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Chapter

Applications of Infrared Spectroscopy and Microscopy in Diagnosis of Obesity

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Abstract

Infrared (IR) spectroscopy is a vibrational spectroscopic technique based on the absorption of infrared radiation by matters that excite vibrations of molecular bonds. It is a powerful method for investigating structural, functional, and compositional changes in biomolecules, cells, and tissues. In recent years, scientific researchers have continued to increase the performance of this technique on clinical cases such as cancers and metabolic disorders. Obesity is one of the main factors that increases the risk of many diseases and contributes to functional disabilities in tissues such as adipose, liver, and muscle. Applications of IR spectroscopic techniques allow identifying molecular changes due to obesity, to understand the molecular mechanism of the disease, to identify specific spectral biomarkers that can be used in diagnosis. In addition, these spectral biomarkers can be used to identify the appropriate drugs and their doses for treatment. In this chapter, applications of IR spectroscopic and microscopic techniques to the characterization and understanding the obesity metabolism will be presented. The discriminatory power of these techniques in diagnosis of obesity will be discussed. In future, these novel approaches will shed light on the internal diagnosis of obesity in clinical application.

Keywords: infrared, spectroscopy, imaging, obesity, adipose tissue, characterization, diagnosis, metabolic disorders

1. Introduction

Biospectroscopy is an analytical multistep process, including sample preparation, spectral acquisition, pre-processing, and computational analysis. Each of these steps plays a critical role for performing experiments accurately in order to obtain reliable results [1]. Since each molecule will have its own unique vibrational characteristics, each molecule possesses a unique IR spectrum. Based on this fact, vibrational spectroscopy has been considered as a golden tool in the characterization of molecular structure. Spectrum as a reflection of molecular structure of sample includes series of peaks/bands with unique characteristic properties such as band position, band width, and band intensity/area. These properties can be used for obtaining functional group information or monitoring molecules in different conditions such as disease states. Disease states are able to induce changes in molecular composition, concentration, structure, and function of biomolecules,

which are directly reflected in the vibrational spectral bands, and therefore, they can be evaluated by using vibrational spectroscopy techniques [2]. Vibrational spectroscopy includes Raman spectroscopy, IR spectroscopy, and Tera Hertz (THz) spectroscopy. IR spectroscopy consists of far, mid, and near IR spectroscopy. Vibrational spectroscopy has been applied widely in biological and medical area. Since, this analytical technique provides qualitative and quantitative information as a rapid, accurate, noninvasive, cost-effective, and operator-independent for identification of the spectral differences arising from pathological or environmental conditions. Here in this chapter, we will discuss the mid and near IR spectroscopy in obesity research.

Obesity is resulted from a chronic imbalance between the level of energy intake and consumption causing extreme weight gain. The prevalence of obesity has increased drastically in recent decades and reached global epidemic dimensions [3, 4]. Unhealthy diet habits, reduced physical activity together with modern lifestyle, urbanization, genetic predisposition, and aging cause an obesity-promoting (obesigenic) environment and contribute to the higher prevalence of these diseases across all age groups [5]. A state of excessive accumulation of body fat results in simultaneous development of a number of metabolic pathologies including insulin resistance, glucose intolerance, diabetes mellitus, hypertension, dyslipidemia, stroke, fatty liver disease, coronary heart diseases, cancer, and metabolic diseases [6–8]. Both genetic and environmental factors contribute to the development of metabolic complications, which result in a significant health and economic burden. Together with excess health care expenditure, obesity also causes decrease in the productivity as a result of lost work days, mortality, and permanent disability [9–11]. Taking into consideration the nonhealth impacts and health risks associated with obesity, there is an urgent need for early diagnosis and treatment of this global burden.

A series of methods have been proposed to define and characterize obesity. Body Mass Index (BMI, also called Quetelet's Index, in kg/m^2) is used widely that is calculated as body weight in kilogram divided by the body height in meters squared. According to this metric, the grading of body fatness can be classified as overweight (BMI: 25–30 kg/m^2), obese (BMI: 30–40 kg/m^2), and morbidly obese (BMI > 40 kg/m^2) [12]. The waist circumference (WC) is also used to monitor central obesity and measured at a level midway between the lowest rib and the iliac crest. The risk of diseases is determined according to cutoff values of 102 cm (40 in) for men and 88 cm (35 in) for females (WHO). Due to its technical problems such as the difficulty in determining bony prominences, this method identifies obesity risk poorly. The dual energy X-ray absorptiometry (DEXA) or magnetic resonance imaging (MRI) have been also used to measure fat pad size and body fat distribution as more reliable techniques [13], however, they are expensive and inaccessible. Other techniques include skinfold thickness, dual energy X-ray absorptiometry, bioelectrical impedance, plethysmography, underwater weighing, computed tomography, and near-IR interactance. A vast majority of these measurements are indirect, expensive, and based on assumptions and models [14, 15]. IR spectroscopy presents the great power in the biomedical research area due to mentioned advantages above, high sensitivity and ease application, Furthermore, IR spectroscopy is concerned as a high throughput technique that is very practical to quantify the alteration of structure, function, composition and distribution, of biologically relevant components in samples.

In this chapter, after mentioning about the basis of IR spectroscopy and microscopy, the sample preparation techniques, spectral analysis methods together with the chemometric tools used in IR spectroscopy, the topic of obesity will be given briefly. Then, how IR spectroscopy can be applied to characterization and diagnosis of obesity will be discussed in detail. Finally, the future direction of the field will be mentioned.

2. Infrared spectroscopy and microscopy in obesity research

2.1 Basis of IR spectroscopy and microscopy

Spectroscopy is based on the interactions between matter (sample analyzed) and energy from the portion of electromagnetic spectrum. IR spectroscopy is one of the most important analytical techniques that almost all type of samples such as liquids, solutions, pastes, powders, gases, and surfaces can all be studied. IR spectrometers have been commercially available since the 1940s [16]. The most significant advance in IR spectroscopy is the introduction of Fourier-transform methodology into the spectrometers. This instrument employs an interferometer and exploits the well-established mathematical process of Fourier-transformation. Fourier-transform infrared (FTIR) spectroscopy allows us to study the samples in its aqueous environment. It has dramatically improved the quality of IR spectra and minimized the time required to obtain data [17]. In addition, with constant improvements in computers, and coupling with microscope to the system, IR spectroscopy has made further great strides. In this section, the basic ideas and definitions associated with IR spectroscopy and microscopy will be described.

