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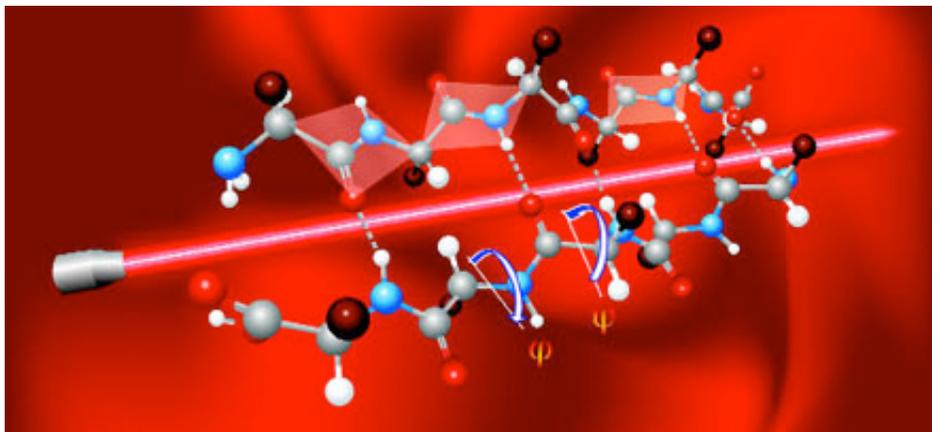
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Tools For Amyloids

Raman and infrared spectroscopic methods give glimpses of difficult-to-see parts of the amyloid formation process

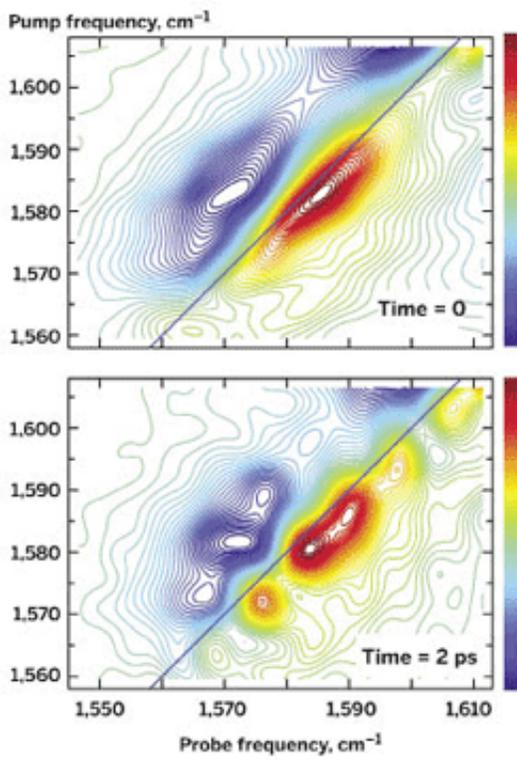
[Celia Henry Arnaud](#)



