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The toxicity of Gold Nanoparticles in relation to their physiochemical properties.

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Abstract

The rapid emergence of gold nanoparticles (AuNPs) technology holds great promise for future applications due to their large volume specific surface areas with high diverse surface activities than bulk gold. These properties have made AuNPs of great importance in the development of excellent nanoelectronic chips, promising vehicle for a wide range of biomedical and environmental applications. However, the huge impact arising from the physiochemical properties has given rise to new concerns for future health status. Currently, there is dearth information on AuNPs health effects and no regulatory safety and guidelines relating their properties to toxicities. This review, therefore, focuses on the potential toxicological aspect of AuNPs experienced so far and their interactions with biological systems. These can be applied as measures to improve their biomedical applications and risk assessment. However, assessing the safety issues of nanoparticles is quite challenging, because of the vast physiochemical properties that confound their biomedical and toxicological profiles. Therefore more research with standardized NPs physicochemical properties is needed based on the different types of AuNPs to establish both *in vitro* and *in vivo* nanotoxicities. The establishment of each size with specific ligand properties will update the complex conflicting ideas emanating from the different AuNPs safety studies thereof.

Keywords: Gold Nanoparticles, Physiochemical Properties, Cells interaction, Toxicity

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Introduction

Nanotechnology is the study of matter at the atomic molecular level with attention focused from 1 to 100 nm diameter nanoscale size [1-3], Other scientist envisage it in terms of its volume specific surface area (VSSA) greater than 60 m^2/cm^3, reflecting the critical importance of surface reactivity of nanomaterials rather than size [4]. Nanomaterials especially engineered gold nanomaterials, hold great promises for future applications due to it large VSSA thereby amplifying their electrical, chemical, mechanical, thermal and optical properties [5-6] that differ from bulk gold. Bulk gold is considered bio-inert, a property found only at the macroscopic level, but at nanoscale size, gold exhibit different properties due to its surface plasmon resonance excitation characteristics [7-9]. Gold nanomaterials are currently used to enhance solar cells [10] and as liquid crystal that acts as flash memory devices [11]. They also have extensive potential biomedical applications in drug delivery, gene therapy, photothermal and radio-therapy, biosensing as well as contrast agents for cancer, diagnostic tracers, immobilization of enzymes and cell imaging [1,6, 12-15]. Other uses include water and hydrogen purification, pollution control and as catalysts in carbon monoxide oxidation [16-19]. However, despite their huge potential benefits in the realm of environmental, biomedical and industrial applications, very little is known about the short and long term health effects in organisms and the environment. Reports show that synthesized NPs can circulate in the body for extended periods of time without being rejected by the body's immune system. All these behaviours are guided by the small size, shape and surface charges. This is of concern because during syntheses and applications, gold nanoparticles (AuNPs) of various sizes, shapes and surface charges are generated that may be of health risk.

Currently, there are very limited data and no safety and regulatory guidelines concerning the manufacture and application of nanomaterials. This review, therefore, focuses on the properties of gold nanomaterials and their interactions with biological systems. This will provide information on how AuNPs physicochemical properties, including those of attached functional groups influence cellular responses both *in vitro* and *in vivo*.

Literature Search Information.

Data for the current study were obtained from various indexing journal sites such as PubMed, Medline, Embase, Global Health, SCOPUS/Elsevier, Web of Science, Springer, Langmuir, Google Scholar, Scientific and peer-reviewed reports, conference proceedings published in English. With the search terms as "gold nanoparticles, production, synthesis, biomimetic synthesis of gold nanoparticles, toxicity, uses of gold nanoparticles, functionalization of gold nanoparticles, types and shapes of gold nanoparticles, toxicity of spherical and rod nanoparticles, toxicity of biomaterials of gold nanoparticles, gold nanoparticles ligands, effect of gold nanoparticle aspect ratio on its toxicity' toxicity of chemically synthesized gold nanoparticle, biologically synthesized gold nanoparticles, effect of gold nanoparticles on cells. Others include toxicity of gold nanoparticles biconjugates, bioaccumulation of aggregation and agglomeration of gold nanoparticles, cellular toxicity of gold nanoparticles'

Synthesis of Gold nanoparticles.