2.1.1 IR spectroscopy

IR spectroscopy is a technique based on the study of absorption of IR radiation, which causes transition between vibrational energy states in the molecule. When the applied IR energy is equal to the energy difference between vibrational states, absorption of IR radiation takes place and a peak is observed. If absorbed energy is plotted as a function of wavenumber, an IR spectrum is observed. IR spectroscopy products measure the transition between stretching vibrations (symmetrical and anti-symmetrical types) and bending vibrations. IR spectrum of a chemical substance is a fingerprint of a molecule for its identification.

2.1.2 IR microscopy

IR microscopy, also called as IR microspectroscopy, is a type of light microscopy. It uses a source that transmits IR wavelengths of light to view an image of the sample. An IR microscope has reflective optics that allows the microscope to cover the entire spectral range of IR light. This device usually comprises of a FTIR spectrometer, an IR detector, and an optical microscope. The IR detector may detect IR light at a single point, a linear array or a focal plane array to view different sections of the sample. This allows both spatial and spectral information about the sample structure to be collected. In FTIR microscopy, the microscope sits above the FTIR sampling compartment. Infrared radiation from the spectrometer is focused onto a sample placed on a standard microscope x–y stage. After passing through the sample, the IR beam is collected by an objective that produces an image of the sample within the point of the microscope [18]. A variable aperture is placed in this image plane. After that, another condenser focuses the radiation on MCT detector. Also, there are glass objectives in order to allow visual view of the sample. In addition, the microscope can be converted from transmission mode to reflectance mode by switching mirrors in the optical train.

3. Experimental sampling protocol of IR spectroscopy and microscopy

The sample preparation methods are chosen according to the size, nature, and condition of the specimen. Particular technique/accessory that is used also plays

an important role for sample preparation. Moreover, useful accessories, such as temperature controller systems and the attenuated total reflectance (ATR) mode are available to increase the fields of applications of FTIR spectroscopy and microscopy. Some popular sampling techniques and accessories will be discussed below.

3.1 IR spectroscopy

3.1.1 Transmission IR spectroscopy methods

Transmission spectroscopy is the oldest and most straightforward method that based upon the absorption of IR radiation at specific wavelengths as it passes through a sample, which can be in liquid and solid samples [17]. Protein in solution and membrane studies are only performed using this method [19].

3.1.1.1 Liquid samples

Various techniques are available for sampling of liquids. For example, fixed-path length-sealed cells are useful for volatile liquids, but cannot be taken apart for cleaning. On the other hand, semi-permanent cells are removable so that the windows can easily be cleaned. Usually, the spacer is made of polytetrafluoroethylene (PTFE, Teflon) with a variety of thickness is used. If quantitative analysis of a sample is required, it is necessary to use a cell of known path length [20, 21]. An important consideration in the choice of IR cells is the type of window material. The material must be transparent to the incident IR radiation and normally alkali halides are used in transmission methods [20, 22, 23]. The cheapest material is sodium chloride (NaCl), but other commonly used materials are potassium bromide (KBr), calcium fluoride (CaF_2) [24], and barium fluoride (BaF_2). A drop of liquid is sandwiched, using suitable spacer, between two IR windows that are then mounted in a cell holder. Before producing an IR sample in solution, a suitable solvent must be chosen. The other factors while choosing solvent are that it should be as nonpolar as possible to minimize solute–solvent interactions; and it should not strongly absorb IR radiation.

3.1.1.2 Solid samples

Alkali halide disk method is one of the most favorable methods to examine solid samples. In order to apply this technique, a solid sample is mixed with a dry alkali halide powder. Then, mixture is ground with an agate mortar and subjected to a pressure of 10 ton in^{-2} (1.575×10^5 kg m^{-2}) in an evacuated die. This sinters the mixture and produces clear transparent disks. The most commonly used alkali halide is KBr, which is completely transparent in the MID-IR region [18]. The ratio of the sample to alkali halide is important; generally, sample to halide ratio is chosen as 1:100. The disk thickness is important; thin disks are fragile and difficult to handle, while thick disks transmit too little radiation. If the crystal size of the sample is too large, excessive scattering of radiation results, especially at high wavenumbers (this is known as the Christiansen effect) [25]. In that case, further grinding is required. Drying process is applied to remove the bulk water from the samples. However, bound water or inter-bilayer water still exists in the biological systems [26]. The presence of bound water molecules is not only required for the structural stability of the lipid bilayer [27], but also to control the dynamics and structure of lipid bilayer phases [28] and proteins [29, 30]. In KBr pellet technique, since the system is not exactly in its natural aqueous environment, some alterations in macromolecular structure may occur. Therefore, only relative information can be deduced. The KBr

pellet technique has been extensively used to study a variety of biological systems in dry stated [31, 32].

3.1.2 Reflectance infrared spectroscopy method

ATR spectroscopy utilizes the phenomenon of total internal reflection. When a beam of radiation enters a crystal, if the angle of incidence at the interface between the sample and crystal is greater than the critical angle, incident radiation will undergo total internal reflection [33]. The crystals used in ATR cells are made from materials that have low solubility in water and are of a very high refractive index. Such materials include Diamond, zinc selenide (ZnSe), germanium (Ge), silicon (Si), and thallium/iodide.

Besides aqueous samples, dry samples, homogeneous soft tissues, or hard tissues can also be studied with the ATR–IR spectroscopy by directly placing a small portion of the sample on the crystal and then applying the same pressure to all the samples. The applied pressure should allow until the strongest spectral bands to appear with an intensity extending beyond 70% T, and care should be given to provide uniform contact of the sample with crystal. ATR is accepted to require little or no sample preparation, and is one of the most versatile sampling techniques.

3.2 IR microscopy

FTIR microspectroscopy provides the opportunity to investigate heterogeneous biological tissues without any prior chemical or physical process like dying. In recent years, there have been considerable advances in FTIR microscopy by reaching sample size at the order of microns. During sample preparation for FTIR microspectroscopy, tissue sections can either be directly obtained using a cryotome or be initially embedded into different kinds of embedding media and then cryo-sectioned. Different kinds of embedding media can be used for soft and hard tissues. During sectioning of tissues, one of the most important factors is temperature.

