Chapter 10

Assessment of Nano-toxicity and Safety Profiles of Silver Nanoparticles

Yasemin Budama-Kilinc, Rabia Cakir-Koc, Tolga Zorlu, Burak Ozdemir, Zeynep Karavelioglu, Abdurrahim Can Egil and Serda Kecel-Gunduz

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http://dx.doi.org/10.5772/intechopen.75645

Abstract

Nanotoxicology, which is related with toxic potentials of nanoparticles (NPs) and their adverse effects on living organisms and environment, is a sub-branch of toxicology discipline. Nano-toxicity of NPs depends on their doses, unique chemical, and physical properties. Nowadays, silver (Ag) NPs are used in many consumer and scientific applications such as antimicrobial and pharmaceutical applications, water purification systems, textile industry, and food packaging processes. However, the information that about their nano-toxic potentials is still not complete, and it is considered that several parameters of Ag NPs such as size, shape, surface, and stability affect the toxic potential in different ways. Nano-toxic potentials of Ag NPs were mentioned as *in vivo*, *in vitro*, and *in silico* the studies. In this chapter, it was evaluated the common unique properties of NPs are related with nanotoxicology such as size, surface area and modifications, shape, agglomeration status, and dose.

Keywords: *in vivo*, *in vitro*, *in silico*, nanoparticles, nano-toxicity, silver

1. Introduction

Toxicology is a discipline that investigates the adverse effects of chemical substances and the interaction mechanisms of these substances on the living organisms. Toxicology is derived from a combination of Greek words which are "toxicos" and "logos" these mean "poisonous" and "subject". Nowadays, modern toxicology concerns with the sources of the poisons, physical, chemical, and biological properties of toxic materials, the alteration of these substances

within organism, and the mechanisms of the actions. At the same time, this concept involves the isolation of the poisons, the analysis of toxic materials as quantitative and qualitative besides that risk analyses, optimization processes, and treatments of poisons [1].

Nanotoxicology is a part of bio-nanoscience, which studies on toxicity of nanoparticles (NPs). The changes in structural and physicochemical characteristics of a material in nano-size compared to micro-size, would lead to number of changes in toxicological impacts [2]. The toxicological potential of a material can be investigated in two subdivisions as health and environmental hazard. The main goal of nanotoxicological studies is the determination of which properties of NPs become a threat for the organisms and environment.

There are several ways of taking NPs from organism, such as dermal, inhalation, oral, intravenous and subcutaneous [3–7]. The skin, lung and digestive tract get contacted with the environment. It is clear that lung and digestive tract are more vulnerable than skin since the skin is forceful barrier against foreign substances in general. On the other hand, injections and implants are the other possible routes for intake of NPs [8, 9]. Due to their ultra-small sizes, NPs can reach tissues and organs through circulatory and lymphatic systems. Thus, they may cause some adverse effects on organism that lead to various problems.

Gold (Au), silver (Ag) and iron oxides (Fe_2O_3 or Fe_3O_4) are extensive metals to be used as a nano-sized form, since they have excellent physicochemical properties such as optical, magnetic activity, high thermal and electrical conductivity as well as their great surface area to volume ratio [10–12]. Among these metals, Ag NPs are more prominent than the others due to their antibacterial, antiviral and antifungal effects [13–15]. Therefore, Ag NPs have become a popular topic among the scientific community. **Figure 1** shows that the number of published research articles in this field within last 8 years [16]. According to graphic, the studies which were carried out with Ag NPs, have been increasing continuously.

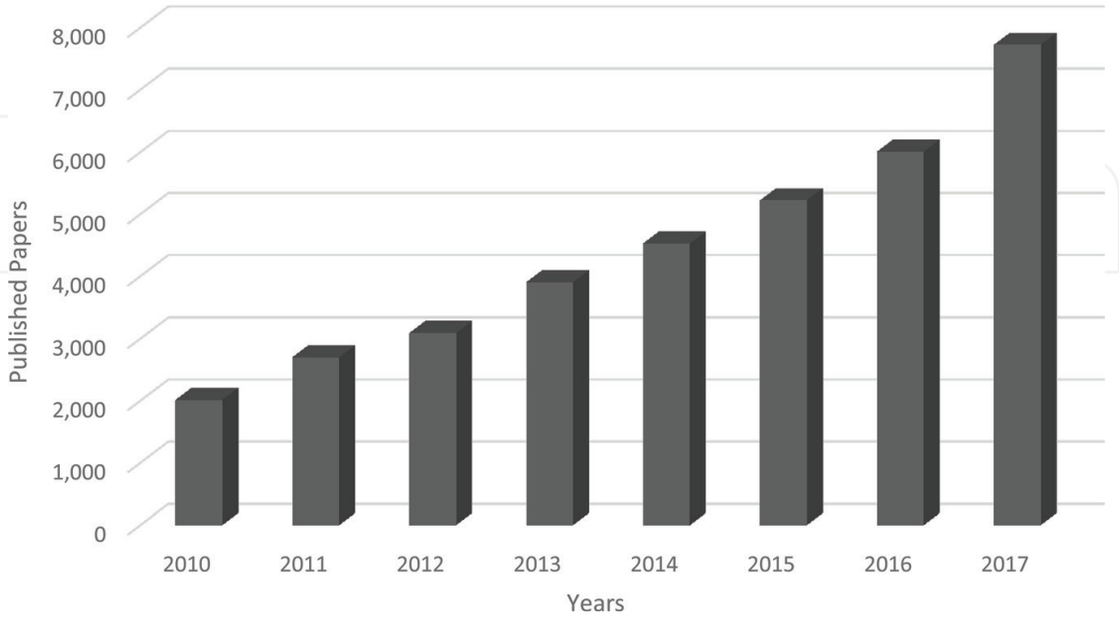

Figure 1. Trend in published research articles on the topic of Ag NPs.

