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Review Article

Vibrational Biospectroscopy in the Clinical Setting: Exploring the Impact of New Advances in the Field of Immunology

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The investigation of pathological diseases largely relies on laboratory examinations. The ability to identify and characterise cells is an essential process for clinicians to reach an accurate diagnosis and inform appropriate treatments. There is currently a gap between the advancement of scientific knowledge on cellular and molecular pathways and the development of novel techniques capable of detecting subtle cellular changes associated with disease. Biospectroscopy is the use of spectroscopy techniques to investigate biological materials. Within a biological sample, important molecules such as lipids, carbohydrates, nucleic acids, and proteins are held together by chemical bonds; these bonds will vibrate following excitation with infrared light. By measuring the vibrational energy of each molecule present in a biological sample, a unique spectrum, known as the “molecular fingerprint” is generated. As disease-related changes in biological samples will be reflected in the molecular fingerprint, biospectroscopy is a well-placed candidate for the investigation of disease. Biospectroscopy has been gaining wider acceptance and application in the clinical setting over the past decade; however, it has yet to reach diagnostic laboratories and healthcare clinics as a routine platform for clinical assessment. Immunological disorders are complex, often demonstrating interaction across multiple molecular pathways which results in delayed diagnosis. Vibrational spectroscopy is being applied in many fields, and here we present a review of its use in cellular immunology. Potential benefits, including an enhanced definition of molecular processes and the use of spectroscopy in disease diagnosis, monitoring, and treatment response, are discussed. The translation of vibrational spectroscopic techniques into clinical practice offers rapid, noninvasive, and inexpensive methods to obtain information on the molecular composition of biological samples. The potential clinical benefits of biospectroscopy include providing a more prompt and accurate disease diagnosis, thus improving patient care and resulting in better health outcomes.

1. Introduction

The process of pathological diagnosis largely relies on the detection of either structural changes to cells and organs or the identification of disease biomarkers in biofluids [1]. There is an ever-expanding gap between the underpinning scientific knowledge base for complex diseases and the availability of new technologies to aid in earlier and more accurate diagnosis. There remain a number of disorders for which a diagnostic test remains elusive, providing clinicians with a diagnostic dilemma and patients with a delay in receiving appropriate care whilst other disorders are

excluded. This is especially true in the field of immunology; current diagnostic laboratory techniques include electrophoresis, nephelometry, western blotting, flow cytometry, enzyme-linked immunosorbent assay (ELISA), and immunohistochemistry. These laboratory techniques are now over 50 years old, and despite significant advancements in scientific knowledge and technology, this has not been reflected in clinical practice, and testing platforms within the diagnostic setting remain limited.

The paucity of available tests for investigating the immune system will be compounded by the fact that the immune system is complex, made up of a large network of

cells of tissue connecting all organ systems throughout the body. Immune-mediated disorders can affect anyone at any age and can impact every organ system in the body. Our current range of tests varies in their specificity and efficacy with each having its own set of clinical and analytical limitations [2]. For example, ELISA tests require expensive cell culture techniques in the manufacture of specific antibody reagents; moreover, the target antigens used may not truly be representative of the autoantigen presented to the immune system *in vivo*, thus leading to false positive or negative results [2].

Vibrational spectroscopy techniques are powerful candidates that could aid in the diagnosis of a variety of complex immune conditions due to their rapid speed of analysis, lack of requirement for test reagents or chemical substances, reduced sample volumes, and high levels of precision and accuracy in the ability to differentiate compounds [3]. Vibrational spectroscopy is a technique that measures how electromagnetic radiation interacts with matter, allowing us to highlight the unique biomolecular features of a given sample in a label-free manner [4]. It is a useful technique to study biological samples such as blood, urine, cerebrospinal fluid (CSF), and saliva and is able to differentiate biomolecules by identifying unique spectral signatures. Each unique spectral signature will vary depending on biochemical changes; this may reflect changes occurring in disease or the increase/decrease of a particular molecule such as DNA [5]. This places vibrational spectroscopy as a potential candidate and novel diagnostic test for a variety of conditions.

2. Biospectroscopy Techniques

Raman spectroscopy is one of the two common types of biospectroscopy methods widely used. It is a powerful analytical tool that delivers a biochemical fingerprint regarding the molecular composition of a biological sample. Inelastic or Raman scattering is a rare event (occurring approximately one in every million photons) following sample excitation by monochromatic radiation such as a laser. Raman scattering alters the frequency of scattered photons with respect to the frequency of i incident photons to provide a quantifiable signal, without causing any damage to the sample.

Raman spectroscopy has been applied in various fields such as forensic science [6] and pharmaceuticals [7]. In the field of medicine, the technique has been applied to monitor diabetes [8], the detection of hyperbilirubinemia [9], the diagnosis of cancer [10, 11], Alzheimer's disease [12], mental health conditions [13], and autoimmune disorders such as systemic lupus erythematosus (SLE) [14].

Raman spectroscopy works on the basis of "Raman shift," which is a measure of the difference of energy between the scattered and incident photons after interaction of the photons with the molecules. The loss of energy from the photon increases its wavelength and is described as Stokes shift. In contrast to this, antistokes signals can be detected due to the gain of energy to the photons following interaction between the photons and the molecule in an excited vibrational state [15, 16]. The signal intensity from the scattered light is proportional to the concentration of a molecule within

a sample; hence, the spectrum created can provide information on the molecular constituents and concentration in a given sample. The unique pattern of Raman shifts within the spectrum differentiates subtle changes between a healthy and pathological samples with each peak intensity varying, depending on the constituents of the sample.

Fourier transform infrared spectroscopy (FTIR) is an analytical technique used to identify functional groups present in a sample by measuring the changes in the vibrational and molecular bonds once irradiated with the polychromatic IR source. In this method, the collected interferogram is transformed using a mathematical model (the Fourier transform) to obtain the infrared spectrum. The Fourier transform is a mathematical tool that breaks a waveform into an alternate representation, allowing it to be deconstructed into its sinusoidal components [17].

Currently, FTIR has been used in the diagnosis of various conditions such as rheumatoid arthritis [18], HIV/AIDS [19], diabetes [20], multiple sclerosis [21], mental health conditions [22], immune deficiency [23], autoimmune vasculitis [24], and multiple cancer types [25–27]. In FTIR spectroscopy, some of the radiation absorbed is converted to vibrational energy within molecules in a sample. Spectral peaks correlate to the frequency of the molecular bonds within a given molecule and can thus be used to identify the biological constituents of a sample alongside subtle changes associated with disease [28].

Like Raman, FTIR has a variety of possible applications due to its high sensitivity in detecting changes in common tissue components such as proteins, nucleic acids, and lipids. In both FTIR and Raman, the spectral bands created are based on the molecular excitations in the form of vibrational energy, depending on the composition of the sample, as illustrated in Figure 1 [29]. FTIR measures how much light energy is absorbed from the original source after being passed through the substance. In comparison, Raman measures the energy that is scattered after being excited by a laser [11].

