Chapter 3

*Structural, Optical, and Biological Characterizations Techniques*

“Outside their laboratories, the physicians and chemist are soldiers without arms on the field of battle.”  
- Louise Pasture
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“Science does not know its debt to imagination.”
- Ralph Waldo Emerson

3.1. Introduction

In this thesis various biological routes like microbial, plant products and fermented product, are explained for the synthesis of silver nanoparticles. The phase analysis, compositional analysis, surface characterization, structural analysis and the toxicity of formed nanoparticles towards pathogenic microorganisms and mammalian cells were characterized by various techniques viz UV-visible (UV-Vis) spectroscopy, Fluorescence Spectroscopy, X-ray diffraction (XRD), Scanning Electron Microscopy (SEM), Energy dispersive microanalysis (EDAX), Transmission Electron Microscopy (TEM), Fourier Transform Infra-Red (FTIR) Spectroscopy. Fluorescence Microscopy, Confocal Laser Scanning Microscopy (CLSM), Dynamic Light Scattering (DLS) and zeta potential, and toxicology Study: cytotoxicity studies. This chapter dedicated to explain of basic principle of the techniques used for characterization.

3.2. Structural Characterization Techniques

3.2.1. X-Ray Diffraction (XRD)

X-ray diffraction is a very powerful and basic technique for the characterization of materials and thin films. It is non-contact, non-destructive technique, and provides useful information, such as grain size and orientation, presence and composition of phases, film thickness, and strain state [1].

The XRD technique mainly depends on the diffraction of x-ray radiation which in general occurs only when the wavelength of the wave motion is of the same order of magnitude as the repeat distance between scattering centers. This condition of diffraction is recognized as Bragg’s law as Bragg scientifically studied the diffraction from crystalline material and formulated the mathematical expression and is represented in equation 3.1,

\[ 2d \sin \theta = n \lambda \]  \hspace{1cm} ............ (3.1)
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where,  \( d \) = Interplanar spacing
\( \theta \) = diffraction angle
\( \lambda \) = wavelength of x-ray
\( n \) = order of diffraction

A typical diffractometer consists of a source of X-ray and for the detection of diffracted X-rays, a detector. Based on the Bragg-Brentano geometry a design of common diffractometer is prepared. Figure 3.1 shows the schematics of the X-ray diffractometer. A crystalline solid is a unit cell where atoms are ordered in a particular repeated pattern referred with its inter-atomic spacing comparable to wave length of x-rays (0.5 to 2.5\( \AA \)) [2]. Hence crystals are the best gratings for the diffraction of x-rays. The directions of diffracted x-rays provide information about the atomic arrangements and hence the phase formation and crystal structure can be confirmed by x-ray diffraction studies.

![Figure 3.1. The typical insight of XRD and image of Rigaku Miniflex 600 diffractometer](http://pubs.usgs.gov/of/2001/of01-041/htmldocs/xrdp.htm)

The way of satisfying Bragg’s condition is formulated and can be done by continuously varying either \( \lambda \) or \( \theta \) during the experiment. The way, in which
these quantities are varied, distinguish the three main diffraction method; Laue method, rotating crystal method, and powder method.

**Phase Identification**

Phases can be identified from the d-spacing in a sample using the standard JCPDS powder diffraction file data provided by the International Center for Diffraction Data (ICDD) and the reflections can be indexed with Miller indice. However, there is no more complete destructive interference at \( \theta \pm d\theta \), if size of the diffracting crystal is little tiny, which broadens the peak corresponding to diffracted beam in proportion to the size of the small crystal. This broadening of the peak can be used for the determination of crystallite size. The equation of calculating crystallite size is given by Scherrer and it is termed as Scherrer equation 3.2;

\[
    t = \frac{0.9 \lambda}{\beta \cos \theta}
\]

Where, \( t \) is particle size, \( \lambda \) the wavelength of X-rays \( \theta \) is diffraction angle, and \( \beta \) line broadening at Full Width at Half Maxima (FWHM).

