

Review

A systematic review on silver nanoparticles-induced cytotoxicity: Physicochemical properties and perspectives



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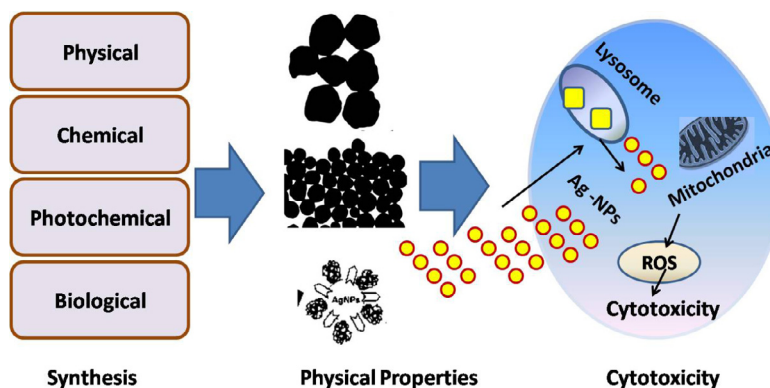
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GRAPHICAL ABSTRACT



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ABSTRACT

With the development of nanotechnology, silver nanoparticles (Ag-NPs) have become one of the most in-demand nanoparticles owing to their exponential number of uses in various sectors. The increased use of Ag-NPs-enhanced products may result in an increased level of toxicity affecting both the environment and living organisms. Several studies have used different model cell lines to exhibit the cytotoxicity of Ag-NPs, and their underlying molecular mechanisms. This review aimed to elucidate different properties of Ag-NPs that are responsible for the induction of cellular toxicity along with the critical mechanism of action and subsequent defense mechanisms observed *in vitro*. Our results show that the properties of Ag-NPs largely vary based on the diversified synthesis processes. The physicochemical properties of Ag-NPs (e.g., size, shape, concentration, agglomeration, or aggregation interaction with a biological system) can cause impairment of mitochondrial function prior to their penetration and accumulation in the mitochondrial membrane. Thus, Ag-NPs exhibit properties that play a central role in their use as biocides

Abbreviations: Ag-NPs, silver nanoparticles; GSH, glutathione; LDH, lactate dehydrogenase; ROS, reactive oxygen species; TMRE, tetramethylrhodamine ethyl ester; NPs, nanoparticles; DNA, deoxyribonucleic acid; TT, toxicity threshold; ppm, parts per million; Ag⁺, silver ions; PVP, polyvinylpyrrolidone.

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along with their applicability in environmental cleaning. We herein report a current review of the synthesis, applicability, and toxicity of Ag-NPs in relation to their detailed characteristics.

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Introduction

Nanomaterials (NPs) have been considered one of the most forefront materials in recent decades. They have been reported to be the “material of the 21st century” because of their unique designs and property combinations compared with conventional materials [1]. There is a wide range of applications of NPs such as in human health appliances, industrial fields, medical applications, biomedical fields, engineering, electronics, and environmental studies [2]. Recently, enormous attention has been focused on the use of nanoparticles (NPs) such as nanotubes, nanowires, fullerene derivatives, and quantum dots to create new types of analytical tools in the fields of life science and biotechnology [3]. Among all of the nanomaterials, Ag-NPs are the most widely used and may be considered one of the most important. They have become a high-demand material for consumer products [4]. Ag-NPs are used in medicine, medicinal devices, pharmacology, biotechnology, electronics, engineering, energy, magnetic fields, and also in environmental remediation [5]. Moreover, because of their highly effective antibacterial activity both in solution and in components, Ag-NPs have gained popularity in industrial sectors including textiles, food, consumer products, medicine, etc. [6]. Currently, Ag-NPs are extensively used in healthcare products, women's hygiene products, the food industry, paints, cosmetics, medical devices, sunscreen, bio-sensors, clothing, and electronics [4].

Their unique physical and chemical characteristics along with their antimicrobial ability, differing largely from bulk materials, make Ag-NPs a high-demand material in different sectors. For example, the high surface area-to-volume ratio enhances the surface properties of Ag-NPs, thereby increasing the interaction with serum, saliva, mucus, and fluid components of the lung lining compared with bulk particles [7]. However, the strong oxidative activity of Ag-NPs releases silver ions, which results in several negative effects on biological systems by inducing cytotoxicity, genotoxicity, immunological responses, and even cell death [8–11]. Unfortunately, the use of Ag-NPs carries a series of unpredictable concerns regarding their interaction with biological systems [7,12]. Therefore, the enormous applications of Ag-NPs raise concerns about human exposure, because they can easily pass through the blood brain barrier (BBB) by transcytosis of capillary endothelial cells or into other critical areas or tissues [13]. According to Aueviriyavit et al., Ag products in colloidal form for medicinal or other purposes have activated Ag⁺ which might have a direct effect on human health [14]. In addition, because of the increased use of Ag-NPs, concentrations of Ag⁺ are increasing in soil and water, which were measured to be 22.7 ppm and 0.76 ppm, respectively [14,15]. Moreover, it is hypothesized that Ag⁺ possesses an enhanced toxicity potential than elemental Ag and Ag-NPs [11]. However, an increasing number of recent occurrences of diseases due to microbial infections has been prevented by the noble metal, with Ag-NPs having a well-documented antimicrobial and disinfectant activity. Very recently, antibacterial activity of green-synthesized Ag-NPs against *Bacillus subtilis* and *Escherichia coli* was revealed [16]. The role of Ag-NPs as an environmental disinfectant and the safe synthesis of Ag-NPs are areas that remain to be explored. Little is known about the diversified mechanisms of action of the cytotoxicity of Ag-NPs, as well as their short- or long-term exposure outcomes, on human physiology [17,18]. The interaction processes of

nanomaterials with biological systems are unknown and consequently might be of great concern [12,19]. The toxicity of other NPs in different organisms has been reported in various studies whereas the toxicity of Ag-NPs has not been extensively explored. For example, titanium dioxide (TiO₂) NPs induce reactive oxygen species (ROS), which further initiate lipid peroxidation, protein dysfunction, and DNA degradation, finally triggering oxidative damage in the mouse brain [20].

It can also be assumed from several studies that the physicochemical characteristics of Ag-NPs solely control the toxicity pathways that they induce. Therefore, the aim of this review was to present and discuss different physicochemical properties (e.g., particle size, dose of NPs, agglomeration of Ag-NPs) that play a vital role in inducing toxicity in different cell lines. Next, toxicology considerations and toxicity initiation pathways are also discussed to outline the Ag-NPs-induced toxicity mechanism.

Synthesis and properties of Ag-NPs

Particles less than 100 nm in at least one dimension are considered NPs [21]. Ag-NPs differ from bulk and micron size silver because of their size, shape, and stability. Currently, Ag-NPs are being fabricated on an industrial scale utilizing physio-chemical techniques such as chemical reduction [8], gamma ray radiation [9], micro emulsion [10], electrochemical methods [11], laser ablation [12], autoclaving [16], microwaving [15], and photochemical reduction [16]. These methods are all effective but suffer from several limitations such as the use of toxic ingredients, high operational cost, and energy needs.

Large amounts of Ag-NPs can be produced using silver nitrate and the reducing agent ethylene glycol along with polyvinylpyrrolidone (PVP) [22]. However, the oleylamine-liquid paraffin system has been used to prepare almost monodisperse Ag-NPs from silver nitrate using oleylamine and paraffin [23,24]. The reduction in different silver salts also results in a colloidal solution of Ag particles, which is followed by both nucleation and subsequent growth. Usually, through the optimization of different parameters such as temperature, pH, precursors, reducing agents, and other experimental conditions, the silver nanocube can be given a definite size [24,25]. Using atmospheric pressure, Ag-NPs can be synthesized by evaporation-condensation, thermal decomposition, the arc discharge method, and the metal sputtering method into the powder form [26–29]. The Ag-NPs can also be produced by photo-induced synthetic strategies, which involve photo-reduction of AgNO₃ using sodium citrate (NaCit) and light sources such as UV, white, blue, cyan, green, and orange light at room temperature [30].

A recent discovery of a methodology for synthesizing green Ag-NPs involves the utilization of bacteria, fungi, yeasts [2], algae, or plant extracts [17] as reducing and/or stabilizing compounds to work on silver salts, which addresses the drawbacks of physio-chemical methods [31]. *Shewanella oneidensis*, *Trichoderma viride* (*T. viride*), *Bacillus* species, *Lactobacillus* species, and some vegetative parts of plants are now being used to produce environmentally friendly Ag-NPs. The association of nanotechnology with green chemistry is thus allowing for the emergence of biologically and cytologically compatible metallic NPs [19,32]. Table 1 shows the size variability of the green-synthesized Ag-NPs from plant and

Table 1

Few works of recent green synthesis of Ag-NPs.

