Chapter 2
Facile Green Biofabrication of Nanocrystallites

Abstract To facilitate widespread applications of engineered nanoparticles, researchers are looking at novel and better synthesis strategies. This brief chapter details the biofabrication/biosynthesis of nanoparticles, advantages of biofabrication over conventional chemical or physical routes of synthesis, and the mechanism involved in biofabrication. An illustration on the biosynthesis of silver nanoparticles using the metal-reducing bacterium *S. oneidensis* will be presented as an example. Further, details on the synthesis methodology, physical characterizations with respect to morphology, crystallinity, surface properties, and size and shape distributions, which will be based on characterization techniques involving UV–Vis and Fourier transform infrared spectroscopy, dynamic light scattering, X-ray diffraction, transmission electron microscopy, and atomic force microscopy measurements will be discussed.

Keywords Biofabrication · Nanocrystallites · Microbial · Mechanism

2.1 Biofabrication

Naturally occurring biological entities such as proteins or peptides, nucleic acids, nucleotides, and plant extracts have been exploited for the production of hierarchically assembled advanced nanomaterials lately. The involvement of biological entities in the growth and nucleation of nanocystallites are gaining tremendous interests for several reasons, most importantly anisotropic metallic structures with size and shape control can be produced, are cost-effective, green, and energy intensive. Different types of engineered nanocrystallites with specific dimensions, shape distributions, forms, and hierarchies have been produced utilizing various biomimetic templates. Fabrication of one-dimensional parallel and two-dimen-

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sional crosses of palladium nanowire arrays, copper nanowires, and silver nanorods were performed on a solid substrate surface templated by DNA followed by reduction of metal ions [1]. It was opined that the formation of unique crystalline nanorods and nanowires arise due to template directed aggregation of small particles and its subsequent re-crystallization, rather than simple agglomeration. Similarly, self-assembled two-dimensional DNA grids were used as templates to grow 5 nm gold nanoparticles into periodic square lattices that have applications in nanospectronic and nanophotonic devices [2]. Silver nanoparticles, nanorods, and nanowires were synthesized by initial complexation of silver with DNA and then reducing the complex with sodium borohydride. CdS quantum dots of narrow size distributions (6 nm) were also fabricated using wild-type tRNA and an unfolded mutant tRNA of similar lengths. One type of DNA molecule was reported to mediate the nucleation and growth of $CaCO_3$ particles of diverse morphologies [2]. The authors suggested that the concentration of DNA significantly influenced the shape distributions of the particles. These and several other demonstrations clearly reveal that biological molecules can definitely have an impact on nanocrystallites size and shape distribution. Under appropriate conditions different segments of the block copolymers have been shown to form regular arrays of cylinders with structures similar to the surfactant used [3]. Different regions of arrayed structures could be designed by selective interaction of functional groups with the precursor metal ions through physical adsorption or chemical bonding followed by its reduction resulting in the formation of one-dimensional nanostructures. By using a wide range of copolymers this templating procedure has also been exploited for the synthesis of silver nanowires, and gold nanowires and nanosheets. Capillaries of single-walled carbon nanotubes have been used for the synthesis of nanowires of gold, silver, platinum, and palladium [4]. Polystyrene mesospheres were also used as templates for the synthesis of silver nanodisks [2]. Silver nanocubes and nanotriangles themselves have also been utilized for the synthesis of nanoboxes and triangular rings of gold [5]. In this approach the synthesis is facilitated by the transmetallation of gold ions by metallic silver.

