ESSENTIAL TECHNIQUES FOR MEDICAL AND LIFE SCIENTISTS: A GUIDE TO CONTEMPORARY METHODS AND CURRENT APPLICATIONS WITH THE PROTOCOLS

PART 2

Editor:
Yusuf Tutar

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Essential Techniques for Medical and Life Scientists: A Guide to Contemporary Methods and Current Applications- Part II

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Foreword

Molecular level understanding of biochemical mechanism requires a thorough knowledge of interdisciplinary sciences. Biomedical applications have been supported by scientific hypothesis for years and innovations have helped scientists to make contemporary research through these cutting-edge technologies. Further, these technologies not only overlap in several aspects but complement each other, supporting and highlights molecular level understanding of biochemical pathways.

Clinical research uses instrumental techniques that have never been used before multiple factors affect pathology and etiology of diseases. For this reason, several instrumental techniques have been emerging and basis of these methods originate from current methodologies. This means that developing instrumentation merges novel scientific data with basic spectroscopic-calorimetric technologies. Therefore, the second volume of this book will help current and future applications of medical and biological data.

As the first volume, the second part of the book provides a training platform and application modules of each technique and may help individual investigators to guide research practices.

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PREFACE

This book is the second volume of "Essential Techniques for Medical and Life Scientists: A guide to contemporary methods and current applications with the protocols". This volume focuses on imaging chemical identification methods, and emission spectroscopy along with a calorimetric method and their applications in medicine - biological sciences.

Chapter 1 explains FTIR and its acquisition of metabolic fingerprint of cells, tissues and biofluids. The method is rapid, simple, and economical, requiring label-free molecules and then sensitive detection.

Chapter 2 describes a common instrument of life science laboratory; High Performance Liquid Chromatography (HPLC). This separation method offers a wide variety of options for purification. Methodology and applications of the technique are explained thoroughly in this chapter.

Chapter 3 is a complementary technique to FTIR. Raman spectroscopy is a molecular spectroscopy and the basis of the method involves scattering of electromagnetic radiation by atoms or molecules. Raman provides fingerprint of an unknown chemical compound.

Chapter 4 explains a unique technique, Circular Dichroism. It is unique since the technique not only measures binding affinity but it also provides secondary structure composition percentage. The chapter further explains conformational analysis upon ligand binding and protein stability.

Chapter 5 and 6 discuss Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy, (SEM) respectively. Microscopy enables direct visualization of macromolecules, cells, organelles, tissues, and organisms. Structure-function relationship can be assigned between macro and/or microscopic structure.

Chapter 7 is based on energy-dispersive x-ray analysis (EDX) technique which is useful in drugs and drug delivery research. SEM-EDX also detects nanoparticles which are generally used to improve therapeutic performance of chemotherapeutic agents. This work elaborates applications of SEM-EDX over compounds in medicinal plants, herbs, and shrubs.

This book is designed not only for early career young scientists (graduate students or postdoctoral associates) but also for scientists who are experts in a particular technique and want to use distinct applications for their experimental set-up. The next volume of the book will provide chapters for different analytical techniques.

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CHAPTER 1

Fourier-Transform Mid-Infrared (FT-MIR) Spectroscopy in Biomedicine

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Abstract: Fourier-transform mid-infrared (FT-MIR) spectroscopy is a powerful technique that probes intramolecular vibrations of almost any molecule, enabling the acquisition of metabolic fingerprint of cells, tissues and biofluids (*e.g.* serum, urine and saliva, *etc.*), in a rapid (in minutes), simple (without or with minimum sample processing), economic (without consumption of reagents), label-free and highly sensitive and specific mode. Due to the flexibility of the technique, there are diverse modes of spectra acquisition, from classical transmission and transflection, to high-throughput measurements using micro-plates in transmission mode, to fiber optic probes coupled to Attenuated Total Reflection (ATR) detection, enabling *in situ* analysis, throughout micro-spectroscopy, with spatial resolution, enabling detection of residual analytes and imaging at the sub-cellular level. Due to the composition complexity of biological samples, the mid-infrared spectra are usually very difficult to interpret without the application of complex and sophisticated mathematical and statistical analysis routines, such as: spectra pre-processing methods to minimize noise and other non-informative data that compromise subsequent pattern recognition models; deconvolution methods to resolve overlapped spectral bands; methods to decrease data dimension and features extraction; supervised and non-supervised pattern recognition methods as those based on support vector machines and artificial neural networks. The present work reviews the main acquisition modes, pre-processing and multivariate spectral analysis used in FT-MIR spectroscopy, followed by the application of FT-MIR for the diagnosis of a multitude of diseases. FT-MIR spectroscopy constitutes one of the most promising biophysical techniques for analyzing biological samples, and consequently may be used for diseases prognosis, diagnosis and even for personalized treatment.

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Keywords: Biomedical sciences, FT-MIR spectroscopy, Medical diagnosis, MIR spectroscopy.

1. INTRODUCTION

Fourier-transform Infrared Spectroscopy (**FT-IRS**) measures vibration modes of molecular bonds, in solid and liquid samples, resulting in dipole moment changes, *i.e.* differences of charges in the electronic field of atoms, and in gaseous phases in rotational modes. With exception of monoatomic (*e.g.* He, Ne) or homopolar diatomic molecules (*e.g.* H_2, N_2, O_2), almost all molecules present a unique FT-IR spectrum, *i.e.* spectrum with an inherent molecular selectivity and specificity. The molecular composition of cells, their surroundings, organisms' biofluids (*e.g.* serum, blood, saliva, urine, spinal fluid) and, other materials (*e.g.* tissue, calculi, feces, cartilage, bone) reflects the underlying metabolic activity. FT-IRS, especially at the mid-region of the spectra, may acquire the whole molecular fingerprint associated with the organism or the tissue's metabolic state in a highly sensitive and specific mode (Fig. **1**, Table **1**).

