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Review Article

A Review of Stabilized Silver Nanoparticles – Synthesis, Biological Properties, Characterization, and Potential Areas of Applications

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Abstract

Nobel metal nanoparticles have drawn significant attention for a wide range of new applications in various fields including biology and medicine. Research work during the last two decades have clearly demonstrated that the properties of nanoparticles, in particular silver nanoparticles are strongly influenced by shape, size and size distribution, which is dictated by the synthetic method adopted. In this review, we enumerate various top-down and bottom up approaches to synthesize nanoparticles. Chemical reduction method is one of the simple and facile approaches for bottom-up synthesis of silver nanoparticles and the stability of the synthesized nanoparticles has been found to be influenced by the type and amount of reducing agent and type of stabilizer used. Some of the capping reagents discussed including citrate salts, oleic acid, amino silanes, and polyelectrolytes so as to stabilize the nanoparticles. Instead of using polyelectrolytes to conjugate nanoparticles, biomacromolecules have been used to stabilize nanoparticles so that it renders the nanoparticles bioactive and biocompatible as well as provides additional functionalities for further biological interactions. Surface modification of nanoparticles with proteins such as Bovine Serum Albumin (BSA) is an effective approach to providing electrosteric stability to silver nanoparticles. We highlight the various pathways by which stabilized nanoparticles promote antibacterial activity and describe the impact of stabilized nanoparticles on mammalian cells. More importantly, in this review we describe the possibility of a concentration window at which nanoparticles are toxic to bacteria and not to mammalian cells, so that the nanoparticles loaded matrix could be designed with the intent that nanoparticles when released in the physiological medium can maintain a sterile environment against microorganisms while not inhibiting the growth of mammalian cells in the site specific region of intended application. Additionally, methodologies used to characterize the composition, morphology and biological properties of synthesized nanoparticles by multiple techniques have been presented.

INTRODUCTION

The effective prevention and treatment of an ever-increasing range of infections caused by bacteria, viruses, fungi, and parasites are a priority to public health officials and a big challenge to pharmaceutical industry [1]. *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* are the etiological agents of several infectious diseases [2]. Infections caused by these and other pathogenic bacteria decreased during the twentieth-century due in part to the discovery and therapeutic use of antibacterial antibiotics [3]. Antibacterial antibiotics are classified by their specific mechanism of action. Examples of antibacterial antibiotics include bacterial cell wall inhibitors, e.g., (beta lactams - penicillins and cephalosporins), vancomycin, cycloserine, and bacitracin; bacterial DNA gyrase/topoisomerase inhibitors, e.g., quinolones; bacterial RNA polymerase inhibitor, e.g., rifampin; RNA elongation inhibitor, e.g., actinomycin; bacterial protein synthesis inhibitors, e.g., 50 S ribosome inhibitors - macrolides and chloramphenicol and 30 S ribosome inhibitors - tetracyclines and aminoglycosides; folic acid metabolism inhibitors , e.g., trimethoprim and sulfonamides; and cell membrane inhibitors, e.g., polmyxins. Moreover, antibiotic resistance has emerged as a prevalent problem due in part to the misuse of existing antibiotics and the lack of novel antibiotics [4,5]. Conventional antibiotics no longer inhibit bacterial growth because bacteria have developed antibiotic resistance via mutational and/or several adaptive mechanisms that include decreasing the antibiotic concentration via efflux pumps (tetracycline efflux pumps), antibiotic inactivation via enzymatic modification, (beta lactamase cleavage of the beta lactam rings present in penicillin and cephalosporins or acetylation of chloramphenicol via chloramphenicol acetyl transferase), and or alteration of the bacterial drug targets, e.g., altered penicillin binding proteins or bacterial ribosome subunits. Additionally, antibacterial resistance genes reside on either the bacterial genome or on extrachromosomal plasmids and these resistance genes may be transferred between bacteria. Increasing antibiotic resistance has emerged as a consequence [4].

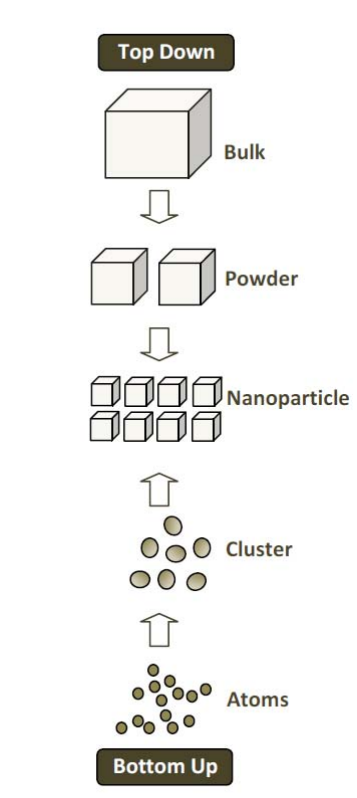
Bacterial resistance to conventional antibiotics has prompted the development of alternative strategies to prevent and treat bacterial infections. Among them, nanoscale materials have

emerged as an alternative approach to treat bacterial infections caused by the antibiotic resistant bacterial strains. Stabilized nanoparticles act on bacteria via multiple mechanisms [6,7]. Bioconjugated nanoparticles are able to attach to the membrane of bacteria by electrostatic interaction and damage the integrity of the bacterial peptidoglycan and/or cell membrane [8].

Among the several metal nanoparticles, silver nanoparticles have received considerable attention due to their broad inhibitory behavior towards nearly 650 species of microbes, and more importantly against antibiotic resistant bacterial strains [9,10]. In one of the findings, it was shown that silver nanoparticles showed superior antibacterial activity against *E. coli* and *S. aureus* when compared to gold nanoparticles [11] Furthermore, the chemistry and morphology of silver nanoparticles can be easily tuned to improve their antibacterial efficacy. The worldwide production of silver nanoparticles is estimated to be 320 tons per year [12].

Synthesis of nanoparticles

A top-down approach to nanofabrication is based on the synthesis of the nanomaterials from the bulk system [13], while bottom-up synthesis of nanomaterials is based on packing of several atoms, or molecules with molecules, or clusters with clusters [14,15]. A representation of the top down and bottom up approach is shown in Scheme (1). **Table (1)** summarizes the advantages and disadvantages of various methods used in the syntheses of nanoparticles. Procedures used in top-down synthesis of nanoparticles include etching, grinding, ball milling,



Scheme 1 Schematic representation of formation of nanostructure via Top-down Vs. Bottom-up.

Table 1: Summary of methods used for the Ag NPs synthesis.