Here in this thesis, the structure and crystalline size of the biological synthesized nanoparticles were recorded by Rigaku Miniflex 600 diffractometer Cu Ka \( (\lambda=1.54 \ \text{Å}) \) with an accelerating voltage of 40 KV in the 2 \( \theta \) range from 20° to 80°.

**3.2.2. Scanning Electron Microscopy (SEM)**

Scanning Electron Microscopy is a powerful tool for surface observation as they provide better resolution and depth of field than optical microscope. It provide images of three dimensional objects because it records the secondary electrons that are released from the sample by the electron beam impinging on it not the electrons passing thorough the specimen (as in TEM) [3]. Through a series of magnetic lenses designed to focus the electrons to a very fine spot the electron beam travels downward. At the bottom, row by row, a set of scanning coils moves the focused beam back and forth across the specimen. As the
electron beam strikes each spot on the sample, the secondary electrons are generated from the surface of sample. A detector counts and sends electrons to an amplifier. From the number of electrons emitted from each spot on the sample the final images are built. The ray diagram of SEM and recorded SEM image of prepared sample is shown in Figure. 3.2. The SEM images of formed nanoparticles have been taken using SEM, JEOL JSM 6360 having resolution of 3 nm, acceleration voltage from ranging 0.5KV to 30 KV and maximum magnification up to X 3,00,000.

![Ray diagram of Scanning electron microscope and image of Ag-alginate biohydrogel](http://zenofstem.com/project/using-the-sem/)

**Figure 3.2.** Ray diagram of Scanning electron microscope and image of Ag-alginate biohydrogel

### 3.2.3. Transmission Electron Microscopy

Transmission electron microscopy is a very powerful technique to study size, shape, morphology, particles size distribution, and crystallinity of the nanoparticles. It provides a real space image of atomic distribution in nanocrystals. It can give atomic resolution chemical information and lattice images at spatial resolution of 1 nm or less than that [4]. TEM consists of objective lens and one or more projector lenses, an imaging system. Objective
determines the degree of resolution in the image. The initial enlarged image of the illuminated portion of the specimen in a plane is formed by objective lens that is suitable for further enlargement by the projector lens. The projector lens projects the final magnified image on the screen or photographic emulsion. In a transmission electron microscope, a high-powered beam is used to essentially shoot electrons through the object. The electron beam first passes through the electromagnetic condenser lens in order to concentrate the beam on the sample. Then the beam penetrates through the object. Some of the electrons pass through whereas others hit molecules in the sample and scatter. The transmitted beam then passes through an objective lens, a projector lens and onto a fluorescent screen where the final image is observed. The pattern of scatter gives the observed and comprehensive view of the interior of the object because the electron beam passes entirely through the object (figure 3.3).

Figure 3.3. The schematic of transmission electron microscopy (TEM) [Source: http://www.ammrf.org.au/myscope/tem/introduction/]

The transmitted beams or some of the forward scattered beams are used in conventional TEM, only to create a diffraction contrast image whereas high
resolution transmission electron microscopy (HRTEM) uses the transmitted and the scattered beams to create an interference image. HRTEM can be used for phase analysis by measuring the interplanar distance. TEM also provides selected area electron diffraction (SAED) pattern similar to XRD pattern. Figure x showing the ray diagram of working of TEM and TEM image of the silver nanoparticles with SAED pattern.

In this thesis, TEM has been extensively used to visualize nanoparticles formed in different biological process. It has also been employed to obtain the SAED patterns that lead to the identification of nanoparticles composition and crystal nature. The TEM measurements were done on a Philips CM200 instrument equipped with a field emission gun electron source operated at an accelerating voltage of 200 kV.