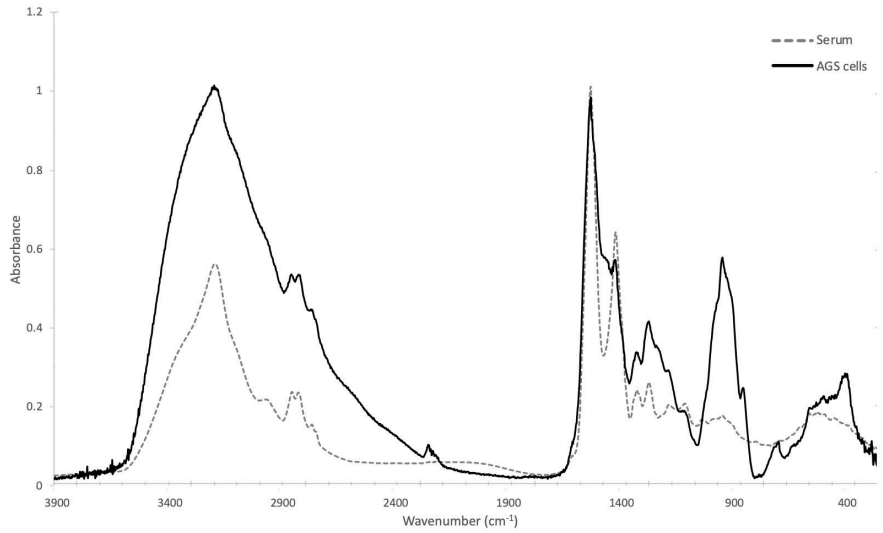

Fig. (1). FT-MIR spectrum of human serum and adenocarcinoma gastric cells.

While the mid-infrared (**MIR**) region of the spectra (2.5-25 μm or 400-4000 cm^{-1}) reflects fundamental vibrations, the near-infrared (**NIR**) region of the spectra (780-2500 nm, or 4000-12821 cm^{-1}) reflects overtones and combinations of vibrations. Therefore, acquisition in the MIR region gives rise to stronger and better-defined absorbance bands compared to NIR, which typically results in weaker and wider spectra. Furthermore, MIR spectra of biological samples are

sensitive to biomolecules with functional groups such as C-C, C=C, C-O, C-N, C-H, O-O, O-P, N-H and O-H bonds, while NIR covers groups exclusively containing the hydrogen atom as C-H, N-H, O-H, and S-H bonds [1, 2]. Consequently, MIR spectra are more informative about the samples' biomolecular composition, and are therefore more appropriate to screen changes in the molecular composition of biological samples, particularly in the following spectral regions: 3600-2000 cm^{-1}, reflecting mainly stretching vibrations between X-H (where X is C, O or N) present in amide A and amide B (~3300 and 3100 cm^{-1}, respectively), and CH_3 (~2960 and ~2872 cm^{-1}) and CH_2 (~2920 and 2850 cm^{-1}) groups of lipids; 1800–1500 cm^{-1}, reflecting mainly stretching vibrations of double bonds (*e.g.* C=O, C=C and C=N), present in amide I (~1655 cm^{-1}) and amide II (~1545 cm^{-1}) of proteins and some secondary structure of proteins, and COOR in phospholipids esters (~1740 cm^{-1}); and 1500-400 cm^{-1}, reflecting a variety of overlapped vibrations due to proteins, lipids, and nucleic acids, designated as fingerprint region [1, 2]. Fig. (**1**) exemplifies FT-MIR spectra obtained from human serum and gastric cells, where Table **1** points out the biochemical significance of major spectral bands.

MIR spectra, in addition to being informative about molecular composition, are also sensitive to the molecules' environment, which may influence vibrations of molecular bonds. Consequently, MIR spectra also reflect conformation changes of biomolecules, as protein folding, *via* vibrational resonance originating from polypeptide backbone or side chains that depends on the protein structure and local interactions such as hydrogen bridges [3, 4], nucleic acids conformation [5 - 7] along with biomembrane organization, fluidity and even biomolecule interactions [8 - 10]. Therefore, MIR spectra present distinctive sensitivity towards biological features, which enables the characterization of metabolic fingerprints of biological samples with high sensitivity and specificity.

Biological samples (*e.g.* cells, tissues or biofluids) present a highly complex molecular composition, ranging from small inorganic and organic molecules to macromolecules, as nucleic acids, polysaccharides, and lipids. MIR spectra result from the complexity of this molecular composition in a highly sensitive and specific mode, which enables monitoring biological processes such as: cell division, differentiation, apoptosis or necrosis, as well as disease progression, prognosis, diagnosis, personalized treatment and even follow-up of drug treatment; some of which will be further discussed in the following sections. The bioassay versatility of this spectroscopic technique can be illustrated by the differences of the second derivative in regions of FT-MIR spectra obtained from adenocarcinoma gastric cells infected or non-infected with *Helicobacter pylori* (Fig. **2A**), the major etiological agent of gastric diseases such as ulcers and cancer. The sensitivity of the technique can also clearly discriminate *H. pylori*

CHAPTER 2

High Performance Liquid Chromatography (HPLC) – Theoretical and Practical Aspects

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Abstract: High Performance Liquid Chromatography (HPLC) is a powerful chromatographic technique to separate, identify and quantify components in a mixture. HPLC has superior features such as versatility, safety, sensitivity, accuracy and low detection capability. It mainly utilizes a column that separates components, a pump that delivers mobile phase through the column and a detector that detects the eluted components from the column. Separation of components occurs based on the interaction between the stationary phase and the components. HPLC is divided into several classes depending on the stationary phase and the separation process such as normal phase, reversed phase, and ion chromatography. It has significantly contributed to many fields of science such as pharmaceuticals, foods, environment and forensics. This chapter mainly focuses on the theoretical and practical aspects of HPLC.

Keywords: High performance liquid chromatography, HPLC analysis, HPLC detector, Mobile phase, Normal phase chromatography, Reversed phase chromatography, Stationary phase.

1. INTRODUCTION

High Pressure Liquid Chromatography (HPLC) is an analytical technique used to identify and characterize components in a sample mixture. In an HPLC system, a solvent (mobile phase) moves through column under high pressure unlike LC (under gravity). This allows to analyze the sample much faster. HPLC is also known as High Performance Liquid Chromatography because of superior features such as speed, sensitivity, accuracy and low detection limit.