Method	Description	Size (nm)	Reference
Evaporation-condensation	Solid bulk material is evaporated under high vacuum, and the vapor-phase molecules are condensed to yield solid NPs.	<100	[16]
Electrochemical	Metal sheet used as anode undergo oxidation to produce metal ions which are reduced at the cathode or in the electrolyte solutions to NPs.	2-20	[17]
Photoinduced reduction	Reduction of silver nitrate with UV irradiation	5-8	[18]
Micro-emulsion	Micro emulsions of metal salt and reducing agent is mixed to produce NPs.	0.5-7	[19]
Chemical reduction	Silver salt solution reduced by inorganic or organic reducing agent.	2-25	[20]
Laser ablation	Vaporization of material by a pulse beam. Vaporized material is condensed in the solvent producing NPs.	1-5	[21]
Microwave assisted synthesis	The electric field developed by microwave produces localized heat for the homogeneous nucleation, and leading to the rapid crystal growth of NPs.	50-130	[22]
Biological	Use of natural materials to reduce, cap, and stabilize such as fungi, bacteria, plant extract.	5-50	[23]

laser ablation, photo-lithography, and electron beam lithography. Unlike top-down approach, bottom up approach is based on organization of small constituents (atoms or molecules). This method is guided by physicochemical interaction of neighboring constituents, the surface chemistry and self assembly principles of the constituents that makeup the nanoscopic material. Bottom-up approach offers a better chance to obtain nanostructures with less defects, more homogeneous chemical composition, potentially better short and long range order. Some examples of bottom-up approach include biological, photochemical, and chemical synthetic routes. Here, we describe the bottom up method which is the primary focus of the study.

Photochemical synthesis of silver nanoparticles

In the photochemical approach, the nanoparticles are synthesized from ionic precursors. For example, when an aqueous solution of silver salt, acetone, propanol and polymer stabilizer is UV irradiated, polymer capped silver nanoparticles are formed. UV illumination is believed to generate ketyl radicals via initial excitation of acetone and subsequent hydrogen atom abstraction from 2-propanol according to **Equation 1**: [24]

$CH_3COCH_3^* + (CH_3)_2CHOH$ -----> $2(CH_3)_2C^{\cdot}OH$ (1)

The short lived radicals serve as strong reductants. It releases electron and a proton in the process of regeneration of acetone. The electrons could subsequently reduce silver salt to form silver atom, according to **Equation 2 and 3**. Polymers effectively stabilize the clusters of silver atoms to form polymer capped nanoparticles.

$(CH_3)_2C^{\cdot}OH$ -----> $CH_3COCH_3 + H^+ + e^-$ (2)

$(CH_3)_2C^{\cdot}OH + Ag^+$ -----> $(CH_3)_2CO + H^+ + Ag^0$ (3)

Alternatively, the synthesis of silver nanoparticles may involve direct photo-reduction of $AgNO_3$ in the presence of sodium citrate with different light sources at room temperature. [25] It was demonstrated that depending upon the wavelength of light source used in photochemical reduction i.e. UV or white or green light, nanoparticle suspension with distinctive optical properties could be formulated. [12] These nanoparticles differed in size and shape.

Occasionally, in a UV photo-activation method, a reagent is used in the preparation of stable silver nanoparticles which serves as reducing agent as well as stabilizing agent. In fact, when silver nanoparticles were prepared along with aqueous Triton X-100, Triton X-100 served the dual purpose of reducing agent and stabilizing agent. [26] Likewise, when silver nanoparticles were synthesized in an alkaline solution of $AgNO_3$/carboxymethylated chitosan (CMCTS) with UV light, CMCTS served the dual role as a reducing agent for silver cation reduction and a stabilizing agent/ surfactant for silver nanoparticles. [27] Studies have shown that surfactants play an important role in the photochemical reduction of silver salt solution to form uniform sized nanoparticles. The surfactant solution acts as stabilizer in the preparation of well-defined nanoparticles (by increasing the surface tension at the solvent– nanoparticles interface). The major merits of the photochemical synthesis route are: (i) clean, (ii) controlled formation of nanoparticles triggered by the photo irradiation and (iii) significant versatility in the photochemical synthesis of nanoparticles in various mediums including emulsion, surfactant micelles, etc. [28]. Some of the factors that can influence the overall composition of synthesized nanoparticles include the wavelength and intensity of irradiation beam, and exposure time of the reagent solution to irradiation. In the absence of proper control, there is a possibility of localized heating of the reagent solution leading to inhomogeneity in synthesized nanoparticles composition.

Biological synthesis of silver nanoparticles

When silver nanoparticles are produced by biological route, the living organisms or proteins act as reducing agent and/or stabilizing agent [29]. Bacteria such as *Shewanella oneidensis* has been noted to interact with silver nitrate solution, resulting in the formation of nearly monodispersed nanoparticles in the size range of 2 to 10 nm with an average size of 4 nm. The bacteria assisted synthesis of nanoparticles is economical, simple, reproducible, and requires less energy when compared to other synthetic routes. [30] Silver nanoparticles have also been synthesized using the *Lactobacillus species* where *Lactobacillus species* serves as reducing and capping agent. Sintubin et al. showed *Lactobacillus species* accumulated and subsequently reduced Ag^+ to produce Ag^0 species inside the cell. [31]. The mean diameter of the biogenic silver nanoparticles produced by this method varied depending upon the *Lactobacillus spp.* used. The recovery of silver nanoparticles and the reduction rate of silver ions were found to be pH dependent. Other researchers have used plant based compounds to synthesize silver nanoparticles. For example, Kumar et al., developed an eco-friendly and sustainable green route for the synthesis of stable silver nanoparticles (AgNPs) using aqueous leaf extract of plants as both reducing as well as a stabilizing agent [32-35]. Most of the AgNPs were spherical and in the range of 8 nm to 24 nm having an average size distribution of 15.5 nm. The biological method of synthesizing silver nanoparticles is a low cost approach and less energy intensive process. Generally, it is not easy to produce a large quantity of silver nanoparticles by using biological approach.

Chemical synthesis of silver nanoparticles

Among the various known methods, the chemical method has been the most widely studied because of the general versatility of the technique. For example, silver nanocubes in large amounts have been synthesized by reducing silver nitrate with ethylene glycol in the presence of stabilizing agent, the so-called polyol process [36]. Ethylene glycol serves as both reductant and solvent. Based on the molar ratio of stabilizer relative to silver nitrate and the experimental conditions used in the synthesis, the geometric shape and size of the nanoparticles could be varied significantly. The polyol process has also been used to synthesize spherical silver nanoparticles with a controllable size and high monodispersity [37].