Courtesy of Igor Lednev

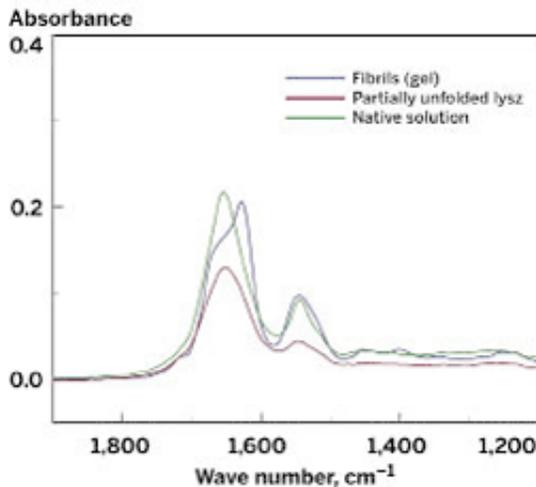
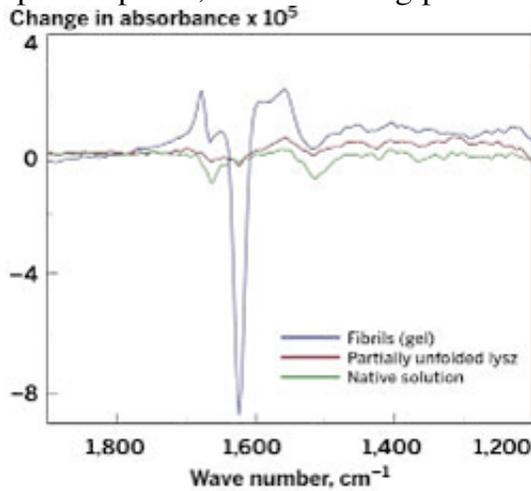
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TO THE CORE Deep-UV resonance Raman spectroscopy, represented by a laser beam, could help determine the dihedral angles of the peptide backbone within the core of amyloid fibrils.



Robin Hochstrasser

RELAXING RESIDUES Two-dimensional IR spectra show the time dependence of amide I vibrations of isotopically labeled leucine-17 in the Alzheimer's amyloid A β 40. The colors denote the intensity of the spectral peaks, with red being positive and blue negative.



Laurence Nafie

BIG CHANGE The magnitude of the vibrational circular dichroism spectrum (top) of lysozyme fibrils

increases greatly relative to that of the native protein. The IR spectrum (bottom) changes less dramatically.

Protein clumps known as amyloids are often bad actors inside the human body and have proven quite elusive to scientists' investigations. To remedy that situation, some researchers have been turning to vibrational spectroscopy techniques such as infrared and Raman spectroscopy, and with these tools they've been seeing amyloids with a new clarity.

Amyloids play a role in neurological diseases such as Alzheimer's and Parkinson's and in many other conditions, including diabetes. They are aggregates of extended fibrils composed of misfolded proteins and polypeptides, and they are rich in β -sheets, a flat type of protein secondary structure. Most infamous and most studied among the amyloids is amyloid- β -40 (A β 40), the one seen in the brains of Alzheimer's patients. Other medically significant ones include human islet amyloid polypeptide (hIAPP) in diabetes and α -synuclein in Parkinson's.

Fibrils of these amyloids are long, thin aggregates of proteins that contain a "cross- β " structure, in which individual β -strands are oriented perpendicular to the direction of fibril growth. After forming by aggregation, fibrils grow by recruiting additional proteins or peptides. Very little is known about the initial stages of fibril formation in particular.

Obtaining structural information about amyloids is challenging, not only because they are macroscopic and insoluble, but also because the most toxic forms in many amyloid-related diseases may be partly folded intermediates rather than fully formed fibrils. Although investigators rely on techniques such as X-ray crystallography and solid-state nuclear magnetic resonance spectrometry to study the structures of fully formed fibrils, these tools are not easily applied to transient intermediates, especially if they are membrane-associated. What is needed is a technique that provides precise structural resolution of membrane-bound proteins on a timescale sufficient to observe the formation of transient intermediates.

That's where vibrational techniques such as IR and Raman spectroscopy come in. From a purely structural standpoint, vibrational methods aren't as powerful as NMR or X-ray crystallography, but they can give researchers insights into the fibrils' properties and the dynamic processes by which they form with residue-by-residue resolution.

Molecular motions associated with the amide in the protein backbone are the key handles for IR and Raman spectroscopy of amyloids. The most common spectroscopic signature of amyloids is the amide I band, 1,600 to 1,700 cm^{-1} , which results from stretching of C–O bonds in carbonyl groups. The amide II band, caused by C–N stretching and C–N–H bending, occurs around 1,550 cm^{-1} .

Amyloid proteins generate complicated spectra that are difficult to interpret. To tease out contributions from individual amino acids, researchers turn to isotope labeling. Usually only one or at most two amino acids are labeled in a given experiment.

