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Infrared multiple photon dissociation (IRMPD) spectroscopy and its potential for the clinical laboratory

Matthew J. Carlo, Amanda L. Patrick *

Department of Chemistry, Mississippi State University, Mississippi State, MS 39762, USA

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ABSTRACT

Infrared multiple photon dissociation (IRMPD) spectroscopy is a powerful tool used to probe the vibrational modes–and, by extension, the structure—of an ion within an ion trap mass spectrometer. Compared to traditional FTIR spectroscopy, IRMPD spectroscopy has advantages including its sensitivity and its relative ability to handle complex mixtures. While IRMPD has historically been a technique for fundamental analyses, it is increasingly being applied in a more analytical fashion. Notable recent demonstrations pertinent to the clinical laboratory and adjacent interests include analysis of modified amino acids/residues and carbohydrates, structural elucidation (including isomeric differentiation) of metabolites, identification of novel illicit drugs, and structural studies of various biomolecules and pharmaceuticals. Improvements in analysis time, coupling to commercial instruments, and integration with separations methods are all drivers toward the realization of these analytical applications. Additional improvements in these areas, along with advances in benchtop tunable IR sources and increased cross-discipline collaboration, will continue to drive innovation and widespread adoption. The goal of this tutorial article is to briefly present the fundamentals and instrumentation of IRMPD spectroscopy, as an overview of the utility of this technique for helping to answer questions relevant to clinical analysis, and to highlight limitations to widespread adoption, as well as promising directions in which the field may be heading.

Introduction

The nexus of mass spectrometry and allied techniques with clinical analysis has a long and noteworthy history [1–3]. While GC–MS [4] and LC-MS/MS [5] reign supreme, recent years have shown growth in the incorporation of complementary methods. Advances in instrumentation and experimental methods have ushered in a new realm of possibilities. In this piece, we will focus on one specific experimental approach, infrared multiple photon dissociation (IRMPD) spectroscopy. In its own right, IRMPD spectroscopy has a rich history of innovation and contributions to our fundamental understanding of gas-phase ion chemistry [6–13]. Recent advances have allowed IRMPD spectroscopy (and closely related gas-phase ion spectroscopy techniques) to gain traction for the analysis of molecules more closely related to those of clinical interest, including peptides [14–17], drug molecules and drug metabolites [18–25], and other metabolites [26–29]. As advances in instrumentation increase accessibility and ease-of-use, IRMPD spectroscopy will no doubt become more common as a go-to structural probe integrated with mass spectrometry experiments.

The purpose of this article is to provide an introduction to IRMPD spectroscopy and its potential utility for the clinical laboratory. We will first walk through the fundamentals, example experiments, and

Abbreviations: 2-AEP, 2-aminoethylphosphonic acid; 2P1EA, 2-phenyl-1-ethanolamine; α-PVP, alpha-pyrrolidinovalerophenone; CIVP, cryogenic ion vibrational predissociation spectroscopy; CLIO, Centre Laser Infrarouge d'Orsay; cw, continuous wave; DFT, density functional theory; FA, fluoroamphetamine; FEL, free electron laser; FELIX, Free Electron Laser for Infrared eXperiments; FMA, fluoromethamphetamine; FTICR, Fourier transform ion cyclotron resonance; GC–MS, gas chromatography-mass spectrometry; GlcNAc, n-Acetylglucosamine; GSNO, S- nitro glutathione; IR, infrared; IR^2MS^3, infrared-infrared double-resonance multi-stage mass spectrometry; IRMPD, infrared multiple photon dissociation (IRMPD); IRMPD-MS, infrared multiple photon dissociation spectroscopy mass spectrometry; IRPD, infrared predissociation spectroscopy; IVR, intramolecular vibrational redistribution; LC, liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MDA, methylenedioxyamphetamine; MDMA, methylenedioxymethamphetamine; MMC, methylmethcathinone; MS/MS, tandem mass spectrometry; MS^n, multi-stage mass spectrometry; NANT, N-acetyl-N-nitrosotryptophan; OPO/A, optical parametric oscillator/amplifier; PTM, post-translational modification; SNOCys, S-nitrosocysteine; UV, ultraviolet; UV-IR, ultraviolet-infrared.

* Corresponding author at: Department of Chemistry, P.O. Box 9573, Mississippi State, MS 39762, USA.
E-mail address: apatrick@chemistry.msstate.edu (A.L. Patrick).

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instrumentation of IRMPD spectroscopy. Then, we will present the current status of the field, as it relates to applications and achievements of potential significance to the clinical science community. Finally, we will highlight exciting advances that are either in their infancy or on the horizon, which will, if successful, make this technique much more widespread and accessible.

Rationale for action spectroscopy and fundamentals of IRMPD spectroscopy

Infrared spectroscopy provides a wealth of information on the structure of a molecule. Subsequently, vibrational spectroscopy has found utility in clinical analysis [30]. Yet, traditional vibrational spectroscopy has limitations, including high limits of detection and matrix interferences. The ability to overcome these limitations, along with the inherent usefulness of the LC-MS/MS platform, make gas-phase infrared ion spectroscopy (i.e., implementation of vibrational spectroscopy within the context of a mass spectrometry experiment) an attractive proposition. Thus, it has, at times, been advantageous to consider coupling mass spectrometry and infrared spectroscopy—but how? How can we measure the infrared spectrum of a mass selected ion within the gas phase of a mass spectrometer?

Fig. 1 shows a conceptual overview of the challenge of coupling mass spectrometry with infrared spectroscopy due to low ion density and the requirements of the absorbance spectroscopy approach. In Fig. 1A, a condensed-phase material is impinged with resonant IR photons, a considerable portion of these photons are absorbed, and the transmitted intensity of the IR beam is measurably reduced, compared to the incident source intensity. In Fig. 1B, the same process is followed, but with a much less dense cloud of gas-phase ions as the absorbing species. In this case, the density of the gas-phase ions is so low that absorption is not measurable—the intensity of the impinging infrared light appears to be the same as the intensity of the transmitted light.

Having established the challenge associated with directly measuring the infrared absorption spectrum of gas-phase ions, one can better understand the rationale behind the solution: action spectroscopy. In general terms, "action spectroscopy" refers to a measurement where absorption of light is inferred via monitoring some absorption-induced change in the sample. In our case, the action to be monitored is infrared multiple photon dissociation (IRMPD). This action is ideal because it is readily implemented within mass spectrometers that are already setup for MS^n experiments, because it is typically background-free (i.e., the mass selected precursor mass spectrum should not already contain IRMPD product ions), and because it is a measurable consequence of resonant IR absorption for a wide range of ionic structures. By monitoring IRMPD yield as a function of wavelength, an infrared *action spectrum* can be constructed.

