THESIS ON

BIOMOLECULAR DETECTION BY SURFACE ENHANCED RAMAN SCATTERING (SERS)

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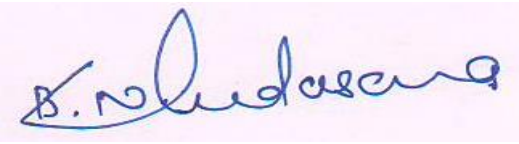
Dedicated To My Parents and My Sister

CERTIFICATE

I hereby certify that the work, which is being presented in this thesis, titled **"Biomolecular Detection Using Surface Enhanced Raman Scattering (SERS)"** in partial fulfillment of the requirements for the award of degree of **Masters of Science** in **Physics** and submitted to the School of Physics and Materials Science, Thapar Institute of Engineering and Technology, Patiala, is an authentic record of her own work, carried out under my supervision. The matter presented in this thesis has not been submitted elsewhere for the award of any other degree or diploma from any institution.



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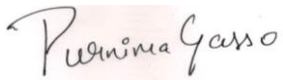

Purnima Gasso

CONTENTS

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1. BIOMOLECULAR DETECTION

There is a need for biomolecular detection for the advances in medical diagnosis. The signature of the disease and its effect should be known for its selective detection. Nowadays for the detection of the specific disease, the signature of the illness, and any information related to its molecular origin are required [1]. There is a great need for low concentration detection in diagnosing the disease. Nowadays multichannel technologies can provide the detection of disease at an early stage. Such technologies give fast, economical, and reliable quantitative detection. Early detection is greatly important in many diseases like cancer as the success of the treatment depends upon its detection at an early stage where the pathogenic signature of the disease is very weak [2].

1.2. CONVENTIONAL PATHOLOGICAL METHODS

There are several techniques proposed for the detection of biomolecules. Based on their levels of sensitivity and quantitative detection various methods that are effective at analyzing clinical samples are given below:

Enzyme-linked immunosorbent assay (ELISA): We can detect proteins and biomarkers using this technique. This technique uses an antibody assay format for detecting the levels of protein biomarkers with perfect sensitivity and specificity. This technique is not able to detect biomarkers having a concentration in the range of femtomolar to attomolar concentrations. Thus improvement in the sensitivity of ELISA is required as potential markers of cancer, neurodegenerative and cardiovascular disease are present at low levels not detected directly by ELISA.

Polymerase chain reaction (PCR) and Enzymatic amplification methods: These techniques are used for the analysis of nucleic acid. These methods use fluorescence or other macroscale techniques. PCR technique is able to detect at the single nucleotide level. However, for detection

of a sample using the PCR method requires efficient lab skills for the correct answer and multiple clinical hours which result in its limitations.

There are many other methods but their use is restricted due to their limitations, for example, the analysis of circulating tumor cells (CTC) allows non-invasive sampling of markers for detecting early cancer and cancerous colorectal lesions. The concentration of such cells is as low as 5 cells per milliliter, hence detection of such low concentration is not possible with such conventional pathological methods [1].

1.3. LIMITATIONS OF CONVENTIONAL PATHOLOGICAL DETECTION

The methods discussed above are fast, sensitive, efficient, and highly specific but they still lack due to their limits to quantitative detection of biomarkers over a large range of concentration [1].

Many methods used nowadays are time-consuming. For the effective and timely diagnosis, this puts in a major limitation. For diagnosis of biomolecules or any fast-spreading pathogens, preparation of culture can take days to weeks. Thus the time-consuming methods for concentrating the sample for the detection result in the limitations of today's technology for clinical diagnosis. The recent clinical identification process takes at least 24 h for the detection of infecting specie and hence delay in determining the treatment [1].

Small amount of samples are used for the detection of biomolecules and some rare, yet specific biomarkers may be missed due to sensitivity limits of pathological methods discussed above [1]. Biological fluids such as blood, urine, and sputum samples are used for the detection of malignant diseases. But the methods and techniques discussed lack in their limit of sensitivity and are unable to detect quantitatively the low abundance of protein or biomarkers in each biofluid specimen [2]. The methods discussed so far use milliliter to microliter volumes of macroscopic patient samples. For low limit detection, further division of sample volume is beneficial, which ensures the presence of only a small integer number of target molecules [1].

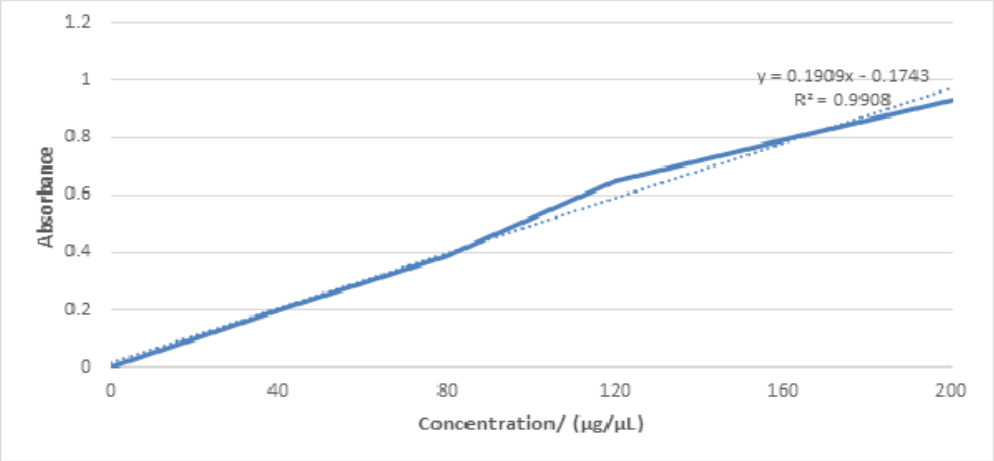

Fig 1: Plot of absorbance of BSA concentration at 0, 40, 80, 120, 160 and 200 µg/µl [3]

Ong et al. in 2015 presented a plot of absorbance of Bovine Serum Albumin (BSA) protein concentration at 0, 40, 80, 120, 160 and 200 µg/µl at 595nm measured using a UV-spectrophotometer. And it can be concluded from the plot that the detection of protein at low concentration is not possible using conventional methods. Thus for the early detection of disease new techniques are required [3].

1.4. MEASURES TO OVERCOME THE LIMITATIONS

Various new approaches are being proposed to overcome the limitations of pathological methods used conventionally. New techniques with improved speed, efficiency, and sensitivity have been proposed to improve the clinical diagnosis. Two main approaches are as follows:

1.4.1 MAGNETIC SEPARATION

Isolation and separation of proteins or biomarkers is a widely used method in biotechnology [4]. For the isolation of target molecules from accompanying compounds present in the medium, new separation techniques are required. Magnetic particles have gained much importance due to their unique binding and separation ability with an external magnetic field [5]. Magnetic separation is

conceptually very easy to understand. The first immobilization of the hydrophobic ligand or ion exchange groups or magnetic biopolymer particles on the magnetic carrier is ensured and is mixed with the sample. After the required incubation period, one can isolate the target biomarker bind to magnetic particles under the influence of magnetic separator (external magnets). Magnetic separation techniques have unique advantages over other separation techniques. It is easy and requires only a few handlings. The steps required can be conducted in a single tube and thus helps in reducing the separation time as compared to other expensive liquid chromatography systems, centrifuges, or other equipment [4].