Sl. no.	Author	Reducing agent	Particle characteristics	Remarks
1	Kathiraven et al. [33]	Filtered aqueous extract of <i>Caulerpa racemosa</i> marine algae	Size—5–25 nm Shape—sph, tri. Structure—FCC	Antibacterial action against <i>P. mirabilis</i> and <i>S. aureus</i>
2	John De Britto et al. [34]	Aqueous filtrate of <i>Pteris argyrea</i> , <i>Pteris confuse</i> , and <i>Pteris biaurita</i>	–	Antibacterial action against <i>Shigella boydii</i> , <i>Shigella dysenteriae</i> , <i>S. aureus</i> , <i>Klebsiella vulgaris</i> , and <i>Salmonella typhi</i>
3	Sant et al. [35]	Aqueous filtrate of <i>Adiantum philippense</i> L.	Size—10–18 nm Shape—anisotropic Structure—FCC Nature—MD	Ag-NPs from medicinally important plants opens spectrum of medical applications
4	Bhor et al. [36]	Aqueous filtrate of <i>Nephrolepis exaltata</i> L. fern	Size—avg 24.76 nm Shape—sph. Structure—FCC	Antibacterial against many human and plant pathogens
5	Ajitha et al. [37]	Filtered aqueous extract of <i>Tephrosia purpurea</i> leaf powder	Size—~20 nm Shape—sph. Structure—FCC	Antimicrobial agents against <i>Pseudomonas</i> spp. and <i>Penicillium</i> spp.
6	Rahimi-Nasrabadi et al. [38]	Methanolic extract and essential oil of <i>Eucalyptus leucoxylon</i> leaf	Size—~50 nm Shape—sph. Structure—FCC	Ag-NPs with biomedical potential
7	Bagherzade et al. [39]	Aqueous extract of saffron (<i>Crocus sativus</i> L.)	Size—12–20 nm	Inhibiting activity against <i>Escherichia coli</i> , <i>Pseudomonas aeruginosa</i> , <i>Klebsiella pneumoniae</i> , <i>Shigella flexneri</i> , and <i>Bacillus subtilis</i>
8	Ashokkumar et al. [40]	Filtered aqueous extract of <i>Abutilon indicum</i> leaf	Size—7–17 nm Shape—sph. Structure—FCC	Antimicrobial action against <i>S. typhi</i> , <i>E. coli</i> , <i>S. aureus</i> , <i>B. subtilis</i>
9	Tagad et al. [41]	Locust bean gum polysaccharide.	Size—18–51 nm	Stability: 7 months, Ag-NPs served in development of H ₂ O ₂ sensor
10	Yasin et al. [42]	Filtered aqueous extract of Bamboo leaf	Size—13 ± 3.5 nm Shape—nearly sph. Structure—cryst	Antibacterial to <i>E. coli</i> and <i>S. aureus</i>
11	Sadeghi and Gholamhoseinpoor [43]	Methanol extracted aqueous filtrate of <i>Ziziphora tenuior</i> leaf	Size—8–40 nm. Shape—sph. Structure—FCC	Stability: 6–12 pH range
12	Chen et al. [44]	Chitosan biopolymer	Size—~218.4 nm Shape—oval and sph. Nature—Ag/chitosan nano hybrids	Antimicrobial to <i>E. coli</i> , <i>S. choleraesuis</i> , <i>S. aureus</i> , and <i>B. subtilis</i>
13	Mondal et al. [45]	Saline washed, filtered aqueous extract of <i>Parthenium hysterophorus</i> root	Shape—spherical	Potential larvicidal for <i>Culex quinquefasciatus</i>
14	Nalwade et al. [46]	Aqueous filtrate of <i>Cheilanthes forinosa</i> Forsk leaf	Size—~26.58 nm Shape—sph. Structure—FCC	Antibacterial action against <i>S. aureus</i> and <i>Proteus morgani</i>
15	Singh et al. [47]	<i>Lantana camara</i>	48.1 nm	Antimicrobial to <i>E. coli</i> and <i>S. aureus</i> . Leakage due to cell wall rupturing
16	Vimala et al. [48]	Leaf and fruit of <i>Couroupita guianensis</i>	Cubic size 10–45 nm 5–15 nm	Water soluble phenolic compounds as reducing and stabilizing agent larvicidal to <i>Aedes aegypti</i> extensive mortality rate (LC90 ~ 5.65 ppm)
17	Cheng et al. [49]	Chondroitin sulfate	Size—20 nm Shape—sph	Stable for 2 months, Served as nano carrier for drug delivery
18	Sadeghi et al. [50]	Filtered aqueous-methanol extract of <i>Pistacia atlantica</i> seed powder	Size—10–50 nm Shape—sph. Structure—FCC	Stability: 7–11 pH range. Antibacterial affect against <i>S. aureus</i> .
19	Zhang et al. [51]	<i>Lactobacillus fermentum</i> . LMG 8900 cells	Size—~6 nm Shape—sph. Structure—FCC	Stable for 3 months. Resist growth of <i>E. coli</i> , <i>S. aureus</i> and <i>P. aeruginosa</i> Act as promising anti-biofouling agent
20	Das et al. [52]	Mycelia of <i>Rhizopus oryzae</i>	Size—~15 nm Shape—sph. Structure—FCC	Stable for 3 months, Antimicrobial to <i>E. coli</i> and <i>B. subtilis</i> , Used for treating contaminated water and adsorption of pesticides
21	El-Rafie et al. [53]	Crude hot water soluble polysaccharide extracted from different marine algae	Size—7–20 nm Shape—sph	Stability: 6 months, Ag-NPs treated cotton fibers antibacterial to <i>E. coli</i> and <i>S. aureus</i>
22	Suresh et al. [54]	Filtered aqueous extract of <i>Delphinium denudatum</i> root powder	Size—85 nm Shape—sph. Structure—FCC Nature—PD	Anti-bacterial against <i>S. aureus</i> , <i>B. cereus</i> , <i>E. coli</i> and <i>P. aeruginos</i> Larvicidal to <i>A. aegypti</i>
23	Zuas et al. [55]	Filtered aqueous extract of <i>Myrmecodia pendan</i> plant	Size—10–20 nm Shape—sph. Structure—FCC	Promising therapeutic value
24	Vijaykumar et al. [56]	Aqueous extract of <i>Boerhaavia diffusa</i> plant powder.	Size—~25 nm Shape—sph. Structure—FCC, Cub	Antibacterial to fish pathogens <i>A. hydrophila</i> , <i>F. branchiophilum</i> , <i>P. fluorescens</i>
25	Elumalai et al. [57]	Filtered coconut water	Size—70–80 nm Structure—FCC Nature—PD	Metabolites and proteins served as capping agents

Note: PD—Polydispersed, MD—Monodispersed, WD—Well Dispersed, Cryst—Crystalline. FCC—Face centered cubic; Tri—Triangular; Sph—Spherical; cryst—crystalline; Cub—cubic.

microbial origins. It is evident from Table 1 that the size of synthesized Ag-NPs ranges from 50 to 100 nm in most of the listed studies. In general, Ag-NPs synthesized using biological reducing and capping agents have shown wide variations in shape and size. The researchers also reported low toxicity levels of these green-synthesized Ag-NPs in comparison to chemically synthesized synthetic Ag-NPs.

The synthesized Ag-NPs vary in size, shape, surface electric charge, and in other physiological characteristics. Nanosized particles are several times more catalytic, have electromagnetic capability, and thus are capable of being more reactive. ROS generation capability could make them more toxic than their bulk counter-

parts [58–60]. Thus, variation in size plays a vital role on nanoparticle activity. NPs agglomeration and concentration range are also two important factors affecting toxicity induction.

Effects of Ag-NP physicochemical properties on cytotoxicity

Effects of particle size variability

The cytotoxicity of Ag-NPs is influenced by the variation in particle size [61]. Ag-NPs showed a vital effect on cell viability, lactate dehydrogenase (LDH) activity [61], and ROS generation [12] in a size-dependent manner in different cell lines. It is evident that

surface area, volume ratio, and surface reactivity can be changed with particle size [12,24,34]. Moreover, sedimentation velocity, mass diffusivity, attachment efficiency, and deposition velocity of NPs over the biological or solid surfaces are considerably influenced by particle size [62–66]. Particle size can also influence the mammalian cell interaction [17]. Several studies have been carried out to determine the particle size effect of Ag-NPs on different cell lines. Table 2 shows some size-dependent studies of Ag-NPs on different cell lines. The studies reported in Table 2 reflect the hypothesis that smaller particles can induce greater toxicity. In support of this statement, Carlson et al. worked with 15 nm and 55 nm hydrocarbon-coated Ag-NPs, and found that the 15 nm Ag-NPs can generate more ROS compared with 55 nm Ag-NPs in a macrophage cell line [12]. Using four cell lines (A549, HepG2, MCF-7, SGC-7901), Liu et al., found that 5 nm Ag-NPs were more toxic than 20 and 50 nm Ag-NPs [66].