The IRMPD action—dissociation of a precursor ion into product ions upon absorption of infrared photons—requires that enough energy is imparted into the precursor ion to induce disruption of, usually covalent, bonds. Hence, the "multiple" in the IRMPD name. As an example, a bond requiring 200 kJ/mol to dissociate (~2 eV) would require, at a minimum, absorption of eight photons at 2000 cm^{-1} (~0.25 eV/photon) or 33 photons at 500 cm^{-1} (~0.06 eV/photon). This illustrates both that multiple photons are required to initiate dissociation of chemical bonds and that the number of photons is dependent on the energy of the photons. Furthermore, an excess beyond that thermodynamic minimum energy input is usually required for bond dissociation to proceed on a mass spectrometric timescale. Thus, we can assume that tens to hundreds of IR photons must be absorbed for dissociation to occur (and an action to be recordable). As is the case for most analytical methods, it is important to measure spectra that are not saturated (e.g., for IRMPD spectroscopy, the precursor ion should not be depleted) for analysis, including comparison to standards or calculations. Currently, attenuators are typically used, along with irradiation time adjustments, to apply appropriate dissociation conditions and, in certain cases, spectra may be recorded under different powers (e.g., if some regions absorb strongly and others weakly). While manual power and irradiation time adjustments are sufficient for the measurement of a few spectra, for any high throughput application an automated system would be needed, somewhat analogous to the implementation of automatic gain control for ion traps [31].

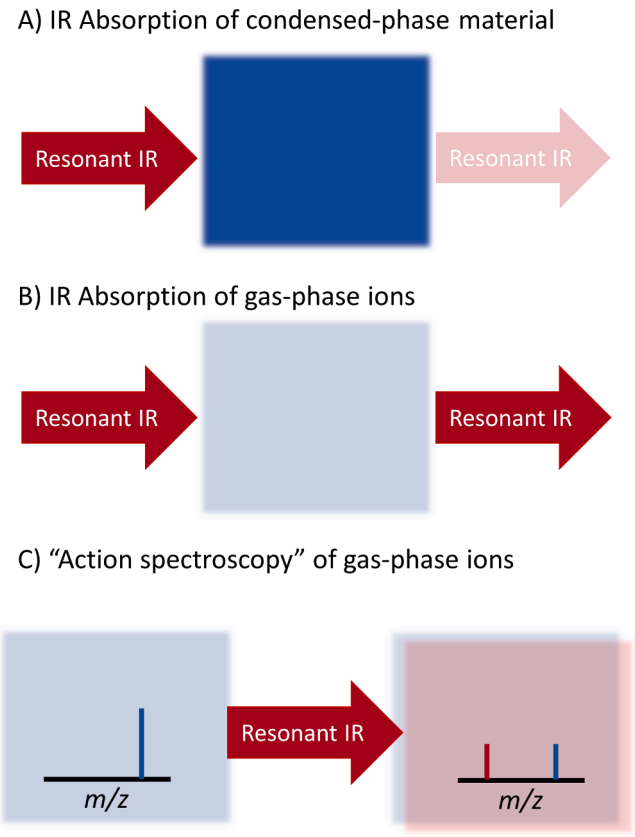

Fig. 1. An overview of the need for an "action spectroscopy" approach to measure the infrared spectrum of gas-phase ions. (A) A typical absorption measurement, where the red arrow represents the intensity of the infrared light (saturation level represents intensity) and the blue box represents the sample (saturation level represents molecular/ion density) for a condensed-phase sample typical of traditional infrared spectroscopy samples, (B) an attempt at conducting the traditional infrared spectroscopy experiment on a much less dense gas-phase ion sample (no measurable decrease in the infrared light intensity upon transmission through the dilute sample), and (C) an illustrative overview of the "action spectroscopy" approach to measuring the infrared spectrum of gas-phase ions, where, upon absorption of infrared photons, some measurable action occurs to the sample (here illustrated as a shift from blue to red by some portion of the ion cloud), rather than attenuation of the light. Ideally, this action produces a background-free measurement by a sensitive technique (i.e., mass spectrometry). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

At any given irradiation wavelength, all the impinging photons have (very nearly) the same energy. In a completely harmonic case, absorption of multiple photons could then proceed via a "ladder climbing" scenario where multiple photons are absorbed nearly instantaneously, allowing the system to "climb" from $v_0 \rightarrow v_1 \rightarrow v_2 \ldots$ until the dissociation barrier is overcome. For real systems, however, there is some degree of anharmonic character, and these levels are not evenly spaced. The photon is then only resonant with a specific transition and a different, slower, mechanism must be at play. A simple overview of the IRMPD process is shown in Fig. 2. The mechanism is a series of steps consisting of excitation of the vibrational mode, followed by redistribution of the energy throughout the molecule's degrees of freedom in a process

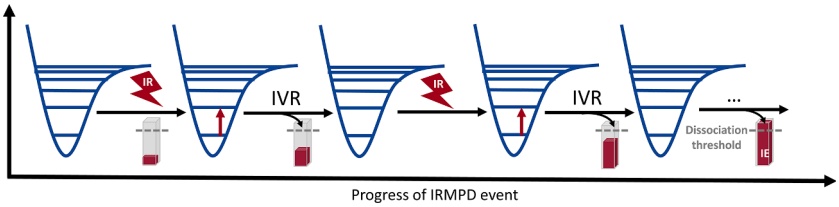

Fig. 2. Overview of the IRMPD process, consisting of irradiation of a molecule, containing a specific vibrational mode, with resonant infrared photons, absorption of a photon causing vibrational excitation, redistribution of the energy via IVR to the internal energy of the molecule causing relaxation of the original electron, and continuation of the cycle until a dissociation threshold is surpassed and dissociation occurs.

known as intramolecular vibrational redistribution (IVR), absorption of an additional photon, and so on until the internal energy of the precursor ion becomes high enough for dissociation to occur. This process still occurs on a sub-second scale and is, thus highly compatible with mass spectrometry. The interested reader can find additional discussion of the IRMPD process and related details in the following references (and citations therein) [10,13,32–37].

Having illustrated the need for an action spectroscopy approach and presented an appropriate action for measuring infrared absorption by mass spectrometry, one can combine these to achieve IRMPD spectroscopy.