Surface-enhanced Raman spectroscopy (SERS) is a form of Raman spectroscopy in which the Raman scattering from molecules is strongly enhanced due to the close proximity of these molecules to a nanostructured metallic surface [30]. In this surface-sensitive technique, the Raman scattering is enhanced by metal structures or nanostructures such as gold or silver nanoparticles [31]. The high enhancement factor (between 10^{10} and 10^{11}) allows for the detection of single molecules [32, 33].

Current research has shown promising results in the application of SERS in various types of cancer such as colon [34], pancreatic [35], oral squamous cell [36], and chronic lymphocytic leukemia [37], with the majority of studies being conducted *in vitro*. The use of SERS to investigate immunological conditions such as autoimmune or immune deficiency disorders has not been widely reported.

Near-infrared spectroscopy (NIRS) is a form of IR spectroscopy based on molecular overtone and combination variations. The resulting wavelength, bands, and intensities are generated based on the anharmonic effects, with intermode anharmonicity playing the most substantial role.

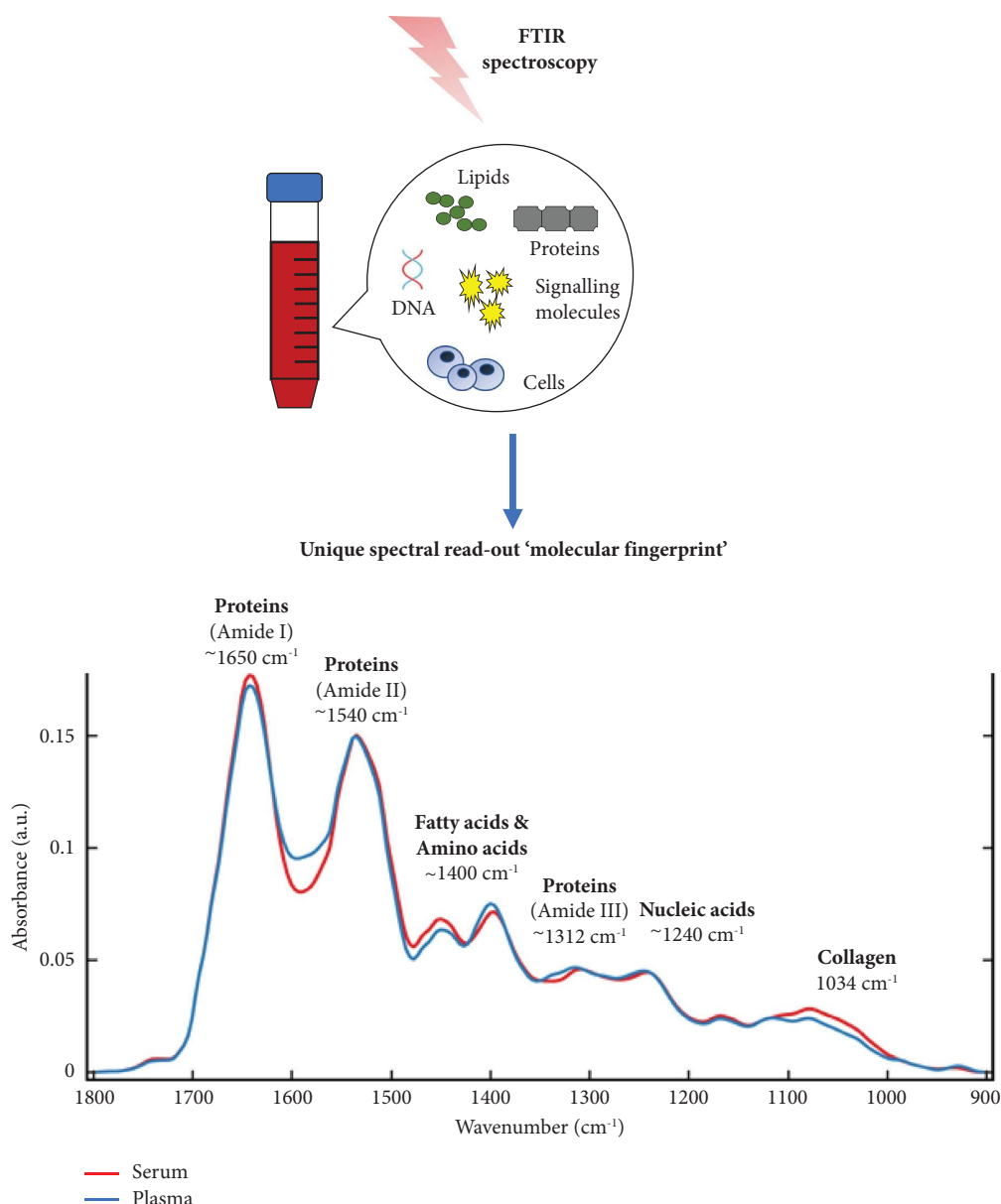


FIGURE 1: Generating unique FTIR spectra for biological samples. Following interaction with IR light, the changes in light absorbance can be measured and a biological spectral fingerprint generated. The characteristic spectrum of blood serum (red) and plasma (blue) are illustrated, with six key wavenumber peaks highlighted alongside their associated biomolecular component assignments. From “vibrational spectroscopy and multivariate analysis techniques in the clinical immunology laboratory: a review of current applications and requirements for diagnostic use,” by Callery and Rowbottom, applied spectroscopy reviews, with permission [29].

NIRS is different from other forms of vibrational spectroscopy as it requires a computationally intensive anharmonic approach compared to the harmonic approach used by FTIR and Raman [38]. Due to a spectral window in the NIR range (650–900 nm), the technique offers further penetration into a sample, where tissue absorption is relatively low [39]. The application of NIRS has been applied to measure glucose [40] and oxygen concentration [41] in blood along with monitoring of hemodialysis [42], but is limited in the field of immunology. NIRS has shown promise in the diagnosis of various types of cancer such as leukemia [43], breast [44], and colon cancer [45].

2.1. Biospectroscopy: Clinical and Diagnostic Application.

In recent years, there has been an explosion of research papers applying vibrational biospectroscopy as an investigative tool in the clinical and laboratory setting, as summarised in Table 1. The major aim of these studies has been to increase the diagnostic capability of testing for pathological disease and thus improve patient care. To date, there has been limited application in the field of clinical immunology; however, the success of this platform in clinical areas with similar sampling requirements (cells, tissues, blood serum, and plasma) has demonstrated the potential for this technique to be translated into the field of immunology.

TABLE 1: Summary of main applications of vibrational spectroscopy in the field of clinical and laboratory immunology.

