The Journal of Chemical Physics

2D-IR spectroscopy of proteins in H_2O—A Perspective

Cite as: J. Chem. Phys. **158**, 030901 (2023); https://doi.org/10.1063/5.0129480
Submitted: 05 October 2022 • Accepted: 06 December 2022 • Accepted Manuscript Online: 27 December 2022 • Published Online: 17 January 2023
Published open access through an agreement with JISC Collections

Samantha H. Rutherford, Matthew J. Baker and Neil T. Hunt

The Journal of Chemical Physics PERSPECTIVE scitation.org/journal/jcp

2D-IR spectroscopy of proteins in H_2O—A Perspective

Cite as: J. Chem. Phys. 158, 030901 (2023); doi: 10.1063/5.0129480
Submitted: 5 October 2022 • Accepted: 6 December 2022 •
Published Online: 17 January 2023

Samantha H. Rutherford,[1] Matthew J. Baker,[2,3] and Neil T. Hunt[4,a)]

AFFILIATIONS

[1] WestCHEM, Department of Pure and Applied Chemistry, University of Strathclyde, Technology and Innovation Centre, 99 George Street, Glasgow G1 1RD, United Kingdom
[2] School of Medicine, Faculty of Clinical Biomedical Sciences, University of Central Lancashire, Preston PR1 2HE, United Kingdom
[3] Dxcover Ltd., Suite RC534, 204 George Street, Glasgow G1 1XL, United Kingdom
[4] Department of Chemistry and York Biomedical Research Institute, University of York, York YO10 5DD, United Kingdom

[a)] **Author to whom correspondence should be addressed:** neil.hunt@york.ac.uk

ABSTRACT

The form of the amide I infrared absorption band provides a sensitive probe of the secondary structure and dynamics of proteins in the solution phase. However, the frequency coincidence of the amide I band with the bending vibrational mode of H_2O has necessitated the widespread use of deuterated solvents. Recently, it has been demonstrated that ultrafast 2D-IR spectroscopy allows the detection of the protein amide I band in H_2O-based fluids, meaning that IR methods can now be applied to study proteins in physiologically relevant solvents. In this perspective, we describe the basis of the 2D-IR method for observing the protein amide I band in H_2O and show how this development has the potential to impact areas ranging from our fundamental appreciation of protein structural dynamics to new applications for 2D-IR spectroscopy in the analytical and biomedical sciences. In addition, we discuss how the spectral response of water, rather than being a hindrance, now provides a basis for new approaches to data pre-processing, standardization of 2D-IR data collection, and signal quantification. Ultimately, we visualize a direction of travel toward the creation of 2D-IR spectral libraries that can be linked to advanced computational methods for use in high-throughput protein screening and disease diagnosis.

INTRODUCTION

The link between protein structure and function is well established, but the fact that biological processes occur predominantly in the solution-phase means that *in vivo* analysis of biological systems requires tools that can probe protein structure under aqueous (H_2O) conditions. The most powerful structural analysis tools that operate on solution phase samples, nuclear magnetic resonance (NMR) and infrared (IR) absorption spectroscopies, both experience challenges that dictate the use of isotope labeling or isotopic exchange of the solvent. In the case of IR spectroscopy, the amide I (ν_{amI}) band of the peptide link, essentially the C=O stretching vibrational mode, reports sensitively on secondary structure.[1–11] This is due to the macroscopic coupling of amide I modes of individual peptide residues that arises from hydrogen bonding patterns and spatial relationships in secondary structural elements, which influences the frequency, width, and shape of the amide I band of the molecule.[1,2,5–8,12] The problem arises because the frequency range of the amide I band of proteins (~1620 to 1680 cm^{-1}) coincides almost exactly with the broad H–O–H bending vibrational mode of H_2O (δ_{HOH}). This frequency overlap means that transmission IR absorption spectroscopy requires very short path length samples to avoid saturation of the δ_{HOH} mode.[13] Even under these conditions or when applying alternate methods such as attenuated total reflection (ATR) spectroscopies, it remains extremely difficult to achieve unequivocal separation of H_2O and protein signals, while the analysis of mixtures of proteins is even more challenging.

The frequency coincidence of the amide I and δ_{HOH} modes has led to the widespread application of H/D exchange in IR spectroscopy, whereby deuterated solvents are used in place of H_2O. The isotope shift of the water-bending mode (δ_{HOH}–δ_{DOD}) is about

400 cm^{-1}, effectively enabling direct measurement of the protein amide I band free of solvent interference. This elegant solution has enabled amide-I-based IR spectroscopy of proteins to become a standard method, leading to a detailed understanding of the spectroscopy and dynamics of proteins in solution.[12,14–24]

There remain, however, applications where IR spectroscopic probing of proteins would be advantageous, but deuteration is either undesirable or impossible. These include the use of IR methods to analyze the protein content of biofluids, where variations in the protein content could be indicative of changes in patient health, but solvent exchange is practically extremely difficult and diagnostically highly undesirable.[25–27] Similarly, IR analysis of protein–drug binding for drug design applications could provide useful insight into the structural and dynamic impacts of drug candidate molecules binding to target proteins, but large-scale sample deuteration is inefficient and economically unviable. The replacement of H_2O with heavy water also raises fundamental questions relating to the impact of the isotopic exchange on biomolecular function and behavior.[28,29] The kinetic isotope effect highlights the fact that deuteration affects the rates of biological reactions, while the importance of H-bonding to protein structures means that heavier solvent molecules are likely to change the rates of structural fluctuations and transitions between structures during function. Finally, the removal of the natural resonance between the δ_{HOH} mode and the amide I band will change the vibrational coupling and energy transfer processes between solvent and solute, meaning that deuterated systems may not perfectly mimic the *in vivo* situation.[28]

It has recently been demonstrated that ultrafast third-order IR spectroscopy methods, such as 2D-IR, can offer a route to studying the protein amide I band in H_2O-rich fluids.[13] The basis for this is the fact that the coupling of amide I modes in secondary structural elements leads to molar extinction coefficients for protein amide I bands that are some two orders of magnitude greater than those of the δ_{HOH} mode.[30] Under normal conditions for IR absorption spectroscopy (<1 mM protein concentration and 10–50 μm path length), the absorbance of a large numerical excess of weakly absorbing solvent molecules far exceeds that of the few, strongly absorbing protein molecules [Fig. 1(a)]. In contrast, the four laser–molecule interactions needed to generate a third-order non-linear optical spectroscopy signal lead to a quadratic dependence of the signal upon the molar extinction coefficient such that the few strongly absorbing protein molecules dominate the signal and the solvent response is much weaker [Fig. 1(b)].[12,13,31]

The use of 2D-IR methods confers other advantages for revealing the information content of the amide I band. The higher-order dependence of the signal on the extinction coefficient means that the measured linewidths in 2D-IR spectra are slightly narrower than those from absorption spectroscopy, increasing spectral resolution.[32] This can be particularly advantageous when attempting to differentiate amide I contributions from different protein secondary structural elements.[33] The 2D-IR signal strength has also been shown to report on the extent of coupling within a macromolecule, which adds information relative to an IR absorption spectroscopy measurement.[31,34] The ability of 2D-IR to spread the amide I band over two frequency axes with the off-diagonal part of the spectrum reporting sensitively on amide I coupling, energy transfer, and structural dynamics means that each protein gives rise to a specific 2D spectral map that is intimately linked to its structure and dynamics in solution. This direct link between structure and spectroscopy confers benefits either for observing small structural and dynamic changes upon perturbation of the protein structure, such as by small molecule binding, or for the separation of signals in mixtures.[35–37] Finally, the use of ultrashort-duration laser pulses to generate the 2D-IR signal enables the measurement of the amide I response in a time-resolved manner. This leads to three benefits. First, the vibrational lifetime of the amide I band of proteins (~1 ps)