"When you label a residue's carbonyl with ^{13}C and ^{18}O , the IR spectrum of that particular residue gets shifted into a region of the spectrum where it's clear of everything else, which means you can study that residue or set of identical residues independently," says [Robin M. Hochstrasser](#), a chemistry professor at the University of Pennsylvania.

With IR techniques, "we can follow the process all the way from monomer states of the protein or polypeptide through soluble aggregates to fibrous aggregates," says [Sean M. Decatur](#), a chemistry professor and dean of the College of Arts & Sciences at Oberlin College, in Ohio. Decatur has used IR methods to study amyloid formation by A β 40, prion proteins, and cataract-causing γD -crystallin.

"We've been able to observe dynamics of rearrangements within amyloid aggregates," Decatur says. β -Sheet structures that form later in the aggregation process have lower energy states than the initial β -sheet structures because polypeptides adopt a more stable "register" between strands as they aggregate. The fibril's register refers to how well equivalent residues in neighboring strands line up along the length of the β -sheet.

To observe these rearrangements, Decatur heats samples to break up the aggregates and then watches them come back together. "Looking at reaggregation gives a clean system for looking at the aggregation kinetics," he says. Depending on how much he heats samples, he can break amyloid systems into monomers or amorphous aggregates. "We can go to different places along the potential-energy surface by controlling how we heat the sample and then follow the kinetics from those particular intermediate points," he says. This trick lets him see early stages of the process that he can't normally see because initial aggregation is very fast for the small peptide fragments he uses.

Decatur finds that there are two mechanisms of reaggregation and alignment, depending on the peptide concentration (*Proc. Natl. Acad. Sci. USA* **2005**, 102, 14272). At low concentrations, strands detach from the aggregate and reattach themselves, sometimes to a different aggregate than the one from which they broke off. At higher concentrations, strands

are less inclined to break off and roam about, so rearrangement tends to be confined within a particular aggregate. At intermediate concentrations, both processes occur.

So far, Decatur has worked only with small amyloid fragments approximately 15 amino acids long. "One of our goals is to go from working with very small fragments to larger systems," Decatur says. But "as you get to longer and longer systems, things slow down, and spectroscopically, they get more complicated," he adds.

One way to deal with the spectroscopic complexity of larger amyloid systems is with two-dimensional IR spectroscopy. Two-dimensional IR reveals which vibrational modes in a sample are close enough to one another to affect each other. Two-dimensional IR spectra are generated by exciting one vibrational mode with a "pump" laser pulse and using a second "probe" pulse to measure the response of that excited mode, as well as all other vibrational modes in the sample. If two vibrational modes are close enough in space to be "coupled," cross-peaks appear off the diagonal "line" that prominently divides 2-D IR spectra.

Researchers such as Hochstrasser, one of the original developers of 2-D IR, and [Martin T. Zanni](#) of the University of Wisconsin, Madison, are using the technique to study the formation of amyloid fibrils.

Hochstrasser's and Zanni's initial studies of amyloid fibrils focused on understanding how the 2-D IR signals correlate with fibrils' structural characteristics and have since moved on to the evolution of those signals at various timescales, including ones that correspond to initial fibrillization.

Hochstrasser has studied A β 40 using 2-D IR and isotope labeling. So far, he has labeled 18 of the amino acids in the 40-residue peptide. In any given experiment, only one or at most two residues can be labeled, because the spectrum otherwise becomes too complicated to interpret.

Early experiments with A β 40 have shown, Hochstrasser says, that the 2-D IR data yield the same repeat distances in various regions of the fibril as have been reported in studies using NMR data (*Proc. Natl. Acad. Sci. USA* **2008**, 105, 7720).