Clinical application	Vibrational spectroscopy method	Sample type	Key findings	References
DBCL and CLL	Raman	Whole blood	Raman shift of 1445 cm^{-1} was increased in DBCL and 1655 cm^{-1} downregulated in CLL. Sensitivity of 92.86%	[46]
PML, BMC and CML cell lines	SERS	Cell lines	Both the diagnostic sensitivity and specificity was 98.3% for differentiating leukemia cells compared to normal cells	[47]
Breast cancer	FTIR	Serum	Differentiating controls and breast cancer patients with a sensitivity and specificity greater than 95%	[48]
Prostate cancer	SERS	Serum	High intensity peaks of 637 cm^{-1} , 808 cm^{-1} , and $1,655\text{ cm}^{-1}$ with a sensitivity of 95.0% and specificity of 93.8% in patients with cancer	[49]
Prostate cancer and BPH	NIRS	Tissue	Spectral ranges of $1055\text{--}1065\text{ cm}^{-1}$ and $1450\text{--}1460\text{ cm}^{-1}$ were found to be higher intensity peaks in prostate cancer patients compared to BPH	[50]
RA	FTIR	Serum	Distinguished between RA patients and controls with a specificity of 100% and sensitivity of 85%	[15]
RA	Raman	Serum	Quantitative levels of IgG, IgA and IgM significantly correlated with the Raman spectra, particularly in the region of $500\text{--}550\text{ cm}^{-1}$	[51]
ANCA-associated vasculitis	Raman	Plasma and serum	Spectral bands 1015 cm^{-1} and 1678 cm^{-1} were higher in plasma of active disease patients (sensitivity 70% and specificity 93.3%)	[21]
			Peak of 1659 cm^{-1} higher in serum of active disease (sensitivity and specificity of 80%)	
SLE	Raman	Serum	Differentiated SLE patients from controls with a sensitivity of 100% and specificity of 99%	[52]
MS	FTIR	CSF	Unique wavenumber of 795 cm^{-1} was identified in patients with MS compared to controls with a sensitivity of 95% and specificity of 92%	[18]
SjS	SERS	Saliva and serum	Peaks at wavenumbers of 1130 cm^{-1} and 1368 cm^{-1} were found to be higher in patients SjS with sensitivity of 96.5% and specificity of 90.5% (salivary samples) and sensitivity of 96.5% and specificity of 100% (serum samples)	[14]
CVID	FTIR	Serum and plasma	Differentiated CVID patients from healthy controls with sensitivities and specificities of 97% and 93%, respectively for serum, and 94% and 95%, respectively for plasma	[53]

DBCL--diffuse B cell lymphoma; CLL--chronic lymphocytic leukemia; BMC--bone marrow mononuclear cells; CML--chronic myelogenous leukemia; PML--promyelocytic leukemia; BPH--benign prostate hyperplasia; RA--rheumatoid arthritis; AAV--ANCA-associated vasculitis; SLE--systemic lupus erythematosus; MS--multiple sclerosis; SjS--sjogren's syndrome; CVID--common variable immune deficiency.

The ability of Raman spectroscopy to measure scattered energy following excitation with a laser has enabled this technology to be used in vivo intraoperatively to establish cancer margins [54]. A study by Jerymn et al. demonstrated the use of a handled contact fiber optic Raman spectroscopy probe alongside magnetic resonance imaging (MRI) to distinguish gliomas from normal brain cells. MRI was used for visualization and for estimating the location of each Raman measurement on the preoperative images. The system was able to measure in vivo spectra and differentiate between normal brain cells and dense cancer cells based on Raman signals with a specificity of 91% and sensitivity of 93% [11].

As FITR measures the light energy from the original source, this limits the in vivo application. However, the applications in cytology seem to be promising. A study by Papamarkakis et al. demonstrated that oral cells could be classified according to principal component cell analysis. Samples of patients with squamous cell carcinoma were differentiated from health samples; spectral changes were also seen in oral cells that had been infected with the herpes simplex virus [55]. Studies have also shown that spectral changes form exfoliated cells in patients with oral dysplasia and healthy samples [56, 57].

The ability to identify and characterise cells is fundamental to the investigation of disease. The differentiation of cell types is used both within the initial diagnosis and in prognostic monitoring, for example, in identifying disease stage and progression in cancerous disease states. Light microscopy, with or without subsequent staining, is one of the most widely used techniques for cellular analysis. The primary limitations of optical microscopes include resolution, magnification, and surface view; subsequent staining processes; and the requirement for highly specialised reader interpretation, further restricting this technique as a high-throughput method.

Biospectroscopy has been applied to the differentiation of cells; Townsend et al. conducted an IR study that could differentiate various types of oesophageal cells such as squamous and Barrett's (with and without dysplastic changes). The accuracy, sensitivity, and specificity varied between the 3 groups; however, an accuracy of 95.1% was achieved in differentiating squamous cells from Barrett's with a sensitivity of 95.5% and specificity of 94.7%, respectively [58]. This demonstrates the potential of this novel technique to detect cancerous and precancerous cells at an earlier time point and thus lead to improved patient outcomes.

Much of the literature to date examines the application of biospectroscopy in the early diagnosis of cancer or in differentiating between types of cancer. This technology has also been applied to the investigation and identification of metastatic cells. A study by Bury et al. compared the use of FTIR vs Raman in the diagnosis of brain metastasis cells from colorectal adenocarcinoma, lung adenocarcinoma, and melanoma. When the two adenocarcinoma groups were combined and compared with the melanoma group, the accuracy of diagnosis of melanoma was 75.4% vs 85% for adenocarcinoma, and the use of FTIR produced an accuracy

of 72% for melanoma vs 96% for adenocarcinoma. However, in this study, spectroscopy was not able to determine the primary tissue origin [59].

2.2. Vibrational Biospectroscopy to Detect and Classify Immune Cells. As highlighted earlier, immune-mediated disorders have interconnectivity with almost all organ systems of the body. There is an unmet need for an improved and holistic approach toward the investigation of immune-associated disorders, whereby simultaneous analysis of multiple immune-mediated components is performed to provide an earlier indication of disease. Direct analysis of the major cell types involved in the pathogenesis of these disorders will be vital, not only to further our understanding of these complex diseases but also to add a highly specific and sensitive means of diagnosis and monitoring which is currently largely unavailable.

