reported data and known crystal structure (Figure 4.4) the orientation of the corresponding Raman tensors relative to the C-NH₃⁺ bond could be qualitatively estimated. We assume that the largest polarizability oscillations occur along the line which is close to being parallel and perpendicular to the C-N bond, for rocking and torsional vibrations, respectively. The relative orientation of polarizability ellipsoids (close to being mutually perpendicular) of torsional and rocking vibrations of NH₃⁺ group is consistent with the reported data and our measurements.
Figure 4.4. The structure of the L-valine crystal shown in the projection along the b-axis. The small a, and c letters denote the crystallographic directions.

Figure 4.1 clearly shows that the 1131-cm$^{-1}$ Raman band is strong in the ZZ spectrum and vanishes almost completely in the case of XX polarization geometry indicating almost perfect orientation of C-NH$_3^+$ bonds parallel to the main axis of the nanotube. At the same time, the higher Raman intensity for the XX geometry of the band at 495 cm$^{-1}$ of the torsional vibration $\tau$(NH$_3^+$) also implies a parallel orientation of C-NH$_3^+$ groups relative to the nanotubes’ main axis.

4.3 Orientation of a dipeptide molecule

The proposed orientation of D-FF molecules with respect to the Z-axis of the nanotube can be seen in Figure 4.5. Dipeptide molecules are organized in such way that the COO$^-$ groups are oriented perpendicular to the nanotube axis (Z) with NH$_3^+$ groups (C-N bonds) running along the axis. At the same time, the suggested orientation of carbonyls is about 54° in the direction of
the nanotube axis. Despite the fact that no quantitative information about the orientation of phenyl ring were obtained, the orientation of rings’ plane were qualitatively estimated to be parallel to the nanotube axis.

**Figure 4.5.** Schematic diagram for the di-D-phenylalanine molecule with respect to the Z-axis based on obtained qualitative and quantitative data. Orientation of phenyl rings is arbitrary.

At this point it should be noted that a relatively broad distribution in case of peptide carbonyls and COO− groups (Figures 4.2 and 4.3) can be explained by the possible existence of more than one specific orientation of dipeptide molecules or/and chemical groups. However, given that there is only one distinct orientation of NH$_3^+$ groups relative to the nanotube main axis, the existence of multiple orientations of dipeptide molecules seems unlikely. In addition, the appearance of Raman bands, which were unambiguously assigned to carboxyl and NH$_3^+$ groups, indicates the water presence within the core of the nanotube, which is in good agreement with the literature data.$^{157}$
In this study, the polarized Raman spectra of well-aligned di-D-phenylalanine nanotubes were obtained to evaluate the orientation of the chemical moieties with respect to the nanotube main axis. Analysis of peak intensities between four different polarization combinations (ZZ, XX, ZX, and XZ) confirmed that the nanotube had cylindrical symmetry as well as multiple highly oriented functional groups. Based on known Raman tensors, it was determined that the orientation angle of the carbonyl bond was 54±3° relative to the nanotube main axis. The band indicative of the NH$_3^+$ rocking mode shows that this group was oriented parallel to the nanotube axis. Finally, the COO$^-$ groups were oriented at 90° relative to the axis of the nanotube.

This study demonstrated a great potential of polarized Raman spectroscopy for determining the orientation of chemical groups in anisotropic samples. As indicated before, amyloid fibrils and peptide nanotubes are too big and non-crystalline, so solution NMR and X-ray crystallography have significant limitations. Polarized Raman spectroscopy could potentially allow determining a complete structure of such well organized and oriented species. The complete approach would involve (1) the acquisition of four polarized Raman spectra and the determination of intensity ratios for individual vibrational modes, (2) calculating optimized molecular conformation and Raman tensors for each mode using density function theory (DFT) and obtaining the order parameters from the experimental intensity ratios, (3) calculating orientation distribution functions for the Raman tensors and corresponding chemical groups, (4) use molecular dynamics (MD) simulation for obtaining atomic-type resolution structure of a fibril or a nanotube based on the optimized molecular conformation, determined orientation of individual chemical groups and intermolecular interactions and packing.
CHAPTER 5. Differentiation of parallel and anti-parallel β–sheet structures

Two manuscripts in preparation for submission.
Valentin Sereda, Stuart Sievers and Igor K. Lednev
Valentin Sereda, Victor Shashilov and Igor K. Lednev

5.1 Introduction

Determination of protein structures is essential for understanding of amyloid fibrils formation and for development of therapeutic approaches against associated diseases. One of the important question concerns the evaluation of β–sheet organization. To be able to distinguish different types of β-sheets, including parallel and anti-parallel β-sheets, using a rapid spectroscopic probe is an important goal.

It is generally believed that two types of β-sheet, parallel and anti-parallel, can be distinguished based on torsional angles, $\Psi$ and $\Phi$, proposed by Ramachandran and Sasisekharan. Based on Asher’s semi-empirical approach which correlates frequency of the amide III Raman band to the peptide bond Ramachandran dihedral angle ($\Psi$), it is expected to differentiate parallel and anti-parallel conformations.

Study of microcrystals, mimicking the fibril core, drastically advanced our understanding of the structure of amyloid fibrils. Eisenberg and coworkers have determined a limited set of possible arrangements of peptides in the cross-β structure depending on the organization of the β-strands and β-sheets with respect to one another. Since microcrystals model the fibril core and structure of microcrystals is known, Raman spectroscopy can be utilized to establish the spectrum-structure relationship and use it for probing the structure of fibrils formed from full-length proteins. The interpretations of vibrational spectra for β-sheets have been the subject of much investigation considering the effects of twisting as well as stacking of multiple sheets.
It is believed that computational methods could enrich our understanding of spectroscopic data for these systems.

5.2 Peptide microcrystals with parallel and anti-parallel β-sheet structures

Herein, the normal (non-resonance) Raman spectroscopy was used for structural characterization of four peptide microcrystals prepared from fibril-forming segments of human lysozyme (IFQINS, TFQINS) and human insulin (LVEALYL, VEALYL) and mimicking the core of the corresponding fibrils.

The Raman spectra of microcrystals are shown in Figure 5.1. The spectra are composed of amide vibrational modes and bands arising from aromatic amino acid side chains. The observed Raman bands and their tentative assignments are summarized in the Table 5.1.

![Raman spectra of peptide microcrystals aligned parallel to the incident light polarization. An excitation wavelength is 785 nm.](image)

**Figure 5.1.** Raman spectra of peptide microcrystals aligned parallel to the incident light polarization. An excitation wavelength is 785 nm.

