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Shining a light on clinical spectroscopy: Translation of diagnostic IR, 2D-IR and Raman spectroscopy towards the clinic

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ABSTRACT

In recent years, the application of vibrational spectroscopy in biomedical research has rapidly expanded; covering aspects of pharmaceutical development, to point-of-care technologies. Vibrational spectroscopy techniques such as Fourier-transform IR (FTIR), and Raman spectroscopy have been at the forefront of this movement, with their complementary information able to shine light onto a range of medical applications. As a relative newcomer to biomedical applications, two-dimensional (2D)-IR is also gaining traction in the field. Here we describe the recent development of these techniques as analytical tools in medical science, and their relative advancements towards the clinic.

Introduction

Vibrational spectroscopy is able to elucidate a wealth of biochemical, molecular and structural information from biological molecules by monitoring the interaction of light with chemical bonds. Using spectrometers, the defined energy required to enable molecular excitation can be recorded as a spectrum, which behaves as a 'biochemical fingerprint' of the sample being investigated, with each band corresponding to a known molecular entity. For biological samples, which are often inherently complex in their biochemical composition, vibrational spectroscopy is able to shine light on subtle chemical differences and holistically characterise the sample under investigation.

The biomedical applications of such vibrational spectroscopy techniques are wide; from monitoring drug efficacy to disease diagnostics [1,2]. A variety of vibrational spectroscopy techniques are able to derive chemical information, each of which with characteristic features that are complementary to clinical translation [3]. Fourier-transform IR (FTIR) spectroscopy and Raman spectroscopy are commonly documented as primary approaches, with each of these techniques having a variety of additional sampling modes that enhance experimental capabilities. This includes suitable analysis of different sample types, including tissues, bodily fluids, and cells; as well as the ability to define the experimental output, such as in the form of spectral images [4].

The potential of such techniques in the field of biomedical science, alongside the development of computational methods for spectral analysis, has spurred an array of proof-of-concept studies [5]. Due to the complexity of a biological dataset, computational approaches have allowed exploratory analysis of data, as well as deep learning and pattern recognition. Ultimately, the goal for many of these studies is translation into the health services, or integration into standard protocols. Whilst many of these studies have advanced the field and brought clinical translation closer, as of yet, no single application has paved the way [6]. It has been suggested that this may be due to lack of understanding of the clinical pathway [7], although is more likely attributed to the multitude of hurdles along this pathway to translation including health technology assessments, regulatory approval, prototype development, clinical feasibility and of course, ever elusive funding (Fig. 1).

As the field of biomedical FTIR and Raman spectroscopies expand, the emergence of complementary techniques have also arisen. Non-linear two-dimensional (2D)-IR for example, has only recently been applied to biomedical investigations and may well follow suit with regards to clinical translation. Here we describe the progress of FTIR, Raman and 2D-IR spectroscopies as medical technologies, with particular emphasis on advanced applications.

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Clinical Question | Technical Approach | Proof-of-Concept | Clinical Studies | Regulatory Approvals | Launch & Sales

Technical Considerations

Clinical Question	Technical Approach	Proof-of-Concept	Clinical Studies	Regulatory Approvals	Launch & Sales
• Define unmet need in clinical field • Determine indication of use *e.g.* diagnostic test, exploratory tool or surgical aid	• Consider which spectroscopic technique best lends itself to the application, *e.g.,* IR, Raman or 2D-IR • Explore workflow considerations • Consider technical developments that are required	• Apply technique to human samples • Test research to find basic utility to defined application • Appropriate choice of sample cohort and size	• Translation to real-world patients • Determine appropriate study type for stage of technology development, *e.g.,* feasibility, validation, diagnostic accuracy or randomized clinical trial	• Obtain clinical data sufficient for regulatory approvals, including trial design and statistical power • Explore relevant regulatory pathway dependent upon technology type and potential market	• Explore procurement and reimbursement process for product launch • Ensuring support from key opinion leaders, clinicians and stakeholders

Fig. 1. Translational research in the field of vibrational spectroscopy. An overview to technical considerations along the pathway to translation.

Fourier-transform infrared spectroscopy

IR spectroscopy is based upon the principle that molecules absorb frequencies of light in the IR range. When this occurs, molecules move from the ground vibrational state ($\nu = 0$) to the first excited vibrational state ($\nu = 1$). This transition requires a quantised amount of energy and thus can only occur when an incident photon exactly matches this energy [8]. Specifically, mid-IR spectroscopy focuses upon the region of the electromagnetic spectrum between 2.5–25 μm, that matches the frequency of known biochemical bond vibrations [9].

Chemical bonds are known to vibrate in their ground state. Photon absorption triggers the molecule to vibrate at a higher vibrational state, causing chemical bonds to stretch apart or bend, altering the bond angle [10]. These distinct vibrations, known as vibrational modes, are dependent upon the structure of the molecule under investigation. A linear molecule with N number of atoms, will exhibit $3N-5$ modes, whereas a non-linear molecule will have $3N-6$ modes [11]. However, not all vibrational modes of a molecule can be identified using IR spectroscopy. To be considered IR active, a molecular vibration must exhibit a change in dipole moment – the charge distribution across the molecule – as it undergoes the transition [12]. For this reason, polar bonds such as those of water are always IR active, whereas non-polar bonds are only active when their vibrations are non-symmetrical.