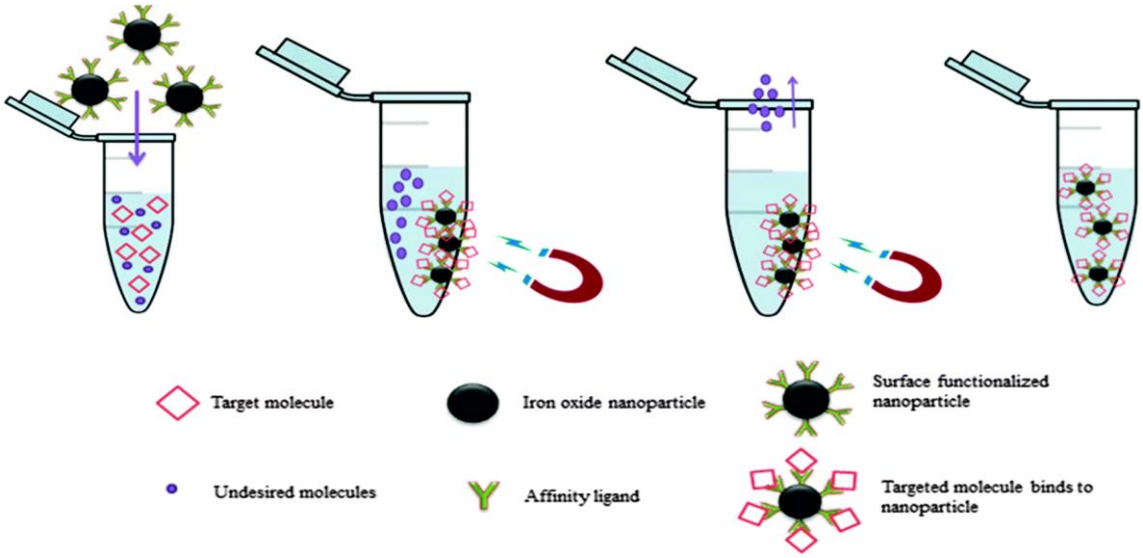

Fig 2: Basic principle of magnetic separation [6]

1.4.2. SURFACE ENHANCED RAMAN SCATTERING

We are unable to detect biomarkers such as proteins or molecules at low concentrations due to their property of inelastic scattering. Surface-Enhanced Raman Scattering (SERS) is one of the techniques through which the detection of a single molecule is possible [7]. First enhanced Raman scattering was observed in 1974 by Fleischman and co-workers due to the enrichment of analyte molecules on a rough surface. Many studies have shown that noble metals are used as a SERS

substrate. The enhancement can go up to 10^{14} corresponding to a cross-section of at least 10^{-16} cm^2 for a molecule [8].

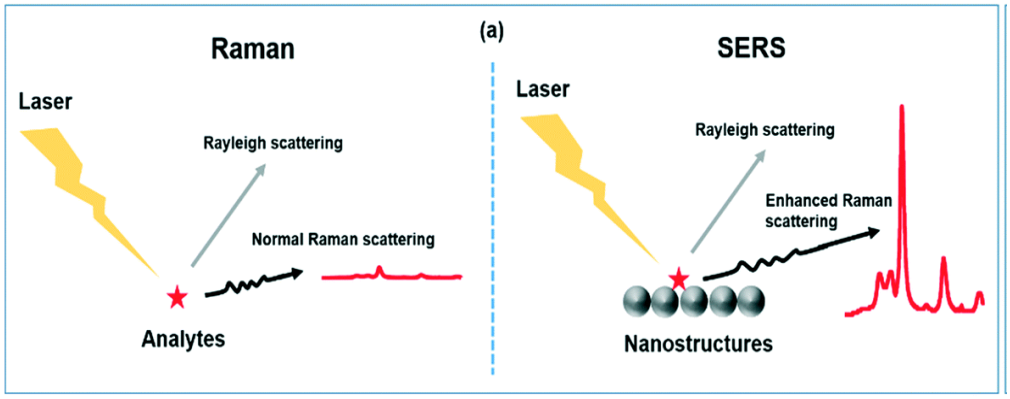

Fig 3: Mechanism of Surface-Enhanced Raman Scattering [9]

Based on various studies related to SERS, it is believed that both the long-range electromagnetic effect and short-range chemical effect result in enhanced signals [10]. When the frequency of the radiations of the electromagnetic field matches with the oscillation frequency of electrons in the nanoparticles, then enhancement of Raman modes from the molecules which are attached with nanoparticles happens. Such surface enhances plasmon resonance is called surface enhance Raman scattering (SERS) [11].

The enhancement of the electromagnetic field from localized surface plasmon result in increased intensity of peaks and thus a quantitative determination of low concentration analyte is possible [12]. Based on these principles, SERS immunoassays were developed that can detector low concentration of 1-12 pg/ml (Grubisha et al., 2003; Gong et al., 2007; Chon et al., 2009) [7].

CHAPTER 2

LITERATURE REVIEW

2.1. ASSESSMENT OF MATERIALS AND PROPERTIES USED IN SERS TECHNIQUE

Surface-Enhanced Raman Spectroscopy (SERS) has a greater potential to overcome the limitations of conventional pathological methods. To understand this effect we should know which materials exhibit SERS and how the shape and the size of the material affect their SERS response. SERS enhancement mostly occurs due to electromagnetic enhancement which further depends upon the size and shape of the material.

Schatz et al. (1986) studied the effect of electromagnetic fields near the surface to determine the field and Raman enhancement for 10 metals in groups 1, 11, 12, and 13. They used a spheroid model to determine field enhancement factor R for modeling single-photon measurements like surface-enhanced photochemistry (SEP), Raman enhancement factor ε for surface-enhanced Raman spectroscopy (SERS) and relevant enhancement factor S for second harmonic generation (SHG) measurements. They also explained the variation of electromagnetic enhancements with particle size and shape and with excitation frequency for different metals. In the case of silver, Raman enhancement occurs for the most prolate spheroids but the EM effects of Ag are quite different from gold and copper. Raman enhancement of Cu is larger than Au by a factor of 3. The order of peak enhancement in the case of noble metals is $Ag \geq Cu > Au$. The comparison of the experiment resulted in fewer values due to optimized peak enhancement. Alkali metals show comparable enhancement with that of noble metals but flatter dependency on frequency in the visible region. Ga gave large enhancement in the IR region, while AL and In show enhancement over the broad region from IR to UV. The enhancement of Cd peaks was very weak but Zn shows moderate enhancement [13].

Castro-Longoria et al. (2013) synthesized bio-inspired gold nanoparticles using Neurospora crassa extract under different environmental conditions of pH and temperature. Nanoparticles of different shapes or sizes such as a sphere, triangle, hexagon, pentagon, quasi-spherical, etc have been synthesized. They used methylene blue as a probe molecule to study the SERS effect and discussed how the size and shape affect the SERS spectra [14].

Li et al. in 2005 studied the effect of three different shapes of silver on Surface Enhanced Raman Spectroscopy using Rhodamine B. Three different types of silver colloids, silver nanowires, silver nanoparticles, and silver triangular nanoplates were synthesized by a solution-based method. The SERS spectra showed that the Raman enhancement of Ag particles was better than that of Ag wires and Ag plates. Also on analyzing the UV-Visible absorption spectra, it was observed that shape affects the absorption peak. Also, they have explained the key factors impacting Chemical enhancement [15].

Schatz [16] compared the SERS effect of noble metals, alkali metals, group 13, and group 14 elements. SERS effect occurs mainly in the visible and NIR range. Thus noble metals: Ag, Au, and Cu are mostly used to study SERS. Cu being unstable and oxidizes readily in the presence of air is hardly used whereas Ag and Au being more stable than Cu are most widely use [16].

Concentrated non-uniform electric field (hot spots) result in enhancement of the electromagnetic field. Shapes with sharp edges (like a cube) result in better SERS enhancement as compared to isotropic particles like spheres. Similarly, enhancement also depends on the size of the nanoparticles. For good enhancement; the size of the metal nanoparticle should be comparable to the wavelength of the incident radiation. Very small size nanoparticles may result in low enhancement due to poor polarization [17].

2.2. STUDIES ON SERS ENHANCEMENT: A REVIEW

SERS enhancement is because of two factors: Electromagnetic enhancement and chemical enhancement. Various studies have been performed on the SERS effect due to its ability to detect

a single molecule. Some reviews are discussed here on SERS based detection along with the lowest limit of detection.

Zhang et al. (2010) synthesized silver nanoparticles using an environmentally friendly and facile green method using $AgNO_3$ aqueous solution and polyethylene glycol 200. PEG200 played an important role in acting both as a mild reducing agent and as protecting agent for stabilizing and preventing aggregation of Ag nanoparticles. TEM and PSA results show that Ag nanoparticles of size less than 5 nm can be prepared using this method. Further, the SERS effect was studied using Ru(bpy) and R6G as probe analytes. The lowest limit of detection of Ru(bpy) was found to be 10^{-14} M using Ag/PEG-NPs as substrate [18].