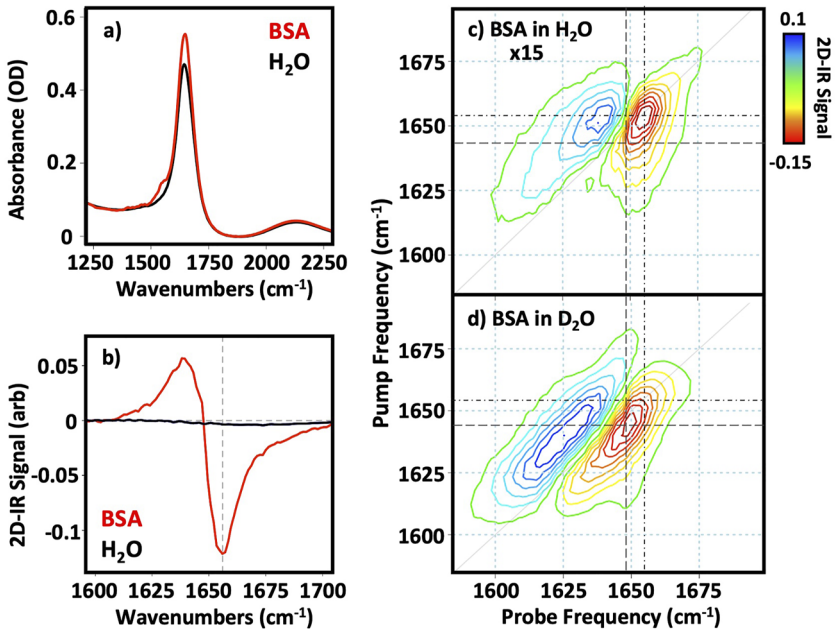

FIG. 1. (a) FTIR spectra of H_2O (black) and a solution of bovine serum albumin (BSA, 30 g/l) in H_2O (red). The protein concentration corresponds to ~0.45 mM, and the sample path length was ~3 μm (b) Pump frequency slices taken from the 2D-IR spectra of H_2O (black) and BSA (30 g/l) in H_2O (red) at a frequency of 1656 cm^{-1}. 2D-IR spectra of BSA (30 g/l) shown in both (c) H_2O with path length ~3 μm and (d) D_2O with path length of 25 μm. Note (c) is scaled by a factor of 15 to enable plotting on a similar scale to that of (d).[13] All 2D-IR spectra were obtained with a waiting time of 250 fs.

is significantly longer than that of the δ_{HOH} mode (~220 fs),[38–40] meaning that the experimental waiting time (analogous to a pump-probe delay time) can be used to separate signals from protein and solvent.[13] Second, a separate signal arising from small residual water heating appears after the protein signal has decayed, and this can be used as an internal standard for signal processing and analysis purposes.[41] Third, it has been shown that the amide I vibrational lifetime depends on secondary structure, which offers an additional possibility for discriminating structure elements.[42]

The ability to measure protein structure and dynamics in H_2O-rich fluids allows the extension of IR methods into analytical applications of the type described above. The purpose of this perspective is to review progress in measuring the 2D-IR spectra of proteins in water, including new data analysis protocols that are made possible by the water response that presents such a problem to IR absorption methods. Thereafter, we will discuss applications of the approach in a range of areas, including the measurement of protein dynamics in H_2O, the observation of intermolecular interactions, and applications of 2D-IR *in vivo*. In doing so, we will consider future directions and the likely result of combining this new spectroscopic approach with high-speed data acquisition strategies for the exploitation of 2D-IR in analytical and biomedical contexts.

METHODOLOGY

The method for acquiring 2D-IR spectra has been described in detail previously,[12] so we will focus specifically on the experimental approach for acquiring 2D-IR spectra of the amide I band of proteins in H_2O-rich fluids.[13] As shown in Fig. 1(a), the IR absorption spectrum of a solution of protein [in this case bovine serum albumin (BSA)] at sub-mM concentrations in an aqueous (H_2O) buffer solution is dominated in the amide I region (near 1650 cm^{-1}) by the absorption band due to the δ_{HOH} mode. Unless the sample path length is restricted, this band will generate an absorbance in excess of unity, which can cause peak shape distortion and reduced signal-to-noise ratios in 2D-IR spectra. To limit the absorbance due to the δ_{HOH} band, samples were prepared with path lengths of ~3 μm.[13] This was achieved by placing a droplet of protein solution (~15 μl) onto a CaF_2 window. The second window was then placed on top of the first and held in place using a standard IR transmission cell arrangement. The only difference from standard sample preparation methods for 2D-IR spectroscopy was the omission of a physical spacer between the CaF_2 plates. Instead, the path length was controlled by adjusting the tightness of the front plate of the sample cell. It was found that adjusting this until an absorbance value of ~0.01 was achieved for the band due to the water $\delta_{HOH} + \nu_{libration}$ combination band near 2100 cm^{-1} corresponded to a path length of ~2.75 μm.[13]

2D-IR data acquisition was achieved using the now-standard approach with three pulses (two "pump" and one "probe") arranged in a pump-probe-style geometry and the pump–pump delay time (τ) being scanned using a mid-IR pulse shaper.[43,44] However, the method would also be applicable using non-pulse shaping methods and boxCARS geometries.[32,45] For the 2D-IR data shown in Fig. 1, τ was typically scanned for 3–4 ps using four frame phase cycling. The acquisition time for a single spectrum was around 60 s.[13] Data were collected at a range of waiting times (T_w) in order to facilitate studies of protein dynamics, though for a purely spectroscopic analysis, the measurement time can be reduced by acquiring only two waiting times of 250 fs and ~5 ps; the former to measure the protein spectrum, the latter to sample the H_2O thermal signal for use in data analysis (see below).[41] The impact of H_2O absorption of the pump and probe beams by the sample as well as the need for a shorter sample path length lead to a slight reduction in the signal-to-noise ratio in comparison to a spectrum of the same protein at the same concentration obtained under deuterated conditions [Figs. 1(c) and 1(d)].

Measuring the 2D-IR spectrum of a sample in which bands due to water (δ_{HOH}) and protein (ν_{amI}) overlap means that signals from both solvent and solute will be generated and therefore detected. This is demonstrated in Fig. 2, where a 2D-IR spectrum of bovine serum albumin (BSA) in H_2O is shown as a function of T_w in order to highlight the changes undergone by the signal. Initially, the signal is dominated by the protein amide I band, though, at T_w values shorter than 250 fs, there is also a contribution from δ_{HOH}, which is

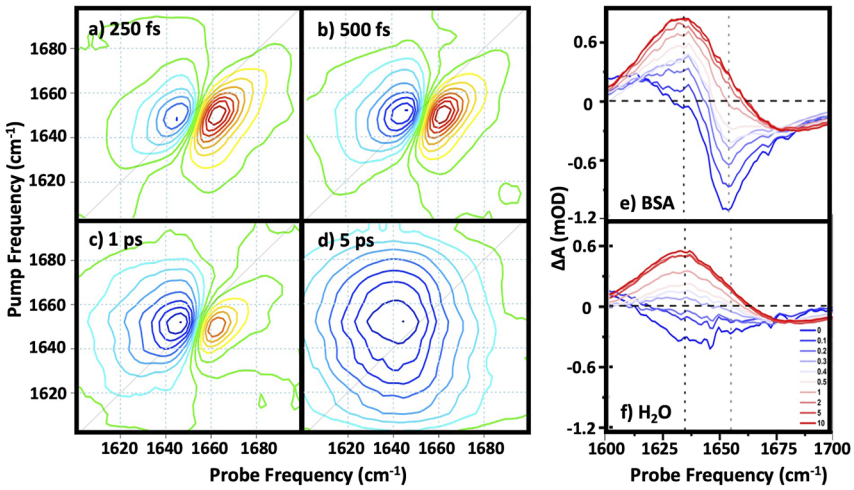

FIG. 2. 2D-IR spectra of BSA in H_2O obtained at the following waiting times: (a) 250 fs, (b) 500 fs, (c) 1 ps, (d) 5 ps. IR pump–probe spectra of (e) BSA and (f) H_2O. Waiting times range from 0 ps (blue) to 10 ps (red).[13] Note: These data were obtained using a different laser/spectrometer to that in Fig. 1.

