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Cytotoxicity Assessment of Plasmonic Nanoparticles: A Review Based on Published Literature

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***Abstract*: Plasmonic nanoparticles (Au-NP, Ag-NP) are of great interest due to their significant potential applications such as Surface Enhanced Raman Spectroscopy, Biological Imaging, Biosensors, or cancer treatment because of their visible and near-infrared surface plasmon resonance spectrum. However, the cytotoxicity and genotoxicity of these limit their potential for such applications. According to several previously reported literature both Au-NP and Ag-NP are handsomely capable of entering living cells and are notorious for their cytotoxicity. Studies show that both these NPs can inhibit cell growth and kill living cells through Oxidative stress and free ion generation. Moreover, Au-NPs can adsorb serum protein and go into cells through the endocytotic pathway, which is more complex and results in greater cytotoxicity with the immunological response than Ag-NPs [1]. Such cytotoxic behavior of these plasmonic nanoparticles is aimed to discuss in this review.**

***Keywords*: Ag-NP, Au-NP, Nanoparticles, Plasmonic NP, Toxicity.**

1. Background

Nanomaterials are referred to as materials with dimensions less than 100 nanometers in either of three directions. At that scale, with the increasing proportions of surface atoms, they show novel and unique properties as opposed to their bulk counterparts. These nanomaterials possess a wide range of tailorability, and they could be engineered to obtain certain properties by controlling the dimensions and parameters. They have found their way out to versatile fields such as energy harnessing from the sun [2]-[7], improving heat transfer[8]-[12], recycling[13] polymers[14], battery, filtration, composite materials[15]-[17], emission control, organics and chemical synthesis and purification, cancer treatments and so on. Many existing technologies have also been optimized by utilizing nanomaterials. While solar energy has been showing great promise for decades, by virtue of nanotechnology, it is much closer to realistic materialization.

Plasmonics is one such unique property that is found at the nanoscale. It's a rapidly growing research field that deals with metal nanoparticles (NPs) of various sizes, shapes, structures, and tunable plasmon resonances over the visible and near-infrared spectrums. Gold (Au) and Silver (Ag) offer the most interest among these NPs as they represent those spectral bands [18]-[20]. Plasmons are collective excitations of conductive electrons in metals. Based on the boundary conditions, classifications are made; bulk plasmons (3D plasma), surface propagating plasmons or surface plasmon polaritons (2D films) [20], and surface localized plasmons (nanoparticles) (Fig. 1). However, the bulk plasmons cannot be excited by visible light because of their longitudinal nature. The surface plasmon polaritons propagate along metal surfaces in a waveguide-like fashion [21].

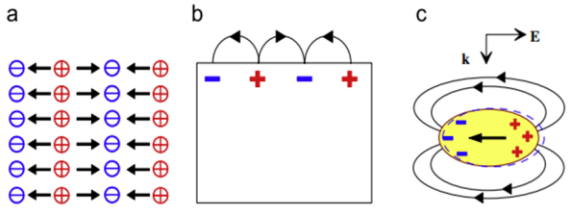

Fig. 1. Schematic of (a) surface propagating (b) and surface localized (c) plasmons. The dashed line shows the electron cloud displacement [21]

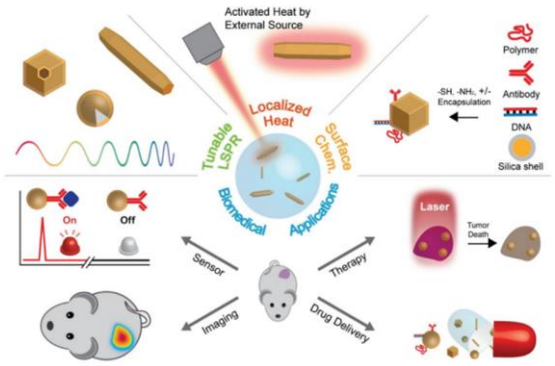

Fig. 1. Plasmonic nanoparticles and their biomedical applications [28]

Surface localized plasmons are of enormous interest due to their variety of bio-medical applications like biomedicine [22, 23], bio-sensing [24], bio-imaging [23], [25]-[27], bio-spectroscopy [26], cancer therapy and so on. For all these applications, nanoparticles must interact directly with biological organisms, tissues, and cells. Therefore, understanding their toxicity mechanism and assessment of cytotoxicity based on previous experimental results remains

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particularly important.