3.3. Spectroscopic Characterization Techniques

3.3.1. Ultra violet- Visible (UV-Vis) spectroscopy

![Figure 3.4](http://teaching.shu.ac.uk/hwb/chemistry/tutorials/molspec/uvvisab3.htm)

**Figure 3.4.** The basic principle of double beam UV-Vis spectrometer and image of Shimadzu UV-1800 [Source: http://teaching.shu.ac.uk/hwb/chemistry/tutorials/molspec/uvvisab3.htm]
UV-Vis spectroscopy refers to absorption spectroscopy or reflectance spectroscopy in the ultraviolet-visible spectral region. UV-Vis spectroscopy has been routinely used not only in analytical chemistry for the quantitative determination of different analytes, such as highly conjugated organic compounds, transition metal ions, and biological macromolecules, but also, in recent years, high-precision, high-energy spectrophotometers are in the field due to the rapid increase in reflection and absorption measurements on solid samples, including semiconductors, films, glass, and absorbing materials. This absorption spectroscopy uses electromagnetic radiations between 190 nm to 800 nm and is divided into the ultraviolet (190-400 nm) and visible (400-800 nm) regions.

Light is a form of energy and so absorption of light by matter causes the energy content of the molecules (or atoms) to increase. The total potential energy of a molecule is given by equation 3.3,

\[ E = hv, \quad v = c/\lambda \]  

UV-visible absorptions in organic molecules occur as a result of transition of valence electrons between molecular orbitals. The total energy change of the molecule, \( \Delta E_{\text{total}} \), is given by equation 3.4

\[ \Delta E_{\text{total}} = \Delta E_{\text{elec}} + \Delta E_{\text{vib}} + \Delta E_{\text{rot}} \]  

where \( \Delta E_{\text{elec}} \), \( \Delta E_{\text{vib}} \) and \( \Delta E_{\text{rot}} \) are the changes in electronic, vibrational and rotational energy respectively. The amount of energy a molecule possesses in each form is not a continuum but a series of discrete levels or states. Among the different states the differences in energy are in the order. Photons of UV and visible light have enough energy to cause transitions between the different electronic energy levels in some molecules and atoms. The energy required to move an electron from a lower energy level to a higher energy level is the wavelength of light absorbed.

The basic principle of the UV-Vis spectroscopy is based on the Beer-Lambert law The Beer-Lambert law provides relation of concentration and light intensity as shown in equation 3.5:
\[ I = I_0 \times 10^{-\varepsilon c l} \] ........................ (3.5)

where \( \varepsilon \) is the molar absorptivity or molar extinction coefficient, and \( l \) is path length. Putting this equation together with that connecting absorbance and light intensity gives the expression equation 3.6:

\[ A = c \varepsilon l \] ........................ (3.6)

where, \( A \) is measured absorbance and \( c \) is the concentration of the analyte.

As explained in the chapter 2, the noble metal nanoparticles show the characteristic surface plasmon resonance where they absorb light strongly in the visible region. So the UV-Vis spectroscopy is the prime characterization technique to analyze the nanoparticles synthesis process [5]. All the UV–Visible absorption spectra existing in the thesis was carried out on Shimadzu UV-1800 dual beam spectrophotometer (figure 3.4).

3.3.2. Fluorescence spectroscopy

When a substance absorbs light energy at a short wavelength and then emits light energy at a longer wavelength, fluorescence is generated. Most molecules rest at the lowest vibrational level of the ground electronic state at room temperature and get elevated to the excited states once absorbs light. The excited molecules rapidly lose its excess of energy by collision and drops to the lowest vibrational level of the excited states. In addition, almost all molecules at electronic state higher than the second undergo internal conversion and pass from the lowest vibrational level of the upper state to a higher vibrational level of a lower excited state, which has the same energy. The molecules again lose energy till reach to the lowest vibrational level of the first excited state. From this level, the molecule can come back to any of the vibrational levels of the ground state where it emits its energy in the form of fluorescence.

The measurement of fluorescence signals provides a sensitive method of monitoring the biochemical environment of a fluorophore. Instruments have been designed to measure fluorescence intensity, spectrum, lifetime and polarization. A typical fluorometer includes a light source, a specimen chamber
with integrated optical components, and high sensitivity detectors (figure 3.5). The most common light source for fluorometers is lamp sources, such as xenon arc lamps. These lamps provide a relatively uniform intensity over a broad spectral range from the ultraviolet to the near infrared. The optical paths of the excitation as well as the detection light paths are along the orthogonal axis. The orthogonal arrangement ensures minimal escape of excitation light into the detection side. High sensitivity photodetectors such as photomultipliers or charge-coupled device cameras are commonly used [6].