"The idea of being able to reproduce at least some main feature of the NMR structure using 2-D IR was an important first step," Hochstrasser says. "The objective of these experiments is to try to discover the microscopic mechanisms of putting together the fibril nuclei and beginning to grow into fibrils. There are models based on kinetic measurements using other observables. Two-dimensional IR has the promise of being the sharpest of all of these."

Zanni has similar goals, but with a different system. He focuses his 2-D IR efforts on hIAPP. This 37-residue polypeptide, which contains two β -strands connected by a loop, is the major component of amyloid fibrils deposited in the islets of Langerhans, the insulin-producing region of the pancreas, in some type 2 diabetes patients. Zanni isotope-labeled six amino acids located at regular intervals along the peptide chain.

Zanni acquired time traces of the evolution of cross-peaks between the labeled amino acids and the amide I band at 1,617 cm^{-1} , which is an indicator of the β -sheet growth. "In our case, all six of our residues show a cross-peak to β -sheet modes, and the time at which each residue is incorporated into the fiber," Zanni says. Lining up the time traces for different amino acids shows the order in which the amino acids of an incoming peptide become part of the β -sheet (*Proc. Natl. Acad. Sci. USA* **2009**, 106, 6614).

Zanni's results suggest that nucleation in hIAPP starts between the amyloid's valine-17 and alanine-25 residues, which are near the loop region of the fibril. The N-terminus β -sheet then forms faster than the C-terminus sheet.

"Our approach of using 2-D IR spectroscopy and isotope labels is not limited to just peptide systems like hIAPP or A β 40, but can also be applied to achieve site-specific structural and kinetic information in much larger protein systems," Zanni says.

Deep-ultraviolet resonance Raman spectroscopy is proving to be another valuable method for studying amyloids. [Igor K. Lednev](#), a chemistry professor at the State University of New York, Albany, uses this method to characterize the structure and fibrillization mechanism of model fibril systems such as lysozyme (an enzyme that attacks bacterial cell walls) and disease-related amyloids such as A β 40 and prion proteins.

He uses a Raman excitation wavelength at 197 nm that overlaps with an electronic transition of the amide chromophore, the building block of the peptide backbone. This resonance enhances the amide's Raman signal 1 millionfold. The deep-UV Raman signature depends on the dihedral angles along the peptide backbone, designated ψ , which determine the 3-D structure of the protein.

"At the moment, we have seven or eight Raman bands that we know how to interpret qualitatively," Lednev says. He notes that [Sanford A. Asher](#) of the University of Pittsburgh developed a semiempirical approach for calculating the ψ angle, but this technique still is severely limited. "If we could get such understanding for all bands," Lednev says, "we would get very nice knowledge of the distribution of dihedral angles and as a result the 3-D structure of the polypeptide backbone."

Lednev combines Raman spectroscopy with hydrogen-deuterium exchange to probe the structure of the fibril nucleus, or core. When the fibril is transferred to D₂O, solvent-accessible protons exchange for deuteriums. Only amides within the fibril core remain undeuterated. "Using statistical methods, we can extract the signature of the protonated part of the fibrils," Lednev says.

"No other techniques at the moment can give direct structural information about the fibril core of full-length proteins," Lednev says. His group reported that the fibril core is extremely well organized (*J. Am. Chem. Soc.* [2007, 129, 11002](#); [2008, 130, 5852](#)). The Raman spectrum of a fibril core has very narrow bands such as those normally observed for microcrystals of small polypeptides, he says.

Obtaining structural information about amyloids is challenging.

The structure of the fibril core depends on both the protein itself and the specific conditions under which it forms. "Fibrils prepared from the same protein but under different conditions form polymorphs," Lednev says. "We can distinguish polymorphs prepared from the same protein. It's well-known that only specific polymorphs are associated with severe diseases like Alzheimer's, Huntington's, and Parkinson's, so it's very important to characterize specific polymorphs."

For example, looking at prion fibril polymorphs, Lednev determined that the hydrogen bonding between β -sheet strands differs in their respective fibril cores. "We interpreted it preliminarily by saying that the different strands are twisted relative to each other," he says.