Recently, Wang et al., found that 20 nm citrate-coated Ag-NPs showed more cytotoxicity than 110 nm Ag-NPs and further generated acute neutrophilic inflammation in the lungs of mice compared with larger Ag-NPs [67]. However, Kaba et al. reported that smaller Ag-NPs do not play a key role in the viability of tumor cells (HeLa and U937 cells) [68]. This might be due to the fact that the interactions of Ag-NPs vary depending on the type of organism. The examination of the toxicity threshold (TT) of different-sized particles showed evidence of size dependency in specific cell types. TT refers to the minimum dose of any substance in which toxicity is first encountered. Doses below the TT dose, referred to as sub-threshold doses, do not induce any toxicity. The TT value does not always depend on particle size (Table 3). Table 3 shows that in the same cell line, the TT value varied. For example, in the A431 cell line, the TT value varied between 1.51 µg/mL and more than 50 µg/mL [76], and in the A549 cell line, the TT value varied from 0.5 µg/mL [77] to 50 µg/mL [76]. This difference in the TT is hardly due to a single factor such as the particle size of Ag-NPs. Thus, the notion that smaller particles show higher biological activity in comparison with the larger ones requires more well established evidence to be accepted. A study reported that for the same cell line, the TT is higher (60 µg/mL) in case of small particle size (2–5 nm Ag-NPs) than in case of the larger ones (TT 20 µg/mL for 10–100 nm Ag-NPs) [78]. Thus, the TT value does not always depend on the particle size.

Different synthesis processes result diverse types of Ag-NPs e.g. spherical, triangular, square, cubic, rectangular, rod, oval and flower (Fig. 1). From the nano-toxicological point of view, it is unknown whether particle shape has any significant effect on the biological system. This might depend on multiple factors rather than a single one. For instance, alveolar epithelial cells (A549) exposed to different shapes of Ag-NPs and Ag⁺ showed agglomeration in the cytoplasm [82,83].

The shape of the Ag-NPs might influence the cellular uptake mechanism, which in turn modulates the cytotoxicity. The shape

of nanoparticles has been reported to show a significant effect on cytotoxic parameters. For example, spherical particles did not show adverse effects on cytotoxic parameters in A549 cells whereas wires induced negative outcomes [82]. The study on different cell lines such as macrophages (RAW 264.7, J774.1), A549, A498, HepG2, and neurons (Neuro 2A) with 5–43 nm Ag-NPs of 2.0 mg/L concentration showed unique results to each cell line, with macrophages exhibiting the highest sensitivity [84]. The internalization of Ag-NPs into macrophages was revealed to occur via the scavenger receptor pathway, and then cytotoxicity is induced in the cytoplasm by employing the release of Ag⁺ [84]. Both Ag-NPs and AgNO₃ are potent, have smaller (average 10 nm) diameters, and are cytotoxic in human lung cells [61]. The solubility of Ag-NPs is another critical toxicity factor in lung epithelial cells. For instance, 20–110 nm Ag-NPs in acidic phagolysosomes exhibited toxicity [85]. Ag-NPs (20 nm) exposed to HepG2 and Caco2 cells caused dose-dependent toxicity, DNA damage, mitochondrial injury, and oxidative stress. Two different-sized Ag-NPs (10 and 100 nm) exposed to HepG2 cells induced the proliferation of cells, activation of mitogen-activated protein kinase (MAPK), and upregulation of c-Jun and c-Fos mRNA [86]. Other cell lines including A2780, MCF-7, and MDA-MB 231 showed differential toxicity when exposed to Ag-NPs (40 nm) at a concentration of 10 µg/mL. The degree of sensitivity to Ag-NPs was as follows: ovarian cancer cells (A2780) > breast cancer cells (MDA-MB 231) > MCF-7 cells. U937 cells showed the highest susceptibility after treatment with 4-nm particles, exhibiting a reduction in cell growth, increase in oxidative stress, and increase in IL-8 p. Upon treatment with greater-sized NPs, U937 cells showed less sensitivity. Treatment with silver-polyvinyl pyrrolidone (Ag-PVP) NPs with sizes of 10, 20, and 80 nm of mouse macrophages resulted in anti-inflammatory effects against *Chlamydia trachomatis*, a very common sexually transmittable infection [87].

Biologically synthesized spherical Ag-NPs that are 50 nm in size and at a 500-nM concentration inhibited cell survival, VEGF-induced cell viability, cell proliferation, and migration through the activation of caspase-3 and suppression of Akt phosphorylation in bovine retinal endothelial cells (BRECs) [88,89]. The exposure of rat brain microvessel endothelial cells to Ag-NPs (25, 40, or 80 nm) resulted in significant BBB inflammation and permeability, suggesting that Ag-NP toxicity may be characterized by the particle size, surface area, dose, and exposure time for the particular cell model [90].

Because of the ability of Ag-NPs to cross the tight junction of the BBB, they are considered a potential neurotoxin. Studies reported BBB inflammation, increased BBB permeability in rat brain microvessel endothelial cells [91], and BBB dysfunction and astrocyte swelling causing neuronal degeneration [92]. The neurotoxicity induced by Ag-NPs has been confirmed by several *in vivo* and *in vitro* studies. Adult male C57BL/6N mice exposed to Ag-NPs showed oxidative stress-induced neurotoxicity in three brain

Table 2
Size dependent effects of Ag-NPs on different cell lines.

Particle sizes (nm)	Cell type	Findings	References
15, 30, 55	Rat Alveolar macrophages	Ag NPs induced size dependent cytotoxicity	[12]
10, 50, 100	HepG2	Ag NPs induced size dependent toxicity through autophagy lysosomal system and inflammasome activation	[18]
5, 20, 50	A549, SGC-7901, HepG2 and MCF-7	EC ₅₀ values were size dependent and smaller particles can enter easily than larger particles	[66]
13 ± 4.7	HeLa and U937	Ag NPs induced cytotoxicity in both HeLa and U937 cell lines	[68]
10	HepG2	Cytotoxicity induced through the oxidative stress	[69]
20, 80, 113	RAW 264.7 & L929	Ag NPs induced cytotoxicity depends on cell type and Np size	[70]
5–10	HepG2	Ag NPs induced Oxidative changes in HepG2 cell	[71]
30–50	A431A549	Ag NP's toxicity depends on particle size and surface potential	[72]
1–10	HIV virus	Interaction of Ag NPs with HIV virus is size dependent	[73]
7–20	A431HT-1080	Apoptosis induced in both A431 and HT-1080 cell lines	[74]
15, 30, 55	Alveolar macrophages cells	ROS and LDH generated in a size dependent manner	[75]

Table 3

Effects of AgNPs on cell viability upon 24 h incubation, adopted from Kaba et al. [68].

Ag NP preparation technique	Particle sizes, nm	Cell type	Cytotoxicity assay	Toxicity threshold, $\mu\text{g/mL}$	Reference
Chemical reduction	30–50	A431 A549	MTT assay	>50	[76]
Unknown (commercial product)	>70 (PVP-coated)	A549	MTT assay	0.5 (Ag NPs) 1 (Ag ⁺)	[77]
Unknown (commercial product)	10	HeLa	CCK-8 (WST-based assay)	10	[78]
	50			20	
	100			20	
Unknown (patented preparation)	7–20	A431 HT-1080	XTT assay	1.56 6.25	[79]
Unknown (commercial product)	<10	HepG2	MTT assay Alamar blue assay	0.5 (Ag NPs) 0.1 (Ag ⁺) 0.7 (Ag NPs) 0.7 (Ag ⁺)	[80]
Unknown (commercial product)	5–10	HepG2	MTT assay	2.0	[80,81]

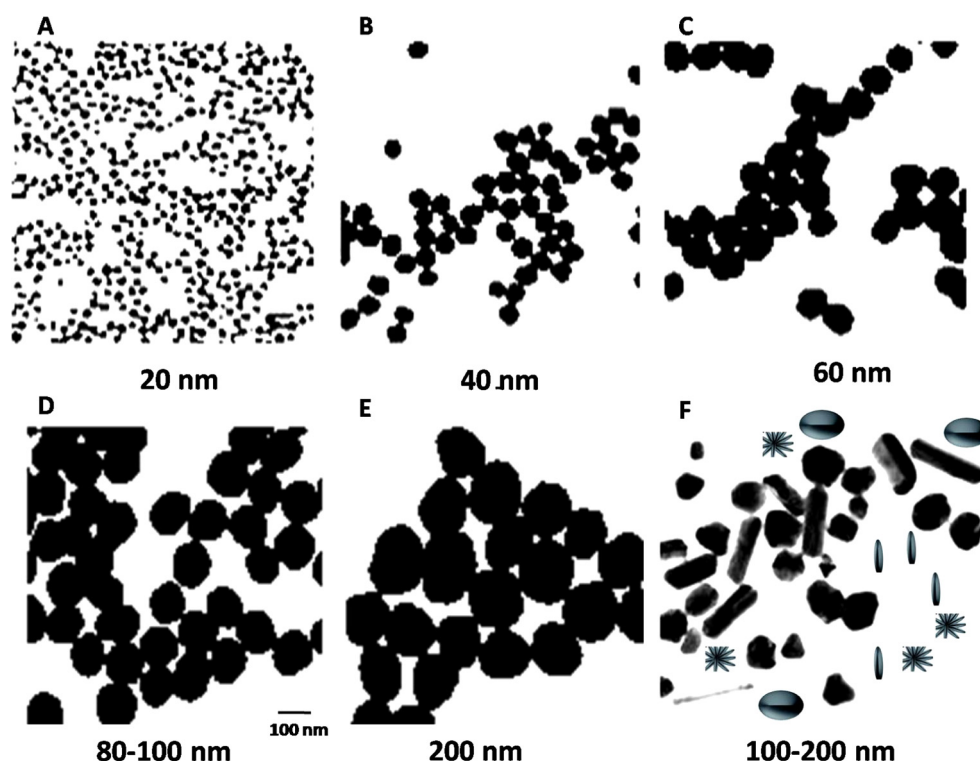


Fig. 1. Transmission electron microscopy (TEM) images of synthesized Ag-NPs with various sizes and shapes (A–F). Spherical, oval, rod and flower shaped Ag-NPs can be obtained from green synthesis. Spherical shaped Ag-NPs mostly obtained by chemical synthesis. The size variability is independent to the synthesis process. Ag-NPs change color as they change their size (color not shown). Scale bars are 100 nm. Modified and redrawn from Stoehr et al. [82] and nanoComposix.com [83].