Here in this study, the fluorescence of the silver nanoparticle colloidal solution synthesized from the cell free extract phenol degraded broth. The measurements were recorded using fluorescent spectrometer (model JASCO) at 23 kV. Emission spectra of AgNPs were observed for the different excitation wavelengths at 390, 400, 420, and 440 nm.

![Figure 3.5](Source: http://spectroscopyinstruments.com/s/spectroscopy-instruments/category/fluorescence-spectrometers/)

**Figure 3.5.** The basic principle of fluorescence spectrometer and image of JASCO fluorospectrometer

### 3.3.3. Fourier Transform Infra-Red (FTIR) Spectroscopy

FT-IR is the analytical technique developed in 1970s to qualify and quantify compounds utilizing infrared absorption of molecules. Absorption occurs when the energy of the beam of light (photons) are transferred to the molecule. The excitation of molecules results in to transfer of molecule to
higher energy state. The energy transfer takes place in different forms like molecular bond vibrations, electron ring shifts, rotations, and translations. IR is mostly concerned with vibrations and stretching. On absorbing infrared energy, the bonds between atoms in the molecule stretch and bend consequently creating the infrared spectrum.

The basic components of an FTIR are shown schematically in figure 3.6. The infrared source emits a broad band of different wavelength of infrared radiation. The IR radiation is passed through an interferometer that modulates the infrared radiation. The interferometer carries out an optical inverse Fourier transform on the entering IR radiation. The altered IR beam passes through the gas sample where it is absorbed to various extents at different wavelengths by the various molecules present. Finally a detector detects the intensity of the IR beam, which is a liquid-nitrogen cooled MCT (Mercury-Cadmium-Telluride) detector. The detected signal is digitised and Fourier transformed by the computer to get the IR spectrum of the sample gas. The unique and main part of an FT-IR spectrometer is the interferometer. Figure 3.6 is showing a Michelson type plane mirror interferometer. Infrared radiation from the source is collected and collimated (made parallel) before it strikes the beamsplitter. One half of the radiation is transmitted by the beamsplitter ideally, and the other half is reflected. Transmitted and reflected beams strike mirrors, reflecting the two beams back to the beamsplitter. Thus, first one half of the infrared radiation that finally goes to the sample gas has been reflected first from the beam splitter to the moving mirror, and then back to the beamsplitter. The remaining other half of the infrared radiation going to the sample has first gone through the beam splitter and then reflected from the fixed mirror back to the beamsplitter. The interference occurs when these two optical paths are reunited at the beamsplitter because of the optical path difference caused by the moving mirror [7].

Here in this study, the Fourier transform infrared spectra were recorded in transmittance mode with Alpha ATR Bruker (Eco ATR 500–400 cm−1)
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spectrum of freeze-dried silver nanoparticle colloidal solution to study the attachment of biomolecules to the silver nanoparticles (figure 3.6).

![FT-IR Spectrometer Diagram]

**Figure 3.6.** The basic principle of FT-IR spectrometer and image of Alpha ATR Bruker FT-IR [Source: http://www.bruker.com/products/infrared-near-infrared-and-raman-spectroscopy/ft-ir-routine-spectrometers/alpha/overview.html]

3.4. Biological Characterization Techniques

3.4.1. Fluorescence Microscopy

In fluorescence microscopy visualization of cells and membranes is done by labeling with fluorescent molecules or by using the natural fluorescent properties of molecules already present in the biological system or a fluorophore such as green fluorescent protein [8]. An illuminating a fluorescent compound with a particular wavelength of light that gets absorbed by the fluorescent compound and observing the emission of light with a longer wavelength from the sample is fluorescence.

