

# Metabolic fingerprinting in disease diagnosis: biomedical applications of infrared and Raman spectroscopy

David I. Ellis\*<sup>ab</sup> and Royston Goodacre\*<sup>ab</sup>

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The ability to diagnose the early onset of disease, rapidly, non-invasively and unequivocally has multiple benefits. These include the early intervention of therapeutic strategies leading to a reduction in morbidity and mortality, and the releasing of economic resources within overburdened health care systems. Some of the routine clinical tests currently in use are known to be unsuitable or unreliable. In addition, these often rely on single disease markers which are inappropriate when multiple factors are involved. Many diseases are a result of metabolic disorders, therefore it is logical to measure metabolism directly. One of the strategies employed by the emergent science of metabolomics is metabolic fingerprinting; which involves rapid, high-throughput global analysis to discriminate between samples of different biological status or origin. This review focuses on a selective number of recent studies where metabolic fingerprinting has been forwarded as a potential tool for disease diagnosis using infrared and Raman spectroscopies.

## Introduction

The ability to detect disease or dysfunction rapidly has manifold and obvious benefits, including early intervention of therapeutic strategies, hopefully in a prognostic fashion, significant reduction in mortality and morbidity, and the freeing up of much needed economic resources within health care systems. In addition, the identification of some indicator of disease could also be used to monitor the progression of therapy. It is also acknowledged that some of the tests currently in use associated with a number of diseases and/or disorders are deemed unreliable, unsuitable or lack the specificity required for routine use in clinical practice. Indeed

in some areas there is certainly room for improvement in the early diagnosis and proper identification and grading (quantitative severity) of several diseases. Probably one of the most well known of these is the prostate specific antigen (PSA) test for prostate cancer, which has received much attention in the literature in relation to its efficacy.<sup>1–4</sup> Indeed, whilst it is known that the PSA test has resulted in an increase in prostate cancer detection, its routine use has been questioned due to a lack of specificity<sup>5</sup> and there is an urgent need for a more suitable test for this particular disease.

One other example, where a multitude of tests currently exist for the detection of one disorder is pre-eclampsia. Pre-eclampsia is a pregnancy-specific multi-system disorder of poorly defined aetiology, which affects at least 3–5% of pregnancies and is a major cause of maternal mortality, perinatal mortality, pre-term birth, and intrauterine growth restriction.<sup>6–10</sup> At present nearly 50 screening tests have been proposed for pre-eclampsia. Although it has been reported that whether clinical, biophysical or biochemical in nature,

<sup>a</sup>School of Chemistry, University of Manchester, Faraday Building, PO Box 88, Sackville Street, Manchester, UK M60 1QD.

E-mail: D.Ellis@manchester.ac.uk; Roy.Goodacre@manchester.ac.uk

<sup>b</sup>Manchester Interdisciplinary Biocentre, University of Manchester, 131 Princess Street, Manchester, UK M1 7ND.

E-mail: Roy.Goodacre@manchester.ac.uk; D.Ellis@manchester.ac.uk



David I. Ellis

David I. Ellis obtained a BSc (Hons) in Environmental Science and a microbiology/FT-IR based PhD, both from the University of Wales Aberystwyth. He now works as an Experimental Officer/Laboratory Manager in Prof. Douglas Kell's Bioanalytical Sciences Group (<http://dbkgroup.org/>) at the Manchester Interdisciplinary Biocentre (MIB), The University of Manchester, on a variety of metabolomics-based projects.



Royston Goodacre

Roy Goodacre is Professor of Biological Chemistry at The University of Manchester. The research group's (<http://www.biospec.net/>) interests are broadly within bioanalytical chemistry, and in the combination of a variety of modern spectroscopies (including MS, IR and Raman) and advanced chemometrics and machine learning to the explanatory analysis of complex biological systems within a metabolomics and proteomics context.

these tests have been inconsistent and contradictory, with the majority deemed to be unreliable or suitable for routine use in clinical practice.<sup>11</sup> Whilst the search continues for the optimum diagnostic test for this particular disorder,<sup>12</sup> a very recent report suggested that Fourier transform infrared spectroscopy has considerable potential as a rapid high-throughput screening method for pre-eclampsia.<sup>13</sup>

It would therefore seem that other options should be considered for diagnostic testing or screening and perhaps a more 'holistic' approach adopted where suitable (and dependent upon the disease in question). Ideally, the method would be rapid, reagent free, non-destructive, high-throughput, relatively inexpensive and require a minimal amount of background training. These requirements could be met by a spectroscopic approach.

## Vibrational spectroscopy

With the requirements for a routine spectroscopic approach that could be made portable and thus applicable to point of care medicine, infrared and Raman spectroscopies appear as obvious candidates. Below first gives details of the physico-chemical mechanisms of these approaches and their implementation within disease diagnosis.

### Fourier transform infrared (FT-IR) spectroscopy

FT-IR spectroscopy is a well established<sup>14,15</sup> and constantly developing analytical technique which allows for the rapid, high-throughput, non-destructive analysis of a wide range of sample types. FT-IR is based on the principle that when a sample is interrogated with an infrared (IR) beam, the functional groups within the sample will absorb the infrared radiation (Fig. 1) and vibrate in one of a number of ways, either stretching, bending, deformation or combination vibrations.<sup>16,17</sup> These absorptions/vibrations can then be correlated directly to (bio)chemical species and the resultant infrared absorption spectrum can be described as an infrared 'fingerprint' characteristic of any chemical or biochemical substance. For most disease diagnoses researchers have concentrated on the mid-IR part of the spectrum (from 4000–600  $\text{cm}^{-1}$ ), because in contrast to near-IR (14000–4000  $\text{cm}^{-1}$ ) the fundamental vibration is seen rather than an overtone or harmonic. Thus the mid-IR spectra contains many sharp peaks and is very information rich. In biological terms the vibrations in the wavenumber region 2800–3050  $\text{cm}^{-1}$ , for example, can