In general, AuNPs are synthesized by the chemical reduction of chloroauric acid ($HAuCl_4$) using various reducing agents [20- 25]. The reduction process causes Au^{3+} to be reduced to neutral gold atoms which further become supersaturated and precipitated as more gold atoms aggregate to form sub-nanogold particles [19]. There are several methods involved in the syntheses of AuNPs, with $HAuCl_4$ as the main source of the gold atoms [22]. These methods include the following modifications (1) The Turkevich method which produces monodispersed spherical AuNPs suspended in water with citrate ions acting as both reducing and capping agents [26]. (2) The Brust method that produces AuNPs in organic liquids which are normally not miscible with water [27]. (3) The Perrault method which uses hydroquinone to reduce $HAuCl_4$ in an aqueous solution to produce AuNP seeds [28]. (4) The Martin method which generates "naked" monodisperse AuNPs in water due to the reduction of $HAuCl_4$ by sodium boron tetrathydride ($NaBH_4$) [29]. (5) The Sonolysis process that produces AuNPs based on ultrasounds, reacting in an aqueous solution of $HAuCl_4$ in glucose using hydroxyl and sugar pyrolysis radicals as reducing agents [30]. (6) Other friendly and cheaper methods of AuNPs synthesis include the use of biological agents such as microbial enzymes, plant phytochemicals or microorganisms such as bacteria and yeast cells [31-32]. For example chickpea leaf reduces 0.1mM HAuCl4 solution to AuNPs at room temperature as well as capping the AuNPs from aggregating [33]. *Escherichia coli* K12 have a fantastical behaviour to biosynthesis AuNPs at room temperature without the addition of growth media, pH adjustments or the inclusion of electron donors and stabilizing agents [34]. This biomimetic processes have revolutionized the nanotechnology field entirely.

The role played by microbial systems in AuNPs synthesis is vital because of their natural ability and mechanism to detoxified metallic gold ions through the reduction process either extracellularly or intracellularly as opposed to chemical synthesis of AuNPs (34-35]. Therefore are more environmentally friendly to exposed gold nanomaterial than chemical synthesis [34,36]. However, microbial and biological synthesis suffers from poor mono-dispersity, random aggregation, non-uniform shapes scale up as compared to chemical synthesis. [36]. Therefore production efficiency and specificity of AuNPs using biological processes is poor and improvement in the design and production of AuNPs by biomimetic technique is needed.

Currently, these methods are being modified to produce AuNPs of various sizes using various reducing agents [22,31, 34,37]. The synthesized AuNPs differ in several forms with some existing as branched nanocrystals of varying shapes i.e. monopod, bipod, tripod or tetrapod structures [38]. After production, these different forms are stabilized from aggregation and agglomeration with organic ligands such as peptides, proteins, fungus [39] and polymers such as polyethylene glycol (PEG) [23,40,41]. Furthermore, these different forms of ligands stabilized AuNPs can be modified by attaching other functional groups based on the application of choice [20,40].

The size of AuNPs in terms of the proportion of width to height that is the aspect ratio (AR) in relation to toxicity is still debatable. Different AR of AuNPs gives different plasmon bands and wavelengths that equally exhibit different colours [42-43]. For example the study by Zhang et al [44] showed that various encapsulated AuNPs surface charges, sizes and shapes to human HEp-2 and canine MDCK cells exhibit different cytotoxic effects. However, the differences were exhibited by the shapes, where CTAB encapsulated gold nanorods (AuNRs) were relatively higher in cytotoxicity than citrate stabilized gold

nano-spheres. Within the AuNRs there was no significant difference between the AuNRs different ARs [44] but increasing AR of AuNPs are difficult in cells uptake than those with lower AR [44-46]. Currently the clearance findings of AuNPs AR by phagocytic cells are still under investigation because of the encompassed complexity exhibited the physiochemical properties of both the AuNPs and their bio-conjugates cellularly. Studies by Qiu et al [47] have shown that cellular uptake is highly dependent on the AR and functional groups attached because ligands such as CTAB can equally enter cells with or without AuNPs, destroy mitochondria, and induce apoptosis [47]. The cationic PDDAC-coated AuNPs with an AR of 4 have been shown to possess both an insignificant toxicity with high cellular uptake, showing excellent photothermal therapeutic properties [47]. The method of AuNPs synthesis also affects toxicity [42-43], however AR in terms of cellular toxicity should be explained with caution because of the dearth information available in terms of AuNPs toxicological considerations. Therefore more systematic AuNPS toxicity studies are essential to decide the role of AR properties relative to cellular responses.

Routes of exposures to Gold Nanoparticles.

Exposure to AuNPs can occur during development, synthesis, and applications by direct injection or ingestion into the system, and waste disposal [48-49]. Exposure can also arise from AuNP-composite bound to consumer products in markets, homes and outdoor activities [50]. Such exposes can account for their accumulation in the soil, water bodies and environment. Other main potential routes of exposure include inhalation, absorption through skin contact and release from implants [49, 51-52]. Furthermore, the approval of AuNPs for various biomedical applications by the Food and Drug Administration (FDA) has led to increased applications as drug carriers, cancer therapy and biological applications [51, 53-55].