In a typical fluorescence microscope have a light source e.g. a xenon arc lamp or mercury-vapour lamp, able to produce various wavelengths for excitation as fluorescent molecules absorb only at specific wavelengths. A special optical filter, an excitation filter, removes any other wavelength of light other than the wavelength used to excite the fluorescent molecule.
A dichroic mirror, which is a special mirror, is able to reflect certain wavelengths of light and let other wavelengths pass through. The filtered wavelength exits the excitation filter and gets reflected onto the sample containing the fluorescent molecules. The absorption of photons and the emission of photons of a shorter wavelength occur subsequently. The design of the dichroic mirror allows them to pass through the dichroic mirror onto the ocular or detector of the microscope as the emitted photons have a shorter wavelength than the absorbed photons (figure 3.7).

Here in this thesis fluorescence microscopy used for the visualization of the toxic effect of nanoparticles on mammalian cells stained with acridine orange and ethidium bromide staining method. The fluorescence microscope used for the study Leica DM6000 FS.

3.4.2. Confocal Laser Scanning Microscope (CLSM)

The technique of laser scanning confocal fluorescence microscopy has become an essential tool in biology and the biomedical sciences, also in materials science due to attributes that are not readily available using other contrast modes with traditional optical microscopy.
Figure 3.8. The schematic representation of Confocal Laser Scanning Microscope (CLSM); [Source: http://www.microscopyu.com]

The basic concept of confocal microscopy was originally developed by Marvin Minsky in the mid-1950s (patented in 1961). Optical sectioning is the key feature of confocal microscopy that is to acquire in-focus images from selected depths. Images are acquired point-by-point with reconstruction with a computer which allows three-dimensional reconstructions of topologically complex objects. Present confocal microscopes are completely integrated electronic systems where a central role is being played by the optical microscope in a configuration that consists of one or more electronic detectors, a image display unit, processing unit, output and storage unit, and several laser systems combined with wavelength selection devices and a beam scanning assembly.

The confocal principle is diagrammatically presented in figure 3.8 of epi-fluorescence laser scanning microscope. Through a pinhole aperture coherent light emitted by the laser system (excitation source) passes which is situated in a conjugate plane (confocal) with a scanning point on the specimen and a second pinhole aperture positioned in front of the detector (a photomultiplier tube). A dichromatic mirror reflects the laser which is then
scanned across the specimen in a defined focal plane, in the same focal plane secondary fluorescence emitted from points on the specimen pass back through the dichromatic mirror and at the detector pinhole aperture is focused. The significant amount of fluorescence emission that occurs at points above and below the objective focal plane is not confocal with the pinhole (termed Out-of-Focus Light Rays in figure 3.8) and forms extended airy disks in the aperture plane. As only a small fraction of the out-of-focus fluorescence emission is carried through the pinhole aperture, most of this extraneous light by the photomultiplier is not detected and does not contribute to the resulting image. The dichromatic mirror, excitation filter and barrier filter execute similar functions to identical components in a wide-field epi-fluorescence microscope. In a confocal microscope, the excitation can be shifted by refocusing objective and emission points on a specimen to a new plane that becomes confocal with the pinhole apertures of the light source and detector [9].

Here in this thesis CLSM used for the visualization of the toxic effect of nanoparticles on mammalian cells stained with acridine orange and ethidium bromide staining method. All the confocal images were captured on an LSM 510 Zeiss workstation (Carl Zeiss Meditec AG, Germany).

3.4.3. Toxicity Studies: In-vitro cytotoxicity assays

To the commercialization of products comprised by nanomaterials is now a reality. Among commercially available nanocompounds, AgNPs are the most used, mainly due to their potent microbicidal activity. The rapid development of nanotechnology has been leading to an increasing concern related to possible toxic effects of nanomaterials, including human health and environmental impact. In this scenario, the detailed investigation of nanoparticles toxicity has been emerging as an important area of research. The great interest of the scientific community by toxicological evaluations of nanomaterials is relatively new, and it has been increasing in recent years. Therefore, the great appeal of nanotechnology can be considered the better
evaluation of the cytotoxicity and genotoxicity of nanoparticles, in particular, biogenic synthesized metallic nanoparticles. Different *in-vitro* toxicity assays have used for the toxicity studies on mammalian cell line and are explained briefly in table 3.1.