A minimum amount of the sample mixture is firstly injected into the loop or put on an autosampler and carried through column by mobile phase. Separation of components in sample mixture occurs in column depending on the interaction bet-

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ween the stationary phase and the components. Affinities of different components (adhesion strengths) in sample mixture towards column lead to separation of the components. Next, each compound is determined by the detector. Signals from the detector are recorded, and then converted to chromatographic data by commercial HPLC software. Each compound is represented by a peak in the chromatogram having a particular retention time to identify and quantify a particular compound. Finally, all peaks are integrated and interpreted.

The HPLC system includes the following basic units (Fig. **1**);

1- Solvent reservoir (Mobile phase)
2- Pump
3- Sample injection system
4- Column
5- Detector

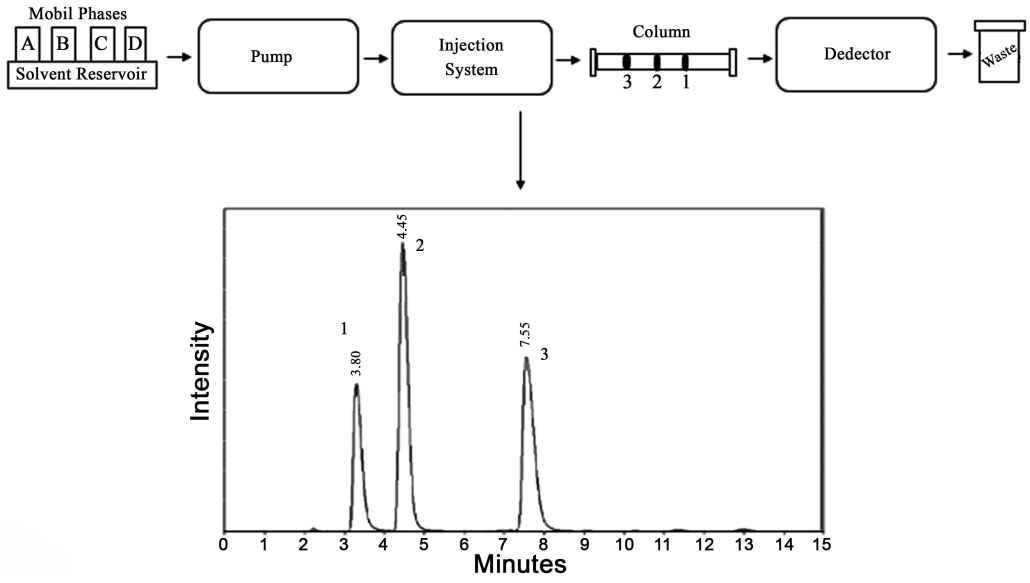

Fig. (1). Basic units of HPLC system and a chromatogram. 1, 2 and 3 represent individual compounds in the chromatogram. Retention times of individual compounds are 3.80, 4.45 and 7.55. Compound "3" eluted from the column later than compounds "1" and "2" due to its stronger affinity to stationary phase.

2. HPLC INSTRUMENT

2.1. Solvent Reservoir (Mobile Phase)

Solvent reservoir holds one or more solvent glass bottles with caps including

mobile phase. Mobile phase is carried to the sample from injector valve to detector. It transports using tubing (inlet line) that is produced from teflon, glass or stainless steel materials. In an HPLC system, the mobile phase is the second important parameter because of its effect on sample separation (the first one is the column itself).

A water-based solvent and an organic solvent are used as mobile phases, mostly with a mixture of both (*e.g.* water-acetonitrile and water-methanol). The mobile phase does not interact with the stationary phase and therefore does not compete with the sample components. However, the mobile phase can also be a non-polar solvent based on structure of the stationary phase (*e.g.*, hexane and methylene chloride). Ratio and type of the mobile phase have important effects on retention of analytes and play key roles in the development of a new method. A buffer solution may be used as coupled with the mobile phase to easily separate some analytes. However, applications of the buffer solution may clog the system lines. The clogging creates a serious problem, especially on pressure and chromatogram. To eliminate this problem, the mobile phase (not containing a buffer solution) should be transmitted to all system lines for a certain period (about 1-2 hours) after using the buffer solution. In addition, it is better to flush the pump thoroughly with pure water.

The mobile phase should not have any air bubbles, which form in cases where mobile phase is super saturated with dissolved air. This may be among the most significant problems in solvent delivery since bubbles can lead to complications on pressure and/or signals. The bubbles can be removed by mobile phase degassing using ultrasonic bath and/or degasser unit that can be found as a module in some improved HPLC.

In HPLC analysis, use of the mobile phase left in reservoir for a long time (overnight) may cause variance in retention and peak area since evaporation and oxidation lead to changes in the composition of mobile phase. Hence, it is suggested that it is freshly prepared for optimum separation and reproducibility.

2.2. Pump (Solvent Delivery System)

The function of pump in HPLC is to deliver the mobile phase to the column at a constant pressure. In the transfer of mobile phase, resistance develops depending on gravity and stationary phase, especially if the stationary phase has very small particle sizes. Hence, a pressure pump is required to flow the mobile phase through the system and carry the analytes. The pressure can change based on pump type and number and/or device type. The pressure can vary between 500-5000 psi in normal analytical operations and 15000-18000 psi in preparative

CHAPTER 3

Raman Spectroscopy and Its Biomedical Applications

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Abstract: Raman spectroscopy is a significant characterization technique using inelastic scattering of light associated with molecular vibration to gather information about chemical fingerprints of tissues, cells or biofluids. Lack of sample preparation including chemical specificity and the ability to use advanced techniques in the visible or near-infrared spectral range have led versatility in biological applications of Raman spectroscopy. By Raman spectroscopy, the changes caused by diseases in tissues and organs can be accurately investigated and it is fast, non-invasive, economic and highly specific in comparison to other diagnostic and imaging techniques. It can provide quantitative molecular information for an objective diagnosis. Raman spectroscopy can measure both chemical and morphological information in samples and provide objective diagnosis for independent tissue samples of new patients. Some specific techniques and applications presented in this chapter which will demonstrate the potential of Raman spectroscopy for medical diagnostics, as well as the versatile interest in healthcare service.

Keywords: Biomedical applications, Instrumentation, Raman spectroscopy, Structure determination.