The amide vibrational modes provide information on the protein secondary structure. Amide III band spans the range of 1220-1250 cm\(^{-1}\) and originates predominantly from C-N
stretching and N-H stretching. In fact, amide III is one of the most structurally informative Raman bands due to its sensitively on the peptide bond Ramachandran Ψ dihedral angle that, in part, determines the secondary structure conformation. Asher et al. have established the dependence of the amide III band position on the Ramachandran Ψ dihedral angle with modest dependence on the Φ dihedral angle.

Table 5.1 Peak assignments for the Raman spectra of peptide microcrystals

<table>
<thead>
<tr>
<th>Raman shift, cm⁻¹</th>
<th>Peak assignment</th>
<th>Raman shift, cm⁻¹</th>
<th>Peak assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>469</td>
<td>τ(NH₃⁺) (Val)¹⁴⁸</td>
<td>940</td>
<td>C-C skeletal str. γ(OH)¹⁶²</td>
</tr>
<tr>
<td>486/489</td>
<td>δ(CCCm)¹⁶⁴</td>
<td>950</td>
<td>v(CC)¹⁴⁸, v(CC)¹¹⁷</td>
</tr>
<tr>
<td>492</td>
<td>δ (struct)¹⁶⁴</td>
<td>985</td>
<td>Ser, v(CO) or v(CC)¹⁶⁵</td>
</tr>
<tr>
<td>540</td>
<td>ω(CO₂⁻)¹⁴⁸</td>
<td>995-1001</td>
<td>v(CC), ν(CN)²⁶²</td>
</tr>
<tr>
<td>621</td>
<td>Phe (v6b)¹⁶⁷</td>
<td>1002</td>
<td>Phe¹⁶⁸</td>
</tr>
<tr>
<td>642</td>
<td>Tyr¹⁴⁶,¹⁶⁹</td>
<td>1010</td>
<td>v(CC)⁹⁵,¹⁴⁸, δ(CH)¹⁶⁵</td>
</tr>
<tr>
<td>665</td>
<td>δ(CO₂⁻)¹⁴⁸</td>
<td>1017-1020</td>
<td>Ca-Cβ str¹⁷⁰, ν(CN), v(CC)¹⁶²</td>
</tr>
<tr>
<td>709</td>
<td>r(CH₂)¹⁷¹</td>
<td>1032</td>
<td>ν(CN)¹⁶³, v(CC)¹⁴⁸,¹⁶²</td>
</tr>
<tr>
<td>760</td>
<td>r(CH₂)¹⁷¹</td>
<td>1046</td>
<td>v(CO)¹⁶⁶</td>
</tr>
<tr>
<td>778</td>
<td>δ(CO₂⁻)¹⁴⁸</td>
<td>1060</td>
<td>v(CN)¹⁴⁸ C-C skeletal str.</td>
</tr>
<tr>
<td>806</td>
<td>ν(CC), γ(CO₂⁻)¹⁶³,¹⁶⁶</td>
<td>1071</td>
<td>v(CN) (Val)¹⁴⁹</td>
</tr>
<tr>
<td>823</td>
<td>Ty¹⁴⁶,¹⁶⁸, γ(CO₂⁻)¹⁶³,¹⁶⁴</td>
<td>1083</td>
<td>v(CN), v(CC)¹⁶²</td>
</tr>
<tr>
<td>825</td>
<td>γ(CO₂⁻)¹⁴⁸</td>
<td>1130</td>
<td>r(NH₃⁺)¹⁶³,¹⁶⁶, v(CN)¹⁶⁸</td>
</tr>
<tr>
<td>827</td>
<td>C-C str (Leu, Val)¹⁴⁹</td>
<td>1179</td>
<td>r(NH₃⁺)⁹⁵</td>
</tr>
</tbody>
</table>
Table 5.1 (continued)

<table>
<thead>
<tr>
<th>Raman shift, cm(^{-1})</th>
<th>Peak assignment</th>
<th>Raman shift, cm(^{-1})</th>
<th>Peak assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>830</td>
<td>Tyr,(^{168})</td>
<td>1206</td>
<td>Phe</td>
</tr>
<tr>
<td>851/853</td>
<td>r(CH(_3))(^{163}) Alanine(^{156})</td>
<td>1210(14)</td>
<td>Tyr, C(\beta)-C1((\gamma))(^{146})</td>
</tr>
<tr>
<td>852</td>
<td>r(CH(_2))(^{162}), r(CH(_3))(^{163})</td>
<td>1236</td>
<td>(\tau(CH_2))</td>
</tr>
<tr>
<td>876</td>
<td>(\nu(CC))(^{163})</td>
<td>1313-1339</td>
<td>w (CH(_2), CH(_3)), (\delta(CH_2, CH_3)), t (CH(_2), CH(_3)), (\delta(CH))(^{168})</td>
</tr>
<tr>
<td>861</td>
<td>Tyr</td>
<td>1447-1451</td>
<td>C-H vibration, (\delta(CH_2)), (\delta_{as}(CH_3))(^{148,168})</td>
</tr>
<tr>
<td>881</td>
<td>r(CH(_2))(^{162})</td>
<td>1555</td>
<td>(\nu(CO_2))(^{95,165})</td>
</tr>
<tr>
<td>903</td>
<td>(\nu(CC))(^{148})</td>
<td>1585</td>
<td>Phe (v8b)(^{167})</td>
</tr>
<tr>
<td>923</td>
<td>(\nu(CC)), (\nu(CN))(^{117,149,162})</td>
<td>1605</td>
<td>Phe (v8a)(^{167})</td>
</tr>
<tr>
<td>940</td>
<td>C-C skeletal str. (\gamma(OH))(^{162})</td>
<td>1614, 1620</td>
<td>Asn, (\delta(NH2))(^{165}), Tyr</td>
</tr>
<tr>
<td>950</td>
<td>(\nu(CC))(^{148}), (\nu(CC))(^{117})</td>
<td>1735</td>
<td>(\nu(C=O))(^{168,172})</td>
</tr>
<tr>
<td>985</td>
<td>(\nu(CO)) or (\nu(CC))(^{163})</td>
<td>2861</td>
<td>(\nu(O-H))</td>
</tr>
<tr>
<td>995-1001</td>
<td>(\nu(CC))(^{164}),(\nu(CC))(^{162})</td>
<td>2874</td>
<td>(\nu(CH_2)) (L-leucine)(^{149})</td>
</tr>
<tr>
<td>1002</td>
<td>Phe(^{168})</td>
<td>2895</td>
<td>(\nu(CH_3))(^{95}) (L-Valine)(^{149})</td>
</tr>
<tr>
<td>1010</td>
<td>(\nu(CC))(^{95,148}), (\delta(CH))(^{165})</td>
<td>2926</td>
<td>(\nu(CH_3))(^{149})</td>
</tr>
<tr>
<td>1017-1020</td>
<td>Ca-C(\beta) str.(^{170}),(\nu(CN)), (\nu(CC))(^{162})</td>
<td>2964-2988</td>
<td>(\nu_{as}(CH_3))(^{149})</td>
</tr>
</tbody>
</table>

