Chapter 2
Analytical and Physical Characterization Techniques Employed to Assess Microbial Toxicity of Nanoparticles

Abstract Various chemical and/or biological surfactants are often employed while synthesizing nanoparticles, which can drastically contribute to their interactions with bacterial cells thereby toxicity. Analytical and physical characterization techniques that can assess toxicity can potentially guide the proper use of these nanoparticles, for instance, to improve drug formulations for the treatment of infections caused by various multi-drug resistance microorganisms, proper disposal of nanoparticles, etc. Bactericidal activity of a material can be evaluated using several toxicity-assessing techniques. The most commonly used analytical techniques (disk diffusion assay, minimum inhibitory concentration, colony-forming units (CFU) and live–dead staining) and physical characterization techniques [fluorescence spectroscopy, inductively coupled plasma mass spectroscopy, ultra-microtome-based transmission electron microscopy, and atomic force microscopy (AFM)] are described in the current chapter.

Keywords Analytical · Physical · Characterizations · Bactericidal toxicity · Techniques

2.1 Analytical Assays to Determine Microbial Toxicity

The antibacterial activity of any cytotoxic formulation, including nanoparticles, can be assessed using several analytical techniques; some of the commonly used analytical techniques are described below.

2.1.1 Disk Diffusion Assay

A filter paper disk impregnated with a formulation possessing toxic potential when placed on Luria–Bertani agar in a Petri dish, the material based on its diffusion rate tends to diffuse from the disk into the agar. This phenomenon of diffusion or

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A.K. Suresh, *Co-Relating Metallic Nanoparticle Characteristics and Bacterial Toxicity*, SpringerBriefs in Biometals,
DOI 10.1007/978-3-319-16796-1_2

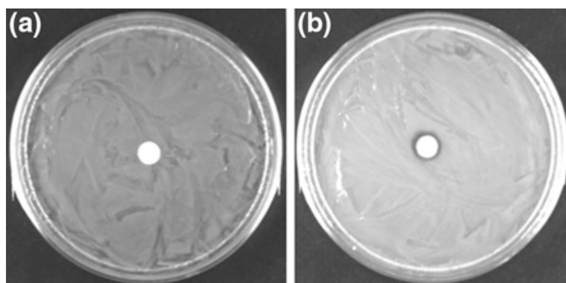

Fig. 2.1 **a** Images of an agar plate containing gold nanoparticle formulations impregnated disks showing the diameter of the inhibition zone for *E. coli* (**a**) untreated controls and treated cells (**b**). Diameter of zone of inhibition can be clearly seen

spread of the material in the agar surrounding the disk can also depend on other factors such as solubility, diffusion rate, and molecular size that ultimately determine the area of the materials infiltrating around the disk. When similar experiments are performed with a bacterium placed on the agar, the bacteria will not grow in the region around the disk if it is susceptible to the material. This area of where there is no bacterial growth surrounding the disk is called the diameter of the zone of inhibition, and this assay methodology is called as the disk diffusion assay. The bacterial sensitivity to different nanoparticles could be tested using disk diffusion assay, for which stocks of equal concentrations (25 μg/mL) of the different types of toxic agents (nano constituents, nanoparticles) were first prepared. Then, small disks of uniform size (6 mm diameter) were placed separately in the nanoparticles stock suspensions for five minutes, and the disks were removed carefully using the sterile forceps. The bacterial suspension (100 μL at densities 10^7 cells/mL^{-1}) was spread-plated uniformly on the Luria–Bertani agar Petri dishes using a sterile spreader under sterile conditions, and the nanoparticles impregnated disks are placed in the middle of the plate. The plates were then incubated at 37 °C for 18 h, after which the average diameter of the inhibition zone surrounding the disks was measured using a ruler of 1-mm resolution. As an example, Fig. 2.1 shows the diameter of zone of inhibition performed for *E. coli* using gold nanoparticle–drug formulations. As can be made out from the image, the *E. coli* cells that were untreated, no diameter of zone of inhibition was observed (Fig. 2.1a), whereas for the *E. coli* cells that were treated with nanoparticle formulation, clear zone of inhibition can be seen (Fig. 2.1b) clearly implying that the formulation has bactericidal or killing effect.