This review intends to explain the significance of plasmonic Au-NPs and Ag-NPs for several modern bio-applications and their related challenges, cytotoxicity, and genotoxicity. In addition, cellular uptake and toxicity generation mechanisms (oxidative stress, free ion generation) will also be discussed. Finally, results from previously reported literature will be presented for cytotoxicity assessment.

2. Cytotoxicity and Nanoparticles

Metallic NPs enter the cell via cell membrane by the process known as endocytosis. During endocytosis, the absorption of NPs occurs through membrane in-folding, budding, and pinching off to form endocytic vesicles later transported to specialized intracellular compartments.

Endocytosis is classified into several types depending on the molecules involved. The two major types of endocytosis are phagocytosis and pinocytosis. Pinocytosis can be divided into four subclasses depending on vesicle and protein size involved in their formation (clathrin-mediated endocytosis, caveolae-dependent endocytosis, clathrin/caveolae independent endocytosis, and macro-pinocytosis). Conversely, phagocytosis - takes place in particular phagocytes, pinocytosis is more predominant. Also, metallic nanoparticles enter the neurons and glial cells by endocytosis - including all its types, even phagocytosis [29].

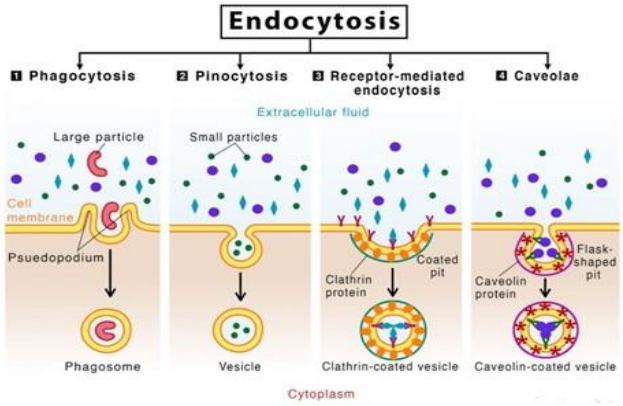

Fig. 2. Types of Endocytosis [30]

Additionally, metallic nanoparticles are among the most used engineered nanomaterials. Different research groups have extensively studied nanoparticles, such as Au-NP, Ag-NP, Cu-NP, Al-NP, Ni-NP, and Co-NP. These metal nanoparticles are significant industrial materials extensively used in drug delivery, cancer treatment, bio-imaging, MRI contrast enhancement, etc. Therefore, identifying common principles of toxic action could help classify the toxic NPs and predict toxicity. Generation of reactive oxygen and nitrogen species (ROS) and release of free ions are common mechanisms for metal and metal oxide NPs. Additionally, protein-nanoparticle interactions: both by changing their physicochemical parameters and as targets for cytotoxicity, play a significant role [31].

3. Reactive Oxygen Species (ROS)

ROS generation and oxidative stress production are predominant mechanisms leading to nanotoxicity, including DNA damage, unfettered cell signaling, changed cell motility, cytotoxicity, and cancer initiation and promotion [32]. The degree of ROS generation by NPs depends on the particles' chemical nature. Compared to their bulk-size counterparts, engineered NPs possess a small size, high specific surface area, and high surface reactivity, producing higher ROS levels and resulting in cytotoxicity and genotoxicity [31]. Reactive oxygen species are one or more oxygen atoms, which are more reactive than oxygen molecules. Some common ROS are superoxide, hydroperoxyl radical, hydroxyl radical, nitric oxide, and hydrogen peroxide. Some reactive nitrogen species are nitrogen dioxide and peroxynitrite, which are normally derived from superoxide and nitric oxide radical reactions. Cells produce ROS from enzymatic activity of myeloperoxidase and cytochrome P450 enzymes, from auto-oxidation of hemoglobin (also called Fenton reaction), oxidases, and flavoproteins in the peroxisomes, riboflavin and catecholamines, transient metals, and from the electron transport chain of the mitochondria. NPs increase ROS levels by reduction of ROS hunters; binding to these scavenger molecules, reacting at the NPs surface, contaminating, and influencing intracellular production (interaction of NPs with lysosomes and mitochondria) [33].