One of the major strengths of the IRMPD spectroscopy approach is that it can be implemented within the ion trap of a mass spectrometer, and, thus, the IR spectrum of mass-selected ions can be recorded. The first step of the IRMPD spectroscopy experiment is to generate gas-phase ions and to select, or purify, the ion cloud, such that only the desired precursor mass-to-charge ratio is present. In addition to diminishing the effects of interfering species, this also provides for a nearly background-free measurement. The next step of the process is to irradiate the ion cloud at a discrete IR wavelength. If this wavelength is not resonant with any vibrational modes of the ion, no absorption and, thus, no dissociation will occur. If the wavelength is resonant with a vibrational mode, IRMPD will occur and a change in the resulting mass spectrum (decrease in precursor intensity, appearance of product ion signal) will be observed. Up to this point in the process, the experiment is essentially a MS/MS experiment wherein IRMPD is the dissociation method. To acquire infrared action spectra, IRMPD-mass spectra are recorded at discrete wavelengths, stepping through the entire range of interest in small steps (e.g., 2 cm^{-1} or so, depending on the resolution of the irradiation source, etc.). Once all the mass spectra have been acquired across the wavelength range of interest, the IRMPD yield can be plotted versus irradiation wavenumber to obtain a spectrum analogous to an infrared absorption spectrum. This procedure is illustrated in Fig. 3.

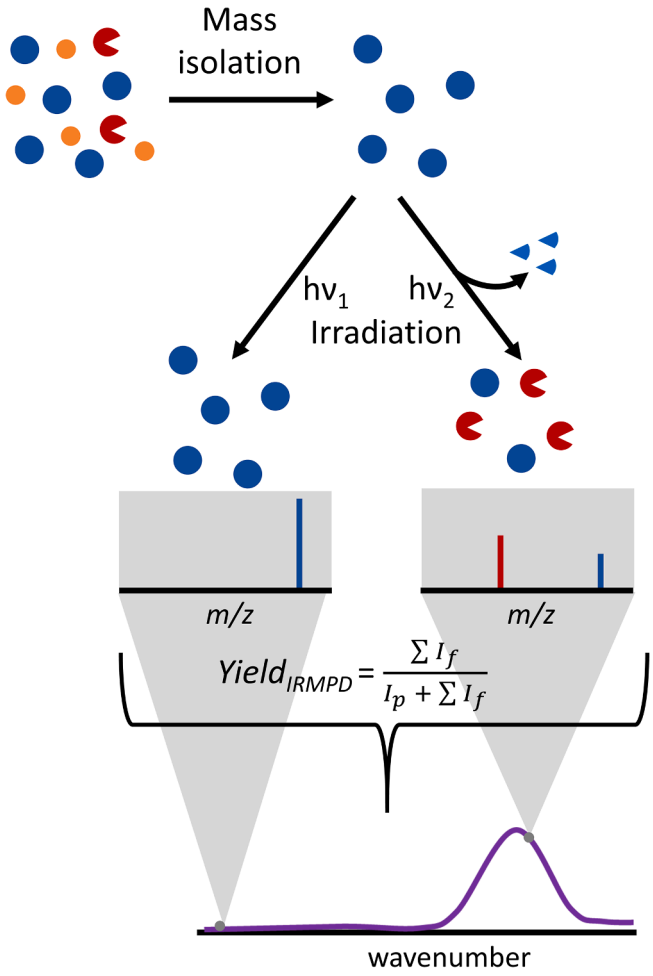

Fig. 3. The IRMPD process. Ions are formed, the desired analyte ion is mass isolated and subjected to irradiation with infrared light at discrete wavelengths. When this light is not resonant with the ions' vibrational modes, no dissociation occurs. At resonant modes, dissociation occurs. By plotting IRMPD yield as a function of irradiation wavelength, an infrared action spectrum is obtained.

Alternative infrared ion action spectroscopy experiments

IRMPD is an "action" readily implemented in ion traps, with minimal modifications. However, other "actions" suitable for measuring gas-phase infrared ion spectra have also been developed. In addition to providing alternative actions, many such approaches measure spectra of ions at reduced temperature, "trapping" ions into specific, stable conformations and improving resolution of the measurement. The experiments and results are sometimes quite pertinent to the present tutorial, so other approaches are briefly described here, along with references to direct the interested reader to more detailed resources. Fig. 4 provides an illustration of the principles of three such approaches.

One approach (Fig. 4A) to measuring cryogenic gas-phase infrared ion spectra is to employ a van der Waals tag, where a gas molecule/atom (e.g., helium) is condensed on to the ion of interest at low temperatures, allowing the disruption of the complex (via loss of the tag, inducing a mass shift) to serve as the measurable action resulting from absorption of an infrared photon. Such an approach may be termed infrared predissociation spectroscopy (IRPD) [38], cryogenic ion vibrational predissociation spectroscopy (CIVP) [39], or generally a "messenger tagging" approach [40]. Compared to the covalent bonds typically dissociated during IRMPD, these van der Waals interactions are much weaker. Thus, disruption of such interactions can often be achieved by absorption of a single photon. However, it is possible that the tag itself could perturb the spectra [39] and instrumentation requirements and complications due to the cryogenic cycling may further limit the widespread adoption of such approaches.

Another approach to overcoming dissociation barriers, while increasing information gained during the experiment, is to implement a two-laser approach. This may take any of several forms (and can even be extended to molecular systems in some cases) [37,41,42]. Here we describe two approaches in quite simplistic terms. For instance, a