~50-times smaller than the amide I band. As T_w increases, the water signal decays rapidly as a result of a vibrational relaxation time of 220 fs.[38–40] This is replaced by a different signal caused by changes in the shape of the δ_{HOH} band due to a small heating effect as the δ_{HOH} mode relaxes. This thermal signal persists to waiting times on the order of milliseconds, but the transition from the δ_{HOH} signal to the thermal one leads to the water response passing through zero when T_w is on the order of 200–250 fs.[13] In contrast, the signal from the amide I mode of the protein decays to the baseline over a period of a few picoseconds, arising from an amide I relaxation time of around 800 fs. This means that the protein signal can be detected with virtually no interference from the H_2O signal by setting T_w to ~250 fs. Moreover, the small size and reproducibility of the H_2O signal mean that it can be reliably scaled and subtracted to reveal the solvent-free protein amide I band at all waiting times.

In addition to reliable solvent subtraction, the small but distinctive water response has been found to have several benefits for 2D-IR implementation. In particular, the size of the thermal response at a fixed waiting time after the protein amide I signal has decayed has been found to provide a means of standardizing the spectra.[41] By making a large number of repeated measurements of the spectrum of the same sample over a period of time, it was found that the size of the H_2O thermal response at longer waiting times [T_w = 5 ps, Fig. 2(d)] correlated linearly with the amplitude of the amide I band at T_w = 250 fs [Fig. 2(a)]. This allowed the thermal response to be used as an internal standard because it provides an *in situ* measurement of the experimental factors such as laser power, beam overlap, and sample path length that affect the magnitude of the 2D-IR signal measured in a given experiment. Path length, in particular, was found to be a source of significant sample-to-sample variation due to the need to manually define the very short path length. Thus, normalization of 2D-IR data to the thermal water response facilitates accurate comparison of different samples as well as the ability to calculate difference spectra in a manner that significantly reduces the impact of experimental errors that can occur when preparing separate samples.[41]

More recently, this process of normalizing the 2D-IR signal to the H_2O thermal response has been extended so that the water signal can be used as a tool to monitor laser bandwidth as well as sample-to-sample variations in alignment and sample cell construction.[46] The bandwidth of the water signal is extremely broad and can exceed the typical laser pulse bandwidth used for 2D-IR data acquisition. This is especially the case for high pulse-repetition rate Yb-based laser systems, which lend themselves to more analytical applications of 2D-IR by virtue of the faster data acquisition rates that they enable.[43,47,48] These systems currently exhibit bandwidths on the order of 80 cm^{-1} in the amide I region. The latter is potentially problematic for protein studies due to the broad nature of the amide I band, and so the ability to monitor the laser bandwidth profile during the measurement provides a guide for correction of baseline fluctuations[49] and the ability to identify regions of the spectrum where reduced pulse energy may be impacting the shape of the recovered amide I signals. Bandwidth correction is achieved by using the shape of the thermal water response as a direct measurement of the laser pulse bandwidth and has been shown to lead to an effective pre-analysis data processing method for 2D-IR data that improves the signal-to-noise ratio and reduces the detection limit for analytical applications of 2D-IR. Overall, standardization of 2D-IR spectra via measurement of the water thermal response will provide an efficient route to data collection in high-throughput measurement protocols.

PROTEIN SPECTROSCOPY

One of the fundamental questions associated with the use of deuteration for amide I IR spectroscopy is the impact of the isotopic substitution upon the observed spectra, for example, in terms of band positions and line shapes. It is widely assumed that the impact is small, and so measurements in D_2O provide a good basis for our understanding of proteins in H_2O.[29] The ability to measure the amide I band in the solution phase free from complications of overlapping water signals now allows this question to be addressed using experimental data. Although few studies in this area have been undertaken so far, 2D-IR measurements of the same proteins in both H_2O and D_2O suggest that changes in both amide I band position and shape may occur [Figs. 1(c) and 1(d)].[13] The impact on the band frequency is modest but clear, with deuteration leading to a frequency down shift of around 10 cm^{-1}. This is consistent with predictions from computational simulations and also with measurements in which ATR-IR absorption was used to subtract the solvent response and implies that there is no dramatic change in amide I behavior with solvent.[29] It is however noticeable that the change in solvent leads to different 2D shapes of the amide I band, with changes in linewidth observed as well as the central frequency.[13] As the 2D-IR linewidths report on inter-peptide vibrational couplings, structural and vibrational dynamics, and energy transfer processes, this observation is worthy of further study because it raises the prospect that, although the amide I band is not significantly affected in terms of its frequency, the underlying molecular dynamics may be perturbed.[28] In any definitive study, it will be essential to ensure complete deuteration of the protein backbone, but the indications that the amide I band shift between H_2O and D_2O is not linear with frequency suggest that a detailed analysis would be warranted. Given the important role that has been played by computational simulations in developing our understanding of the link between structure and 2D spectroscopies,[2,6,50–53] supporting these experimental developments with parallel computational investigations of the impact of H_2O/D_2O exchange will be essential.

PROTEIN DYNAMICS

In addition to work on the amide I spectroscopy of proteins, there have been studies that show an impact of the isotopic composition of the solvent on their vibrational relaxation behavior. In a study that measured the vibrational lifetime of the amide I band of several globular proteins, it was found that replacing D_2O with H_2O resulted in a 10% reduction in relaxation time on average (Fig. 3).[13] It has been hypothesized that the resonance of the δ_{HOH} mode with the amide I band, along with the propensity for direct hydrogen bond contacts to occur between the solvent and the peptide C=O unit, would lead to an efficient vibrational relaxation mechanism, similar to that observed for other chemical systems where an analogous resonance exists.[54,55] The 10% reduction observed for the studied proteins would seem to be much less dramatic than might be expected based on these studies of solutes, which exhibit far less structural complexity. However, in the case of proteins, it must be

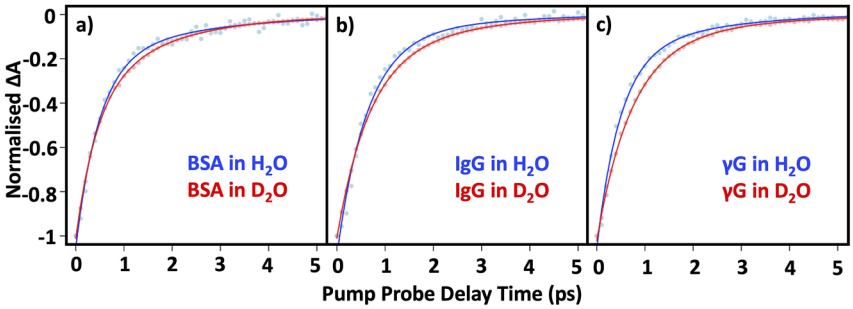

FIG. 3. Temporal variation of the amide I v = 0–1 transition for (a) BSA, (b) Immunoglobulin G, and (c) γ-globulins in both D_2O (red) and H_2O (blue) solvents. Solid lines denote tri-exponential fits for H_2O and single exponential decays for D_2O.[13]

taken into account that (i) only a fraction of protein residues will be fully exposed to the solvent, (ii) each protein will differ in terms of its structure and solvent interactions, and (iii) the process of intramolecular relaxation within secondary structural units might be expected to be rapid due to H-bonding and associated vibrational coupling, and this may compete effectively with solvent-mediated relaxation. Thus, a careful study of relaxation behavior as a function of secondary structure and solvent exposure would be beneficial in addressing these open questions.