Abbreviations: t, torsion; \(\delta\), bending; r, rocking; w, wagging; \(\nu\), stretching; s, symmetric; as, asymmetric.
To obtain the structural information in terms of dihedral angles we have analyzed the spectral range between ~1200 and 1270 cm$^{-1}$. It should be mentioned that some vibrational modes of aromatic amino acid sidechains (Table 5.1), which are not related directly to the secondary structure, contribute to this spectral range too. We deconvoluted each Raman spectrum assigned bands at ~1220 cm$^{-1}$ and ~1230 cm$^{-1}$ to the amide III mode (Figure 5.2). ~1205 and ~1210 cm$^{-1}$ bands most probably resulted from the vibrational modes of tyrosine and phenylalanine sidechains.$^{117,155}$

**Figure 5.2.** Band decomposition of the amide III region of Raman spectra of peptide microcrystals.

As shown by Mikhonin et al., the average $\Psi$ angle of the amide group can be calculated based on the amide III frequency with an error of $\pm8^\circ$. Authors proposed a set of equations
describing correlation between amide III frequency and \( \Psi \) Ramachandran angle with detailed considerations of the peptide bond – water hydrogen bonding, and the peptide – peptide bond hydrogen bonding. Knowing the hydrogen bonding pattern for the microcrystals, dihedral angles were obtained from amide III frequencies using the following equation:

**Equation 53**

\[
\nu_{AmIII}^\beta = [1255\text{cm}^{-1} - 54\text{cm}^{-1}\sin(\Psi + 26^\circ)]
\]

\( \Psi \) angles calculated from Raman spectra of all four microcrystals are shown in Table 5.2. We compared the obtained values with the set of \( \Psi \) angles (Table 5.2) determined from the atomic-resolution structures of the microcrystals reported based on X-ray crystallography. For all microcrystals, the calculated values of \( \Psi \) angles were within an expected error (\( \pm 8^\circ \)) with respect to the crystallographic values.

**Table 5.2** Amide III Frequencies and \( \Psi \) Ramachandran angles for peptide microcrystals

<table>
<thead>
<tr>
<th>Microcrystal</th>
<th>( \Psi ), crystallographic</th>
<th>( \Psi ), calculated</th>
<th>Amide III</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFQINS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>115°, 121°</td>
<td>113±8°</td>
<td>1221 cm&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>127°, 131°</td>
<td>129±8°</td>
<td>1232 cm&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>TFQINS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>119°, 122°, 123°</td>
<td>114±8°</td>
<td>1220 cm&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>131°</td>
<td>125±8°</td>
<td>1229 cm&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>LVEALYL&lt;sup&gt;a&lt;/sup&gt;</td>
<td>118°, 122°, 124°</td>
<td>119±8°</td>
<td>1224 cm&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>130°, 139°</td>
<td>129±8°</td>
<td>1232 cm&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>VEALYL&lt;sup&gt;b*&lt;/sup&gt;</td>
<td>99±7°, 118±6°</td>
<td>117±8°</td>
<td>1222 cm&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>126±8°, 131±6°</td>
<td>129±8°</td>
<td>1232 cm&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>-parallel, <sup>b*</sup>-anti-parallel. *Average values and standard deviation are shown.
Amide I is also a sensitive marker of peptide secondary structure.\textsuperscript{103,108,116} For the three microcrystals (IFQINS, TFQINS and LVEALYL), the amide I peak is centered at $\sim$1666 cm\textsuperscript{-1} and only for the microcrystal VEALYL, the amide I band is at 1672 cm\textsuperscript{-1} (Figure 5.3). The position of the amide I Raman band indicates that peptide microcrystals have well-organized $\beta$-sheet structure which is in agreement with crystal structures.

\textbf{Figure 5.3.} Amide I region of peptide microcrystals’ Raman spectrum.

It has been reported that the profile of amide I Raman peak is a good tool in discriminating between parallel and anti-parallel $\beta$-sheets.\textsuperscript{160,161} The frequency of amide I normal vibrational mode depends on the $\text{C=O}$ hydrogen bonding and the vibrational coupling between adjacent amide units. Both these factors depend on the three-dimensional structure of the
polypeptide backbone.\textsuperscript{103-105} Because of a strong effect from the number and the strength of hydrogen bonds,\textsuperscript{175} the amide I position for anti-parallel β-sheets might be upshifted relative to that for parallel β-sheets because of weaker hydrogen bonding in the latter case. However, this difference does not normally allow one to distinguish between them in experimental studies due to dependency of the amide I position on the twisting and bending as well as on the number of strands in the β-sheet.\textsuperscript{161} However, in the case of microcrystals such kinds of deformations are not expected. Indeed, our results clearly show that the amide I band is upshifted in Raman spectra of the microcrystal which possess an anti-parallel β-sheet structure (VEALYL) in comparison to amide I position in spectra of microcrystals with parallel β-sheets (IFQINS, TFQINS and LVEALYL).

It is accepted in biochemistry literature that $\Psi$ Ramachandran angles for anti-parallel and parallel β-sheets are around +135° and +113°, respectively.\textsuperscript{158} The assignment of distinct ideal values for parallel and anti-parallel β-strands can be misleading because of these Ramachandran angles are characteristic of the “classical,” flat β-sheet conformations only.\textsuperscript{176} Salemme and Weatherford\textsuperscript{177,178} have shown that a deviation from the “classical” structure can result in significant changes in $\Psi$ dihedral angles. As a result, knowing only values of dihedral angles does not allow for answering the question whether polypeptides of interest possess parallel or anti-parallel structural organization of β-sheets.

\textbf{5.3 Organization of β-sheet in amyloid fibrils and microcrystals}

In this work, we utilized polarized Raman spectroscopy of microcrystals and aligned amyloid fibrils to differentiate the parallel and anti-parallel β-sheet structures of microcrystals and fibrils with application of advanced chemometrical methods.
Series of polarized Raman spectra from microcrystals and fibrils were recorded over the range from 0 to ≈120° by the samples rotation relative to the direction of polarization of the incident light. Fibrils prepared from human, bovine insulin, hen egg white lysozyme, Aβ34-42 and Aβ16-22 fragments were aligned as described in Chapter 2.