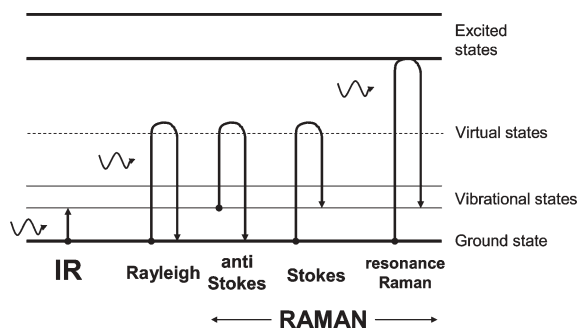


Fig. 1 Virtual energy levels for infrared and Raman spectroscopies.

be ascribed to  $\text{CH}_2$  and  $\text{CH}_3$  stretching vibrations from fatty acids, whilst those in the wavenumber region 1500–1750  $\text{cm}^{-1}$  (the amide I and II bands) are ascribable to C=O, NH and C–N from proteins and peptides.<sup>18</sup> Whilst not as specific and sensitive as some techniques, such as GC-ToF-MS,<sup>19,20</sup> the rapidity and reproducibility of FT-IR cannot be overstressed and due to its holistic nature it has been recognised as a valuable tool for metabolic fingerprinting/footprinting, owing to its ability to analyse carbohydrates, amino acids, fatty acids, lipids, proteins and polysaccharides simultaneously.<sup>18,21,22</sup> However, one potential disadvantage of FT-IR in the mid-IR is that the absorption of water is very intense, but this problem can be overcome in one of several ways such as; dehydration of samples, subtraction of the water signal, or by application of attenuated total reflectance (ATR).<sup>17,23,24</sup> In addition, another perceived disadvantage is that as a holistic measurement is made with biochemical information spread across the whole of the IR spectrum, validated and robust chemometrics must be used in order to turn data into information.

### Raman spectroscopy

By contrast, Raman spectroscopy measures the *exchange* of energy with electromagnetic (EM) radiation of a particular wavelength (Fig. 1; usually a laser in the visible to mid-IR part of the EM). This exchange of energy results in a measurable Raman shift in the wavelength of the incident laser light (or what is also known as the inelastic light scattering effect);<sup>25–27</sup> this shift is complementary to IR absorption and one can also construct a Raman 'fingerprint' of the same sample (for typical FT-IR and Raman spectra of human serum see Fig. 2). For example, in addition to observing peaks for Amide I and II in Raman spectroscopy the Amide III band at 1240–1300  $\text{cm}^{-1}$  is also observed, which is attributable to secondary amide vibrations; in addition phenylalanine has a very strong vibration at  $\sim 1005 \text{ cm}^{-1}$  due to its benzene ring.

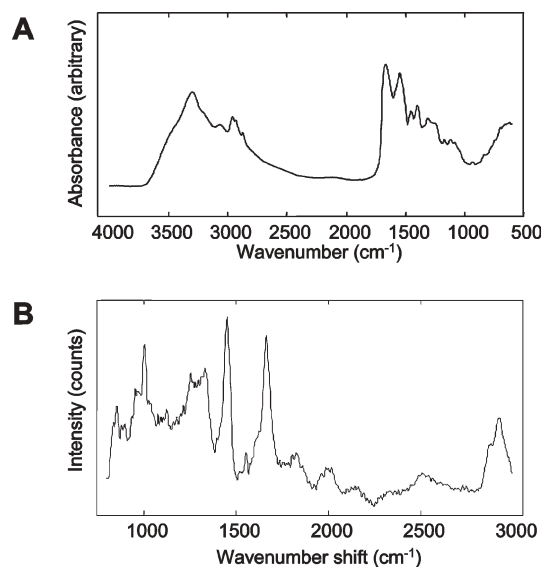


Fig. 2 Typical FT-IR absorbance (A) and Raman (B) spectra of human serum.

Unlike FT-IR, Raman spectroscopy does not suffer from water interference as water is a very weak scatterer. Therefore Raman measurements can be made directly from biofluids and there are even reports of *in vivo* measurements from the bladder and prostate,<sup>28</sup> oesophagus,<sup>29–31</sup> skin,<sup>32–34</sup> the cervix<sup>35,36</sup> and arteries.<sup>37</sup> However, the Raman effect is very weak and typically only 1 in  $10^6$ – $10^8$  photons undergo an inelastic light scattering event; therefore collection times tend to be relatively long. In addition, Raman spectra collected with a visible laser from biological samples can often be plagued with fluorescence which, whilst much broader than the sharp Raman peaks, can dominate the spectra and often need to be removed mathematically. The Raman signal can be enhanced by one of two methods. The first is based on resonance enhancement and this occurs because the laser wavelength used to excite the Raman spectrum lies under an intense electronic absorption band of a chromophore. When this occurs an enhancement of Raman scattering is observed so that some of the band intensities are increased by a factor of  $10^3$ – $10^5$ . As this method uses chromophores, resonance Raman spectroscopy can be directed to certain chemical species; for example in the deep UV, 227 nm is used to enhance selectively aromatic amino acids, whilst 244 nm causes enhancement predominantly from nucleic acids.<sup>38–40</sup> The second method of enhancement is that of surface enhanced Raman spectroscopy (SERS). In SERS one relies on either the adsorption or close proximity of an analyte to a metal substrate. The substrate can take the form of a roughened metal surface, a colloidal solution or a roughened electrode.<sup>41–44</sup> The enhancement is in the order of  $10^3$ – $10^6$ , and can be combined with a chromophore to effect surface enhanced resonance Raman spectroscopy (SERRS).<sup>45</sup>