In the future, Lednev plans to use deep-UV resonance Raman to compare in vitro fibrils with disease-related fibrils from patients. He is currently collaborating with J. Andrew Carlson and Jiang Qian at the [Albany Medical Center](#) to obtain fibrils from patients for these studies.

Lednev also hopes to determine whether the current picture of fibril cores is correct. "The majority of the structural information we have about the fibril core is based on short-peptide microcrystals and short-peptide fibrils," he says. Researchers determine the segment of the fibril that makes up the core and then use just that short fragment to make crystals for X-ray analysis. They then extrapolate the structure of the entire fibril on the basis of data from this fragment.

There's no way to know whether this extrapolation is valid, Lednev says. "With our method, we can compare Raman spectra from both the microcrystals and full-length proteins," he notes. "We will determine how relevant this microcrystal structure is to the structure of the corresponding full-length proteins."

[Laurence A. Nafie](#), a chemistry professor at Syracuse University, is applying yet another vibrational technique, vibrational circular dichroism (VCD), to amyloids. In VCD, a sample is interrogated with circularly polarized IR light that alternates between left and right circular polarization. Every band in a sample's IR spectrum has a corresponding VCD band, the sign of which depends on the motions of the electrons and nuclei as the molecule vibrates.

The VCD spectrum is typically much weaker than the IR spectrum, but Nafie, collaborating with Lednev, has found unusual features in the VCD spectra of amyloids (*J. Am. Chem. Soc.* [2007, 129, 12364](#)). "When we looked at our first fibril-solution spectrum, the signal was enormous," Nafie says. "We measured the lysozyme protein VCD, and it was a normal size VCD." However, as aggregation began, "suddenly the fibril VCD was between 10 and 100 times larger, depending on the fibril state and how long the fibril had been developing," he says.

The group has seen similarly large VCD spectra with insulin fibrils. Insulin and lysozyme are "quite different proteins with somewhat different secondary structure, but their fibril spectra are very similar and very large," Nafie says. "Both of these proteins go into the fibril state and have a structure that's very similar."

The main protein regions probed by VCD are the same ones probed by IR spectroscopy, the amide I and amide II bands. The shape, position, and relative size of those bands in VCD convey information about the protein's normal secondary structure.

Nafie finds that every fibril VCD spectrum has five main bands. A large central negative band is located at about 1,624 cm^{-1} . Two positive bands occur at lower frequencies, and two positive bands occur at higher frequencies. The frequencies

of each of the peaks in the VCD spectra of insulin and lysozyme fibrils differ only slightly, and their relative intensities depend on what's happening to the sample, he says.

"The evidence is growing that as the fibril forms and develops, these five major VCD features are reporting on the helical supermolecular structure of the protein's fibril," Nafie says.

He uses VCD to watch the real-time evolution of fibrils. The central VCD band becomes increasingly negative as the fibril forms, for example, even though the IR spectrum doesn't change. Also, the ratio of the magnitudes of the VCD and IR peaks is roughly equal to the ratio of the amplitude of the vibrational motion to the wavelength, Nafie explains. From this, he can eke out details about structural motifs that are in motion as fibrils form.

Such large VCD peaks suggest very large concerted motions of the aggregate, Nafie says. "The VCD is on the order of what you see in the UV" with electronic circular dichroism, he adds. "It just shouldn't be that way unless you have a very large structure. We think we're reporting on much longer dimensions of amyloid fibrils or much richer structural geometry."

More and more researchers are entering the amyloid field and bringing new techniques with them, observes [Ronald B. Wetzel](#), a structural biologist at the University of Pittsburgh. Some are drawn in by the protein chemistry and some by the potential medical payoffs of learning more. "We're still in a growth phase where we're hungry for new techniques that allow us to look at different aspects," he says. "There's definitely a place for these innovations that are happening in vibrational spectroscopy."

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