The spectra were treated with advanced statistical methods in order to show the potential of Raman spectroscopy for distinguishing between parallel and anti-parallel β-sheet structures. Analyses included a principle component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA). At first, classification analysis was performed using PLS-DA method which is a special form of PLS for a classification purpose. This method is useful for dealing with data sets with nonlinear relationships between variables and helpful to highlight the significant differences between samples.

PLS-DA as implemented in PLS Toolbox 8.0 by Eigenvector, Inc. were employed to discriminate parallel and anti-parallel β-sheet of crystals and fibrils using polarized Raman spectra of six microcrystal and five fibril samples. The raw experimental spectra were baseline corrected, mean centered and trimmed to include only the spectral region of 1190-1780 cm⁻¹ encompassing amide III and amide I bands. Raman spectra of fibrils and crystals were treated separately. Each spectrum was assigned to one of the two known classes – parallel or anti-parallel, and PLS-DA was used to build the latent variable model and predict class assignment.

Figure 5.4 shows the results of PLS-DA. The plot is an illustration of the predicted class anti-parallel β–sheet for each experimental spectrum. All spectra were assigned to the correct class.
Figure 5.4. Classification (PLS-DA) of Raman spectra collected from (A) microcrystals (anti-parallel β-sheet – red circles, parallel β-sheet - black diamonds) and (B) amyloid fibrils (anti-parallel β-sheet - green squares, parallel β-sheet - blue triangles). The x-axis plots a specific Raman spectrum, while the Y-axis corresponds to the predicted “class values” of Raman spectra, or the probability of a spectrum to be identified as anti-parallel. The horizontal red line is the threshold of anti-parallel β-sheet identification estimated using Bayes’ theorem. Each symbol corresponds to a single experimental spectrum.

Figure 5.5 shows the Variable Importance Projection (VIP) scores obtained for PLS-DA modeling in the spectral region 1190–1780 cm$^{-1}$. The magnitudes of the VIP scores can be employed to detect variables that influence mostly on the model and to highlight differences in Raman band shapes and position that significantly contribute when discriminating between sample groups.$^{180}$

It is clearly visible that the major spectral zone, between 1635 and 1700 cm$^{-1}$, can be distinguished to be important for optimal PLS-DA model performance. Several other regions show a weaker contribution to the VIP scores. Contributions of other regions, including the
amide III, to the PLS-DA regression coefficients are weaker relative to the spectral contribution of the amide I region (Figure 5.5), which suggests that the amide I region provides the greatest selectivity in discriminating parallel and anti-parallel β-structures.

![Variable Importance in Projection (VIP) scores for the investigated spectral region 1190–1800 cm\(^{-1}\).](image)

**Figure 5.5.** Variable Importance in Projection (VIP) scores for the investigated spectral region 1190–1800 cm\(^{-1}\).

Next, PCA analysis was performed on each data set for the amide I region only. The optimal number of principal components was determined through cross-validation. Results of PCA analysis of spectral data obtained for microcrystals (Figure 5.6) and fibrils (Figure 5.7). The first two components were used to create scores plots.
Figure 5.6. (A) PCA scores calculated for polarized Raman spectra of plots of microcrystals (1635-1700 cm$^{-1}$). Each data point is the representation of one Raman spectrum. (B) Spectral components calculated by PCA (PC1 blue, PC2 green).
The analysis of the data showed a distinct separation between Raman spectra collected from microcrystals with different types of β-sheet organization (parallel and anti-parallel). First group included Raman spectra from TFQINS, IFQINS, YTIAAL and LVEALYL microcrystals (parallel β-sheet).\textsuperscript{4} Second group, comprised only spectra from VAEALYL microcrystal (anti-parallel β-sheet).\textsuperscript{140} In other words, Raman spectra of microcrystals with anti-parallel structural organization fall apart from Raman spectra of the microcrystal which possess an anti-parallel β-sheet structure (VEALYL).

It should be mentioned that the overlapping of two data sets can be explained by fact that points in overlapping regions represent Raman spectra where the amide I peak have minimum intensity or even vanishes. This was the case when microcrystals were oriented near perpendicular (90°) to the direction of the electric field component of the polarized laser radiation.

Spectral data obtained for aligned fibrils also show clustering in two groups as presented on two dimensional component scores plot (Figure 5.7). It is apparent that the group contained Raman spectra of insulin (human and bovine)\textsuperscript{181,182} and HEWL fibrils\textsuperscript{183} (parallel β-sheet) is well separated from the group comprising spectra of Aβ\textsubscript{34-42} and Aβ\textsubscript{16-22} fibrils (anti-parallel β-sheet).\textsuperscript{184,185}
Figure 5.7. (A) PCA scores calculated for polarized Raman spectra of plots of aligned fibrils (1635-1700 cm\(^{-1}\)). Each data point is the representation of one Raman spectrum. (B) Spectral components calculated by PCA (PC1 blue, PC2 green).

Loadings of the first two principal components (Figure 5.6B and 5.7B) confirm that the difference in the shape and position of the amide I band are most sensitive to the type of β-structure.
Due to the strong dependency of the amide I local mode frequency on the number and the nature of hydrogen bonds,\textsuperscript{175} it is expected that the amide I position for anti-parallel β-sheets might be upshifted in contrast to parallel because of weaker hydrogen bonding in the parallel β-sheets. Our results show that the amide I band is indeed upshifted in Raman spectra of microcrystals which possess an anti-parallel β-sheet structure in comparison to amide I position in spectra of microcrystals with parallel β-sheets. However, fibrils with parallel β-sheet organization have a higher amide I wavenumber with respect to anti-parallel. In this instance, the difference in the pattern of hydrogen bonding might not play a major role in the variance of the amide I peak position.