1. INTRODUCTION

Raman Effect was discovered by C.V. Raman (Nobel Laureate) in 1928 [1]. It is an important tool for nondestructive analysis. However, there are a number of challenges such as lack of good Raman source, lack of good detector, and interference from fluorescence.

In 1986, the first Fourier-Transform (FT) Raman spectroscopy instrument was developed. It provided a new platform of break-through to make it more convenient and sophisticated for scientists [2]. Dispersive Raman spectroscopy

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instrumentation [3] was developed in 1990 with the advancement of compact near-infrared (NIR) lesser, multichannel detectors and fiber optic probes. The advent of portable integrated dispersive Raman spectroscopy systems was also investigated during this period. All these investigations led to Raman spectroscopy to gain importantance and value for vast applications of multidimensional fields.

Raman spectroscopy is a special form of molecular spectroscopy that depends on the scattering of electromagnetic radiation by atoms or molecules. It represents vibrational, rotational and other low-frequency modes of molecules, which is observed as inelastically scattered light. Whenever a beam of radiation passes through a transparent substance, a small amount of the radiation energy gets scattered. If monochromatic radiation is used, the frequency of scattered energy is almost equal to the frequency of incident radiation. This phenomenon is known as Rayleigh scattering.

In case the frequencies of scattered energy lie below or above the frequency of incident radiation, this phenomenon is known as Raman scattering. Raman spectroscopy is a nondestructive technique with a simple sample preparation. The analysis can be carried out directly through transparent containers such as glasses, jars, plastic bags, cuvettes, *etc.* The technique is applicable for both qualitative and quantitative analysis. It is highly selective due to its ability to differentiate molecules in similar chemical species and is insensitive to aqueous absorption bands. The analysis time is relatively short. Basically, this spectroscopy deals with excited rotational or vibrational energy levels of electronic ground state of molecules. Therefore, Raman spectroscopy is also known as vibrational spectroscopy. Vibrational spectroscopy deals with absorption and inelastic scattering of photons, informs about the complex structure and molecular dynamics of polyatomic systems. It provides valuable information pertaining the nature including magnitude of several physical parameters as intra and intermolecular forces, bond length, bond angle, identification of functional groups in compounds and thermodynamic properties. Spectroscopic studies of large molecules such as proteins, peptides and nucleic acids have been extensively investigated.

The process of absorption is used in acoustic (with sufficiently small energy difference between ground and excited states) and X-ray absorption spectroscopy (with a large energy difference). There are different processes of absorption methods such as NMR (Nuclear Magnetic Resonance), EPR (Electron Paramagnetic Resonance), infrared absorption, electronic absorption and fluorescence emission, and ultraviolet (UV) spectroscopy. Fig. (**1**) depicts the classification of different types of radiations on the basis of wavelength.

Wavelength (λ) is the characteristic of radiation. However, in spectroscopic calculations energy is often expressed in terms of frequency (ν) or wave number (ϖ). The relationships are $\lambda=c/\nu$, $\nu = \Delta E/h$, $\varpi = \nu/c = 1/\lambda$ where c is the velocity of light and ΔE is the energy difference and h is the plank's constant.

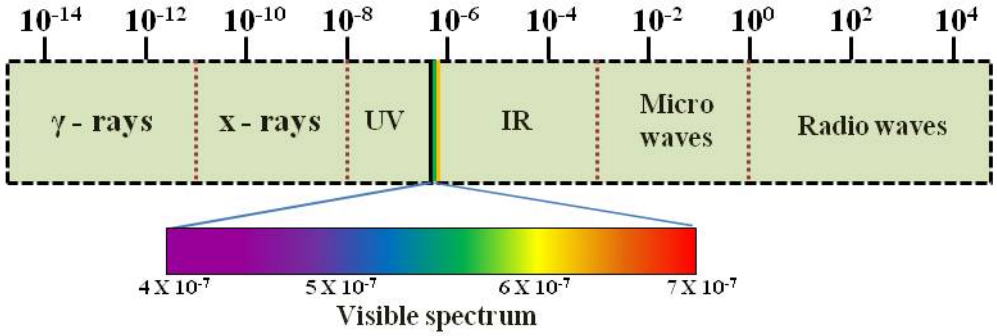

Fig. (1). The electromagnetic spectrum on the wavelength scale in meters.

All the mentioned equations indicate the direct proportionality of energy to the reciprocal of wavelength.

There is a difference in the processes in which radiation is used in IR and Raman spectroscopy. In infrared spectroscopy, energy is directed on the sample and absorption takes place where the frequency of incident radiation matches with the energy difference of vibrational ground and excited states. The absorption of this frequency from incident radiation is recorded. While a single frequency of radiation is used for irradiation of sample in Raman spectroscopy, scattered from the molecule, one vibrational unit of energy different from the incident beam is detected. Thus, matching of the incident radiation to the energy difference between the ground and excited states is not required.

2. THEORETICAL BACKGROUND

2.1. Raman Scattering

Molecules may fall to different excited vibrational or rotational levels of electronic ground state giving rise to secondary emission of different frequencies when the excited frequency of the electromagnetic radiation lies in the UV-vis range. This phenomenon is called the Raman effect. During Raman scattering, photons of excited radiation interact with the molecules of the medium. The energy of inelastically scattered photons may either be decreased or increased relative to excited photon energy by quantized increments corresponding to

CHAPTER 4

Circular Dichroism

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Abstract: Circular Dichroism (CD) is an absorption spectroscopy technique and measures left and right circularly polarized light difference of optical activity of asymmetric macromolecules. Unequal absorption of left and right circularly polarized light provides information about macromolecules depending on the spectral region. The far UV region provides data for secondary structure composition of proteins whereas near UV region provides data for tertiary structure of proteins. Furthermore, denaturation experiments provide stability parameters of proteins.

Keywords: Circular Dichroism, Far UV region, Folding, Kinetic measurements, Near UV region, Stability.

1. INTRODUCTION

Absorption spectroscopy is a powerful method to reveal structural information from macromolecules. Polarization of circular light provides precise results for CD experiments such as structural symmetry. This makes CD spectroscopy highly sensitive as the difference between right and left polarized light of a protein sample with one absorbance unit can be as small as one in a million [1]. In contrast, other structural techniques require higher protein concentration. CD can monitor protein conformational changes as well as thermal, pH, ionic strength or solvent dependent stability measurements. Although, CD can be employed for lipids, carbohydrates, and DNA, it has become the standard technique to measure protein absorption. Therefore, this chapter will deal mainly with the protein applications. Secondary structure composition determination, ligand binding effect determination, and protein stability measurements will be covered.