3.2.1 Soft tissues

The infiltration and embedding medium for soft tissues is paraffin. However, for the evaluation of certain proteins or other cellular components, routine formaldehyde fixation and paraffin-based histology are not suitable. In these situations, sections should be embedded in optimum cutting temperature (OCT) medium and cryo-sectioned to retain the most sensitive and undisrupted insight into tissue architecture. Bubble formation should be avoided while applying OCT media. After embedding in a material, generally, 6–10 micron tissue sections are obtained [34, 35]. Finally, tissue samples are thaw-mounted on IR-transparent CaF_2 or BaF_2 windows for IR mapping and data processing [36].

3.2.2 Hard tissues

Hard tissues are first exposed to different concentrations of ethanol, methyl methacrylate, and benzoyl peroxide for fixation and infiltration, and then are embedded into polyethylene methyl methacrylate (PMMA) along with a polymerization accelerator [37]. After the embedding procedure, 2-micron tissue sections are obtained with a cryotome [38, 39] and tissue samples are thaw-mounted on IR-transparent CaF_2 or BaF_2 windows for IR mapping and data processing.

4. Data analysis of IR spectroscopy and microscopy

4.1 Preprocessing methods

Preprocessing is essential to extract the relevant chemical information by applying chemometric techniques to remove baseline deviations, to normalize sample to the same thickness/quantity of material, and to reduce noise or contaminants. It is also useful to sort out bad quality spectra (spectra showing signs of saturation, spectra that are too weak, spectra that are too distorted) and remove outliers. It is applied before data analysis but should be kept minimum not to introduce some artifacts and should performed in the right order. First, visual inspection of the spectra should be done to remove bad quality spectra. If there is a distortion in the spectral bands that may be encountered in cell studies, Mie scattering correction should be performed. Then, smoothing is applied if necessary and baseline correction is applied before normalization and derivatives [1].

Multiple methods exist to perform baseline correction. Offset correction can be applied by putting the lowest point to zero and linear baseline correction can be applied by subtracting a linear baseline as shown in **Figure 1** or polynomial baseline correction may be preferred by subtracting a polynomial baseline of required order.

Normalization is the mathematical function to set all the spectra to the same norm and scale. It is mandatory to correct differences in spectral intensity that arises from heterogeneity in sample thickness or inaccuracies in positioning. It should be performed before any scaling or other preprocessing steps, but after baseline correction. Common normalization methods are min-max and vector normalization. In min-max normalization, the minimum and the maximum intensities of the whole spectrum are scaled to zero and one, respectively. In vector normalization, the sum of all intensity values squared of normalized mean-centered spectrum is equal to 1. Derivatives are used for finding precise peak positions, which are mostly computed by a Savitzky-Golay filter. There are several orders of derivative; first order is according to slope of the peak and second order is according to curvature radius. Smoothing is used to reduce noise in data. However, smoothing also induces distortion in peak shape and may erase small peaks and shoulders [40].

4.2 Data analysis

The band positions and bandwidths of the spectral bands are measured from the center of weight (0.80× peak height position). The position of the spectral bands
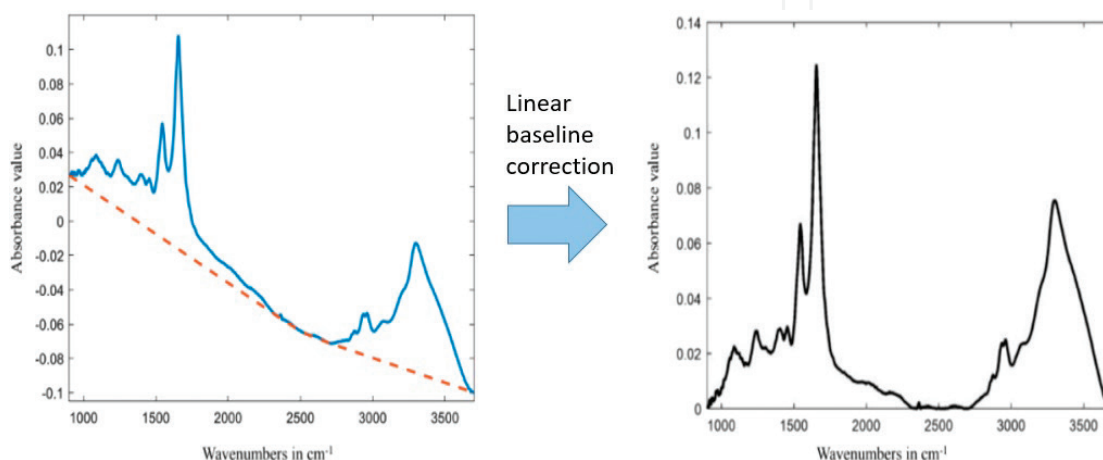
Figure 1.
Representation of a spectrum before (left) and after (right) linear baseline correction.

allows us to do band assignments. The bandwidth of the CH_2 stretching bands gives lipid fluidity information. The shift in the positions of the bands gives structural information. For example, the shift in the position of CH_2 symmetric and anti-symmetric bands monitors lipid order/disorder, which gives information about the flexibility of the lipid acyl chains [20, 24]. The shift in the position of the C=O stretching and the PO_2^- anti-symmetric double stretching bands gives information about hydration status of these functional groups [20, 24]. The shift in the position of the amide I band and ratio of the amide I to amide II give information about the variations in protein secondary structure [22]. According to Beer–Lambert law, the signal intensity or more accurately the area under the bands gives concentration information. In order to remove any artifact that arise from the thickness of the sample, generally in cell and tissue studies, area ratios of the specific bands are used [41, 42]. In membrane studies, since the OH stretching bands (3400–3200 cm^{-1} and 1800–1500 cm^{-1}) due to the buffer, these bands overlap with the bands of interest. Therefore, the spectrum of the buffer is subtracted from the spectrum of liposomes at corresponding temperatures. The subtraction process is performed until the bulk water located around 2100 cm^{-1} is flattened using the spectrometer's software program. The detailed analyses are performed from the subtracted native spectra.