Several research groups have successfully applied biospectroscopy techniques to the analysis of immune cells in a label-free manner, negating the requirement for expensive reagents and alleviating restrictions on the number of cell markers studied. A study by Ramoji et al. was able to differentiate between lymphocytes and leukocytes (neutrophils and eosinophils) by looking at the differences of the Raman bands and at the variations in wavelength that arises due to differences in chemical composition with an accuracy of 82.3% [60]. In a study by Borek-Doros et al., Raman spectroscopy could successfully differentiate between T and B cells by using principal component-linear discrimination (PCA-LDA) analysis. The presence of peaks associated with beta-carotene was determined to be a major difference, with significantly higher levels observed in T cells [61]. Raman spectroscopy has shown the possibility to differentiate between T and B cells while also monitoring the activation of T cells from their naïve state. The study by Ichimura et al. found unique peaks between 1000 and 1150 cm^{-1} that represented B cells and peaks between 1300 and 1400 cm^{-1} that represented T cells. Activation of T cells were monitored over the course of 48 hours and found an abundance of cytochrome C in activated T cells, indicating higher metabolic activity [62]. Wesełucha-Birczyńska et al. looked at the activation of B cells using Raman spectroscopy and distinct peaks that separate activated and intact cells. However, the peak of 521 cm^{-1} was identified as an important marker of B cell activation; this sharp peak suggests that disulphide linkages are in similar geometries in the activated cell. It is believed that the formation of immunoglobulins and the following aggregation during B cell activation is the cause of the remarkable increase in the intensity of 521 cm^{-1} marker bands [63].

Differences between various types of T cells were also identifiable using this technique. In a recent study by Chaudhary et al., B cells, T cells, and monocyte cell lines could each be identified and differentiated. Differences were seen in Raman peaks associated with DNA and histone levels, proteinase-K, and lipid synthesis. The study was also able to differentiate between activated- and nonactivated immune cells. The unique Raman spectra produced by

activated T cells and monocytes contained strong discrete bands which had molecular associations with nucleic acids and proteins. Conversely, in this study, activated B cells did not produce suitably strong spectral differences to enable differentiation from nonactivated cells [64].

A further study by Chen et al. supported the use of Raman spectroscopy techniques for the label-free identification of immune cells by successfully distinguishing between CD4⁺ T lymphocytes, CD8⁺ T lymphocytes, and CD56⁺ natural killer cells. Various unique wavelengths were able to differentiate between CD4⁺ and CD8⁺ cells, including: 645 cm⁻¹ (C-C twist in tyrosine), 1259 cm⁻¹ (Amide III), 1378 cm⁻¹ (thymine/adenine/guanine), and 1665 cm⁻¹ (Amide I). Unique wavelengths were also found that differentiated T cells and NK cells, such as 621 cm⁻¹ (C-C twist in phenylalanine), 725 cm⁻¹ (adenine ring), and 1304 cm⁻¹ (adenine/amide III) [65], as illustrated by Figure 2.

Supplementary evidence to support the use of Raman spectroscopy as a tool to differentiate between different lymphocyte subsets includes a study by Hobro et al. This study successfully used cell lines to differentiate between B and T cells based on single-point spectra collected from the centre of each lymphocyte. Most differences appeared in the wavenumber region between 750 and 900 cm⁻¹, with peaks predominantly seen at 755 cm⁻¹ (cytochrome c), and 840 cm⁻¹ (B-form DNA). Smaller differences were noted in the region of 1200–1400 cm⁻¹, as well as between 2800 and 3000 cm⁻¹ [66].

FTIR spectroscopy methods have also successfully demonstrated the ability to differentiate between B cells, cytotoxic, and helper T cells. A study by Verdnock et al. found that lymphocytes have a spectral difference over the entire IR region. Particular peaks in the 2800–3000 cm⁻¹ region highlight a change in the lipid/protein ratio between T and B lymphocytes. The study looked at assignment scores for successfully differentiating cells with a score of 91.8%, 87.6%, and 87.7% for CD4⁺, CD8⁺, and CD19⁺ lymphocytes, respectively [67]. Another study by Krafft et al. used FTIR to identify anatomical features of the spleen such as primary and secondary follicles, T zones, arteries, and spleen red pulp. The study also compared spectra between lymphocytes in blood and those in spleen tissue. Unique wavelengths were identified for various anatomical features; for example, peak wavelengths of 1,171, 1,304 (amide III), 1,392 (COO⁻), 1,454 (CH₂/CH₃ deformation), 1,543 (amide II), and 1,654 cm⁻¹ (amide I) represented red pulp. Similar unique wavelengths were also identified for lymphocytes as well. This study showed that while the sensitivity and specificity in identifying B and T cells of FTIR are similar to current staining techniques, the speed, data throughput, and minimal sample preparation are advantages that FTIR has over current staining techniques and highlight the possibility of using biospectroscopy in a clinical setting [46]. SERS has also shown the ability to differentiate between T cells and other blood cells.

A study used “composite organic-inorganic nanoparticles” (COINs) Raman nanoparticles, which possess unique Raman spectra, to previously demonstrate this. The study detected proteins expressed on the surface of cells that

were able to distinguish T cells among other blood cells. Furthermore, intracellular phosphorylation of Stat1 and Stat6 was measured using SERS, with results comparable to flow cytometry techniques [68].

2.3. Biospectroscopy: Use in Cancer

2.3.1. Immune Cell Cancers. Biospectroscopy has demonstrated advantages as a key diagnostic tool for cancer compared to conventional methods, due to easier sample handling, more rapid analysis, and increased specificity towards diagnosis. A range of studies have examined the use of biospectroscopy across a wide range of cancer types, including solid tumours as well as immunological cancers. A study by Féré et al. compared the blood samples of patients with B-chronic lymphocytic leukaemia (B-CLL) to those of healthy patients. They found unique Raman spectra that were able to differentiate protein, DNA, and cytochrome-c bands, suggesting their potential use as diagnostic biomarkers [47]. Another study looked at the diagnosis of diffuse B-cell lymphoma (DBCL) and chronic lymphocytic leukemia (CLL) with promising results, differentiating patients with DBCL or CLL from controls, with sensitivities of 92.86% and 80%, respectively. The study further established distinct Raman peaks that could differentiate CLL from DBCL. An intensity shift at 1445 cm⁻¹ (related to collagen and lipids) was increased in DBCL, whereas a shift at 1655 cm⁻¹ (related to proteins, alpha helix, and collagen) was found to be upregulated in DBCL but downregulated in CLL [69]. In another study, similar intensity changes were found at 1456 cm⁻¹ and 1666 cm⁻¹ that differentiated CLL from normal cells. Specifically, a significant red shift of 5 wavenumbers was observed for the phenylalanine band in the mean spectrum of the CLL patients. Finally, an increased peak was observed at 2954 cm⁻¹ in the CLL spectrum (associated with increased stretching vibrations of CH₃ bonds) compared to healthy controls, illustrating future diagnostic biomarkers in haematological malignancy [70].