Table 1
FTIR sampling modes used for analysing biological materials.

Sampling Mode	Positive Features	Negative Features	Tissue Analysis	Biofluid Analysis
Transmission IR Beam Sample To Detector	High quality spatial information High quality signal-to-noise ratio (SNR)	Susceptible to water interference Need for IR transparent substrates Sample thickness restrictions	[23] M.J. Baker et al., Journal of Biophotonics, **2** (2009) 104-113. [24] P. Lasch et al., Biochimica et Biophysica Acta, **1688** (2004) 176-186. [25] M.J. Pilling et al., Analyst, **140** (2015) 2383-2392.	[26] H. Fabian et al., Journal of Biomedical Optics, **10** (2005) 1-10. [27] C. Hughes et al., Journal of Biophotonics. **7** (2014) 180–188. [28] E. Scaglia et al., Analytical and Bioanalytical Chemistry. **401** (2011) 2919–2925.
Transflectance Reflective Substrate	Good quality spatial resolution Relatively low-cost reflective substrates High quality SNR data	Susceptible to water interference Spectral artefacts from reflective substrates Sample thickness restrictions	[29] K.R. Bambery et al., Analyst. **137** (2012) 126–132. [30] P. Bassan et al., Analyst. **135** (2010) 268–277. [31] B. Bird et al., Analyst. **134** (2009) 1067.	[32] E. Staniszewska-Slezak et al., Analyst. **140** (2015) 2412–2421.
ATR Evanescent Wave IRE	Minimum sample preparation Substrate not necessary Shorter pathlength allowing analysis of water containing samples	Fixed point of analysis limiting throughput Sample thickness must be greater than penetration depth	[33] C. Lima et al., International Journal of Molecular Sciences. **16** (2015) 6621–6630. [34] G. Theophilou et al., Analyst. **141** (2016) 585–594. [35] M.J. Walsh et al., Vibrational Spectroscopy. **60** (2012) 23–28.	[36] K. Gajjar et al., Analyst. **138** (2013) 3917. [37] J. Hands et al., Journal of Biophotonics. **7** (2014) 189-199. [38] S. Roy et al., Analytical Chemistry. **89** (2017) 5238-5245.

Fourier-transform infrared spectroscopy: instrumentation

Traditionally, these bond vibrations were analysed using dispersive IR instruments. In such systems, the sample is first exposed to polychromatic light generated by a globar source, which is subsequently directed to a diffraction grating where the individual wavelengths of light would be separated out towards the detector [13]. As each wavelength is measured one at a time, this process was slow and also suffered from throughput losses. However, the development of FTIR spectrometers in the 1960's dramatically changed the approach to IR spectroscopy, improving analysis times as well as instrument sensitivities [14]. Spectra are obtained in the frequency domain in dispersive systems, whereas in FTIR spectrometers spectra are obtained in the time domain, which allows all the wavelengths of radiation to be monitored at once [15]. This is possible due to the integration of a Michelson interferometer as well as the significant advancements in computer technology [16,17].

The development of additional sampling modes and acquisition capabilities has meant that FTIR spectroscopy is now considered a powerful and versatile analytical tool. The user generally has the choice between three major sampling modalities; transmission, reflectance, and attenuated total reflection (ATR) approaches (Table 1) [18]. Transmission FTIR spectroscopy observes energy absorption from the sample by directing the IR beam through the sample, and consequently monitoring the energy change. This simple approach can yield high quality IR spectra in a non-destructive manner, although it is also reliant upon the sample being placed on IR transparent substrates, which are often expensive in comparison to low emissivity slides used in transflection mode [19]. Furthermore, as light needs to fully traverse the sample, there is a limit to the sample thickness to allow sufficient detection of the signal [9]. The preparation steps required for samples in this case can often be time consuming, and not conducive towards clinical translation.

Contrastingly, transflection FTIR spectroscopy observes the transmission of IR light through the sample; however, by placing the sample on a reflective substrate, the light is in fact reflected back through the sample toward the detector. Transflection has become popular due to the relative low cost of substrates (aforementioned low emissivity slides), as well as the higher absorbance obtained due to a double pass through the sample [20]. However, as with transmission FTIR spectroscopy, there is a limit to sample thickness, and also the possibility of spectral artefacts due to the employment of reflective substrates [21].

Alternatively, an internal reflection approach offers the possibility of substrate-free sample analysis. ATR-FTIR spectroscopy is based upon the principal of total internal reflection, where the IR beam is reflected within a fixed crystal, or internal reflection element (IRE), made of a IR transparent material with a high refractive index (such as diamond, germanium or zinc selenide) [22]. Traditionally, a sample is placed in contact with this IRE eliminating the need for a sample substrate. At the point of reflection within the IRE, the difference in the diffractive indices of the IRE and sample results in the creation of an evanescent wave at the IRE-sample interface. This wave penetrates into the sample to a defined depth allowing photon absorption in the sample which will thus attenuate the incident light [18]. With a smaller pathlength of light, defined by the evanescent wave penetration depth, one benefit of using ATR-FTIR spectroscopy is the ability to analyse water containing samples, as less water is sampled. Additionally, as the sampling area is defined by the IRE, the spectra obtained are from a larger sample area. For heterogenous samples with inherent architecture, such as tissue samples, spatial information is not as readily accessible using this sampling mode, however could provide a 'macro' measurement across a defined area.