4.3 Chemometrics

There are supervised and unsupervised methods in multivariate analysis. Compared to unsupervised methods (simple clustering methods), supervised methods (classification and identification methods) can be used to train a model to create classes or in order to predict the class of unknown samples. Because they are supervised methods, there is a need for cross-validation and independent validation dataset. Since there is no application of supervised methods to obesity yet, only unsupervised methods will be discussed here.

4.3.1 Principle component analysis (PCA)

PCA is an unsupervised analysis method based on the decomposition of variance in a new multidimensional space. It is a well-known technique for reducing the dimensionality of multivariate data while preserving most of the variance [43]. This technique can reduce a large scale of spectra to a point in a space by using linear transformation. PCA can be used to define groups based on spectral similarity/variance as classification method. PCA can cluster spectra by similarity, detects outliers, finds correlations between variables and can decorrelate different sources of variability as a data exploration method. Each PC describes the spectral variability among samples in decreasing order. Thus, the first principle component, i.e., PC1, expresses most of the variance in the data; PC2 expresses the second largest variance in the data and so on [44]. Furthermore, PCA is capable to obtain loadings plots to identify the main contributory variables (i.e., wavenumbers) that determine the discriminating wavenumbers throughout the IR spectrum.

4.3.2. Hierarchical cluster analysis (HCA)

Among the different methods used in cluster analysis, generally Ward's algorithm together with Euclidian distances is used to construct the dendrograms, which gives one of the best predictions [45]. In this method, the two spectra with the highest similarity (i.e., spectra with the smallest spectral distance) are merged

into a cluster and then the distances between this cluster and all other spectra are calculated. This process continues until the two spectra (spectrum/spectrum or spectrum/cluster or cluster/cluster) are merged into a new cluster. This procedure is repeated until only one big cluster is left. Different from other methods, Ward's algorithm tries to find as homogeneous groups as possible. By the way, HCA builds the hierarchical tree of dissimilarity between data points that is called dendrogram. Well-known parameters of clinical studies such as sensitivity and specificity can be used to describe the performance of the methods in disease diagnosis. Explanation of sensitivity, specificity, positive (true and false), and negative (true and false) values can be found in Severcan et al. [22].

5. Obesity

Stability of body fat is key point to maintain metabolic homeostasis. Excess amount of energy is stored in the form of triglycerides in the lipid droplets of adipocytes and result in the increase in the number of adipocyte (hyperplasia) or enlargement in the size of adipocytes (hypertrophy). Adipose tissue depots are classified into white adipose tissue (WAT) and brown adipose tissue (BAT), each of them has unique structure and function. They differ in their degree of vascularization and innervation. BAT is rich in protein content and mitochondria than that of WAT. Brown adipose tissue (BAT) is highly specialized in thermogenesis and maintaining body temperature [46]. WAT is involved in the regulation of energy homeostasis, body thermal insulation and present active endocrine organ functions via production and secretion of adipokines such as leptin, adiponectin, IL6, IL10, TNFα, etc. Both tissues are capable to store triglycerides [47]. White adipose tissue has been organized in different anatomical location as visceral white adipose tissue (VAT) and subcutaneous white adipose tissue (SCAT). VAT has higher amount of plasminogen activator inhibitor-1 (PAI-1), IL6 and higher expression of glucocorticoid, androgen, AT_1 and beta-3 adrenergic receptor than in the subcutaneous tissue. The products of VAT are driven to the liver via portal vein system, affecting its function. SCAT possesses higher concentrations of leptin and adiponectin. The secretions of SCAT are released into the general circulation [48]. Therefore, WAT paves the way for different metabolic diseases and presents great detrimental effect to human health [49, 50].

Adipose tissue responds to excess triglycerides accumulation by inducing an immune response. Excessive fat accumulation results in changes in cellular and structural remodeling processes and causes alterations in the endocrine and metabolic systems. This inflammatory state is associated with increased accumulation of macrophages in adipose tissues along with the production of inflammatory cytokines and their secretion into the circulation (e.g., adiponectin, leptin, resistin, IL6, IL10, TNFα, macrophage migration inhibitory factor [51], and components of the renin-angiotensin system [52]). These cytokines mediate inflammation or play an active role in immune and inflammatory responses to obesity. Increased circulating levels of inflammatory markers are associated with obesity-related pathologies. For example, TNFα and IL6 contribute to the induction of insulin resistance [53] and are associated with increased glucose levels and altered lipid metabolism [54, 55]. Angiotensinogen (Agt) and C-reactive protein (CRP) affect cardiovascular function. Plasminogen activator inhibitor 1 (PAI1) causes impaired fibrinolysis and promotes atherosclerosis [56, 57]. These associations are affected by adipose tissue distributions. In the literature, visceral adipose tissue has been implicated as a major risk factor for insulin resistance, type 2 diabetes [48, 58], cardiovascular disease [59], stroke [60], and metabolic syndrome [61]. Therefore, visceral fat is thought to be the more dangerous

adipose tissue. The liver is the major organ of lipid metabolism and can store lipids, which could lead to fatty liver syndrome. Fatty liver, itself, stimulates a local subacute inflammatory state by production of inflammatory mediators such as TNFα or IL-6 that contribute to hepatic and systemic insulin resistance [62].

Obesity-induced inflammation is emerged as a result of change in the cytokine profile, in particular, decrease in adiponectin and increase in leptin levels, mitochondrial dysfunction, accelerated adipocyte death and endoplasmic reticulum (ER) stress. Specific signaling pathways, such as Jun N-terminal kinase, IκB kinase β/nuclear factor κ-light-chain-enhancer of activated B cells, inositol requiring kinase 1, pancreatic ER kinase, activating transcription factor 6, etc., also are contributed to inflammatory state [63]. Endoplasmic reticulum is a major site for protein and for lipid synthesis. In the ER, proteins are folded into their native confirmation and undergo posttranslational modifications, which are important for their structure and activity. In the case of nutrition overload, function of ER is impaired, protein folding is disturbed, ER stress develops and activates a signaling network called the unfolded protein response (UPR) to restore ER homeostasis [64, 65]. It is known that ER stress is linked with obesity-related pathologies including insulin resistance, type 2 diabetes, hypertension, and nonalcoholic fatty liver disease [5, 65, 66]. Consequently, adipose tissue serves as a main site for activation of the inflammatory response in obesity.