Zhang et al. (2016) synthesized carbon nanotubes (CNTs) decorated with silver nanoparticles to study Surface-Enhanced Raman Scattering (SERS). The average size of Ag nanoparticles on the surface of CNTs was determined to be 32.5 nm. Rhodamine 6G (R6G) at different concentrations was used to study the sensitivity of SERS and the lowest limit of 10^{-14}M was observed. Also to determine the changes in Raman intensity during the evaporation process of probe molecule, time-course SERS mapping was done. Further, the reproducibility of SERS signals was estimated using relative standard deviation and strong SERS signals were observed on increasing the number of Ag nanoparticles [19].

Tong Lee et al. (2010) synthesized zinc oxide (ZnO) nano-needles and nanorod arrays using aluminum-doped zinc oxide as a substrate by chemical vapor transport and condensation technique. ZnO/Au composites were prepared by the hydrothermal method. Rhodamine 6G (R6G) was used as a probe molecule to study the Surface-Enhanced Raman Scattering (SERS) effect. The enhancement factor of nano-needle and nanorod arrays was 1.2×10^7 and 6.4×10^6, respectively. It was determined that the density of Au nanoparticles on the surface of ZnO nanoarrays and morphology of ZnO/Au nanoneedle arrays changes on altering the concentration of Hydrogen tetrachloroaurate (Ⅲ) tetrahydrate ($HAuCl_4$). Further on increasing the $HAuCl_4$ a redshift from 526 to 538 nm of the plasmon resonance band was observed. Detection of melamine using ZnO/Au composite nanoarrays yielded high SERS signals. Also, they determined the presence of melamine in the complex egg white solution [20].

2.3. BIMETALLIC NANOPARTICLES FOR SERS ENHANCEMENT

Bimetallic nanoparticles are those which are composed of two metals of different configuration. Two main configurations are alloyed and core-shell nanoparticles. The alloyed nanoparticles are composed of homogeneous distribution of two metals at an atomic scale. While the core-shell nanoparticles are formed due to the inhomogeneous mixing of two metals. These bimetallic nanoparticles result in enhanced SERS activity than the traditional noble metals based substrates [16]. Some of the review papers discussing the role of bimetallic nanoparticles are discussed below:

Hu and Li et al. (2014) studied the fabrication and applications of metal-oxide framework embedded by gold nanoparticles (AuNPs/MIL-101). The average diameter of the AuNPs was found to be 50 nm. UV-visible absorption spectra revealed that the intensity of absorption peak increases on increasing the density of AuNPs that is easily controlled by changing the amount of chloroauric acid. The SERS activity of the synthesized AuNPs/MIL-101 nanocomposites was studied using Rhodamine 6G (R6G) and a series of aromatic amine compounds. The molar detection limit of R6G was found to be 41.75 fmol and 0.54 fmol for benzidine. They have also studied the Sieving effect of the SERS measurement using pyridine derivatives as analytes and stability and reproducibility of AuNPs/MIL-101. Further Surface-Enhanced Raman Scattering (SERS) detection of p-phenylenediamine in environmental water and SERS-ELISA based detection of Alpha-Fetoprotein in Human serum [21].

Zhou et al. (2020) reported a bio-inspired synthesis of pomegranate like Silica@Gold nanoparticles (P-SiO_2@Au). The synthesis occurred in the presence of arginine in aqueous solution from chloroauric acid and tetraethyl orthosilicate. Thiophenol (TP) was used as probe analyte to study SERS spectra. Finite-Difference Time-Domain (FDTD) simulations were performed to reveal the optical contribution of P-SiO_2@Au NPs of different sizes. UV-visible spectra showed a red-shift of plasmon peak from 580 nm to 730 nm on increasing $HAuCl_4$ concentration. The enhancement factor was experimentally determined to be $4.3 \times\times 10^7 - 8.5 \times 10^7$ and 1 ng mass detection limit was determined by paper-based SERS measurement [22].

Zhu et al. (2013) discussed the preparation and measurements of Surface-Enhanced Raman Scattering (SERS) activity of the multifunctional Fe_3O_4@Ag/SiO_2/Au core-shell microspheres. Fe_3O_4 is used as a core that helps in recycle the novel metals by magnetic means. Fe_3O_4 microspheres were prepared by solvothermal reaction; the Ag shell coated on Fe_3O_4 were prepared through thermal reduction, further shell of SiO_2 was synthesized via the sol-gel process and gold particles were coated using interfacial growth method. The microspheres had a uniform size and high magnetization. Rhodamine-b (RdB) was used as a probe molecule to detect the enhanced SERS spectra of these microspheres. The enhancement factor (EF) was estimated to be 2×10^4 for RdB at 1652 cm^{-1} [23].

From the studies, we have concluded that both alloyed and core-shell nanoparticles result in enhancement of the SERS spectra. But the magnetic core-shell nanoparticles have additional advantages than the non-magnetic core-shell nanoparticles. Due to the presence of magnetic material, magnetic core-shell nanoparticles can be easily isolated using magnets thus resulting in less time consumption as compared to old techniques.

2.4. BIOMOLECULAR DETECTION USING SERS SUBSTRATES

Surface-Enhanced Raman Scattering (SERS) plays a very important role in the detection of biomolecules and pathogens. SERS technique is non-destructive and sensitive for the detection of biological samples. Its ability to detect biomolecules or pathogens at an early stage resulted in improvement in disease diagnosis [24]. Some of the studies related to the detection of biomolecules or pathogens using SERS method are given below:

You et al. (2018) performed Surface-Enhanced Raman Scattering (SERS) detection of uric acid and creatinine using superhydrophobic silver films (AFS). Super-hydrophobic AFS was fabricated using the silver mirror reaction and modified with different thiols. The particle size of the Ag nanoparticles prepared was 20-80 nm. R6G was used as a probe molecule for SERS measurement. Strong signals were obtained on AFS-Dodec as a substrate which was further used

to detect uric acid and creatinine. An increase in the concentration of creatinine increased the intensity of Raman peaks [25].

Lu et al. (2020) carried out Surface-Enhanced Raman Scattering (SERS) detection of dopamine (DA) using dual molecule recognition. They proposed the coupling of silver nanocubes (AgNCs) with nanoporous silver film (AgNF) which resulted in effective low-level detection of dopamine with a LOD of 40 fM. AgNF was functionalized with mercaptopropionic acid (MPA) for specific capturing of DA. While DA was detected by 4-mercaptobenzene boronic acid (MPBA)-functionalized AgNC SERS substrate. SERS detection showed good sensitivity and reproducibility. Further detection of DA in the biological fluids such as human serum and urine samples was carried out resulting in excellent selectivity of DA [26].

J.Halas et al. (2008) studied Surface-Enhanced Raman Spectroscopy (SERS) of thermally treated single-stranded DNA (ssDNA) and thermally treated prehybridized double-stranded DNA (dsDNA) oligomers. The changes in SERS spectra due to the chemical modification of the adsorbate molecules were studied with the help of spectral correlation function (SCF). Further SERS spectrum of the interaction of DNA with cisplatin in comparison with transplatin was studied. The authors also explained the importance of adenine base in exhibiting single-molecule detectability [27].

Wang et al. (2019) synthesized amorphous shell and crystalline core Black-TiO_2 (B-TiO_2) nanoparticles using a solid-state method. Several analytes were used to study the SERS effect using B-TiO_2. Limit of detection (LOD) for dye probe molecules (R6G, CV) was determined to be 10^{-7} M. Enhancement factor (EF) of B-TiO_2 nanoparticles was calculated to be 4.3×10^5. They further discussed the advantages of synthesized crystal amorphous – core-shell B-TiO_2 nanoparticles in the diagnosis of cancer cells. MCF-7/ADR breast cancer cells were easily diagnosed due to the high sensitivity of synthesized B-TiO_2 nanoparticles [28].

CHAPTER 3

SYNTHESIS AND CHARACTERIZATION TECHNIQUES

3.1. SYNTHESIS OF NANOPARTICLES EXHIBITING SURFACE PLASMON RESONANCE

Nanomaterials are synthesized using two methods: top-down method and the bottom-up method. In the top-down method, nanoparticles are formed by the reduction of bulk materials. While in the bottom-up method, nanoparticles are formed from atom to clusters. These methods are further characterized into three processes: physical, chemical, and biological methods [29].