regions including the caudate nucleus, frontal cortex, and hippocampus [93]. Furthermore, synaptic degeneration, neuronal degeneration, and astrocyte swelling were reported in the rat brain due to a low dose of Ag-NP exposure via oral and intragastric administration [94,95]. The exposure of PC12 cells to 15 nm NPs at a concentration of 10 $\mu\text{g/mL}$ for 24 h exhibited the involvement of silver in both induction of oxidative stress and enzymatic dysfunctions that play a crucial role in the depletion of dopamine [96]. The cytotoxicity of Ag-NPs was further confirmed in cerebellum granule cells (CGCs). The toxicity was dose-dependent and occurred via induction of caspase-3 activation, oxidative stress, reduction of anti-oxidants, and intracellular calcium levels; however, it did not damage the cell membrane [97].

Furthermore, Ag-NPs exhibited increased toxicity in stem cells. For instance, murine spermatogonial stem cells had less cell viability, LDH leakage, and prolonged apoptosis after Ag-NPs exposure [98]. The biocompatibility of Ag-NPs (100 nm) in human mesenchy-

mal stem cells (hMSCs) was examined and there was a dose-dependent effect on cytotoxicity [69]. In addition, male somatic Leydig (TM3) cells, Sertoli (TM4) cells, and spermatogonial stem cells (SSCs) showed similar effects using Ag-NPs of varied sizes. Ag-NPs therefore, exert a significant amount of negative effects on neurogenesis.

Effects of concentration

The concentration of NPs is another important factor affecting toxicity. It is critical to determine the minimum concentration level of NPs that induces toxicity and its variation in different subjects. Mostly, Ag-NPs showed cytotoxicity in a concentration-dependent manner. In RAW 264.7 cells, 0.2 ppm Ag-NPs reduced cell viability by 20%, whereas 1.6 ppm of Ag-NPs reduced viability by 40% [70]. The same trend was also observed in human Chang liver cells, where cell viability decreased in a concentration- and

dose-dependent manner [60]. In a rat liver cell line (BRL 3A), 25 ppm of Ag-NPs was reported to be the most toxic concentration, with toxicity observed at concentrations ranging from 1 to 25 ppm. Depending on the cell type, Ag-NPs cytotoxicity varies significantly, and this should be taken into consideration for their application in consumer products and in examining environmental effects.

Induction of toxicity varies with different concentrations of Ag-NPs in different cell lines. Thus, the TT for Ag-NPs is dependent on the tested cell line. In HeLa and U937 cells, the TT of Ag-NPs was measured as 2.0 ppm for both types of cells after 4 h of treatment. The TT value was same for HeLa cells after 24 h of treatment, whereas for the U937 cell line the TT value was 0.05 ppm. Cell viability started to decrease at concentrations of 2.0 ppm and 0.05 ppm [68]. However, in HepG2 cells, no toxicity was found at concentrations from 0.01 ppm to 5 ppm at any exposure time [18]. In addition, Ag-NPs showed complete cytotoxicity against *E. coli* at a concentration of 8 µg/mL [12,99].

The concentration range of NPs that can induce toxicity depends on the particle size, type of medium, temperature, surface functionalization, particle crystallinity, etc. [100]. For example, Ag nano prisms and spherical Ag-NPs at a concentration of 100 ppm were not cytotoxic to HaCaT keratinocytes after 48 h [101]. While exposing a normal human lung bronchial epithelial cell line (BEAS-2B) to Ag-NPs at a range of concentrations (0.01–10 mg/mL for 24 h), endocytic vesicles-induced genotoxic effects were observed via ROS induction, micronuclei formation, and DNA damage [102].

Ag-NPs exhibited increased toxicity under a hypoxic environment at exposure levels 3 and 50 µg/mL in A549 cells, normal lung epithelial cells (L132), human ovarian cancer cells (A2780), and human breast cancer cells (MCF-7 and MDA-MB 231) [103]. Pre-exposure to hypoxic conditions could induce hypoxia-inducible factor (HIF)-1 α , which eventually neutralizes the Ag-NP-induced oxidative stress in cells to protect them. However, prolonged exposure to hypoxia may induce cell death [104,105]. Ag-NPs (10–75 µg/mL) caused survival inhibition. A 10-fold increase in oxidative stress levels corroborated this inhibition. Macrophages exposed to water-dispersible Ag-NPs (50–500 µg/mL) exhibited vesicle expansion, membranolytic action, and inflammatory outcomes [106]. At higher NP doses, NM300K cells exhibited an altered cell shape, and the production of vacuoles was induced along with enhanced cytokine and ROS induction, with DNA damage and cell apoptosis. This is due to the fact that Ag⁺ released from NPs by dissolution might be the initial factor for toxicity induction [107].

Human umbilical vein endothelial cells (HUVECs) treated with biologically synthesized Ag-NPs showed no toxicity response compared to chemically synthesized Ag-NPs [108]. The latter inhibited the proliferation of the cell cycle, disruption of the cell membrane, cellular apoptosis, and upregulation of cytokines, adhesion mole-

cules, and chemokines in HUVECs via NF- κ B pathways [109]. Identical effects were observed in primary NHEK cells treated with Ag-NPs [110].

Examining the effect of Ag⁺ and Ag-NPs on human dermal fibroblasts (NHDF) and NHEKs revealed that silver ions were significantly more toxic than Ag-NPs to both cell types. Likewise, the neurotoxicity is also furnished by Ag⁺ more than Ag-NPs [61]. During the exposure of rat cortical cells to various concentrations of Ag-NPs (1–50 µg/mL), the inhibition of neurite outgrowth and the cell survival of premature neurons and glial cells was lowered via mitochondrial dysfunction and loss of cytoskeleton proteins including β -tubulin and filamentous actin (F-actin). Similarly, neural stem cells (NSCs) showed an increase in cell death, leakage of LDH, induction of ROS, upregulation of pro-apoptotic Bax protein, and increased in apoptosis when exposed to various concentrations of Ag-NPs [111]. Table 4 shows the effects of Ag-NPs at different concentration ranges on different cell lines.

Taken together, it can be concluded that cytotoxicity of Ag-NPs varies from cell to cell. Moreover, the cell type, particle size, and exposure time also play vital roles in cytotoxicity. However, the minimum or highest concentration of Ag-NPs needed to induce toxicity is not fixed and might vary based on the organism.

Effects of coatings

To prevent aggregation of Ag-NPs, coating is a way to produce electrostatic as well as electrosteric repulsions between particles, which further helps to stabilize the NPs. Uncoated Ag-NPs significantly decreased cell viability in a time- and dose-dependent manner, and coating is used to provide protection against cytotoxicity. The type of coating depends on the capping agent properties such as organic capping agents (polysaccharides, citrates, polymers, proteins, NOM, etc.) and inorganic capping agents (sulfide, chloride, borate, and carbonate). Since the capping material plays a role in maintaining the surface chemistry of Ag-NPs by stabilizing, giving a definite shape, and reducing Ag⁺, the potentiality of modulating the bioactivity of coated Ag-NPs is significant. In this section, we discuss the possible effects of Ag-NP coatings on their toxicological phenomena. Ag-NPs-induced cytotoxicity may vary depending on several factors including the type of coating materials. Usually the processes involved in toxicity induction involve ROS generation, depletion of antioxidant defense systems, and loss of mitochondrial membrane potential. Surface coating of Ag-NPs can affect shape, aggregation, and dissolution ratio. However, the method and extent of Ag-NPs toxicity varies based on the coating materials. For example, chitosan-derived polysaccharide-coated Ag-NPs showed antimicrobial activity with no toxicity to eukaryotic cells [115].

Table 4
Effects of Ag-NPs of different ranges of concentration on different cell lines.