Although CD may not provide residue specific information of macromolecules as NMR and X-ray crystallography, structural alterations upon ligand binding or

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environmental changes (pH, ionic strength, and temperature) can be monitored readily. Polarized light is sensitive to molecule dynamics and the difference between left and right polarized light provides direct information on conformation that is not directly available by other techniques. CD is sensitive to changes in conformation and most solvents can be conveniently employed. Samples used in CD measurements are not destroyed or denatured during the experiment and can be further used for other purposes.

CD applications of biomolecules are as follows: i. Predicting protein secondary structure from far-UV spectra; ii. Detecting changes over biomolecules (protein, nucleic acids) introduced by pH, salt, and solvents, and determining structural analysis of chimeric and mutant structures; iii. Monitoring temperature or denaturant dependent protein-nucleic acid folding to understand biomolecule structural features and stability parameters; iv. Monitoring binding interactions between biomolecules (protein-ligand, protein-protein, and protein-nucleic acid); v.Determining kinetics of biomolecule interactions and folding. These applications may provide functional understanding of biomolecules in biochemical pathways.

2. INSTRUMENTATION AND SAMPLE PREPARATION

CDs from different sources provide high quality data and offer accessories for kinetics-temperature dependent experiments. However, solvents employed in the instrument must be high grade in purity. Pure, oxygen-free nitrogen gas must be used ahead of and during the experiment for purging. Maintenance of the instrument provides reliable and precise results.

Samples must also be in high purity since strong signals originating from impurities may perturb biomolecule signals. Further, total absorbance must not exceed one absorbance unit to avoid back-light scattering. Factors perturbing CD signal (high concentration of chloride, nitrate, HEPES, PIPES, chelators, and reducing agents and solvents as dioxane and DMSO) must be carefully controlled [2].

Peptide bonds absorb at far-UV (170-250 nm) and aromatic amino acids absorb at near UV (250-300 nm) region. Therefore, measurements in far-UV region employ short path length cuvettes to reduce absorption of intense signals. But, for near-UV region similar conditions are used as in absorbance measurements. At this region, nucleic acids have better signals than proteins [3].

Accurate sample concentration is essential for CD measurement especially for far UV region. Absorption spectroscopy is a common method if extinction coefficient

of the sample is known. The spectrum should be recorded under temperature control with baseline subtraction to accurately determine the concentration. Filtering and centrifugation of sample solution clarify the sample from potential scattering agents. Significant background absorption above 315 nm indicates light scattering and must be corrected.

3. DATA COLLECTION AND PROCESSING

Signal to noise ratio of the instrument correlates with square root of scan numbers and square root of time constant. Therefore, either small number of slow scans with a long time constant or multiple fast scans with a short time constant must be measured for acceptable signal to noise ratio [4].

Increasing the spectral bandwidth and therefore the light throughput may reduce the noise, but 2 nm must be an upper limit to prevent distortion of the spectrum. 1 nm bandwidth is commonly employed.

Since temperature is an important factor for macromolecular behavior, temperature-controlled CD measurements will reflect a true conformational alteration and eliminate any undesired effects. Baseline recording using the same cuvette, buffer, and instrument setting will prevent noise in the scan [2].

After subtracting the baseline from the sample scan, the spectrum can be converted to desired unit (molar extinction coefficient or mean residue CD extinction coefficient) for convenience. Absorption (A) can be converted to molar extinction [(A*M)/(C*L*1000)], degrees [A*32.98], molar ellipticity [(A*M*3298)/(L*C)] where C is concentration in g/L, M is molecular weight in g/mol, L is path length of cell in cm [1].

4. APPLICATIONS

4.1. Secondary Structure Content Of Proteins

Proteins consist of different secondary structural elements and CD data in the far-UV region can be used to determine the composition of these secondary structures. If the structural elements are additive, then measured signal (*i.e.* molar ellipticity, Θ) at any wavelength can be defined as:

$$\Theta(\lambda) = \sum_{i=1}^{n} f_i \Theta_i(\lambda) \quad \textbf{(1)}$$

CHAPTER 5

Transmission Electron Microscopy: Theory and Applications

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Abstract: Transmission Electron Microscopy (TEM) is a useful technique to explore the molecular structure, interactions and processes including structure-function relationships at cellular level using a variety of TEM techniques with resolution of one angstrom (Å) 1 to 1000 Å. Developments in modern science and technology, especially in the material science, depend significantly on microstructure characterization. In this context, novel characterization techniques are crucial to understand the properties of materials. The quality of TEM results is dependent on preparation of TEM grid. Experts are constantly working on optimization of milling parameters to reduce the potential artifacts. The resolution power of TEM makes it possible to visualize different objects with high quality images to study complex structures and tissue morphology. Thus, TEM has made a milestone towards the understating of cellular structure. This chapter includes introduction and theory of TEM including its instrumentation, sample preparation, and working applications.

Keywords: Backscattered electron, Instrumentation, Sample preparation, Transmission electron microscope.

1. INTRODUCTION

The use of electronic microscopes is significant due to their capability to determine the details of an object by imaging. But the extent of resolution is a function of wavelength of the corresponding wavelength of light to get a highly accurate image. This concept was first proposed by Ernst Abbe in 1893. In 1932, Ernst Ruska tried to construct a new electron microscope which could produce a direct image of specimens. In the following year, by improving the resolution, normal imaging of object was attained using the electron beam [1]. Interest in electron microscopes with higher magnifications increased and the idea of developing TEM appeared. The first TEM was demonstrated by Max Knoll and Ernst Ruska with high resolution (greater than that of light) electronic microscopy

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in 1933 and the first commercial TEM was launched in 1939. In 1986, Ernst Ruska was awarded the Nobel Prize in Physics for the development of TEM [2]. Later in 2008, Jannick Meyer *et al.* explained the direct visualization of light atoms such as carbon, hydrogen and a graphene sheet [3] and opened the door to various field applications.