Alternatively, spherical silver nanoparticles can be synthesized using oleyl amine - liquid paraffin mixture [38]. The use of a high boiling point liquid e.g. paraffin, offers the flexibility to effectively use reaction temperature to generate silver nanoparticles of varying size without changing the solvent. The size of nanoparticles in the solution is strongly dependent on the duration of the individual stages of synthesis i.e., synthesis of silver nuclei and subsequent growth accompanying nuclei formation. For the synthesis of monodispered silver nanoparticles with uniform size distribution, it is preferable to form the nuclei at similar time. The initial nucleation and the subsequent growth step of initial nuclei can be controlled by adjusting the reaction parameters such as reaction temperature, pH, type of metal precursors, reducing agents (e.g. $NaBH_4$, ethylene glycol, glucose) and stabilizing agents (e.g. sodium citrate) [39-41].

Reduction of silver salts to form nanoparticles has been achieved using sodium citrate and/or borohydride. The use of sodium borohydride (a strong reductant) usually results in the formation of somewhat monodispersed smaller sized silver nanoparticles while the use of only citrate (a weaker reductant) usually results in the formation of somewhat polydispersed larger sized silver nanoparticles because of slower reduction rate [42]. Reduction of silver ion by sodium citrate is shown below [43]

$4Ag^+ + C_6H_5O_7Na_3 + 2H_2O$ ----->

$4Ag^0 + C_6H_5O_7H_3 + 3Na^+ + H^+ + O_2\uparrow$ (4)

Reactions 5, 6, and 7 provide the individual steps and overall reaction step in the formation of silver nanoparticles upon reduction with sodium borohydride.

$$2Ag^{+} + 2e^{-} \text{ -----> } 2Ag \quad (5)$$

$$2BH_4^{-} \text{ -----> } B_2H_6 + H_2 + 2e^{-} \quad (6)$$

$$2AgNO_3 + 2NaBH_4 \text{ -----> } 2Ag + 2H_2 + B_2H_6 + NaNO_3 \quad (7)$$

Solomon et al. [44] proposed a mechanism of nanoparticle formation based on sodium borohydride reduction and stabilization (without stabilizing agent). The nanoparticle formation is based on the temporary stabilization of smaller sized silver nanoparticles by excess BH_4^- species. **Figure 1** shows structure of stabilized silver nanoparticles with a shell of excess borohydride anion. However, with time, there is the collapse of the stabilized shell around the nanoparticles that causes the nanoparticles to aggregate which is largely attributed to the degradation of BH_4^- accompanied by hydrogen gas evolution as mentioned in **equation 8**.

$$BH_4^{-} + 4H_2O \text{ -----> } B(OH)_4 + 4H_2 \quad (8)$$

Given the borohydrydate anion degradation in sodium borohydride capped nanoparticles, a number of alternative capping agents have been studied to stabilize nanoparticles with or without dispersants. A nice review on the common capping agents commonly used in nanoparticle synthesis and their impact is presented by Niu and Li et al. [45]. Recently aminosilanes have been used as capping agent to stabilize the nanoparticles as well as serve as coupling agent to couple with other moieties [46]. Li et al., took a different approach to stabilize nanoparticle by dispersing oleic acid capped silver nanoparticles with different dispersion agents. Interactions between dispersant and capping agent determine the extent of dispersion of silver nanoparticles. H-bonding between dispersant and capping agent effectively results in enhanced agglomerations of Ag nanoparticles [47]. Other studies have evaluated the stability of stabilized silver nanoparticles at various pH conditions. It was established that citrate anion as capping molecule may not be enough to maintain the stability of citrate stabilized nanoparticles over a wide pH range [48]. There is a strong likelihood for the nanoparticles to aggregate depending upon the pH despite the nanoparticles stabilized by small molecules such as citrate anion. Therefore alternative routes to stabilize nanoparticles have been studied.

Stabilization of silver nanoparticles

It is well known that nanoparticles in its free form are thermodynamically unstable due to high surface energy. Due to Brownian motion, the high surface energy nanoparticles collide and the final state of nanoparticles is dictated by the type of interaction between the colloidal nanoparticles [49]. When interaction between nanoparticles is dominated by attractive forces, the colloidal particles will adhere with each other until particle stabilization occurs. When repulsive forces dominate between nanoparticles, the colloidal particles are rather stable in the dispersed state. Van der Waals forces are the primary source of attraction between colloidal particles. When strong repulsive force (Born repulsion) counteracts the van der Waals attraction, the nanoparticles remain in dispersed state. Counteractive repulsive forces can be enhanced by charge repulsion and steric hindrance. **Figure (2)** is a pictorial representation of electrosteric stabilization of nanoparticles, where the presence of bulky and highly charged adsorbent molecules provides stability to the nanoparticles. Badawy et al. compared the stability of electrostatically stabilized silver nanoparticles (citrate capped silver nanoparticles) with those of electrosterically stabilized silver nanoparticles (using branched polyethylenimine (BPEI)-coated silver nanoparticles) and noticed a more significant stabilization effect in nanoparticles stabilized by electrosteric repulsion [50]. The citrate coated silver nanoparticles aggregated at higher ionic strengths (100 mM $NaNO_3$) and/or acidic pH (3.0). As for BPEI-coated silver nanoparticles, the ionic strength, pH, and electrolyte type had lesser impact on the state of aggregation of the electrosterically stabilized silver nanoparticles.

Instead of using polyelectrolytes to conjugate nanoparticles, antibodies, antigens, and proteins have been used to electrosterically stabilize nanoparticles. The rationale for stabilizing nanoparticles with biomacromolecules is that it renders the nanoparticles bioactive and biocompatible as well as provides additional functionalities for further biological interactions or coupling [51]. Metal nanoparticles have been stabilized by peptides (Arginylglycylaspartic acid (RGD), antigenic peptides), proteins (bovine serum albumin, transferrin, antibodies, lectins, cytokines, fibrinogen, thrombin), polysaccharides (hyaluronic acid, chitosan, dextran, oligosaccharides, heparin), polyunsaturated fatty acids (palmitic acid, phospholipids), DNA, plasmids, and siRNA [52,53]. Among these stabilized nanoparticles, BSA conjugated nanoparticles has received special importance because of its broad applicability for drug delivery applications and its stability over a range of intracellular pH [54,55]. Therefore, BSA conjugated nanoparticles is the primary focus of the review article.