Concentration range	Effects of Ag-NPs in different ranges	References
25–75 µg/mL	In rat alveolar macrophage cell line, cytotoxicity increase in a concentration dependent manner	[12]
5, 15, 40, 125 µg/mL	Cytotoxicity occurred through mitochondrial depolarization	[14]
10–50 g/mL	Induce cytotoxicity in BRL 3A rat liver cell through ROS generation GSH depletion and reduction of mitochondrial membrane potentiality	[60]
20 µg/mL	Induce mitochondrial swelling in HSCs cell line after giving treatment for 2 days	[82]
20–250 µg/mL	Apoptosis and necrosis induced in HSCs cell line	[84]
40–80 µg/mL	40 µg/mL was considered as IC50 value for MCF-7 cell line and apoptosis occurred at the concentration of 80 µg/mL. More than 80 µg/mL induce necrosis when percentage of apoptosis being decreased	[86]
10–25 µg/mL	In MDA-MB- 231 cell line, DNA damage occur in presence or absence of concurrent radiation treatment	[87]
50 µg/mL	Antioxidant capacity increased in Caco-2 cells	[88]
1, 2, 4 µg/mL	Cell viability decreased in a concentration dependent manner	[96]
10–50 µg/mL	In THP-1-derived human macrophages cell line cell viability decreased in a concentration dependent manner	[112]
5 µg/mL	Promote epigenetic dysregulation in HT22 cells through cell proliferation, DNA damage response and DNA methylation	[113]
0.4 and 0.8 µg/mL	Arrest G1 phase in cell cycle in RAW 264.7 cell line	[114]

Polystyrene-coated Ag-NPs caused fewer changes in genetic induction and repression compared to Ag-NPs and AgCO₃ in HepG2 cells [116]. Furthermore, citrate- and polyvinylpyrrolidone (PVP)-coated Ag-NPs were tested to compare their toxicity with uncoated Ag-NPs using J774A.1 macrophages and HT29 epithelial cells [117]. Both citrate and PVP-coated Ag-NPs proved to be less cytotoxic than uncoated ones in tested cell lines. Cytokine expression as well as oxidative stress pathway analysis corroborates the possible mechanism of toxicity induction in epithelial cells and macrophages. Citrate coatings can improve the stability of colloidal Ag-NPs and decrease their toxicity. In contrast, PVP-modified Ag-NPs maintain good stability and cause negligible toxic effects in human skin HaCaT keratinocytes. However, no significant changes were observed between uncoated and PVP- and oleic acid-coated Ag-NPs in terms of bioaccumulation and toxicity in earthworms (*Eisenia fetida*) [118]. In contrast, polysaccharide-coated Ag-NPs resulted in greater DNA damage than uncoated Ag-NPs by increasing the likelihood of entering into the mitochondria and the nucleus [119]. The stability of thiol-coated Ag-NPs reported by Andrieux et al. [120] was due to their corrosive properties and affinity for the cell membrane proteins [120]. It is evident from the above discussion that coating materials and their characteristics play a vital role in Ag-NPs induced cytotoxicity.

Effects of agglomeration

NPs have high potential to aggregate or agglomerate in solution and in ambient air. The interaction potentiality of NPs with cells is dependent on diffusion, gravitation, and convection forces [121,122]. The agglomeration process might be affected by the pH, electrolyte or salt content, and protein composition in the culture medium [123]. Several studies showed that the binding capacity of NPs with protein is different based on the composition of both the NPs and protein [124–126]. Agglomeration states of Ag-NPs in medium depend on treatment preparation. A study by Lank-off et al. revealed that 20 nm and 200 nm-sized Ag-NPs aggregated in culture medium, and the aggregation range changed depending on the NPs suspension preparation. Depending on the suspension preparation, the hydrodynamic diameter of Ag-NPs could be larger than the nominal size of the particles [71]. Finally, more aggregated particles showed fewer effects on the cellular level [71].

Cellular localization of NPs may depend on the agglomeration states of the NPs [71]. For example, under the same conditions, Ag-NPs seem to aggregate very loosely compared with TiO₂ NPs. Therefore, Ag-NPs were observed in the cytoplasm, nucleus, and mitochondria with a slight agglomeration whereas clusters of agglomerated TiO₂ were mainly distributed in the vacuole [71]. This occurs because intracellular localization of Ag-NPs and TiO₂ NPs depends on the interaction of the particles with protein and DNA inside the cell, which also initiates toxicity [127].

Ag-NPs have a high agglomeration tendency in culture medium because of their high surface area [128]. This agglomeration may induce toxicity rather than the ionic metal-induced toxicity. Sometimes, aggregation plays a role in the various types of intracellular responses. Hence, from the point of view of toxicological interest, it is very important to know how agglomeration or aggregation states of NPs affect different biological responses [71,129].

Like other NPs, agglomeration is a common phenomenon observed for Ag-NPs. As agglomeration and aggregation are barriers to cytotoxicity measurement, usually a different surface coating is used on the NP surface. However, the surface coating materials, such as organic (citrate, PVP) and inorganic coatings (sulfide, chloride), potentially interfere with cytotoxicity measurements [68]. In addition, easy penetration of agglomerated Ag-NPs into mesenchymal stem cells and the nuclei was made evident by several studies [130,131].

Effects of surface corona, charge, and hydrophobicity/hydrophilicity

Nanomaterials have the potential to be utilized in biological systems for different purposes such as in biomedical applications. It is generally agreed that the mixing of nanomaterials with biological entities may exert detrimental effects on biological systems as a result of nano-bio interfacial interactions. In this interaction, DNA, proteins, membranes, cells, and organelles usually play the vital role of providing access to the nanomaterials through their natural boundaries fueled by colloidal forces. Every biological entity eventually forms a surface corona in the nano-bio boundary region which is adverse in nature. Among all surface coronas, the protein corona is considered as an emerging entity in nanobiointerface.

Ag-NPs also have received an immense amount of attention owing to their complicated interaction with proteins [132]. Implementation of AgNPs in different sectors such as medical, biological, chemical, and electronical make them potential agents for inducing adverse human health effect, especially cardiovascular, central nervous system, malfunction, neurotoxicity, or immunotoxicity [133,134]. The process of corona formation depends on the capability of the protein to get adsorbed onto the surface of NPs and therefore, the presence of a protein corona could greatly influence biological activity. Many *in vitro* and *in vivo* experiments were conducted worldwide to understand the interactions between NPs and biological fluids. Almost all experiments show that the surface between cellular systems and the nanoparticles establishes the corona formation.

The corona significantly affects the biological response. Particle size [87], particle shape [97], particle surface properties [98], and biological fluid properties and composition affect the corona composition and thus the adverse effects on human health and the environment [135,136]. Based on the surface affinity and exchange rate, the corona can be divided into two forms: hard corona and soft corona. The soft corona proteins are 'vehicles' for the silver ions whereas the hard coronas are rigid for the trespass into the cellular system. Various investigations have been conducted on various types of corona effects, examining the interactions of structure based (cube, sphere, wire, and triangle) silver nanoparticle with fetal calf and bovine serum (FBS), bovine serum albumin (BSA), human blood plasma, human serum albumin (HSA), tubulin, ubiquitin (cytoskeletal protein), and hyaluronan-binding protein *in situ*. This research aimed to measure and understand protein enrichment on the surface of different silver NPs. More than 500 proteins were identified and isolated that were directly related to the corona formation among which 50% would be found on the NPs regardless of their surface coating or size. The studies with BSA indicated that NPs could be strongly affected by the presence of polymer coatings and the surface charge of the nanoparticles. In some cases, BSA exhibits a relatively low affinity for the electrostatically stabilized NPs, demonstrating the importance of interactions between electrostatic and hydrophobic elements in the protein corona formation. This affinity and electrostatic stabilization mainly controls the toxicological aspects of nanoparticles and thus the corona itself. In addition, uncoated and surfactant-free Ag-NPs promoted a maximum protein (BSA) coating due to increased changes in entropy and a lower affinity for electrostatically stabilized NPs due to the constrained entropy changes. The studies with FBS indicated that, in a protein-free solution, hard protein corona could be sustained in their final form for a long time, undergoing a stabilization process. A typical nanoparticle protein corona consisting of HSA, immunoglobulins, fibrinogen, apolipoproteins, transferrin, complement proteins, and hemoglobin causes certain illnesses to develop and progress. In an HSA study, it was found that the interaction of protein coronas with lipid vesicles could enhance their fluidity. Usually, cellular uptake

is reduced by the incubation of silver with albumin, which significantly alters the association of the particle with the membrane. The biological activities of the surface corona were also studied to understand their antibacterial activity and cytotoxicity. It is evident from the literature that the antibacterial activity of Ag-NPs mostly depends on the capping agents and the route of administration into the organism e.g., orally or intravenously. The toxicity of the protein corona is controlled in most cases by particle coatings and is induced by oxidative stress through cell surface receptors. The corona may affect the ability of the NPs to dissolve into silver cations (Ag^+), which impacts the toxic effect.

Different functional groups present on the particle surface along with the protein charges regulate the cytotoxic properties of the corona. The functional groups play a key role in the formation of the nanoparticle-protein corona. Positively and negatively charged Ag-NPs showed the highest and lowest bactericidal activity, respectively. In both cases, surface charge plays an important role in bactericidal activity of Ag-NPs against both gram-positive and gram-negative bacteria [137].