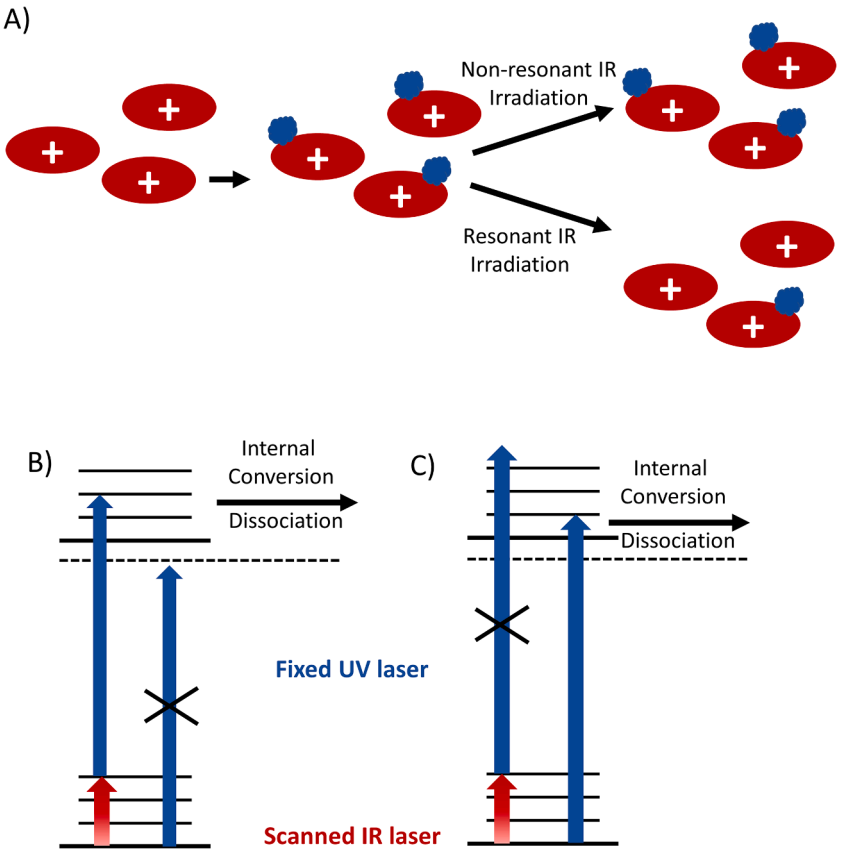

Fig. 4. Approaches to cryogenic infrared ion spectroscopy, (A) messenger tag approach and (B, C) UV-IR approach.

selected UV photon energy may be of sufficient energy to induce photodissociation only from the first vibrational excited state (Fig. 4B). In this case, by scanning the IR laser wavelength and following each IR laser pulse with a UV pulse of the required energy, a spectrum can be generated by plotting photodissociation as a function of IR wavelength. In an almost reverse situation, a UV wavelength can be chosen such that absorption occurs from the vibrational ground state, but not the excited state (Fig. 4C). In this case, scanning the IR photon energy produces a depletion spectrum, where photodissociation is absent at resonant IR bands. These methods can be highly selective (for instance, in some cases contributions from different conformations can be resolved) [43] and high-resolution IR action spectra can be obtained. However, these methods are not without their own drawbacks, including the requirement for cold ion generation (of ions with desirable UV absorption properties), the need for two tunable lasers, and specifications for laser overlap, timing, and other factors.

Instrumentation for IRMPD spectroscopy

At its heart, the IRMPD spectroscopy experiment is simply a specific type of MS^2 (or MS^n, if dissociation products are being spectroscopically probed) experiment. Thus, the required mass spectrometer is similar to those used for MS^n experiments. Key requirements/considerations that are beyond the scope of typical mass spectrometry experiments include:

- An ion trap (rather than a beam-type) mass spectrometer to ensure sufficient irradiation time prior to mass analysis and to facilitate laser overlap with the ion packet;
- An optical path through the ion cloud, thus optical windows must be integrated into the chamber and often holes must be drilled into the actual ion trap;
- For higher pressure ion traps (e.g., quadrupole ion traps), a pulsed trapping gas or a lowered bath gas pressure may be required or useful; [44–46] and
- In some cases, a multi-pass optical path must be incorporated to provide sufficient beam overlap with the ion cloud for enough dissociation to occur [9,11].

Given these requirements, the most commonly used setups are Fourier transform ion cyclotron resonance (FTICR) mass spectrometers [47–50] and quadrupole ion trap mass spectrometers [35,51,52].

In addition to the modified ion trap mass spectrometer, the other required piece of instrumentation is the tunable infrared laser. In choosing a tunable light source, power, tunable range, pulse length (if not continuous), and resolution are all considerations that must be made. A general overview of light sources for photodissociation and spectroscopy of mass-separated biomolecular ions has been published [53]. Details on how each light source works is beyond the scope of this work, thus we here focus on the advantages, disadvantages, and applications of some common systems. A table comparing common light sources for IRMPD spectroscopy has also been published [10]. Common types of light sources implemented for IRMPD spectroscopy are CO_2 lasers, free electron lasers (FELs), and optical parametric oscillators/amplifiers (OPO/As).

CO_2 lasers were employed early on for IRMPD spectroscopy and are still used routinely for IRMPD as an activation method for multistage mass spectrometry experiments. The main advantage of CO_2 lasers is their inherent power for benchtop systems, making high IRMPD yields possible within a typical laboratory setting. However, the tunable range is rather limited (~925–1085 cm^{-1}) [10] and, thus, only appropriate for some applications. Applications of note include selective dissociation of phosphorylated peptides [54] and characterization of saccharides [55–57].

FELs have the advantage of high peak power and wide tunability over a useful infrared range (~500–2000 cm^{-1} for typical IR spectra, with options to extend the range possible). Much of the work pushing the application of IRMPD spectroscopy to new analytical applications is being done with FELs, yet these lasers require huge commitments of space, know-how, and start-up capital, among other resources. Typically, FELs are operated as dedicated shared facilities that are, in some cases, open to outside users. Two FELs that are open for user-proposed IRMPD spectroscopy experiments are the Free Electron Laser for Infrared eXperiments (FELIX) [6,58] at Radboud University in the Netherlands and the Centre Laser Infrarouge d'Orsay (CLIO) facility in France [12]. One can envision a future where foundational work (e.g., establishing vibrational modes for moieties of interest) and one-off experiments (e.g., structural elucidation of a specific feature of interest) are performed at such user facilities, but an alternative benchtop option used for routine home institution applications and screenings.

Benchtop OPO/A systems are now readily available, covering the infrared region corresponding to the X—H stretching region (where X = O, N, C) and can provide a good deal of structural information about ions. Various suitable OPO systems are available, including both continuous wave (cw) and pulsed systems. Specific examples of OPO systems that have been used for IRMPD spectroscopy include a Lockheed-Martin Argos cw OPO [59,60], a LINOS OS4000 cw OPO [45], and a pulsed LaserVision OPO [60,61]. Down conversion crystals are available, allowing the tunable region to extend into the fingerprint region of the spectrum, but at present this typically produces insufficient power to induce multiple photon dissociation and is, thus, currently only implemented in alternative ion action spectroscopy experiments. In general, the relatively low power and limited range are the major disadvantages to OPO/As, while their compact size and relative affordability are primary advantages.

Historically and presently, there are many examples of mass spectrometers coupled to tunable infrared light sources for implementation of IRMPD spectroscopy experiments. FTICR instruments have been widely used for coupling with tunable infrared light sources, including CO_2 lasers, OPOs, and FELs, for IRMPD spectroscopy measurements; [6,9,12,62–64] quadrupole ion trap mass spectrometers (e.g., Bruker Esquire series, Bruker amaZon series, Thermo LCQ) have been coupled to both benchtop and FEL light sources in several instances, with some cited here [60,65–67]. Additionally, hybrid instruments, where the trapping component and the mass analysis component are separated in space, have also been used for IRMPD spectroscopy [45]. Notably, cryogenic ion spectroscopy using messenger tagging and similar approaches have been implemented on a modified Orbitrap [68] and a custom version of a 2-D linear ion trap [69]. These advances further suggest the promise of more widespread implementation of infrared ion spectroscopy on commercial instruments in the future.