Prasad et al. have reported the formation of BSA-directed gold, silver, platinum, gold–silver, and silver–platinum nanoparticles [56,57]. Yang et al. have successfully fabricated BSA conjugated Ag_2S nanorods [58]. Generally, BSA conjugated nanoparticles have been noted to be stable over a variety of conditions, such as pH and electrolyte concentrations [59]. The stability comes from several amino acids present in BSA such as histidine, cysteine, aspartic and glutamic acid, which promote binding with transition metal salts. In particular, residues of BSA including sulfur-, oxygen-, and nitrogen-bearing groups can stabilize the nanoparticles. Of these, thiol bearing cysteine residues are likely to interact with silver nanoparticles more strongly via direct chemical bonding and provide steric stabilization due to bulky protein molecules. Additionally, metal nanoparticles, such as silver and gold are expected to show strong affinity towards amino groups of proteins due to its large complexation constant for noble metal amines [60]. During the process of stabilization and capping, the macromolecules (BSA) is believed to retain its overall structural integrity while inducing biocompatibility characteristics to the silver nanoparticles [61]. Stabilization of silver nanoparticles by BSA is also believed to not to interfere with the original antibacterial properties of nanoparticles [62].

Biological properties of stabilized silver nanoparticles

Particles of nanoscale dimension are noted for their enhanced surface area and high reactivity. More importantly, the physical,

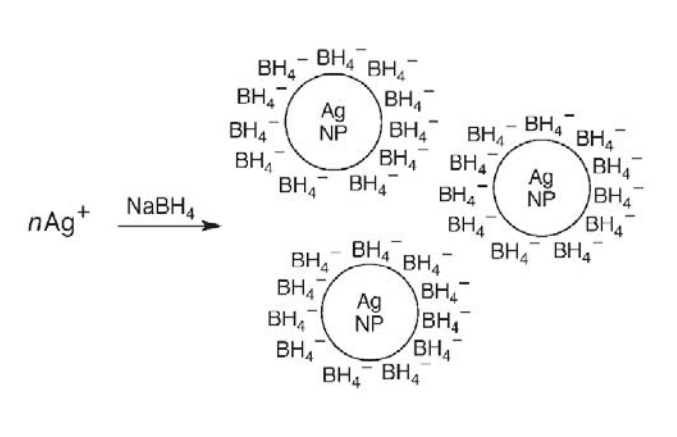


Figure 1 Silver nanoparticles stabilized by repulsive forces generated by borohydride anion. (Adopted from Ref. 44).

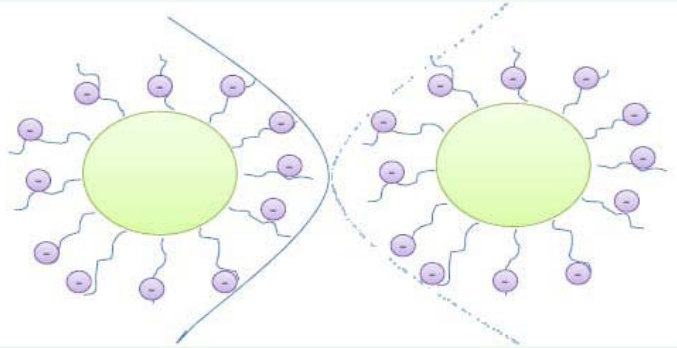


Figure 2 Electrosterically stabilized silver nanoparticles.

biological, and chemical properties of silver nanoparticles are far different from bulk counterparts. In this overview, we are interested in reviewing the biological activity of the silver nanoparticles towards microbes and the cytotoxicity of silver nanoparticles towards mammalian cells.

Nanoparticles show antimicrobial activity against a range of bacteria. Among the various nanoparticles, silver nanoparticles exhibit broad inhibitory behavior towards nearly 650 species of microbes, and more importantly against antibiotic resistant bacterial strains [9,10,63]. In one of the findings it was shown that silver nanoparticles showed superior antibacterial activity against *E. coli* and *S. aureus* when compared to gold nanoparticles [11].

In general, the antibacterial properties of nanoparticles are dictated by dimensional characteristics and chemical composition [64-66]. Pal et al. demonstrated that silver nanoparticles undergo shape dependent interaction with *E. coli*. They reported that truncated triangular silver nanoparticles showed stronger antimicrobial activity than spherical and rod-shaped geometry [67]. Equally, particle size plays an influential role in the antibacterial properties of silver nanoparticles, with smaller particles exhibiting improved activities [61, 67, 68]. However, it must be noted that the smaller nanoparticles have a tendency to agglomerate in a media with high electrolyte content resulting in a loss of antibacterial effectiveness [61]. The anti-bacterial properties of silver nanoparticles are also dependent on the surface oxidation state of silver and the polydispersion of silver in the medium. The levels of chemisorbed Ag^+ ion that form on the surface of nanoparticles during oxidation influence the extent of antibacterial activity [61]. Also, the antimicrobial efficacy of nanoparticles depends on the concentration of nanoparticles used in the biological growth media.

Sondi et al. reported that silver nanoparticles of concentration of about 20 and 50–60 ppm are 100% inhibitory towards 10^4 CFU and 10^5 CFU of *E. coli*, respectively [64]. SEM images clearly showed nanoparticles accumulated on the bacterial membrane of *E. coli* that was exposed to nanoparticles. Morones et al. have reported that in gram-negative bacteria the nanoparticles mainly attach to the surface of the cell membrane and influence the normal functions of cells such as permeability and respiration [68]. Also, nanoparticles that penetrate inside the bacteria through accumulation on the bacterial membrane can cause further damage by possibly interacting with sulfur- and phosphorus-containing molecules such as protein or DNA.

Kvitek et al. reported that the antibacterial activity of silver nanoparticles is also dependent on the surface modifiers (surfactant/polymers) used in the stabilization of smaller sized nanoparticles [69]. Three stabilized silver nanoparticles (with sodium dodecyl sulfate-SDS and polyoxyethylenesorbitane monooleate-Tween 80, and polymer (polyvinylpyrrolidone-PVP

360)) were tested for their antimicrobial activity with *S. aureus, E. faecalis, E. coli,* and *P. aeruginosa,* and other strains isolated from human clinical samples such as *P. aeruginosa,* methicillin-susceptible *S. epidermidis,* methicillin-resistant *S. epidermidis,* methicillin-*resistant S. aureus*, vancomycin-resistant *E. faecium* and *K. pneumoniae.* The results showed that the minimum inhibitory concentration (MICs) of silver nanoparticles fall in the range of 1.69–13.5 ppm, depending upon the type of bacterial strain, and the type of surfactant/polymer used to stabilize silver nanoparticles. In particular, among the three stabilized nanoparticles systems studied, the antibacterial activity of the silver nanoparticles was highest for the SDS modified silver nanoparticles.

To date, significant studies have been conducted on evaluating the effects of silver nanoparticles against different bacterial strains. However, there is no clear understanding as to how silver nanoparticles exhibit strong antimicrobial characteristics. Explanations have been put forth to describe the bacterial growth inhibition and the lethal effect of silver nanoparticles [64,70,71], including several pathways/mechanisms by which silver nanoparticles may influence the antibacterial activity.