Adaptations to these standard FTIR spectroscopy methods have also been further developed this technique in the biomedical field. The addition of a microscope for instance has allowed the focusing of IR light on microscopic sample areas, providing high spatial resolution data [39,40]. The use of alternative light sources, such as broadband radiation with high flux density from a synchrotron source or quantum cascade lasers (QCL) that can provide rapid discrete frequency sampling, can also provide greater sensitivity when measuring biological samples. The development of imaging technologies, largely based upon the detector capabilities in the spectrometers, have also allowed greater flexibility in spectral acquisition [41].

Fourier-transform infrared spectroscopy: clinical applications

There have been many proposed clinical applications of FTIR spectroscopy, yet the majority have been limited in their development. Here we describe the most advanced of these applications and present their progress towards translation into the clinic.

One such application is the development of spectral histopathology. Histopathology is a process within disease diagnosis pathways where a trained pathologist observes changes in tissue structure at a microscopic scale. Whilst this is considered gold standard, the subjective nature of this approach often results in a lack of consensus between pathologists [42–44]. Integration of 'spectropathology' into the current clinical pathway may reduce this operator variability, particularly in marginal cases, whilst also improving time efficiency during the process. The imaging capabilities of FTIR spectroscopy allow visual classification of tissue samples, with clear differentiation between tissue features, as well as the presence of diseased or cancerous tissue. This approach has proven valuable in exploring prostate tissue, differentiating between benign and malignant epithelium, as well as other tissues including colon, lung and brain tissues [45,46–48]. However, it is worth noting that tissue analysis often requires significant sample preparation steps, particularly when using pathlength dependent FTIR modalities – such as transmission or transflection.

The rapid determination of cancerous tissue is useful for determining tumour margins and could be a useful intra-operative aid [48]. However, it is noticeable that FTIR spectroscopy have been relatively limited to *ex vivo* applications, although novel light sources may extend this technique to other applications [49]. QCLs as an example, have significant speed advantages over traditional thermal light sources; allowing discrete frequency scanning [50]. A significant hurdle in translation of this application is the choice of an appropriate sample substrate that results in spectra of sufficient quality and also fits within cost restrictions in health services. IR transparent (or reflective) substrates, such as calcium fluoride windows, provide optimum spectral quality yet fail with regards to cost-effectiveness [19]. A recent study presented the analysis of routine pathology samples obtained from prostate samples; sectioned onto glass slides with coverslips and stained with eosin and haematoxylin [51]. This promising study shows a non-disruptive application of spectroscopy that is complementary to traditional pathology; minimising widespread changes to the clinical pathway. Whilst disruptive technologies may initially excite the markets they enter, in the case of medical technologies disruption to the current clinic practise can become a significant barrier to translation [52].

Another established application is in field of spectral cytology, or "spectrocytology", where FTIR spectroscopy has been explored as a complementary, or even alternative, technique to cytological screening [49]. A number of early studies investigated FTIR spectroscopy as a tool for aiding the detection of cervical cancer. This area has since expanded dramatically with more recent studies achieving greater granularity, such as determining the spectral response of cervical cancer cell lines to human papillomavirus [53–56]. Furthermore, cells exfoliated from oral tissues have also been shown to be effective in the diagnosis of oropharyngeal and laryngeal cancers [57]. A number of aspects of spectrocytology make it well suited to the clinic; samples are generally easy to obtain and the opportunity to adjunct onto existing screening programmes make it an attractive target for translation. However, there remains no clear advancement towards a clinical setting. Whilst it is possible to obtain high quality spectra across most modalities of FTIR spectroscopy, barriers to translation could potentially be; (i) a requirement for sample

preparation to account for inherent water content in cells, (ii) requirement for sample substrates that may incur costs too high for health service screening, and (iii) spectral distortions that may arise as a consequence of Mie scattering [58].

The relative amount of light absorbed by a sample is directly proportional to the concentration of its molecular components, following the principles of Beer-Lambert Law. As such, IR spectroscopy can derive quantitative information which alongside multivariate analysis has allowed many applications across the clinical field. It has been shown that analytes within biofluids, such as blood serum, plasma and urine can be quantified with direct clinical applications [59–61]. For instance, total protein content of blood serum, which is routinely used as a marker of disease or infection, can be elucidated using ATR-FTIR spectroscopy; potentially allowing rapid, and label free monitoring of patient health [62].

The analysis of biofluids is a significant portion of the clinical IR spectroscopy field, with a wide range of diagnostic applications [63]. Biofluids represent an ideal medium for diagnostic tests as they can be acquired without significant invasion (in comparison to tissue biopsies, for example), and can provide localised or globally representative information of patient health. Saliva, cerebrospinal fluid, and urine are examples of organ and system specific biofluids; whereas blood and its derivatives, such as plasma and serum, can provide body-wide information. Due to this, liquid biopsies are largely considered the ‘holy grail’ of early disease detection; with increasing scientific research and media coverage in recent years, particularly in the area of cancer diagnostics [64]. However, the majority of research in the field of liquid biopsy is centred around discrete biomarker identification; the search for a known entity that is confirmatory of disease [65]. Whilst this has been shown to be diagnostically accurate, particularly in the case of circulating tumour DNA, there are a number of limitations to this approach [66]. Significantly, it requires *a priori* knowledge of the specific biomarker, and is also limited by population variance that can lower diagnostic performances [67].