### Multivariate data analysis

The result of FT-IR and Raman spectroscopy is either an infrared absorbance spectrum (wavenumber *vs.* relative absorbance) or a Raman profile (wavenumber shift *vs.* photon count). These contain many overlapping bands and so data interpretation can not be made by simple visual inspection and alternative approaches are needed. Thus it is essential that these vibrational spectroscopic measurements are combined with any of the rapidly growing arsenal of multivariate analysis strategies, in what appears to be the exponential phase of computational bioanalysis.<sup>46–51</sup>

Multivariate data such as those generated from an infrared or Raman experiment consist of the results of observations of many different variables (wavenumbers or wavenumber shifts) for a number of individuals (objects; *e.g.* diseased or healthy subjects). Each variable may be regarded as constituting a different dimension, such that if there are  $n$  variables (IR or Raman bands) each object may be said to reside at a unique position in an abstract entity referred to as  $n$ -dimensional hyperspace. This hyperspace is necessarily difficult to visualise, and the underlying theme of multivariate analysis (MVA) is thus *simplification* or dimensionality reduction. This dimensionality reduction occurs in one of two ways; either using an unsupervised or supervised learning algorithms. Please see Fig. 3 for a summary of the main methods and the following reviews.<sup>52–59</sup>

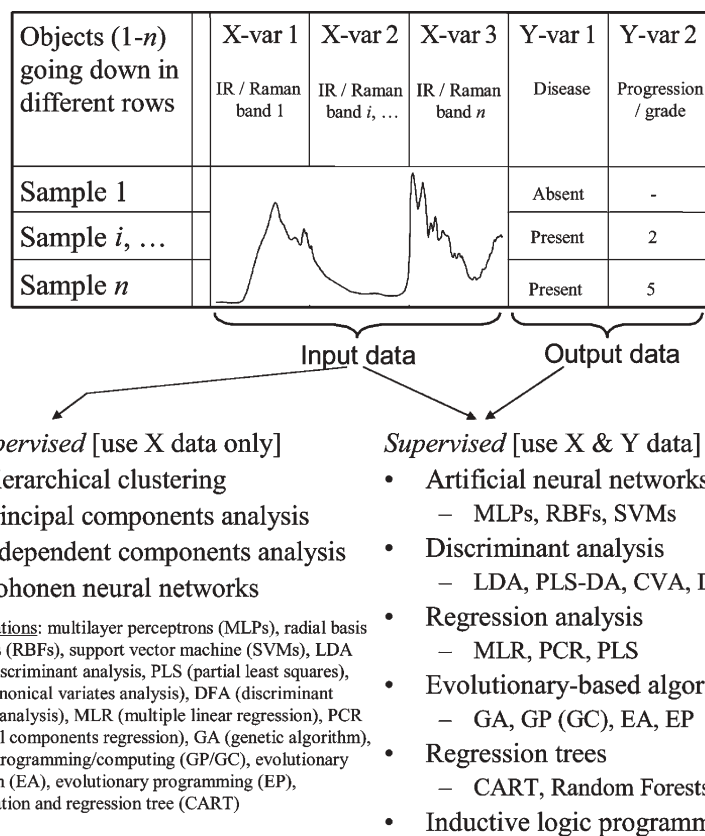
In general unsupervised methods such as principal component analysis (PCA) and hierarchical cluster analysis (HCA) are used to look at the ‘natural’ differences and similarities between these spectra. For most infrared and Raman spectra the number of observations (samples) in the analysis is very small compared to the number of variables (wavenumbers or wavenumber shifts), which may be 100s–1000s. In this case multivariate analysis by PCA and HCA is most appropriate. These methods are employed to discover structure in these data and can be used to ‘cluster’ samples into groups by producing scatter plots (PCA) and dendrograms (tree-like figures; HCA).<sup>52,54</sup> By contrast, supervised methods like discriminant analysis (DA) and artificial neural networks (ANNs) are ‘calibrated’ with some known answer from existing knowledge of the sample. That is to say, one set of spectra were generated from the serum of people with cancer, *versus* another set of spectra from healthy age-matched, gender-matched *etc.*, controls. This *a priori* knowledge is used in the construction of the DA or ANN model, and must therefore be correct else the model will not generalise well, and one is reminded of the adage ‘garbage in – garbage out’.<sup>60</sup> Thus one must suitably validate these methods.

### Biological applications of vibrational spectroscopy

Within the biosciences, the applications of FT-IR have been numerous and diverse. In the field of microbiology for example, FT-IR has been used for the rapid and accurate identification of bacteria to the sub-species level,<sup>61</sup> differentiation of clinically relevant species,<sup>62</sup> rapid enumeration of food spoilage bacteria *in situ*,<sup>23,63,64</sup> metabolic fingerprinting of tryptophan-metabolism mutants<sup>21</sup> and discrimination or identification of a range of bacterial genera, such as *Bacillus*,<sup>65,66</sup> *Micromonospora*,<sup>67</sup> *Acinetobacter*,<sup>65</sup> *Streptomyces*<sup>68</sup> and *Listeria*.<sup>69</sup>

However, during the last decade it has been recognised that FT-IR, in combination with the appropriate multivariate analysis strategies, has considerable potential as a metabolic fingerprinting tool for the rapid detection and diagnosis of disease or dysfunction, and indeed, a significant number of studies have been undertaken on tissues, cell and biofluids in an emergent area of research termed ‘infrared pathology’ by Diem and co-workers,<sup>70</sup> and now more commonly referred to as metabolic fingerprinting.<sup>58</sup> The remainder of this review will concentrate on some of the many spectroscopic studies undertaken thus far, related in the main to human disease, concerning analysis of cells, tissues and biofluids.