Other mode of AuNPs exposures include airborne and surface materials adherence which sometimes are difficult to detect. They can therefore, persist and bioaccumulate in such environment making them readily to translocate into the food chain thereby influencing both biotic and abiotic processes [56]. This enhances the uptake of AuNPs by other environmental organisms such as algae and fish which can further be consumed by animals and humans.

The effect of size and shape of Gold nanoparticles toxicity.

Synthesized AuNPs come in a variety of sizes and shapes ranging from 1 nm to 500 nm: some as rods, spheres, tubes, wires, ribbons, plate, cubic, hexagonal, triangular, tetrapods, etc [38, 57]. The small size and their 'needle-like' penetrating ability into cells have also made AuNPs excellent carriers in biomedical and molecular biology techniques [58]. This needle like feature as reported by De Jong et al [53] have ease the absorption, penetration, circulation and distribution of AuNPs in bio-systems as a size dependent factor. These findings were similar to those earlier reported by Connor et al [59] who found that AuNPs of approximately 18 nm in diameter could penetrate the cells without cell injury and toxicity. A study by Tsoli et al [60] also demonstrated that AuNPs of approximately 1 nm in diameter could penetrate the cell and nuclear membranes and attach to DNA without cell injury and cell death. The mechanism of entry into cells without cell injury has not been elucidated, but it seems the small nanosize plays a major role. The small size of the AuNPs therefore, facilitates their incorporation into biological systems for subsequent probing and modification [61]. These unique features of AuNPs have led them to various chemical properties transducing into dissimilar cellular studies where some are reported either as toxic or non toxic. Some display size dependent toxicity due to the presence of coated surface ligands [5,62-63], while others because of their large surface area to volume ratio provide platforms for increase surface particle activity [54]. This therefore, contributes an easy flexible pathway of penetration and reactivity in biological system than bulk gold material.

In terms of size, De Jong et al [53] found that 10 nm AuNP when administered to experimental animals can circulate more within 24h than other sizes. The mechanism of this 10 nm AuNP widespread has not been elucidated. Apart from the fact that AuNPs circulations in the system are highly size dependent, earlier findings by Hauck et al [64] also showed that other sizes such 50 nm AuNPs when exposed within 30 min can be the most abundant cellular AuNPs in the a system.

The interactions of AuNPs with biological systems are often related to their physiochemical characteristics which enable them to be internalized within cells, a situation which is not possible for larger particles. This is one of the reasons why AuNPs may be toxic than larger particles when compared on a mass dosage. This emphasizes lies in the importance of their dimension, the large surface area to volume ratio which enables them applicable in biomedical systems [65,66].

Table 1. *Summary of some selected in vitro gold nanoparticles cytotoxicity studies.*

Type of cell line	Size of AuNP	Dose	Shape, Surface group	Type of Test	Biological effect	\Ref
Human leukemia cells (K562 leukemia cell line)	≈4, 12 or 18 nm in diameters	25mM-250mM	Citrate coated	MTT assay	Non toxic to K562 cells	[59]
Mytilus edulis	750 ppb gold-citrate nanoparticles 1, ~13 nm,	1 mM	Citrate coated	Oxidative stress, Catalase activity, neutral red retention time assay, 2DE gels	AuNPs induced oxidative Stress in bivalves, especially in digestive gland	[73]
Human dermal fibroblast-fetal	10-50 nm	10, 50, 100, 200, 300μM	Spherically citrate coated	MTT	20 nm were non toxic even at 300 μM	[74]
Healthy volunteer blood specimens	30-50 nm	0.450 & 0.420 mg/mL	Colloids citrate-stabilized	2D PAGE, AFM, DLS, and TEM	69 different proteins bound to the surface of AuNPs. No detectable platelet aggregation, Change in plasma coagulation time, and complement activation.	[75]
The human umbilical vein endothelial (ECV-304) cells	100 nm in diameter	20 μL	Spherically bare and PCL-coated gold particles	Microtubule staining	The AuNPs were found in the endosomes or lysosomes, cytoplasm, nucleic envelope, and nucleus. Bare were slightly toxic while PCL coated had no effect	[76]
Human hepatocellular carcinoma HepG2 cells	25±3.5 nm	1.0 nmol /L AuNP, 1.2_mol/L Paclitaxel (T)	Spherical particles	MTT, quartz crystal microbalance (QCM) and flow cytometer assay	AuNPs show low cytotocxicity but can disrupt adhesion and enhance apoptosis of HepG2 cell. Paclitaxel plus AuNP inhibits the growth of HepG2 cell more effectively than Paclitaxel alone	[77]
Human skin cell line HaCaT keratinocytes	1.5 nm diameter	10 μl	Spherically and nanorods CTAB coated	MTT	Spherical AuNPs were non toxic. AuNP Nanorods were highly toxic due to presence of CTAB coat layer used for the synthesis of nanorods.	[78]
Human prostate carcinoma PC-3 cells	30-90 nm diameter	1.5nM	Spherical AuNPs	MTT and LDH assay	No LDH leakage observed up to 34 nM. Plain spherical 50 and 90 nm in diameter induced the proliferation of PC cells	[79]
Pancreatic carcinoma cell line (EGFR-1, Panc-1, and Cama-1)	20-nm spherical	100-nmol	Spherical L cetuximab-conjugated AuNPs (L C225-AuNP)	Flow cytometry	Panc-1 had viability of 46% ± 12%, Cama-1 cell had a viability of 92% ± 2%	[80]
Optical cells	20 nm	2 mM	protein-coated	Optical images	AuNP was found to disrupt the mixed phospholipid/cholesterol monolayer	[81]
MRC-5 human lung fibroblasts	20 nm in diameter	1nM	FBS coated AuNPs	Oxidative stress PCR array, Lipid hydroperoxide assay, Western blotting	Oxidative damage, induced upregulation of antioxidants, stress response genes and protein expression	[70]
Mammary adenocarcinoma (SKBR3), Human leukemia cells (HL60)	Length= 44.8±2.8 and 41.8± 3.3 Width = 18.5 ±1.6 and 11.7 ±1.4 respectively	20ul	PEG coated nanorod and PSS-coated nanorods	MTS assay	PEGylated particles did not induce toxicity to all the cells tested. PEGylated gold nanorods also exhibited better dispersion stability, PSS-coated rods tended to flocculate or cluster with induced toxicity	[82]
A549 cells, a human alveolar epithelial-like cell	15 nm	200-2000 μg	AuNPs	Real-time PCR, ELISA	No adverse effects from AuNPs were observed. No induction of oxidative stress markers and inflammatory cytokines	[83]
Human Sperm	9 nm AuNPs +	500 μL	AuNPs	media	The AuNPs penetrated the sperm cells head and tails. 25% of the sperm became non motile as compared to 95% control	[84]