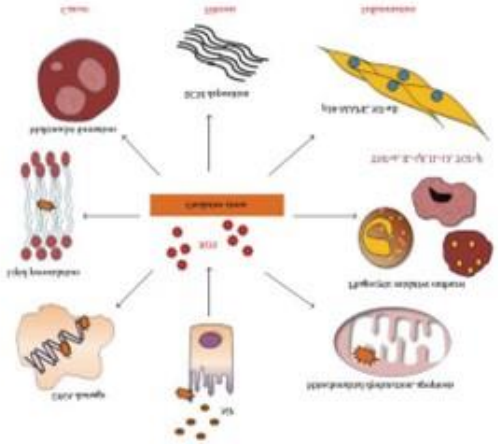

Fig. 3. Effect of ROS Generation [34]

4. Free Metal Ions

For several metal NPs, cytotoxicity cannot be accredited to ROS generation exclusively. The NPs act mostly by free ions. NPs are dissolved and intracellular concentrations can rise higher than the respective IC50 values for the free ions. The discharge of metal ions also influences gene transcription.

For Instance, in the presence of water Ag^+ can be released from the surface of nano-Ag by surface oxidation. The ion release rate depends upon the size of the nano-Ag particles, sulfur concentrations contained in the nano-Ag, temperature, O_2, pH, and light. Later the reaction of Ag^+ ions with molecular oxygen produces superoxide radicals with other ROS - resulting in apoptosis and the expression of stress-response-related genes [35].

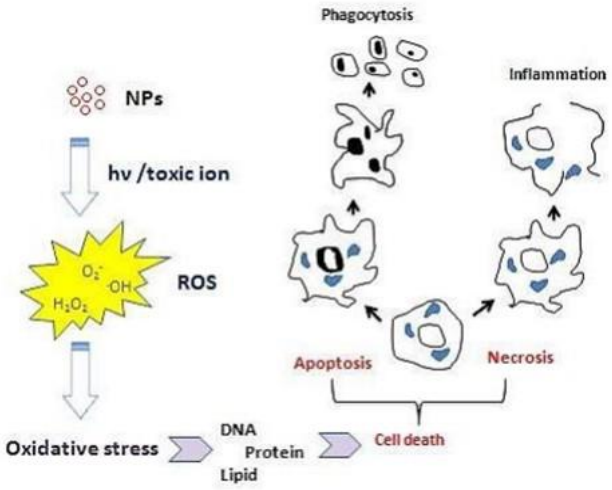

Fig. 4. Mechanism of ROS Generation [31]

5. Plasmonic Nanoparticles

Although few metal NPs exhibit surface plasmon resonances (SPR), Gold and silver are always idiosyncratic when compared due to visible plasmonic spectrum, high resonance intensity, and better biocompatibility. Therefore, most of the bio-applications are based on Au-NPs and Ag-NPs, making them significant for risk assessment. In this section, the cytotoxic behavior of both these nanoparticles is discussed. However, the discussion point of view will be neutral – both positive and negative sides of cytotoxicity are added.

6. Cytotoxicity of Au-NP

Luo et. al. (2011) developed a smart drug carrier, an aptamer (ap)/hairpin (hp) DNA - gold nanoparticle (apt/hp-Au NP) conjugate for targeted delivery of drugs. The DNA aptamer sgc8c, was assembled onto the Au NP surface. The repeated d (CGATCG) sequence within the hp-DNA on the Au NP surface was used to load the anticancer drug doxorubicin (Dox). After optimization, Dox molecules were successfully loaded onto the surface of AuNP (13 nm). The binding capability of apt/hp-Au NP conjugates toward targeted cells was examined by flow-cytometry and atomic absorption spectroscopy, which showed that the aptamer-functionalized nano-conjugates were selective for targeting cancer cells.

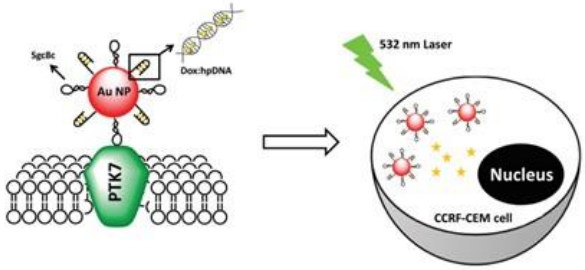

Fig. 5. Light-induced Dox release from Dox:apt/hp-Au NP nanocomplexes inside targeted cancer cells [36]

Cell toxicity assay also demonstrated that these drug-loaded nano-conjugates could kill targeted cancer cells more effectively than non-targeted (control) cells. Most importantly, when illuminated with plasmon-resonant light (532 nm), Dox: nano-conjugates exhibited higher antitumor efficacy with few side effects. The authors also marked the releasing of Dox from these nano-conjugates in living cells which was examined by collective fluorescence signals upon light exposure [36].

