Ultraviolet Raman spectroscopy is uniquely suitable for studying amyloid diseases

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Amyloid fibrils associated with numerous degenerative diseases are non-crystalline and insoluble, and thus are not amenable to conventional X-ray crystallography and solution NMR, the classical tools of structural biology. We have recently demonstrated that deep UV resonance Raman (DUVRR) spectroscopy combined with hydrogen–deuterium exchange and advanced statistical analysis, 2D correlation in particular, allow for quantitative characterization of protein structural evolution at all stages of fibrillation in vitro. The application of DUVRR spectroscopy for studying the fibrillation of lysozyme is briefly overviewed here.

Keywords: Amyloid fibril, chemometrics, protein structure, Raman spectroscopy, two-dimensional correlation spectroscopy.

Introduction

Toxic misfolded proteins are associated with numerous debilitating diseases such as Alzheimer’s disease (AD), Parkinson’s disease (PD), Huntington’s diseases (HD), prion disease, amyotrophic lateral sclerosis (ALS), familial amyloid polyneuropathy (FAP), senile systemic amyloidosis and type II diabetes1,2. Misfolded proteins can adopt many conformations and assemblies, including small aggregates, fibrils and large inclusion bodies3. These large inclusions are striking features of multiple neurodegenerative diseases and their appearance correlates with pathology4. At the same time, there has been increasing evidence for the cytoprotective role of large inclusion bodies5. It has been reported recently that amyloidogenic peptides are abundant in semen and promote sexual transmission of HIV/AIDS6. In particular, fragments of prostatic acidic phosphatase form amyloid fibrils that capture HIV particles and drastically enhance HIV infection6. All known amyloids, including pathogenic and non-pathogenic forms of prion precursor (PrP) proteins display functional and structural heterogeneity (polymorphism)7,8. Fibril morphology could be potentially manipulated by small molecules, which might provide the basis of therapeutic approaches to suppress the formation of toxic aggregates9,10. The systematic rational design of successful therapeutic strategies necessitates: (i) having a robust method for structural characterization of amyloid polymorphs, (ii) comprehending the nature of the polymorphs and their toxicity, and (iii) eventually being able to control (suppress) the formation of toxic polymorphs.

Full-length amyloid fibrils are non-crystalline and insoluble, and thus are not amenable to conventional X-ray crystallography and solution NMR, the classic tools of structural biology. X-ray microcrystallography11,12 and solid state NMR13,14 utilized for short-peptide microcrystals and amyloid-like fibrils have contributed greatly to our knowledge about the fibril core structure. Several novel methods15–19 have been developed recently for structural characterization of fibrils, but they either require protein labelling or provide limited information about the fibril core structure. Molecular dynamics simulations of several PrP proteins have been successfully utilized for constructing models of the infectious aggregates16,19. Yet, there is currently a serious gap between our extended knowledge in the structural variations of model short-peptide microcrystals amenable to X-ray crystallography and the core structure of amyloid fibrils prepared from entire proteins.

Raman spectroscopy has been proven to be an efficient technique for characterizing highly scattering gelatinous and solid samples. Raman spectroscopy, in general, and resonance Raman spectroscopy, in particular, have been widely used for structural characterization of biological systems20–23. We have recently demonstrated that deep UV resonance Raman (DUVRR) spectroscopy is a powerful tool for protein structural characterization at all stages of fibrillation23–27. In particular, this method is capable of (i) detecting structural intermediates at early stages of fibrillation and determining their sequential order using 2D correlation analysis22; and (ii) characterizing the cross-β core structure of amyloid fibrils prepared from entire proteins by the hydrogen–deuterium exchange combined with DUVRR spectroscopy and advanced statistical analysis26. DUVRR spectroscopy is a novel method to acquire quantitative information on the peptide backbone conformation in large fibrillar aggregates. This method does not require isotope labelling and, consequently, opens the opportunity for comparative characterization of β-sheet structure in fibrils prepared from entire proteins and short peptides as well as in model segment microcrystals suitable for X-ray crystallography. In addition, the proposed method complements X-ray crystallography and

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solid-state NMR by providing the opportunity for real-time kinetic study of amyloid fibril formation, including the process of peptide aggregation, protofibril formation and fibril maturation.