Enzymes, nature's catalysts, and peptides provide functional building blocks for the development of advanced materials and tend to perform reactions much faster, under mild conditions in a highly specific manner. The oxidation/reduction mechanism involved in the formation of nanomaterials coupled with the self-assembling ability of enzymes to carry out such reactions have prompted investigators to examine the role of proteins and enzymes in the biotransformation of metals. Engineered proteins and peptides that recognize inorganic surfaces were also proven to be successful in the generation and assembly of various inorganic nanostructures. For example, in vitro synthesis of magnetite nanocrystallites of uniform size distributions of $\sim$30 nm were achieved using the Mms6 protein purified from the magnetotactic bacteria [6]. Similarly, silicateins are known to promote biosilica formation in nature; silicatein filaments have been shown to produce different forms of nanoparticles like the titanium dioxide, gallium oxohydroxide, and γ-gallium oxide in vitro [7, 8]. An enzymatic biocatalyst purified from marine sponge, *Tethya aurantia* was used as a catalyst as well as a

template to hydrolyze and condense molecular precursor $BaTiF_6$ at low temperatures to produce $BaTiOF_4$ nanocrystallites [9]. In another report, standard lab protein bovine serum albumin has been shown to act as both a reducing as well as a stabilizing agent for the synthesis of various metal (Au, Ag) and alloy (Au–Ag) nanoparticles [10]. Brown using the gold binding peptides, identified from a cell surface library, synthesized very big platelets of gold nanocrystallites [11]. Similarly, nanometer-scale thick gold nanocrystalline platelets with high yields were synthesized using a protein purified from the green algae, *Chlorella vulgaris* [12]. In two independent investigations, Kumar et al. showed that enzymes, nitrate reductase, and sulfite reductase purified from the plant pathogenic fungus, *Fusarium oxysporim*, can be used for the production of silver and gold nanoparticles respectively [13, 14]. In another investigation, the authors further showed that using a similar enzyme (sulfite reductase), chemically difficult to fabricate nanoparticles of metal sulfides can also be synthesized [15]. Similarly, Liu et al. using the enzyme phytochelatin synthase and the capping agent phytochelatin purified from the model bacterium, *E. coli*, showed the synthesis of small CdS quantum dots of less than 5 nm [16]. Recently, gold nanocrystallites of various anisotropic size and shape distributions were synthesized using the dodecapeptide, Midas-2, selected from a phage-displayed combinatorial peptide library [12]. The authors further claimed that changes in the single amino acid in the peptide, which are in turn controlled by the pH, were responsible for the production of diverse shapes such as nanospheres, nanoribbons, nanowires, and nanoplatelets. For a detailed understanding of the various polypeptides used to produce different types of nanoparticles, the readers can refer the cited review article [17].

Lately, several microbial systems including fungi, yeast, and bacteria have been utilized as environmentally benign nano-factories for the production and assembly of various types, size, and shape distributions, and bimetallic alloy nanoparticles. Microorganisms have long been known to develop resistance to metal ions either by sequestering metals inside the cell or by effluxing them into the extracellular medium. The ability of microorganisms to produce inorganic materials either intra- or extracellularly has been known for more than 30 years. This unique microbial behavior has been re-exploited lately, for the production of diverse engineering nanostructures of metals, metal oxides, alloys, and other complex structures. There are several reports on the microbial-based biosynthesis of different types of nanoparticles, viz. gold, silver, platinum, palladium, magnetite, cadmium sulfide, cadmium selenide, etc. using microorganisms such as *Fusarium, Enterobacteria, Shewanella, Geobacter, Pseudomonas, Cyanobacteria, Bacillus, Escherichia,* and *Aspergillus*. In these studies biosynthesis completely relies on the microorganism's reductive capabilities and intrinsic metal resistance mechanism to quell metal ion stresses [2]. The source of inspiration for biofabrication has always been nature. Many naturally existing organisms are capable of producing inorganic structures for protection, survival, tools, or weapons for self-defense, etc. For example, mollusks produce hard shells or nacres made of crystalline calcium carbonate for protection against predators. Mangetotactic bacteria are known to produce magnetite nanoparticles, well-assembled in the subcellular membrane that