Raman spectroscopy has charted similar areas, particularly within microbiology (for an excellent review see ref. 71). The use of resonance Raman spectroscopy in the deep UV was first used by Nelson, Sperry and colleagues<sup>72–75</sup> in the early 1990s for the characterization of bacteria. This approach has recently been taken further with the development of suitable chemometric processing that have allowed for the identification of bacteria<sup>76</sup> and the effect of antibiotics on the bacterial cell.<sup>77</sup> Raman spectroscopy in the visible to mid-IR part of the EM spectrum has also been used for the characterization of microorganisms<sup>78–80</sup> including single cells.<sup>81</sup> Enhancement methods based on surface enhanced Raman spectroscopy



**Fig. 3** When learning is unsupervised, the algorithm is shown a set of inputs and then left to *cluster* the IR or Raman data into groups. For MVA this optimization procedure is usually *simplification* or dimensionality reduction. This means that a large body of IR or Raman data (*x*-data) are summarised by means of a few parameters with minimal loss of information. When one knows the desired responses (*y*-data) associated with each of the IR or Raman data inputs (*x*-data) then the system may be supervised. The goal is to find a mathematical transformation (model) that will correctly associate all or some of the inputs with the target traits. This trait can be categorical (*e.g.*, disease *vs.* healthy) or quantitative (*e.g.*, grade of cancer, response to therapy). In its conventional form this is achieved by minimising the error between the known target and the model's response (output).

have also been investigated for the identification of bacteria.<sup>82,83</sup> Finally, due to its ability to collect vibrational spectra from aqueous environments Raman spectroscopy has also been used for the at-line and on-line analysis of microbial fermentations.<sup>84–86</sup>

## Disease diagnostics

Globally, cancers are the most obvious and major threat in terms of morbidity and mortality and the rapid and early diagnosis of malignant neoplasia would be extremely beneficial in many respects, not least of which would be proper identification and early diagnosis leading to a desirable prognosis. Whilst a significant number of studies have been undertaken using infrared or Raman spectroscopy in attempts to detect several forms of cancer, the caveat must always be 'proceed with caution'. Diem and co-workers for example have stated that 'the level of activity in a field is no measure of the quality of the work published'.<sup>70,87</sup> In addition, doubts have been expressed as to whether some of the studies are supported with sufficiently validated pathological data on the assayed biological samples,<sup>18</sup> and researchers should obey the 3 Vs when using multivariate analysis – validation, validation and validation. It is also of paramount importance

that characteristic infrared absorptions, for example, are correctly assigned.<sup>88</sup>

## Cervical cancer

Cervical cancer, particularly invasive squamous cell carcinoma, is the second most prevalent cancer in females after skin cancer. Cervical cancer is a major public health problem worldwide and one of the major causes of mortality in women, with estimated mortality rates of approximately 30%.<sup>89,90</sup> Whilst widespread screening programmes exist for cervical cancer it has been stated that the present screening method, based on PAP (Papanicolaou) smear and histopathology, makes it tedious and prone to human errors.<sup>91</sup>

Over the last few years spectroscopic studies have been undertaken in relation to cervical cancer and other gynaecological disorders, and indeed, spectroscopy has been recognized as an emergent technology in cervical cancer screening.<sup>92</sup> These studies have included analysis of cell maturation in cervical tissue where changes in glycogen concentration could be associated with the different stages of cell maturation.<sup>93,94</sup> Cohenford and co-workers have also undertaken several studies including analysis of cervical smears<sup>95</sup> and demonstrated that FT-IR, as well as aiding in the diagnosis of cervical

neoplasia, could also provide information into its pathogenesis.<sup>96</sup> This same group also analysed FT-IR spectra of over 2000 individual cells. Results from chemometric analysis of the data showed a continuum of changes which they said paralleled the transition from normalcy to malignancy, which as well as assisting diagnosis could also aid in the classification of cervical disorders.<sup>97</sup> For example, in cancerous samples the ratio of the peak at  $1026\text{ cm}^{-1}$  was less than half that of the normal group and these ratios were observed to progressively decrease from normal (1.77) through dysplasia (1.17) to cancer (0.77).<sup>97</sup> Other studies of interest which have validated earlier work include microspectroscopic analyses of individual cultured cervical cancer cells,<sup>98,99</sup> and spectral mapping of the squamous and glandular cervical epithelium and the cervical transformation zone.<sup>100</sup> Mahadevan-Jansen and colleagues have investigated Raman spectroscopy with linear discriminant analysis for its ability to differentiate between cervical precancers from normal tissues, and to diagnose low-grade from high-grade precancers.<sup>35,36</sup> Utzinger *et al.* reported that the ratio of Raman peak intensities at  $1454\text{ cm}^{-1}$  to  $1656\text{ cm}^{-1}$  were greater for squamous dysplasia than all other tissue types, whilst the ratio of peak intensities at  $1330\text{ cm}^{-1}$  to  $1454\text{ cm}^{-1}$  was lower for samples with squamous dysplasia than all other cell types.<sup>36</sup> A simple algorithm based on these two peak intensity ratios was able to separate high-grade squamous dysplasia samples from all others, misclassifying only one sample from a total of 24 *in vivo* measurements from each of 13 patients.