Table 2. *Summary of some selected in vivo gold nanoparticles toxicity studies.*

Type exposed organism	Size of AuNP	Route of exposure	Dose	Surface coating	Test	Biological Effect	Ref
Male WU Wistar-derived rats	10, 50, 100 and 250 nm,	Intravenously	One ml	Spherical AuNPs	Organ Index	The AUNPs were found in the liver and spleen. 10 nm was present in all blood, liver, spleen, kidney, testis, thymus, heart, lung and brain. Larger particles were only detected in blood, liver and spleen.	[53]
Female mice	2, 40 and 100 nm	Intratracheal	1.4-1.6 mg/kg	Negatively charged monodisperse and spherical AuNPs-	Organ index (liver)	AuNPs found in the liver and macrophages	[88]
Mice	13.5 nm in diameter	Oral, intraperitoneal routes and the tail vein intravenous injection	137.5–2200 µg/kg	Spherically and citrate-coated	Animal survival, weight, hematology, morphology, and organ index	High AuNPs induced decrease in body weight, red blood cells. No effect at low level. Oral admin caused a significant decrease in body weight, spleen index, and red blood cells.	[14]
BALB/C Mice	Naked 3 to 100 nm	Intraperitoneal	8 mg/kg/week	Naked colloids AuNPs	Physical and behavioral examination.	AuNPs of 8,12,17, 37 nm induced fatigue,loss of appetite, change of fur color, and weight loss. Most died within 21 days. 3-5 nm did not induce sickness.	[68]
Male Wistar rats	20 nm	Tail-vein intravenous injection	0.2-mL (0.01 mg/kg)	AuNPs	Organ index	AuNPs accumulated and persisted in the liver and spleen than other organs. Many up and down regulated genes were expressed.	[37]
Male Mice C57/BL6	12.5 ± 1.7 nm	Intraperitoneal	40–400 µg/kg/day	Colloids citrate coated AuNPs	Animal survival, weight, hematology, morphology, and organ index	The AuNPs were able to cross the brain barrier and accumulate in neural tissues but No toxicity was evident. But there was uptake in the spleen, kidney and liver	[87]
Rat	5 nm	Tail vein intravenous and intratracheal	570–870 µg/kg	PEG Coated AuNPs	Organ index	PEG Au NPs accumulated mostly in liver and spleen.	[90]
Male CD1 mice	15±1 nm	Tail vein intravenous injection, scanned with the eXplore Optix to observe the distribution pattern (brain uptake)	150–200 µL	Human serum albumin (HSA), polyallylamine hydrochloride, polystyrene-4-sulfonate coated AuNPs	Organ index	Functionalized AuNPs accumulate in the hippocampus, thalamus, hypothalamus, and the cerebral cortex.	[91]
BALBc/c AnNHsd female mice	2.5±1.0nm	Subcutaneous injection	200µl	PEG-TMPC coated	Organ index	100% survival at all the different concentrations of PEG-TMPC and TMPC. Particles present in the organs but TMPC is not suitable for in vivo studies	[92]