The aforementioned ‘biomolecular fingerprint’ provided by FTIR and other vibrational spectroscopies may provide an alternative approach for liquid biopsies. The spectra obtained are representative of the entire composition of a given biofluid, including any potential diagnostic biomarkers. In conjunction with multivariate analysis techniques, this holistic approach is less reliant upon a single biomarker and observes widespread changes and interactions of diagnostically relevant molecules. This has been shown across a range of biofluids including the detection of; malignant biliary tissue from bile [68], lung cancer using sputum [69], and bladder cancer using urine [70]. It is worth noting that biofluid analysis using IR-based spectroscopies is somewhat limited due to the inherent water content of the fluids, which can often mask significant biological information. For this reason analysis of dried biofluids is preferable [9]. That said, the complex drying patterns that arise from air-dried biofluid drops, such as the widely-known coffee ring effect [71], have inhibited successful translation thus far, as the surface inhomogeneity can cause peak shifts and alterations in band intensities [72]. Hence, it is vital the drying conditions are controlled and optimal protocols are developed in order to obtain an even deposition across the sample [73,74].

Perhaps the most advanced application of FTIR spectroscopy, is the analysis of blood serum for the detection of brain tumours using ATR-FTIR spectroscopy [75]. This is due in part to the unmet clinical need for a rapid diagnostic, which would prevent repeat visits to the general practitioner (GP) due to misinterpreted symptoms, which often leads to diagnoses being made in an emergency. Based upon ATR-FTIR spectroscopy specifically, a small aliquot of serum is placed upon the IRE, spectra are collected, and the subsequent dataset is then analysed using machine learning. This approach yields a diagnostic result dependent upon the clinical question (for example, cancer versus non-cancer, primary versus secondary cancers etc.) alongside a prediction of accuracy, or confidence in the given result, to aid in the clinical decision making [76].

An important consideration for applications such as this is the indication of use. In this example, the implications of an absolute diagnostic versus a triage method have been explored by mapping the diagnostic pathway for brain tumours, with the opportunities as a triage tool in primary and secondary care measured in terms of cost savings to the health service as well as the impact on quality of life [77]. In primary care, such a test would allow a GP to investigate a patient’s symptoms, without immediate referral to secondary care, where there is already increased demand on expensive neuro-imaging equipment such as CT and MRI scanners. In secondary care, this test would allow patients with suspected brain tumours to be imaged more rapidly, allowing faster diagnosis and consequently prompt treatment. Interestingly, this approach moves away from point-of-care testing, where health economic benefits could potentially be negligible [7].

This is one example where research has been actively commercialised for the purpose of improving the clinical pathway and impacting patient health; in this case, a Scottish based university spin-out called ClinSpec Diagnostics Ltd. There has also been the formation of several other commercial entities employing IR technologies for similar purposes, including; CIRECA (US), for FTIR-based tissue imaging and biomarker identification [78], Beamline Diagnostics (UK) for ATR-FTIR spectroscopy of bulk tissue analysis in the operating theatre [79], Glyconics (UK), a point-of-care diagnostic for COPD using FTIR-based analysis of mucus [80], and also Biotech Resources (Aus) for infectious disease detection [81].

This latter commercial entity has recently made significant advancements towards translation, with exciting studies on malarial detection in the field [82]. The technology employs ATR-FTIR spectroscopy for the analysis of whole blood samples, which are freeze-thawed, and deposited upon a glass slide [83]. This approach has been shown to not only indicate presence of infection, but also provide a quantitative estimate of malarial parasitaemia, as well as blood urea and glucose levels [38]. An interesting aspect to this approach is the use of glass slides, which negates the need for expensive IR substrates that would otherwise inhibit uptake in remote locations.

Raman spectroscopy

Raman spectroscopy (RS) relies on the inelastic scattering of incident light upon a sample. In biological samples, such as tissue and biofluids, the majority of incident light on the sample is elastically scattered known as Rayleigh scattering [84]. A small proportion of the light (around 1 in 10^7) is inelastically scattered, resulting in a difference in energy between the incident photon and the scattered photon [85]. The energy difference in the scattered light corresponds to the transitions between vibrational energy levels within the molecules, and the bands that are observed in Raman spectra relate to the different vibrational and rotational modes of the molecules present within the sample.

Similar to IR, a Raman spectrum provides a wealth of biochemical information from a sample. Due to different selection rules that govern the scattering process – for example, change in polarizability vs change in dipole moment – Raman spectroscopy can yield complementary information to IR spectroscopies. This is significant when considering that water is a strong IR absorber, whereas water molecules have a small Raman cross section when interrogated with visible wavelength excitation. The minimal water interference within Raman spectra has allowed Raman spectroscopic analysis of clinical samples such as liquid biopsy specimens [86].