As the diameter of the zone of inhibition measurements are performed on an agar Petri dish and the inhibition zone being measured using a ruler, there is a possibility of error; however, the method illustrates the potential analytical assay techniques to assess the toxicity of various toxic agents on different bacterial strains.

2.1.2 Minimum Inhibitory Concentration

Minimum inhibitory concentration is defined as the lowest concentration of a formulation with potent bactericidal activity that can inhibit the visible growth of bacteria

after overnight treatment with the formulation. Assessing minimum inhibitory concentration is considered crucial in diagnostic and research purposes to not only confirm the resistance of an organism to the formulation or chemotherapeutics but also to monitor the activity of novel drug formulation. In the standard methodology, to evaluate the minimum inhibitory concentrations of various drug formulations or biologically significant material, the test bacterium was maintained on Luria–Bertani agar Petri dishes, bacterial growth was performed by inoculating a single bacterial colony from the agar plates into fresh liquid Luria–Bertani medium in a culture tube and incubated at 37 °C on a rotary shaker (200 rpm) until the bacterium attains the required growth, which is usually performed by measuring the absorbance at 600 nm. Two hundred microliters of the overnight grown bacterial cells (0.8–1.2 optical density) was inoculated into 10 mL of fresh Luria–Bertani medium. The reaction was performed by transferring 200 μL/well of the above medium into a sterile 100-well bioscreen microtitre or ELISA plate along with the drug formulation, for which the bactericidal activity is desired, at various increasing concentrations separately. The treatments are usually performed in octuplet, and every experiment was repeated at least three times to ensure reproducibility and consistency. The plate was then placed into the bioscreen or ELISA plate reader, and the bacterial growth was monitored every 15 min for 12 h at a wavelength of 600 nm, where the bacteria can be read. Experiments with no drug formulations will be used as control reactions. A greater lag phase and lower maximum absorbance@ 600 nm should be observed for the drug formulation with an increase in the concentration for the bacteria, if the formulation is toxic to that particular bacteria. For example, Fig. 2.2 illustrates the diameter of zone of inhibition performed for the bacterium, *E. coli* using gold nanoparticle–drug formulations at various increasing concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 μm). As can be seen from the image, the *E. coli* cells that were untreated had no growth inhibition, where the bacteria are happily growing (Fig. 2.2, bacteria alone line), whereas for the *E. coli* cells that were treated with various concentrations of nanoparticle formulation, bacterial inhibition was observed (Fig. 2.2).

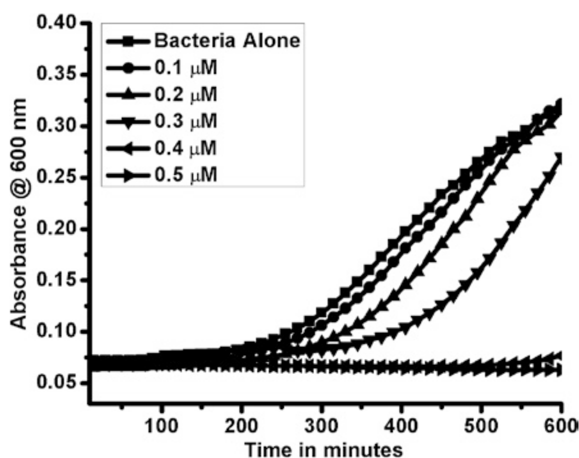

Fig. 2.2 Bacterial dynamic growth curve for *E. coli* treated with gold nanoparticle formulation at various increasing concentrations

As the concentration of formulation is increased, more amount of particle will available to get adsorbed on to the surface, thereby enhancing the microbial activity. Therefore, a concentration-dependent killing of the bacteria should be observed. However, while performing these bactericidal assays, several other factors from the formulation, such as their size and shape distributions, such as the surface coat, surface charge, and surface properties can contribute and might play a predominant role in dictating potential toxicity by toxic formulate.