Figure (3) summarizes the various mechanisms by which silver nanoparticles can interact with the microbial cells [70]. One of the mechanism presented by Sondi et al. (2005), is that the silver nanoparticles adhere to the bacterial cell wall and subsequently penetrate the cell wall by forming 'pits', thereby causing structural changes to the cell membrane. In this mechanism, the size of nanoparticles plays an influential role in its diffusion into the microbial cell membrane. This process exposes the cell organelles to the medium present in the extracellular environment and impacts the normal functioning of the cell. This mechanism is broadly categorized as the direct impact of nanoparticles on microbial cells.

A slight modification to the proposed mechanism was presented by Danilcauk et al., (2006) and Kim et al., (2006) where formation of free radicals by silver nanoparticles was used to describe the damage to the cell membrane [72, 73]. Once the cell membrane is damaged, the cell wall becomes permeable to the extracellular medium and the bacterial cell becomes vulnerable to damage. Alternatively, the ion channels present in the cell can facilitate the diffusion of oxidized silver species (oxidation product of silver nanoparticles) from the extracellular environment into the cell and promote interaction with enzymes thus causing damage to the cell. Silver ions can specifically interact with thiol groups to inactivate bacteria [68,74,75] and/or interact with respiratory chain enzymes, nucleic acids and/or cytoplasmic components [76].

The third mode by which the nanoparticles inhibit bacterial growth is by the generation of reactive oxygen species which influences respiratory enzyme functioning. The reactive oxygen species can destruct the cell [76,77].

Lastly, the silver nanoparticles can interact with the cell based on its tendency to react with soft base. The cells have several constituents which are made up of sulfur and phosphorus which can serve as soft bases [68]. The interaction of the silver nanoparticles with DNA can lead to the suppression of DNA replication of the bacteria and thus inhibition of bacterial growth [78,79].

Cytotoxicity of silver nanoparticles

Silver nanoparticles have not only been noted to show broad antimicrobial activity but also broad cytotoxicity towards mammalian cells [80, 81]. Cytotoxicity studies of nanoparticles towards mammalian cells have focused on (i) uncapped silver nanoparticles, (ii) chemically capped silver nanoparticles and (iii) biogenic capped silver nanoparticles [80]. The biogenic capped nanoparticles represent protein capped nanoparticles. The review focuses initially on the cytotoxicity of uncapped silver nanoparticles, and then describes the cytotoxicity of biomolecule conjugated silver nanoparticles.

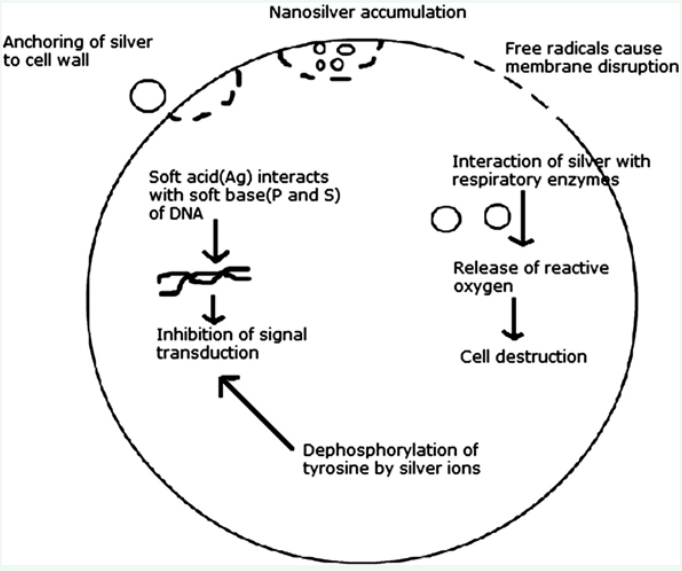


Figure 3 Mechanism of antimicrobial action of silver nanoparticles." Modified from Reference 70".

A number of studies have been conducted to investigate the impact of silver ions and silver nanoparticles on the viability and differentiation of bone cells (osteoblast/osteoclast cells). In an in vitro study conducted to investigate the effect of silver ions and nanoparticles on the activity of primary osteoblasts (OBs) and osteoclasts (OCs) cells [82]. It was established that the cytotoxicity effect of silver nanoparticles is dependent on the particle dose and the size of nanoparticles (ranging from nanometer to micron sized silver particles). When comparing the mean half maximal inhibitory concentration (IC50), the nanometer sized silver nanoparticles with an average size of 50 nm, surface area 11.54 m^2/g exhibited stronger inhibition than micron sized silver particles (3 m). These results are in general agreement with the results reported by Liu et al.; Hussain et al; Carlson et al.[83-85] Liu et al., conducted cytotoxicity measurements of silver nanoparticles of various size ranging from 3 nm to 25 nm towards L929 fibroblast cells [83]. Cytotoxicity was noticed at concentrations of 10 ppm for the smaller-sized silver nanoparticles while for the larger nanoparticles even at 10 ppm, no significant cytotoxicity was noticed. It should be noted that the cytotoxicity studies were performed on nanoparticles not stabilized by capping agent and the methodology used to prepare nanoparticles were by physical methods [86].

Carlson et al. investigated the viability of macrophages as a function of dose concentration (10-75 g/mL) and size of nanoparticles (Ag-15 nm and Ag-30 nm nanoparticles) [85]. A more than 10-fold increase of Reactive Oxygen Species (ROS) levels in the cells exposed to 50 ppm of Ag-15 nm was observed suggesting that the cytotoxicity of Ag-15 nm is likely to be mediated through oxidative stress. The physicochemical properties of nanoparticles can produce pro-oxidant environment in the cells, causing an imbalance in the cellular energy which can lead to adverse biological consequences, ranging from the initiation of inflammatory pathways through to cell death.

Similar toxic effect of smaller-sized silver nanoparticles towards mammalian cells has been reported previously [83]. It is believed that smaller silver nanoparticles can easily diffuse the mammalian cell membrane, get internalized and because of their enormous surface area they are able to interact with cellular organelles and this causes cytotoxicity. [81] Also, the smaller silver nanoparticles have higher surface area to volume ratio compared to the larger silver nanoparticles, which can contribute to an enhanced release of toxic Ag^+ ions to the surface of nanoparticles. Several studies have shown that both the silver nanoparticles and ionic silver can exhibit cytotoxic effects in different types of cells [87,88]. The release rate of silver ions from nanoparticles is dependent also on other factors such as temperature, oxygen, pH, and UV light. Ag^+ ion formation leads to the production of superoxide radicals and other reactive oxygen species via a reaction with oxygen, inducing cellular apoptosis and the expression of stress response-related genes. In one study it was reported that when eukaryotic cells (MC3T3-E1) were exposed to various cations, silver ion was found to be one of the most cytotoxic followed by Cu^+ ions than Co^{+2} ions than Ni^{+2} ions than Fe^{+3} ions [89].