### Leukaemia

Leukaemia is the most frequent form of cancer in children and adults below the age of 30, and whilst the differentiation between normal and leukaemia lymphocytes is a routine procedure; the differentiation between the subforms or clones of leukaemia is time consuming, labour intensive and specialized.<sup>101</sup> Investigations into the diagnostic screening potential of FT-IR with regard to leukaemia have been ongoing for the last decade through analysis of normal and leukaemic lymphocytes,<sup>102–109</sup> with few reports using Raman spectroscopy.<sup>110</sup> Schultz and co-workers have undertaken extensive spectroscopic investigations of the most common form of leukaemia in Western Europe and North America, chronic lymphocytic leukaemia (CLL). This group compared CLL cells to normal cells and observed differences in the amide region as well as a reduction in lipid content and major spectral differences were observed primarily from absorptions ascribed to the DNA backbone region ( $900\text{--}1300\text{ cm}^{-1}$ ).<sup>102</sup> Additionally, it was shown that it was possible to separate the CLL cells further into subclusters based on their different DNA content and from this suggested that this may provide a useful diagnostic tool for staging (disease progression) and multiple clone detection.<sup>102,104</sup> Liu and colleagues have also been active in this area with particular reference to the effect of chemotherapeutic agents such as etoposide,<sup>109</sup> and have suggested that infrared spectroscopy has the potential clinical utility for the fast, reagent-free assessment of chemotherapeutic efficacy in leukaemia patients.<sup>108</sup> Liu and Mantsch observed several features in the difference spectra which discriminate control cells from those treated with

etoposide; such as a shift from  $1635\text{ cm}^{-1}$  in control cells to  $1657\text{ cm}^{-1}$  in treated cells, and significant changes in the region of the amide I band (shift from  $\beta$ -sheet to unordered structure), the amide II band ( $\sim 1545\text{ cm}^{-1}$ ) and the band at  $1517\text{ cm}^{-1}$ , arising from tyrosine in protein side chains.<sup>109</sup>

One further study has investigated the use of FT-IR as a tool to discriminate between sensitive and drug resistant leukaemia cells.<sup>111</sup> In contrast to leukaemia, where unrestrained proliferation of white blood cells occurs, a group of diseases known as myelodysplastic syndrome (MDS) involves the disruption in the production of any, and sometimes all, blood cells by the bone marrow. Recent work has used statistical models based on FT-IR spectra to discriminate between the DNA structure of normal granulocytes and those obtained from patients with MDS.<sup>112</sup> The models allowed for the high sensitivity and specificity prediction of which DNA came from normal granulocytes and granulocytes from MDS patients, which could be used as a basis for the development of a diagnostic blood test for MDS.<sup>112</sup>

### Prostate cancer

Prostate cancer is a major cause of mortality worldwide and in the United Kingdom it is the second most common cause of cancer related mortality in males with 10 164 recorded deaths in 2003.<sup>113</sup> As already mentioned, the current diagnostic method for prostate cancer is a physical examination followed by measurement of PSA in blood serum. However, PSA levels can be influenced by factors other than the presence of prostate cancer, which complicates diagnosis. It has also been stated that there is considerable interest in developing methods to identify the more aggressive cancers, either by elucidating the biochemical features of metastases or by finding chemical markers which identify changes in the underlying biochemistry associated with the development and progression of malignancy.<sup>114</sup>

Several studies have been undertaken on tissues, cell lines and DNA from subjects with normal and malignant prostates, and benign prostate hyperplasia (BPH) using FT-IR microspectroscopy<sup>114–119</sup> and Raman spectroscopy.<sup>28,120–125</sup> Gazi and co-workers studied prostate cancer cell lines derived from different metastatic sites and tissue samples from BPH and Gleason-graded malignant prostate tissue.<sup>114</sup> It was found that the ratio of peak areas at  $1030\text{ cm}^{-1}$  and  $1080\text{ cm}^{-1}$ , ascribed to glycogen and phosphate vibrations respectively, suggested a potential method for the differentiation of benign from malignant cells. The use of this ratio, in association with FT-IR spectral imaging was said to provide a basis for estimating areas of malignant tissue within defined regions of a specimen.<sup>114</sup> Subsequent results from further investigations by the same group suggested that the extent to which clusters were separated from each other may be associated with the invasive properties of each cell line.<sup>117</sup> They further suggested that the cluster plot could be used to determine whether inorganic ions have a negative or positive effect on invasiveness as a consequence of ion uptake, which could be subsequently confirmed and quantified through imaging ToF-SIMS.<sup>117</sup>

Crow and colleagues have been investigating prostate cancer using Raman spectroscopy using a  $785\text{ nm}$  and  $830\text{ nm}$  diode

laser for excitation. In these studies they have primarily concentrated on the differentiation between different cell lines using principal components analysis with linear discriminant analysis.<sup>121,122,125</sup> In further studies these authors have investigated the ability to grade cancers and achieved an overall accuracy of 89%.<sup>124</sup> Finally, they have also investigated the exciting ability for *in vivo* measurements of the prostate using fibre optic delivery of the laser light and the same probe for collection of the Raman scattered light.<sup>28,120</sup>

In other studies, infrared investigations of prostate DNA suggested that the progression from normal prostate tissue to BPH to prostate cancer involves structural alterations. It was stated that significant separation of the sample groups was possible using two regions of the infrared spectra, 1174–1000  $\text{cm}^{-1}$  (assigned to strong stretching vibrations of the  $\text{PO}_2$  and C–O groups of the phosphodiester-deoxyribose structure) and 1499–1310  $\text{cm}^{-1}$  (assigned to weak NH vibrations and CH in-plane deformations of nucleic acids) and that these mutagenic alterations were due to the hydroxyl radical.<sup>115</sup> One very recent study coupled high-throughput FT-IR imaging with statistical pattern recognition. This study demonstrated the potential for automated histopathologic characterization of prostatic tissue, without any requirement for dyes or molecular probes, which was able to differentiate benign from malignant prostatic epithelium.<sup>119</sup>