Other studies of size-dependent cytotoxicity have been demonstrated in triphenylphosphine stabilised AuNPs using four cell lines such as tissue fibroblasts (L929), epithelial cells (HeLa), macrophages (J774A1) and melanoma cells (SK-Mel-28) [67]. Data obtained from these studies shown that cellular response is size dependent. For example 1.4 nm AuNP was observed as the most toxic responsible for rapid cell death by necrosis [67] as compare to 15 nm which was shown to be non-toxic [68]. This suggests that "larger" NPs are non-toxic *in vitro*. Furthermore, other *in vitro* studies on AuNPs of 20 and 100 nm in diameters have been shown to have no apparent effect on viability of human retina microvascular endothelial cells [69]. Although some studies have shown AuNPs not having an effect on cell viability, it is important to note that genotoxicity can occur without cytotoxicity and may result in genetic damage and transcription alterations which are not phenotypically expressed. A study on the effect of 5nm to 20 nm AuNPs on MRC-5 human fetal lung fibroblast cells have showed no influence on the viability of MRC-5 treated cells [70-71]. However, cell proliferation was inhibited which was

linked to downregulation of cell cycle genes. More so, oxidative DNA damage has been observed in conjunction with a downregulation of DNA repairs [71]. Furthermore, other reports have revealed that AuNPs of 2-4 nm, 5-7 nm and 20-40 nm are non-toxic to MRC-5 cells however when they were ≥ 10 ppm induced apoptosis and up-regulated the expressions of pro-inflammatory genes interlukin-1 (IL-1), interlukin-6 (IL-6) and tumor necrosis factor (TNF-alpha) [72]. Table 1 summaries some AuNPs *in vitro* toxicity studies that examined size, type of cell culture and their biological effects.

The effect of AuNPs shows that the smaller the AuNP the higher the probability of it to cause toxicity as well as bind easily on cellular surfaces. For example 1.4 nm AuNP in diameter was found to bind with DNA and affect genes (mutation) as comparable to their larger ccounterparts.

Some studies have shown that AuNPs between 30 and 110 nm when exposed to rats for up to 15 days, can accumulate in the lungs, olfactory bulb, spleen, oesophagus, tongue, kidney, aorta, heart, septum and blood. This was quantified by means of inductively coupled plasma mass spectrometry (ICP-MS) [85]. The results obtained so far were similar to those earlier reported by Takenaka et al [86] where engineered gold nanomaterials of 5-8 nm were found retained in rat's lungs before translocating into other tissues. These were also in agreement with those reported by Lasagna-Reeves et al [87] who used ICP-MS and GF-AAS to determine a significant amount of AuNPs in the liver, blood, brain, kidney, spleen, and lungs. Sadauskas et al [88] also demonstrated the amount of 2, 40 and 100 nm AuNPs in mice with similar tools with the liver as site of huge bioaccumulation, with only a small fraction translocating into the blood circulation and macrophage endosomes. The 2 nm AuNPs were further found to be the most translocated particles within the liver cells. The *in vivo* behavioural activities of these particles were due to their large surface area per unit mass. This shows that AuNPs bio-distribution vary with different sizes due to the availability of atoms ready to take part in various chemical reactions.

The mechanisms of biodistribution of AuNPs so far described are via endocytotic-exocytotic activity and to a lesser extend by paracellular transport (transport of molecules around cells and via tight junctions of epithelial cells) [88-89]. Such mechanisms are due to differences in the surface properties of the AuNPs, the type of animals used and the route of exposures. Table 2 summaries some of the *in vivo* AuNPs toxicity studies which examine the size, route of exposure and biological effect. Gosens et al [93] believes that single AuNPs can pose greater health effects than their agglomerates and aggregates counter parts. Because of the agglomerates and aggregates relative larger sizes, they are restricted from translocating easily across membranes as compared to single nanogold particles. Although, when Gosens et al [93] intratracheally instilled AuNPs agglomerates and spherical single dose of 1.6 mg/kg AuNPs (50 nm or 250 nm) into rat lungs, both particles gave mild pulmonary inflammation at the same dosage. Meanwhile, earlier reports by Mühlfeld et al [89] and Sadauskas et al [88] showed that when AuNPs are inhaled and deposited in the lungs, only a small fraction (both single and agglomerates) can be phagocytozed with a small part translocated across the alveolar epithelium. Nevetheless, the nanosize factor is a major significant feature in determining the deposition, translocation, distribution and fate of AuNPs. These facilitate the crossing of the blood brain barrier by AuNPs, which accumulate in neural tissues as well as in the placenta and fetus [87, 94-95]. Earlier reports by Takahashi and Matsuoka [94] reported the uptake of colloidal AuNPs of 5 and 30 nm after maternal intravenous injection in rats. Other studies by Lee et al [96] and Myllynen et al [95] have also showed the internalization of 10-30 nm PEGlyated AuNPs in the placental cells which are comparable to immunoglobulins that cross the placenta (IgG). The findings from these studies also showed AuNPs with sizes up to 240 nm crossing the human placental barrier without affecting the viability of the placental explants. Other findings by Sadauskas et al [88], however, showed that AuNPs of 2 and 4 nm when injected intravenously or intraperitoneally respectively did not seem to penetrate either the placenta barrier or the blood - brain barrier but were found in the macrophages and Kupffer liver cells. Information from literature envisages size as the most significant physical property responsible for inducing AuNP toxicities [97].