2.1.3 Colony-Forming Units

CFU refer to the individual colonies of a microorganism (bacteria, fungi, yeast, or mold). This is used as a measure to assess the number of colonies present in or on the surface of a sample and may be referred as CFU per unit weight, CFU per unit area, or CFU per unit volume depending on the type of sample assessed. To evaluate the number of CFUs, a sample is prepared and spread or poured uniformly on a surface of an agar plate followed by incubation at suitable temperature for few days, until the single colony is grown. To evaluate the effects of any materials on the CFUs, the viability was performed in liquid cultures after treatment with various concentrations of the material. Aliquots will be collected at different time intervals, for example, 0, 2, 8, 12, 18, and 24 h, and serially diluted in the appropriate growth medium, and the dilutions were plated onto Luria–Bertani agar Petri plates. After overnight incubation at 37 °C, the numbers of colonies were counted manually and assessed for the number of colonies grown (CFUs).

2.1.4 Live/Dead Toxicity Assay

The LIVE/DEAD Bacterial Viability assay utilizes mixtures of our SYTO® 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide. These stains differ both in their spectral characteristics and their ability to penetrate healthy bacterial cells. When used alone, the SYTO 9 stain generally labels all bacteria in a population; those with intact membranes and those with damaged membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. Thus, with an appropriate mixture of the SYTO 9 and propidium iodide stains, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. The excitation/emission maxima for these dyes are about 480/500 nm for SYTO 9 stain and 490/635 nm for propidium iodide. To determine the LIVE/DEAD assay for the different nanoparticle samples, bacterial cultures were grown for 3 h in Luria–Bertani medium and subsequently treated with different concentrations of nanoparticle samples for 3–16 h, After which, the microbial suspension

and the stain solution were added to each well of a 96-well micro plate, the plate was incubated at room temperature in the dark for 15 min, and to quantify the live and dead cells, the relative fluorescence intensities were measured using a fluorescence plate reader (excitation at 485 nm; emission at 525 and 625 nm). To assess the percent viability, using the same set wavelength filters, color images can also be captured using an epi-fluorescence microscope. Green fluorescence represents the living bacterial cells, and compromised bacterial cells with membrane damage are stained red. The percent viability of the bacterial cells could then be obtained from the images using Image J (version 1.4.3) software.

2.2 Physical Characterization Techniques to Ascertain Bacterial Nanoparticle Interactions

The depths of interactions of nanoparticles with bacterial cells can be assessed using several physical characterization techniques that give away information on several aspects of nanoparticle bacterial interactions. For instance, nanoparticles bound to the surface or nanoparticle internalized, probable mode of killing of the bacteria, biodistribution of nanoparticles within the bacterial cells, mode of damage caused to the bacterial cell (membrane integrity, bacterial membrane rupture, lesions, and nodules appearing on the bacterial surface, bacterial DNA damage, etc.). Some of the commonly used physical characterization techniques including their principle and experimentation details are illustrated below.

2.2.1 Fluorescence Spectroscopy

In fluorescence spectroscopy, the species is first excited, by absorbing a photon, from its ground electronic state to one of the various vibrational states in the excited electronic state. Collisions with other molecules cause the excited molecule to lose vibrational energy until it reaches the lowest vibrational state of the excited electronic state. The molecule then drops down to one of the various vibrational levels of the ground electronic state again, emitting a photon in the process. As molecules may drop down into any of several vibrational levels in the ground state, the emitted photons will have different energies, and thus frequencies. Therefore, by analyzing the different frequencies of light emitted in fluorescent spectroscopy, along with their relative intensities, the structure of the different vibrational levels can be determined. In a typical fluorescence (emission) measurement, the excitation wavelength is fixed and the detection wavelength varies, while in a fluorescence excitation measurement, the detection wavelength is fixed and the excitation wavelength is varied across a region of interest. An emission map is measured by recording the emission spectra resulting from a range of excitation wavelengths and combining them all together. This is a three-dimensional surface

data set: emission intensity as a function of excitation and emission wavelengths and is typically depicted as a contour map.