A detailed understanding of amide I dynamics in H_2O would also provide a valuable link to studies that employ vibrational probe groups with mode frequencies that are shifted away from those of the amide I band to interrogate protein structural dynamics. Common examples include azido, thiocyanate, and their derivatives, among others.[49,56–70] Such studies have provided a wide range of site-specific insights into the inherent dynamics of proteins and their solvent interactions. Because they exploit probes with vibrational mode frequencies in the IR window around 2000 cm^{-1}, well separated from backbone and solvent modes, these studies are often performed in H_2O.[58] Currently, these data have to be correlated with amide I dynamics derived from measurements in D_2O, which represents a fundamental difference between the samples that could now be addressed.

INTERMOLECULAR INTERACTIONS

Another area in which the ability to measure the amide I band in H_2O is expected to be of benefit is in the field of biological intermolecular interactions. For example, a recent publication has shown the kinetics of amyloid fibril formation, which involves the formation of large aggregates, to be slower in D_2O as compared to H_2O.[71] To study fibril formation in H_2O, 2D-IR spectra were obtained at regular intervals over the course of amyloid fibril formation, with signals due to β-sheets, which are used as a marker for fibril formation, appearing earlier in H_2O than D_2O.[71] It was also suggested that the initial structures from which the fibrils form could be different,[71] though with the caveat mentioned above that fundamental differences in the amide I spectroscopy of the same molecule in H_2O and D_2O are not yet fully understood.

This work builds on a number of studies that established the methodology for following processes such as fibril formation on biological timescales using 2D-IR spectroscopy, including developing the use of isotope labeling to reveal molecular details of the fibrillation pathway.[21,23,24,72–79] This has recently been extended by a novel approach to studying fibril formation in blood serum, where different fibril structures were observed as compared to those found in aqueous buffer solutions. In this study, the natural protein content of the serum samples was depleted via antibody-based methods prior to freezing, lyophilization, and resuspension in D_2O. In this manner, the study was able to evaluate the impact of additional molecular components of blood serum, such as salts, lipids, and sugars upon the fibrillation pathway, albeit still in a largely deuterated solvent.[80]

A particularly interesting outcome of these fibrillation studies is the observation that intermolecular processes between biomolecules may be influenced by solvation in an isotopically labeled solvent. There have been studies that suggest that the nature of D_2O, in terms of its structure, H-bonding, and ability to solubilize biomolecules, may be different from that of H_2O.[28] If this is found to fundamentally alter intermolecular processes, then it will have implications for studies of complex biomolecular mechanisms, such as transcription, replication, or aggregation, which often involve the formation of clusters or assemblies.

The choice of solvent could also impact small molecule binding to proteins. A small number of studies have highlighted changes in the structural dynamics of proteins upon binding of molecules ranging from substrates to inhibitor analogs and drug molecules, and these have shown a general trend toward complex formation leading to an increase in stiffness or reduced dynamic fluctuations in the protein backbone.[35,36,56,66] As some of these studies have been performed using site-specific vibrational probes in H_2O, the implication is that this effect is not exclusive to a particular solvent, though the extent of the effect may differ between H_2O and D_2O if the solvent has a direct impact on the native structural dynamics of the protein, as has been demonstrated.[69] A detailed study of the impact of small molecule binding on amide I spectroscopy and dynamics that would confirm this is yet to be undertaken, though it has been shown that 2D-IR spectroscopy can detect spectroscopic changes in the serum albumin protein when paracetamol is introduced into blood serum samples (Fig. 4).[36] The changes observed included a shift in the amide I band position and changes in amplitude of the 2D-IR signal, which are suggestive of an impact upon the vibrational coupling within secondary structure elements of serum albumin. These could imply altered structural dynamics caused by drug binding but will require further investigation. Given the central position in biological function occupied by intermolecular interactions of all types, this area is one that will clearly benefit from more experimental data.

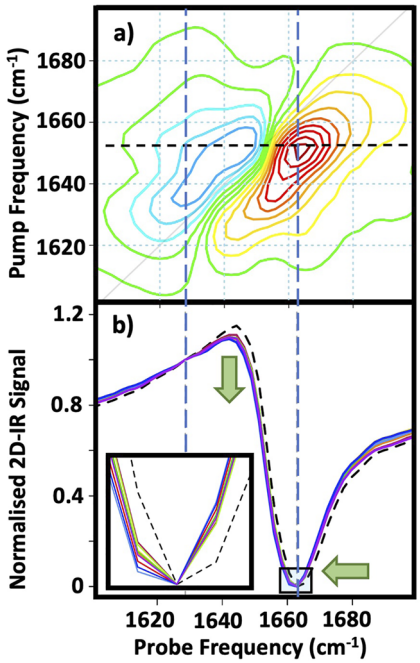

FIG. 4. (a) 2D-IR spectrum of blood serum. Black dashed horizontal line at frequency of 1652 cm^{-1} is used for further analysis in (b). (b) Slices through the 2D-IR spectra of a number of serum-paracetamol mixtures at a range of paracetamol concentrations. The black dashed curve denotes the neat serum spectrum obtained in the absence of the drug. For clarity, the slice is base-lined at 1661 cm^{-1} and normalized to the globulin contribution at 1629 cm^{-1}, as indicated by the blue vertical dashed lines. Green arrows indicate line shape changes with increasing paracetamol concentration. Inset shows the v = 0–1 peak expanded for clarity, denoted by the black box in the main figure. It is noted that the 2D-IR signal from free paracetamol is not detectable at the concentrations used in the study.[36]

PROGRESS TOWARDS *IN VIVO* 2D-IR

One of the most exciting new avenues of inquiry that the ability to measure amide I protein spectroscopy in H_2O opens up is the ability to study systems *in vivo*. This has been an active area of study since the first demonstration of the method, with studies focusing on measuring the 2D-IR spectra of proteins in blood serum.[13,36,37,41,46,80]

The analysis of blood serum is technically challenging because it consists of a complex mixture of many proteins in an aqueous matrix that also contains lipids, sugars, ions, and nucleic acids. However, the fact that blood serum encounters most of the major organs in the body means that it has the potential to provide a chemical snapshot of metabolic processes and so could provide useful data on patient health or an early warning of disease onset. The protein component, in particular, has been shown to fluctuate in terms of composition and the concentration ratios of certain proteins in the event of ill health.[13] This presents an opportunity for 2D-IR to contribute to biofluid diagnostics because measuring protein concentrations currently requires antibody panels, which cannot address large numbers of proteins and must be directed toward certain biomedical questions rather than providing a general screening tool.

Vibrational spectroscopy, in general, is attractive as a means of biofluid analysis because, in principle, each protein provides a distinct, secondary-structure-related amide I band.[25–27,81] However, absorption spectroscopy of biofluids has required sample drying to remove the H_2O contribution, while reliable mixture analysis using IR absorption has proved elusive due to the broad overlapping nature of the bands of different proteins. This highlights a potential advantage for 2D-IR spectroscopy because of its abilities to bypass the H_2O problem, produce a structure-specific 2D signature for each protein, and also offer better resolution via the slight band narrowing in comparison to IR absorption spectroscopy mentioned above.