Furthermore, the influence of AuNP toxicity has also been shown to vary due to the different particle shapes. Among the shapes, rods shaped AuNPs have been reported to demonstrate more toxicity than their spherical counterparts. Research on gold nanorods has shown that they are more toxic to human keratinocyte cells (HaCaT) as compared to spherical gold nanomaterials [62]. The mechanisms of less toxicity of spherical AuNPs compared to nanorods are yet to be demonstrated; however, they are all engulfed on their surface properties. Studies investigating the cytotoxicity and cellular uptake of gold nanorods on human breast adenocarcinoma cell line (MCF- 7) also reported loss of mitochondrial integrity in cells treated with nanorods [47] as compared to spherical

shapes [77]. Li et al [70] also showed that naked AuNPs (20 nm in diameter) when taken up by MRC-5 human lung fibroblast *in vitro* can induce autophagy (degradation of a cell's own components via lysosomal machinery) concomitant with oxidative stress, stimulating up-regulation of antioxidants, stress response genes and protein expression. Other studies have also shown nanorods toxicity to be highly associated with surface layer used for the synthesis of nanorods such as CTAB [97]. Therefore the association of surface stabilizers and functional ligands chemistry or composition should not be overlooked. Also as the application of AuNPs are increasing in medicine to diagnose and treat diseases detailed data on the possible toxic effect of various sizes, shapes and ligands of the AuNPs are needed because the current available information are limited and inconsistent.

The effect of ligands and bio-conjugates on the toxicity AuNPs.

In nanotechnology, ligands are functional groups attached onto the surfaces of NPs thereby modifying their surface activities. The functional groups are usually attached either covalently or non-covalently onto the NPs by chemical processes (98-99). Place-exchange reaction is the most versatile and widely used method for introducing functional groups to AuNPs (57,92). The widely attached functional groups onto AuNPs are highly available for further conjugation (57). Polyethylene glycol (PEG), poly-L- lysine (PLL), poly- D- L- lactic-co- glycolic acid (PLGA) and their co- polymers have been successfully applied to develop novel biocompatible AuNPs [14]. Of these, PEG has gained popularity as a modifying agent due to its amphiphilic and solubility characteristics [12].

Hetero-functionalized PEGylated mono protected clusters (MPCs) with a thiol group on one terminal and a reactive functional group on the other have become popular for AuNP applications [100]. Preferred end groups for hetero- functional PEG AuNPs are maleimide, vinyl sulfones, pyridyl disulfide, amine, carboxylic acids, hydroxyl, methoxy and esters [101]

Functionalization of AuNPs increases the circulation period of the NPs in the blood stream [12]. A study investigating the bio- distribution of PEG modified and non- modified gold nanorods in mice reported a larger percentage of modified AuNPs in the blood in contrast to unmodified particles over the same time period [102]. Other data have shown that surface modified AuNPs have the ability to reduce cellular toxicity associated with chemical surfactants used during the synthesis of the NPs [42, 92]. Takahashi et al [94] reported the modifying of gold nanorods with phosphatidylcholine to reduce cytotoxicity associated with the CTAB molecule on the nanorods surfaces. Another study by Goodman et al [103] investigated the hazardous effect of AuNPs modified with an amine and carboxyl groups on Cos-1 cells, red blood cells, and *E. coli* cells. Their results showed that anionic AuNPs species are non-toxic to cells, whereas cationic species can cause moderate toxicity in all cells lines. The authors suggested that toxicity was related to the interaction of a positive charge on the ammonium species with a negative charge on the lipid bilayer of cell membranes.

Other findings reported by et al [67] showed that 1.4 nm AuNPs in diameter capped with triphenylphosphine monosulfonate can cause necrosis via the oxidative stress and mitochondrial damage, while Gu et al [104] found that 3.7 nm AuNPs in diameter modified poly(ethylene glycol) (PEG) were non toxic when internalized in the cell nucleus of human cervical cancer (HeLa). This shows that functionalized molecules play a significant role in AuNPs toxicity.