Raman spectroscopy: instrumentation

One of the first instrumental developments for clinical applications was the coupling of a Raman spectrometer to a microscope for applications to pathologic specimens in the 1970s [87]. Through advances in laser excitation sources and improvements in charge-coupled

device (CCD) detectors, Raman microspectrometers are now commercially available and have been applied to a range of different biomedical applications; such as live cellular imaging, pathologic tissue analysis and biofluid analysis [88–91].

As Raman microspectroscopic imaging commonly uses dispersive detection, which is rather inefficient when collecting images (minutes per frame), it can limit real time analysis of dynamic processes, particularly across large areas and/or over short periods of time. Moreover, microscopy-based techniques rely on light being collected through objective lenses, which typically have a smaller field of view than the sample being imaged (e.g. tissue sections). This latter point is not exclusive to RS, with FTIR microspectroscopy approaches also encountering this limitation. To combat these problems, coherent Raman spectroscopy (CRS) techniques have become of interest in the field [92]. Two of these processes, stimulated Raman scattering (SRS) and coherent anti-Stokes Raman Scattering (CARS), can overcome issues with Raman imaging of biological systems due to their potential speed and spectral resolution. The technological developments in SRS and CARS imaging for biological systems have been reviewed in depth elsewhere [92–94]. Furthermore, a recent review article on the potential for transmission Raman highlights the use of spatially offset Raman spectroscopy (SORS) and surface enhanced spatially offset Raman spectroscopy (SESORS) for completely non-invasive imaging and disease monitoring at depth through tissue [95].

An alternative method for analysing biological samples is through Raman fibre-optics. Fibre-optic systems have great potential for rapid, non-destructive, real-time in-vivo and ex-vivo tissue analysis, due to their ability to effectively excite samples and collect a spectrum from a single point. The flexibility of fibre-optic systems has enabled them to be developed and integrated into a range of medical systems, such as contact probes, endoscopes and needle based probes [96]. Weaknesses in signal strength in Raman instrumentation can be overcome by using enhanced Raman techniques such as resonant Raman (RR) and surface enhanced Raman spectroscopy (SERS) [97]. RR takes advantage of the internal resonance of molecules within a biological sample to the excitation wavelength to enhance scattering effects [98,99]. SERS utilises plasmonic nanostructures to enhance the Raman response of a given analyte. RR and SERS have both also been developed for a range of clinical applications in conjunction with different Raman instrumentation [100].

Raman spectroscopy: clinical applications

Much like FTIR spectroscopy, the majority of RS clinical research has been aimed at tissue analysis, because of the ability to generate images representative of tissue morphology and structure. However, due to having a small water cross-section, there is the potential to apply RS to tissues *in vivo*. An early application of RS for *in vivo* analysis was based on squamous cell carcinoma characterisation of skin using a microspectrometer [101]. More recently, there are a number of clinical applications of RS that are based upon fibre-optic Raman probe systems for diagnostic applications. Fibre-optic RS systems have been applied to many *in vivo* and *ex vivo* tissue diagnostic studies, such as the detection of cervical cancer [102,103], digestive cancers and precursor lesions [104–107], oesophageal cancers [108–110] and real-time diagnosis of nasopharyngeal cancer [111]. The application of rapid real-time feedback from fibre-optic systems highlights the potential use as a method of surgical guidance. A study by Jermyn et al. reported a needle-based Raman probe for inter-operative use in brain tumour resection [112], with the group later reporting the potential for the system to be used for targeted *in vivo* brain cancer biopsies [113]. SORS has also shown to be useful in the non-invasive determination of breast cancer margins [114]. The developments in non-linear instrumentation has led to faster imaging times, meaning RS techniques are now somewhat comparable with rapid IR imaging technology, such as QCL-based spectrometers. Recently, SRS imaging was applied to resected brain tissue, for the detection of tumour infiltration, on a small cohort of 22 patients [115].

As with FTIR spectroscopy, RS is well suited to spectrocytology applications, with cervical and oral cytology studies dominating the field [116–118]. The major benefit of employing RS in cytology samples, is that *ex vivo* analysis is possible due to RS being less sensitive to water content, compared to FTIR spectroscopy approaches. The ability to automate instruments, coupled with fast analysis times, means that RS would lend itself well to screening applications whilst providing sensitive data on disease status and progression that could potentially improve patient time-to-diagnosis and ultimately survival [119]. Perhaps the biggest challenge in this field is competition with relatively inexpensive screening pathways that are well established in the developed world.

RS has also been applied to the field of liquid biopsies, having the potential to become a rapid, minimally invasive diagnostic tool for various types of disease. One significant advantage of RS over FTIR-based techniques, is that biofluids can be analysed in a liquid state, without the need for drying. The first application of RS for biofluid analysis was carried out by Berger et al., where RS was used to analyse whole blood [120]. Due to the large Raman scattering cross-section of red blood cells in whole blood, most Raman biofluid studies now focus on serum and plasma. The potential of RS to differentiate malignant diseases from healthy control samples using either serum or plasma has been repeatedly demonstrated. Since early studies from Li et al., who used serum-based RS and fluorescence for GI cancers [121], there have been numerous proof-of-concept studies for a range of malignant diseases including breast cancer [122,123], head and neck cancers [124], colorectal cancer [121], cervical cancer [125] and hepatocellular carcinoma from cirrhotic patients [126]. Sahu et al. initially analysed serum samples to try to differentiate between oral cancer patients and controls [127], then the group later presented the application of serum RS to detect recurrence of oral cancers, although this study only included a small number of patients [128]. The development of high-throughput Raman instrumentation for diagnosis of prostate cancer in plasma samples has also been reported for a small patient cohort, which demonstrates the potential for rapid analysis with high sensitivity and specificity (96.5 % and 95 %, respectively) [129]. Furthermore, malaria parasites have also been shown to be measurable through both RS, with some studies reporting high sensitivity and specificity for detecting low levels of parasitaemia [82].