The nano-toxic effects of Ag NPs should be investigated properly because the numerous usage areas of these NPs such as pharmaceutical applications [17], water purification systems [18], textile industry [19] and food packaging processes [20] make them as an outstanding material for humankind and for environment. A comprehensive investigation about toxicity of Ag NPs will provide useful information in risk management for present and future issues.

In this chapter, nano-toxic potentials and common unique properties of Ag NPs related with toxicology such as size, surface area and modifications, shape, agglomeration status and dose were evaluated.

2. The properties affect the nanotoxicology

The physicochemical properties which are related with nanotoxicology can be classified as size-dependent [21], surface-dependent [22], shape-dependent [23], aggregation or agglomeration-dependent [24] and dose-dependent [25]. These properties may change the nano-toxic potentials of NPs in different ways as indicated below.

2.1. Size-dependent toxicity

NPs are defined as materials which are at least one-dimensional and range in 1–100 nm. According to studies, the size of NPs may alter toxicological effects on organism [26].

Toxicological properties of NPs may be induced when the particle surface interacts with cellular components [27]. Therefore, surface area of NPs is depended on their diameters [28] and is enlarged exponentially when the diameter drops off [29]. This situation means that NPs may have several levels of toxicity based on their particle sizes and surface reactivates even if they have same compounds and crystalline structure [30]. Moreover, sizes of NPs increase significantly cellular uptake mechanisms and distribution in the body [31].

Some studies showed that NPs need to migrate across the epithelial barriers to cause toxicity and inflammatory response in animal models [32, 33]. NPs can diffuse into the lung parenchyma when they are inhaled [34, 35]. Different sizes of NPs indicate the special dispersion patterns in the respiratory tract. Stokes number and Reynolds number affect the dispersions of NPs. At the beginning, dispersion of NPs is highly stable in the gas phase. However, their dispersion stabilities may be changed in liquid phase of respiratory fluids depending on the numbers that was mentioned above [36, 37]. Thus, dispersion patterns of NPs are a crucial consideration to determine nano-toxicity [38]. It was reported that kidneys cannot excrete the NPs which were bigger than 6 nm and accumulate some specific organs such as liver and spleen till the clearance of this accumulation by mononuclear phagocyte system [39]. Many NPs cause important adverse effects by accumulation in the liver and spleen [40].

At cellular level, uptake mechanisms and efficiency of NPs are important factors which affect toxicity. NPs penetrate the cell through several ways such as phagocytosis and pinocytosis depending on their particle size and surface properties [41, 42]. The range of 10–500 nm is suitable size for uptake by cells and 5 mm is upper limit for this. The bigger NPs are swallowed

with the help of macro-pinocytosis. The size of vesicle of clathrin-mediated endocytosis is about 100 nm, meanwhile the size of vesicle of caveolae-mediated endocytosis is about 60–80 nm [27].

The size of Ag NPs not only changes with the uptake mechanism but also with the cytotoxicity potential of them [36]. In one study, researchers suggested that Ag NPs have an adverse effect, dependent on size, on lactate dehydrogenase (LDH) activity, cell viability and reactive oxygen species (ROS) generation in different cell lines [36]. In another study, Carlson et al. investigated that 55 and 15 nm of hydrocarbon coated Ag NPs for generating ROS in macrophage cell line. The results showed that the generation of ROS levels with 15 nm of Ag NPs was higher than 55 nm Ag NPs [43]. Wang et al. reported that 20 nm of citrate-coated Ag NPs had more toxicity potential than 110 nm of Ag NPs and 20 nm of citrate-coated Ag NPs have more capacity for generating acute neutrophilic inflammation in the lungs of mice when compare with 110 nm of Ag NPs [44]. However, Kaba et al. showed that smaller Ag NPs do not have a crucial role in the viability of tumor cells [45].

2.2. Surface-dependent toxicity

Surface area and charge of NPs have also important role in biological toxicity. Some studies were reported that a large surface area causes alterations in band gap, decreased melting points and higher reactivities which have critical adverse effects including inflammation, toxicity and cytotoxicity [46–48]. The NPs that have bigger surface area can interact with the other particles which are nearby, and may cause the higher reactivity. Thus, NPs with higher reactivity induces harmful effects in cosmetic products and drug carrier components when used as fillers [49]. From this point of view, it can be inferred that when size of NPs are decreased, biological activity of them are increased, substantially [50].

Some researchers investigated that effect of different surface areas and specific reactivities of NPs in lung for understanding connection between surface area of NPs and their potential toxicities [51]. The result of a research shown that the nano-toxicity which depends on different sizes were not occurred significantly, however, it was suggested that total surface area had an important role to consist of lung inflammation [52, 53]. Particle surface reactivity can be easily determined by single particle aggregate [54–56].