SERS has been used in the differentiation and diagnosis of immunological cancers. An in vitro study demonstrated that SERS were able to differentiate between 60 acute promyelocytic leukemia cells (HL60 cell line), 60 chronic myelogenous leukemia cells (K562 cell line), and 60 normal human bone marrow mononuclear cells (BMC). Intensity shifts at 1053 cm⁻¹ and 1296 cm⁻¹ were found to exhibit higher SERS signals in HL60 and K562 compared to BMC, with shifts of 670 cm⁻¹ and 896 cm⁻¹ exhibiting lower intensities in cancerous cell lines compared to BMC. Post-analysis yielded a diagnostic sensitivity and specificity of 98.3% for differentiating leukemia cells compared to normal cells [71]. Another study compared blood samples from 98 patients with acute myeloid leukemia (AML) and 48 controls. The SERS intensity shift at 636 cm⁻¹, 766 cm⁻¹, 1069 cm⁻¹, and 1367 cm⁻¹ was found to significantly differentiate between AML patients and control ($p < 0.05$). However, the study was not able to demonstrate significant spectral variances to differentiate between AML subtypes [72].

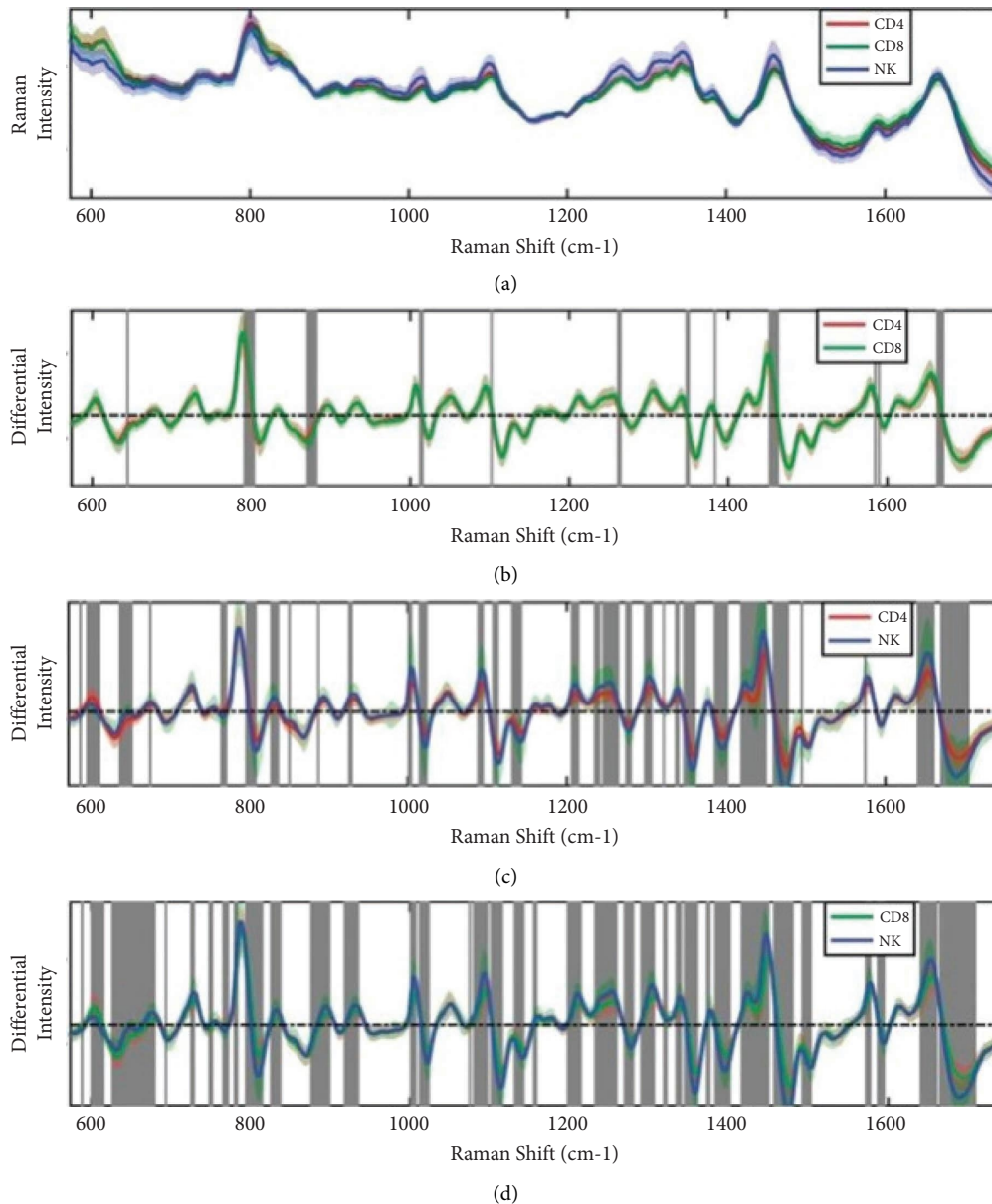


FIGURE 2: Raman spectra produced to differentiate 3 groups of lymphocytes. (a). Unique spectra generated and compared between CD4 and CD8 cells (b). Unique spectra generated and compared between CD4 and NK cells (c). Unique spectra generated and compared between CD8 and NK cells (d). From “the use of wavelength modulated Raman spectroscopy in label-free identification of T lymphocyte subsets, natural killer cells and dendritic cells” by [57], PLOS [35].

There is limited research on the applications for the use of NIRS in leukemia. One study looked at the use of NIRS for early screening of leukemia via the least angle regression (LAR) and partial least squares (PLS) regression model. The study found that the LAR-PLS model had a correlation coefficient of 0.949 for the classification of leukemia, demonstrating an advantage over other regression models such as principal component regression [48]. A study by Lanfranconi et al. used NIRS in the detection of oxidative skeletal muscle impairment in children with acute lymphoblastic leukaemia (ALL) [73]. The study found skeletal muscle damage in children with ALL compared to healthy controls by looking at the oxygen uptake and tissue oxygen

status of the vastus lateralis muscle. A statistically significant linear regression was found between the oxygen uptake peak and tissue oxygenation status in children with ALL compared to controls, highlighting the possible use of NIRS in monitoring disease-associated changes in patients with leukemia.

2.3.2. Nonimmune Cell Cancers. The use of Raman spectroscopy as a diagnostic technique has been successfully demonstrated across several nonimmune cell cancers. A study was able to differentiate between healthy and cervical cancer cells based on glycogen storage variations with a sensitivity of 95%, with the most distinctive spectral

changes observed at a phenylalanine peak at 1004 cm^{-1} [74]. FTIR spectroscopy has also been used. A study analysing the spectra of serum samples for the detection of breast cancer reported sensitivities and specificities of greater than 95%, supporting the use of FTIR as a future diagnostic tool [75].

Blood spectral biomarkers were also identified for bladder cancer in a FTIR study conducted by Ollesch et al. Using random forest classification techniques, patients with bladder cancer could be distinguished from patients with bladder inflammation with sensitivities of 93% [49].