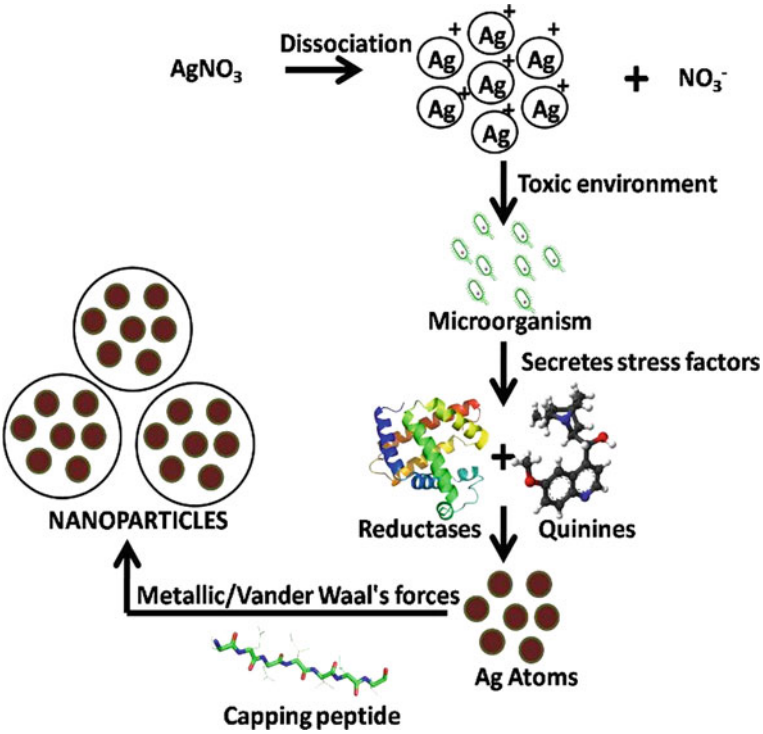

Fig. 2.1 Schematic mechanism for the biotransformation of metal ions into stable nanocrystallites by microorganisms, illustrated using the biosynthesis of silver nanoparticle by the fungus, *Fusarium oxysporum*

recognize magnetic currents for migration and alignment purposes [18]. Utilizing the biofabrication technique, and using the metal-reducing bacterium, *S. oneidensis* nearly monodispersed nanoparticles have also been produced [19].

Monodispersed nanoparticles with specific size and shape distributions are important for various applications in biological and chemical sensing, optical, medical, and electronic devices due to their unique optical features [19]. Nanomaterials created through biosynthesis have the advantages of being highly reproducible, water soluble, and environmentally benign since the use of toxic surfactants and chemicals is unnecessary. The process involves cellular secretion of NADH-dependent reductases to quell metal ion stresses. As illustrated in Fig. 2.1, demonstrating the biosynthesis of silver nanoparticles as an example, the possible synthesis mechanism might involve an enzymatic reduction of metal ions utilizing NADH-dependent reductases to convert toxic metal ions (M^+) into stable metal atoms (M^0) and subsequent stabilization using the capping protein/peptide secreted by the bacteria under metal stress. Several studies performed by independent investigators have demonstrated that the biotransformation might involve a complex of either reductases and capping peptides, quinines or

cytochromes, phytochelatins or electron shuttles that are known to reduce various metal and metal oxides [20]. Apart from metallic nanoparticles, semiconductor CdS quantum dots were also synthesized utilizing the above-mentioned method using the enzyme sulfite reductase [15]. These in vitro syntheses of metallic gold, silver, and semiconductor CdS nanoparticles utilizing the enzymes confirms the mechanism proposed for the in vivo conversion of metal ions into nanoparticles by the microorganism. The defense mechanism definitely can be and has been exploited as a means for the production of diverse nanoparticles, and has overcome the disadvantages of similar chemical/physical routes of nanoparticle synthesis. Biosynthesis also avoids the use of toxic surfactants and solvents, is environmentally benign, highly reproducible, and produces hydrophilic and biocompatible nanoparticles.

Detailed below is an illustration of the process of biofabrication, monitoring, and evaluation of the biosynthesis of silver nanoparticles by the well-studied metal-reducing bacterium, *S. oneidensis* [20].

2.1.1 Biofabrication of Silver Nanoparticles

Silver nanocrystallites are among the many nanoparticles of particular interest because of their well-known bactericidal and fungicidal properties and are therefore widely used in consumer products such as medical devices, textiles, food packaging, and health care and household products. One type of silver nanomaterial from namely silver sulfadiazine is used clinically to reduce burn or wound infections caused by multi-drug resistant bacteria and fungi. Silver nanoparticles are also used in several water purification and air quality management systems. Due to the widespread use of these nanomaterials, there is ever-growing need regarding their synthesis; for large-scale production, addressing economic concerns, and most importantly environmentally benign green synthesis procedures.