Some findings showed that functionalized AuNPs are not cytotoxic but can cause a slight reduction in the reactive oxygen and nitrite species [59, 63,105]. However, according to the findings by Bar-Ilan et al and Pan et al [61,67], functionalized AuNPs with a concentration of 62.5 mg mL^{-1} of triphenylphosphine monosulfonate (TPPMS) utilized as ligand is non-toxic, whereas concentration higher than 625 mg mL^{-1} can result in morphological malformations of zebrafish embroyos. Earlier studies by Tsoli et al [60] and Pan et al [67] revealed that 1.4 nm AuNPs functionalized with TPPMS are able to bind on dsDNA major groove and disrupt cellular function. However, their findings failed to indicate whether the effect was specific to gold 1.4 nm or to all AuNPs coated with TPPMS. In other related study it was found that the toxicity and biodistribution of PEG-coated AuNPs 20 nm with TA-terminated PEG5000 has more stability with lower toxicity than 40 or 80 nm AuNPs functionalized with TA-terminated PEG5000 [90]. It is therefore, evident that functional groups on AuNPs affect toxicity. An investigation into the effect of size and the presence or absence of sodium citrate residues on the cytoxicity and uptake of AuNPs in alveolar type-II cells has showed the reduction in cell viability due to sodium citrate residues on AuNPs [52].

However, when Boca et al [106] examined the cytotoxic effect of chitosan capped-AuNPs on chinese hamster

ovary cells *in vitro*, the conjugated particles were found to tranverse the cell membrane by endocytosis; and using dark field microscopy imaging, it revealed ≥ 85% of the cells were viable even after long period of exposure. This, therefore, shows that chitosan-conjugated AuNPs can be deemed to have great potential in cellular imaging or photothermal therapy as they are non-toxic compared to other coated AuNPs.

Oberdorster et al [107] earlier showed that, partial surface composition coupled with size of the NP is accountable for the observed toxic effects. However, other studies by Bar-Ilan et al [61] demostrated that zebra-fish embryo toxicity depends more on its surface chemical composition rather than on particle size. This implies that surface functionalization or coatings of AuNPs has a very huge impact on the toxicity of nanomaterials.

Furthermore, Cho et al [108] showed that PEG-coated 13 nm AuNPs when injected intravenously in to BALB/C mice can elicit an immune response and apoptosis with further accumulation in the liver and spleen after a week of administration. The findings of Wang et al [97] involving the intravenously injected CTAB- capped gold nanorods into rats circulated in blood as the main route of bio-distribution. Similarly, Hirn et al [109] also found the accumulation of AuNPs mainly in the liver, spleen and to a lesser extend in the kidney, brain, muscle and bone. Those found in the spleen and Kupffer cells lymphocytes form aggregates within the lysosomes [97]. The formation of aggregation scenario can result into complex biological system of an unknown response and toxicity *in vivo*. This indicates the need to study the biological effect of NPs in biological systems.

The effects of surface charge on the toxicity of gold nanoparticles.

Surface charge which is measured by zeta potential is one of the major physical characteristic influencing AuNPs toxicity [108]. The application of zeta potential provides useful information on the stability of colloid nanomaterials. It is thus, essential to always state whether the zeta potential of colloid NPs is positively or negatively charged. Surface charges determine the properties and functions of NPs. AuNPs have charged (negatively or positively) surfaces which make them highly reactive and receptive to surface modifications due to either cations or anions interaction, thus, creating a net surface charge [40]. Based on surface charges, AuNPs can promote protein refolding through electrostatic interactions between the exposed charged residues on the un folded protein and the oppositely charged ligands on the AuNPs [110]. The overall high negative charge of the NP-protein complex prevents the proteins from aggregating; the NP thereby promotes refolding which can be used to refold proteins in a chemical denatured state [110].

It is important to note that modifications of NP surfaces may cause undesirable ionic interactions with biological systems [73], due to changes in surface charges. Many AuNPs are stabilized with surface charges to prevent aggregation via electrostatic repulsion [42], playing a significant role in toxicity of the NP. Aggregated AuNPs have modified surface charges which intend influence changes of cellular environment and thus altering the cellular behaviour and cellular toxicity [79].