3.1.1. PHYSICAL METHODS

The physical methods mechanism works on a top-down approach. This method results in the production of uniform monodisperse nanoparticles and results in a contamination-free solvent. Mechanical pressure, high energy radiations, thermal energy, or electrical energy are used for the production of nanoparticles through physical methods. It is an expensive method [30]. The most commonly used physical methods are given in the flow chart (figure 4) and few are explained as follows.

a) **HIGH ENERGY BALL MILLING (HEBM):** HEBM was developed by John Benjamin in 1970. It is an energy-efficient method. In this method, the kinetic energy of moving balls is transferred to milled material resulting in the breakage of bonds and the formation of small particles of diverse shapes and dimensions. Physical and morphological properties of particles prepared depend on the milling media, milling speed, type of energy mill, milling atmosphere, the ball to powder weight ratio, dry or wet milling, and duration of the milling [29, 30].

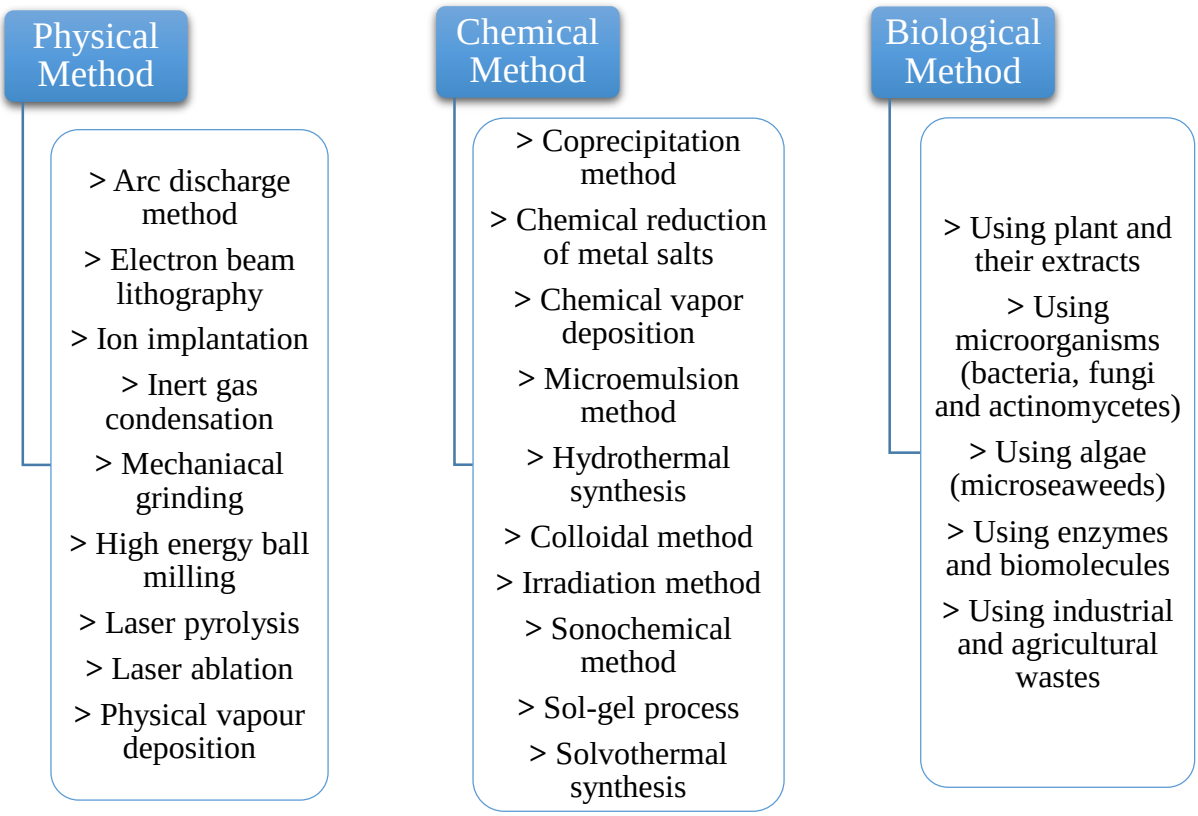

Fig 4: Flow chart on the different types of synthesis methods for the production of nanoparticles.

b) **INERT GAS CONDENSATION (IGC):** Inert gas condensation is a widely used physical method. In this process evaporation of matter under ultra-high vacuum takes place and then these vapors are transported with inert gas (He or Ar) and condensed onto the surface of a collection device attached with liquid nitrogen. Particles are collected in the form of a powder of low density on the collection device. Liquid nitrogen is used for cooling the device for the condensation process to occur. Morphology, crystallinity, and size distribution of the particles produced depend on the temperature mass of inert gas and gas pressure. This method is slow and not used for industrial production [30, 31].

c) **PHYSICAL VAPOR DEPOSITION (PVD):** Physical vapor deposition is an environmentally friendly method. This method comprises a set of steps for the production of nanoparticles and deposit thin films. These steps include vaporization of matter from a solid surface, transportation of vaporized material, and then nucleation to generate nanoparticles.

Nanoparticles of few nanometers to several micrometers can be synthesized by this method. Most commonly used methods that come under this category are [30]:

- Electron beam evaporation
- Pulsed laser deposition
- Vacuum arc method
- Sputtering techniques

d) LASER PYROLYSIS: Laser pyrolysis method results in the synthesis of a variety of oxide, non-oxide, and ternary composites. CO_2 laser pyrolysis work on the vapor phase process. Condensable products are generated due to the interaction between the interface of the laser beam and the gaseous phase reactants. For better interaction and energy flow to occur between laser beam and precursor (liquid or vapor), chemicals like ammonia, sulfur hexafluoride, etc. are used. It is a highly localized, simple, and cost-efficient method for large scale production [29, 30].

e) LASER ABLATION: The laser ablation method is an alternative to the conventional chemical reduction method. In this method, nanoparticles are synthesized by irradiation of the metal substrate by a laser beam. Nanoparticles synthesized by this method are stable in organic solvents and water. If the laser flux is low then the nanoparticles are synthesized by heating the material, which results in evaporation of the solid material. On the other hand, high laser flux results in plasma formation. This method is based on the evaporation-condensation technique. Noble metal nanoparticles with high melting points are generally synthesized using this method under the top-down mechanism [29-32].

3.1.2. CHEMICAL METHOD

The chemical method is a cost-effective method that results in the formation of a colloidal dispersion of nanoparticles or clusters in water or organic solvents [32]. Sol-gel method, microemulsion method, hydrothermal synthesis, co-precipitation method, chemical vapor deposition (CVD), and chemical reduction methods are few chemical methods, which are explained below.

a) **SOL-GEL METHOD:** Sol-gel method is a wet chemical method that follows the bottom-up approach for the synthesis of nanoparticles. Sol is defined as a solution of solids suspended in a liquid. While a gel is defined as solid macromolecules. Hydrolysis and condensation are used in the sol-gel method. In host liquid, one adds precursors (metal oxide, chloride, etc.) resulting in sol-gel formation. This method is assisted through the steps of sol evolving to gel, phase separation through centrifugation or drying to eliminate liquid phase, and at last heat treatment to favor polycondensation. This method is a low cost, a low-temperature, time-consuming technique [29-33].

b) **MICROEMULSION TECHNIQUE:** Microemulsion results in the formation of uniform and monodispersed nanoparticles. It is defined as thermodynamically stable, isotropic liquid mixture (oil and water) under the influence of surfactant, which is one of the main components besides the polar phase (water) and non-polar phase (hydrocarbon liquid or oil). It is of three types: oil dispersed in water (o/w), reversed microemulsion (w/o), and bicontinuous microemulsion (both water and oil in continuous phase). This technique is easy to use, result in minimum agglomeration of nanoparticles and high control on particle size and composition. The major drawback of this technique is: it needs a large number of surfactants and control on external factors for reproducible results [29-33].

c) **HYDROTHERMAL SYNTHESIS:** Hydrothermal method is mainly used for the synthesis of ceramic and inorganic materials. Thermal treatment of substance undergoing chemical reaction above ambient temperature and pressure is required for the synthesis of nanoparticles. Crystal growth is conducted in an autoclave, a steel pressure vessel in which water and minerals are provided for the reaction. This process is assisted by maintaining a temperature gradient. The solubility of minerals in hot water results in the dependency of single crystal growth. This process results in precise and easy control of particle size, shape, and crystallinity. Also using this method one can synthesize particles of the intermediate state, metastable state, and other specific phases [30, 31].