2.1.1.1 Biosynthesis Methodology

The methodology illustrated here is for the production of silver nanoparticles using the metal-reducing bacterium *S. oneidensis* [20]. A single bacterial colony from an overnight grown bacterial colony in Luria–Bertani agar Petri dish was inoculated into 100 ml of the fresh growth medium, Luria–Bertani in a 500 ml Erlenmeyer flask and was incubated at 30°C on a shaker at 200 rpm for 24 h. The bacteria grown were collected by spinning down the bacteria by centrifugation ($5{,}000 \times g$, 20 min); the obtained bacterial cells were washed thoroughly with distilled water under sterile conditions, to get rid of the leftover nutrient medium and cellular secretions if any. In another 500 ml Erlenmeyer flask $\sim$3–5 g wet bacterial biomass was suspended in 100 ml aqueous solution containing 1 mm $AgNO_3$ and

incubated under the same conditions mentioned above. The formation of silver nanoparticles was monitored based on visual inspection as well as by performing UV–Vis absorption measurement for 1 ml aliquots over a spectral range of 200–700 nm at regular intervals. After completion of the synthesis process ($\sim$48 h), the reaction mixture was first centrifuged (5,000$\times g$, 20 min) to remove the bacteria, filtered using a sterile 0.2 μm syringe filter, to get rid of high molecular weight cellular secretions, and the particles were collected by high speed ultracentrifugation (100,000$\times g$, 45 min). The obtained silver nanoparticles were washed a couple of times with Milli Q water and the biogenic-Ag nanoparticles were further characterized for their purity, crystallinity, surface characteristics, size and shape distributions using various advanced analytical tools and techniques as described below.

2.1.2 Characterization of the Silver Nanoparticles

The first and the foremost indication for the formation of nanoparticles is the change in its color (see Fig. 2.2a) that originates from their size- and shape-dependent surface plasmon resonance (SPR). SPR is a unique phenomenon for investigating several catalytic and oxidation/reduction reactions, sensing, alloying, and electrochemical processes. Not all nanoparticles have SPR, the very few metals with SPR include gold, silver, copper, tin, lead, mercury, cadmium, indium, and the alkali metals. The SPR varies with the nanoparticle type and therefore has its own characteristic color, for example gold; ruby red, silver; brown, cadmium; yellow, magnetite; black; platinum, brownish red. For a given particular type of nanoparticle, change in the color can also occur due to the changes in the size and/or shape distributions of the particles. Therefore, UV–Vis spectra of the same nanoparticles, with different size distributions, should reveal little shift in the surface plasmon resonance (SPR) band. UV–Visible spectra in this study were recorded on a CARY 100 Bio spectrophotometer (Varian Instruments, California) operated at a resolution of 1 nm. The SPR for small silver nanoparticles, of sizes $\sim 4 \pm 2.5$ nm, showed an intense band centered at 410 nm, which is due to the excitation of SPR in the metal nanoparticles, as can be seen in Fig. 2.2b, and suggests the presence of silver nanoparticles.

Irrespective of the methodology employed to synthesize nanoparticles, most of the nanoparticles, if not encapped by a stabilizing molecule tend to aggregate or agglomerate, due to weak Wander Wall's forces or intermetallic interactions, and if not bothered they eventually form big clumps and precipitate at the bottom, and such aggregated nanoparticles do find applications where no surface coat is required such as in thin films, electric annealing, etc. However, for most biomedical and analytical applications, highly stable and soluble nanoparticles are desired. And as described earlier, to achieve such stable nanoparticles, various researchers incorporate different additives in the form of capping agents, solvents, and templates, based on the specific application the particles are desired for.