It is well known that the strands can be in- or out-of-register, the separate sheets within fibrils can also be aligned with the same or reverse sense or with an angular alternation produced by or contributing to the fibril helicity. Such deformations, like twisting and bending, are important for fibrils.\textsuperscript{186} Schweitzer-Stennert has shown\textsuperscript{161} that simulated Raman amide I profiles of anti-parallel and parallel β-sheets vary depending on numbers of strands. Author stated that decreasing the number of strands in the model, from 12 to 1, causes upshift of the amide I peak for parallel β-sheets with negligible changes in the peak position for anti-parallel β-sheets. Also, even 3° twisting and/or 2° bend per strand causes detectible upshift of the amide I band for parallel β-sheet organization. We assume that an opposite behavior, in the amide I band position for anti-parallel and parallel β-sheet organization between fibrils and microcrystals, results from deformations such as bending and/or twisting.
CHAPTER 6. Tip-enhanced Raman spectroscopy of protein aggregates

One manuscript in preparation for submission.
Valentin Sereda and Igor K. Lednev

6.1 Introduction

Tip-enhanced Raman spectroscopy (TERS) has been applied to characterize the amino acid residue composition and secondary structure of amyloid fibrils, and for a comparative TERS study of the surfaces of proto-filaments and mature fibrils.\textsuperscript{112,113,187-189}

Despite significant interest in this novel and promising technique, our understanding and interpretation of TER spectra remains challenging. The reproducibility of TERS performed in different laboratories has been recently reported,\textsuperscript{190} and analysis of biological samples by TERS (in the ‘gap mode’), conventional Raman and SERS compared the spectroscopic characteristics of samples acquired by these three Raman methods.\textsuperscript{191} Van Duyne and coworkers used a selective tip enhancement of vibrational modes of copper phthalocyanine and interpreted variations in the corresponding TER spectra.\textsuperscript{192} Two different types of TER spectra have been reported for biological samples, proteins and protein aggregates in particular: spectra that resemble the normal Raman spectra acquired for the same sample\textsuperscript{193-195} and spectra with different relative intensities and peak positions.\textsuperscript{113,187,196} To the best of our knowledge, only one type of TER spectra has been reported to date in any individual work and no comparative discussion of the appearance of the two types of TER spectra has been offered. So it would beneficial to compare two types of TER spectra obtained using the same tip and experimental conditions for the same sample and/or chemically related samples.
6.2 LVEALYL microcrystal

Here, we report TER spectra for a LVEALYL peptide microcrystal mimicking the structural organization of the cross-β core of the insulin fibril. A single LVEALYL microcrystal was deposited onto a coverslip. First, a topography image was acquired to locate the area of the interest on the substrate. Atomic force microscopy (AFM) was conducted on the microcrystal using the same tip, which was later used for the TERS measurements. Figure 6.1 shows the AFM image of a selected 6μm×5μm area at the edge of the microcrystal. According to the height profile generated along the line in Figure 6.1A, the microcrystal was uniformly ~3 μm thick.

![Figure 6.1](image)

**Figure 6.1.** (A) AFM image of the microcrystal (LVEALYL) on the glass coverslip. The white dashed-line rectangle encloses the area used for TERS mapping. (B) Height profile of the microcrystal (LVEALYL) obtained along the solid white line in Figure 6.1A. Scale bar is 1 μm.

TERS mapping was conducted within the 1 μm × 5 μm rectangle enclosed by the white dashed line in Figure 6.1. The lateral distance between the adjacent TERS measurements inside the mapping region was set to 50 nm. Individual TER spectra were acquired in noncontact mode with an acquisition time of 0.2 sec and a laser power of 0.1 mW. A total 2000 TER spectra were measured in a one-hour experiment. Remarkably, the vast majority of TER spectra acquired on the top side of the microcrystal were similar. Three representative TER spectra are shown in
Figure 6.2, which shows a TERS map of a single microcrystal based on the area of Raman peaks in the 2800-3000 cm\(^{-1}\) region. The main contribution to the Raman signal in this spectral range is from CH, CH\(_2\) and CH\(_3\) symmetric and antisymmetric stretching vibrations.\(^{95}\) TERS maps of the microcrystal, built using other Raman spectral areas, had similar appearances to the map shown in Figure 6.2. As expected, there was good agreement between the AFM image of the microcrystal and its TERS map.

![Figure 6.2](image)

**Figure 6.2.** (A) Representative TER spectra recorded on the top side of the LVEALYL microcrystal and a TERS image of the microcrystal obtained by integration of the 2800-3000 cm\(^{-1}\) area. The color chart represents the relative Raman signal intensity in the 2800-3000 cm\(^{-1}\) area. (B) A normal Raman spectrum (blue) of the microcrystal (LVEALYL).

A relatively small group of TER spectra of the microcrystal had different spectral profiles. The majority of these spectra appeared at the “edges” of the crystal instead of its “flat”
top surface. To further investigate this result, two additional sets of TERS measurements were measured with a longer accumulation time (5 sec) at 0.03-mW laser power along two straight lines. First, we collected 12 TER spectra (Figure 6.3) on the flat top of the microcrystal along the line parallel to the main axis and in the center of the crystal with approximately 0.5-μm steps. All spectra showed remarkable reproducibility and resembled the TER spectra acquired during the mapping experiment (Figure 6.2).
Figure 6.3. TER spectra measured at different points along the line shown on the right. For every point, three consecutive measurements were taken (a, b, c). Scale bar is 1 μm.
These results indicate that the flat surface of the microcrystal was highly homogeneous in terms of TERS. This is not a trivial result and indicates that an individual TER spectrum is collected from an area larger than that occupied by a single crystal cell (a = 49.48, b = 4.84, c = 19.44 Å). In other words, the spatial resolution of TERS should be larger than 20 nm when measurements are taken on a flat crystal surface.

For the second set of measurements, 12 TER spectra with a 0.5-μm step (Figure 6.4) were acquired along the line perpendicular to the main axis of the microcrystal and at the edge of the crystal.
Figure 6.4. TER spectra obtained along the line perpendicular to the main axis of the microcrystal. Capital letters (A-D, red crosses) indicate the position on the sample. The active TERS tip was moved from point A to D. Three measurements were taken (a, b, c) at each individual point. Scale bar is 1 μm.
Figure 6.5 shows selected TER spectra acquired at four different positions on the line. The spectra at the edge of the crystal (spectra A-C, Figure 6.5) varied significantly from point to point and contained a number of additional bands in the fingerprint region relative to the characteristic TER spectrum of the flat top surface of the crystal (spectrum D, Figure 6.5). The edge spectra were also consistently more intense than the flat-top surface TER spectra.

**Figure 6.5.** Representative TER spectra obtained along the line perpendicular to the main axis of the microcrystal. TER spectra obtained on the edge (A-C) and flat top surface (D) of the microcrystal. The spectra are offset for clarity, but their relative intensities are as measured. Scale bar is 1 μm.
To verify the tip enhancement effect and ensure that the tip was not contaminated, the tip was retracted several micrometers to record control spectra. The latter spectra (Figure 6.6) showed no enhancement of any Raman bands, indicating that the initial spectra were actual TER spectra.