However, fluorescence spectroscopy can be used an analytical tool only to assess the interaction of selected forms of nanoparticles called the quantum dots that retain the above properties. And is usually based on quenching, refers to any process which decreases the fluorescence intensity of a given substance. A variety of processes can result in quenching, such as excited state reactions, energy transfer, complex formation, and collisional quenching. Quenching is the basis for Forster resonance energy transfer (FRET) assays in biomedical applications, and interaction with a specific molecular biological target is the basis for activatable optical contrast agents for molecular imaging.

2.2.2 Inductively Coupled Plasma Mass Spectroscopy for Quantitative Uptake

Inductively coupled plasma mass spectrometry (ICP-MS) is an analytical technique used for elemental determinations. For the ICP-MS measurements, the sample is usually introduced into the ICP plasma as an aerosol, either by aspirating a liquid or dissolved solid sample into a nebulizer or using a laser to directly convert solid samples into an aerosol. Once the sample aerosol is introduced into the ICP torch, it is completely desolvated and the elements in the aerosol are converted first into gaseous atoms and then ionized toward the end of the plasma. Once the elements in the sample are converted into ions, they are then brought into the mass spectrometer via the interface cones. The interface region in the ICP-MS transmits the ions traveling in the argon sample stream at atmospheric pressure (1–2 torr) into the low-pressure region of the mass spectrometer ($<1 \times 10^{-5}$ torr). The ions from the ICP source are then focused by the electrostatic lenses in the system, and the ions coming from the system are positively charged, so the electrostatic lens, which also has a positive charge, serve to collimate the ion beam and focus it into the entrance aperture or slit of the mass spectrometer. Once the ions enter the mass spectrometer, they are separated by their mass-to-charge ratio. The resolving power (R) of a mass spectrometer is calculated as $R = m/(|m1 - m2|) = m/\Delta m$, where m1 is the mass of one species or isotope and m2 is the mass of the species or isotope it must be separated from; m is the nominal mass.

To determine the nanoparticle concentrations pure nanoparticles suspensions, the sample is directly injected into the ICP plasma, as mentioned above. However, for the bacterial that were treated with nanoparticles, either attached to the surface or internalized, the cells need to be processed. To perform this, the bacterial cells treated with various nanoconstructs are usually exposed for up to 12 h, after which the medium was aspirated, the cells were collected by centrifugation and washed once with PBS, acid digested overnight using 1 mL of 67–70 % HNO_3 until the cell are digested, and analyzed using ICP-MS upon appropriate dilution. Certified standard suspensions (0, 5, 10, 50, 100, 250, 500, 1000, and 2000 ppb) should be run for each experiment as calibrant suspensions.

2.2.3 Dark Field Microscopy

The ability to observe and characterize nanoconstructs and their interaction with biological materials is crucial for nano drug delivery research. Dark field microscopy is an optical microscope technology with optimized focus and alignment of oblique angle illumination (dark field) that can produce a very high signal to noise ratio image. This enables fast observation of the Rayleigh scatter from a wide range of nanoscale materials. For the dark field microscopy assessments, bacterial cells grown overnight, treated with the nanoparticles construct are fixed on a glass slide and mounted with the coverslip. Dark field hyperspectral imaging was performed using a CytoViva dark field microscope system equipped with CytoViva Hyperspectral Imaging System 1.2. Spectral mapping was accomplished using customized ENVI hyperspectral analysis software provided by the manufacturers. First, a library of spectra for particles alone was generated. Each spectra included in the library was sampled from a single pixel imaged with a suitable objective. This library was then mapped onto images of interest by false-coloring a pixel red if it was within 0.1 rad of one of the spectra in the library.