6. Application of IR spectroscopy and microscopy in obesity research

Biological samples contain biochemical substances such as lipids, proteins, carbohydrates, proteins, and nucleic acids, and these biomolecules have their unique vibrational fingerprints. Therefore, disease-induced changes in the position, bandwidth, signal intensity, and area of the spectral bands of the system of interest, can be monitored by IR spectroscopy. In current chapter, the applications of IR (mid and near), spectroscopic and microspectroscopic techniques in obesity research will be discussed in detail.

Recent studies pointed out the importance of adipose tissue in diagnosis and treatment of obesity and obesity related diseases. Therefore, adipose tissues draw attraction in obesity research. **Figure 2** shows a representative IR spectrum of a human adipose tissue. This spectrum is quite complex and contains several bands, which arise from different functional groups belonging to biomolecules of the system. The relevant band assignments of the bands are given in **Table 1** [67, 68].

It is known that obesity constitutively results in storage of triglycerides in different adipose tissues [2]. A recent article discussed the potential of ATR-FTIR spectroscopy to monitor obesity from the triglyceride spectral band located around 1730 cm^{-1} [43]. In this study, hierarchical cluster analysis (HCA) and principal component analysis (PCA) were applied to the spectra of triglyceride bands of subcutaneous (SCAT) and visceral (VAT) adipose tissues. The adipose tissue samples were obtained from 10 weeks old male control (DBA/2 J) (n = 6) and four different obese BFMI mice lines (n = 6 per group). As seen from **Figure 3**, successful discrimination of the obese, obesity-related insulin resistant and control groups was achieved with high sensitivity and specificity. Based on the spectral differences, the results revealed the power of FTIR spectroscopy coupled with chemometric in internal diagnosis of abdominal obesity. This work also indicated that SCAT and VAT were affected from obesity at different rates and ATR-FTIR spectroscopy coupled with chemometric analysis could sensitively differentiate these effects of obesity in adipose tissues. In conclusion, the VAT and especially, the SCAT samples can be easily examined by ATR-FTIR spectroscopy coupled with
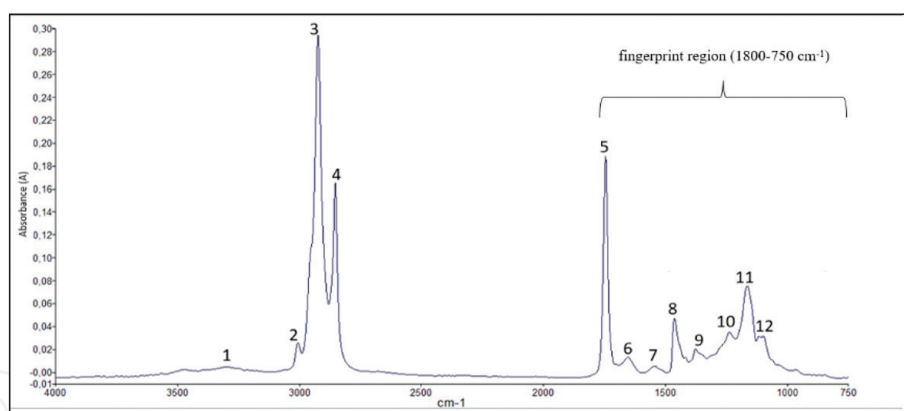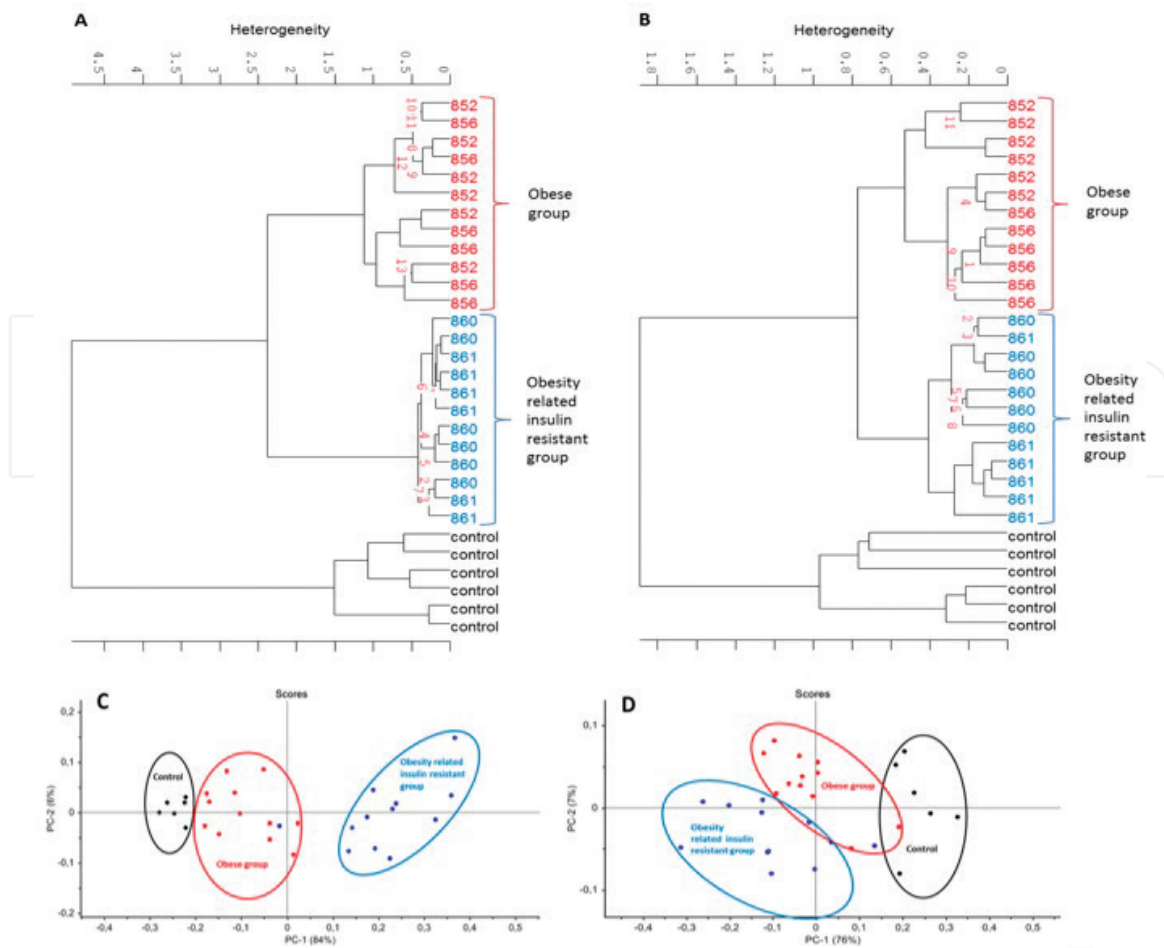
Figure 2.
A representative baseline corrected normalized FTIR spectrum of a human adipose tissue in the 4000–750 cm^{-1} region.