The surface charge of NPs can affect the distribution stability in aqueous solutions and for this reason; it may cause dramatic effects on biological systems and organisms. The surface charge may represent the surface of native NPs and adsorption capacity of ions and biomolecules at their interface [57]. In one study, researchers investigated the bacterial activity of Ag NPs positively and negatively charged. In the result of this study, it showed that positively charged Ag NPs have higher bactericidal activity than negatively charged ones. In either case, bactericidal activity against both Gram-positive and Gram-negative bacteria can change according to the surface charge [58]. Cytotoxic properties of NPs can also be affected by different functional groups on the particle surface and they are associated with protein charges. These different functional groups have important role in forming the NP-protein corona [59].

2.3. Shape-dependent toxicity

There are several chemical and physical synthesis methods of Ag NPs. These differences about synthesis method cause different types of Ag NPs such as spherical, triangular, square, cubic, rectangular, rod, oval and flower. It is still unclear that which critical factors of Ag NPs are playing a role in the formation of particles for toxicity and how they are affecting the biological systems. This situation may occur based on multiple factor. In one study, researchers investigated effect of different shapes of Ag NPs on alveolar epithelial cells (A549) and it was reported that agglomeration of Ag^+ ions occurred in the cytoplasm in the result of the study [60]. In another study, shape of NPs affects cellular uptakes. Gratton et al. showed that nano-rods has the highest uptake potential and nano-spheres, -ylinders and -cubes are followed it, respectively [39, 61]. In the other study, the researchers used NPs which were smaller than 100 nm. In the result of this research, nano-spheres had a significant advantage over rods. The study also showed that total cell uptake of nano-rods decreased when the aspect ratio of them increased [62, 63].

2.4. Aggregation or agglomeration-dependent toxicity

Aggregation or agglomeration potentials of NPs are very high in solution and air. The parameters such as diffusion, gravitation and convection forces can affect the interaction between NPs and the cells [64, 65]. The agglomeration can increase or decrease association with pH, electrolyte or salt content, and protein composition in the culture medium [66]. Some studies reported that binding capacity of NPs with protein can be changed depending on both composition of NPs and protein [67–69].

It is also known that preparation methods influence the agglomeration status of Ag NPs in medium. Lankoff et al. investigated that the aggregation ranges of Ag NPs using Ag NPs at 20 and 200 nm sizes in culture medium. The results showed that range of aggregation changed based on the culture medium preparation. The hydrodynamic diameter of Ag NPs could also change based on the culture medium preparation and it could be larger than nominal size of NPs. In conclusion, more aggregated particles have lower nano-toxic effect on the cells [70]. Ag NPs may show a high agglomeration tendency in culture medium because Ag NPs have high surface area. Occasionally, aggregation may play a vital role in the several types of intracellular response. Therefore, in terms of toxicological interest, agglomeration or aggregation states of NPs are very crucial for understanding different effects of biological responses [71].

2.5. Dose-dependent toxicity

The dose of NPs is one of the critical factors affecting toxicity. To determine the minimum dose of NPs which is induces toxicity, dose is very important. In one study, 0.2 ppm of Ag NPs decreased cell viability by 20%, meantime 1.6 ppm reduced Ag NPs viability by 40% [72]. Similarly, in human Chang liver cell, cell viability was reduced based on concentration and dose. In another study, researchers investigated toxicity potential of dose range of between 1 and 25 ppm. Result of this study showed that 25 ppm of Ag NPs was the most toxic dose [73].

There is still a problem about dose-dependent issues, which is crucial for understanding and comparing toxicological data. In many studies, which are carried out *in vitro*, doses of NPs are given as mass per volume (μg/ml) due to different experimental setup of studies [74]. Mass per surface area or particle number per surface area is alternative units which were given in some studies. Additionally, there are some differences between nominal dose and theoretical mass which is applied, delivered dose and targeted dose, cellular dose and internalized mass. For example, the deliver dose is related to the stability of NPs in the biological ambient and the viscosity of the dispersion medium [75].

3. *In vivo* toxicological information and experiments about silver NPs

In vivo toxicological studies are carried out with animals. Especially, mammalians such as mice, rat and rabbit are preferred by the researchers because they have the similar biological structure as humans. Over the last decade, the number of *in vivo* studies that examined the toxic effects of NPs has increased. This is due to the presence of NPs in many consumer products. However, the limitations of *in vivo* nanotoxicological studies which are carried out with these products still make it impossible to understand full toxicity profiles of Ag NPs.

Ag NPs naturally use three exposure ways into the body: (1) dermal, (2) inhalation and (3) oral route [76, 77]. In this regard, Ag NPs can make transition to circulatory system and may accumulate in various tissues and organs such as spleen, liver and brain. In recent years, use of Ag NPs in topical antibacterial formulations has caused skin interaction as a primary exposure route [78]. Skin, which constitutes 10% of the total body mass, exhibits a barrier property against external threats and maintains the special feature with various physical, immunological and metabolic activities. In this way, it can also resist particulate factors, especially various microorganisms, and keep the factors out of the body. The role of Ag NPs in the healing of skin wounds by dissociating into Ag^+ ions has made these materials as one of the most successful topical application materials [79]. However, their nano-toxic potentials that exhibit during topical application remains a question mark. From this point of view, *in vivo* animal models are confronting and helping us to test the nano-toxic activities of Ag NPs on the skin. For this purpose, porcine skin is an ideal *in vivo* model for acute nano-toxicity studies. This model is preferred due to its similarity to human skin in terms of either thickness or absorption rate [80]. Rats are also used further as *in vivo* skin nanotoxicology models. However, nanotoxicology studies which are carried out with both models have shown that Ag NPs have not toxic effects on the skin, surprisingly [81]. This may indicate that Ag NPs are using the skin as a transit route, not as a point where can exhibit their toxic abilities [3]. Extra small dimensions of Ag NPs are the biggest factor in achieve this passing.