TEM is a microscopic technique in which an electron beam is transmitted through a sample (substrate) for imaging. The sample mostly has an ultra-thin (100 nm) foil or a suspended layer on a grid. As the electron beam is transmitted through the specimen, electrons interact with sample to produce an image focused on an imaging device after magnification. The quantum mechanical behavior of electrons is considered to develop TEM. Characteristics of electron, a quantum mechanical object, results in interaction of materials with it. Compared to that of light microscopy, TEM can produce high resolution images capturing fine details of atoms. Electrons have higher resolution capabilities because of their dual nature, and smaller De Broglie wavelength than that of light. Materials with different thicknesses and compositions lead to differential absorption of electrons resulting in image contrast. At high magnifications, complex wave interactions modulate the image intensity, necessitating expert analysis of observed images.

An image is created by interaction of transmitting electrons with the sample, and is focused on an imaging device. Alternative modes of use allow observation of sample structure.

TEM is a major analytical method in material and biological sciences. It can be applied in environmental, physical, biological, and chemical fields, and information on crystal structure, symmetry, and orientation of materials, and chemical composition can be obtained. The possible interaction between a specimen and an electron is given in Fig. (**1**).

2. THEORY

Working principle of TEM is similar to that of a projector. In a projector, beam of light is transmitted through a slide. The patterns painted on the slide permit certain parts of the light beam to pass through. In this manner, patterns are replicated on the slide by the transmitted beam and then it falls on the screen, forming the magnified image of the slide. In case of TEM, a beam of electrons (parallel to light in a projector) passes through the specimen (similar to slide). The electron beam depends on the properties of material being investigated such as density, composition, *etc.* More electrons pass through low-density materials while less electrons pass through high-density materials leading to differentiating materials of non-uniform density. The transmitted beam of electrons is projected

on a screen. Theoretically, the maximum resolution, *d*, obtained in light microscope is given by the relation.

$$d = \frac{\lambda}{2nsin\theta} \approx \frac{\lambda}{2NA}$$

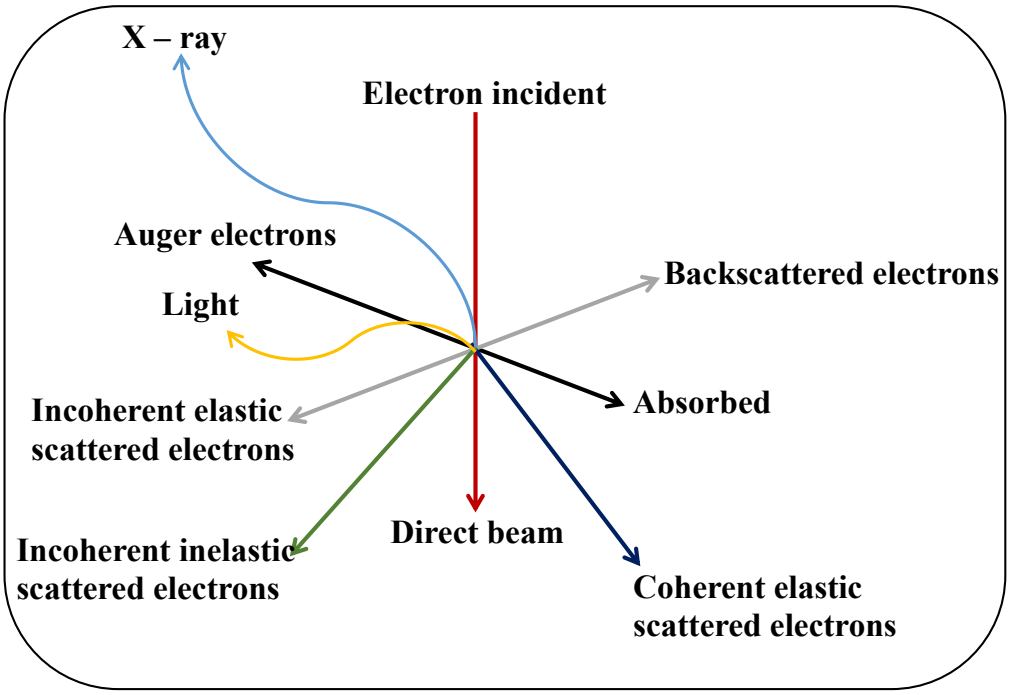

Fig. (1). The possible interaction between a specimen and an electron [4].

In the last century scientists theorized described the limitations of using large wavelengths of visible radiation, by replacing light beam with electrons [5]. Electrons have dual nature, and an electron beam behaves like a beam of electromagnetic radiation due to its wave-like nature. The wavelength of electrons can be evaluated using De Broglie equation. As velocity of electron is comparable to velocity of light, an additional correction to account for relativistic effects can be made from the following equation.

CHAPTER 6

Scanning Electron Microscopy: Theory and Applications

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Abstract: In the present chapter, brief history, working principle, instrumentation and, recent applications of Scanning Electron Microscopy (SEM) have been enlightened. SEM is a highly sensitive and efficient magnification tool that exploits focused beams of electrons to obtain information allied to topography, morphology, and composition of materials. Utilization of SEM techniques in different fields, like various domains of materials science, forensic investigation, mechanical engineering, biological, and medical sciences has been discussed.

Keywords: Electron beam, Scanning electron microscopy.

1. INTRODUCTION

High-energy electron beam is used to generate signals to scan the surface of solid specimen. Manfred von Ardenne [1] in 1937 developed scanning electron technique capable of magnifying the several folds of object. SEM developed by Ardenne was free from chromatic aberration. It was developed using several detection modes and theories [2] with the capability of high magnification [3]. Zworykin's group [4] worked on further modifications. Later, in 1950s and early 1960s, Charles Oatley [5 - 8] and his coworkers put their efforts to develop the first commercial SEM. The instrument was fabricated by Cambridge Scientific Instrument Company in 1965 and delivered to DuPont.

The human eye is capable of distinguishing two points that are 0.2 mm apart, by avoiding use of additional lenses. This distance is termed as resolving power of the eye. The device used to increase the resolving power is known as microscope. Advanced microscopes can achieve magnification up to 1000x. The magnification power depends on the lens quality and radiation wavelength (λ) used. The wavelength of visible light falls in the range of 400 nm to 700 nm while electrons possess relatively short wavelengths, leading to improved resolution.