In a study of the 2D-IR spectrum of blood serum, it was shown that clear resolution of amide I bands from the majority serum albumin protein, which makes up between 43% and 70% of the protein content,[82] and that due to the γ-globulins, which constitute the remainder of the proteinaceous fraction, is possible. [13] The separation of these signals was facilitated by the fact that serum albumin is largely α-helical in terms of structural composition while the globulins contain a large proportion of β-sheet structures, but a similar result was not possible using IR absorption spectroscopy. In a demonstration of the potential relevance of 2D-IR to biofluid analysis, the albumin and globulin peak amplitudes were used to determine the albumin-to-globulin concentration ratio (AGR).[13] The AGR has been shown to vary in the presence of disease as well as with metabolic activity, so it provides a useful piece of diagnostic data. It was determined that the accuracy with which the AGR could be established with 2D-IR (±4%) was larger than that from laboratory assays (±1%), but the lack of complex sample preparation showed that the potential exists for this to be a realistic screening approach.

The utility of 2D-IR lineshapes was demonstrated when changes in the γ-globulin signal were detected upon spiking serum samples with different quantities of the specific globulin proteins IgA, IgM, and IgG.[13] These proteins constitute the bulk of the γ-globulin fraction of serum and have similar β-sheet-rich secondary structures, but showed differences in their spectral signatures, which allowed them to be detected separately in serum spectra. This proof-of-concept study suggests that as the 2D-IR method evolves in terms of measurement sensitivity and as data analysis becomes more sophisticated, it may be possible to use 2D-IR to determine the concentrations of a number of important proteins in a single measurement rather than just the AGR ratio.[46,83] Note that the protein concentration can be quantified because the H_2O thermal response can be used as an internal standard, as discussed above.

It has already been mentioned above that blood serum analysis using 2D-IR has encompassed a study of drug binding to the albumin fraction.[36] As well as offering a means to understand the fundamental nature of protein–drug binding, this could be a useful tool for both biomedical and pharmaceutical applications. Albumin is a promiscuous binding molecule and acts as a reservoir for small molecules in the blood stream. As such, drugs will enter an equilibrium between albumin-bound and free states. The position of this equilibrium can impact the efficacy of a drug molecule because it is generally the quantity of the drug that is free in solution that is active. Similarly, in the event of a patient overdose, the albumin-bound drug fraction can act as a reservoir, maintaining high levels of free drug over a long period. Thus, whether for

drug metabolism and pharmacokinetics (DMPK) applications or biomedical diagnostics, the ability to measure the albumin-bound drug fraction is likely to be important.[84–86]

Currently, methods exist that can measure the free component of drugs, most notably electrochemical approaches can operate using a drop of whole blood.[84] However, direct measurement of the bound fraction is challenging, requiring relatively slow wet chemical analyses that frequently involve protein denaturation. There is thus the possibility that 2D-IR could play a role in measuring the albumin-bound fraction of drug molecules. However, the single study to date cannot reveal whether the effect observed for the paracetamol test-case was a specific spectral signature caused by paracetamol binding or a generic effect caused by a small molecule binding to one of the many binding sites in the albumin structure.[36] Further work will be required to understand how specific 2D-IR will be able to be in this respect.

Another promising area for 2D-IR biofluid diagnostics could be the detection of the low molecular weight content of blood serum. It was shown that the structure-specific off-diagonal peak patterns, which occur in the 2D-IR spectrum of a molecule due to mode coupling, can be used to quantify the presence of small molecules. The specific example used glycine as a model peptide, but the method could be extended to other molecules, including lipids, nucleic acids, and sugars.[37] For more challenging applications, methods such as those developed to enable the study of fibril formation, described above, in which the main protein content can be depleted to focus on minor components could be used in the future. Although there must be a focus on methods that minimize sample handling or pre-preparation in order to optimize spectroscopy for diagnostics, which may limit the utility of such methods directly, there will be a broad scope for the use of depleted serum as the authors suggest for studies of biomedical or biophysical phenomena in the most physiologically relevant environment possible.

Moving away from blood serum toward tissue, there have been a number of studies using 2D-IR to observe the presence of fibrils in cataracts.[72,87,88] To date, these have been completed using dried specimens, but the potential for future studies in wet tissue would be an exciting development, especially if used in harness with 2D-IR imaging modalities.[89,90] IR imaging has been applied effectively as a hyperspectral tool using FTIR for the diagnosis of cancerous tissue,[91] so it will be interesting to judge whether the added information content of a 2D-IR spectrum can provide useful data regarding the proteinaceous content of tissue samples.

Finally, in an application to 2D-IR on *in vivo* systems other than blood serum, it has been shown that amide I spectroscopy can be used to differentiate species of bacterial spores.[92] Differences in the amide I bands of *Bacillus atrophaeus and B. thuringiensis* spores indicated that changes in the protein content of the spore coat may be used as a marker for discrimination. These studies also raise the prospect of using 2D-IR to probe vegetative cells and cells of other organisms in the future.

CLINICAL TRANSLATION OF 2D-IR

In recent years, infrared absorption spectroscopies have increasingly been employed in clinical research settings, with studies on disease diagnostics using biofluids showing great promise.[93–96] However, there are many challenges to overcome before the translation of 2D-IR from the research lab to the clinic can occur. In particular, the standardization of data collection and development of spectral pre-processing are vital to aid comparisons between different spectrometers and to help improve data interpretability. Additionally, while 2D-IR spectra can be acquired in under a minute, this leads to large and complex datasets that take significant time to analyze. For the realization of 2D-IR in clinical settings, it would be prudent to learn from processes devised within the IR absorption spectroscopy community and employ multivariate analytical (MVA) techniques to analyze large spectral datasets. To achieve this, further efforts in applying statistical analysis combined with predictive modeling will be necessary. This will require the generation of large spectral libraries and patient data banks to train machine learning algorithms to diagnose particular diseases, but the spectrometer technology has now developed to such a position as to make such datasets viable in the short term.

The harnessing of 2D-IR spectral libraries to advanced data processing will be assisted by the availability of computer software packages that have been developed for processing and analysis of spectroscopic datasets. Two of note are the PRFFECT processing package and hyperSpec.[97,98] Pre-processing and Random Forest Feature Extraction Combination Tester (PRFFECT) provides a full data work-up, requiring very little input from the user. Essentially a "black-box," this package allows a user to apply a range of advanced spectral processing and analysis tools, making techniques available that were once only accessible to those with specialist knowledge. Similarly, hyperSpec can be used to apply many different algorithms written using R software to multiple types of spectroscopic datasets with little user input. It is anticipated that the advent of large protein data libraries will drive the production of similar automated packages for the handling of complex 2D-IR datasets.

As described above, the development of methodologies for processing the spectra of H_2O-rich fluids can provide reductions in spectral noise, increasing signal-to-noise ratios, and minimized measurement-to-measurement fluctuations across instruments. While a step in the right direction for the translation of 2D-IR to clinical applications, additional experimentation on the capabilities of 2D-IR when combined with disease classification algorithms and the progression of 2D-IR toward human data will be essential.

CONCLUSIONS AND FUTURE PERSPECTIVES

Given that the first demonstration of using 2D-IR to study protein amide I spectroscopy in H_2O was published in 2019,[13] it is encouraging that examples of its application already span fundamental protein structure and dynamics, protein analysis, and biomedical diagnostics. They also indicate that this is an area with the potential for further growth in the years to come.

The likely extent of its impact will vary between these areas of application. For example, the current high level of understanding of protein structure and dynamics obtained via extensive studies carried out in D_2O alongside computational simulations means that amide I spectroscopy in H_2O is likely to provide only a fine-tuning of these models. However, there are early indications that the solvent has a considerable impact on intermolecular and solvation processes, and the impact of these is yet to be fully appreciated.