d) **CO-PRECIPITATION METHOD:** It is a chemical solution method, which produces homogeneous mixture and good stoichiometric control. The co-precipitation method undergoes the processes of nucleation, growth, and agglomeration. Supersaturation condition

is required for the product formation under the co-precipitation method. This method results in the production of particles in the presence of at least two different kinds of elements. It is a simple method works at low temperature. Co-precipitation method is time consuming method and is not used when uncharged species participates [31-34].

e) **CHEMICAL VAPOR DEPOSITION (CVD):** Chemical vapor deposition follows bottom-up approach and is performed at very high temperature. CVD is deposition of solid film from vapor phase. We can synthesize nanoparticles instead of solid films using CVD method by altering the conditions that are temperature, supersaturation, residence time, etc. This process is then named as CVS. Using CVS we can synthesize pure, uniform, strong and multicomponent or doped nanoparticles using different precursors. This method requires the use of special equipment's and the gaseous byproducts. It is highly toxic [29, 30].

f) **CHEMICAL REDUCTION:** Chemical reduction method is method widely and preferentially used method for the synthesis of noble metal nanoparticles. In this method reduction of ions is done using various reducing agents such as sodium borohydride, citrate, elemental hydrogen, etc. In chemical reduction method agglomeration of nanoparticles occur easily, thus a stabilizing agent is required [33, 35].

3.1.3. BIOLOGICAL SYNTHESIS

Biological synthesis is green approach for the synthesis of nanoparticles. This synthesis approach is slow but result is ecofriendly, stable and less toxic nanoparticles. This method requires no use of chemical reagents and complex apparatus. Some of the biological methods are summarized here:

a) **BIOLOGICAL SYNTHESIS USING MICROORGANISMS:** This method uses microorganisms such as bacteria, actinomycetes, fungi, algae and yeast for the synthesis of metal or metal oxide nanoparticles. These microorganisms interact with the metal ion to form elemental metal through enzyme reactions. This synthesis process consist of two methods: intracellular and extracellular method. When the metal ions are trapped inside the microbial cell to form nanoparticles in presence of enzyme, this method is known as intracellular method.

In extracellular method the metal ions are trapped on the surface of microbial cell. Synthesis of nanoparticles using fungi as a mediator has many advantages such as it is economical, has higher bioaccumulation and simple biomass holding [30, 31, 35].

b) **BIOLOGICAL TEMPLATES TO SYNTHESIZE NANOPARTICLES:** Biomolecules such as nucleic acids, membranes, viruses and diatoms are used as templates to synthesize nanoparticles. Based on strong interaction with metal ions, DNA and proteins are widely used for the synthesis process. Biological membranes such as rubbers are also used due to the presence of their ultra-fine pores. It has been observed that on using viruses as templates we can synthesize uniform size and morphology of nanoparticles [30, 31, 35].

c) **NANOPARTICLE SYNTHESIS USING PLANT EXTRACTS:** Synthesis of nanoparticles using plant extracts or plant biomass is an effective, safe, rapid and eco-friendly process. In this method plant extract are used as capping ligands and reducing agents to synthesize metallic nanoparticles. We can synthesize metal oxides, noble metals, bi-metallic alloys etc. using plant extracts [30, 31, 35].

3.2. CHARACTERIZATION TECHNIQUES

3.2.1 X-Ray Diffraction

X-Ray diffraction method is most widely used method for the determination of crystal structure. This method was developed in 1912 for material characterization. X-Ray diffraction method is of two types: Spectroscopic technique and Photographic technique. Spectroscopic technique is mostly used and known as X-Ray powder diffractometry in which sample in the form of powder is used. Commonly instead of powder, polycrystalline aggregate solids are used as sample. The instrument for X-Ray diffraction method is called an X-Ray diffractometer and consist of X-Ray source, specimen, slits and detector. This method works on the principle of diffraction of X-Rays. When the X-Ray beam at different angles is made to fall on the sample, it results in the formation of spectrum of diffraction intensity versus the angle between incident and diffracted beam. The spectrum observed is compared with known database of crystalline substances to determine the

crystal structure and quality of the sample thus analyzed. Diffracted beams should fulfill the Bragg's equation stated as,

$$n\lambda = 2d \sin\theta$$

Where, n = an integer (order of reflection)

λ = wavelength of X-Rays

d = spacing between parallel crystal planes

θ = incident angle

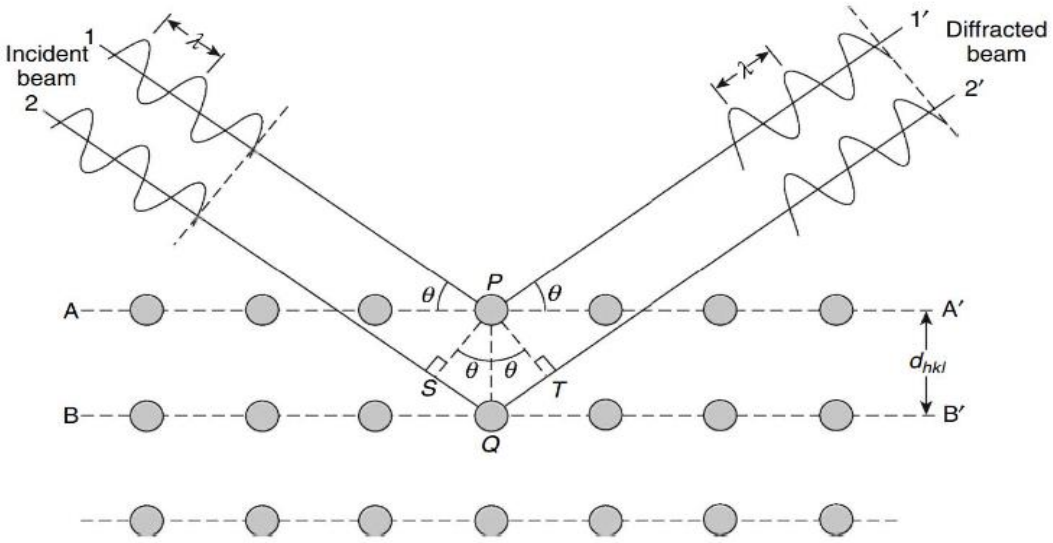

Fig 5: Crystal planes showing Bragg diffraction [36].

In the figure, two incident X-ray beams (1 and 2) are diffracted by the crystal planes (A and B). The diffracted beams (1′ and 2′) result in constructive interference only if they satisfy the Bragg's equation. Knowing the inter-planar distance we can calculate lattice parameters thus can determine the crystal structure [36]. We can also calculate the crystallite size using Scherrer equation given

by Paul Scherrer and Peter Debye. The mean particle size of the crystal can be calculated by observing intensity of peaks in XRD spectra. The Scherrer equation is given by,

$$D_{hkl} = K\lambda / (B_{hkl} \sin\theta)$$

Where, (hkl) are the miller indices, B_{hkl} is the full width half maxima (FWHM) of X-ray diffraction peak corresponding to a particular Bragg angle (θ). K is numerical factor known as crystallite shape factor, which depends on crystallite shape, width and vary according to the definition of average crystallite size [37].

3.2.2 Scanning Electron Microscope (SEM)

Electron microscopy uses short wavelength electrons to study the microstructure of the material. Scanning electron microscope is a type of electron microscopy. We can determine surface topography, composition and other properties of the material under study using SEM. It consist of electron gun, a number of electromagnetic lenses or condenser lens, specimen stage, secondary electron collector and signal processing system [36]. And to avoid the deviation of electron beam on striking with air particles SEM is operated at high vacuum. Beam of accelerated electrons are generated using tungsten filaments in most of the SEM used nowadays since they are economical. Field emission gun is also used as they result in high brightness of beam more than that of tungsten [38].