In this article, the in vitro amyloid fibril formation of hen egg white lysozyme (herein referred to as lysozyme) is discussed based on DUVRR spectroscopic study. Hen egg white lysozyme was chosen because well-accumulated data on lysozyme structure and the ability to form amyloid fibrils in vitro make this protein an excellent model for studying protein structural transition during fibril formation.

**Early events of lysozyme fibrillation: probing a fibrillation nucleus directly by 2D correlation DUVRR spectroscopy**

Studying the early events of fibrillation is crucial for understanding the molecular forces driving the pathogenic process at its nucleation step. It is well established now that fibrillation is initiated by the partial destabilization of protein structure and proceeds through the nucleus formation followed by the rapid elongation of a fibril. The understanding of the nucleation mechanism is crucial for developing inhibitors for a limiting stage of the pathological process. So far, the proposed nucleation models have been verified by correlation of the fibrillation lag time or the rate of fibril accumulation with protein concentrations and other experimental conditions. The nucleation step of fibrillation remains poorly characterized as no experimental technique allows for direct monitoring of nucleus formation. We applied DUVRR spectroscopy combined with advanced statistical analysis, including 2D correlation spectroscopy, independent component analysis (ICA), and pure variable methods, to study nucleus formation during the fibrillation of lysozyme.

Fibrils were prepared by incubating a solution of lysozyme at pH 2.0 and 65°C for different times. Three sets of spectra (over 20 spectra each) were obtained at three different initial lysozyme concentrations, i.e. 70, 14 and 1.4 mg/ml. The time-dependent lysozyme DUVRR spectra are shown in Figure 1. The spectra exhibit pronounced amide bands, which report on the protein secondary structure. In particular, Cα-H bending band is strong in deep UVRR spectra of random coil and β-sheet, where the adjacent Cα-H and N-H bending vibrations are coupled, and is weak for peptides in the α-helical form. The frequency of Cα-H bending mode is different for β-sheet (~1396 cm⁻¹) and random coil (~1387 cm⁻¹) conformations. This band is especially sensitive to secondary structural transformations of proteins. The Cα-H bending band intensity increased with lysozyme incubation time, indicating the melting of the α-helix and the formation of the β-sheet and random coil. The evident decrease in the 1000 cm⁻¹ phenylalanine band intensity during the incubation corresponds to the tertiary structural changes of lysozyme. At the early stages of fibrillation, the melting of native lysozyme and the formation of a partially unfolded intermediate has been reported to be accompanied by a small β-sheet contribution. Classical factor analysis suggested the existence of only two principle components, indicating that the formation of β-sheet and the unfolding of native lysozyme are highly correlated.

We utilized 2D correlation Raman spectroscopy to distinguish these correlated processes and establish their sequential order. Synchronous Φ(ν₁, ν₂) and asynchronous Ψ(ν₁, ν₂) 2D Raman spectra were calculated following Noda’s approach:

\[
Φ(ν₁, ν₂) + iΨ(ν₁, ν₂) = \frac{1}{\pi(T_{max} - T_{min})} \int \hat{F}_1(ω) \cdot \hat{F}_2^*(ω) dω,
\]

where \( \hat{F}_1(ω) \) and \( \hat{F}_2^*(ω) \) were calculated based on experimental spectral intensities \( \bar{y}(ν, t) \) for all wave-numbers ν and incubation times t.

The 2D Raman correlation maps are shown in Figure 2 for the Cα-H bending region. The synchronous spectrum consists of two auto-peaks at 1387/1387 cm⁻¹ and 1396/1396 cm⁻¹, corresponding to random coil and β-sheet respectively, and two positive cross-peaks at 1387/1396 cm⁻¹ and 1396/1387 cm⁻¹, which show positive correlation between β-sheet and random coil changes. The asynchronous spectrum exhibits two opposite-sign areas at

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**Figure 1.** Experimental (blue) and modelled (red) DUVRR spectra of lysozyme incubated for different various times (adapted from Shashilov et al.)