One area where the benefit of the ability to work in H_2O-rich fluids may be felt is in the use of 2D-IR for protein drug screening. The removal of the need to deuterate samples will overcome a major economic barrier to the implementation of the technology, while fast data acquisition means that structural and dynamic information on a large number of protein–drug combinations could be acquired relatively quickly. These data could then form part of a pre-screen for more detailed structural analysis using tools, such as NMR, crystallography, or cryo-EM, which provide atomistic insight but take considerably longer to acquire and analyze data, thus streamlining the process of candidate molecule selection and optimization. This discussion leaves aside the technical challenges for implementation of 2D-IR spectroscopy in a commercial setting, which is outside the scope of this article, but it is clear that the technology is improving steadily in terms of reliability, ease of implementation, and speed of data acquisition. Such advances will be accelerated if a broad potential user base for the end product is identified.

Biomedical diagnostics also seems to be a promising route for application of 2D-IR methods that has been opened up by the ability to study proteins in H_2O. The spectroscopic methodology required to obtain spectra has already been developed in a number of areas, while data preprocessing tools should continue to improve sensitivity. This latter issue is a potential barrier to progress for 2D-IR, largely because IR methods generally lack the sensitivity to interrogate molecules at physiological concentrations, which lie in the mM to nM range. While some molecules are clearly accessible, and we highlight examples of protein content analysis and paracetamol binding, which operated within physiologically relevant parameters, other cases, such as the detection of glycine, have not yet been able to reach physiological concentrations. It is anticipated that laser systems and detectors will continue to evolve, allowing ever-improving sensitivity and lower detection limits, but other methods also offer promise. A very recent proof-of-concept study involving the use of silicon-based metasurfaces for the enhancement of IR signals in diluted solutions is one such example.[99] In this study, nanostructured surfaces were able to deliver 100-times improvement in the IR absorption signal and an order of magnitude improvement over ATR spectroscopy methods. The relevance to biofluid diagnostics is that Si surfaces produce an enhancement effect that probes deeper into the analyte than plasmonics-based methods, which have been demonstrated for thin films and adsorbed analytes.[100–105] This offers the scope for IR enhancement in dilute solutions. Although these have been demonstrated only for IR absorption methods so far, if the approach is transferrable to ultrafast and non-linear spectroscopies, then it will provide another route to improving the sensitivity of detection with 2D-IR.

Whether for use in drug screening or biofluid diagnostics, it is clear that opportunities exist in relation to the exploitation of large quantities of 2D-IR data alongside sophisticated data analysis tools. As 2D-IR data collection on a large scale becomes economically viable as well as technically possible, there is scope for the creation of protein spectral libraries under H_2O-rich conditions.[46,83] This will serve the purposes of providing standards for use in analytical methods but also for the development of computational tools and could, in principle, mirror the success of the protein databank and other sources of open-access data relating to protein structure. Previously, the lack of standardization across instruments and measurement protocols would have made cross-comparisons extremely problematic, but the water thermal response could provide a useful tool for standardization that, with time and development, could overcome many of these issues. In this, it is essential that progress toward analytical applications learns from work in other disciplines, where the need for standardization of methods and data analysis are already under discussion.[106]

ACKNOWLEDGMENTS

Grant funding from the Engineering and Physical Sciences Research Council of the UK (EPSRC: Grant Nos. EP/T014245/1 and EP/T014318/1) is gratefully acknowledged.

AUTHOR DECLARATIONS

Conflict of Interest

The authors have no conflicts to disclose.

Author Contributions

Samantha H. Rutherford: Writing – original draft (equal); Writing – review & editing (equal). **Matthew J. Baker**: Project administration (equal); Writing – original draft (equal); Writing – review & editing (equal). **Neil T. Hunt**: Conceptualization (equal); Project administration (equal); Writing – original draft (equal); Writing – review & editing (equal).

DATA AVAILABILITY

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

REFERENCES

[1] E.-L. Karjalainen, T. Ersmark, and A. Barth, J. Phys. Chem. B **116**, 4831 (2012).
[2] A. V. Cunha, A. S. Bondarenko, and T. L. C. Jansen, J. Chem. Theory Comput. **12**, 3982 (2016).
[3] C. R. Baiz, M. Reppert, and A. Tokmakoff, J. Phys. Chem. A **117**, 5955 (2013).
[4] A. W. Smith and A. Tokmakoff, J. Chem. Phys. **126**, 045109 (2007).
[5] N. S. Myshakina and S. A. Asher, J. Phys. Chem. B **111**, 4271 (2007).
[6] T. Hayashi and S. Mukamel, J. Phys. Chem. B **111**, 11032 (2007).
[7] I. V. Rubtsov and R. M. Hochstrasser, J. Phys. Chem. B **106**, 9165 (2002).
[8] H. Torii and M. Tasumi, J. Chem. Phys. **96**, 3379 (1992).
[9] A. W. Smith, C. M. Cheatum, H. S. Chung, N. Demirdoven, M. Khalil, J. Knoester, and A. Tokmakoff, Biophys. J. **86**, 619A (2004).
[10] N. Demirdöven, C. M. Cheatum, H. S. Chung, M. Khalil, J. Knoester, and A. Tokmakoff, J. Am. Chem. Soc. **126**, 7981 (2004).
[11] C. M. Cheatum, A. Tokmakoff, and J. Knoester, J. Chem. Phys. **120**, 8201 (2004).
[12] P. Hamm and M. T. Zanni, *Concepts and Method of 2D Infrared Spectroscopy* (Cambridge University Press, Cambridge, 2011).
[13] S. Hume, G. Hithell, G. M. Greetham, P. M. Donaldson, M. Towrie, A. W. Parker, M. J. Baker, and N. T. Hunt, Chem. Sci. **10**, 6448 (2019).
[14] N. T. Hunt, Chem. Soc. Rev. **38**, 1837 (2009).
[15] L. P. Deflores, Z. Ganim, R. A. Nicodemus, and A. Tokmakoff, J. Am. Chem. Soc. **131**, 3385 (2009).
[16] Z. Ganim, H. S. Chung, A. W. Smith, L. P. Deflores, K. C. Jones, and A. Tokmakoff, Acc. Chem. Res. **41**, 432 (2008).
[17] I. V. Rubtsov, J. Wang, and R. M. Hochstrasser, J. Chem. Phys. **118**, 7733 (2003).
[18] M. T. Zanni, J. Stenger, S. M. Decatur, and R. M. Hochstrasser, Biophys. J. **80**, 9A (2001).