The above discussions on FT-IR and Raman investigations into analysing prostate and cervical cancer and leukaemia highlight the excitement and intense activity in these areas. In addition, these vibrational spectroscopic approaches have been used to investigate the diagnosis of other cancers. Infrared spectroscopic analysis has included studies into thyroid tumours,<sup>126,127</sup> imaging of colorectal adenocarcinoma sections<sup>128,129</sup> and the potential for infrared diagnosis of gastric cancer.<sup>130,131</sup> Whilst for Raman, gastric cancer has also been investigated,<sup>132</sup> as has *in situ* measurements for Barrett's oesophagus (a pre-indicator for oesophageal cancer)<sup>31,123</sup> and studies of pre-cancers and cancers of the larynx.<sup>133–135</sup> Others have investigated imaging of colorectal cancer with Raman spectroscopy.<sup>136</sup> Finally, Haka *et al.*<sup>137</sup> have developed Raman spectroscopy with chemometrics for diagnosing benign and malignant lesions in human breast tissue. For general background for *in vivo* cancer diagnostics using Raman spectroscopy please refer to ref. 138.

### Reproductive biology

In addition to infrared analysis of tissues and cells, FT-IR has also been used to acquire metabolic fingerprints from a wide range of biological fluids, and indeed, it can be said that the majority of metabolomic investigations for biomarker discovery involve biofluid analysis<sup>18</sup> as this is less invasive than tissue biopsies for example. Thomas and co-workers<sup>139</sup> used FT-IR to analyse follicular fluids from large and small natural luteinized antral follicles. Characteristic and reproducible infrared absorption spectra were observed from all samples with recognizable amide I protein vibrations and acyl vibrations from fatty acids. Chemometric analysis (utilizing both unsupervised and supervised learning approaches)

showed that follicular fluid from large follicles formed a homogenous closely related cluster, indicative of close biochemical similarity, as opposed to follicular fluid from small follicles, where greater heterogeneity was observed but which were still generally distinct from the large follicles. More in-depth analysis of the spectra showed that inter- and intra-specific differences within the follicular fluids did not correlate with measured distributions of steroids, indicating that FT-IR could elucidate other aspects of follicular biochemistry that were important. It was suggested that the differences observed in the biochemical nature of the fluids may be reflective of the developmental capacity of the oocyte, suggesting that FT-IR could provide a biomarker related to oocyte quality.<sup>139</sup>

### Arthritis

It has been recognized that the complexity of diagnosis for osteoarthritis (OA) and rheumatoid arthritis (RA), and the limitations of single entity markers, has led to the investigation of synovial fluid from the joints of both normal and arthritic subjects.<sup>140</sup> Eysel *et al.* demonstrated the ability to determine a number of differences in synovial fluids resulting from disease processes, and moreover, associated specific sub-regions of the infrared spectra with significant discriminatory power. Of these spectral sub-regions it was found that the region with the maximum diagnostic potential was 3500–2800  $\text{cm}^{-1}$ , dominated by  $\text{CH}_x$  stretching vibrations from all synovial fluid components, including those typically found in serum, as well as, and perhaps not surprisingly, glycosaminoglycans (GAGs). The observed discrimination between control, OA and RA subjects was said to have shown excellent agreement with clinical diagnosis and the authors stated that it was evident that FT-IR spectra of synovial fluids could be used as a diagnostic aid for arthritic disorders.<sup>140</sup>

For the analysis of synovial fluids FT-IR has significant advantages over other metabolomic analytical platforms and methodologies.<sup>19,141</sup> Synovial fluid is a viscous substance making it somewhat intractable to solubilization rendering mass spectrometry techniques difficult. In addition, techniques such as NMR can require sample volumes measured in 100s of  $\mu\text{l}$  in comparison to FT-IR where samples can be  $<1 \mu\text{l}$ .<sup>18</sup> That being said, it would obviously be advantageous to establish biomarkers for arthritis in other biofluids, such as blood products or urine. One study using infrared spectroscopy has used this approach and investigated serum for differentiating between rheumatoid arthritis and healthy subjects with a sensitivity and specificity of 84% and 88% respectively, purely on the basis of spectral classification. With sensitivity and specificity improved by 1% and 6% respectively, upon inclusion of rheumatoid factors levels for the selection of more discriminatory infrared windows.<sup>142</sup>

Whilst infrared and Raman spectroscopy are usually used to generate holistic measurements of biological samples, followed by some multivariate analysis (chemometrics), these approaches can be used to detect specific chemicals. This has been demonstrated for measuring hyaluronic acid, a lubricating polymer that leaks from the joint's synovial fluid in osteoarthritis, in the bloodstream using Raman spectroscopy.<sup>143</sup>

## Diabetes

Many diseases are a result of metabolic disorders, therefore it is logical to investigate methods that generate metabolic fingerprints since these approaches are more likely to detect changes in metabolism. Thus many other studies have been undertaken with the aim being to link infrared or Raman spectra to a disease related interpretation in a process described as 'disease pattern recognition' (DPR).<sup>22,144–147</sup> Petrich and colleagues using diabetes mellitus as an example, collected infrared spectra of serum from healthy volunteers and type 1 and type 2 diabetics, and with the successful application of multivariate statistical analyses, these spectra could be directly related to the donors' disease state. Results showed that in supervised classification (using linear discriminant analysis) of any pair of the disease sets within the data set, specificities and sensitivities of  $\geq 80\%$  were achieved, illustrating a clear correlation between a patient's disease state and the mid-IR spectra of the patient's serum sample.<sup>144</sup>