chemometrics in human studies and this combined technique will shed light on the internal diagnosis of obesity in medical research [43].

In the other study of the same group, lipid profiles in terms of the content and structure of skeletal muscle and adipose tissues were determined to understand better the characteristics of juvenile-onset spontaneous obesity without high fat diet induction. Biomolecular content, concentration, and structure were determined by ATR-FTIR spectroscopy. The muscle (longissimus, quadriceps) and adipose (inguinal, gonadal) tissues of 10-week-old male DBA/2 J and Berlin Fat Mouse inbred (BFMI) lines (BFMI856, BFMI860, BFMI861) fed with a standard breeding diet were used. Spectral differences indicated differences in lipid structure and content of BFMI lines, which may originate from different insulin

| Band number	Band position (cm^{-1})	Definition of band assignment	References
1	3290	N–H and O–H symmetric stretching: mainly N–H stretching (amide A) of proteins with the little contribution from O–H stretching of polysaccharides, carbohydrates and water	[67]
2	3006	Olefinic = CH stretching vibration: unsaturated lipids, cholesterol esters	[68]
3	2924	CH$_2$ anti-symmetric stretching: mainly lipids, with some contribution from proteins, carbohydrates, nucleic acids	[68]
4	2854	CH$_2$ symmetric stretching: mainly lipids, with some contribution from proteins, carbohydrates, nucleic acids	[68]
5	1744	Carbonyl C–O stretch: triglycerides	[68]
6	1654	Amide I (protein C–O stretching)	[67]
7	1547	Amide II (protein N–H bend, C–N stretch)	[67]
8	1469	CH$_2$ bending of the acyl chains of lipids	[67]
9	1375	C–N Stretching	[67]
10	1238	Asymmetric PO$_2$ stretching	[67]
11	1164	C–O stretching (in normal tissue)	[67]
12	1100	Stretching PO$_2$ symmetric (phosphate II)	[67]

Table 1.
General band assignment of the FTIR spectrum of an adipose tissue.

Figure 3.
Hierarchical clustering of control, obese and obesity related insulin resistant groups in SCAT (A) and VAT (B) in the 1770–1720 cm^{-1} spectral region. Control group (black), obese group (BFMI 852–856) (red), obesity related insulin resistant group (BFMI 860–861) (blue). PC1 versus PC2 scores plot of the second derivative vector normalized spectra in the same range of SCAT (C) and VAT (D). Control group (black), obese group (BFMI 852–856) (red), obesity related insulin resistant group (BFMI 860–861) (blue).

sensitivity levels of the lines. This makes them promising animal models for spontaneous obesity. The referred study will shed light to the understanding of the generation of insulin resistance in obesity without high fat diet induction [69]. A recent FTIR microspectroscopic study of the same group aimed to characterize and compare VAT and SCAT in terms of macromolecular content and investigate transdifferentiation between white and brown adipocytes [34]. In order to achieve this, the gonadal (visceral) and inguinal (subcutaneous) adipose tissues of male BFMI lines, which are spontaneously obese were studied. The prominent increase in the lipid/protein ratio (**Figure 4**), accompanied with a decrease of UCP1 protein content might be due to the transdifferentiation of brown adipocytes to white adipocytes in obese groups. When the amount of BAT was decreased, lower unsaturation/saturation ratio, qualitatively longer hydrocarbon acyl chain length of lipids and higher amount of triglycerides were obtained in both adipose tissues of mice lines as seen in **Figure 4**. The results also showed that SCAT was more prone to obesity-induced structural changes than VAT. This could originate from it possessing a lower amount of brown adipose tissue. The current study clearly revealed the power of FTIR microspectroscopy in the precise determination of obesity-induced structural and functional changes in inguinal and gonadal adipose tissue of mice lines [34].

In another obesity research [68], which targets to identify specific gene loci, regulating obesity in BXD recombinant inbred (RI) strains of mice, ATR-FTIR
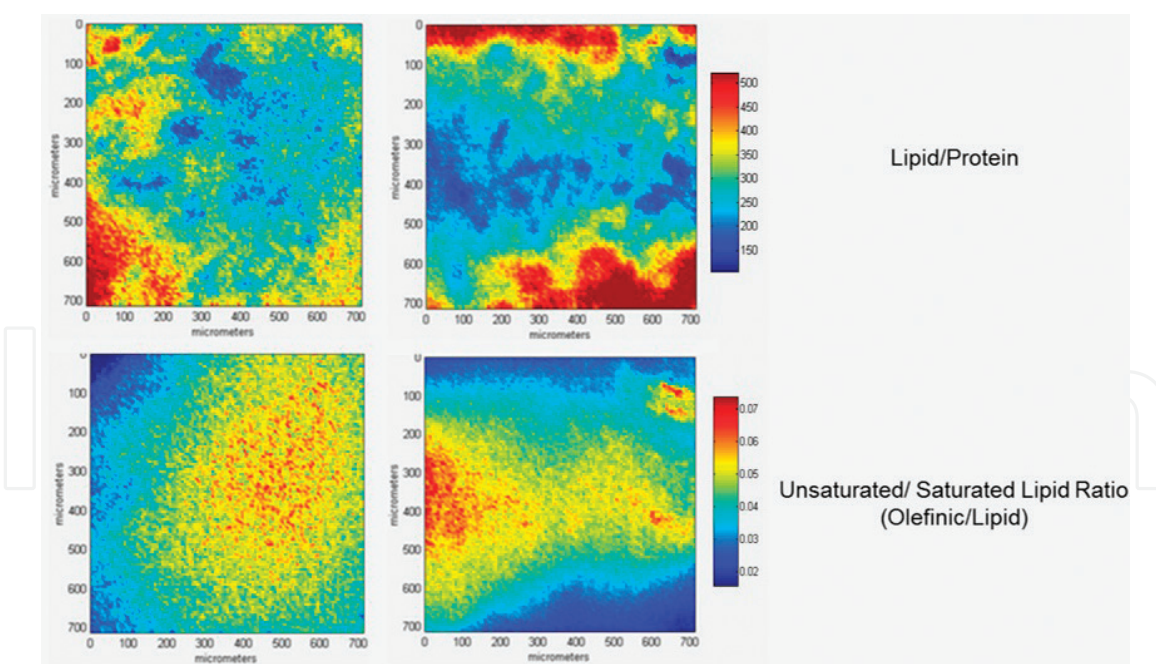
Figure 4.
The representative spectral maps of lipid/protein and olefinic/protein ratios belong to the inguinal adipose tissue of control (left side) and obese (right side) male mice lines. The absorbance in the spectral maps was represented in color-coded images, where low absorption was represented in blue and high absorption was represented in red color. (Adapted from [34], with permission from Royal Society of Chemistry).