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**Figure 2.** Synchronous (a) and asynchronous (b) 2D correlation Raman spectra of lysozyme.
1387/1396 cm$^{-1}$ and 1396/1387 cm$^{-1}$, indicating asynchronous formation of random coil and $\beta$-sheet. The valley at 1396/1378 cm$^{-1}$ shows that the $\beta$-sheet appeared after the random coil. These data can be used to distinguish between two alternative mechanisms of $\beta$-sheet formation. In a parallel process mechanism, random coil and $\beta$-sheet are produced directly from the native protein and should be completely correlated. In a step-by-step mechanism, the $\beta$-sheet develops from the partially unfolded intermediate (Scheme 1). In the latter case, formation of the $\beta$-sheet and the partially unfolded intermediate could correlate, but only partially. Consequently, the step-by-step mechanism proposed by Booth et al. was in complete agreement with our analysis above. Following the proposed step-by-step mechanism, the newly formed $\beta$-sheet in the solution part of the incubated samples could be assigned to the fibrillation nucleus. To further support this assignment, the supernatant of a lysozyme sample incubated for 48 h was used for seeding the fibrillation of fresh lysozyme. The seeding was successful and the fibrillation lag phase was eliminated. The newly formed $\beta$-sheet may only be a portion of the nucleus, leaving the rest of the protein unchanged. Similar to differential spectroscopy, our method specifically probes and characterizes protein structural transformations by eliminating the contribution of the unchanged parts. All experimental Raman spectra were fitted with three pure component spectra, i.e. spectra of nucleus $\beta$-sheet and partially unfolded intermediate calculated by ICA, and the experimental spectrum of native lysozyme. A mixed soft–hard modelling approach provided the refined DUVRR spectra of $\beta$-sheet and partially unfolded intermediate, kinetic profiles for all three species and the characteristic times (indicated in Scheme 1) for each step of lysozyme transformation. The independence of the characteristic times on protein concentration indicated that the early stages of lysozyme fibrillation, irreversible partial unfolding and nucleus formation were intramolecular processes.

**Characterizing the structure of amyloid fibril core by hydrogen–deuterium exchange deep UV resonance Raman spectroscopy**

Studying the structure of amyloid fibrils is important for the detailed understanding of fibrillogenesis at a molecular level. Amyloid fibrils are non-crystalline and insoluble, which limits the application of conventional X-ray crystallography and solution NMR, the classical tools of structural biology. Wide-angle X-ray scattering from flow-oriented fibrils has been utilized to estimate inter-strand and inter-sheet spacing in cross-$\beta$ structures. Solid-state NMR probes inter-atomic distances and torsion angles, which define local secondary structure and side-chain conformations. This technique, however, requires site-specific $^{13}$C and/or $^{15}$N labels, and is limited...
to short synthetic peptides and proteins. We have recently developed a new method based on the combination of hydrogen–deuterium exchange (H/D) and deep UV resonance Raman spectroscopy for characterizing the structure of fibrils prepared from natural proteins.

We hypothesized that amide N-H protons in unordered fragments of amyloid fibrils should exchange readily, whereas those hidden from water in the cross-β structure will remain protonated. Spectroscopically, HIX causes a down-shift of the amide II DUVRR band from ~1555 cm⁻¹ to ~1450 cm⁻¹ (amide II') and the virtual disappearance of the amide III band (Figure 3 a) originating from an unordered protein. DUVRR amide bands of the protonated cross-β core will remain unchanged (Figure 3 b). Thus, the HIX–DUVRR spectroscopic method will resolve the spectroscopic signature of the cross-β core from that of water-accessible moieties, including unordered structures and β-turns. The Bayesian approach was utilized for the mathematical separation of overlapping DUVRR bands of the deuterated, unordered moieties and the protonated cross-β core. The resolved DUVRR spectrum of the lysozyme fibril cross-β core (Figure 4) is dominated by sharp amide I (1674 cm⁻¹), amide II (1559 cm⁻¹), C=O-H (1400 cm⁻¹) and amide III (1224 cm⁻¹) bands. Approximate D₂ symmetry of the anti-parallel β-sheets gives rise to four vibrational modes in the amide I region, A (1675 cm⁻¹), B (~1690 cm⁻¹, not observed), B₂ (1633 cm⁻¹) and B₃ (unknown). Two of them, A (1675 cm⁻¹) and B₂ (1635 cm⁻¹), are well resolved in the cross-β core spectrum, indicating that the anti-parallel β-sheet structure dominates the lysozyme fibril core. The other two amide I bands may be obscured by the strong 1675 cm⁻¹ band resonantly enhanced via Albercht A term. The C=O-H band is composed of at least three narrow sub-bands centered at 1400, 1377 and 1356 cm⁻¹. Two of them, 1400 and 1356 cm⁻¹ are observed in the β-sheet region of poly-L-lysine spectra and originate from C=O-H in-plane and out-of-plane vibrations. This assignment was made based on MP2/TZV(d,p) normal mode analysis followed by the resonance Raman enhancement calculation of 2-amino-N-methylacetamide (NH₂CH₂CONHCH₃, ANMA), a well-accepted model for the protein amide group. In addition, we constrained the Ψ dihedral angle of ANMA to 135° to mimic the geometry of amide motifs of the anti-parallel β-sheet. The origin of the 1376 cm⁻¹ band is yet to be clarified. The amide III band is dominated by a ~1224 cm⁻¹ band with a small shoulder at 1255 cm⁻¹. Both bands originate predominantly from C-N stretching and N-H stretching. The former is strengthened because of the coupling with C=O-H bending, as evident from the MP2 calculation on the ANMA model.