It is not clear from a number of these studies whether silver nanoparticle-related cytotoxicity is mediated by the nanoparticles themselves or by the ions released by the nanoparticles during dissolution/oxidation because the results suggest that ionic as well as the nanoparticles contribute to silver nanoparticle-associated cytotoxicity [64].

Apart from a significant number of studies focused on cytotoxicity of nanoparticles, limited studies have focused on establishing the concentration window of nanoparticles i.e., where nanoparticles exhibit microbial inhibition without nanoparticles exhibiting cytotoxicity. Flores et al. reported that cubic Ag nanoparticles of 6 nm exhibit inhibitory effects towards *S. aureus* and *P. aeruginosa* at concentration less than 4 μM (~0.43ppm) while at concentration above 50 μM (~5.40 ppm), the Ag nanoparticles exhibit cytotoxicity towards UMR-106 cell lines [90].Their results suggested that there exist a concentration window (between 4 μM and 50 μM) of Ag NPs at which no cytotoxic effect towards UMR-106 cell line was observed while a sterile environment was maintained against microbial agents [90]. However, Albers et al. reported that primary mouse osteoblasts and osteoclasts were more susceptible to silver treatment than *Staphylococcus epidermidis*, a bacterial skin commensal that may infect joint prosthesis. Moreover, it was reported that the MICs of Ag+ deriving from AgNO3 or silver nano particles to inhibit *S. epidermidis* bacteria growth were two to four times higher than the minimal Ag+ concentration required to decrease the viability and proliferation of primary osteoblasts and osteoclasts [91].

In contrast, other studies have suggested that at concentrations of silver nanoparticles and ionic silver where an antibacterial effect is noticed, there may not be a cytotoxic effect on eukaryotic cells [81,82]. In addition, Ag NPs has been shown to exhibit anti-inflammatory properties and expedited wound healing with minimal tissue scarring. [92,93] Ag NPs coated onto the surface of absorbable suture showed an improved anastomosis healing and significantly less inflammation [94]. Collectively, these studies strongly suggest that the type and concentration of silver (silver ions or aggregated silver) may not be the only factors that plays an important role in the concentration window, other factors such as the procedure used to synthesize nanoparticles, presence or absence of capping agent, type of capping agent, and grafting density of capping agent on nanoparticles are all integral aspects in dictating the extent of antimicrobial and cytotoxic effect on functionalized nanoparticles.

As mentioned previously, silver nanoparticles are often stabilized with reagents such as citrate [95], chitosan [95], polyethylenimine [64], polyvinylpyrrolidone(PVP) [72], polysaccharides [95], carbon , hydrocarbons [62,72,96], starch [67], peptides [96], and bovine serum albumin [66]. The capping agent could introduce various surface chemistries on silver nanoparticles in solution [66].

Capping agent used to stabilize silver nanoparticles can have an effect on inducing oxidative stress, DNA damage, and apoptosis of mammalian cells. Ahamed et al. compared the performance of uncapped and polysaccharide capped silver nanoparticles of similar sizes and found that polysaccharide-capped silver nanoparticles was lethal towards mouse embryonic stem cells and fibroblasts and showed extensive genotoxic DNA alterations and apoptotic changes [97]. On the other hand, carbon-coated silver nanoparticles were found to be less lethal than uncoated

silver nanoparticles of similar sizes in mouse macrophages [98]. This suggests that the capping agent functionality is critical to cytotoxicity of silver nanoparticles. Further studies are needed to determine the influence of capping agent and surface charge on silver nanoparticles -induced cytotoxicity.

Only to a limited degree, silver nanoparticles capped with biogenic compounds have been examined for cytotoxicity. Albumin-capped silver nanoparticles showed more genotoxicity than polysaccharide-capped nanoparticles. For example, silver nanoparticles capped with albumin (size 70 nm) have been found to be more genotoxic on a mouse peritoneal macrophage cell line (genotoxicity at around 2ppm) [97], compared with silver nanoparticles capped with polysaccharides (size 25 nm) which exhibited genotoxicity at 50ppm on mouse embryonic stem and fibroblasts [84,99]. Also, it has been reported that peptide-coated silver nanoparticles can be more toxic to macrophages and can show enhanced expression of redox-sensitive HO^{-1} than that of negatively charged citrate-coated silver nanoparticles of an equivalent size [100]. El Badawy et al., [101] and Cho et al., [102] results suggest that the lower toxicity of citrate-coated silver nanoparticles may be a result of the high level of repulsion between the negatively charged silver nanoparticles and the cellular membrane of mammalian and bacterial species.

Since studies on cytotoxicity and antimicrobial aspect of biogenic nanoparticles are dependent on the nanoparticle surface charge, shape, size, composition of capping agent, density of capping agent, a thorough characterization of synthesized nanoparticles becomes highly important. Furthermore, the cytotoxicity and antimicrobial results of biogenic nanoparticles are sensitive to the methodologies used in the evaluation, and the strain/ culture of cells. Additionally the microbial strains/ cultures used for toxicity evaluation are very different, thus direct comparison of the toxicity results obtained by various research groups for a set of biological conditions becomes extremely difficult. In this context, the last phase of the review will deal with laying out well-established protocols/techniques for characterizing nanoparticles and procedures for evaluating antibacterial activity and cytotoxicity level of bioconjugated silver nanoparticles.

Characterization of silver nanoparticles

A variety of analytical instruments have been used for the physical and chemical characterization of silver nanoparticles and they include electron microscopy, zeta-sizer, dynamic light scattering (DLS), ultraviolet–visible (UV–Vis) spectroscopy and inductively coupled plasma (ICP)-based mass spectrometry. Transmission electron microscopy (TEM) has been used not only to determine the size, shape but also the crystal structure of metal in the nanoparticles [103]. A broad review of the various techniques used to characterize nanoparticles is presented by Cho et al., [104].

Surface charge of nanoparticles, expressed as zeta potential, is especially important because nanoparticles interact with the external medium. It is often believed that particles with zeta potential greater than +30 mV or more negative than −30 mV are commonly stable due to the electrostatic repulsion. Also the zeta potential measurement depends on the ionic strength of the medium in which the nanoparticles are suspended. Some of the factors that influence the zeta potential include pH, temperature and composition of the medium.