Oral route is the one of the most important ways for nano-toxic effects of Ag NPs in the physiological systems. According to the Center for Food Safety (CFS), it has been reported that Ag NPs are included in various food additives, baby products and kitchen utensils [82] and this enhances the oral intake of Ag NPs. Since Ag NPs does not have any vital effect on human physiology as an essential metal, the intake into the body is also an undesirable situation [83].

Nonetheless, the studies have reported that the amount of Ag NPs from 0.4 to 27 μg per day can be taken orally by the human body [84–86]. It is known that the microparticulate sizes of silver cause argyria disease, which triggers pigment changing on the skin [87, 88]. However, it is not known whether Ag NPs provoke such a disease. It was shown that 10 nm of citrate-stabilized Ag NPs accumulation leads to oxidative stress in brain [89]. This suggests that Ag NPs may pass through the blood-brain barrier (BBB). BBB is the one of the most important physiological barriers that prevents the passage of various chemical agents and toxic substances into the brain. However, localization of Ag NPs in brain by passing the barrier and, moreover, having the nano-toxic potential to cause loss of function are highly thought provoking. Another *in vivo* study which was carried out by rats showed that it is also sufficient to localize 50–100 nm of Ag NPs in the brain by subcutaneous administration [90]. Intake of Ag NPs by oral administration may cause not only accumulation in brain but also in spleen, liver, kidney, stomach, salivary gland, skin and heart [91–94]. Although the inhalation route does not directly induce the nano-toxic effect, it causes Ag NP accumulation in various tissues and organs via the circulatory system, and indirectly supports to emerge of nano-toxic effects when compared to the other two main exposure routes.

Inhalation exposure is another key route for intake of Ag NPs. Since Ag NPs participate in the construction of various hygiene sprays, it is not difficult for the body to intake by inhalation route [95]. Therefore, it is useful to examine the nano-toxic potentials of Ag NPs on respiratory system. An acute inhalation nano-toxicity study, which was carried out with Ag NPs indicates that the NPs have diameters of 18–20 nm, generated nano-toxic effects at higher doses greater than 3.1×10^6 particles/cm^3 [96]. However, another study using Ag NPs have 12–15 nm indicates that nano-toxic effects did not occur even at higher doses than 1.32×10^6 particles/cm^3 [97]. A fundamental question arises here because these studies were acute and chronic inhalation toxicity studies, respectively. The differences between acute and chronic toxicity studies may help to determine the nano-toxic potentials of Ag NPs. It was observed that Ag NPs accumulated in two major organs such as lung and liver. Changing of lung function was occurred after 90 days in the sub-chronic inhalation nano-toxicity studies that were carried out with *in vivo* rat models [98, 99]. The dose-dependent nano-toxicity is another factor that affects the accumulations of Ag NPs in various tissues and organs. Kim et al. (2011) reported that there was not a significant weight gain of Ag NPs in the organs such as brain, stomach, liver, lungs and kidneys of both male and female rats at the end of 90 days in the lower doses, while the higher doses was effective to accumulating in these organs [100]. This situation can be explained as inhalation exposure leads to a rapid Ag NPs transition to the circulatory system and causes to accumulate of the NPs in various organs. The accumulations can induce to uptake of Ag NPs due to their size, stability, shape and surface activity to the cells which is building blocks of the higher organisms, and cause to loss of function of vital biochemical structures such as DNA and RNA [36, 101–103].

An *in vivo* sub-acute immunotoxicity study which was carried out with rainbow trout showed that approximately 12 nm of Ag NPs caused immunosuppression and inflammation-inducing effects on the fish after 96 hours [104]. In another *in vivo* study using 20–100 nm of Ag NPs, it was observed that Ag NPs almost completely suppressed natural killer (NK) cell activity and decreased the production of interferon-γ, interleukin (IL)-10 and IL-6 at the end of the 28 days in rats [105].

The adverse effects of Ag NPs on the cardiovascular system are still debated [106–108]. It is also possible to use different exposure routes to investigate the nano-toxic potentials of Ag NPs on the cardiovascular system [109]. Tang et al. (2009) reported that Ag NPs could transit directly to the cardiovascular system and cause adverse effects by accumulating in various organs [110]. Researchers also investigated the nano-toxic potentials of Ag NPs on heart in *in vivo* study which carried out with rats and it showed that approximately 20 nm of Ag NPs localized in the myocardium and caused to disorders in cardiac physiology by generating oxidative stress [111, 112]. Another *in vivo* study which used rainbow trout suggested that 50–60 nm of Ag NPs could produce cardiotoxicity in the fish [113].

Nano-toxic effects of Ag NPs on development and reproduction in animal models are another problem. An *in vivo* acute toxicity study that was carried out with male rabbits showed that 45 nm of Ag NPs were detected in acrosome and semen axonemal after intravenous injection [114]. Another study which was conducted with male rats revealed that 60 nm of Ag NPs occurred sperm abnormalities after 23–55 days [115]. The situation is the same for the female individuals. The study which was carried out with female rats showed that intake of 15 nm of Ag NPs orally induced decrease in body weight and increase in the number of atretic and degenerated follicles [116]. Another study also reported that 20 nm of Ag NPs influenced many gene sets including the genes which control the circadian clock regulation and photoreception in zebrafish [117].