![Figure 6.6](image)

**Figure 6.6.** A control experiment demonstrating that the TERS tip was not contaminated. (A) TER spectrum obtained on a flat surface of the microcrystal and (B) measured immediately after the tip was retracted.

A normal Raman spectrum of the LVEALYL peptide microcrystal is shown in Figure 6.2B and was acquired at 532-nm excitation with 10-mW laser power for 30 sec of accumulation time. These normal Raman spectra closely resemble the TER spectra, which is important for the mechanistic understanding of TERS of biological systems, specifically for proteins and protein aggregates. The normal Raman spectrum represents an average Raman spectroscopic signature of the entire microcrystal, and the TER spectra of the microcrystal’s flat top surface should also represent the bulk crystal. This outcome is in contrast to the results reported for insulin fibrils, in which TER spectra represented the fibril surface. A hypothetical mechanism explaining this difference is discussed below.
Normal Raman spectroscopy of peptides and proteins is well understood, and LVEALYL peptide Raman bands could be confidently assigned based on literature data. The majority of TER spectra were very similar to the corresponding normal Raman spectra (Figure 6.2B), which allowed us to assign the TER spectra based on conventional Raman data. Bands in the 1600 – 1690 cm\(^{-1}\) region correspond to the amide I vibrational mode,\(^{108}\) and the position (1666 cm\(^{-1}\)) and shape of the amide I Raman band indicated that LVEALYL was a well-ordered \(\beta\)-sheet. Bands at 1450 and 832 cm\(^{-1}\) were assigned to C-H vibration\(^ {95,149}\) and tyrosine out-of-plane ring breathing\(^ {95,117}\) modes, respectively. The peak at 1227 cm\(^{-1}\) was assigned to the amide III vibrational mode, which is one of the most structurally informative parts of the protein Raman spectrum. It involves significant C-N stretching, N-H bending, and C-C stretching vibrations.\(^ {173}\) Bands at approximately 2600-3000 cm\(^{-1}\) were assigned to CH, CH\(_2\) and CH\(_3\) symmetric and antisymmetric stretching.\(^ {95}\) The peak assignments of the microcrystal are summarized in Table 6.1.
**Table 6.1** Band Assignment for TERS, and Raman Spectra of LVEALYL microcrystal

<table>
<thead>
<tr>
<th>Raman shift, cm$^{-1}$</th>
<th>Peak assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>823</td>
<td>Tyr$^{95}$</td>
</tr>
<tr>
<td>851/853</td>
<td>r(CH$_3$)$^{148,169}$</td>
</tr>
<tr>
<td>852</td>
<td>r(CH$_2$)$^{165}$</td>
</tr>
<tr>
<td>995-1001</td>
<td>ν(CC), ν(CN), ν(CC)$^{148,165}$</td>
</tr>
<tr>
<td>1017-1020</td>
<td>Cα-Cβ stretching, ν(CN), ν(CC)$^{165,168}$</td>
</tr>
<tr>
<td>1036</td>
<td>ν(CN), ν(CC)$^{149,165}$</td>
</tr>
<tr>
<td>1060</td>
<td>ν(CN)$^{149}$</td>
</tr>
<tr>
<td>1083</td>
<td>ν(CN), ν(CC)$^{165}$</td>
</tr>
<tr>
<td>1122-28</td>
<td>ν(CN)$^{95}$</td>
</tr>
<tr>
<td>1179</td>
<td>r(NH$_3^+$)$^{146}$</td>
</tr>
<tr>
<td>1313-1339</td>
<td>w(CH$_2$, CH$_3$), δ(CH$_2$, CH$_3$), t(CH$_2$, CH$_3$), δ(CH)$^{95}$</td>
</tr>
<tr>
<td>1447-1451</td>
<td>C-H vibration, δ (CH$<em>2$), δ$</em>{as}$(CH$_3$)$^{95,149}$</td>
</tr>
<tr>
<td>1735</td>
<td>ν(C=O)$^{95,170}$</td>
</tr>
<tr>
<td>2895</td>
<td>ν(CH$_3$)$^{146}$</td>
</tr>
<tr>
<td>2926</td>
<td>ν$_s$(CH$_3$)$^{172}$</td>
</tr>
<tr>
<td>2964-2988</td>
<td>ν$_{as}$(CH$_3$)$^{172}$</td>
</tr>
</tbody>
</table>

Abbreviations: t, torsion; δ, bending; r, rocking; w, wagging; ν, stretching; s, symmetric; as, asymmetric.
6.3 Insulin fibrils and the cast film of native insulin

TER spectra of insulin fibrils have been reported previously,\textsuperscript{113,187} but repeating these measurements in the current study using the same TERS instrument and tips for both the fibrils and microcrystal was important. This is because TERS is a relatively new technique and the reproducibility of data obtained at different laboratories using different instruments and tips is still a concern.\textsuperscript{190}

AFM topography was conducted with TERS probes to locate deposited fibrils and determine the area for TERS mapping. TERS spectra were measured within the $10 \text{ nm} \times 100 \text{ nm}$ area at a 1-nm step producing 1000 total spectra. The acquisition time was 0.2 sec per spectrum with a laser power of 0.1 mW. The map region was selected to contain a zone with part of the insulin fibrils and areas with substrate only. The areas containing only substrate (i.e., without the fibril) allowed us to detect and eliminate artifacts such as tip contamination and SERS on the tip. Figure 6.7A shows representative TER spectra of the insulin fibrils acquired in the mapping experiment. In agreement with our previous reports,\textsuperscript{113,187} these spectra vary significantly from point to point, indicating significant surface heterogeneity of the insulin fibrils.
Figure 6.7. Representative TERS spectra of insulin fibrils (A) and native insulin (B).