**Table 3.1.** The overview of *in-vitro* toxicity assays used in toxicity studies of nanoparticles [10]

<table>
<thead>
<tr>
<th>Assay with Detection Principle</th>
<th>Purpose</th>
<th>Advantages</th>
<th>Used for nanoparticles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetrazolium salts (MTT, MTS, WST, XTT)</td>
<td>Cell viability/cell growth (Cell metabolic activity)</td>
<td>1) Real time assay results using low cell numbers</td>
<td>Silver nanoparticles; carbon nanoparticles; Fullerenes</td>
</tr>
<tr>
<td>Principle: mitochondrial activity is determined colorimetrically and by visible lightspectrometer</td>
<td>2) Provides simple method for estimation of live cell number in order to assess rate of cell proliferation and to screen cytotoxic agents 3) Non radioactive 4) Inexpensive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutral red assay</td>
<td>Cell viability (Lysosomal activity)</td>
<td>1) Quantitative estimation of the number of viable cells in a culture 2) One of the most used cytotoxicity tests with many biomedical and environmental applications</td>
<td>Carbon nanotubes; Silver, molybdenum, aluminum, iron oxide and titanium dioxide nanoparticles;</td>
</tr>
<tr>
<td>Principle: Colorimetric detection of intact lysosomes and detected via fluorescence or absorption measurement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate dehydrogenase (LDH)</td>
<td>Cell viability</td>
<td>Reliability, speed and simple evaluation</td>
<td>Carbon Nanoparticles; ZnO nanoparticles; Iron Oxide Nanoparticles</td>
</tr>
<tr>
<td>Principle: Detection of LDH release colorimetrically</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypan blue</td>
<td>Cell viability</td>
<td>Conveys the actual number of viable cells and</td>
<td>Gold nanoparticles;</td>
</tr>
</tbody>
</table>
Here in this thesis, two classical assays viz MTT assay and NRU assay have used to evaluate the in-vitro cytotoxicity of biological synthesized nanoparticles to mammalian cell line (L929). The necrotic and apoptotic effect of silver nanoparticle was visualized by acridine orange and ethidium bromide staining method. These methods are described in briefly.
3.4.3.1. MTT

Measurement of cell viability and proliferation forms the basis for numerous in vitro assays of a cell population’s response to external factors. The reduction of tetrazolium salts is widely accepted as a reliable way to examine cell proliferation. The yellow 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (tetrazolium MTT) is reduced by metabolically active cells in turn by dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The reduction process forms intracellular purple formazan which can be solublized and quantified by spectrophotometrically [11]. The MTT assay measures the cell proliferation rate when metabolic events lead to necrosis or apoptosis, the reduction in cell viability. In the absence of cells, the MTT reagent doses not yield high background absorbance values.

3.4.3.2. Neutral Red Uptake (NRU) Assay

The neutral red uptake assay is a quantitative estimation of the number of viable cells in a culture. It is been most used cytotoxicity tests with many biomedical and environmental applications. The basis of this assay is the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes. Most primary cells and cell lines from varied origin can be used for this assay. Cells are grown in 96-well tissue culture plates and are treated for the appropriate period. The plates are further incubated for 2 h with a medium containing neutral red. Viable cells will take up the dye by active transport and incorporate the dye into lysosomes, whereas non-viable cells are unable to take up the dye. Then cells are subsequently washed, the dye is extracted in each well and the absorbance is measured using a spectrophotometer. The procedure is simple, cost effective and more sensitive than other cytotoxicity tests (tetrazolium salts, enzyme leakage or protein content). Once the cells have been treated, the assay can be completed in <3 h [12].
3.4.3.3. Acridine orange and ethidium bromide (AO/EB) staining assay

The AO/EB double stained method is used to visualize the apoptotic and necrotic effect of nanoparticles to mammalian cells. Acridine orange (AO) is permeated by all cells and makes the nuclei appears green. Whereas Ethidium bromide (EB) is only penetrate inside cells when cytoplasmic membrane is damaged, which stains the nucleus red. AO is dominated by EB. Thus live cells are with a normal green nucleus; early apoptotic cells display bright green nucleus with condensed or fragmented chromatin; late apoptotic cells have condensed and fragmented orange chromatin. Cells died from direct necrosis have a structurally normal orange nucleus.