Whilst it is known that early diagnosis of type 2 diabetes is possible by monitoring blood glucose levels, it is also known that diabetics have additional metabolic disorders, with the result that several parameters need to be measured for a suitable appraisal of the specific disease state which may target therapy more accurately. Therefore, sera samples from the FT-IR analysis were also measured by a clinical analyzer for glucose, triglycerides and cholesterol. When the clinical analyzer results were compared with those based on infrared spectra, the latter were said to exhibit a superior correlation in the assignment of true disease state. The authors of this study believe that a more generalised view of the results of the spectroscopy of any biofluid (or tissue) may be much more closely related to the actual disease state, than an interpretation in terms of an individual molecule.<sup>144</sup> Further, they state that a successful interpretation of the spectra using pattern-recognition methods may be possible even when a lack of information (be it partial or complete) exists concerning the underlying molecular components and processes.<sup>144</sup> It could be said that this study suggests that there is no absolute requirement for any in-depth knowledge into an underlying metabolic disorder at the molecular level when using the DPR approach. However, as this approach uses supervised learning methods the disease must be categorised properly and is unlikely to work for disease classes that are multifactorial.

## Scrapie/BSE

Prions are self-replicating proteins implicated in transmissible spongiform encephalopathies (TSEs) in animals and humans. These proteins can undergo conformational change to a protease-resistant pathological form which allows the protein to aggregate and it is this form that causes TSEs.<sup>148</sup> This group of related infectious degenerative brain disease includes scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and new variant Creutzfeldt-Jakob disease (vCJD) in humans.<sup>149</sup> It has been suggested that the human form of this disease can be transmitted by consumption of infected animal tissue<sup>150,151</sup> and also poses a potential risk of infection through blood and plasma derived blood products.<sup>152</sup>

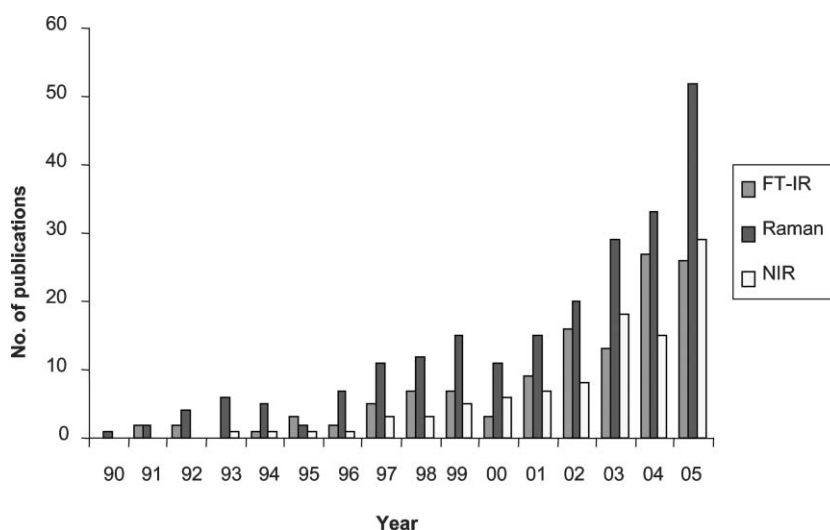
Several investigations have been undertaken using FT-IR to detect scrapie and BSE in tissue and serum.<sup>146,153–156</sup> Kneipp *et al.*<sup>153,154</sup> used infrared microspectroscopy to detect disease-associated molecular changes in central nervous system (CNS) tissue acquired from case and control hamsters. This was undertaken to assess the efficacy of FT-IR as a potential diagnostic tool in the terminal, early, and, if possible, pre-clinical stages of scrapie. It is known that complex histological and molecular differences occur in TSE-affected nervous tissue, such as changes in protein expression, alterations in gene expression, and composition of membrane systems.<sup>154</sup> The authors therefore applied FT-IR to this problem as this technique is able simultaneously to detect changes in multiple biomolecules rapidly and *in situ*.

Spectra of tissue were collected and analysed from 90 and 120 days post-infection, and in the terminal stage of orally transmitted scrapie. Perhaps not surprisingly, results showed that prominent variations in the spectra were obtained from scrapie-infected nervous tissue at the terminal stage. However, differences in spectra between healthy and diseased tissue were observable in the 1060–1040  $\text{cm}^{-1}$  region which were traceable back to 90 days post-infection, clearly observable in the preclinical stage. Whilst these changes were said to be small, a clear progression toward the terminal stage of the disease was seen, resulting in a pronounced shoulder in the spectra at  $\sim 1050 \text{ cm}^{-1}$ .<sup>154</sup> The observed changes in this region were assigned to sugar moieties of nucleic acids, to sugar molecules involved in metabolism, such as glucose in the cells, or to other events which have yet to be described.<sup>154</sup> From this the authors concluded that FT-IR could be used as to further investigate TSE pathogenesis and potentially be developed into a rapid post-mortem diagnostic screening method. More recent studies of bovine sera in relation to BSE<sup>146,156</sup> and sera from scrapie infected hamsters<sup>155</sup> used a DPR approach (*vide supra*) and have proposed FT-IR as a potential method for ante-mortem TSE testing on living animals.