Applications of potential clinical relevance

Peptides and Post-Translational modifications

IRMPD spectroscopy has already made useful contributions to our understanding of peptide fragmentation pathways relevant to MS-based peptide sequencing and proteomics experiments [14]. As an extension of this work, applications of IRMPD spectroscopy to post-translational modification (PTM) analysis has also proliferated [70]. Table 1 summarizes many of the key IRMPD spectroscopic signatures for PTMs from the literature.

Beyond the direct application to PTM analysis, this body of work also provides proof-of-principle evidence for the application of IRMPD spectroscopy more broadly for functional group identification. For instance, the phosphopeptide and sulfopeptide signatures reported in the literature (3670 and 3590 cm^{-1}, respectively) [71] match closely with signatures for phosphorylated and sulfated glucosamine (3666 and 3595 cm^{-1}, respectively) [66]. Signatures for phosphorylation versus sulfation are especially useful, as the two modifications (-SO_3H and -PO_3H_2) are isobaric, and, thus sometimes difficult to distinguish via mass spectrometry alone. Similarly, differentiation of isomeric hydroxyproline amino acids has been shown for both protonated [72] and

Table 1
Examples of IRMPD signatures of PTMs.

PTM/Functional Group	Notes	Reference
Hydroxylation of proline	$[hPro + H]^+$	[72]
	Carbonyl stretching mode shifts from 1750 to 1770 cm^{-1} when moving from the S,R to S,S diastereomer of 4-hydroxyproline	
	$[hPro + Li]^+$	[73]
	1734 cm^{-1} corresponds to *cis*-3-hydroxyproline, 1718 cm^{-1} to *cis*-4-hydroxyproline, and 1640 cm^{-1} to *trans*-4-hydroxyproline	
O-Sulfation	$[sYG + H]^+$, $[GsYR + H]^+$, $[sYGGFL + H]^+$	[71]
	~3590 cm^{-1}, sulfate-OH stretching	
	$[sSer - H]^-$	[77]
	1732 cm^{-1}, C = O stretching	
	1207 cm^{-1}, antisymmetric SO_3 stretch coupled to NH_2 twisting	
	1297 cm^{-1}, antisymmetric SO_2 stretching	
	$[Ser-H]^-$ displays a C = O stretching mode at 1330 cm	
	$[sSer + H]^+$	
	1022 cm^{-1}, SOH bending coupled to S-OH stretching	
	3562 cm^{-1}, sulfate-OH stretching	
	Hydroxyl side chain signature at 3660 cm^{-1} is absent upon O-sulfation	
Phosphorylation	$[pSer + H]^+$	[78]
	920–970 cm^{-1}, P-OH stretching and 1228 cm^{-1}, P = O stretching	
	$[pThr + H]^+$	
	967 cm^{-1} P-OH stretching and 1216 cm^{-1}, P = O stretching	
	$[pTyr + H]^+$	
	946 cm^{-1}, P-OH stretching and 1265 cm^{-1}, P = O stretching	
	$[pYG + H]^+$, $[GpYR + H]^+$, $[pYGGFL + H]^+$	[71]
	~3670 cm^{-1}, phosphate-OH stretching	
S- and N-Nitrosylation	$[SNOCys + H]^+$ (S-nitrosocysteine)	[74]
	1783 cm^{-1}, SN-O stretching	
	$[SNOCys - H]^-$	
	1460–1488 cm^{-1}, SN-O stretching	
	$[GSNO + H]^+$ (S-nitroso glutathione)	[75]
	1622–1690 cm^{-1}, SN-O stretching	
	$[NANT-H]^-$ (N-acetyl-N-nitrosotryptophan)	[76]
	1463 cm^{-1}, SN-O stretching	

lithiated species via IRMPD spectroscopy [73].

As summarized in Table 1, IRMPD spectroscopic investigations of several other PTMs, including nitrosation (or nitrosylation) of cysteine [74], glutathione [75], and acetyltryptophan [76] have also been reported. Spectral differences reported between non-modified versus modified amino acids suggest utility of the approach for identifying ions with specific functional groups and, potentially, targeted screenings of mixtures for specific moieties.

Electrospray ionization and protonation site isomers

There is an ongoing debate on whether gas-phase structural probes reflect solution-phase realities and whether such gas-phase measurements are biologically relevant [79,80]. While this discussion is often targeted at larger molecules with broad conformational space (e.g., proteins, complexes), which is beyond the scope of the present discussion, a related question of protonation site isomers is quite relevant to MS-based analysis of small molecules (e.g., metabolites, pharmaceuticals). Protonation site identification is not just a physical chemistry point of interest; in some cases, the site of protonation can affect the observed dissociation patterns and, troublingly, the solvent system can play a role in the observed protonation site (typically when either different protonation sites are thermodynamically favored in one solvent versus the other, or when different sites are preferred in the solution versus gas phases). For instance, mobile phase composition has been shown to affect MS/MS spectra of some drugs and drug-like compounds [81]. The effects of solvent systems on protonation site have been probed by infrared ion spectroscopy for various molecules [82–84]. While protonation site elucidation is not directly relevant to clinical laboratory analysis, an understanding of how protonation site is affected by experimental conditions and, in turn, affects observed dissociation patterns, is relevant in controlling clinical laboratory protocols and potential pitfalls that may be encountered.