Chueh et al. (2013) described the impacts of Au-NPs in different mammalian cell models using an automatic and dye-free method for continuous checking of cell growth based on the measurement of cell impedance. Numerous well-established cytotoxicity assays were used for comparison. Au-NPs prompted a concentration-dependent reduction in cell growth. This inhibitory upshot was linked with apoptosis induction in Vero cells but not in MRC-5 or NIH3T3 cells. Excitingly, cDNA microarray studies in MRC-5 cells supported the association of DNA damage and repair responses, cell-cycle regulation, and oxidative stress in Au-NP induced cytotoxicity and genotoxicity. Moreover, autophagy played a role in Au-NPs-induced reduction of cell growth in NIH3T3 cells [37].

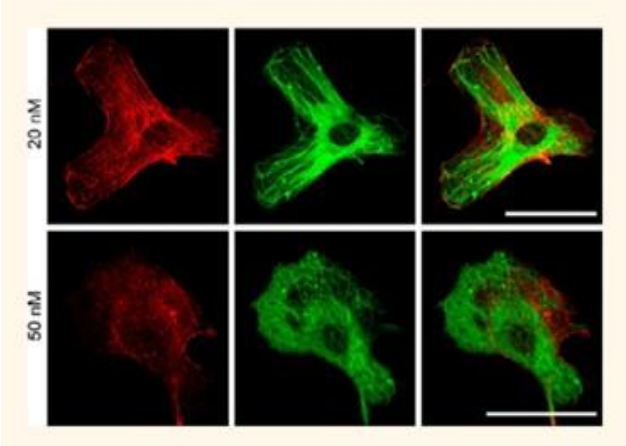

Fig. 6. Reduced Cell Growth at Higher NPs Concentration [38]

Soenen et al. (2012) evaluated the effects of poly-methacrylic acid-coated 4 nm diameter Au NPs on an assortment of sensitive cell types (C17.2 neural progenitor cells, human umbilical vein endothelial cells, and PC12 rat pheochromocytoma cells) using a multi-parametric approach. Using various NP concentrations and incubation times, the authors performed a stepwise examination of the NP effects on cell viability, reactive oxygen species, cell morphology, cytoskeleton architecture, and cell functionality. Their data showed a reduction in cell viability at higher NP concentrations (200 nm) primarily through induction of reactive oxygen species; significantly induced at concentrations of 50 nm Au NPs or higher. The NPs significantly obstructed neurite outgrowth of PC12 cells up to 20 nm concentrations. However, at 10 nM, they observed no significant effects on any cellular parameter [38].

Kong et al. (2008) studied cell uptake and radiation cytotoxicity enhancement in breast-cancer cells. The researchers found that cancer cells take up thioglucose functionalized gold nanoparticles (GNPs) significantly more than naked GNPs. Their results also showed that GNPs significantly increased the cytotoxicity of X-rays. The authors

listed three benefits to such an approach: firstly, compared to naked GNPs, localized delivery produces a higher local concentration of GNPs in target locations; Secondly, GNPs can increase the cytotoxicity of radiation. Therefore, lower doses of radiation can be used, avoiding the risk of side effects; and finally, local tissue damage surrounding the cancer is decreased [39].

However, Connor et al. (2005) found interesting results when they examined a series of gold nanoparticles for uptake and acute toxicity investigation in human leukemia cells. The results showed that though some precursors of nanoparticles may be toxic, the nanoparticles themselves are not necessarily detrimental to cellular function. Nevertheless, the authors suggested that the results only apply to short-term exposure [40].

7. Cytotoxicity of Ag-NP

Austin et al. (2011) exploited the plasmonic scattering property and the ability of nuclear-targeted silver nanoparticles (NLS/RGD-AgNPs) to incite programmed cell death to the image in real-time behavior of human oral squamous carcinoma (HSC-3) cell communities during and after the induction of apoptosis. Plasmonic live-cell imaging exposed that HSC-3 cells behave as amateur phagocytes. The induction of apoptosis in some cells is directed to the attraction of and their subsequent engulfment by adjoining cells. The grouping of the cellular community causes attraction to apoptotic cells. Cell imaging also exposed that, as the initial concentration of NLS/RGD-AgNPs increases, the rate of self-killing upturns and the degree of grouping decreases [27].

However, Song et al. (2013) exposed Ag-NP to Caco-2 cells. They concluded that Caco-2 cells treated with Ag-NPs showed zero cellular oxidative damage. Moreover, they found cells' antioxidant capacity increased and reached the utmost level when the concentration of Ag-NPs was 50 micro-g/mL. Based on this, they termed Ag-NPs as safe antibacterial material [41].