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2. INSTRUMENTATION

A focused beam of electrons is used to scan the specimen surface to form a magnified image (Fig. **1**). The signals produced by electron-sample interaction give an idea of surface morphology, composition *etc.* An electron beam generated from electron gun passes through the column of the SEM, electromagnetic lenses are used to focus and direct the beam towards the specimen. The beam scans the sample surface after passing through deflector plates and lens [9]. SEM can achieve resolution more than 1 nm. In conventional SEM, specimens are observed under vacuum. High-energy electrons produce signals due to electron-sample interactions. These signals give information about various properties of the sample including morphology, texture, surface topography, chemical composition, electrical conductivity, *etc.*

The working principle of SEM is based on scanning a focused beam of electrons on the sample. Its main components include:

1. Source of electrons (electron gun).
2. Column (with electromagnetic lenses).
3. Electron detector.
4. Sample chamber.
5. A recorder (Generally a computer and a display for image viewing).

An electron beam is produced by a source (electron gun) and potential difference of 25 KV is applied for its acceleration. The electron beam accelerated due to potential difference moves down through the column and passes through electro-magnetic lenses and apertures resulting in a focused beam of electrons to strike the specimen. The electron beam first passes through condenser lens and results in an electron beam spot. It then passes through another lens known as objective lens, and then through a beam deflection coil to get deflected. As soon as the beams interact with the sample, the secondary electron beams are ejected to form secondary electron image and give information about surface morphology of the sample. The signals generated by a SEM includes secondary electrons, back-scattered electrons (BSE), characteristic X–rays, light (cathodoluminiscence), specimen current, and transmitted electrons that will be discussed briefly. The sample is mounted on a stage in the chamber area. Unless the microscope is designed to operate at low vacuums, both the column and the chamber are evacuated by a combination of pumps. The vacuum level depends on the microscope design.

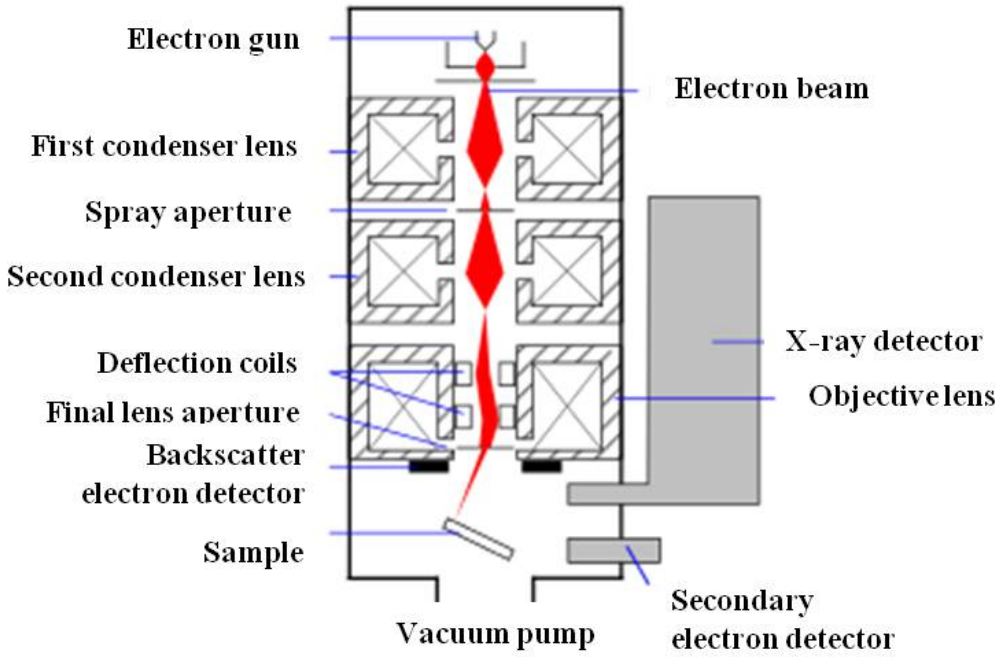

Fig. (1). Schematic diagram of SEM.

I) Secondary Electrons: The widely used imaging mode collects low-energy secondary electrons ejected from the k-orbitals of the specimen atoms by non-conserved energy (inelastic) scattering interactions with the beam electrons. Owing to their low energy, these electrons originate within a few nm from the sample surface. The secondary electrons yield high-resolution images of the surface, revealing details about less than 1 to 5 nm in size. Due to the very narrow electron beam, SEM micrographs have a large depth of field yielding a characteristic three-dimensional appearance useful for understanding the surface structure of the sample.

II) Backscattered Electrons (BSEs): BSEs are reflected from the sample by elastic scattering. The intensity of BSEs depends on the mean atomic number (Z) of the sample. Images created by BSEs give information about element distribution; heavy elements (high atomic number) can backscatter the electrons stronger in comparison to lighter nuclei (low atomic number) forming comparatively bright images. BSEs give information about differences in chemical composition throughout the specimen surface. Therefore, they are used for imaging of colloidal gold immuno-labels of 5 or 10 nm in diameter. They also

CHAPTER 7

SEM-EDX: A Potential Tool for Studies of Medicinal Plants

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Abstract: Naturally obtained compounds from aromatic and therapeutic plants, herbs and shrubs have been playing a vital role in providing medicinal benefits for humans since the prehistoric period. Ancient Unani manuscripts, Egyptian papyruses, and Chinese writings have described the use of medicinal plants, herbs, and shrubs. Shreds of evidence can be found from Unani hakims, Indian Vaids, and European and Mediterranean cultures using herbs in medicine for over 4000 years. India has been known to be a rich repository of medicinal plants. The forests in India are the principal repository of various medicative and aromatic plants that are mostly collected as raw materials for the production of diverse medicine and perfumery merchandise. Treatments with these drugs are considered safe as there is minimal or no side effects. The Energy-dispersive x-ray analysis (EDX) technique is useful in the study of drugs and drug delivery. It detects nanoparticles, which are generally used to improve therapeutic performance of chemotherapeutic agents. EDX is also used for characterization of minerals accumulated in tissues. It can also be considered as a useful tool in element determination, endogenous or exogenous in the tissue, cell or any other samples. In the present chapter, potential applications of SEM-EDX in the study of valuable compounds present in medicinal plants, herbs and shrubs have been highlighted. Special reference was given to the rich biodiversity of medicinal plants found in the state of Jharkhand and the need for preserving it for the well-being of human-kind. The study provides information about the availability of some crucial minerals and phytoconstituents, which can be used to provide dietary elements and may also help in emerging new drug formulations. This chapter further highlights the role of electron microscopy coupled with analytical analysis, particularly SEM-EDX, in characterization of various primary and secondary elemental compositions. Since medicinal products based on the extracts from plants, herbs, and shrubs are eco-friendly, there is urgent need to promote extensive research in the field.