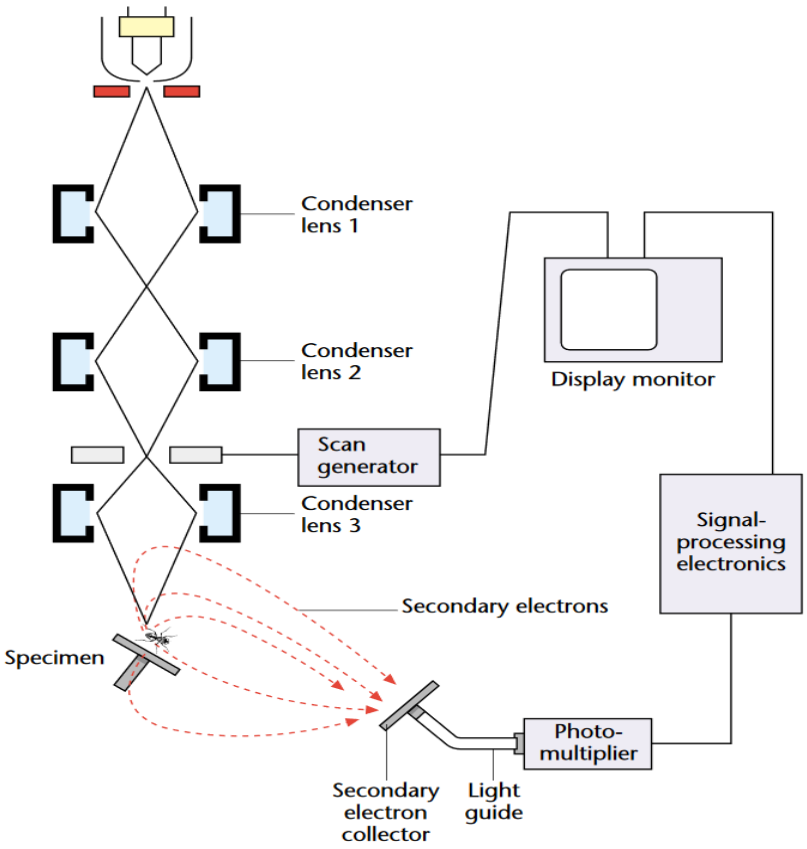

Fig 6: Schematic diagram of scanning electron microscope (SEM) [38].

On the interaction of incident electron beam with the sample, two types of electrons are detected by SEM. If the interaction is elastic in nature then the electrons detected are called backscattered electrons (BSE). By detecting BSE we can differentiate between the atomic number of different elements as we observe bright image if the atomic number is higher as compared to the atomic number of other element present in the sample and vice-versa. One can detect secondary electrons (SE), if the interaction between the incident beam and sample is inelastic [39]. BSE are produced from depth whereas SE is produced from the surface of the sample and therefore we can study the topography of the sample on studying SE. In general SEM can resolve particle sizes upto 10 nm. The electron beam is focused on very tiny spots of the specimen and result in 3D image formation because of its large depth of field. The surface area of the sample is scanned in raster scan pattern [36].

3.2.3 Transmission Electron Microscope (TEM)

Transmission electron microscopy is the study of electrons that are transmitted from the specimen. TEM helps in the characterization of morphology, crystallographic and atomic structure of the sample under study. TEM consist of:

- Electron gun
- Condenser lenses
- Specimen stage
- Objective lenses
- Projector lenses
- Fluorescent screen / CCD device

A beam of accelerated electron beam in TEM is generated by providing negative potential and heating the tungsten filament. The electron beam is further focused on the specimen using condenser lenses. The interaction of the electron beam with the specimen results in two dimensional image formation. TEM is operated under high vacuum. We can depict the mass of component by observing the TEM image. If deflection of electrons occurring over an area with large mass result in formation of dark image. And when deflection from area of small mass occurs then a brighter image is observed [38]. TEM has higher resolution than SEM. The resolution of TEM is 0.1 nm if lens aberrations are minimized [36].

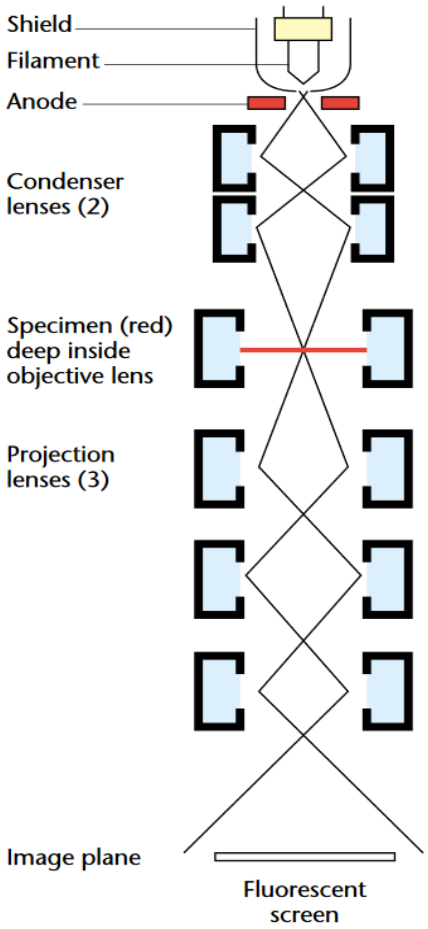

Fig 7: Schematic diagram of transmission electron microscope (TEM) [38].

3.2.4 Ultraviolet-Visible (UV-Vis) Spectroscopy

Ultraviolet visible spectroscopy is a method that study the interaction of electromagnetic field with the material under study. The components of UV-Visible spectrophotometer are:

- Light source
- Monochromator
- Cell holder
- Detectors

This method study the absorption of light that passes through a dilute solution contained in a cell. There are two type of light source used. For the production of visible light, tungsten filament is

used. And deuterium arc lamp is used for the generation of ultraviolet radiation. Since, monochromatic light is required to study, a monochromator is used to convert polychromatic light into monochromatic light.

Fig 8: UV-Visible spectrometer UV-2600 from Shimadzu [40]

The electromagnetic radiations are partially absorbed by the solution resulting in increase of energy content of the molecules. Thus absorption of the radiations results in transition of electrons in the outer orbitals of the molecule. UV-Visible spectroscopy is also known as electron excitation spectroscopy, as the energy of the absorbed radiations is given by the energy difference between the energy levels of two orbitals. The radiations absorbed result in an absorption spectra. These sharp lines corresponds to the energy difference between the energy levels of two orbitals. The intensity of light absorbed is related to the concentration and path length of the solution. The relation is known as Beer-Lamberts law or Bouguer-Lambert law given as,

$$\boldsymbol{A = \log(\frac{I_0}{I}) = \varepsilon c L}$$

Where, I_0 = intensity of incident light

I = intensity of transmitted light

ε = molar absorptivity

c = concentration

L = optical path length

Knowing the value of absorption of light, we can determine the concentration of the solution under study. Thus UV-Visible spectroscopy is useful in determining the concentration of material by studying the absorption of the electromagnetic radiations [41].

3.2.5 Fourier Transform Infrared (FTIR) Spectroscopy

Fourier transform infrared spectroscopy is one of the vibrational spectroscopy that uses Fourier transform to obtain an infrared spectrum. FTIR deals with the study of chemistry of chemical bonding. The main component of FTIR is Michelson interferometer and other components are:

- Infrared light source
- Beam splitter
- Infrared detector
- Fourier transform

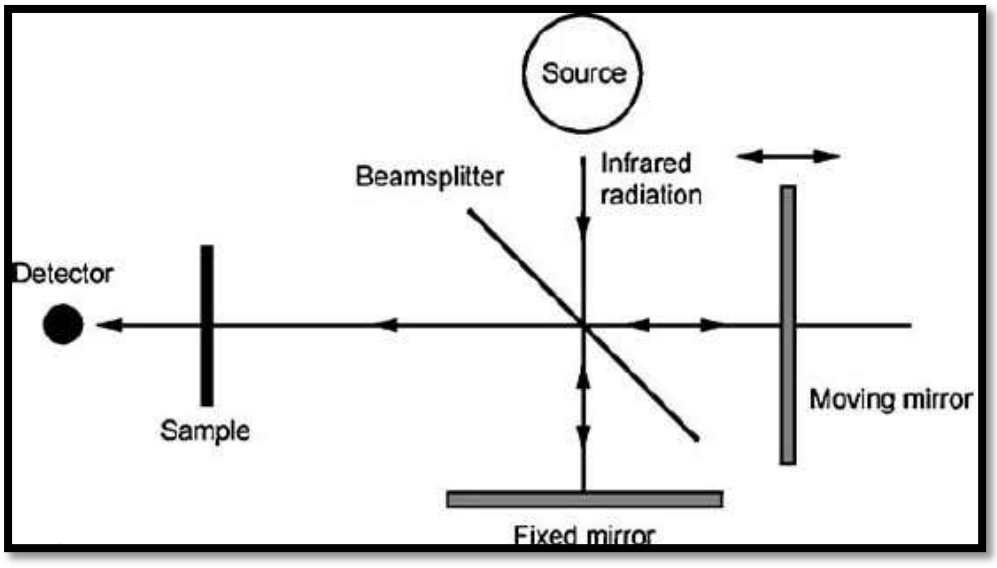

Fig 9: Michelson interferometer diagram [37].