4. *In vitro* toxicological information and experiments about silver NPs

The physicochemical and structural features of Ag NPs have an important role in their associations with cells. These different features can bring about different toxicity effects. For this reason, the physicochemical properties of Ag NPs are fundamental parameters in risk assessments and health studies. The assays which are used for predicting of nano-toxic potential of Ag NPs, their toxicity mechanisms and *in vitro* effects of Ag NPs were mentioned below.

4.1. *In vitro* assays for determining of nano-cytotoxicity and -genotoxicity

Cell viability test is the most commonly used method which evaluates the toxicity of Ag NPs. Typically, the percentage of dead cells is directly commeasurable to the toxicity of Ag NPs. Generally, cell viability tests are comprised of chemicals and they are based on differential inclusion, exclusion or transformation of dye or dye precursor which can only be enzymatically converted to detectible dye in living cells. In addition, the toxicity of Ag NPs can be identified by taking into consideration of morphological alterations in cells, cell viability, metabolic activity and oxidative stress. In the present case, nano-toxicity potential of Ag NPs can be evaluated by some assays such as MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide), 96AQueous One (96AQ), alamarBlue, LDH, live/dead and neutral red. Due to ability of Ag NPs to adsorb the chemicals onto their surface area, they may interact with dyes or assay reagents, and this situation may lead to incorrect results. Thus, wrong results may occur [118].

As a one of the most used method, the main goal of MTT assay is to measure cell viability in 96 well plates without the necessity of exhaustive cell counting. In brief, the principle of MTT assay is based on mitochondrial activity of viable cells, since decrease or increase of living cell number is directly related with mitochondrial activity. The mitochondrial activity of cells is reflected by the transformation of the tetrazolium salt into formazan crystals which should be dissolved for homogenous evaluation. In this way, any increase or decrease of viable cell number can be determined by measuring formazan concentration reflected in optical density utilizing a plate reader at 540 and 720 nm [119].

The neutral red assay is another cytotoxicity test which is used for measuring cell viability. According to this assay, viable cells are capable of binding the supravital dye neutral red in the lysosomes. This assay may be applied successfully for most of the primary cells and different cell lines. This weakly cationic dye penetrates cell membranes by non-ionic passive diffusion and concentrates in the lysosomes, where it binds by electrostatic hydrophobic bonds to anionic and/or phosphate groups of the lysosomal matrix [120–122]. Then, the absorbance of the solubilized dye which is extracted from the viable cells using an acidified ethanol solution, is quantified using a spectrophotometer.

On the other hand, alamar blue assay is a fluorometric method to determine metabolic activity of cells. The method depends on reduction of resazurin to resorufin via mitochondrial enzymes which carry diaphorase activity, like NADPH dehydrogenase [123]. Resazurin is blue and optical, it has poor fluorescent property. Via cells, resazurin is incrementally converted into the resorufin which is red and highly fluorescent. Fluorescence of resazurin and resorufin can be observed at 530–560 nm stimulation wave length, in addition emission wave length and oxidized form does not fluoresce much at 590 nm. Absorbance value can be observed at 570 and 600 nm, respectively, for the oxidized and reduced forms [124].

In addition to these, genotoxicity tests are also important for evaluating of nano-toxic potential of Ag NPs. These tests are implemented to determine potential genotoxic carcinogens and germ cell mutagens. Ames test (*Salmonella*/Microsome test) is known as the most exact and frequently used step to determine genotoxic carcinogens which cause base pair substitution mutation and small frameshift mutation [125]. The Ames test can be utilized as an indicator of the carcinogenic potential in mammals and it utilizes bacterial strains of *Salmonella typhimurium.* Because of the existence of mutations in the histidine operon, *these strains* are auxotrophic for histidine (his^-) (i.e., it cannot grow in a minimal culture medium without histidine). Base pair substitutions, frameshift types and gene mutations can be detected via these strains [126]. Although Ames test is generally preferred as first method to determine genotoxicity, there are a lot of studies suggesting that Ames assay is not a proper test method to evaluate the genotoxicity of NPs because Ames assay is mainly negative on NPs. Contrastingly, although many NPs are negative in the Ames assay, they generate positive genotoxic response in comet assay and micronucleus (MN) assay which are two of the *in vitro* mammalian cell test systems [125].

Comet assay is a quick and sensitive test which can determine the DNA damage at the level of individual eukaryotic cell. To perform this test, the cells are fixed in agarose gel on microscope slides and lysed under mild alkaline conditions to discard the cellular proteins. Then, slides are exposed to alkaline conditions to induce the DNA to unwind and electrophoresis.

During the electrophoresis, the migration of the undamaged super coiled DNA is slow, and it is close to the nucleoid, however, the migration of broken DNA fragments and relaxed chromatin is faster and further away from the nucleoid toward the anode. Thus the appearance of a "comet tail" is occurred. The DNA is marked with a fluorescent dye, so the DNA damage can be determined under a fluorescence microscope by visual scoring or via computerized image analysis [127]. In addition, this assay is the one of the most commonly used tests for determining the genotoxicity of NPs and also this test gives the most positive outcomes [125]. Genotoxicity of Ag NPs was evaluated by alkaline comet assay in human peripheral blood cells [128]. After exposure for 3 hours, the results demonstrated that Ag NPs (50 and 100 g/ml) lead to DNA damage. Besides, a short exposure of 5 minutes also demonstrated DNA damage too. To sum up, the study has demonstrated that the synthesized Ag NPs induced DNA damage in human peripheral blood cells and it was detected by the alkaline comet assay. Moreover, results showed that there was no inducing of any DNA damage in the presence of hydrogen peroxide, when the cells were exposed to Ag NP's.