spectroscopy was performed as a novel phenotyping tool. The epididymal adipose tissue, liver, and muscle of 29 BXD recombinant inbred mouse strains fed with high fat diet were used to analyze the role of white adipose tissue as a mediator of inflammation. The content of total fat, unsaturated fat, lipid to protein ratio, and collagen and collagen integrity were measured in tissue of interest and the results of IR analysis were revealed differences in the biomolecular composition of adipose and liver tissues among high fat diet-fed BXD RI strains that reflect genetic variation. Rsad2, which may modulate lipid droplet contents and lipid biosynthesis, and Colec11, which may play a role in apoptotic cell clearance and maintenance of adipose tissue were suggested as potential quantitative trait candidate genes. This study showed the efficiency and suitability of ATR-FTIR spectroscopy to identify quantitative trait loci (QTLs) that influence various traits and heightened the power of gene mapping studies.

Bortolotto et al. [70] determined the total content of trans fatty acids (TFA) in subcutaneous, retroperitoneal, and visceral fat of morbidly obese and nonobese patients subjected to bariatric surgery or plastic and abdominal surgery. TFA were measured by FTIR-ATR spectroscopy. TFA content in all adipose tissues analyzed was higher than reported in other countries. They showed more TFA in VAT than in other abdominal fat (subcutaneous and retroperitoneal) stores. The TFA depot in visceral fat was higher than other fatty tissues for morbidly obese and nonobese patients. Gazi et al. [71] demonstrated lipid-specific translocation between adipocytes and tumor cells and the use of FTIR microspectroscopy to characterize various biomolecular features of a single adipocyte without the requirement for cell isolation and lipid extraction. Stimulating increased thermogenic activity in adipose tissue is an important biological target for obesity treatment. Aboualizadeh et al. [72] investigated the biochemical changes caused by cold exposure using FTIR imaging technique in the brown and subcutaneous white adipose tissues (BAT and s-WAT) of 6-week-old C57BL6 mice exposed to different temperatures for 10 days. Protein to lipid ratio, calculated from the ratio of the integrated area from 1600 to 1700 cm^{-1} (amide I) to the integrated area from 2830 to 2980 cm^{-1} (saturated

lipids), was increased as temperature decreased. The degree of unsaturation was obtained from the ratio of the integrated area of unsaturated lipids (2992–3020 cm^{-1}) to the integrated area of saturated lipids (2830–2980 cm^{-1}), showed stepwise decreases going from colder-exposed to warmer-exposed BAT. Complementary H-1 NMR measurements confirmed the findings from this ratio in BAT. PCA analysis successfully operated to groups. This study revealed that, FTIR imaging is a promising technique to quantify cold-induced biochemical changes in BAT and s-WAT in a label-free manner [72].

Nonalcoholic fatty liver disease (NAFLD) is a frequent lesion associated with obesity, diabetes, and the metabolic syndrome. The potential of mid-IR fiber evanescent wave spectroscopy was used as a minimum-invasive method for evaluating the liver status during NAFLD. In the study, 75 mice were subjected to a control, high-fat or high-fat-high carbohydrate diets. The mid-IR spectra were acquired from serum, and then analyzed to develop a predictive model of the steatosis level. Animals subjected to enriched diets were obese. The relationship between the spectroscopy-predicted and observed levels of steatosis, expressed as percentages of the liver biopsy area, was not linear. The data suggest that mid-IR spectroscopy combined with statistical analysis allows identifying serum mid-IR signatures that reflect the liver status during NAFLD [73]. In another work [74], the potential of FTIR microspectroscopy for grading steatosis on frozen tissue sections was evaluated. The use of the bright IR source emitted by synchrotron radiation (SR) allowed the investigation of the biochemical composition at cellular level. PCA method was applied to spectral data. The results showed that the progression of steatosis corresponds not only to the accumulation of lipids but also to significant changes in the qualitative composition of the tissue. Indeed, as the grade of steatosis was lowered, a decrease in glycogen content with a concomitant increase in lipids was observed in comparison to normal liver. Intermediate steatosis exhibited an increase in glycogen amount and major changes in lipids, with a significant contribution of esterified fatty acids with elongated carbon chains and unsaturated lipids. These features were more pronounced in a high grade of steatosis. The dramatic biochemical changes occurred in the nonsteatotic part of the tissue, suggested that the whole tissue reflects the grade of steatosis [74].