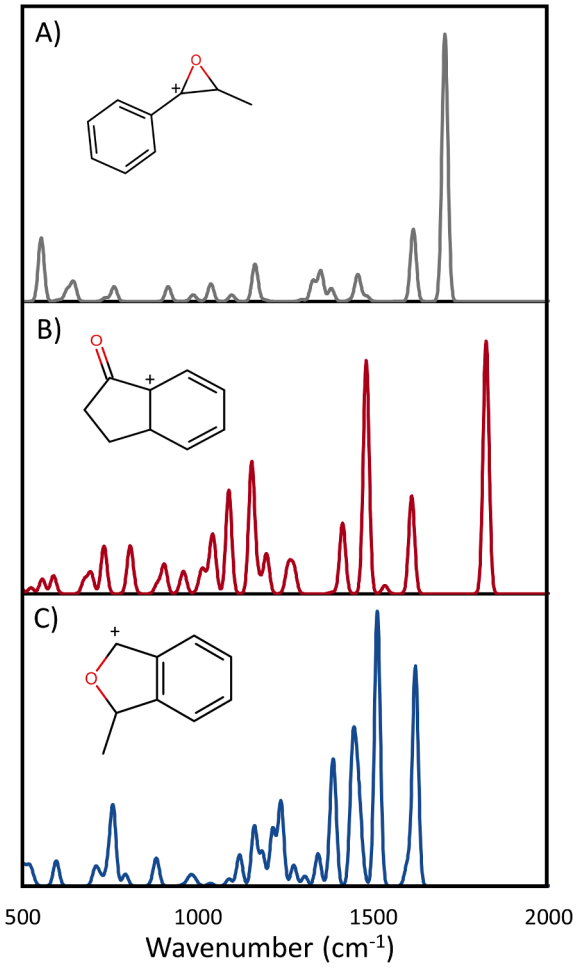

Fig. 5. Theoretical infrared spectra of three candidate structures for *m/z* 133 product ions of α-PVP (alpha-Pyrrolidinopentiophenone). Coordinates of structures denoted therein as 133a, 133b, and 133c (from bottom to top of Fig. 5), provided in Reference [85], were used to generate these spectra. This illustrates that infrared spectra fingerprints are sensitive to ion structure and that theory can be used to predict IR spectra of various structures. Theoretical calculations were performed at the B3LYP/6–31++G(d,p) level of theory, with a 0.975 scaling factor. Note, that the y-axis scale for each panel is chosen to best illustrate the fingerprint and is not identical between the three spectra.

Structural elucidation and isomer differentiation

IRMPD spectroscopy is a powerful method for structural elucidation and isomer differentiation. Through comparison of experimental IRMPD spectra of mass-isolated analyte ions to either experimental spectra obtained for synthetic standards, or to theoretical spectra obtained through computational chemistry for candidate ion structures, the structural identity of the analyte ion can be determined. Fig. 5 shows theoretical IR spectra for three candidate structures for an ion appearing at *m/z* 133 from α-PVP (alpha-pyrrolidinovalerophenone) calculated based on structures and coordinates previously reported by others [85]. These theoretical spectra illustrate how three isomeric ions each have unique infrared spectra. This concept can be generalized toward applicability in identifying drug molecules, pharmaceutical metabolites, endogenous metabolites, and other compounds of clinical significance.

Clinically relevant small molecules beyond amino acids

The IR spectra of many metabolites, pharmaceuticals, illicit drugs, and similar compounds as ions in the gas phase have been reported, as summarized in Table 2. While many of these studies provide important benchmarks for theoretical spectral predictions, fundamental insights into gas-phase ion structures, and vibrational signature determination, a few of the included studies stand out as making great strides toward the analytical (rather than fundamental/physical) utility of infrared ion spectroscopy.

Saccharides are notoriously difficult to study by mass spectrometry due to subtle structural characteristics including differences in glycosidic linkages and the presence of stereocenters. Stefan and Eyler utilized a lithium-tagging approach for the structural differentiation of disaccharides containing glucose [55]. In that work, a method was developed using a tunable CO_2 laser to differentiate disaccharides with varying glycosidic linkages by comparing peak heights of selected product ions. Moving closer to a routine analytical technique, Schindler et al. combined LC-MS with IRMPD for the online analysis of select mono- and disaccharide standards [86]. Isomeric saccharides may exhibit very similar fragmentation patterns; to overcome this challenge IRMPD spectroscopy was used to observe diagnostic fingerprints, allowing GlcNAcβ1,4GlcNAc, GlcNAcβ1, and 6GlcNAc to be differentiated in the region between 2700 and 3700 cm^{-1}.

A wide range of pharmaceuticals and drug-like compounds have also been studied with IRMPD spectroscopy. For example, IRMPD studies have been carried out on cisplatin, a prominent anticancer drug. De Petris et al. sought to characterize a key intermediate in the hydrolysis of cisplatin, where previous attempts had failed due to complex processes occurring in solution [19]. Here, the group was able to obtain previously unreported vibrational spectral features of this intermediate, free from solvent interferences, which was only possible by gas-phase action spectroscopy techniques. Corinti et al. employed IRMPD spectroscopy and tandem mass spectrometry methods toward elucidating the mechanisms behind the binding of cisplatin with uracil and thiouracil, with the aim of aiding the understanding of drug mechanism and design [21].

IRMPD spectroscopy has been used for the characterization and

Table 2
Example applications of gas-phase infrared action spectroscopy to metabolites, pharmaceuticals, and potentially clinically relevant small molecules.

Target Compounds	Notes	Reference (s)
2-Fluoromethamphetamine, 4-Fluoromethamphetamine (2-FMA, 4-FMA)	Single-laser IR^2MS^3 method utilized to match FA standards to a confiscated street sample	[87]
2-phenylethylamine	IRMPD used to explore the effects of fluorine substitution	[25]
α-PVP (alpha-Pyrrolidinopentiophenone) and fragment ions	IRMPD along with DFT calculations used to structurally characterize fragment ions of α-PVP	[85]
Atorvastatin (Lipitor) & metabolites	LC used to separate positional isomers of hydroxy-atorvastatin, structural characterization by IRMPD	[22]
Ciprofloxacin	IRMPD utilized to characterize the structure of this quinoline complexed with different metal centers	[88]
Cisplatin	IRMPD utilized to study the binding of cisplatin with uracil and arginine-linked cisplatin	[21,89]
Disaccharides	Lithium tagging of analytes	[55]
Fluorinated nucleosides	IRMPD used in various studies of fluorinated nucleosides for structural characterization and effects of fluoro-substitution	[90,91]
Glutaric acid and ethylmalonic acid	IRMPD used to characterize and differentiate these isomeric metabolites that are well-known biomarkers for metabolic defects	[28]
Isomers of FA (fluoroamphetamine), MMC (methylmethcathinone), MDA (methylenedioxyamphetamine), and MDMA (methylenedioxymethamphetamine)	IRMPD used to identify unknown substances present in confiscated street sample; analyzed in conjunction with reference standards	[18]
Lysine and pipecolic acid	Reference-standard free comprehensive workflow described; applied to detection of metabolites from a patient suffering from hyperlysinemia	[27]
MDMA & metabolites	Cryogenic ion trap and tagging approach utilized	[23]
Mono- and disaccharides	LC used to separate isomeric mixtures of saccharides, structural characterization by IRMPD	[86]
N-acetylhexosamines	IRMPD used to differentiate enantiomers of these amide sugar derivatives in human body fluids	[29]
Penicillamine	IRMPD used to differentiate L- and D- pencillamine encapsulated by β-cyclodextrin	[20]
Platinum anticancer drugs	IRMPD used to characterize anticancer drugs containing platinum	[19]
Tacrolimus	IRMPD used to probe preferred calcium binding sites of this immunosuppressant	[92]
Tyramine, taurine, 2-aminoethylphosphonic acid (2-AEP), 2-phenyl-1-ethanolamine (2P1EA), p-, o-, m-aminobenzoic acid, salicylamide, 3-pyridylacetic acid	IRMPD and IRPD used to identify functional groups of isobaric metabolites, and the differentiation of isomeric metabolites	[26]
Unmodified amino acids	IRMPD used for the analysis of protonated and deprotonated amino acids in various applications	[93–96]
Unmodified nucleosides	IRMPD used for various applications of unmodified nucleosides	[97–99]