Michelson interferometer consists of a beam splitter, which transmits half of the infrared beam from the source. FTIR uses carborundum (SiC) as infrared light source. The infrared beam obtained after splitting is reflected from two mirrors which are part of Michelson interferometer. After reflection from the mirror, the radiations are combined at the beam splitter to irradiate the sample. The two split beams result in constructive and destructive interference depending upon the path difference (δ) produced by moving the mirror away from the beam splitter. Interference intensity as a function path difference results in a plot formation known as interferogram. The study of interferogram transmitted or reflected from a sample is done using FTIR. The interferogram detected is not an infrared spectrum thus Fourier transform is used to transform it. Thee interferogram obtained is a plot of light intensity versus wavenumber. Transmittance and absorbance of the sample is studyed with the help of interferogram. The absorbance spectrum is mostly used when dealing with quantitative analysis. The absorbance is calculated from the transmittance. The equation for calculating the absorbance is given as,

$$A = -\log T$$

Where, T is the transmittance defined as ratio of intensities [37]

$$T = I / I_0$$

3.2.6 Dynamic Light Scattering (DLS)

Dynamic light scattering technique is an optical technique that studies the frequency fluctuations in scattered light of a dispersive system. DLS is used for the analysis of particle size. DLS make use of Brownian motion for the determination of distribution of particles hydrodynamic equivalent diameter. The hydrodynamic size is not the actual size of the particles under study but rather include the size of the outer radius of the particle or particle agglomerates. DLS consist of a laser beam, which is used for illuminating the test sample. The laser beam is scattered through the sample at a particular angle (θ). The signals are detected by a detector resulting in the formation of a plot of scattered signal versus time. The fluctuations occurring due to the thermal motion of

scattering material, which occur at small time scale of microsecond or nanosecond is studied through DLS.

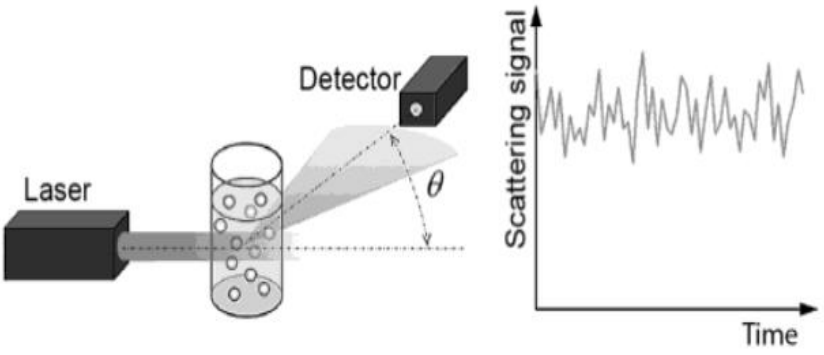

Fig 10: Diagram showing principal of DLS and plot of intensity of scattered light versus time [42].

The particle size of the sample can be determined by calculating the particle diffusion coefficient D_P given as,

$$D_P = (K_B T) / (3\pi\eta x_{h,t})$$

This equation is known as Stokes-Einstein equation. Here, K_B and T are Boltzmann's constant and temperature respectively, while η is the dynamic viscosity. And $x_{h,t}$ is the hydrodynamic diameter of translational motion [42].

CHAPTER 4

BIOMOLECULAR DETECTION USING SURFACE ENHANCED RAMAN SCATTERING

Detection of biomolecule plays vital role in health care. It has applications over wide range of fields from forensic to environmental research. The need to detect biomolecules especially at low concentration is increasing day by day. The old pathological methods have their limitations as discussed in chapter 1, thus the introduction of surface-enhanced Raman scattering (SERS) is required. Owing to its high sensitivity, we can detect biomolecules at low concentration using noble metal nanoparticles [43]. The enhancement occurring due to the electromagnetic effect and little contribution of chemical effect of the noble metal nanoparticles are the main factor resulting in the enhancement of spectra of SERS. Till now various studies have been conducted to study SERS effect using different biomolecules. We are thus going to discuss about the detection of some biomolecules in order to understand the need and importance of SERS.

DETECTION OF DEOXYRIBONUCLEIC ACID (DNA)

DNA is an important biomolecule and its detection plays an important role. From diagnosis for healthcare to forensic analysis, DNA detection has applications over a wide area. Polymerase chain reaction and microarray method are some of the old methods used for the detection of DNA but are time consuming, costly, results in loss of chemical structure and many other limitations restrict their use [44, 45]. SERS technique eliminate the limitations of traditional methods and grant additional advantages.

As observed by Green et al. (2005) who has fabricated highly sensitive SERS platform, which resulted in enhancement factor of 10^8 compared to traditional fluorescence imaging used to identify the labeled analyte. SERS platform was formed typically of silver on a SiO_2/Si substrate. The substrate was synthesized using island lithographic technique whereas galvanic exchange method was used to deposit silver. Stabilizing agent was required due to unstable torus

structure of silver. Initially immobilization of oligonucleotide probe and its hybridization effect on SERS was studied. Study on 18 different bases such as oligo A, oligo C, oligo T etc., as well as various equi-molar mixed solution was carried out. The spectra of these bases taken at the same time was easily distinguishable as compared to the other Si colloids or SERS substrates. Then further SERS spectra of single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) for adenine base and cytosine base showed different intensities before and after hybridization. SERS spectra of in-situ that is when probe is present with target was also studied. They observed appropriate Raman signal around 5×10^{-8} to 1×10^{-7} M. It was also observed that to detect low signals the total amount of target DNA should be greater than the total amount of probe DNA [45].

Similarly, Braun et al. (2007) studied the detection of single stranded DNA (ssDNA) using SERS by self-assembling silver nanoparticles to a smooth silver film. They synthesized a label free bio-sensor. The diameter of silver nanoparticles was determined to be around 15 nm and thickness of Ag film was 100 nm. They have also used 24-mer oligonucleotide as a model analyte. To prevent non-specific attachment of nanoparticles or impurities, Ag film was coated with 6-mercaptohexanol (MCH) and b-Ag film was treated further with SAMSA fluorescein (F). The surface density of silver nanoparticles was about 3 particles/μm^2. Signals of very strong intensity were observed when SERS spectra of a-Ag nanoparticles were recorded. Featureless spectra was observed when b-Ag Film-F combined with a-Ag nanoparticles. Thus on coating and using metal film for the assembly of nanoparticles we can get more efficient and better substrates for the detection of DNA using SERS [46].

Marini et al. (2014) discussed a novel approach for the detection of DNAs on suprehydrophobic (SuperHyPho) substrate. Using surface enhanced Raman scattering (SERS) technique, DNA of low concentration upto 60 picomolar was detected. SuperHyPho substrate were employed owing to the low scattering shown by DNA. They were made by optical lithographic technique and reactive ion etching of microPillars over silicone surface. Lithographic technique was particularly used to employ regular patterns of disks. Raman spectra of single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) were recorded on separate SuperHyPho surfaces. The spectral range was 2500-3500 cm^{-1} for the Raman mapping and spectra was observed for very

low concentration of ssDNA. Good reproducibility was also observed. Clear spectral difference between the Raman spectra of ssDNA and dsDNA was also observed in the spectral range of 900-1350 cm^{-1} [43].

Xu et al. (2015) conducted detection of single base DNA in aqueous solution using iodide coated silver nanoparticles which showed good reproducibility of SERS signals. Listing the limitations of pathological methods they proposed of coating iodide on chemically synthesized Ag nanoparticles. The coating resulted in clear surface in addition to modified structure of nanoparticles. $MgSO_4$ was also added to improve the interaction of negatively charged DNAs. Further, they have also studied the spectra of DNA bases adenine and cysteine and polynucleotides. Hybridization effect was also observed [44]. From these studies we conclude that SERS effect result in better detection of low concentration of DNA and better results can be observed when using a hydrophobic substrate.