Biospectroscopy has further advantages in that it can be applied to the analysis of several types of biofluids and is not solely restricted to blood samples. Other biofluids, such as urine, saliva, (CSF), ascites fluid, and sputum have been examined by FTIR spectroscopy for the detection of biomarkers for pathological disease. A study by Yap et al. applied FTIR spectroscopy analysis to urine samples for the investigation of prostate cancer. The group used principal component analysis combined with a linear discriminant analysis (PCA-LDA) to create a classification model that achieved a specificity of 60% and a sensitivity of 83% in the diagnosis of prostate cancer [76]. Furthermore, ascites fluid from 45 patients with varying stages of ovarian tumours has been examined using FTIR spectroscopy. Giamougiannis et al. performed partial least squares discriminant analysis (PLS-DA) to generate a classification model able to distinguish between benign pathologies, borderline categories, and confirmed ovarian tumour samples with 93% specificity and 79% sensitivity [77].

SERS has been extensively studied for its application in the diagnosis of prostate cancer, with sensitivities varying depending on the biofluid analysed. A study compared the blood serum samples of patients with benign prostate hyperplasia (BPH) and prostate cancer with those of healthy controls. Patients with prostate cancer showed higher intensities at peaks 637 cm^{-1} , 808 cm^{-1} , and $1,655\text{ cm}^{-1}$ with a sensitivity of 95.0% and specificity of 93.8% [78]. Application of SERS to other biological samples such as tissue [79], urine [80], and seminal fluid [81] has shown promising results in the detection of prostate cancer.

Use of SERS in other types of cancer has also demonstrated encouraging results. A study found unique peaks at 1009 cm^{-1} and 1096 cm^{-1} in the detection of breast cancer cell lines. This study used gold nanoparticles to identify cells through the interaction between a specific aptamer (Apt1) and the overexpressed protein mucin 1 (MUC1) on the surface of the tumour cells [50]. Other studies have used SERS in combination with gold nanoparticles to detect heavy metals in breast cancer cells [82] and in combination with silver nanoparticles to differentiate between HER2+ breast cancer cells [83].

The use of NIRS for its potential use in the diagnosis of cancer is limited. Vermassen et al. compared tissue samples of patients with BPH and prostate cancer and found a significant difference in N-linked glycosylation between the 2 groups. Spectral ranges between 1055 cm^{-1} and 1065 cm^{-1} and $1450\text{--}1460\text{ cm}^{-1}$ were found to result in higher peaks in patients with prostate cancer compared to BPH [84].

2.3.3. Metastatic Cancer. Due to the fact that different cell types have unique infrared spectral features, FTIR spectroscopy is well placed for the identification and differentiation of specific cell types. This has been demonstrated in the clinical setting in a study successfully differentiating nonmetastatic from metastatic cells in the lymph nodes of patients with melanoma. Wald et al. were able to identify melanoma cells from healthy cells with specificities and sensitivities of 85.7% and 87.1%, respectively. Key differentiating features included unique wavenumbers between 1620 and 1670 cm^{-1} , and specifically, a peak of 1638 cm^{-1} (collagen). The study was also able to differentiate between lymphocytes and other cell types with unique wavenumbers between 1080 and 1230 cm^{-1} corresponding to stretching of the phosphate groups [85].

In a similar study, Mazur et al. used Raman spectroscopy to look at the activation of B lymphocytes in the lymph nodes of patients with colon, stomach, or oesophageal cancer. Unique spectral peaks were found for each cancer type; in oesophageal squamous cell carcinoma, a distinct peak at 1120 cm^{-1} was identified, whereas the remaining two carcinomas displayed a smaller shoulder on the 1090 cm^{-1} DNA band. These changes were indicative of B cell proliferation, with spectral variances most prominent in the DNA/RNA regions. Further analysis revealed similar unique peaks could be observed during B cell activation, specifically wavenumbers 1164 cm^{-1} (phosphate group), 1090 cm^{-1} , and 1655 cm^{-1} (amide band I) [51].

2.4. Biospectroscopy: Use in Autoimmune Disorders. There is limited evidence in the literature on the use of biospectroscopy for the diagnosis of autoimmune disorders. It is well recognised that current serology testing for autoimmunity has limitations, which can lead to long diagnostic delays for patients. Most tests are based on immunoassay techniques; however, even within the same disease cohort, there are significant variations between the autoantibodies produced by different patients. This can result in false positive and negative results or difficulties monitoring patients, as an individual's autoantibodies can also change over the course of disease. This unmet need for improved autoantibody testing is likely to be answered by novel testing platforms that are not restricted to single antigen-, antibody-, or biomarker analysis. Biospectroscopy is an ideal candidate, with increased research emerging in recent years.

A pilot study by Lechowicz et al. used FTIR spectroscopy for the diagnosis of rheumatoid arthritis (RA). They used RA patient blood serum and found significant spectral differences in the range of $3000\text{--}2800\text{ cm}^{-1}$ and $1800\text{--}800\text{ cm}^{-1}$, capable of discriminating between patients and controls with a specificity of 100% and sensitivity of 85%. Of interest, a specific peak at wavenumber 1424 cm^{-1} was most effective in differentiating patients. This wavenumber vibration is indicative of two key amino acids, tryptophan and proline. These findings are in line with other studies in RA; tryptophan had previously been reported at higher levels in RA patients [18], and proline is one of the amino acids in collagen type 1 and hydroxyproline which is a marker for bone collagen degradation [86].

Another study looked at the use of Raman to screen patients with early RA and found a number of key peaks were responsible for the differentiation of controls from early RA. Increased peaks at 744 cm^{-1} (ν carbonyl, ν (C-S)), 953 cm^{-1} (benzene ring torsional vibration), and 1079 cm^{-1} (C1-H5, C2-H6 symmetric bend) were identified in the early RA group compared to the healthy control group. Peaks of 1374 cm^{-1} (hemoglobin (oxyhemoglobin) methemoglobin), 1390 cm^{-1} (COO⁻ symmetric stretching), 1530 cm^{-1} (C=C stretching of carotenoid), and 1715 cm^{-1} (C=O stretch) were able to differentiate between the early RA group and non-early group. These peaks are attributable to many proteins such as anticitrullinated protein antibodies (ACPA), serum albumin, and rheumatoid factor [52].

More recently, Raman spectroscopy has been applied to the diagnosis of RA. A study by Cao et al. examined the Raman spectra of serum from 130 patients: 70 with RA, 30 with control, and 30 with osteoarthritis (OA). Using LDA, they found that the spectral regions of $300\text{--}900\text{ cm}^{-1}$ were able to differentiate between the three groups and could aid in the diagnosis of both anticitrullinated protein antibodies (ACPAs) positive and negative patients. The study found that quantitative levels of IgG, IgA, and IgM significantly correlated with the Raman spectra, particularly in the region of $500\text{--}550\text{ cm}^{-1}$ ($p < 0.05$) [87].