Amyloid fibrils consist of a well-organized fibril core with a cross-β structure and an unordered protein on the surface of the fibril composed of various secondary structural elements. We used a cast film of native insulin to mimic the unordered parts of the fibril. The protein solution was deposited on a substrate with gold nanoplates and dried. TERS mapping of the cast film was conducted over a 1 μm x 1 μm area with 25-nm steps using 0.1-mW laser power and a 0.2-sec accumulation time. Representative TERS spectra collected from the map are shown in Figure 6.7B. Similar to the spectra of the insulin fibrils, the TERS spectra of native insulin varied significantly in terms of the Raman band positions and relative enhancement (Figure 6.7).
TER spectra of the fibrils and native insulin contained a number of characteristic Raman protein bands. Prominent bands corresponding to amide I (1630-1675 cm\(^{-1}\)) and amide III (1220-1280 cm\(^{-1}\)) modes, CH\(_2\) scissoring modes (~1450 cm\(^{-1}\)), C–H stretching vibrations (1440–1470 cm\(^{-1}\)) and ring C–C vibrations (~1604 cm\(^{-1}\)) are evidence that the spectra are in good agreement with our earlier reports.\(^\text{113,187}\)

### 6.4 Hypothetical mechanisms of TERS by peptides and proteins

The set of TERS experiments on protein and peptide aggregates resulted in good quality reproducible TER spectra that could be separated into two groups based on their appearance. Relatively weak spectra measured at the top flat surface of the peptide microcrystal did not vary from point-to-point on the crystal surface and were completely reproducible when consecutive accumulations were recorded from the same point. Most importantly, these TER spectra were identical to the normal Raman spectrum of the microcrystal, indicating that these TER spectra reflected the bulk properties of the crystal rather than specific information about the microcrystal surface.

Conversely, relatively intense TER spectra measured at the microcrystal edge, insulin fibrils and native insulin cast film varied significantly from point-to-point and when consecutive accumulations were recorded from the same point. These TER spectra did not resemble the corresponding normal Raman spectra, although they contained several common Raman bands that could be assigned to the same protein/peptide vibrational modes.\(^\text{113,187,189,197}\)

Comparison of TER spectra in terms of their relative intensities requires a special discussion, and quantitative analysis of tip enhancement of the Raman signal is a complicated problem.\(^\text{198-201}\) However, we believe that there is strong evidence to differentiate TER spectra assigned to the two groups based on their intensity. Specifically, all microcrystal spectra (Figure
6.4) measured along the line perpendicular to the main axis were recorded under the same conditions and using the same tip. This experiment was reproduced three times, and the spectra from the top flat surface were always less intense than the spectra from the microcrystal edge. Comparison of the top surface spectra (Figure 6.3) with the TER spectra of the fibrils and cast films (Figure 6.7) is more challenging because these spectra were acquired in separate experiments, even though we attempted to preserve the experimental conditions. Again, multiple experiments showed that normal-Raman-type TER spectra of the top surface of the microcrystal were typically less intense than the spectra of the fibrils and the protein cast film.

The two types of TER spectra identified in this study are not an exception, but have already been reported by other research groups using TERS in various systems. To the best of our knowledge, only one type of TER spectra, either similar to or very different from normal Raman-type spectra, has been reported in any individual study. Specifically, in the investigation of nanoscopic biological objects or even single molecules, TER spectra were considerably different from conventional Raman spectra.\textsuperscript{37,189,202,203} However, TER spectra of benzenethiol\textsuperscript{194} and amyloid nanotapes on an Au substrate\textsuperscript{195} agreed well with their conventional Raman spectra. The TER spectra of calcium oxalate monohydrate crystals\textsuperscript{193} also resembled a normal Raman spectrum, which is in agreement with our data and consistent with the assumption about the influence of sample surface “roughness” on the appearance of certain types of TER spectra. The described effect should depend on the properties of the TERS tip in addition to the sample surface characteristics. For example, it might be possible to manufacture a tip with an active site located on the apex that would chemically enhance the Raman signal over a short range even on a flat crystal-like surface. Experimental verification of this hypothesis is currently not practical
due to the lack of control over the positioning of a hot spot during tip manufacturing, but this could be possible in the future.

Both types of TER spectra, (i) relatively weak, normal Raman-type and (ii) relatively strong, and different from the normal Raman spectrum, could be obtained in an individual experiment using the same tip and different parts of the same sample (i.e., a top flat surface vs. an edge of a single microcrystal). This result indicates that a certain property of the sample, such as roughness of the probed surface at the molecular level, could determine the type of TER spectra. We propose a hypothetical mechanism for tip enhancement of Raman scattering in proteins and peptide aggregates that is consistent with our data and all reported literature data to the best of our knowledge. Similar to SERS, we hypothesize that there are two different mechanisms for tip enhancement of Raman scattering based on the proximity of the sample to the “hot spot”.\textsuperscript{204,205} As with chemical enhancement (CM) in SERS, the strongest enhancement occurs when a chemical group comes into immediate van der Waals contact with the hot spot on the TERS tip (Figure 6.8B). We call this the short range mechanism of tip enhancement. In this case, a TER spectrum is affected by the metal-chemical group interaction, including a possible charge transfer, and appears different relative to the normal Raman spectrum of the entire sample or even a molecule. A limited number of vibrational modes could be enhanced because of this short-range mechanism. In contrast, when the chemical group is not in immediate contact with the hot spot, but is within range of the hot spot’s electromagnetic field (could be several nm apart), TER scattering is also observed, and the spectrum resembles the normal Raman spectrum (Figure 6.8A). We would call this phenomenon the long-range mechanism of tip enhancement of Raman scattering. This type of tip enhancement is similar to the electromagnetic mechanism (EM) in SERS.
Figure 6.8. A schematic representation of the hypothetical mechanism of tip enhancement of Raman scattering of peptides and proteins. (A) Long-range tip enhancement of Raman scattering on the top flat surface of the LVEALYL peptide microcrystal: the electromagnetic field of the tip probes multiple peptide molecules in the crystal, resulting in an average TER spectrum that resembles the normal Raman spectrum of the crystal. (B) Short-range tip enhancement of Raman scattering by a polypeptide chain segment, which comes into immediate contact with the tip’s hot spot. The arrows indicate a possible movement of the peptide chain to and from the contact with the hot spot.

Figure 6.8 schematically illustrates our hypothesis on the tip enhancement of Raman scattering in peptides and proteins. We assumed that the active hot spot is located at some distance from the AFM tip apex. As a result, direct van der Waals contact between the hot spot and chemical groups on the sample surface is not possible on a plain microcrystal surface. This situation leaves the long-range mechanism for tip enhancement playing the predominant role, in which the tip-enhanced electromagnetic field of the tip spreads up to ~10-30 nm depending on the theoretical model\textsuperscript{206-209} and consequently probes multiple peptide molecules in the crystal.
This outcome results in a TER spectrum averaged over a significant portion of the microcrystal that resembles the normal Raman spectrum of the crystal.