The affinity for water is another key factor impacting Ag-NP effectiveness and toxicity that has gained serious attention from researchers worldwide. To protect against viral-mediated diseases, Ag-NPs act as anti-viral agents which will eventually be utilized in antiviral therapy. The antiviral activity of Ag-NPs is largely controlled and regulated by increased membrane hydrophilicity [19]. Nanosilver incorporation also increased membrane hydrophilicity, reducing the potential for other types of membrane fouling. In addition, Katherine et al. indicated that the decrease in hydrophobicity can be potentially beneficial for preventing chemical fouling [138].

Huge efforts were made to convert hydrophilic Ag-NPs to hydrophobic Ag-NPs [139]. Both hierarchical surface structures (micro/nano-scale roughness) and a low surface energy layer are required for the conversion of a hydrophilic surface into a hydrophobic surface. Hydrophilic Ag-NPs (5–30 nm) in the presence of cationic surfactant could be transferred to an organic phase by solvent exchange induced by inorganic salts with a high transfer efficiency (>95%). The hydrophobic Ag-NPs are stable and suitable for long-term storage without loss of their original particle integrity [140].

Effects of Ag-NPs on degradation of non-biodegradable dyes

Silver in the nanoparticle form is extremely valuable for industrial, electrical, mechanical, and biomedical uses, because of its antimicrobial and catalytic properties. Non-biodegradable dyes are currently a great environmental health and pollution concern. UV-light degradation, carbon sorption, flocculation, and redox treatments are the most widely practiced methods for the removal of dyes. However, they are mostly ineffective and a better approach is needed. Nevertheless, it is difficult to remove these dyes from water because of their aromatic structural stability. Ag-NPs show catalytic properties in the field of dye detoxification and its removal from textile and paper industry effluent. Biosynthesized Ag-NPs are highly effective in comparison to the synthetic Ag-NPs as catalysts in the process of degradation of hazardous dyes in a cost-effective manner. The degradation efficiency of Ag-NPs is greater because of their very high surface area, high migration rate of electrons to the surface of the NPs, accelerated kinetics, independency of size and shape, etc. This makes them compatible, efficient, economic, and eco-friendly for dye removal from industrial effluent [141].

Different researchers have focused on the photocatalytic activity of the Ag-NPs for the detoxification of Safranin O (SO), Methyl red (MR), Methyl orange, Congo red (CR), and Methylene blue (MB), etc. under sunlight for a particular period of time. MB, an aro-

matic cationic dye, is present in contaminated wastewater and might lead to eye, gastrointestinal tract, and skin irritation [142,143]. Generally, the maximum absorption band of MB in aqueous solution is observed at 665 nm owing to the $n-\pi^*$ transition of the MB [62,144]. The photocatalytic degradation of the MB solution could be determined by the decreasing intensity of the absorption band with respect to time while exposed to sunlight. The surface plasmon resonance (SPR) property of the Ag-NPs could be responsible for the decrease in MB in solution at about 6–72 h [145]. CR, a secondary diazo anionic dye, is a carcinogenic metabolite that can cause bladder cancer and undergoes photocatalytic degradation spectrophotometrically within 20 min by Ag-NPs [146].

SO is a derivative of phenazine that affects aquatic biodiversity and can be successfully catalyzed using photocatalysts like Ag-NPs. It has a high surface-to-volume ratio, non-toxicity, cost effectiveness, and provides a novel way of treating several dye pollutants [147]. Jyoti et al. showed that catalytic activity can be strongly dependent on the crystal structure, morphology, and size of the particles. Methyl red and Methyl Orange can also be photochemically degraded in the presence of Ag-NPs as photocatalysts [141]. Some NPs are known to induce endoplasmic reticulum (ER) stress, leading to cell death. Jean et al. reported that Ag-NPs target and induce ATF-6 degradation, leading to activation of the NLRP-3 inflammasome and pyroptosis, which provides a new link between ER stress and activation of the NLRP-3 inflammasome. Kalantari et al. fabricated Ag-NPs by treated alkaline tapioca starch, which showed good catalytic activity in the degradation of 4-NP by sodium borohydride within a short time [148]. They also reported the antioxidant activity of Ag-NPs for the treatment of some diseases caused by oxidative stress, which lead to them being labeled as green particles and making them a biocompatible and low-cost candidate for commercial and biomedical applications.

Biocidal applications of Ag-NPs based on physical properties

The physicochemical properties of Ag-NPs (e.g., size, shape, concentration, and electrochemistry) largely direct the Ag-NP applications in industrial, medicinal, and environmental sectors. Both gram-negative and gram-positive pathogenic bacterial strains can be destroyed by Ag-NPs. The particle sizes along with the surface stability of Ag-NPs are the major factors regulating the effectiveness of Ag-NPs as a biocide. Evidently, the Ag-NPs damaged and destroyed bacterial cells by penetrating and accumulating in the bacterial membrane. The penetration of Ag-NPs largely depends on the size of the particles, for example, 1–100 nm Ag-NPs can easily penetrate into gram-negative bacteria and 10–15 nm-sized Ag-NPs can inhibit non-resistant and drug-resistant bacteria [131]. In addition, a low concentration of Ag-NPs (3.3–33 nM) can completely inhibit *E. coli* and *S. aureus* [149]. Other than the size, shape and surface modifications also impact the effectiveness of Ag-NPs as biocides. For example, the truncated triangular Ag-NPs exhibit stronger biocidal activity than the rod-shaped and spherical shaped NPs, and ionic silver [150]. The surface modification of Ag-NPs with sodium dodecyl sulfate-SDS, polyoxyethylene sorbitan monooleate-Tween 80, and polyvinylpyrrolidone-PVP 360 significantly raised the antibacterial activity of the Ag-NPs against *E. coli*, *P. aeruginosa*, *E. faecalis*, *S. aureus*, *P. aeruginosa*, methicillin-susceptible *S. epidermidis*, methicillin-resistant *S. epidermidis*, methicillin-resistant *S. aureus*, vancomycin-resistant *E. faecium*, and *K. pneumonia* [72,151]. This characteristic of Ag-NPs along with the identification of the exact particle size distribution for establishing Ag-NPs as an antibacterial agent led to its use as an air disinfectant in air filters, preventing bacteria from colonizing filters. The presence of Ag-NPs in the air filters prevented the colonization

of bacteria such as *Micrococcus luteus*, *Micrococcus roseus*, *B. subtilis*, and *Pseudomonas luteola*. The presence of *E. coli* and other pathogenic microbes in the drinking water is another health and social concern worldwide, especially in poor countries. Ag-NPs functionalized with polyurethane (PU) can contribute a —COO— carboxylic functional group which in turn exhibits effective anti-infection activity against two types of gram-negative bacteria (*E. coli* and *P. aeruginosa*) and two types of gram-positive bacteria (*B. subtilis* and *S. aureus*). The release of Ag^+ into water is considered the key component needed to kill pathogens. Thus, the contamination of water with ionic Ag may trigger other health concerns, which should be addressed in future research [152].

Ag-NPs also have anti-fungal properties against *Trichophyton rubrum*, *Trichophyton mentagrophytes*, and *Candida albicans* at different sizes and concentrations. Ag-NPs work best against fungal strains at a size of ~ 100 nm, with the IC_{50} value ranging from 1 to $7 \mu\text{g mL}^{-1}$ [16,153,154]. The most recent discovery of Ag-NPs as biocides involves its potency as an antiviral agent against viral infectious diseases such as SARS-Cov, influenza A/H5N1, influenza A/H1N1, Dengue virus, HIV, HBV, and new encephalitis viruses. Ag-NPs ranging in size from 1 to 10 nm inhibit HIV-1, whereas 10–80 nm particles can inhibit other viral strains by binding to the outer proteins of the viral particles. The exact mechanism of Ag-NPs as an antiviral agent has not been elucidated. Further research into this mechanism will help in the fight against harmful viruses in the near future [73,155,156]. Ag-NPs are now used in the industrial sector to form antimicrobial paints, functionalized plastics, medicinal gels, preservatives, packaging materials, fabrics, etc. The sustainable functionality of effluent treatment plants in some major industrial zones can be assured by intensive characterization and modification of this novel nanoparticle.

Cytotoxicity of Ag-NPs

Mechanism of toxicity induced by Ag-NPs

Despite the wide applications of Ag-NPs, little research has been conducted concerning their impact on human health and the environment. The toxicological mechanism is still unclear. Regardless, there is a number of publications available describing both *in vitro* and *in vivo* NP toxicity experiments. Results showed that the cytotoxic and genotoxic effect of Ag-NPs is dependent on their concentration, size, exposure time, and environmental factors. In addition, nanosilver surface-coating agents, such as citric acid, amino acids, acetyl trimethyl ammonium bromide, and sodium dodecyl sulfate are noncovalently attached to nanosilver particles and can be released into the environmental and biological media with or without interaction with biological macromolecules, and inorganic and organic ions cause the NPs to be unstable in media [157,158]. Additionally, particle aggregation, surface oxidation to form silver oxide, and oxidation of silver oxide release both Ag^+ and Ag^0 into the media, which eventually results in accumulation of ionic silver in the environmental media, biological media, and inside the cell through diffusion or endocytosis, causing mitochondrial dysfunction [159]. Ag-NPs then interact with cell membrane proteins and activate signaling pathways to generate reactive oxygen species (ROS), leading to damage of proteins and nucleic acids caused by the strong affinity of silver for sulfur and finally causing apoptosis and inhibition of cell proliferation [160]. Most of the research has pointed to the above-mentioned cytotoxicological pathways of Ag-NPs.