2.2.4 Scanning Electron Microscopy

Scanning electron microscopy (SEM) is a microscope that uses electrons instead of light to form an image is one of the most widely used technique for the characterization of nanostructures. The SEM has many advantages over traditional microscopes, has a large depth of field and higher resolution, which allows larger specimen to be in focus at one time simultaneously closely spaced specimens can be magnified at much higher levels. As SEM uses electromagnets instead of lenses, there is much more control in the degree of magnification. Unlike optical microscopy, this technique not only provides topographical information but also can analyze the chemical composition near the surface. The interaction between the electron beam and the sample gives different types of signals providing detailed information about the surface characteristics, structure, and morphology. When an electron from the beam encounters a nucleus in the sample, the resultant columbic attraction will lead to deflection in the electron's path known as Rutherford elastic scattering. A fraction of these electrons are then backscattered resulting in reemergence from the incident surface. Since the scattering angle depends on the atomic number of the nucleus, the primary electrons arriving at a given detector position produce image yielding topological and compositional data. The high-energy incident electrons can also interact with loosely bound conduction band electrons in the sample. However, the amount of energy given to these secondary electrons as a result of such interactions is small with a very limited range. The secondary electrons produced within a very short distance from the surface escape from the sample giving high-resolution topographical images.

Bacterial treatments with the nanoparticles for the SEM imaging should be performed in a similar manner prepared for TEM imaging and gives information consistent with TEM, the morphological changes of bacterial cells. For SEM analysis, cells (at densities ~10^6 CFU/mL) were treated with desired concentrations of nanoparticle samples for 1–3 h and centrifuged at 3000× g for 30 min. The cell pellets were washed with phosphate-buffered saline (PBS) at least three times and fixed using 2.5 % glutaraldehyde for 30 min. The fixed bacterial cells were washed twice with PBS and treated with 1 % osmium tetroxide for 1 h. After washing with PBS three times, the samples were dehydrated using 30, 50, 70, 80, 90, and 100 % of ethanol treatments, respectively, were dried and gold coated using ion sputter and were imaged under SEM.

2.2.5 Transmission Electron Microscopy

Transmission electron microscope (TEM) operates on the basic principles as for the light microscope, however, uses electrons instead of light. And because TEM uses electrons as “light source,” the lower wavelength from the electron source makes it possible to get a resolution thousand times better than the light microscope. TEM is often used for high-resolution imaging of thin films of solid samples for structural and compositional analysis. The technique involves (i) irradiation of a thin film by a high-energy electron beam, which is then diffracted by lattices of the crystalline material and propagates in different directions, (ii) imaging and angular distribution analysis of the forward-scattered electrons, and (iii) energy analysis of the emitted X-rays. The topographic information obtained by TEM, in the vicinity of atomic resolution, can be utilized for structural characterization and identification of various forms of nanomaterials, viz. and can discriminate various crystal lattices such as hexagonal, cubic, or lamellar. There are two ways by which one can ascertain nanoparticles interaction with bacteria using TEM; direct imaging and imaging of ultra-thin sections of cell mounted in resin upon ultra-microtome, and are described in detail in the following sections.

2.2.5.1 Direct Imaging Using TEM

The interaction between the bacteria and the nanoconstructs can be illustrated by direct imaging of the bacterial using bright-field TEM imaging of the bacteria treated with the nanoconstructs. Figure 2.3 describes the interaction of silver nanoparticles with the laboratory-standard bacterium *E. coli*. As can be made out from the Fig. 2.3, most of the silver nanoparticles were found attached to the surface of the bacterial cell wall, implying their higher affinity toward the cells. It was obvious that the silver nanoparticles were bound very well throughout the surface of the bacteria and were also able to penetrate the bacteria. Interestingly, it was also observed that the silver nanoparticles looked well separated and spread throughout