In vitro micronucleus (MN) assay swiftly determines small membrane-bound DNA fragments which are located in cytoplasm of interphase cells [125]. This assay detects the genotoxic damage in interphase cells and it is also an alternative to chromosome aberration test. The evaluation of micronuclei can be counted faster, thanks to the ability of the assay to investigate cells during interphase. Micronuclei may be the result of aneugenic and clastogenic (chromosome breakage or whole chromosome) damage [129]. Li et al. [130] used 5 nm of Ag NPs to determine their genotoxicity via *in vitro* micronucleus assay. Frequency of micronucleus was increased by the Ag NP exposure and increase of micronucleus is dependent on dose of Ag NPs. At the concentration rate of 30 μg/ml (with 45.4% relative population doubling), Ag NPs induced a significant 3.17-fold increase with a net increase of 1.60% in micronucleus frequency over the vehicle control, a weak positive response by criteria of the study. These results showed that 5 nm of Ag NP are genotoxic on TK6 cells.

4.2. Nano-toxicity mechanism and *in vitro* toxic effects of Ag NPs

There are various types of nano-toxicity mechanisms which are suggested for Ag NPs. However, toxicity of this material is fundamentally associated with reactions such as the surface oxidation, Ag ion release and interaction between biological macromolecules and Ag NPs [131]. AshaRani et al. (2008) suggested that deformation of the mitochondrial respiratory chain via Ag NPs raised ROS generation, and interruption of ATP synthesis [101]. Thus, DNA was damaged due to this situation. Ag NPs can interact with membrane proteins and activate signaling pathways. Hence, they lead to inhibition of cell proliferation. It is also suggested that Ag NPs can uptake the cell via diffusion or endocytosis, and they may cause some disorders such as mitochondrial function disorder, generation of ROS, damaging of the proteins and nucleic acids and inhibition of cell proliferation [101]. Hsin et al. (2008) were studied about nano-toxicity mechanisms of Ag NPs in NIH3T3 fibroblast cells [132]. They have discovered that exposing Ag NPs induced the releasing of cytochrome C into the cytosol and increasing of translocation of Bax to the mitochondria. It is the fact that Ag NPs may induce apoptosis via the mitochondrial pathway while acting through ROS and C-Jun N-terminal kinase. In addition to this situation, interaction of Ag NPs with DNA can cause cell cycle

arrest at the G2/M phase [132, 133]. The antibacterial property of Ag NPs makes them lethal to bacteria, besides it makes nano-toxic effects on human cells. For instance, lethal concentration (LC) of Ag NPs for bacteria is also lethal for keratinocytes and fibroblasts [133]. AshaRani et al. (2009) have investigated the antiproliferative activity of Ag NPs and they proposed a mechanism of toxicity as shown in **Figure 2** [134]. Ag NPs can cause cell proliferation interacting with membrane proteins and activating signaling pathways [135]. Besides, the Ag NPs can enter into the cell via different ways such as diffusion and endocytosis. After entering into the cell, mitochondrial dysfunction and generation of ROS are occurred, proteins and nucleic acids inside the cell are damaged and finally, it results inhibition of cell proliferation [131].

Traditionally, easily ionized nanoparticles such as silver nanoparticles induce toxicity by a Trojan-horse type mechanism [72, 95]. Phagocytosis of Ag NPs stimulates inflammatory signaling via the generation of ROS in macrophage cells, following that the activated macrophage cells induced secretion of TNF-α. The increasing level of TNF-α leads to damage of cell membrane and apoptosis. All these results seemed to be caused by ionization of Ag NPs in cells which is expressed by a Trojan-horse type mechanism.

As a rule, the change of cell shape or morphology in a monolayer culture is the first and easily perceptible effect after exposing of toxic materials with cells. According to the microscopic observations, exposed cells with Ag NPs showed that significant morphological alterations which are hints of unhealthy cells, whereas control appear normal. In comparison to control group, the cells which were exposed with Ag NPs appeared to be clustered with a few cellular extensions and cell spreading patterns were limited. Those can be examined by deformations in cytoskeletal functions which are result of Ag NPs exposure [101].