Generally, in *in vitro* tests, Ag-NPs are highly toxic at concentrations ranging from 5 to $10 \mu\text{g mL}^{-1}$ and sizes from 10 to 100 nm, and they disrupt mitochondrial function [74,161]. It can be assumed from several studies that Ag-NPs are transported across

cell membranes, especially into the mitochondria, but it is unknown whether nanomaterials target the mitochondria directly or enter the organelle secondary to oxidative damage [162]. Hasse et al. reported that the cytotoxicity of Ag-NPs was mainly induced through the mitochondrial pathway by reducing glutathione (GSH), high lipid peroxidation, and ROS responsive genes causing DNA damage, apoptosis, and necrosis [160]. On the other hand, a few *in vivo* studies showed that Ag-NPs cause adverse effects on reproduction, malformations, and morphological deformities in different non-mammalian animal models, in addition to the above-mentioned *in vitro* effects [163].

There is another debate regarding whether Ag-NPs or Ag^+ induce toxicity in biological systems. Ag^+ is released through the surface oxidation and then reacts with biological molecules [5]. Though it is controversial, there is strong evidence supporting the idea that it is Ag^+ that is responsible for the Ag-NPs-mediated toxicity and not the NP itself [164]. A recent study revealed that cytotoxicity of Ag-NPs occurs due to the minimum release of Ag^+ [7,165–167]. Therefore, distinguishing the part of the Ag-NPs that leads to toxicity is challenging.

Uptake mechanism of Ag-NPs

Uptake of Ag-NPs into cells may differ from cell to cell. Diffusion, phagocytosis, and endocytosis are some potential methods [168]. In human macrophages, Ag-NPs can enter cells in phagocytic and non-phagocytic ways [112]. In medium, some Ag-NPs aggregate and enter into the cells through phagocytosis, but other particles that are not in an aggregated form enter through alternate ways. Sometimes, Ag-NPs are engulfed by mammalian cells, and the uptake range of NPs depends on the particle size and type of cell [169–171]. The membrane flip flop mechanism or direct penetration via the ion channel is another possible route of Ag-NPs uptake. In this case, active transport also exists with passive transport [112]. Intracellular uptake of Ag-NPs was confirmed in the HT22 cell line even 96 h after removal of Ag-NPs from the medium [113].

ROS generation in Ag-NPs-induced toxicity

Most of the cellular and biochemical alterations in the cells are caused by ROS-mediated toxicity, and this has been confirmed by several *in vitro* models [172]. Oxidative stress is considered as the probable mechanism of Ag-NPs-induced toxicity. Superoxide radical (O_2^-) and H_2O_2 can act as ROS, which are essential for maintaining normal physiological processes. However, excessive ROS can collapse the antioxidant defense system, leading to the damage of DNA, proteins, and lipids [75]. Mitochondria mainly release ROS, leading to oxidative stress, disruption of ATP synthesis, DNA damage, and eventually apoptosis [173]. Likewise, Ag-NPs usually generate ROS after entering into the cell [172]. As ROS levels increase, the GSH level decreases dramatically and at the same time LDH increases in the medium, which ultimately induces apoptosis [174]. Increased levels of ROS ensure oxidative stress that might cause calcium dysregulation or neurodegeneration in neuronal cell [96,175]. Oxidative stress resulting from Ag-NPs can damage the antioxidant defense capacity of the cell, damage DNA, and finally lead to apoptosis, especially in human cell lines [172,174,176–179]. Intracellular oxidative stress causes MMP3 to secrete a specific amount of MMP, an extracellular matrix (ECM) digester protease [180,181]. Moreover, ROS generation also affects redox homeostasis at the intracellular level, and as a result, lipid peroxidation and protein carbonylation occurs. At the same time, the glutathione level and antioxidant enzyme activity are decreased. Thus, glutathione level, antioxidant enzyme activity, and protein bound sulfhydryl group depletion promote apoptosis [182]. Therefore,

the main cytotoxic effect of Ag-NPs is apoptosis-mediated cell death [152].

Different toxicological pathways of Ag-NPs

Ag-NPs induce cytotoxicity following different pathways. Several studies have shown that Ag-NPs induced toxicity is triggered by the increase of ROS generation [183]. *In vitro* instillation of Ag-NPs into the cell could generate overproduction of intracellular ROS, which activates cell death-regulating pathways such as p53, AKT, and MAPK signaling apoptotic pathways [184]. Over production of ROS causes the down regulation of total AKT, which increases the expression of proapoptotic kinase p38. Meanwhile, decrease in PARP (poly ADP ribose polymerase) expression resulting significant increase of caspase-3, H2X, p-p53, and total p53 expressions [184]. Thus nanosilver can induce apoptosis following p53 signaling pathway (Fig. 2).

Mitochondrion is an important centre of apoptosis signal. Effect of Ag-NPs on mitochondrial membrane permeability could cause loss of mitochondrial integrity, which may regulate JNK mediated caspase dependent apoptosis [60]. Loss of mitochondrial membrane potential ($\Delta\Psi$) regulate down-regulation of Bcl-2, up-regulation of BAX and release of cytochrome c into the cytosol. Down-regulation of Bcl-2 can be influenced by JNK (Jun amino – terminal kinases). JNK is a member of MAPK family, which participate in apoptosis via phosphorylation of Bcl-2, consequences inactivation of Bcl-2. Release of cytochrome c into the cytosol initiates a cascade that leads to the initiation of caspase 3 through apaf-1 and caspase 9 [185]. Thus Ag-NPs can induce apoptosis via mitochondria and caspase dependent pathway mediated by JNK (Fig. 3). Epigenetic dysregulation can also be induced by Ag-NPs, which may have long term effects on gene expression reprogramming. [113]. Ag-NPs could have effect on the cell cycle and induc-

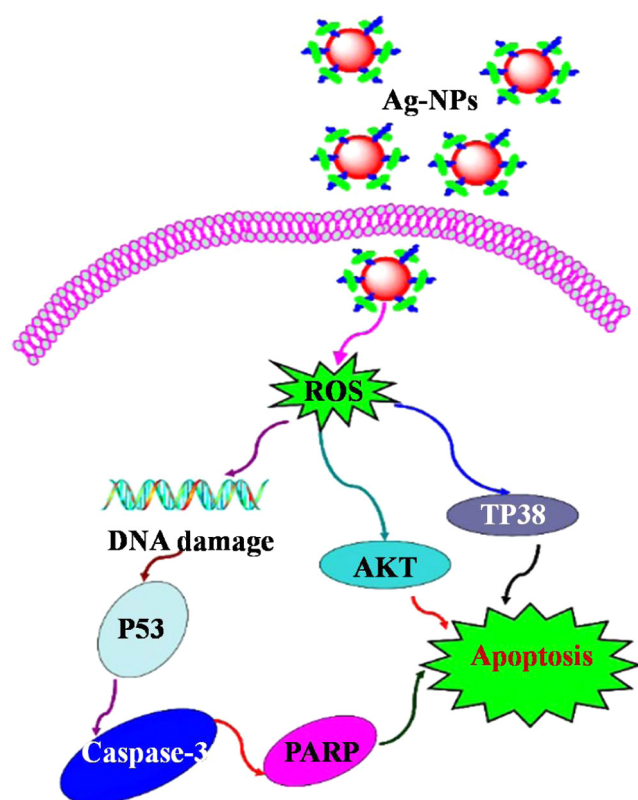


Fig. 2. Apoptosis inducing signaling pathway mediated by p53, AKT, MAPK activation to suppress ROS generated by Ag-NPs [184].

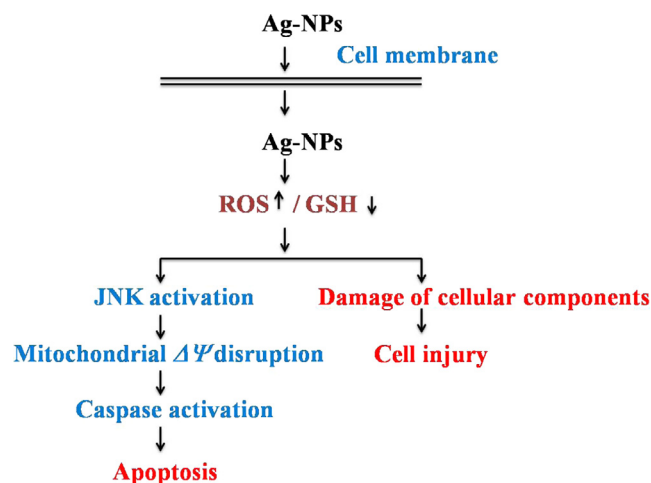


Fig. 3. A proposed pathway for Ag-NPs induced ROS generation and intracellular GSH depletion, damage to cellular components, and apoptosis [60].

tion of DNA hypermethylation following the p53 or p21 pathway, which may have effect on epigenomic level [113].