Apart from earlier reports that cationic are moderately toxic than anionic AuNPs [103], other reports by Schaeublin et al [111] have shown that both cationic and anionic AuNPs are toxic to cells. Schaeublin et al [111] further showed that both positively and negatively charged AuNPs can alter the mitochondrial membrane potential resulting into oxidative stress. The oxidative stress according to current findings by Oikawa et al [112] enhances the production of reactive oxygen species, various immunologic stimuli, inflammation, some human diseases such as neurodegenerative disorders, and cancers. Apart from these the anionic and cationic surface charges of AuNPs can stimulate lymphoid cells phagocytosis to an extend greater than neutral AuNPs [79]. Therefore, the wider the charge differences on the AuNP surfaces, the greater the opportunity for it phagocytic activities and the inflammatory responses.

Other studies have shown that surfaces charges of NPs enhance their uptake into cells. For example findings by Chithrani et al [62] using incubated citric acid coated AuNPs have shown that NPs surface charges can potential influence their uptake by mammalian cell line HeLa. Furthermore, He et al [113] also found that although the surface charges play a significant role in phygocytoses they also aid phagocytic clearance due to the NPs small size and high diffusible nature. The interaction of AuNPs with serum proteins therefore alter the physiochemical properties of the NP, which can intend affect uptake and target drug delivery processes [114]. Other factors such as ionic strength (charges) of AuNPs can also affect their biocompatibility, thereby interfering with the biokinetics of the cells, resulting in a reduction in cell viability [75,115].

However, citrate stabilized AuNPs toxicity test using MTT assay have shown that 20 nm AuNPs at a concentration of 300 µM have no significant effect on cell human dermal fibroblast-fetal [74] which was similar to

earlier findings by Connor et al [59]. In other study purified and citrate sterilized AuNPs have shown rather milder cytotoxicity in A549 and NCIH441 cells as compared to the particles with excess citrate. This indicates that functionalized side chain can interfere with the activities of the AuNPs depending on the shape and surface charge [62,103]. This, therefore, indicates that further more *in vitro* studies on cell viability concerning charge AuNPs properties are required to ascertain their toxicity.

As discussed only a few studies of AuNPs toxicities have been conducted using transformed cells lines with only a limited number using primary cell cultures which are prototype systems closer to *in vivo* studies. AuNPs surface modifications (surface charges) have been found to influence particle uptake *in vitro.* Some of the reports have shown that *in vitro* studies are the easiest toxicology studies which can be used to better understand the molecular events underlying cellular effects [66] as shown in Table 1. However, Donaldson et al [66] have stressed that *in vitro* studies on cell culture alone are highly limited due to narrow range of biological effects which do not reflect the range of pathological effects observed *in vivo*. Apart from that, the issue of NPs translocation into host tissues and their toxicokinetics *in vivo* is an important underlying principle in understanding nanotoxicology because *in vitro* testing has shown less convincing results. Apart from that there are few *in vivo* studies on the biodistribution and biological effects of AuNPs that can serve as a basis for assessing its health impact due to surface charges. Furthermore, *in vivo*-methods are very important because they determine the whole body health effects in animals although this will depend on the route (nasal, oral or dermal) of exposure (49). The biodistribution and biological processes after exposures are all tied down to the surface physiochemical properties that make them chemically reactive upon interaction with biological systems [93]. Therefore, the attributes of AuNPs and its coated surface charges must be examined with care to ascertain toxicities.

Conclusion and Challenges

In AuNPs and gold nanomaterials applications, the most important features stimulating their compositions array are sizes, shapes, surface area/porosity, surface charges, aggregation, surface modifications and host cell interactions. We can say these unique properties of AuNPs profiles are size-dependent and provide the challenge of determining their biological toxicities. However, for a better understanding of the acute, subchronic and chronic health effects of AuNPs toxicity, recommendations of methods for testing of reproductive toxicity, genotoxicity or carcinogenicity effects have been made available by the Organization for Economic Cooperation and Development (OECD). Furthermore, studies gathered so far show that AuNPs nanotoxicity studies and their health effects/implication are currently limited and insufficient to determine their health status to both human and the environment. Therefore, more *in vitro* and *in vivo* AuNPs and gold nanomaterials toxicity studies are highly recommended to augment the current limited controversial data (whether toxic or no-toxic). This is due to the fact that gold nanotechnology is a relative new field and its findings are budding. Also their easy aggregations arising from the fragile capped stabilized surfaces make physical handling difficult thus limiting applications. Different sizes, shapes and surface ligands exhibit different properties, therefore, when considering toxicity testing for AuNPs these factors should be taken into account. Furthermore, in depth research should be done to understand the chemical processes of size, shape and or surface ligands because they exhibit different properties both *in vitro* and *in vivo*. In addition, before, AuNPs and other NPs application are safely and widely applied in biomedical systems or at best in clinical trials, information on their biocompatibility, bio-distribution and biodegradability nature of Nanomaterials when applied into biological systems. Also it will be of paramount importance to understand the long term persistent behavior of AuNPs *in vivo* before their applications in biological settings.

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