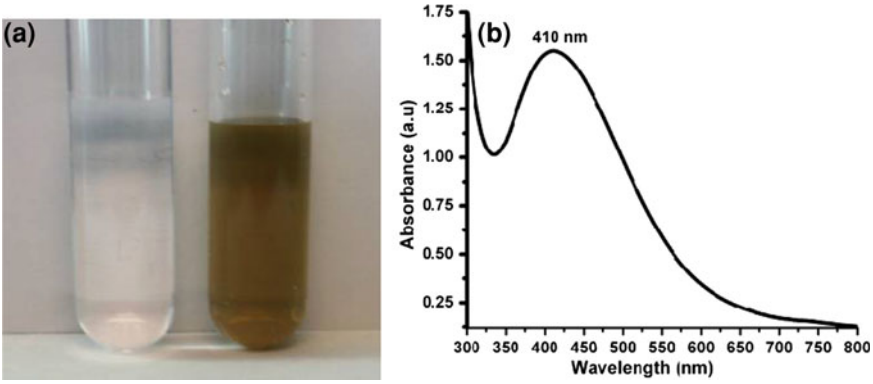

Fig. 2.2 **a** Image of the test tubes containing $AgNO_3$ solution before (test tube on the *left*) and after (test tube on the *right*) the formation of silver nanoparticles by the *S. oneidensis* biomass. **b** UV–Vis spectra of the silver nanoparticles with intense plasma peak at 410 nm

However in this case, as the particles are synthesized using microbial-based biosynthesis, the stabilizing molecule encapping their surface is not completely understood yet. Therefore, to partially elucidate the nature of stabilizing molecule surrounding the biogenic-Ag nanoparticles produced by the microorganism, Fourier transform infrared spectroscopy measurement was performed. As mentioned earlier, FTIR is a basic analytical tool that analyses the specific functional moieties associated within the given sample. FTIR analysis of the samples dried on a ZnSe window was performed on a Nicolet Magna-IR 760 spectrophotometer at a resolution of 4 cm^{-1}. As seen in Fig. 2.3, FTIR spectroscopy for the biogenic-Ag nanoparticles revealed the presence of vibration bands centered at regions 1,057, 1,398, 1,653, 2,360, and 2,930 along with an intense broad band at 3,292 cm^{-1}. The band corresponding at 1,653 cm^{-1} arises due to the presence of carbonyl (–C–O–C– or –C–O–) stretch and –N–H stretch vibrations in the amide linkages, the small peak at 1,398 cm^{-1} was also observed from amide III, clearly indicating the involvement of protein or a peptide on the surface that likely appears to be acting as a capping/stabilizing molecule. The vibration bands at 1,057 cm^{-1} correspond to the carbonyl group and alcoholic groups respectively. The band at 3,292 cm^{-1} is characteristic of the hydroxyl functional group in alcohols and phenolic compounds. We have also demonstrated that the capping protein, which may contribute to the overall stability and integrity of the nanoparticles, can be removed from the surface of the particles by a detergent (e.g. sodium dodecyl sulfate) treatment, followed by boiling for half an hour. Such a treatment may be necessary for specific applications where a surface coat is not desired, such as in particle annealing and thin film formation.

Another important characterization technique used to assess the purity, structure, and crystallinity of the nanoparticles is the X-ray diffraction (XRD). Every type of nanocrystalline material has its own characteristic Braggs reflections or

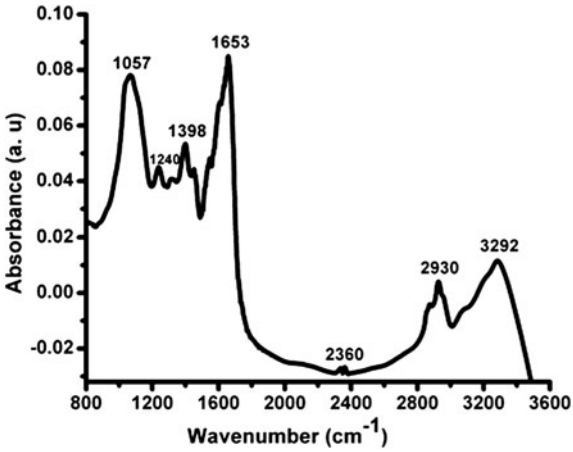

Fig. 2.3 Fourier transform infrared spectra of the biogenic silver nanoparticles. Significant vibration bands are labeled