[19]C. Kolano, J. Helbing, M. Kozinski, W. Sander, and P. Hamm, Nature **444**, 469 (2006).
[20]A. Ghosh, J. S. Ostrander, and M. T. Zanni, Chem. Rev. **117**, 10726 (2017).
[21]L. E. Buchanan, E. B. Dunkelberger, H. Q. Tran, P.-N. Cheng, C.-C. Chiu, P. Cao, D. P. Raleigh, J. J. de Pablo, J. S. Nowick, and M. T. Zanni, Proc. Natl. Acad. Sci. U. S. A. **110**, 19285 (2013).
[22]A. M. Woys, Y.-S. Lin, A. S. Reddy, W. Xiong, J. J. de Pablo, J. L. Skinner, and M. T. Zanni, J. Am. Chem. Soc. **132**, 2832 (2010).
[23]S.-H. Shim, R. Gupta, Y. L. Ling, D. B. Strasfeld, D. P. Raleigh, and M. T. Zanni, Proc. Natl. Acad. Sci. U. S. A. **106**, 6614 (2009).
[24]D. B. Strasfeld, Y. L. Ling, S.-H. Shim, and M. T. Zanni, J. Am. Chem. Soc. **130**, 6698 (2008).
[25]L. Lovergne, P. Bouzy, V. Untereiner, R. Garnotel, M. J. Baker, G. Thiéfin, and G. D. Sockalingum, Faraday Discuss. **187**, 521 (2016).
[26]M. J. Baker, S. R. Hussain, L. Lovergne, V. Untereiner, C. Hughes, R. A. Lukaszewski, G. Thiéfin, and G. D. Sockalingum, Chem. Soc. Rev. **45**, 1803 (2016).
[27]F. Bonnier, F. Petitjean, M. J. Baker, and H. J. Byrne, J. Biophotonics **7**, 167 (2014).
[28]G. Sieler and R. Schweitzer-Stenner, J. Am. Chem. Soc. **119**, 1720 (1997).
[29]K. Chelius, J. H. Wat, A. Phadkule, and M. Reppert, J. Chem. Phys. **155**, 195101 (2021).
[30]S. Y. Venyaminov and F. G. Prendergast, Anal. Biochem. **248**, 234 (1997).
[31]M. Grechko and M. T. Zanni, J. Chem. Phys. **137**, 184202 (2012).
[32]M. Khalil, N. Demirdöven, and A. Tokmakoff, J. Phys. Chem. A **107**, 5258 (2003).
[33]C. R. Baiz, C. S. Peng, M. E. Reppert, K. C. Jones, and A. Tokmakoff, Analyst **137**, 1793 (2012).
[34]L. Minnes, D. J. Shaw, B. P. Cossins, P. M. Donaldson, G. M. Greetham, M. Towrie, A. W. Parker, M. J. Baker, A. J. Henry, R. J. Taylor, and N. T. Hunt, Anal. Chem. **89**, 10898 (2017).
[35]D. J. Shaw, R. E. Hill, N. Simpson, F. S. Husseini, K. Robb, G. M. Greetham, M. Towrie, A. W. Parker, D. Robinson, J. D. Hirst, P. A. Hoskisson, and N. T. Hunt, Chem. Sci. **8**, 8384 (2017).
[36]S. H. Rutherford, G. M. Greetham, M. Towrie, A. W. Parker, S. Kharratian, T. F. Krauss, A. Nordon, M. J. Baker, and N. T. Hunt, Analyst **147**, 3464 (2022).
[37]S. H. Rutherford, G. M. Greetham, P. M. Donaldson, M. Towrie, A. W. Parker, M. J. Baker, and N. T. Hunt, Anal. Chem. **93**, 920 (2021).
[38]D. Kraemer, M. L. Cowan, A. Paarmann, N. Huse, E. T. J. Nibbering, T. Elsaesser, and R. J. D. Miller, Proc. Natl. Acad. Sci. U. S. A. **105**, 437 (2008).
[39]S. Ashihara, N. Huse, A. Espagne, E. T. J. Nibbering, and T. Elsaesser, Chem. Phys. Lett. **424**, 66 (2005).
[40]L. Chuntonov, R. Kumar, and D. G. Kuroda, Phys. Chem. Chem. Phys. **16**, 13172 (2014).
[41]S. Hume, G. M. Greetham, P. M. Donaldson, M. Towrie, A. W. Parker, M. J. Baker, and N. T. Hunt, Anal. Chem. **92**, 3463 (2020).
[42]C. T. Middleton, L. E. Buchanan, E. B. Dunkelberger, and M. T. Zanni, J. Phys. Chem. Lett. **2**, 2357 (2011).
[43]P. M. Donaldson, G. M. Greetham, D. J. Shaw, A. W. Parker, and M. Towrie, J. Phys. Chem. A **122**, 780 (2018).
[44]S.-H. Shim and M. T. Zanni, Phys. Chem. Chem. Phys. **11**, 748 (2009).
[45]M. C. Asplund, M. T. Zanni, and R. M. Hochstrasser, Proc. Natl. Acad. Sci. U. S. A. **97**, 8219 (2000).
[46]S. H. Rutherford, G. M. Greetham, A. W. Parker, A. Nordon, M. J. Baker, and N. T. Hunt, J. Chem. Phys. **157**, 205102 (2022).
[47]K. M. Farrell, J. S. Ostrander, A. C. Jones, B. R. Yakami, S. S. Dicke, C. T. Middleton, P. Hamm, and M. T. Zanni, Opt. Express **28**, 33584 (2020).
[48]P. Hamm, J. Chem. Phys. **154**, 104201 (2021).
[49]K. C. Robben and C. M. Cheatum, J. Chem. Phys. **152**, 094201 (2020).
[50]F. Dahms, B. P. Fingerhut, E. T. J. Nibbering, E. Pines, and T. Elsaesser, Science **357**, 491 (2017).
[51]R. Costard, T. Tyborski, and B. P. Fingerhut, Phys. Chem. Chem. Phys. **17**, 29906 (2015).
[52]T. la Cour Jansen, A. G. Dijkstra, T. M. Watson, J. D. Hirst, and J. Knoester, J. Chem. Phys. **125**, 044312 (2006).
[53]C. R. Baiz, B. Błasiak, J. Bredenbeck, M. Cho, J.-H. Choi, S. A. Corcelli, A. G. Dijkstra, C.-J. Feng, S. Garrett-Roe, N.-H. Ge, M. W. D. Hanson-Heine, J. D. Hirst, T. L. C. Jansen, K. Kwac, K. J. Kubarych, C. H. Londergan, H. Maekawa, M. Reppert, S. Saito, S. Roy, J. L. Skinner, G. Stock, J. E. Straub, M. C. Thielges, K. Tominaga, A. Tokmakoff, H. Torii, L. Wang, L. J. Webb, and M. T. Zanni, Chem. Rev. **120**, 7152 (2020).
[54]J. T. King, M. R. Ross, and K. J. Kubarych, J. Phys. Chem. B **116**, 3754 (2012).
[55]D. Czurlok, J. Gleim, J. Lindner, and P. Vöhringer, J. Phys. Chem. Lett. **5**, 3373 (2014).
[56]J. N. Bandaria, S. Dutta, M. W. Nydegger, W. Rock, A. Kohen, and C. M. Cheatum, Proc. Natl. Acad. Sci. U. S. A. **107**, 17974 (2010).
[57]G. W. Tumbic, M. Y. Hossan, and M. C. Thielges, "Protein dynamics by two-dimensional infrared spectroscopy," Annu. Rev. Anal. Chem. **14**, 299 (2021).
[58]M. C. Thielges, J. Chem. Phys. **155**, 040903 (2021).
[59]J. M. Schmidt-Engler, S. von Berg, and J. Bredenbeck, J. Phys. Chem. Lett. **12**, 7777 (2021).
[60]J. M. Schmidt-Engler, R. Zangl, P. Guldan, N. Morgner, and J. Bredenbeck, Phys. Chem. Chem. Phys. **22**, 5463 (2020).
[61]S. Ramos and M. C. Thielges, J. Phys. Chem. B **123**, 3551 (2019).
[62]S. Ramos, A. L. Le Sueur, R. E. Horness, J. T. Specker, J. A. Collins, K. E. Thibodeau, and M. C. Thielges, J. Phys. Chem. B **123**, 2114 (2019).
[63]P. Pagano, Q. Guo, C. Ranasinghe, E. Schroeder, K. Robben, F. Häse, H. Ye, K. Wickersham, A. Aspuru-Guzik, D. T. Major, L. Gakhar, A. Kohen, and C. M. Cheatum, ACS Catal. **9**, 11199 (2019).
[64]J. J. Humston, I. Bhattacharya, M. Jacob, and C. M. Cheatum, J. Chem. Phys. **150**, 234202 (2019).
[65]P. Pagano, Q. Guo, A. Kohen, and C. M. Cheatum, J. Phys. Chem. Lett. **7**, 2507 (2016).
[66]M. C. Thielges, J. K. Chung, and M. D. Fayer, J. Am. Chem. Soc. **133**, 3995 (2011).
[67]S. Bagchi, B. T. Nebgen, R. F. Loring, and M. D. Fayer, J. Am. Chem. Soc. **132**, 18367 (2010).
[68]H. Ishikawa, I. J. Finkelstein, S. Kim, K. Kwak, J. K. Chung, K. Wakasugi, A. M. Massari, and M. D. Fayer, Proc. Natl. Acad. Sci. U. S. A. **104**, 16116 (2007).
[69]I. J. Finkelstein, H. Ishikawa, S. Kim, A. M. Massari, and M. D. Fayer, Proc. Natl. Acad. Sci. U. S. A. **104**, 2637 (2007).
[70]K. Adamczyk, N. Simpson, G. M. Greetham, A. Gumiero, M. A. Walsh, M. Towrie, A. W. Parker, and N. T. Hunt, Chem. Sci. **6**, 505 (2015).
[71]S. Y. Chun, M. K. Son, C. R. Park, C. Lim, H. I. Kim, K. Kwak, and M. Cho, Chem. Sci. **13**, 4482 (2022).
[72]A. M. Alperstein, J. S. Ostrander, T. O. Zhang, and M. T. Zanni, Proc. Natl. Acad. Sci. U. S. A. **116**, 6602 (2019).
[73]J. P. Lomont, K. L. Rich, M. Maj, J.-J. Ho, J. S. Ostrander, and M. T. Zanni, J. Phys. Chem. B **122**, 144 (2018).
[74]T. O. Zhang, A. M. Alperstein, and M. T. Zanni, J. Mol. Biol. **429**, 1705 (2017).
[75]A. Abedini, A. Plesner, P. Cao, Z. Ridgway, J. H. Zhang, L. H. Tu, C. T. Middleton, B. Chao, D. J. Sartori, F. L. Meng, H. Wang, A. G. Wong, M. T. Zanni, C. B. Verchere, D. P. Raleigh, and A. M. Schmidt, eLife **5**, e12977 (2016).
[76]S. D. Moran, T. O. Zhang, and M. T. Zanni, Protein Sci. **23**, 321 (2014).
[77]S. D. Moran and M. T. Zanni, J. Phys. Chem. Lett. **5**, 1984 (2014).
[78]E. B. Dunkelberger, L. E. Buchanan, P. Marek, P. Cao, D. P. Raleigh, and M. T. Zanni, J. Am. Chem. Soc. **134**, 12658 (2012).
[79]S.-H. Shim, D. B. Strasfeld, Y. L. Ling, and M. T. Zanni, Proc. Natl. Acad. Sci. U. S. A. **104**, 14197 (2007).
[80]C. R. Fields, S. S. Dicke, M. K. Petti, M. T. Zanni, and J. P. Lomont, J. Phys. Chem. Lett. **11**, 6382 (2020).
[81]F. Bonnier, G. Brachet, R. Duong, T. Sojinrin, R. Respaud, N. Aubrey, M. J. Baker, H. J. Byrne, and I. Chourpa, J. Biophotonics **9**, 1085 (2016).
[82]J. T. Busher, *Clinical Methods: The History, Physical, and Laboratory Examinations* (Butterworths, 1990).