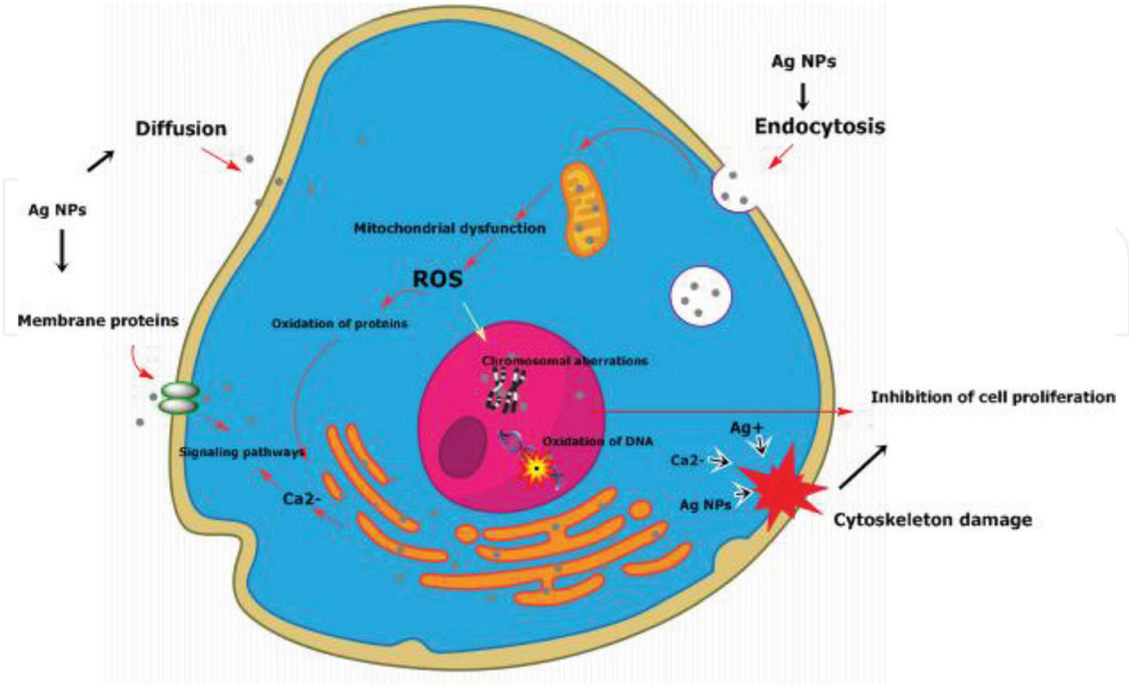

Figure 2. Proposed antiproliferative activity and nano-toxicity mechanism of Ag NPs.

Size effect of the Ag NPs is the one of the most important parameters which affects nano-toxicity. In a study using Ag NPs with different sizes (10, 40 and 100 nm), it was reported that all types of Ag NPs showed powerful cytotoxic activity at lower concentrations and they lead to overproduction of ROS at concentrations, which are lower than cytotoxic ones. Ag NPs, which are smaller than 10 nm, are the most toxic ones. According to this study, the nano-cytotoxicity of Ag NPs is related to production of ROS [136]. Another study suggested that the nano-toxicity potentials of Ag NPs which were coated similarly and had different sizes including 10, 20, 40, 60 and 80 nm were investigated on bacteria, yeast, algae, crustaceans and mammalian cells *in vitro*. According to the study, cells of *Daphnia magna* were the most sensitive cells to Ag NPs. *Pseudokirchneriella subcapitata, Escherichia coli*, *Pseudomonas fluorescens, Saccharomyces cerevisiae* and lastly mammalian fibroblast cells followed them, respectively. Also, researchers reported that as the size of particles is decreased, their toxic effect is increased. In addition, the toxic effect difference between 10 and 80 nm of Ag NPs was the biggest for *D. magna* and the smallest for mammalian fibroblast cells [137].

The shape of the Ag NPs is also another important parameter which affects nano-toxicity potentials of Ag NPs. In a study, differences between toxicity of Ag nano-spheres (30 nm) and nano-wires (length: 1.5–25 μm; diameter 100–160 nm) were investigated by using alveolar epithelial cells. In conclusion, the nano-wires had powerful impact on the alveolar epithelial cells, while the nano-spheres had no specific effect [60]. In another study, nano-toxic differences of Ag NPs which had nano-spheres (diameter 40–80 and 120–180 nm; two different samples), nano-platelets (20–60 nm), nano-cubes (140–180 nm) and nano-rods (diameter 80–120 nm, length > 1000 nm) were investigated. As the result of study, all NPs which exposed to human mesenchymal stem cells were cytotoxic at concentrations greater than 12.5 mg/ml. However, particle shape had no distinct cytotoxic effect toward the cells. On the other hand, the nano-toxicity against *Staphylococcus aureus* is increased by a higher dissolution rate and this situation was suggested that dissolved Ag ions were one of the toxic species against the bacteria. The particles, which had higher specific surface area, were more toxic against the bacteria in comparison to particles, which had lower specific surface area. The differences in the solution rate may be utilized to practice Ag NPs with a comparatively higher bacterial effect with a lower cytotoxic effect toward tissue [138].

Coating is another factor which affects nano-toxicity to the cells. Samberg et al. (2010) determined the nano-toxicity of Ag NPs using human epidermal keratinocytes *in vivo* and *in vitro* [81]. The cells were exposed to varied concentrations of uncoated and carbon coated NPs, individually. Viability of the cells which were exposed to uncoated Ag NPs decreased due to the doses. On the other hand, there was no toxic effect was observed in the cells treated with carbon coated Ag NPs [139]. In an *in vitro* study on yeast cells and lung cells (A549) showed that Ag NPs, which were coated with positively charged bPEI, were more toxic toward yeast cells in comparison to Ag NPs, which were coated with negatively charged citrate. Besides, the researchers determined that positively charged Ag nanoparticles (10 and 80 nm) adsorbed onto the surface of the yeast cell. In the lung cells, 10 nm of Ag NPs, which were coated with positively charged bPEI, were more toxic than Ag NPs, which were coated with negatively

http://dx.doi.org/10.5772/intechopen.75645

charged citrate. In addition, positively and negatively charged Ag NPs were adsorbed onto the cell surface of the lung epithelial cells [140].