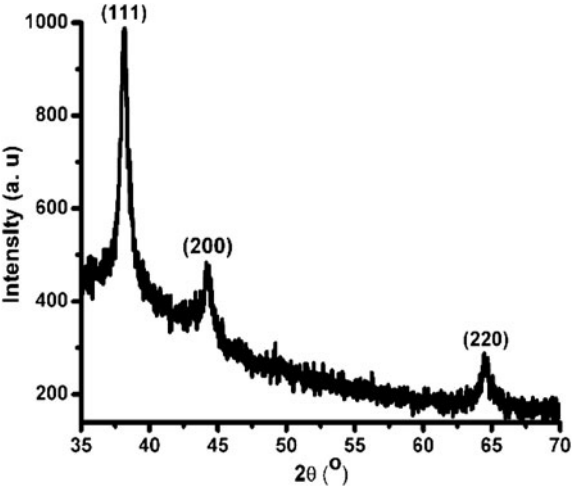

Fig. 2.4 X-ray diffraction analysis of the silver nanoparticle powder

Bragg peaks that are documented in a book called the Methods in X-ray Crystallography often used as a standard reference. Depending on the compatibility of the XRD machine, samples for the XRD can be prepared in several ways; for example, a thick layer can be coated onto a solid Si(III) wafer; sample can be dried to powder and can be used directly. In this study, XRD of dried silver nanoparticle powder was performed on a Discover D8 X-ray diffractometer with an Xe/Ar gas-filled Hi Star area detector and an XYZ platform, operated at 40 kV and at a current of 40 mA. XRD of biogenic-Ag nanoparticle powder showed intense Bragg peaks at (111), (200), and (220) in the 2θ range of 35–70θ (see Fig. 2.4) and agree with the values that are reported for silver nanocrystals, thereby confirming the purity and crystallinity of the biogenic-Ag nanoparticles.

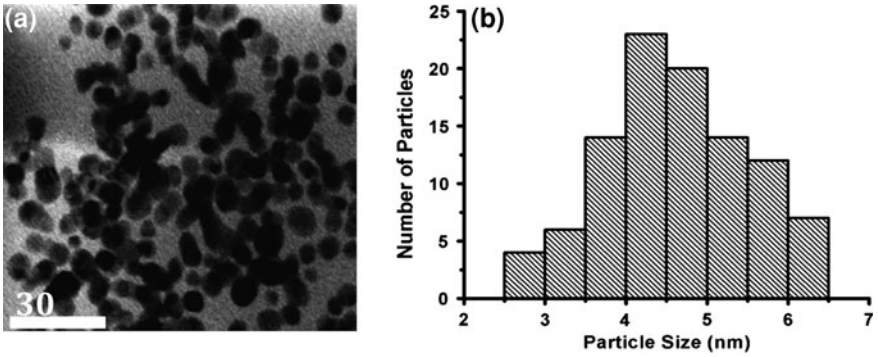

Fig. 2.5 **a** TEM image of the biogenic silver nanoparticles. **b** Histogram particle size distribution measurements made from the TEM image by counting ~100 particles in order to obtain average particle diameter