Both FTIR and Raman have shown promising results for the diagnosis of ANCA-associated vasculitis (AAV), a disease with a high mortality rate. Currently, there is no reliable biomarker for AAV: however, a study by Morris et al. successfully used Raman spectroscopy to differentiate patients with ANCA-associated vasculitis (AAV). In total, 73 patients were characterized as having either active disease, disease remission, or healthy controls. For the discrimination between active disease and disease remission, key spectral bands in plasma were 1015 cm^{-1} and 1678 cm^{-1} , which were higher in active disease patients, while 1446 cm^{-1} was higher in disease remission patients. Similar analysis was done on serum with peaks of 1659 cm^{-1} higher in active disease and peaks at 1311 cm^{-1} , 1441 cm^{-1} , and 1524 cm^{-1} higher in disease remission. This study was able to use Raman spectroscopy for both plasma (specificity 93.3%, sensitivity 70%) and serum (specificity 80%, sensitivity 80%) towards the diagnosis and treatment of AAV [24]. Morris et al. performed a similar study to assess the use of FTIR spectroscopy in the diagnosis of AAV. Following the analysis of plasma samples from patients with active disease, disease remission, or healthy controls, they found key discriminating wavenumbers at 1040 cm^{-1} , 1540 cm^{-1} , and 1612 cm^{-1} alongside various peaks between 1620 and 1716 cm^{-1} which were found to be higher in active disease patients compared to patients in remission [88]. These studies provide evidence to support the translation of biospectroscopy into the clinical setting for complex autoimmune disorders.

Systemic Lupus Erythematosus (SLE) is a complex autoimmune disorder which presents heterogeneously across the patient cohort. This heterogeneity, in addition to the previously described limitations of the current immunoassay-based testing platform, results in an average

diagnostic delay of 6.4 years in the UK [89]. A recent study by Callery et al. investigated the application of Raman spectroscopy for the diagnosis of Systemic Lupus Erythematosus (SLE). The study successfully differentiated SLE patients from controls with a sensitivity of 100% and a specificity of 99%. The group identified 12 significant discriminatory peaks ($p < 0.001$) which contributed to the separation between the two groups. The study further divided the SLE patients into three subgroups based on results for antidouble-stranded DNA (anti-dsDNA) antibodies obtained from the two routine diagnostic tests for SLE: enzyme-linked immunoassays (ELIA) and semiquantitative Crithidia Luciliae indirect immunofluorescence (CLIFT). Raman spectroscopy and subsequent multivariate analysis demonstrated a reduced separation between the subgroups, further suggesting that current assays are not sufficiently detecting SLE biomarkers for all patients. While this study was limited by sample size ($n = 12$), it set precedence for future work on the use of Raman for diagnosing patients with SLE and the possibility of using it in future clinical practice [14].

FTIR has also been applied to CSF samples in the diagnosis of multiple sclerosis (MS). MS ranks as one of the most prevalent neurodegenerative diseases and causes disability among adults worldwide, while the exact aetiology of the disease is poorly understood. The pathogenesis involves the immune-mediated degradation of myelin coating around nerve fibres of the central nervous system. The diagnosis of MS is difficult due to overlapping clinical and radiological feature with other diseases; therefore, improved diagnostic measures are needed. A study using FTIR spectroscopy examined CSF samples from MS patients and controls. Further, the spectrum of patients with relapsing-remitting MS (RRMS) was compared with patients that experience a single demyelinating event known as clinically isolated syndrome (CIS). A unique wavenumber of 795 cm^{-1} was identified in patients with MS compared to controls with a sensitivity of 95% and a specificity of 92% [21]. Whilst a unique wavenumber capable of discriminating between the two subgroups could not be identified, spectral features associated with biomolecular changes in lipids and the Z conformation of DNA were able to discriminate between the RRMS and CIS groups in 70% of cases. Their findings suggest that FTIR and multivariate analysis could not only be used in the diagnosis of MS but also in monitoring the progression of the disease. Clinical translation of biospectroscopy could greatly impact patient prognosis and quality of life, as studies have shown that early diagnosis of MS leads to better patient outcomes [90].

An in vivo study by McQueenie et al. used SERS to detect early stages of inflammation by measuring expression of intercellular adhesion molecule 1 (ICAM-1). This was achieved by applying antibody-conjugated SERS to detect ICAM-1 expression on endothelial cells. This study found that SERS had double the sensitivity for the measurement of ICAM-1 expression compared to flow cytometry, proposing a potential future use of SERS for the in vivo detection of inflammation [91]. Another study examined the use of SERS in patients with Sjogren's syndrome (SjS), using saliva and

TABLE 2: FTIR vs Raman spectroscopy: advantages and disadvantages.

Technique	Advantages	Disadvantages
FTIR	(i) High sensitivity and speed (ii) No labels, minimal sample preparation (iii) Enable all frequency that measures metabolites simultaneously (iv) Nondestructive	(i) Constraints on sample thickness and uniformity (ii) Interference from water (iii) Complex mixtures can lead to misleading spectra (iv) Limited to IR-active molecule (with dipole moment)
Raman	(i) Minimal sample preparation and setup (ii) Minimal interference from water (iii) Can be used for analysis of all molecules (iv) Suitable on any surface (v) Little-to-no sample preparation (vi) Can be nondestructive (when used with low power lasers or short exposure times)	(i) Autofluorescence may interfere with the ability of taking Raman spectra in biological samples (ii) Sophisticated data analysis (iii) Weak Raman signal can lead to long acquisition times (iv) Initial setup cost and instrumentation can be costly (v) Destructive-biological samples can be sensitive to high power laser and extended exposure times
SERS	(i) Higher sensitivity (ii) Minimal sensitivity to water (iii) Highly specific (iv) Multiplexing capability (v) Faster results	(i) Higher cost (ii) Background signal from interference (iii) Intricate sample preparation (iv) Poor measurement reproducibility
NIRS	(i) No sample preparation (ii) Low cost per sample: no solvents needed (iii) Rapid analysis (iv) Versatile (v) Faster results	(i) Low sensitivity due to lower absorption coefficients (ii) Complex data interpretation (iii) Environmental factors (like temperature and humidity) can affect the accuracy and reproducibility of measurements (iv) Indirect method hence a development of multivariate calibration model required

serum samples from patients with SjS and healthy controls. SERS spectra were acquired using silver nanoparticles synthesised with reduction by hydroxylamine hydrochloride. Unique spectral peaks at wavenumbers of 1130 cm^{-1} and 1368 cm^{-1} were found to be higher in patients with SjS, with sensitivities and specificities of 96.5% and 90.5%, respectively, in salivary samples and 96.5% and 100%, respectively, in serum samples. SERS has also shown promising results in the diagnosis of rheumatoid arthritis (RA), using autoantibodies against citrullinated peptide (anti-CCP) as a biomarker. The SERS nanotags were comprised of anti-human IgG conjugated gold nanoparticles. The anti-CCP serum levels were determined by observing Raman intensities corresponding to gold nanoparticles. The results were compared to an ELISA-based assay. A good correlation was found in patients with positive anti-CCP results, with the performance of the SERS technique superior to the ELISA method for the anti-CCP negative group [53]. These studies highlight the future application of SERS in the diagnosis of auto-immune disorders, particularly in seronegative patient groups [92].