Several studies have compared the toxicity mechanism of Ag-NPs with the Trojan-horse-type molecular pathway [70]. For instance; Ag-NPs can be phagocytosed by RAW 265 cells, making them available in the cytosol and culture medium of active cells, but not in damaged cells. It is possible that NPs released from the damaged cell into the culture medium promote a further biological response referred to as a “Trojan-horse-type” mechanism. Disappearance of Ag-NPs inside the damaged cells suggests that the NPs were ionized inside the cell resulted to cell damage. It is also worth noting, phagocytosis of AgNPs can generate ROS which stimulate inflammatory signaling $\text{TNF-}\alpha$. The increase of $\text{TNF-}\alpha$ causes the damage of cell membrane and apoptosis. Thus it is speculated to be caused by ionization of AgNPs in the cell which is expressed as Trojan-horse type mechanism [70].

Like other nanoparticles, Ag-NPs also provoke oxidative stress into the cell through ROS generation [58]. Moreover in Ag-NPs treated cells, generation of ROS can be decreased by pretreatment of cells with NAC, suggesting involvement of intracellular antioxidant defense system [60]. GSH is one of the major endogenous antioxidant scavengers that able to bind to and reduce ROS. Thus GSH mediated antioxidant scavenge system is considered as a critical defense system for cell survival [186]. GSH is formed in two steps by γ -GCL and GSS. First, γ -GCL catalyses and produce glutamylcysteine in the process of cellular GSH biosynthesis. Then finally glutamylcysteine is catalyzed by GSS and adds a glycine residue to form glutamyl cysteinyl glycine or glutathione [60]. Ag-NPs raised intracellular ROS by the reduction of GSH level through the inhibition of GSH synthesizing enzyme [60]. However superoxide dismutase and catalase is also intracellular antioxidant defensive enzyme.

Nrf2 is another defensive pathway which plays an important role in preventing cellular stress. Nrf2 can play a central role in protecting the cell from oxidative, electrophilic, and nitrosative stress, especially in the intestinal cell, through the induction of antioxidant-responsive genes and genes of the phase II detoxifying enzyme [187–190]. Oxidation of Keap-1 dissociates Nrf2 and it is then translocated into the nucleus and ultimately activated [191]. Thus, activation of Nrf2 influences the generation of cytoprotectors such as HO-1. HO-1 is an enzyme of heme catabolism, which counteracts cell death by producing equimolar quantities of Fe^{2+} , biliverdin, and carbon monoxide to neutralized ROS [192,193].

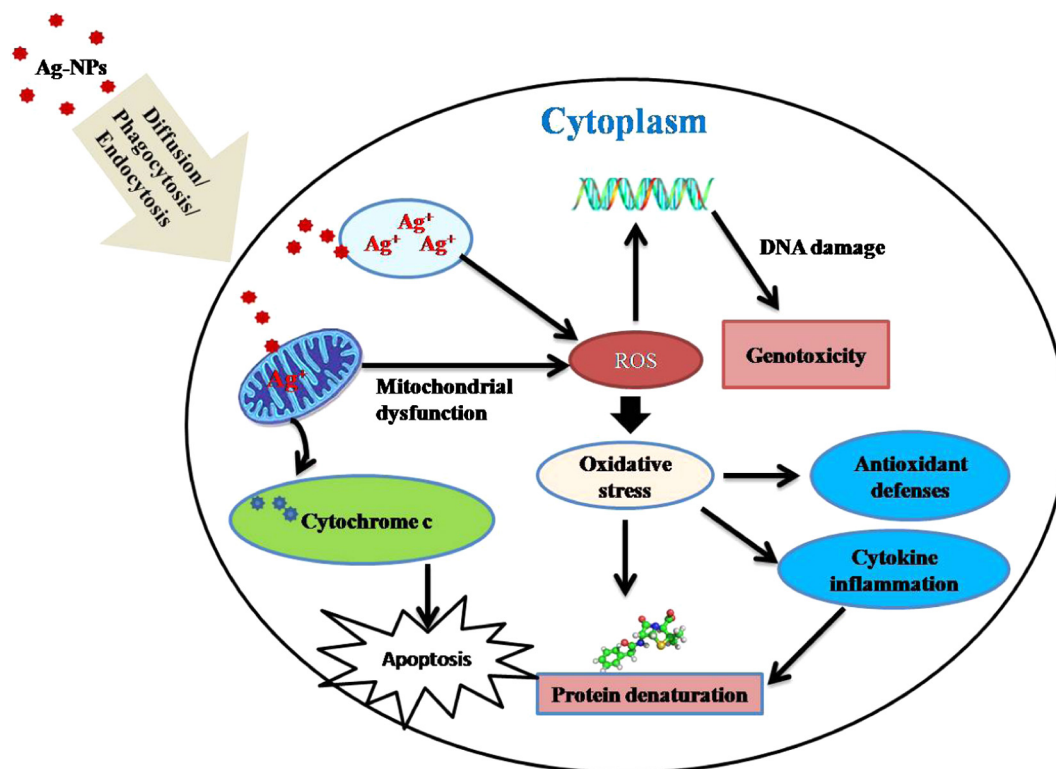


Fig. 4. Possible uptake process and mechanism of cytotoxicity induced by Ag-NPs in different cell lines based on the metadata from several studies.

In summary Ag-NPs can enter into the cell through the process of diffusion, phagocytosis or endocytosis. Inside the cell, Ag-NPs itself or ionized Ag^+ generate ROS cause oxidative stress. Over production of ROS can denature different antiapoptotic proteins and initiate proapoptotic proteins expression. Thus expressions of apoptotic proteins initiate apoptosis signaling pathway (Fig. 4).

Dosimetry in Ag-NPs-induced cytotoxicity

Growing evidence suggests that human exposure to engineered nanomaterials (ENMs) can lead to adverse health effects, but the underlying toxicity mechanisms are not currently well-understood [190]. So, an efficient and cost-effective toxicological screening method is needed for characterizing the relationships between ENM physicochemical properties including size, morphology, surface chemistry, and crystallinity, and their biological effects on an organism *in vitro* [191]. Dosimetry is the measurement of the absorbed dose delivered by ionizing radiation or another source that is received by the human body. Dosimetry of nanomaterials could be used for rapidly assessing nanomaterial toxicity [192]. Nanomaterials exhibit a transition between bulk materials and atomic or molecular structures where quantum effects lead to the occurrence of specific physicochemical properties (e.g., malleability, electrical conduction, magnetism) and also exhibit specific toxic effects. A prevailing view is that nanoparticle surface area, gravitational settling, diffusion, sedimentation, agglomeration, mobility, mass, particle size, shape, exposure time, and dose are important determinants of toxicity and could be examined to determine dose metrics [193]. Considering particle migration, it was estimated that the active fraction of particles might be extremely low or even negligible for particles with nanoscale dimensions.

We did not find any concrete research describing an integrated methodology for *in vitro* Ag-NPs dosimetry with accurate determination and reporting of delivered cell dose metrics. It is suggested

that researchers determine relevant doses to deliver into the cells rather than relying on the typically reported administered doses of particles in suspension. There is a need for more work to resolve this issue and to determine the required doses. In addition, the effects of particle size should be considered, specifically in different cell lines. Consequently, nanotoxicologists should carefully consider the nominal doses of nanomaterials for *in vitro* experiments.

Conclusions and future perspectives

The physicochemical attributes of Ag-NPs mainly distribute and categorize major toxicological concern, and establish the ladder of toxicity framework while imposing into the biological system. Till now, studies are not enough to get a concrete idea on the cytotoxicity of Ag-NPs and also the mechanism behind the toxicity. But on the basis of above discussion it is evident that cytotoxicity of Ag-NPs can be considered as dependent on different kinds of properties such as nanoparticle size, shape, concentration, agglomeration or aggregation. In this review, we provide some comprehensive idea about particle size and toxicity, that is, less particle size is responsible for high toxicity. Aggregation and sedimentation lead to a decrease in the activity of biologically active particles. However, Ag-NPs can agglomerate frequently that's why surface coating is used in toxicity measurement, which is also contradictory. Moreover, aggregation of Ag-NPs cannot prevent penetration into the stem cells in mesenchyma including its nucleus. That is supposed to be another interesting contradiction. In addition, cytotoxicity not only depends on NPs properties, but also organism's variation plays a vital role. All cell line does not show same types of responses. Finally, Ag-NPs can induce cytotoxicity through oxidative stress by the generation of ROS. ROS generation initiate a pro-inflammatory protease, caspase-1 activation that regulates apoptosis or cell death. For the better understanding of cytotoxicity mechanism of Ag-NPs further studies are needed.

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Conflict of interest

The authors declare no conflict of interest.

Compliance with Ethics Requirements

This article does not contain any, studies with human or animal subjects.

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