As mentioned earlier size and shape are the most important characteristics considered for any nanomaterial before it can be implemented in an application, as the properties of nanoparticles primarily depend on their size and shape distributions, and are determined based on transmission electron microscopy measurements. Samples for the TEM measurements were prepared by drop coating the nanoparticles sample on carbon coated copper TEM grids followed by air drying the grids for a couple of hours. TEM measurements were performed on a LIBRA® 120 PLUS transmission electron microscope (Carl Zeiss, Germany). TEM images of the biogenic-Ag nanoparticles revealed a large number of nearly monodispersed particles as shown in Fig. 2.5. Closer inspection of the particle morphology, at higher magnifications and different locations of the grid showed spherical single well dispersed nanoparticles as well as aggregates. An estimate of the size of the particles was also made from the line broadening of the (112) reflection pattern using Debye–Scherrer's formula ($D = 0.94\ \lambda/b\cos\theta$), and are in good agreement with the nanoparticles size estimated by TEM analysis. The particle size distribution was also performed using dynamic light scattering measurements. However, based on dynamic light scattering the hydrodynamic sizes of the nanoparticles appeared to be much larger (~82.5 nm) when compared to the TEM measurements. This may be attributed to overlapping particles and the electrical double layer phenomenon that occurs with charged particles which can affect DLS measurements, while TEM imaging allows latitude for eliminating aggregated particles from the analysis. A particle size histogram plot (plotted using Image J software) from the TEM image showed the size distribution of biogenic silver nanoparticles to be in the range between ~2 and 11 nm with the largest number of particles being 4 ± 1.5 nm (see Fig. 2.5b). The particles were negatively charged with a zeta potential of −12 ± 2 mV, which can be one of the reasons for their long-term stability; the electrostatic repulsive forces between the nanoparticles might protect them from association and thereby prevent agglomeration or clumping in aqueous suspension. The dynamic light scattering and the

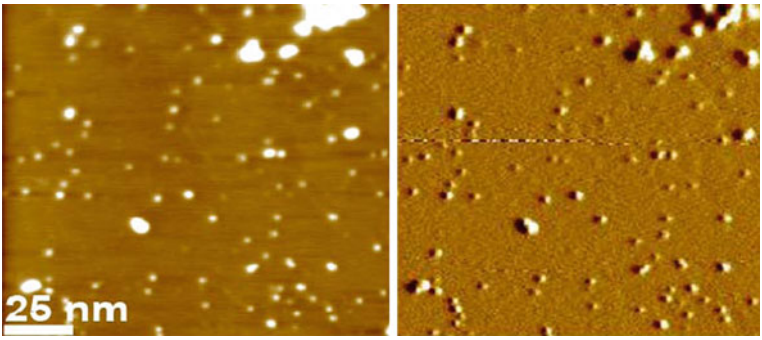

Fig. 2.6 Atomic force microscopy analysis of the biogenic silver nanoparticles. AFM topographical (*left*) and deflective (*right*) images. The deflection is shown to differentiate single particles and clumps of particles

zeta potential measurements were performed on a Brookhaven 90 Plus/BI-MAS Instrument (Brookhaven Instruments, NY).

The size and shape distribution of the nanoparticles was also confirmed by performing the atomic force microscopy (AFM) analysis. As can be seen in Fig. 2.6, AFM revealed uniformly shaped well dispersed nanoparticles with a particle height ranging from ~2 to 11 nm, similar to the size distribution observed in TEM measurements. The samples for the AFM analysis were prepared by coating a drop of the nanoparticle sample onto plain mica and drying overnight; the particles dispersed well on mica surface, characteristic of a hydrophilic protein surface coat. For AFM, samples were imaged in either contact or intermittent contact mode with a PicoPlus AFM (Aligent Technologies, Tempe, AZ) using a 100 μm scanning head at 128–512 pixels per line scan and a scan speed of 0.5 line/s. The cantilevers used were Veeco silicon nitride probes (MLCT-AUHW, Veeco, Santa Barbara, CA).

2.2 Summary

For the past few decades, there has been a tremendous progress in the biofabrication of engineered nanosturctures of controlled morphology and size distributions at the nanometer scale. Microorganisms are best known machines that function at the nanoscale, performing numerous operations ranging from energy production to target material extraction with very high efficiency. Microorganisms have been successfully implemented for the synthesis, nucleation, and assembly of various metal, metal oxide, and metal sulfide nanocrystallities under ambient conditions. The produced particles have interesting properties like hydrophilic nature, high stability, biocompatibility, high surface area. Additionally, biological entities have also been shown to have an influence on the size and shape distributions, and surface properties of diverse nanoparticles for various intended

applications. With the continued interest and ingenuity of researchers from various disciplines, the future materials promise to be green, exciting, with selectively designed nanoparticles for diverse applications.

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