It has been shown previously that the TERS enhancement factor (EF) can be estimated using the engaged \( I_{\text{engaged}} \) and retracted intensities \( I_{\text{retracted}} \), estimated laser spot size \( R_{\text{focus}} \) and tip radius \( R_{\text{tip}} \). Alternatively, the average number of molecules \( N_{\text{normal}} \) contributing to the normal Raman signal and the number of molecules probed in TERS \( N_{\text{TERS}} \) can be used\(^{192,198,210,211}\) (Eq. 54):

\[
\text{Equation 54}
\]

\[
EF = \left( \frac{I_{\text{engaged}}}{I_{\text{retracted}}} \right) - 1 \left( \frac{R_{\text{focus}}}{2R_{\text{tip}}} \right)^2 = \left( \frac{I_{\text{engaged}}}{I_{\text{retracted}}} - 1 \right) \frac{N_{\text{normal}}}{N_{\text{TERS}}}
\]

However, due to the strong dependence of electromagnetic enhancement on the distance \( r \) between the surface and the adsorbate, the TERS intensity \( I \) as a function of the average size of the field-enhancing features on the surface \( a \) also have to be taken into account.\(^{194,212}\)

\[
\text{Equation 55}
\]

\[
I \propto \left( \frac{a + r}{a} \right)^{-10}
\]

The surfaces of the fibrils and protein cast film are not plain at the molecular level and contain segments of the polypeptide chain that could come into direct contact with the hot spot, as illustrated in Figure 6.8B. A similar situation could occur when the TERS tip is placed at the microcrystal edge, where either surface roughness or inclination allows for direct contact between the chemical groups (i.e., amino acid residues) and the tip hot spot. This contact results in the short-range enhancement of Raman scattering from relatively small chemical groups,
which are in immediate contact with the hot spot. These chemical groups might change from point-to-point on the surface of the fibrils, protein cast film and crystal edge, resulting in significant variations in the TER spectra. In addition, more than one hot spot could be active on the TERS tip, and different hot spots could come into close contact with the chemical groups on a rough sample surface or at the edge of a crystal. The latter effect could be further strengthened by tip oscillations near the edge of the crystal, which could bring different hot spots in immediate contact with different spots on the sample, while consecutive TERS measurements are made from the “same” tip position at the edge of a crystal.

It is difficult to make any convincing quantitative comparison between the possible contributions of long- and short-range mechanisms of tip enhancement at this time. According to the SERS literature, EM can greatly enhance in the range of $10^4$ to $10^8$ for Raman scattering, compared to $\sim 10^0$ to $10^3$ for CM enhancement, and the contribution of CM is typically negligible relative to EM. However, the TERS hot spot could be distant from the entire sample and, at the same time, be in immediate contact with a small individual chemical group (e.g., a peptide segment). The chemically-enhanced Raman signal from this group might dominate the TER spectrum in this case. The latter TER spectrum could be affected by molecular resonance, and charge transfer or nonresonant chemical mechanisms could be different than the normal Raman spectrum of the sample. Consequently, we suggest that although long-range enhancement is always present, short-range enhancement dominates the TER spectrum if there is direct contact between chemical groups and the tip’s hot spot.
CONCLUSIONS

The conversion of proteins from their soluble functional states into structures referred to as amyloid fibrils has been implicated in a large number of diseases. A rich diversity of nanostructures has been described in the last two decades, such as nanorods, nanotubes, and nanosphers. The self-assembly of short peptides into molecular nanostructures has received a significant amount of interest due to their biocompatibility, ability for molecular recognition, and ease of chemical modification. However, atomic-level structural characterization of such species is challenging because of the limitations of solution NMR and X-ray crystallography when applied to insoluble, non-crystalline samples.

Polarized Raman spectroscopy is uniquely suitable for probing amyloid fibrils. We found that a simple approach based on the so-called “coffee stain” phenomenon is highly efficient for preparing well-oriented fibrils. This work demonstrates that the order parameters \( \langle P_2 \rangle \) and \( \langle P_4 \rangle \) of the orientation distribution function can be obtained solely from the measurement of changes in Raman scattered intensity as a function of the angle between the incident laser polarization and the aligned fibrils.

We developed a method that provides unique quantitative information regarding the orientation of selected chemical groups, which is not easily available from other structural methods. This approach offers additional constraints for building structural models. In the case of amyloid fibrils, the analysis of the obtained data revealed a high level of orientation of β-strands perpendicular to the long axis of the fibril and showed that the α-helical portions were also oriented, although to a lesser extent. We demonstrated that the two inter-chain disulfide bonds are an integral part of the fibril core. The acquired data were compared with proposed structural models of insulin fibrils. The orientation of C═O groups in the core of insulin fibrils
was in excellent quantitative agreement with Eisenberg’s model. A significant difference was found for the orientation of α-helices and disulfide bonds. Two of the three insulin disulfide bonds were found to be oriented nearly parallel to the fibril axis as predicted by the qualitative Dobson model.

We investigated diphenyl nanotubes using the same approach. It was found that the nanotube had cylindrical symmetry as well as multiple highly oriented functional groups. We determined the orientation angles of the carbonyl bond, NH$_3^+$ and COO$^-$ groups relative to the nanotube main axis.

In conclusion, we demonstrated that, at least for the limited dataset employed in the present work, non-resonance Raman spectroscopy in combination with Asher’s approach allows evaluating dihedral angles from the amide III band in peptide microcrystals, although parallel and anti-parallel β-sheet conformations cannot be distinguished. At the same time, parallel and anti-parallel β-sheet structures can be discriminated by using Raman spectroscopy in combination with advanced chemometrical methods. The differences in parallel and anti-parallel structural organization can be illustrated by means of calculated spectral components. In the case of microcrystals, a higher wavenumber of amide I band corresponds to an anti-parallel β-sheet, as opposed to a fibril, for which the upshifted amide I band can be assigned to a parallel β-sheet.

In this study, two types of TER spectra were acquired from a small peptide microcrystal, insulin fibrils and a native insulin cast film depending on the sample’s surface characteristics. We proposed a simple hypothetical mechanism that attributes these two different types of TER spectra to short-range chemical and long-range physical mechanisms for tip enhancement of Raman scattering. These results enhance our understanding and facilitate the interpretation of TER spectra of biological samples in general and protein aggregates in particular.
Future directions

NMR and X-ray crystallography have limited capabilities to elucidate complete structural information about amyloid fibrils and peptide nanotubes. A joint approach based on theory and experimental polarized Raman spectra should be able to solve this problem. At the early stages density function theory will be used to calculate optimal molecular geometry and polarizability tensors. The latter will be used with the experimental spectra to obtain the orientation distribution functions of corresponding chemical groups. Finally, molecular dynamics simulations will optimize the overall structure based on the determined orientation of individual chemical groups, optimized molecular conformation and intermolecular interactions.
REFERENCES


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