3.5. Size Distribution and Colloidal Stability Characterization Techniques

The dynamic light scattering (DLS) and zeta potential have great importance in the determination of the biomedical application of nanoparticles in the biomedical filed. The use of this technique provides information regarding colloidal stability, charge on the surface and aggregation of nanoparticles.

3.5.1. Dynamic Light Scattering (DLS)

Dynamic Light Scattering or Photon Correlation Spectroscopy is one of the most popular methods used to determine the size of nanoparticles in the colloidal solution. On incidence of a monochromatic light beam, such as a laser, onto colloidal solution of nanoparticles in Brownian motion causes a Doppler Shift where the light hits the moving particle and further changes the wavelength of the incoming light. The change is solely dependent on the size of the nanoparticles (figure 3.9). The sphere size distribution and the particle’s motion in the solution are possible to compute by measuring the diffusion coefficient of the particle, using the autocorrelation function. On comparison with size obtained from TEM, size obtained from DLS is slightly higher. As DLS provides the information of inorganic particles along with any coating material and solvent layer attached to particles which moves under the
influence of Brownian motion. Whereas TEM gives the information regarding the size of only inorganic particles. A shot experimental time and an automatized system minimizing mistakes occur due convectional cumbersome measurements are the major advantage of this system.

![Image](image_url)

**Figure 3.9.** The basic principle of DLS and images of NICOMP 380 ZLS particle sizing system (Santa Barbara, CA, USA) with output of the experiment [Source: http://pssnicomp.com/products/nicomp/nicomp-380-zls-seeing-nanoparticles-from-all-angles/]

Dynamic light scattering of the powder were measured using a PSS/NICOMP 380 ZLS particle sizing system (Santa Barbara, CA, USA) (figure 3.5) with a red He–Ne laser diode at 632.8 Å in a fixed angle 90° plastic cell All measurements were carried out at 25.0 ± 0.1°C using a circulating water bath. 10 mm diameter cylindrical cells were used in all of the light scattering experiments. DLS is used throughout the experiments to analyze the change in the particle size as the change in the experimental conditions.

### 3.5.2. Zeta potential

The physical mechanism that is used to stabilize most aqueous colloidal systems is electrostatic repulsion. The charged colloidal particles are resulting in their mutual repulsion at extended distances. In other cases, the colloidal
particles may already carry specific groups that are covalently bound to their surfaces and are ionizable. They carry a net positive or negative charge, or are neutral, depending on the pH of the surrounding aqueous solvent. Hence, the pH of the suspension will strongly influence the net charge of the colloidal particles and therefore their stability against aggregation. This charge on the particle is determined by zeta potential. The potential at the surface of shear for a particle is defined as the zeta potential. The zeta potential of colloidal dispersions is routinely measured using the technique of micro electrophoresis. A voltage is applied across a pair of electrodes at either end of a cell containing the particle dispersion. Charged particles are attracted to the oppositely charged electrode and their velocity is measured and expressed in unit field strength as their mobility. Dispersion gives the possibility of controlling the electrostatic interactions in the dispersion, and hence controls the stability of the emulsion or dispersion [13].

Herein this thesis, the zeta potential of biological synthesized nanoparticles is measured using a PSS/NICOMP 380 ZLS particle sizing system (Santa Barbara, CA, USA) with a red He–Ne laser diode at 632.8 Å in a fixed angle 90° plastic cell. The zeta potential measurements were performed at 25°C after a temperature homogenization time of 5 min. The magnitude of zeta potential gives an indication of the potential stability of colloidal system. If the particles in suspension have a large negative or positive zeta potential then they will tend to repel each other which in results lower the tendency to flocculate. However, if the particles have low zeta potential values then there is no force to prevent the particles aggregation. The general dividing line between stable and unstable suspensions is generally taken at either +30mV or - 0mV. Particles with zeta potentials more positive than +30 mV or more negative than -30mV are normally considered stable.
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