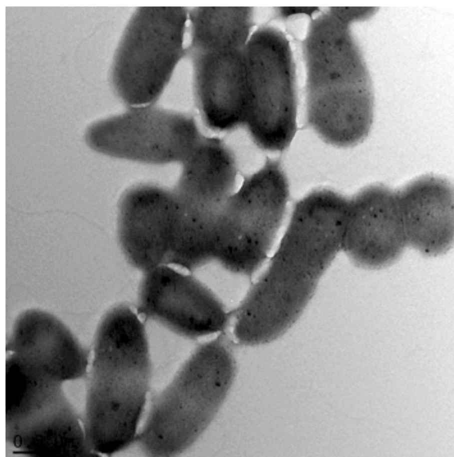

Fig. 2.3 Representative TEM image showing the interaction of *E. coli* with silver nanoparticles. It can be clearly seen that the particles apparently stick to the bacterial surfaces and also might have internalized

the TEM grid prior to bacterial incubation. Upon incubation with the bacteria, most of the nanoparticles tend to agglomerate and mostly found attached to the surface the bacteria. As demonstrated by electron microscopy, interaction with silver nanoparticles resulted in perforations in the cell wall, contributing to the enhanced antibacterial effects of the nanoparticles. Additionally, clusters of particles are seen throughout the bacterial surface. However, using direct imaging technique, the biodistribution or cell distribution of the particles within the cells cannot be assessed, therefore trans-sectional TEM, where the nanoconstructs treated cells are mounted into resin, ultra-thin sections of which is made using platinum blade and then imaged under TEM. Details of which are illustrated in the later section.

2.2.5.2 Ultra-Microtome-Based Trans-Sectional Imaging Using TEM

Trans-sectional-based transmission electron microscopy is also becoming an emerging tool to assess the local biodistribution of the various nanoparticle constructs within cells or biological tissues. After uploading of the cells or tissue with the desired nanovectors, the cells or tissue are sectioned using a very fine blade (diamond) that can cut or make very thin sections of the cells or tissues up to nanometer scale lengths. For these experiments, the bacterial cells treated with nanoparticle constructs were collected by centrifugation, followed by washing with PBS, fixed with 2 % glutaraldehyde in 0.1 M cacodylate buffer ($Na(CH_3)\cdot 2AsO_2\cdot 3H_2O$), pH 7.2, at 4 °C overnight. The following day, the bacterial cells were washed three times with 0.1 M cacodylate buffer, post-fixed with 1 % OsO_4 in 0.1 M cacodylate buffer for 30 min and washed three times with 0.1 M cacodylate buffer. The samples were then dehydrated using 60, 70, 80 and 95 % ethanol and 100 % absolute ethanol (twice), propylene oxide (twice) and were leveled in propylene oxide/eponate (1:1) overnight at room temperature under a sealed environment. The following day, the vials were level open until the propylene

oxide evaporated (2–3 h). The samples were infiltrated with 100 % eponate and polymerized at 64 °C for 48 h. Ultra-thin Sections (70 nm thick) were cut using a Leica Ultra-cut UCT ultra-microtome equipped with a diamond knife, and the sections were picked up onto 200 mesh copper TEM grids. The grids were stained with 2 % uranyl acetate for 10 min followed by Reynold's lead citrate staining for a minute and were imaged using a TEM.

2.2.6 Atomic Force Microscopy

Atomic force microscope (AFM) is a form of scanning probe microscopy (SPM) wherein a small probe is scanned across the sample to obtain information about the sample's surface. The information gathered by such interaction can be as simple as physical topography or as diverse as measurements of the material's physical, magnetic, or chemical properties. AFM has the advantage of imaging almost any type of surface such as thick film coatings, ceramics, composites, glasses, synthetic and biological membranes, microorganisms and cells, biomaterials, metals, and polymers. AFM is being applied to study various phenomena such as the abrasion, adhesion, cleaning, corrosion, etching, friction, lubrication, plating, and polishing. The AFM probe has a very sharp tip, often less than 100 Å diameter, at the end of a small cantilever beam. The probe is attached to a piezoelectric scanner tube. Interatomic forces between the probe tip and the sample surface cause the cantilever to deflect as the sample's surface topography or other properties change. A laser light reflected from the back of the cantilever measures the deflection of the cantilever. This information is fed back to a computer, which generates a map of topography and/or other properties of interest. However, based on the type of application, different operation modes of AFM are used like the contact mode, where the AFM measures the hard-sphere repulsion forces between the tip and the sample; non-contact mode, where the AFM derives topographic images from measurements of attractive forces; the tip does not touch the sample; and the tapping mode, where the cantilever is driven to oscillate up and down at near its resonance frequency by a small piezoelectric element mounted in the AFM tip holder similar to non-contact mode. To be able to perform AFM, the bacterial cells should be mounted or immobilized onto a solid mica substrate coated with gelatin, so as to aid imaging, details of which is described below.