DETECTION OF DOPAMINE (DA)

Dopamine (DA) is the one of the important neurotransmitter in our central nervous system. The decrease in its amount in humans can result in serious diseases such as Parkinson's disease (PD). Various methods such as fluorescence, colorimetric, and electrochemical methods have been employed for the detection of DA [26]. In 2009, Gu et al. [47] conducted an experiment for the volumetric detection of DA. Volumetric measurement is one of the electrochemical method used for the detection of bio molecules. They made use of gold nanotadpoles synthesized using seeding approach. On observing the cyclic voltammetry (CV) measurement it was found that the anode peak current depends linearly on the concentration of DA. The range of concentration was of $3 \times 10^{-5} \sim 1.8 \times 10^{-4}$ M with correlation factor of 0.999 and detection limit was 0.1 μm. One of the limitations of this method is that we are not able to distinguish between the biomolecule and the coexistence of interfering molecules, effecting the sensitivity and selectivity [47]. Also, the detection of single molecule is not possible using these traditional methods. As, the concentration of DA is found to be very small in biological fluids, ranging from 1 nm to 1 μm.

Thus using SERS substrates we can attempt to detect low concentration of DA. Wang et al. (2015) conducted detection of dopamine and serotonin using graphene - gold nanopyramid heterostructure as surface enhanced Raman scattering (SERS) substrate. Gold nanopyramid were fabricated by lithography. Graphene was synthesized using CVD method and transferred on Au nanopyramid using poly(methyl methacrylate) (PMMA). Enhancement factor of over 10^{10} was observed on studying the micro-Raman spectra. EFs of dopamine and serotonin were determined to be ~ 2×10^8 and 10^9. Finite difference time fdomain (FDTD) analysis was also conducted to study the electric field enhancement of Au nanopyramids. They were able to detect dopamine and serotonin upto 10^{-10} M concentration using SERS. Detection upto concentration of 10^{-9} M was also done in cell culture medium DMEM with 10% fetal bovine serum (FBS) or simulated body fluids (SBF) with 10% FBS [48].

Jiang et al. (2015) performed the SERS detection of DA using silver nanoparticles on the surface of metal-organic framework. Metal-organic framework are good absorbent, thus can be used to develop SERS substrates. In situ synthesis of silver nanoparticles was carried out by using tannic acid (TA) as a reducing agent. High density of Ag nanoparticles on the surface of metal-organic framework MIL-101(Fe) resulted in increment of Raman hot spots. DA in human urine at low concentration was detected with Ag nanoparticles as SERS substrate and good reproducibility of SERS signals using malachite green (MG) was observed. SERS effect was studied using a number of common analytes and enhancement factor of Rh6G was found to be 1.8×10^5. They have also observed reproducible detection of 105.4 pM of DA in urine [49].

Lu et al. (2020) used silver nanocubes (AgNCs) coupled nanoporous silver film (AgNF) as SERS substrate. Silver nanocubes due to their anisotropic property resulted in SERS enhancement. Silver films due to their high porosity and surface area on combining with Ag nanocubes resulted in high sensitivity detection of dopamine. Ag nanocubes were synthesized by chemical reduction of $AgNO_3$ by glucose while Ag nanofilm was prepared by silver mirror reaction. 4-MPBA-functionalized AgNC SERS substrate resulted in efficient and selective detection of DA. R6G (10^{-6} M) was used as probe molecule for the substrate of AgNCs on AgNF showing considerable enhancement. Enhancement factor of 5.5×10^{10} was calculated for

"AgNCs on AgNF". Limit of detection (LOD) for DA was determined to be 40 fM. Detection of DA in biological fluids, blood and urine was also conducted. The intensity of Raman peaks were observed to decrease with the decrease in concentration from 10^{-8} M to 10^{-12} M [26]. From these studies it is concluded that pathological methods are not able to detect low concentration of DA. While different SERS substrates can result in DA detection at very low concentrations. In most recent work by Lu et al. [26] detection of DA as low as 40 fM was successfully carried out by using heterogeneous substrate.

DETECTION OF URIC ACID (UA)

Uric acid (UA) or 2, 6, 8-trihydroxypurine is main product of purine in humans. Deviation in concentration of uric acid can cause the development of various diseases such as gout, diabetes, hyper-uricaemia, Lesch-Nyan disease, chronic impairment, high cholesterol, pre-eclampsia, and many others. It is an important biomolecule referred as biomarker in urine and serum samples. The concentration of UA under normal conditions lies between 120 – 380 µM while its concentration in blood lies in the range 208.2 – 416.4 µM and 951.8 – 5948.5 µM per 24 hr in urine [50, 51, 52]. Lakshmi et al. (2010) studied various approaches for the detection of uric acid using electrochemical technique. The electrochemical method is relatively faster method in comparison with other laboratory techniques. They studied the electrochemical oxidation of UA under various conditions. But a modified electrode is required to avoid interference of ascorbic acid (AA) in UA detection. The limit of detection of UA on using preanodised nontronite coated carbon electrode was 0.42 μmol^{-1}. Sulfur adlayer modified gold electrode detected 0.4 µmol L^{-1} UA in human urine. Pencil graphite electrodes detected 1.5 nmol L^{-1} of UA. Further studies using electrodes modified with polymeric films were carried out. Their major advantage is they performed electro-catalysis and prevent surface fouling. Molecular imprinted polymers had detected UA upto the detection limit of 10^{-5} mol L^{-1} – 10^{-7} mol L^{-1} [50]. The electrochemical method is able to detect UA better but it is not sensitive for UA detection at very low levels. Thus, surface enhanced Raman scattering is employed for the detection of UA in place of these traditional methods.

Lu et al. (2018) conducted the detection of uric acid and creatinine using superhydrophobic silver films as SERS substrate. Superhydrophobic substrate resulted in high density hotspots enabling the detection at low concentrations. Silver mirror reaction method was used for the synthesis of Ag film (AFS) and was functionalized using thiol. They have also studied the condensation effect of superhydrophobic surfaces and how it effects the detection sensitivity of SERS. The SERS effect was studied by using Rhodamine 6G (R6G) as probe molecule. It was found that the intensity of Raman peaks increased on increasing the carbon chain length of thiol and decreased on decreasing the concentration of R6G. The concentration of R6G detected between 10^{-8} to 10^{-11} M. Further, the linearity between the Raman intensity of the peak and concentration in case of uric acid and creatinine was achieved in the range of 5∼1000 μM. The limit of detection of UA on using superhydrophobic-AFS was determined to be 5 μM [51].

Alula et al. (2019) conducted the detection of uric acid with LOD of 0.365 μM at 1145 cm^{-1}. They used silver nanoparticles coated ZnO/Fe_3O_4 composite that resulted in such a sensitive detection of UA. The introduction of magnetic particles resulted in additional benefits by reducing the filtration or centrifugation. The silver particles were deposited on ZnO/Fe_3O_4 using thermally induced chemical reduction method. ZnO/Fe_3O_4 was used as catalyst and ethylene glycol as solvent cum reducing agent. Hydrothermal method was used for the synthesis of Fe_3O_4 nanoparticles, while Ag nanoparticles were prepared in water bath at temperatures below 100°C. Raman spectroscopy was used for the study of SERS effect using p-aminothiophenol (pATP), p-nitro-thiophenol (pNTP) and Rhodamine 6G (R6G) as probe molecules. They studied the effect of concentration of silver nitrate on SERS intensity of pATP at constant relaxation time and temperature. Selective and sensitive detection of UA and UA in urine was carried out using Ag/ZnO/Fe_3O_4 composite. Saturation of UA with Ag nanostructure occurred above the concentration of 15 μM [52].

CONCLUSION:

The improvement in the clinical diagnose is needed for early and selective detection of diseases. With the current disruptive life style there is an increment in the number of people being affected with life threatening diseases which are difficult to detect in their early stages. Traditional methods

used in pathology are not appropriate to meet these challenges as they are time consuming, cannot detect very low concentration of molecules and many other limitations restricting their use. From this study, we are able to understand the importance of surface enhanced Raman scattering (SERS) for low level detection of biomolecules. By using SERS technique, we can eliminate these limitations. SERS provides fast low level detection of various important biological / pathological biomarkers.

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