As shown by Asher and co-workers, the average Ramachandran Ψ dihedral angle of the amide group can be calculated based on the band frequency of the amide III vibrational mode. For a multistranded β-sheet structure:

\[ \nu_{\text{Amid}}^\beta (\Psi) = 1244 \text{ (cm}^{-1}) - 54 \text{ (cm}^{-1}) \cdot \sin (\Psi + 26^\circ). \]  

Based on eq. (2), we estimate a Ψ angle of 133° for the cross-β core structure. This value falls within the range 129–133° that we estimated using the atomic resolution X-ray structure (2OMP) of the anti-parallel β-sheet fragment of the insulin fibril. It is worth noting that the DUVRR spectrum of insulin fibrils is also similar to that of lysozyme reported here, despite the completely different mechanisms of fibrillation of the two proteins.

In conclusion, 2D correlation deep UV Raman spectroscopy has been demonstrated to be a powerful tool for protein structural characterization at early stages of fibrillation. In particular, the formation of fibrillation nucleus has been directly monitored and quantitatively characte-
rized. Hydrogen–deuterium exchange DUVRR spectroscopy combined with Bayesian statistics allowed for structural characterization of the highly ordered cross-$\beta$ core of lysozyme fibrils. The resolved Raman signature indicated that the anti-parallel $\beta$-sheet is the dominant secondary structural conformation of the core.

Future development and application of Raman spectroscopy

Raman spectroscopy in general and ultraviolet Raman spectroscopy in particular have become powerful tools for protein structural characterization. It is especially valuable for studying aggregated proteins when the application of conventional X-ray crystallography and solution NMR, the classical tools of structural biology, is limited. Up to now, X-ray diffraction studies of the amyloid fibril core have been performed only on microcrystals grown from short fibril-forming segments. Hydrogen–deuterium exchange DUVRR should allow for comparative characterization of the cross-$\beta$ core structure in the model short-peptide fibrils and those formed from an amyloidogenic protein. This study is ongoing in our laboratory.

One of the major goals of Raman spectroscopy is to provide structural information about molecules. There has been great advancement in this respect recently, especially for relatively small molecules limited to approximately 20 heavy atoms. To further expand the capability of Raman spectroscopy to quantitatively report on the structure and conformation of large biopolymers, proteins and DNAs, is an immerging task for the coming years.

Significant advances in laser technology and the development of novel light detectors have dramatically improved spectroscopic methods for molecular characterization over the last decade. Although the accumulation time is significantly reduced in a typical measurement, the amount of information hidden in digital datasets composed of hundreds and thousands of points is dramatically increased. The once golden rule of previous generations of spectrosopists – that if you do not see a change in the spectrum by naked eye, then you are chasing a ghost – no longer applies. Further development and application of advanced statistical methods for Raman data analysis will allow for retrieving qualitative and quantitative information that is not evident otherwise.


SPECIAL SECTION: RAMAN SPECTROSCOPY


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