There are limited reports in the literature on the application of NIRS to the diagnosis of autoimmune disorders. A study by Nojima et al., used visible NIR spectroscopy and PCA to diagnose specific antibodies in SLE patients using plasma samples. The study demonstrated successful detection of antiphospholipid antibodies (aPL) in 42 of 48 (87.5%) aPL-positive patients and the absence of the antibodies in 33 of 36 (91.7%) aPL-negative patients. This suggests a future possibility of using visible NIR spectroscopy in the diagnosis of SLE in favor of ELISA, which is more expensive, time-consuming, and limited by the analytical restrictions of the target antigen [93].

2.5. Biospectroscopy: Use in Immune Deficiency Disorders.

The use of biospectroscopy in the field of immune deficiency is limited. However, a study conducted by Picard et al. looked at the use of FTIR spectroscopy for the investigation of patients with common variable immune deficiency (CVID). CVID is a heterogeneous disease which lacks a clinical or laboratory pathognomonic feature. This has resulted in significant diagnostic delays (approximately 5 years) and thus poor patient outcomes [94]. The study compared CVID patients ($n=21$) and controls ($n=30$), analysing both serum and plasma samples by FTIR spectroscopy. Application of multivariate analysis revealed spectral features capable of stratifying CVID patients from healthy controls with sensitivities and specificities of 97% and 93%, respectively for serum, and 94% and 95%, respectively, for plasma. Furthermore, several discriminating spectral biomarkers were identified: wavenumbers in regions indicative of nucleic acids (984 cm^{-1} , 1053 cm^{-1} , 1084 cm^{-1} , 1115 cm^{-1} , 1528 cm^{-1} , 1639 cm^{-1}), and a collagen-associated biomarker (1034 cm^{-1}), which may represent future candidate biomarkers and provide new knowledge on the aetiology of CVID. This proof-of-concept study provides a basis for developing a novel diagnostic tool for complex immunodeficiency disorders [23].

There have not been any reports in the literature directly demonstrating the application of SERS to the investigation of immune deficiency disorders. However, the technique has been used for the quantification of immunoglobulins, a key laboratory test to aid in the diagnosis of primary and secondary immune deficiency disorders. A study by Kunushpayeva et al. compared the use of gold nanoparticles to silicon wafer to measure human IgG levels [95]. The study used two lasers (633 nm and 785 nm) and found an increase in Raman intensity with increasing antigen concentrations for both SERS-active substrates. The correlation between the surface concentration of extrinsic Raman label (ERL) calculated from atomic force microscope (AFM) maps and the measured background-corrected Raman intensities was high, with an average R^2 of 0.98. The Raman signal per nanoparticle was higher on gold by an order of one magnitude compared to silicon, with a relative signal standard deviation for silicon of approximately half (5.5%) compared to signal the standard deviation for gold (12.1%). Hence, the study concluded that silicon could be used as the substrate of choice for future SERS analytical methods.

In a study by Kasemsumran et al., NIRS was applied to determine the presence of human serum albumin, γ -globulin, and glucose in a control serum solution. A chemometric model called the combination moving window partial least squares (SCMWPLS) was used to determine the concentrations of each analyte. The spectral region of $4789\text{--}4619\text{ cm}^{-1}$, $4594\text{--}4502\text{ cm}^{-1}$, $4478\text{--}4472\text{ cm}^{-1}$, and $4357\text{--}4287\text{ cm}^{-1}$ provided very good prediction results for γ -globulin with a correlation coefficient of 0.999 [96]. The ability to detect and quantify γ -globulin (IgG, IgA, and IgM) by NIRS could provide a future analytical technique for the diagnosis and monitoring of immune deficiency and autoimmune disorders.

3. Conclusion and Future Prospective

The application of IR spectroscopy has developed substantially over the past 60 years. Alongside instrument advances, developments associated with spectral data analysis have increased exponentially in recent years; Table 2 summarises the major advantages and disadvantages associated with the described biospectroscopy techniques. Computational advances have changed how spectroscopists analyse data; the availability of a wide range of approaches for preprocessing and multivariate analysis (chemometrics) has enabled spectroscopists to uncover subtle but relevant spectral information from large datasets in a timely manner. This had a far-reaching impact on the popularity and application of biospectroscopy, which is now being used across several sectors including chemistry, biology, pharmaceuticals, food and drink, materials science, healthcare, and forensics, among many others.

For clinicians, having vibrational spectroscopy as an additional tool in their armoury of investigations would be ground-breaking, particularly when applied to complex diseases which may have previously required many clinical and laboratory investigations, some of which were highly invasive (such as tissue biopsies). Diagnostic delays could be

significantly improved, cost-savings could be made, and the patient pathways for a wide repertoire of diseases could be transformed. The increasing number of studies reporting excellent sensitivities and specificities, alongside the low cost of biospectroscopy analysis has already highlighted the potential use of this platform within large-scale screening programs [97, 98].

As biological samples between individuals will share numerous constituents and thus spectral features, the application of chemometrics to identify subtle disease-associated peaks is paramount for the successful use of this analytical platform. There are many chemometric methods that can be applied to biospectroscopy data analysis; however, a consensus on a standardised approach has yet to be established. It is important for researchers to understand the suitability of both preprocessing techniques (such as smoothing, baseline correction, and normalisation), and subsequent multivariate analysis and machine learning applications for specific datasets. In the absence of a standardised protocol for spectral preprocessing and chemometric analysis, researchers should evaluate the suitability of available techniques in relation to the data set and research question. A critical appraisal of the machine learning and statistical techniques available for vibrational biospectroscopy has been published in review papers by Trevisan et al. [99] and more recently by Meza Ramirez et al. [100].

These papers provide a means for researchers to understand the mathematical algorithms and chemometric processes required for spectral data analysis, with the aim to bridge the knowledge gap between spectroscopists (who understand chemometrics) and data scientists (who have an advanced knowledge of machine learning). Further guidance to support accurate and robust vibrational spectroscopy research in the clinical setting can be sought from the literature [101–104].

In the field of immunology, the potential clinical impact and new knowledge acquisition gained from the translation of biospectroscopy into a routine clinical laboratory test would be extensive. Research groups in the field continue to publish extensively on this subject, with a shared aim to support the development of a standardised process and facilitate clinical translation [105–107]. An extensive critical appraisal of the development of biospectroscopy, the application of chemometrics, and the challenges facing the translation of biospectroscopy into the clinical setting is available by Callery and Rowbottom [29].

In summary, vibrational spectroscopy has made huge strides in becoming an analytical technique that can be translated into the healthcare setting. In line with this, and the increasing interest in and application of immunology research, we envisage that biospectroscopy methods will become a routine platform in healthcare testing in the near future.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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