5. *In silico* toxicological information and experiments about silver NPs

Determination of the toxicity of chemicals used as active substance in medicine is very important for the detection of harmful effects on people, animals, plants or environment. Although the animal models for toxicity determination have been used for a very long time but the long duration of these experiments, ethical issues, financial burden and animal damage make these models unfavorable. For this reason, computerized calculation methods have begun to gain attention for toxicological studies. *In silico* toxicology is a type of toxicity assessment method used to estimate the toxicity of chemicals, and through these computational method toxicities of chemicals are modeled, analyzed and identified. The computational methods, which are also complementary to *in vivo* and *in vitro* toxicity tests, aim to minimize the need for animal testing with the reliability of toxicity determination and reduce the cost and time. Another advantage of computational methods is that they can predict the toxicity of chemicals before further synthesis takes place [141].

The relationship between structure and toxicity has led to the creation of a new model called quantitative nanostructure-toxicity relationship (QNTR), which provides us with NPs and their toxic properties. In this model, the mathematical objects, which are called descriptors, are described. These descriptors must be computable sizes and they are related with some properties of NPs such as the chemical and structural properties, particle shape, size, surface area, ionization potential, formation heat, zeta potential and physicochemical properties of molecules that was attached to NPs surfaces. Subsequently, a subset of the identifiers associated with most biological properties (e.g. cell apoptosis, metabolism or signaling pathway modulation) is selected and modeled using mathematical techniques. In statistical modeling, neural networks are often used and a mathematical model that links the biologic activity and the identifiers is created. Finally, the robustness and adequacy of models are assessed and interpreted using statistical cross-validation techniques without anticipating the properties of new materials [142]. Although the determination of the *in vivo* effects of NPs via experiments is very laborious and difficult, it is possible to create fingerprints of NPs on the organisms, while estimating with obtained from the QNTR models. Use of molecular descriptors and *in vitro* assay results of NPs is also an effective method for the prediction *in vivo* toxicities of these materials [143]. Quantitative structure-activity relationship (QSAR) is a model that is used to estimate the toxicity of chemicals. QSAR modeling tools include statistical methods such as multiple linear regression, polynomial and kernel regression, as well as machine learning methods such as artificial neural networks and clustering methods like random forest and decision trees [144–146]. These methods have revealed that there is a mathematical relationship between the physicochemical or molecular properties of NPs and their biological activities. These associations are often very complex. However, thanks to the QSAR and QNTR methods, toxicity can be predicted for drugs, which is used on humans and animals, or chemicals, which is used in industry [147]. The obstacles that make it difficult to implement QSAR methods; insufficiency

of modeling the biological properties of NPs and experimental data on the bio-corona composition, and unpredictability of *in vivo* effects of NPs compared with *in vitro* studies.

Nano-QSAR is a QSAR method that is used as the descriptor of NPs such as size, surface area, solubility, protein corona, zeta potential, bio-distribution and shape. There are not so many researches about nano-QSAR method that was carried out with Ag NPs in the literature. Therefore, it can be helpful to look up the study which was conducted by Silva et al. [148]. In this research, nano-QSAR method was used for predicting the organo-coated Ag NPs such as citrate-coated, polyvinylpyrrolidone-coated and branched polyethyleneimine-coated. These NPs were applied to two model organisms, *E. coli* and *D. magna*, and nano-toxic potentials of Ag NPs were predicted. However, it is the fact that there is more study about computational predicting of nano-toxic effects of Ag NPs needed.

6. Conclusion

NPs are commonly used in many different areas such as technology, health, transportation, construction, information and communication. Ag NPs, which have antibacterial, antiviral and antimicrobial properties are used in many area and highly preferred compared to other NPs due to their physical properties, such as high biocompatibilities, unique electronic and catalytic properties. However, Ag NPs may appear to be potential risk to the environment. Size, shape, surface area and dose are the most important factors which affect the toxic potential. Toxicity of Ag NPs can be determined via *in vivo* and *in vitro* assays, and *in silico* models. *In vitro* toxicity assays are more sensitive and rapid than *in vivo* assays. However, *in vitro* toxicity assays can be contaminated by external factors such as microorganisms and different particulate matters. On the other hand, *in vivo* toxicity assays show more realistic results, but they take a long time. Besides, different doses unit such as ppm, mass per volume or mass per unit of NPs may be altered the result of toxicity assays. Therefore, *in silico* methods can be replaced to other methods in the future because of *in silico* methods are rapid and they predict the adverse effects of NPs, correctly as well as *in vivo* and *in vitro* assays. One of the main goals of the nanotoxicological studies is preventing animal sacrificing. Thus, *in silico* models can overcome this issue. However, it is disadvantage that use of computational methods for predicting to determine the toxic potentials of NPs is relatively new and their usage is quite restricted. Evaluation of complete toxicological profile of Ag NPs depends on the development of combined and strong nanotoxicological assays.

Author details

Yasemin Budama-Kilinc[1]*, Rabia Cakir-Koc[1], Tolga Zorlu[1], Burak Ozdemir[1], Zeynep Karavelioglu[1], Abdurrahim Can Egil[1] and Serda Kecel-Gunduz[2]

*Address all correspondence to: yaseminbudama@gmail.com

1 Yildiz Technical University, Bioengineering Department, Esenler, Istanbul, Turkey

2 Istanbul University, Physics Department, Vezneciler, Istanbul, Turkey

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