identification of street drugs. In one study, the novel psychoactive substances (NPSs) of a street sample were subjected to IRMPD spectroscopy, where the active compounds were identified as 2- and 4-flouromethamphetamine (FMA), and the presence of 3-fluoromethamphetamine was ruled out [87]. Specifically, in this study, an approach dubbed "IR^2MS^3", based on hole-burning (setting the laser at one specific frequency to "burn away" one type of conformer/structure), was implemented. In addition, (room temperature and/or cryogenic) infrared ion spectroscopy has been applied to MDMA and similar synthetic drug isomers [18,23]. Studies of drug molecules, including illicit drugs and especially novel designer drugs, have potential utility in various fields including toxicology, forensic science, and sports anti-doping science.

Recent innovations and areas for future focus

IRMPD spectroscopy has significant unrealized potential application in the clinical analysis field. From providing foundational understanding of underlying methods (e.g., protonation site formed during ESI or benchmarking theoretical calculations) to characterizing novel street drugs, differentiating isomeric metabolites, and shedding light on pharmaceutical binding motifs, there are many paths by which IRMPD spectroscopy could make valuable contributions in the clinical laboratory. Several areas where progress and improvements are being made highlight the challenges and opportunities of this technology.

Instrumentation and light sources

As introduced earlier, implementation of IRMPD spectroscopy on commercial ion trap instruments is increasing. This means that, with minor alterations, familiar user interfaces and hardware can be used to make ion spectroscopy more accessible. Increasing support of ion spectroscopy on commercial systems may be expected to contribute to reaching a tipping point that will lead to resonant IR photodissociation and infrared ion spectroscopy's widespread use. While ion traps are becoming ever more accessible, the lack of powerful, tunable light source options at the desired wavelength ranges is a current limitation. While innovations in tunable, powerful light sources is beyond the scope of this discussion, it is worth mentioning that there are promising avenues of innovation being pursued for improved power over relevant ranges, with OPOs, quantum cascade lasers, and other technologies potentially playing future roles in making IRMPD spectroscopy more routine and widespread [53]. Of note, a recent report demonstrates the implementation of a hollow-core fiber-optic cable to deliver infrared light from a cw CO_2 laser to an Orbitrap Fusion Lumos mass spectrometer; this approach represents an exciting step toward increasing safety and robustness when coupling laser light to mass spectrometers [100].

Complex mixtures

Many biological matrices are inherently complex. Mass isolation within an ion trap is limited, given isobaric interferences, and space charge limits require that injected ions be relatively pure to maintain an adequate ion population after isolation. Thus, with the rise in "real samples", it is increasingly necessary to explore coupling of separations methods with IRMPD spectroscopy to exploit mass spectrometry's power.

LC separations can be performed offline or online. Offline, there are few worries with integrating with IRMPD spectroscopy, so long as sufficient sample is available. Online integration is a bit trickier, with analysis timescale compatibility becoming relevant. Over the past few years, great strides have been made toward full integration of infrared

ion spectroscopy with LC-MS. Using a stop flow approach and a 6-minute IRMPD analysis time, online separation and identification of disaccharide regioisomers and monosaccharide anomers have been demonstrated [86]. A tutorial perspective has recently been published covering some of the finer details of the LC-infrared ion spectroscopy coupling [101]. Excitingly, such approaches have begun offering insights into real health issues. Analytical methods incorporating LC and infrared ion spectroscopy have been used to characterize lysine in the plasma of a hyperlysinemia patient [27], identify the Phe-glucose Amadori rearrangement product as a signature for phenylketonuria [102], and discover new biomarkers associated with pyridoxine dependent epilepsy [103]. Contributions of IRMPD spectroscopy supporting metabolomics and biomarker discovery can be expected to increase. Likewise, LC-coupled IRMPD spectroscopy will likely also make important contributions to clinically relevant realms in the near future.

While LC is one of the most established separations methods, ion mobility spectrometry (IMS) methods have increasingly found utility in combination with mass spectrometric analyses. Some early iterations of coupling IMS with infrared ion spectroscopy have been made, and as with LC integration, such successes are expected to continue and accelerate. Notable reports include coupling of high resolution IMS with (cryogenic) infrared ion spectroscopy for glycan mixture analysis [104] and the coupling of FAIMS with IRMPD spectroscopy for the analysis of molecular isoforms, namely a mixture of paracetamol and 2-phenylglycine [105]. As IMS and IRMPD spectroscopy methods are each refined toward clinically relevant applications, their combination will be a powerful tool.

Analysis timescales

Because wavelength-resolution is required, measuring IRMPD spectra is inherently slower than typical MS/MS experiments (though on-par with energy-resolved CID). Thus, one approach to making infrared ion spectroscopy more analytically viable is to improve the amount of information obtained in a given time. One approach is to exploit the multiplexing advantage, and another is to focus solely on wavelengths where specific information can be obtained.

Multiplexing is not intuitive with IRMPD spectroscopy, given that dissociation pathways are not known a priori (at least for unknowns) and product ions must be included in the yield calculations. In cases where a single, known loss channel can be devised, multiple analytes could be measured simultaneously, as long as their masses do not overlap. The most straightforward approach to this would be to tag molecules, similar to how cold ion messenger tagging is achieved, with a tag of known mass, which would be lost upon irradiation with resonant photons. At room temperature, this has been demonstrated with crown ethers bound to peptides [106]. Since a non-selective room-temperature tag is not accessible, this approach to multiplexing is generally more suitable for cold ion spectroscopy approaches, with intermediate tags (for instance acetonitrile, with binding affinity between the very cold He, N_2 tags and the selective room temperature crown ether tags) having been proposed [26]. A promising, segmented ion trap approach to multiplexing (cryogenic) infrared ion spectroscopy has recently been introduced toward high-throughput IR fingerprinting of IMS-separated glycans [107]. Extensions of this approach will no doubt provide additional paths forward toward more rapid, parallelized measurements of infrared ion spectra.