RS has also been shown to be an efficient tool for detecting non-malignant diseases. Differences in the Raman spectra of blood serum collected from Hepatitis C and healthy patients were observed through principal component analysis (PCA) [130]. Likewise, patients with rheumatoid arthritis have been successfully separated from healthy controls, with a sensitivity and specificity of 96 % and 88 %, respectively [131].

Despite potential issues with reproducibility and reagent shelf life, SERS methods for malignant disease detection using biofluids have been widely reported using both targeted and un-targeted methods [132–135]. A large SERS serum assay for liver disease involving over 700 patients was recently reported by Shao et al., with an accuracy of 81.76% [136]. SERS has also been successfully used for the detection of prostate cancer and UTI identification in urine [137]. Chemical quantification within biofluids is also possible with RS, which could be a potential route to clinical translation. Early chemical concentration measurements in blood serum and urine using liquid-core optical fibre were performed by Qi et al. [138]. RS has previously been used to measure concentrations of blood analytes, like cholesterol, in both serum and plasma [139]. The detection and analysis of carotenoids, such as lycopene and β-carotene, in blood plasma has also been investigated [140]. Promisingly, SERS-based immunoassays hold potential for ultrasensitive detection of blood analytes such as chemokines outperforming standard ELISA tests [141]. Glucose monitoring in *ex vivo* serum samples has been possible using both RS and SERS [142,143], meanwhile a recent study has directly compared RS and FTIR for glucose measurements in liquid and dried serum samples, respectively [144]. Liquid biopsies are often described as non-invasive, but there is an argument that repeatedly extracting blood

for chemical monitoring could still raise issues for clinical translation. As such, SORS and SESORS have been used for sub-epidermal glucose monitoring tools, which if successful could have huge potential for truly non-invasive biofluid applications [145–147].

Promising results for biofluid RS applications have been reported in many studies, but few groups have reported developments past proof-of-concept stages. To push liquid biopsy RS towards translation there will undoubtedly need to be further development of high-throughput techniques, as well as larger patient cohort/multi-centre clinical studies in line with those currently underway for IR applications [77]. Further to academic publishing, patents have been filed for the application of RS to colorectal cancer using dried serum samples. CanSense Ltd (UK) are pursuing this application commercially, with clinical studies currently underway, displaying one of the first RS based companies working in the clinical sphere.

2. D-IR spectroscopy

Whilst Raman spectroscopy may overcome some of the challenges of water containing samples, it is clear that IR absorption methods can be hindered by intense absorptions of water, which may limit *ex-* and *in vivo* investigation. In particular, the H−O−H bending vibration near 1650 cm^{-1} obscures the protein amide I mode that is highly sensitive to secondary structure. As the protein content of biofluids, such as serum, provides a useful indicator of metabolic function, the ability to differentiate between different proteins in a complex aqueous mixture without significant sample preparation would be a major advance. Recently, it has been shown that the technique of ultrafast 2D-IR spectroscopy is not only capable of measuring the amide I band of proteins in transmission mode in aqueous solvents, but is capable of differentiating and quantifying the albumin and globulin fractions of as-received blood serum, suggesting that the method may have considerable potential for biofluid diagnostic applications.

Since its development in 1998 [148], 2D-IR has been widely used to investigate the structure and ultrafast dynamics of biomolecules in solution [149–171]. As a technique, 2D-IR can be thought of as an IR analogue of 2D-NMR spectroscopy, utilising a train of ultrafast IR laser pulses to excite molecular vibrations in place of radiofrequency radiation interrogating nuclear spins. In common with 2D-NMR methods, 2D-IR places the IR absorption spectrum of a molecule along the diagonal of the 2D plot, while the off-diagonal region reports on couplings between the vibrational modes [148]. This is important for studies of proteins because secondary structure elements within proteins give rise to unique 2D spectral patterns via intramolecular couplings of the peptide carbonyl groups, meaning that the 2D-IR signal is sensitive to secondary structure to a greater degree than IR absorption spectroscopy. The short duration of the laser pulses also enables measurement of sub-picosecond molecular dynamics. Of particular relevance to applications to biofluids is the fact that, as a nonlinear optical spectroscopy, the 2D-IR signal scales quadratically with the vibrational transition dipole moment of the mode being studied, whereas the IR absorption signal scales with the second power [171,172]. For an aqueous protein solution this is important, because in an absorption measurement, the response from the many, weakly absorbing water molecules overwhelms that from the few strongly absorbing proteins (the molar absorption coefficient of the amide I mode of a typical protein is two orders of magnitude larger than that of the H−O−H bending mode). In a 2D-IR experiment, the situation is reversed, meaning that the protein signal dominates, allowing measurement of protein content of blood serum without drying or alternate detection geometries. We return to the clinical relevance of this measurement below following a discussion of the method of acquiring a 2D-IR spectrum.