Sierra et al. (2011) exposed human periodontal tissue to 0–1,000 μM silver nanoparticles of each size for 24, 72, and 168-hour periods. Then evaluated their cytotoxicity with a nonradioactive, soluble MTS/PMS assay. The authors found that silver nanoparticles sizing less than 20 nm increased cytotoxicity in human periodontal fibroblasts in a dose- and time-dependent manner. However, the 80–100-nm-sized nanoparticles never changed the viability of human primary culture cells [42].

Corroborating results were observed by Katsumiti et al. (2015). Maltose-stabilized Ag NPs showed size-dependent cytotoxicity in their experiment - smaller (20 nm) NPs being more toxic than larger (40 and 100 nm) NPs [43].

Park et al. (2011) reported size-dependent cytotoxicity of silver nanoparticles. Effects of silver nanoparticles of increasing sizes (20, 80, 113 nm) were compared in in-vitro assays for cytotoxicity, inflammation, genotoxicity, and developmental toxicity. The authors observed that silver nanoparticles most pronouncedly affect the cellular metabolic activity and membrane damage. Nanoparticles of 20 nm size were found to be the most toxic. Their results also suggested that the most prominent effect of silver nanoparticles is causing damage to a range of different cell types, potentially resulting in a myriad of secondary effects, such as generation of ROS, DNA damage, and inhibiting stem cell differentiation. Finally, the authors concluded that the influence of silver nanoparticles to induce cell damage is cell type and particle size dependent [44].

AshaRani et al. (2009) evaluated the toxicity of Ag-NPs using changes in cell morphology, cell viability, metabolic activity, and oxidative stress. The authors suggested that Ag-NP reduced the ATP content of the cell, caused damage to mitochondria, and increased the production of reactive oxygen species (ROS) in a dose-dependent manner. As measured by single cell gel electrophoresis (SCGE) and cytokinesis blocked micronucleus assay (CBMN), DNA damage was also dose-dependent and more projecting in the cancer cells. Further analysis indicated that the presence of Ag-NP inside the mitochondria and nucleus is directly involved in mitochondrial toxicity and DNA damage. Authors proposed a possible mechanism that involves disruption of the mitochondrial respiratory chain by Ag-NP, leading to the production of ROS and interruption of ATP synthesis, which in turn cause DNA damage [45].

Foldbjerg et al. (2010) investigated the effects of well-characterized PVP-coated Ag NPs and silver ions (Ag^+) in the human alveolar cell line, A549. Dose-dependent cellular toxicity caused by Ag NPs and Ag^+ was demonstrated by atomic absorption spectroscopy and flow cytometry. They detected DNA damage induced by ROS in bulky DNA adducts. Their results also suggested Ag NPs as a mediator of ROS-induced genotoxicity [46].

Table 1
Comparative cytotoxicity of Au-NP and Ag-NP

NP	Size (nm)	Concentration	Duration of Exposure	Comment	Ref.
Au	12.5	40, 200, and 400 μg/kg/day	Daily for 8 days	Au NPs crossed blood brain barrier and accumulated in the neural tissue; yet no evidence of toxicity.	[47]
Au	10/30	70 μg/kg /day	Daily for 8 days	Inhibition of catalase activity and energy metabolism was observed in the hippocampus, striatum and cerebral cortex.	[48]
Ag	10	1, 3, 6 μg/ml	24h/48h	Ag NPs induced of ROS and NO production in astrocytes and microglia: trigger apoptosis of neurons	[49]
Ag	20	1, 5, 10, 50 μg/ml	2-3 days	Ag NPs induced toxicity in neurons: expressed as degradation of cytoskeleton components, perturbations of pre- and post-synaptic proteins expression, and mitochondrial dysfunction.	[50]
Ag	20/100	6.25, 12.5, 25, 50, 100 μg/ml	24 h	Higher cell susceptibility to the cytotoxic effects of smaller Ag-NPs.	[51]

8. Conclusion

Both Au-NP and Ag-NP possess a handsome amount of cytotoxic behavior. This cytotoxicity is driven mainly by the generation of reactive oxygen species (ROS) and free ion generation, which may even lead to DNA damage - genotoxicity. However, cytotoxicity has both positive and negative aspects. For Instance, it can be employed to kill malignant cancer cells. But negative aspects must be considered seriously while implementing applications like bio-imaging, bio-sensing, or drug delivery. For the latter case, concentration, size, shape, and purity must be defined precisely to engineer their cytotoxic nature. Additionally, comprehensive research must be conducted to determine these nanoparticles' concentration, size, shape, and purity-based cytotoxicity for precise cytotoxic information and data collection.

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