The size of nanoparticles can be used to assess the uniformity of synthesized nanoparticles and stability of nanoparticles. Commonly DLS is used to measure the hydrodynamic diameter of hydrated nanoparticles. If the synthesized nanoparticles are of nearly uniform size then the changes in nanoparticles can be interpreted as an indication of nanoparticles dissociation or instability in the medium. On the other hand, if the size of hydrated nanoparticles increases with time in aqueous solution it is often interpreted as swelling of nanoparticles. Finally, if the NPs showed constant particle size and turbidity at pH 7 or higher but the turbidity and size at pH below 7 increased, this suggests that the nanoparticles are aggregating due to hydrophobic interactions.

UV-Vis can be used to obtain the size, aggregation state, and population of nanoparticles of a particular size. The position of plasmonic peak in the UV-Vis spectrum depends on average particle size, whereas its full width at half-maximum depends on the extent of polydispersity of nanoparticles [105].

Bulk composition of silver nanoparticles samples can be obtained using ICP-optical emission spectrometry and ICP-mass spectrometry. The high precision and large linear range of ICP make the technique popular for the quantification of total metal content in nanoparticles [106,107]. ICP-MS because of its high sensitivity and selectivity is able to provide information on nanoparticle size, size distribution, elemental composition, and number concentration in a single, rapid analysis.

Biological assay of nanoparticles

Since, gram-positive and gram-negative bacteria respond to nanoparticles differently, therefore antibacterial studies of synthesized nanoparticles often include at least one gram-positive species and one gram-negative species. The antibacterial activity of silver nanoparticles towards gram-positive and gram-negative bacteria has been commonly evaluated by performing *in vitro* experiments. While numerous techniques have been developed to determine the antibacterial activity of nanoparticles, many of the techniques have some drawbacks. Some of the techniques provide information about total cells without discriminating live from dead cells, while other techniques provide indirect information about live cell content by measuring the enzymatic activity. Therefore, multiple standard microbial techniques are often used in conjunction for drawing complete information. **Table 2** summarizes various techniques used to assess antibacterial and cytotoxicity activity of nanoparticles [108-122].

Different experimental techniques have been developed to study and quantify bacterial adhesion and antibacterial activity on material surface [109,110]. Some of the techniques include Colony forming units (CFU) plate counting, Kirby-Bauer disc diffusion assay, Resazurin assay, SEM, Confocal laser scanning microscope (CLSM), optical density measurement, and atomic force microscopy (AFM) to mention a few [95,109-113].

CFU plate counting is the basic method used for estimating the number of viable bacterial cells in a sample. Cell viability is

defined as the ability of the cells to multiply via binary fission under controlled conditions. To determine the number of colony forming units, a bacterial suspension is prepared and spread uniformly on the agar plate and then incubated at suitable temperature for a defined duration. The viable bacteria cells grow with time and form isolated colonies that are counted. Colony forming units may be recorded as CFU per weight, CFU per unit area, or CFU per unit volume, depending on the type of sample tested [110,111].

Kirby-Bauer disc diffusion is a method used to determine the sensitivity of bacteria to specific antimicrobial agent. Greater antimicrobial efficacy yields broader bacterial-free-zones (zone of inhibition) surrounding the antimicrobial disc after incubation [114-115]. In this method, a disc containing the antimicrobial agent is mounted on the bacterial lawn agar plate. The plate is incubated. During incubation, the antimicrobial active ingredient in the disc diffuses out and inhibits the growth of bacteria surrounding the disc. This method can be performed under standard conditions by comparing the zone of inhibition size with standard antimicrobial agents [114-116].

In general, the antimicrobial activity of silver nanoparticles is proportionate to size of nanoparticles and nanoparticles concentration. Nanoparticles can either show inhibitory or lethal effect towards bacteria, depending upon the nanoparticles concentration [117]. Minimum Lethal Concentration (MLC) is defined as the minimum concentration of the nanoparticles that will produce lethal effect on bacteria (99.9% of the original bacterial colony is destroyed) upon 24h incubation at 37°C. In contrast, the Minimum Inhibitory Concentration (MIC) is the lowest concentration of the nanoparticles that when used inhibits bacterial colony formation by two orders of magnitude compared to the positive control sample upon 24h incubation at 37°C.

Additionally, the nanoparticles present in a medium that may be lethal or inhibitory to microorganisms, may also be toxic to eukaryotic cells depending upon the nanoparticle concentration [26]. The cytotoxicity of the nanoparticles towards eukaryotic cells is expected to influence the viability of adherent cell lines. Eukaryotic cell viability can be measured in terms of a loss/decrease of metabolic activity. Also, there is the possibility of loss/retention of membrane integrity of the cells when treated with nanoparticles, due to the nanoparticles damaging the eukaryotic cellular organelles.

Cell cytotoxicity assays are often performed to screen compounds or materials to determine whether the test compounds or materials exhibit cytotoxicity or inhibit eukaryotic cell growth and proliferation. These assays are also used to monitor organelle function or cellular transport [117]. There are different assay methods available that can be used to measure eukaryotic cells viability in a medium or on a polymeric scaffold. These methods include tetrazolium reduction (MTT, MTS, XTT,

Table 2: Techniques to evaluate *in-vitro* Antibacterial Activity & Cytotoxicity [108-122].

Assay	Advantages	Disadvantages
Optical density measurement	Quick, no reagents required	Spectrophotometer required, low accuracy, cannot discriminate between viable and non-viable cells
Cell counting devices Hemocytometer Coulter Counter Microplate Reader	High accuracy Inexpensive, rapid cell proliferation assy	Best used for eukaryotic cells. Require vital dye, e.g., trypan blue, to discriminate between viable and non-viable cells, requires a microscope Expensive device, require vital dye and specific wavelength detection to discriminate between viable and non-viable cells.
Spread-plate (bacterial colony counts on agar)	High accuracy	Determines viable CFU count but not total cell population, time consuming, sterile agar and materials are required, cells must be removed from surfaces for measurement
Crystal violet staining	Quantifies biofilm formation Rapid Assay	Spectrophotometer required, not suitable for planktonic bacteria growth
Live/dead vital fluorescent stain (Calcein AM) (Resazurin-Alamar Blue Assay)	Fluorescence allows visualization of viable cells on sample surface May be used for Qualitative or Quantitative detection of viable cells. Very sensitive assay, a small number of viable cells may be detected or measured	Costly reagents, fluorescent plate reader or microscope required
Protease Viability Assay	Measures cellular viability via cell protease activity	Costly reagents, fluorescent plate reader required. Measurement dependent upon active cellular proteases.
MTT/MTS/XTT/WST assays	Measures cell viability on surfaces and in solution with vital dye. Cell reduced MTS, XTT, and WST soluble formazan-based dyes may be directly measured from culture media. Reduced MTT is stable	Spectrophotometer or plate reader required, costly reagents Reduced MTT formazan-based dye must be extracted from cells with solvents. Cells must be metabolically active Reduced MTS and XTT should be assayed by four hours

and WST-1), resazurin reduction, protease markers, and flow cytometry [118-122].