2. D-IR spectroscopy: instrumentation

A full description of the theory and experimental details of 2D-IR spectroscopy measurements have been described in several review articles and so we will focus on the state-of-the-art methodology most relevant to biofluid analysis [148,172–175].

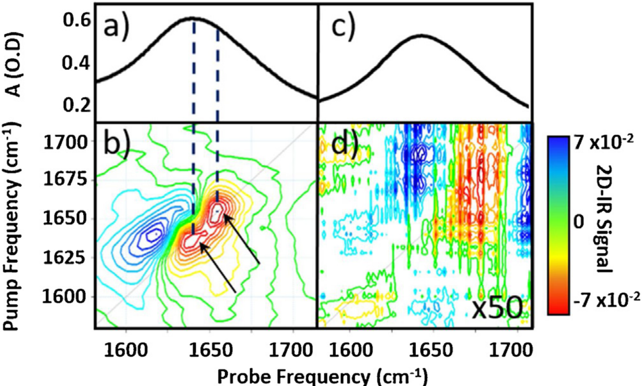

Fig. 2. (a) FTIR and (b) 2D-IR spectra of the amide I band of horse serum. (c) FTIR and (d) 2D-IR spectra of H_2O under the same conditions. The H_2O 2D-IR signal (d) has been magnified 50× and is shown on the same scale as the serum spectrum (b). Figure reproduced from ref. [179] with permission from the Royal Society of Chemistry.

The 2D-IR method employs a sequence of three IR pulses. Two ‘pump’ pulses and one ‘probe’ pulse are overlapped in a liquid sample that is held between two IR transmissive windows. The pulses are typically around 50–100 fs in duration, meaning that they cover a spectral bandwidth of ~200−400 cm^{-1} and are centred at the wavelength region of interest e.g. the amide I band. The current state of the art technology involves the use of 100 kHz pulse repetition rate lasers combined with pulse-shaping to control the timing of the pump pulse pair [176–178].

The combination of the three pulses results in the emission of a signal pulse from the sample, which is directed into a spectrometer and dispersed via a grating onto a multi-element array detector. This frequency dispersal generates the ‘probe’ frequency axis of the 2D-IR spectrum. By recording the signal as a function of the delay time between the pump pulse pair (τ) and performing a Fourier Transformation, the second ‘pump frequency’ axis of the spectrum is generated. The resulting 2D spectrum is thus a correlation map of pump frequency versus probe frequency. The delay time between the pump pair and the probe pulse, called the waiting time, can also be altered to interrogate the sample at different waiting times after excitation. Under these conditions, a 2D-IR spectrum typically requires less than a minute to acquire, comparable with the time needed for a benchtop FTIR spectrometer.

2. D-IR spectroscopy: clinical applications

Traditionally, 2D-IR spectroscopy studies of biomolecules in aqueous solutions are conducted using deuterated solvents (D_2O), in order to prevent overlap between the amide I and the O−H bending mode of H_2O at around 1650 cm^{-1}. Whilst this allows detailed study of the amide I band, free from water absorption, studies in D_2O are not without issue; this non-physiological solution may not be representative of the biological sample due to the impact of heavy isotopes on dynamic behaviour. Furthermore, deuteration is impractical to implement in biofluids and costly on an industrial scale.

However, it has been shown recently that 2D-IR measurements of the protein amide I band in aqueous solutions is possible [179]. The FTIR and 2D-IR spectra of the amide I band of a sample of horse serum are shown in Fig. 2((a) and (b)). Two clear features are visible on the diagonal of the serum 2D-IR spectrum, marked by black arrows in Fig. 2(b), while the corresponding water signal (Fig. 2(d)) is significantly weaker. The two diagonal features were assigned to the ν = 0–1 transitions of the amide I modes of the albumin and globulin protein fractions of the serum. The two amide I bands are separated in frequency by the differing secondary

structure composition of the proteins (albumin is largely alpha helical, whereas globulins are dominated by beta sheets). Importantly, the two features were not separable in the FTIR spectrum (Fig. 2(a)) which shows only a broad band consisting primarily of the water response (Fig. 2(c)). The reason for this is that, as well as amplifying the protein signal, the 2D-IR method leads to a narrower linewidth than FTIR spectroscopy, enabling greater spectral resolution [172].

Using these diagonal bands, 2D-IR spectroscopy enabled the albumin to globulin ratio of blood serum to be determined. The 2D-IR results were compared with current standard measurements for obtaining protein concentrations and were found to be accurate to within ± 4 % across a clinically relevant range. It was also shown that the unique 2D signatures that derive from protein secondary structure enabled differentiation and quantification of the similarly structured globulin proteins, IgG, IgA and IgM in serum [179].