For cases where specific functional groups are of interest, one can envision a single (or few)-mode IRMPD experiment allowing for analyte ions containing that specific mode to be illuminated from the background signal. Such resonant IRMPD-MS could be exploited to scan, for instance, an LC trace for regions wherein analytes with specific functional groups are eluting. Fig. 6 provides an illustration of this type of approach in two ways. The boxes with colored elliptical moieties illustrate how the precursor ion would appear as an identical signal for both "isomers", whereas when a wavelength was chosen that produced

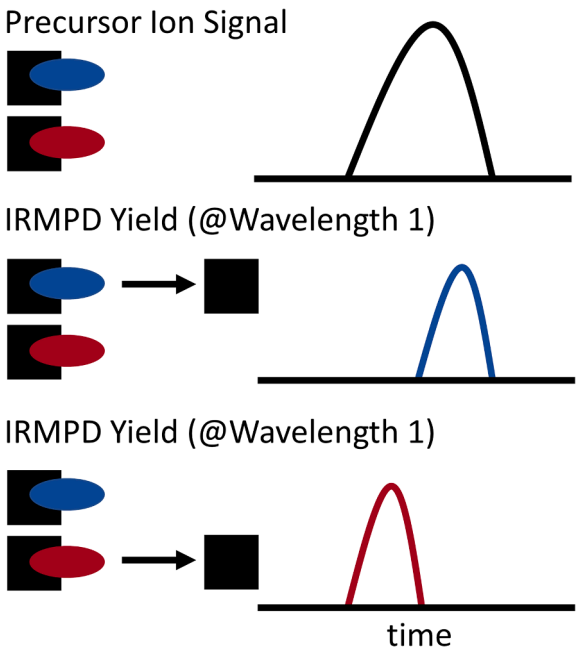

Fig. 6. Rapid resonant IRMPD integration with a separation method (e.g., IMS or LC). Here, a precursor ion made up of two isomers (schematically represented as a black block having either a blue or red moiety) produces a single convoluted peak during the separation process. By measuring IRMPD yield at either wavelength 1 (where the blue moiety absorbs and leads to IRMPD) or wavelength 2 (where the red moiety absorbs and produces IRMPD), the relative contributions of the two isomers can, in principle, be separated by plotting resonant IRMPD yield (at a mode-specific wavelength) versus time. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

IRMPD for the blue mode, the middle chromatogram (plotted as IRMPD yield vs time) would illuminate the portions of the signal that arose from ions containing the blue moiety and, likewise, when a second wavelength was used for irradiation, this time resonant with the red moiety, the portion of the initial signal containing said moiety could be deduced (bottom panel). This approach would be especially beneficial to isomeric/isobaric species and cases where isomers may have a propensity to dissociate via similar pathways/neutral losses. For this approach to work, the modes of interest need to remain in a relatively narrow region of the IR spectrum, regardless of chemical environment within the scope of the class(es) of compounds of interest for the studies. Proof-of-principle versions of this approach have been demonstrated, for example, for phosphorylated and sulfated peptides [71]. Notably, similar differentiation between sulfated and phosphorylated carbohydrates has been reported [66], illustrating how this type of approach may be feasible across various compound classes, including those of potential clinical relevance.

Knowledge transfer and collaboration

One hurdle, more human than technological, to overcome is transferring knowledge and know-how between research communities and facilitating communication. Much of ion spectroscopy has historically been focused on fundamental physical chemistry/chemical physics questions, like identifying interstellar bands [108–111] and understanding mechanisms of chemical reaction [13,37,112–114]. However, as outlined in Tables 1 and 2, and the references provided therein, biochemical and pharmaceutical systems have recently begun to gain traction as systems of study for ion spectroscopy, with many recent examples. Looking at the authors' lists of some of these most recent papers, it is also remarkable that collaborative works between

researchers and practitioners at hospitals, forensic science units, and similar collaborations are beginning to emerge. Additional collaborations including those from fundamental biology fields, as well as more applied areas such as toxicology, forensic science, and sports anti-doping science, are expected to continue to rise in the future. As this type of interdisciplinary collaboration and cross-pollination continues to grow, so too will the clinical (and clinically adjacent) applications of this budding and valuable technique.

While not discussed at length here, it is worth noting that such knowledge transfer and collaboration can also lead to answering more fundamental questions of biomolecular structure and interactions in the gas phase, which may one day have important impacts on our understanding of biochemical systems at the root of various diseases and treatment approaches. A critical review on this topic, highlighting the importance of fostering improved communication and collaboration between molecular physics, biochemistry, and molecular biology communities as it relates to vibrational spectroscopy of biomolecules in the gas phase, has been published [115].

Outlook

While IRMPD spectroscopy is not without challenges for full realization of its potential to positively influence mass spectrometry-based clinical analysis, it has been illustrated herein that it provides a powerful structural elucidation tool for gas-phase ions, which has already provided a wealth of relevant fundamental understanding of gas-phase ions' behaviors, and which holds even greater promise for the future.

Short-term outlook

Currently, IRMPD spectroscopy finds utility and demonstrates great promise as an orthogonal structural characterization technique; for example, IRMPD spectroscopy has potential for speeding up biomarker structural elucidation—which could then be used to inform the development of more traditional clinical analysis method development. IRMPD spectroscopy can exploit the sensitivity of mass spectrometry, the mixture analysis power of LC-MS platforms, and the ability to predict gas-phase IR spectra *in silico* without the need for reference standards, making it a powerful structural elucidation tool for low-abundance analytes of interest.

Long-term outlook

While there are still many practical challenges hindering the widespread adoption of IRMPD spectroscopy into most mass spectrometry-based workflows, there have been incredible advances in the past 20 or so years and it is likely that the next decades will usher in new technologies and experimental approaches that allow it to flourish further. Historically, technology has risen to new challenges repeatedly. Notable examples with regard to mass spectrometry include the shift from slow, scanning methods, like magnetic sector mass spectrometry, to rapid mass analysis using time-of-flight based mass spectrometers and the ongoing developments of liquid chromatography from relatively slow HPLC to fast, high resolution modern UPLC and beyond. Infrared ion spectroscopy will likewise march toward more widespread utility, as acquisition time is reduced, multiplexing is mastered, and automation is integrated.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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