The MTS assay gives a more direct measure of the impact of nanoparticles on cell viability. MTS assay is based on measuring the intensity of formazan product formed upon viable cells interaction with the MTS reagent. It is assumed that in viable healthy cells, the eukaryotic mitochondrial dehydrogenase or bacterial dehydrogenase activity will be significant. Therefore, there will be conversion of MTS to MTS formazan. Fujihara et al., applied the MTS assay to measure osteoblast cell attachment and proliferation on bone regenerative membrane made of polycaprolactone/calcium carbonate composite nanofibers by monitoring the intensity of formazan product as a function of time [123].

The final section of this review addresses the broad use of nanomaterials in biology and/or medicine. Biological applications of nanomaterials include the development of fluorescent biological labels to detect specific proteins and to probe DNA structure. Moreover, nanomolecules have been developed to enhance separation and purification of biological molecules and cells, specific/targeted drug and gene delivery, biodetection of pathogens, tissue engineering, tumor destruction via localized heating (hyperthermia), MRI contrast, and phagokinetic studies [124]. Especially, silver nanoparticles have found use in medical devices and supplies such as wound dressings, scaffold, sterilized equipment, medical catheters, bone prostheses, artificial teeth, and bone coatings. Additionally, silver nanoparticles have found uses in cosmetics, lotions, creams, toothpastes, laundry detergents, soaps, surface cleaners, room sprays, toys, antimicrobial paints, and home appliances (e.g., washing machines, air and water filters).

Summary of literature review

Silver nanoparticles have received considerable attention due to the strong toxicity to a wide range of microorganisms, including gram-positive and gram-negative bacteria. The properties (size, shape, morphology, composition, aggregation level) of silver nanoparticles play an important role in the nanoparticles antibacterial activity. Properties of nanoparticles can be influenced by a number of factors such as the method of selection for synthesis of nanoparticles and type of stabilizer used. Generally, there are two basic methods of synthesis of nanoparticles. They are classified as top-down and bottom-up approaches. Several methods of synthesis of silver nanoparticles have been reported in the literature, ranging from photochemical reduction, biosynthesis, γ irradiation to chemical reduction. Chemical reduction method is one of the common approach for bottom-up synthesis of silver nanoparticles, and is simple and facile. The stability of the nanoparticles is important so that the nanoparticles can be used for their intended application. The stability of the synthesized nanoparticles can also be affected by the type and amount of reducing agent and type of stabilizer used. For example, the size of nanoparticle core can be tuned from few nanometers to greater than 10nm based on the composition of reagent used in the synthesis. Nanoparticles corona can be modified either through adsorption or in-situ method with ligands/biomacromolecules so as to create surface specific receptors for further conjugation with other biomolecules or other ligands.

There are three modes of stabilizing the nanoparticles: electrostatic charge stabilization, steric stabilization and their combination electrosteric stabilization. Electrosteric stabilization is the most preferred method of stabilization of nanoparticles especially when dealing with high ionic strength biological medium. However, electrostatic stabilization may not be enough to maintain the stability of the nanoparticles over a variety of conditions such as variation in pH value and electrolyte concentration that is especially found in biological medium [45].

Surface modification of nanoparticles with proteins such as biomacromolecules is an effective approach to providing electrosteric stability to silver nanoparticles. Among the wide range of biomolecules used to functionalize nanoparticles, Bovine serum albumin (BSA) is one of the widely studied protein. This is because serum albumin is the most abundant protein in blood plasma, which transports hydrophobic molecules such as bilirubin and fatty acids, and aids in regulating blood pH. During the process of stabilization and capping, the macromolecules (BSA) is believed to retain its overall structural integrity while inducing biocompatibility characteristics to the silver nanoparticles [61]. Stabilization of silver nanoparticles by BSA is believed to not to interfere with the original antimicrobial properties of nanoparticles [62].

There are various mechanisms by which nanoparticles promote antibacterial activity ranging from (a) formation of pits in cell wall, (b) disruption of cell membrane via free radical formation by nanoparticles and inhibition of respiratory enzymes by free oxygen species produced by silver nanoparticles and silver ion, and (c) binding of silver nanoparticles with soft basic residues within the cell, e.g., DNA. Depending upon their size, shape, and composition, they are capable of penetrating the cell membrane and influencing the intracellular processes.

Silver nanoparticles have not only been noted to show broad antimicrobial activity but also exhibit cytotoxicity towards mammalian cells [80,81]. Cytotoxicity of nanoparticles was noticed against fibroblast cells at concentrations of 10 ppm for the small-sized bear silver nanoparticle (3 and 5 nm) while for the larger sized bear nanoparticles (25 nm) even at 10 ppm, no significant cytotoxicity was noticed. Until recently, very limited studies have been conducted to evaluate the cytotoxicity of bioconjugated nanoparticles towards osteoblast cells. Owing to the variation in size, shape, composition, and capping agent used in the formulation of nanoparticles, it is very difficult to obtain a general trend of silver nanoparticles cytotoxicity.

An evaluation of concentration of bioconjugated nanoparticles at which nanoparticles are toxic to bacterial cells and not to the mammalian cells would be highly useful. If indeed there exists a concentration window at which nanoparticles is toxic to bacteria and not to mammalian cells, then nanoparticles loaded matrix could be designed with the intent that nanoparticles be released in the physiological medium so as to maintain a sterile environment against microorganisms while not inhibiting the growth of mammalian cells in the site specific region of intended application.

The characterization of bioconjugated nanoparticles is equally important in formulating nanoparticles of defined morphology in a reproducible manner. Generally, characterization of nanoparticles are done with multiple tools, so as to provide information about size, shape, the size distribution of nanoparticles, morphological information, state of aggregation, and surface charge. The physical and chemical properties dictate the biological activity of the nanoparticles, and ultimately its therapeutic utility.

Nanoparticles of well-defined chemistry and morphology can be used in broad biomedical applications, especially in bone tissue engineering applications. For example, the BSA of Ag/BSA nanoparticles could interact with the collagen of collagen immobilized PHBV film by electrostatic interaction so as to form Ag/BSA nanoparticles loaded collagen immobilized PHBV film. The pH of local environment in the region of infection could be used to trigger the release of Ag/BSA nanoparticles bound by electrostatic interaction from the nanoparticles loaded collagen immobilized PHBV film [125].

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