The ability to differentiate small changes in protein secondary structure using 2D-IR has been demonstrated and this may be powerful in determining structural changes of proteins during disease states or allosteric affects due to drug binding. A recent study of the calcium-binding messenger protein calmodulin (CaM) has shown the capability of 2D-IR to detect small secondary structure changes upon binding of Ca^{2+} ions [180]. 2D-IR measurements measured a thermally induced reduction in α-helical content of apo (no Ca^{2+}) CaM. The observed 15 % loss of helical structure was benchmarked using circular dichroism spectroscopy, which measured a 13 % change [181]. Moreover, the ability to detect a Ca^{2+}-induced change in structure affecting only seven residues of the ~150 that make up the protein was reported. These changes in the amide I band are detectable using IR absorption methods, but they lack the additional information content of 2D-IR spectroscopy that allows definitive assignment of the structural origin of the changes. 2D-IR has also been used to quantitatively determine the secondary structure of a library of 16 proteins using the 2D amide I lineshape. These measurements were taken without the use of sample labelling and were compared positively with circular dichroism results [182].

2D-IR has been used to study the effects of drug binding upon protein structure and dynamics in InhA, an enzyme partly responsible for the maintenance of cell walls in the bacterium *Mycobacterium tuberculosis*. Changes in the off-diagonal portion of the amide I lineshape were found to correlate with drug-induced inhibition of activity [183]. The technique has also been shown to reveal amyloid β-sheet-like secondary structures in ex-vivo porcine cataracts using both off and on diagonal 2D-IR features, where the current standard technique - transmission electron microscopy (TEM) - failed to resolve them [154]. Again, this highlights the potential use of 2D-IR to understand how diseases are manifest, allowing more accurate and effective treatments. Moving away from proteins to other biofluid components, the off-diagonal features in a 2D-IR spectrum that link coupled vibrational modes can be diagnostic of the presence and quantity of e.g. phospholipids or nucleic acids. For example recent work on DNA showed the presence of cross peaks linking vibrational modes that are diagnostic of double stranded DNA [184,185].

Although spectra obtained in 2D-IR spectroscopy experiments can be obtained in under a minute, giving access to high throughput screening applications, this can lead to large, complex datasets that require new approaches to display and analyse the information. In order to allow translation of 2D-IR towards clinical purposes, handling of the data obtained has to be efficient in order to return accurate results in a timely manner. Principal component analysis (PCA) has seen increasing usage as a tool for extracting information from 2D-IR data sets, such as changes in structure of proteins or following thermal denaturation of DNA duplexes [180,184]. A recent study on DNA-ligand binding builds on these previous studies by combining recent technological advances in 2D-IR measurement with PCA [186], demonstrating that large dataset analysis is achievable. These studies show the potential of the rapid, information rich 2D-IR spectra equipped with the right analytical tools to examine complex structures, paving the way to determine between disease and non-disease states in biofluids.

Together, the advances in 2D-IR spectroscopy measurement protocols and the subsequent analysis of 2D-IR spectra present the possibility of translating 2D-IR spectroscopy towards medical applications. On the road to achieving this, a number of important milestones have been reached. Reliable information can now be extracted from large and complex biological systems even those that exhibit very small fluctuation in molecular structure or composition. Further to this, large and complex datasets can be analysed in a timely manner which is necessary when processing large numbers of patient samples. The third hurdle crossed is that of measuring 2D-IR spectra of biological systems in H_2O, meaning that rapid disease diagnosis using unlabelled, as-received serum could be a future possibility. However, obstacles still need to be overcome in order to present 2D-IR in a medical setting. Currently, state-of-the-art instrumentation is expensive, large and requires constant expertise to maintain good quality spectra. However, with the continuing development of 2D-IR and growing of the accompanying community, continual advances can be made in order to allow 2D-IR to make a significant impact in medicinal research.

Conclusions

The translation of vibrational spectroscopies towards the clinic has been advancing alongside technological developments, with proof-of-concept studies highlighted here providing evidence of numerous clinical applications. In recent years, there has been a clear movement towards clinically relevant studies and commercialisation of these technologies; particularly in the case of FTIR and Raman spectroscopy.

Automated or high-throughput instrumentation would be best suited to clinical settings, in order to minimise pressure on personnel resource. For example, ATR-FTIR spectroscopy is inherently limited to a single, fixed point of analysis, which restricts the overall sample throughput, particularly when taking into account cleaning the crystal between measurements. However, the development of low cost IREs may provide a disposable substrate for ATR-FTIR spectroscopy, similarly enabling batch processing of samples and high-throughput spectral acquisition alongside the development of novel instrumentation [187].

The analysis of tissue for the detection of malignant diseases has yielded a potentially powerful tool to complement traditional histopathology, that can rapidly extract molecular information from heterogeneous samples. The major hurdles for such an approach remain disruption to the current clinical pathway and also the health economic arguments for implementation.

The analysis of biofluids presents a promising application of both FTIR and Raman spectroscopy. These homogenous samples are significantly easier to extract, require minimal preparation prior to analysis, and can also provide a global signature of a patient that is able to derive disease specific information. As discussed in this article, there is now great potential for 2D-IR spectroscopy to follow suit, requiring further research and understanding in how to apply to the field of clinical spectroscopy. Going forward, the results of pivotal clinical trials will be telling for the first of these technologies to translate into the clinic.

Declaration of Competing Interest

Matthew J. Baker and Holly J. Butler are directors of ClinSpec Diagnostics Ltd.

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