[83]B. R. Smith, K. M. Ashton, A. Brodbelt, T. Dawson, M. D. Jenkinson, N. T. Hunt, D. S. Palmer, and M. J. Baker, Analyst **141**, 3668 (2016).
[84]N. Wester, B. F. Mikladal, I. Varjos, A. Peltonen, E. Kalso, T. Lilius, T. Laurila, and J. Koskinen, Anal. Chem. **92**, 13017 (2020).
[85]P. Daneshgar, A. A. Moosavi-Movahedi, P. Norouzi, M. R. Ganjali, A. Madadkar-Sobhani, and A. A. Saboury, Int. J. Biol. Macromol. **45**, 129 (2009).
[86]T. P. Milligan, H. C. Morris, P. M. Hammond, and C. P. Price, Ann. Clin. Biochem. **31**, 492 (1994).
[87]A. M. Alperstein, K. S. Molnar, S. S. Dicke, K. M. Farrell, L. N. Makley, M. T. Zanni, and U. P. Andley, PLoS One **16**, e0257098 (2021).
[88]A. M. Alperstein, J. S. Ostrander, T. Q. O. Zhang, and M. T. Zanni, Biophys. J. **114**, 560A (2018).
[89]C. R. Baiz, D. Schach, and A. Tokmakoff, Opt. Express **22**, 18724 (2014).
[90]A. L. Serrano, A. Ghosh, J. S. Ostrander, and M. T. Zanni, Opt. Express **23**, 17815 (2015).
[91]F. Grosserueschkamp, T. Bracht, H. C. Diehl, C. Kuepper, M. Ahrens, A. Kallenbach-Thieltges, A. Mosig, M. Eisenacher, K. Marcus, T. Behrens, T. Bruning, D. Theegarten, B. Sitek, and K. Gerwert, Sci. Rep. **7**, 44829 (2017).
[92]B. Procacci, S. H. Rutherford, G. M. Greetham, M. Towrie, A. W. Parker, C. V. Robinson, C. R. Howle, and N. T. Hunt, Spectrochim. Acta, Part A **249**, 119319 (2021).
[93]A. Sala, J. M. Cameron, C. A. Jenkins, H. Barr, L. Christie, J. J. A. Conn, T. R. J. Evans, D. A. Harris, D. S. Palmer, C. Rinaldi, A. G. Theakstone, and M. J. Baker, Cancers **14**, 3048 (2022).
[94]K.-Y. Su and W.-L. Lee, Cancers **12**, 115 (2020).
[95]S. Mordechai, E. Shufan, B. S. P. Katz, and A. Salman, Analyst **142**, 1276 (2017).
[96]S. L. Haas, R. Müller, A. Fernandes, K. Dzeyk-Boycheva, S. Würl, J. Hohmann, S. Hemberger, E. Elmas, M. Brückmann, P. Bugert, and J. Backhaus, Appl. Spectrosc. **64**, 262 (2010).
[97]B. R. Smith, M. J. Baker, and D. S. Palmer, Chemom. Intell. Lab. Syst. **172**, 33 (2018).
[98]C. Beleites and V. Sergo, hyperSpec: A package to handle hyperspectral data sets in R′, R package version 0.100.0, https://github.com/r-hyperspec/hyperSpec.
[99]S. Kharratian, D. Conteduca, B. Procacci, D. J. Shaw, N. T. Hunt, and T. F. Krauss, Chem. Sci. **13**, 12858 (2022).
[100]L. Chuntonov and I. V. Rubtsov, J. Phys. Chem. C **126**, 3314 (2022).
[101]L. Chuntonov and I. V. Rubtsov, J. Chem. Phys. **153**, 050902 (2020).
[102]R. T. Mackin, B. Cohn, B. Engelman, A. Goldner, I. V. Rubtsov, and L. Chuntonov, J. Phys. Chem. C **123**, 24731 (2019).
[103]R. T. Mackin, B. Cohn, A. Gandman, J. D. Leger, L. Chuntonov, and I. V. Rubtsov, J. Phys. Chem. C **122**, 11015 (2018).
[104]A. Gandman, R. T. Mackin, B. Cohn, I. V. Rubtsov, and L. Chuntonov, ACS Nano **12**, 4521 (2018).
[105]A. Gandman, R. Mackin, B. Cohn, I. V. Rubtsov, and L. Chuntonov, J. Phys. Chem. Lett. **8**, 3341 (2017).
[106]J. M. Cameron, C. Rinaldi, S. H. Rutherford, A. Sala, A. G. Theakstone, and M. J. Baker, Appl. Spectrosc. **76**, 393 (2022).