Other FT-IR studies of interest include a significant body of work by Petibois and colleagues concerning the metabolic profiling of athletes.<sup>157–162</sup> These studies applied FT-IR to the analysis of blood, serum and plasma for the monitoring of athletes in terms of endurance, overtraining and doping from a diverse range of athletic disciplines such as rugby, rowing, swimming and cycling. The results of triglyceride and glycerol concentration measurements from athletes after endurance testing and at rest for example demonstrated that FT-IR without any chromatographic separation could be applied to routine clinical analyses.<sup>163</sup> Other metabolomics-based studies include non-invasive multi-component assays of metabolites in urine using ATR,<sup>164</sup> diagnosis and monitoring of the metabolic disorder alcaptonuria,<sup>165</sup> identification of the genetic disorder beta thalassemia,<sup>166</sup> as well as studying idiosyncratic toxicity of drugs causing hepatotoxicity in rats by FT-IR on urine.<sup>22</sup>

## Near infrared

The potential of near infrared (NIR) spectroscopy as a metabolic fingerprinting tool in disease diagnosis should also be recognised, and indeed the number of NIR publications in



**Fig. 4** Results of bibliometric analysis of the number of publications per year listed on ISI Thomson Web of Science<sup>®</sup> (<http://wos.mimas.ac.uk/>), using the search terms (Raman OR FT-IR OR NIR) AND (cancer).

relation to cancer for example follow similar trends to both FT-IR and Raman spectroscopies, as can be seen in Fig. 4. Studies in this particular area include those related to breast cancer detection involving a novel NIR breast detector pad,<sup>167</sup> and use of an NIR probe to determine concentrations of deoxy, oxyhemoglobin, water and lipids in the breast, with results suggesting that the additional information collected with NIR may prove useful in managing breast disease.<sup>168</sup> Others have used a prototype optical mammography system designed to image the interior of the breast assisted by NIR light transmission measurements,<sup>169</sup> and a novel hybrid system utilising magnetic resonance imaging (MRI) and NIR has been applied to the non-invasive study of breast cancer *in vivo*.<sup>170</sup> The authors of the MRI-NIR system suggest that this technology can be used to characterize functional parameters of cancers with diagnostic and potential prognosis and that it could possibly play a major role in functional activation

studies of the brain and muscle.<sup>170</sup> Several additional NIR studies of interest include NIR fibre-optic detection of pancreatic cancer,<sup>171</sup> transmission and spectral imaging measurements of prostate cancer<sup>172</sup> and analysis of atherosclerotic plaques in post-mortem human subjects with the aim to develop a NIR catheter system to detect vulnerable coronary plaques in living patients.<sup>173</sup>

## Conclusion

Fourier transform infrared (FT-IR) spectroscopy is a rapid, robust and highly reproducible analytical technique. With transmission-based 96 and 384 location sampling<sup>22</sup> FT-IR has considerable potential for use in routine clinical analyses, in particular, for high-throughput screening and examples of sampling approaches for infrared and Raman spectroscopies are shown in Table 1. Once the wavenumber region, or specific

**Table 1** Sampling approaches for infrared and Raman spectroscopies

| Method  | Properties  |
|---|---|
| Fourier transform infrared spectroscopy in the mid-IR |   |
| Transmission and reflection based                     | Sample is dried onto a sample carrier made of either ZnSe or Si. Sample acquisition is rapid (10–60 s per sample) and is automated in batches of 96 or 384. <sup>22</sup>   |
| Attenuated total reflectance                          | Sample is analysed directly by intimate contact with an ATR crystal. Crystals can be KRS-5, ZnSe, Ge or C. Penetration is within the evanescent field which can be controlled and allows measurement from aqueous body fluids.  |
| Imaging   | Point mapping of ( <i>e.g.</i> ) tissues is possible but very slow. This can be extended by focal plane array technology (typically 64 × 64 pixel IR detection arrays).   |
| Near infrared spectroscopy                            |   |
| NIR   | Water does not cause a problem so can be used for body fluids. Direct measurements from living tissue possible.   |
| Raman spectroscopy                                    |   |
| Normal  | Need to choose laser excitation, usually in the visible to near IR range (514.5–830 nm). In the visible part of EM radiation can be plagued by fluorescence.  |
| Resonance enhanced                                    | Sample is excited with a frequency of light that is within the molecular absorption bands of the sample. Excitation of this type is in resonance with the electronic transition. Enhancements over normal Raman scattering of typically 10 <sup>4</sup> .   |
| Surface enhanced                                      | Requires close proximity/adsorption onto a roughened metal surface, a colloidal solution or a roughened electrode (usually Ag or Au). Enhancement explained by two processes; an <i>electromagnetic enhancement</i> effect (thought to dominate) and a charge transfer mechanism, known as <i>chemical enhancement</i> . Has a fluorescence quenching effect. Can tune to a specific chromophore for additional resonance enhancement (known as SERRS). Enhancements over normal Raman scattering of typically 10 <sup>3</sup> –10 <sup>6</sup> . |

wavenumbers of interest, relevant to the detection of a particular disease has been established, then this information can be exploited. Optical technology is rapidly developing and instruments are already available commercially as portable, hand-held, and micro- devices. These include infrared filterometers<sup>174,175</sup> and infrared<sup>176–179</sup> and Raman<sup>180</sup> mobile microspectrometers, which can be used when it is not practical or economical to utilize sophisticated and more costly instruments such as those used in research laboratories. These, and other devices (also known as selective wavelength devices) operate across a fixed or variable wavenumber range (significantly more reduced than that of research grade spectrometers), or are calibrated to detect specific wavenumbers, or even ratios of wavenumbers.

Moreover, as more in-depth understanding of disease progression (e.g., grade of cancer) becomes realised it will become possible to generate both qualitative and quantitative predictions from infrared and Raman spectroscopies, leading to the potential ability to detect the stage of progression of a particular disease and not just its presence or absence. Beyond medical diagnostics discussed above FT-IR and Raman are exploited in very diverse fields including chemical and biochemical analysis, environmental monitoring, field applications, process control monitoring and imaging amongst many others. The manifold advantages of such approaches based on vibrational spectroscopy include low cost, small size, compactness, robustness, lightweight, as well as mass producibility. Finally, as they generally require a minimum of background training to operate, it is likely that an optically based fingerprinting approach could be developed for routine use in a clinic, ward, medical practitioners surgery, or taken to wherever required for a rapid measurement patient by patient, for so-called point of care diagnoses.

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