Obesity might alter the electric activity in brain areas triggering appetite and craving. In a recent study, electrical activity was induced by transcranial direct current brain stimulation (tDCS). FTIR spectroscopy and synchrotron X-ray fluorescence (SRXRF) microprobe techniques show that brain areas implicated appetite control, upon experimental treatment by either anodal-type tDCS (atDCS) or cathodal-type tDCS (ctDCS). The C-H stretching, amide I-IV, and PO_4^{2-} anti-symmetric stretching regions were investigated. The most dramatic lipid-related changes were found for $\gamma_{as}(CH_2)$ at 2930 cm^{-1}, whereas the protein secondary changes occurred at the amide I band, for α–helix and β-sheet located at 1660 and 1630 cm^{-1}, respectively. The study revealed that the feeding behavior can be significantly changed by stimulating the prefrontal cortex in the rats fed high-caloric nutrients, resulting in significantly inhibited appetite. Both, atDCS and ctDCS produced significant molecular changes involving qualitative and structural properties of lipids, whereas atDCS induced more significant effect on protein secondary structure in all the brain areas investigated [75].

Alterations in dietary fatty acid composition can influence the regulation of metabolism in adipose tissue. Bizeau et al. [76] studied the effect of dietary fatty acid composition on lipolysis. The rats were fed with diets containing menhaden oil (MO) or coconut oil (CO) for 4 weeks. FTIR spectroscopy was used to examine the effect of altered plasma membrane fatty acid composition on membrane physical properties. The results of this study suggested that membrane order is not responsible for the lower rate of lipolysis in animals fed

coconut oil when both variables are measured at $37°$C. In another recent study, rats fed with a high-fat diet were treated with resistant starch (RS), chitosan oligosaccharide (COS), and a combination of these complexes with the aim of determining their effect on controlling blood glucose levels and improving insulin resistance. Cross-linking between RS and COS was confirmed by FTIR spectroscopy analysis. For COS analysis, in IR spectroscopy, the absorption peak at 3416.33 cm^{-1} belonged to O-H and N-H stretching vibrations, 2922.80 cm^{-1} corresponding to a C-H stretching vibration, and 1566.70 cm^{-1} originated from -NH$_2$ absorption peak were used. The RS spectra showed an absorption band at 3416.50 cm^{-1}, (O-H stretching) and 2926.85 cm^{-1} by C-H stretching vibration. A new absorption peak at 1560.88 cm^{-1} appeared in the COS-RS complex spectra, and the shape of typical absorption peaks for starch from 1180 to 953 cm^{-1} also changed. The results suggested that the consumption of COS-RS complexes exerted a more efficient recovery of insulin sensitivity compared to individual treatments [77]. Herbal supplements are currently available as a safer alternative to manage obesity. Many chemical drugs on the market are designed to prevent or manage obesity whose high cost, low efficacy, and multiple side effects limit their use. El-Menshawe et al. [78] evaluated nano lipo-vesicles phytosomal thermogel of Soybean [*Glycine max* (L.) Merrill], for its anti-obesity action on body weight gain, adipose tissue size, and lipid profile data by FTIR spectroscopy. Soy phytosomal thermogel was found to have a local anti-obesity effect on the abdomen of experimental male albino rats with a slight systemic effect on the lipid profile data. In another study, FTIR spectroscopy was used to determine physio-chemical properties of pectin extracted from the apple pomace that was proposed to inhibit pancreatic lipase (steapsin). The study showed the extracted pectin has potential use in the anti-obesity formulations [79]. Dunkhunthod et al. [80] used FTIR microspectroscopy coupled with chemometrics to monitor and discriminate biomolecular changes due to the anti-adipogenesis effect of baicalein on adipogenesis in 3 T3-L1 cells. The signal intensity and the integrated areas of glycogen and carbohydrate of baicalein-treated 313-L1 adipocytes were found to be significantly less than the untreated 3 T3-L1 adipocytes. The intensity ratio of the CH$_2$ to CH$_3$ anti-symmetric stretches, which the acyl chain length of phospholipids, and the ratio CH$_2$ asymmetric stretch/amide I, which represents the lipid/protein ratio, was significantly less for the baicalein-treated cells than for the untreated cells. The findings provided evidence for the development of IR biochemical obesity markers and showed the potential of FTIR microspectroscopy in the evaluation of the effectiveness of the drug in the treatment of obese patients [33].

7. Conclusion and future directions

FTIR spectroscopy is a rapid, very sensitive, cost-effective, and easy-use technique. Especially in ATR mode, samples are directly put on the crystal without any sample preparation step. In addition, results are not dependent on the operator but to computational analyses of the spectra. This property gives an important advantage to this technique. Furthermore, FTIR spectra may give information about the disease-induced changes at very early stage, earlier than they become visible. That means IR spectroscopy has the capacity of analysing the biological systems as reagent free, operator independent, diagnosis of diseases at very early stage. It also allows the detection of lower concentrations of biological components ranging from biomolecules, membranes, cells, tissues, and body fluids. With these state-of-the-art technology, IR spectroscopy coupled with chemometric tools have taken part in the field

of biomedicine especially in determination of disease-induced spectral biomarkers, disease diagnosis, and treatment-oriented monitoring in clinical investigations.

The IR spectroscopy results of the animal studies on adipose tissues can be transferred to medical research to be used in the diagnosis of obesity. Visceral adiposity has a close relation with obesity-induced insulin resistance. This effect of obesity on the VAT highly correlates with the SCAT. Since the SCAT is more accessible than the VAT for medical interventions, this type of adipose tissue can be used preferably in biopsies and bariatric operations [43]. In recent years, since BAT have been proposed as a potential therapeutic target for obesity and related metabolic diseases, targeting BAT, thermogenesis and monitoring BAT metabolism might hold a possible therapeutic potential for these metabolic diseases. Recent innovations in spectroscopy and microspectroscopy contributed to evaluation, characterization, and differentiation of the obesity. However, application of IR spectroscopy to obesity research is quite limited so far. There are several issues in obesity area that need to be clarified. For example, the mechanism of the protective role of fatty acids, the effect of macrophage localization and origin to induce inflammation in adipose tissue and ER stress induced response of different organs such as liver, adipose tissue, anti-obesity effect of diets, therapeutic effect of drug, structural characterization of obese human membranes, tissues and organs are still not exactly known and further researches are required. In terms of structural studies, there is no information yet in the literature about obesity-induced protein structural changes. Furthermore, supervised analysis methods have not been applied to IR spectra yet for the obesity differentiation. Promising new experimental tools and models will help for elucidating remaining questions related to obesity and may be critical for the development of the new therapeutic strategies.

Conflicts of interest

The authors report no financial conflicts of interest. The authors are only responsible for the content and writing of this chapter.

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