Keywords: Herbal drugs, Medicinal plant, Pharmacology and ailments, SEM-EDX.

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1. INTRODUCTION

Plants are a great source of medicinal products, particularly traditional medicine, which can be beneficial in treatment of various diseases [1]. Medicinal plant is defined as encompassing various constituents that can be used for therapeutic purposes and precursors for the synthesis of useful life drugs [2]. Such plants are still a major source of human life-saving drugs. According to WHO (World Health Organization), it is predicted that more than 80% of the world population in developing countries still use herbal medicines for their rudimentary health care [3]. Thus, medicinal plants play a vital role and make a significant contribution to health sector. They are distributed around the world but are abundant in tropical countries. Approximately 25% of all modern medicines are obtained from higher plants directly or indirectly. But in certain cases, those prescribed in anti-tumor and antimicrobial medicines are 60% available in the market derived from natural products, mostly from higher plants [4]. Natural products and related drugs are used to treat nearly 87% of all categorized human diseases, including immunological disorders, bacterial infections, and even cancer [5]. A report from the WHO reveals that around 65-80% of the world's populations in developing countries live below poverty and lack modern medical facilities therefore rely on traditional, predominantly herbal medicine to meet their primary health care needs [6, 7]. Plants have been studied to evaluate their quality, safety, and efficacy [8]. The demand for herbal medicine in the world is growing rapidly [9]. The market is estimated to grow about 20% annually for Ayurvedic medicinal products in India [10], while in a single province of China (Yunnan) the market of medicinal plants has grown 10 times over the last 10 years [11]. India comprises of forest and other trees, 67.83 M ha (20.68%) and 9.99 M ha (3.04%) of the geographical area. Thus, the total forest and other tree coverage are figured as 77.82 M ha, which is 23.68 percent of the total geographical area [12]. In Indian civilization, use of medicinal plants along with herbal medicines has been very popular. For several hundred years, tribal life, tradition, and culture have remained almost static. The knowledge acquired by the tribes through generations shows a profound understanding of forest resources [13]. There are 17,000 species of higher plants in India, 7500 of which are known for their medicinal uses [14]. The proportion of medicinal plants is the highest in the world (Table **1**). More than 43% of total flowering plants in India were reported to be of medicinal importance [15]. Singh *et al*. [16] studied 22 different medicinal plant species and assayed for antiplasmodial activity in which the highest anti-plasmodial activity (Pf3D7IC50r5 mg/ml) was seen in leaf ethanol extracts of Corymbia citriodora (Hook.) K.D.Hill & L.A.S.Johnson, *Calotropis procera* (Aiton) Dryand. and *Annona squamosa* L. and bark ethanol extract of *Holarrhena pubescens* Wall. ex G.Don. Leaf ethanol extract of *H. pubescens*, and bark ethanol extract of *Pongamia pinnata* (L.). These results confirmed the traditional use of medicinal

plants against malaria in parts of Ranchi in Jharkhand. Similarly, Nguyen *et al*. [17] investigated nematicidal properties of different medicinal plants such as *Terminalia nigrovenulosa, Cinnamomum camphora, Jasminum suptriplinerve, Premna integrifolia, Treptocaulon Juventas* and found the extracts could be used as biocontrol of plant-parasitic nematodes, especially root-lesion nematode populations. Extraction of medicinal herbs and their mixtures were found to contain toxic elements such as Pb, Al, Hg, Cd, and Cr harmful to human health depending on their oxidation and concentration [18 - 20]. Therefore, scientific studies of these medicinal plants are required to assess their effectiveness [21, 22]. Under these conditions, analytical tools like Scanning Electron Microscopy (SEM) coupled with Energy-Dispersive X-ray Spectroscopy analysis (EDS) could be a useful tool for characterising trace elements [23].

Table 1. Comparative distribution of medicinal plants.

Country	Total Number of Native Species in Flora	No of Medicinal Plant Species	Medicinal Plants (%)	References
World	297000	52885	10	38
India	17000	7500	44	41

2. DETAILS OF SEM-EDS

SEM uses a focused beam of high-energy electrons to generate variety of signals at the surface of solid specimens. Electron and sample interaction emits a signal that provides sample information about its external morphology (texture), composition, crystalline structure, and orientation. It is also capable of analyzing the location of selected points on the sample. Hence this approach is useful in determining chemical compositions qualitatively or semi-qualitatively. Energy dispersive X-ray spectrometry is a popular method to determine the trace elements in geological and environmental samples. Morphological characters obtained from SEM are supported by EDS micro analysis device, making it possible to identify the elements present in it [24]. SEM-EDX uses several analytical techniques for elemental analysis which is highly quantified for the identification and quantification of different elements present in medicinal plants for their biological and environmental importance. Developments in this field of mineral elements have taken place in the chemical, biochemical and immunological areas of research [25]. Flegler *et al*. [26] reported that SEM is a powerful tool for investigating surface structures of herbal plants of leaves, pollen grains and seeds. This technique provides a large depth of field, which means, the area of the sample that is viewed in focus at the same time is actually quite large.

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Dr. Tutar received 15 awards, including the National Young Scientists Award-Turkey, FEBS-EU, Teach Academy-USA, the Honor Society Membership-Texas etc. He is the author of 80 articles, 24 book/book-sections and EBM of Bentham Medical Books, Anticancer Agents in Medicinal Chemistry, and Medicinal Chemistry journals.