2.2.6.1 Immobilization of Bacteria onto Gelatin-Coated Mica

Immobilization is a commonly used technique for the physical or chemical fixation of materials (e.g., cells, organelles, proteins, molecules) onto a solid substrate, into a solid matrix, or retained by a membrane, in order to increase their stability and make possible their repeated or continued use. Here, we immobilize bacterial cells on to gelatin-coated mica so as to detect them using the AFM, for which

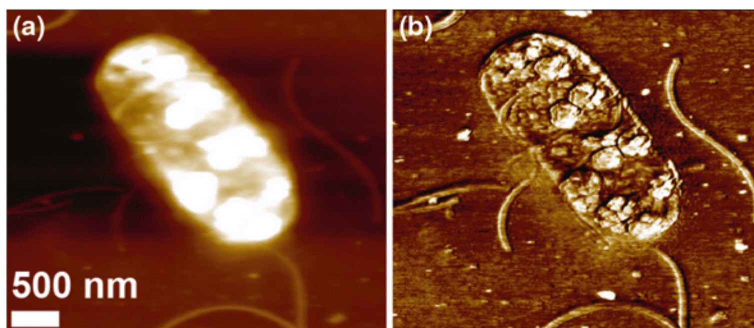

Fig. 2.4 AFM images showing the interaction of the bacterium, *E. coli*. **a** Topographical. **b** Deflective images with silver nanoparticles

freshly cleaved mica surfaces were dipped into 0.5 % gelatin prepared in Milli Q water at 60 °C and dried overnight. A total of 100 μL of the sample analyte was applied to the gelatin-coated mica surface, allowed to stand for 10 min, rinsed in Milli Q water, and placed in the cell for AFM imaging.

AFM measurements were performed to clearly understand the depth of interaction between the bacteria and the silver nanoparticles. Several reports exist on the suitability of AFM for investigating cell structure and morphology of both human cells as well as bacterial cells. AFM is an appropriate technique for elucidating the action of bactericides on bacterial cells. In fact, several investigations performed by various groups suggested noticeable significant changes in the cell membrane morphology upon treatment with different bactericidal agents and could be monitored with ease using AFM. Bacteria with no nanoparticles should be used as controls where the cells looked healthy, intact with no perforations. Figure 2.4 shows the interaction of laboratory-standard *E. coli* with silver nanoparticles; as assessed from the figure, *E. coli* cells were significantly damaged with several perforations and pitches throughout the surface upon treatment. The Ag nanoparticles made long scars on the bacterial surface completely tearing the membrane showing its high killing efficiency. Despite the fact that the mechanism of this interaction is still unanswered, the nanoparticles might cause perforations thereby structural changes, degradations, and finally cell death.

2.3 Summary

The most commonly used analytical (disk diffusion assay, minimum inhibitory concentration, CFU and live–dead staining) and physical characterization (fluorescence spectroscopy, inductively coupled plasma mass spectroscopy, ultra-microtome-based transmission electron microscopy, and AFM) techniques that will shed information on the probable modes of interaction of nanoparticles

with bacterial cells are discussed. The detailed methodologies of each and every technique including sample preparation and processing illustrating suitable examples are presented. A better understanding on the interaction of nanoparticles with bacterial cells are considered crucial for the intended proper use of nanoparticles either as better bactericidal agents and or for biomedical purposes.

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Co-Relating Metallic Nanoparticle Characteristics and Bacterial Toxicity
Suresh, A.K.
2015, XII, 47 p. 13 illus., 5 illus. in color., Softcover
ISBN: 978-3-319-16795-4