Nanotoxicity of Inert Materials: The Case of Gold, Silver and Iron

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ABSTRACT - Nanotechnology has opened a new horizon of research in various fields including applied physics, chemistry, electronics, optics, robotics, biotechnology and medicine. In the biomedical field, nanomaterials have shown remarkable potential as theranostic agents. Materials which are considered inert are often used in nanomedicine owning to their nontoxic profile. At nanoscale, these inert materials have shown unique properties that differ from bulk and dissolved counterparts. In the case of metals, this unique behavior not only imparts paramount advantages but also confers toxicity due to their unwanted interaction with different cellular processes. In the literature, the toxicity of nanoparticles made from inert materials has been investigated and many of these have revealed toxic potential under specific conditions. The surge to understand underlying mechanism of toxicity has increased and different means have been employed to overcome toxicity problems associated with these agents. In this review, we have focused nanoparticles of three inert metallic materials *i.e.* gold, silver and iron as these are regarded as biologically inert in the bulk and dissolved form. These materials have gained wider research interest and studies indicating the toxicity of these materials are also emerging. Oxidative stress, physical binding and interference with intracellular signaling are the major role player in nanotoxicity and their predominance is highly dependent upon size, surface coating and administered dose of nanoparticles. Current strategies to overcome toxicity have also been reviewed in the light of recent literature. The authors also suggested that uniform testing standards and well-designed studies are needed to evaluate nanotoxicity of these materials that are otherwise considered as inert.

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INTRODUCTION

Nanotechnology has emerged as one of the exciting and novel field of science in last few decades. The history of nanoparticles traces back to ninth century when metallic nanoparticles, not realized at that time, were used as paint to decorate ports and windows, making them distinguishable among other subjects (1, 2) . Nanoparticles are the particles with size less than 100 nm in any single dimension (3). This definition is based on the fact that particles in this size range possess unique structural properties that differ significantly from their bulk and dissolved counterparts. However, this definition may not serve well for nanoparticles in biomedical applications where pharmacological and chemical aspects are of pivotal consideration along with size and structure. Nanoparticles that show improved characteristics with size below 100 nm are used majorly in diagnostic, like quantum dots, metal nanoclusters and paramagnetic particles while those for therapeutic applications has size usually above 100 nm like micelles, dendrimers, liposomes and polymersomes all loaded with drug (4, 5). Nanoparticles for drug delivery are designed to carry payload in desired temporal and spatial specifications.

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They may be considered acceptable with size up to 300 nm which is sufficiently small to avoid reticuloendothelial (RES) systems and blocking of blood vessels when administered intravenously (6). They present immensely large surface area and surface modification opportunities which has made them an attractive tool to deliver bioactive molecules specifically to target pharmacological sites in body (7, 8). In some cases, specific characteristics may be imparted to drug delivery nanoparticles e.g. lipophilic surface modifications can make them cross blood brain barrier-suitable for CNS drug delivery (9, 10). Nanoparticle, after intravenous administration, are rapidly taken up by reticuloendothelial (RES) system which lead to their elimination from the body. This problem can be overcome by incorporating stealth property to nanoparticles (11). Polyethylene glycol (PEG is most widely used polymer for stealth coating which attracts water on nanoparticles surface to prevent opsonization and escape immune system (Figure 1).

Achieving different pharmacological and pharmaceutical milestones are possible by nanoparticles engineering and encapsulating drug inside it, without any chemical modifications in structure of drug molecule which may compromises its optimum pharmacological and toxicological balance. Nanoparticles have been modified in different ways to ensure site specific drug release, sparing the rest of the body cells from unwanted exposure. The slightly acidic pH (~6) of tumor microenvironment from rest of physiological pH (7.4) is targeted by making acid cleavable ligation of drug on surface of nanoparticles. Another strategy is to exploit overexpressing receptors on cancer cells like folic acid, hyaluronic acid and transferrin. These molecules if conjugated on surface of nanoparticles, will drive it directly to the cancer cells (12). Immune system recognize and produces antibodies against infectious organisms and tumor cells. Attempts have been made to decorate these antibodies on surface of nanoparticles for tumor targeting and utilizing their specificity in this regard. Monoclonal antibodies, a purified form of antibodies against single cancer epitope, can be covalently bound to nanoparticles that will circulate throughout the circulation and target only cancer cells (13, 14). Cancer tissues are rapidly proliferating and need large supply of blood to bring nutrients and carry away wastes. For this reason, cancer cells have leaky vasculature to allow permit free movement of substance in and out of cancer. This provides a passive targeting mechanism to nanoparticles as they are can cross leaky vasculature of cancer tissues more easily, a process known as enhanced permeation and retention (EPR) effect (15). The newly formed blood vessels also offer resistance to the blood flow in this area hence increasing the retention of nanoparticles in the tumor mass (16).

More recently, research interest has shifted to devise theranostics nanoparticles making them immensely attractive in diagnosis and treatment (10). Gold, silver and iron are three widely used materials that are considered inert to biological systems because they are biocompatible and lack toxicity. These nanoparticles are also supposed to have biological activity like silver nanoparticles (AgNPs) have very well (9) documented antibacterial activity (17), gold nanoparticles (AuNPs) have cytotoxic, oral bioavailability enhancing and immunomodulatory effects, which may be an added advantage of these particles in treatment of diseases like multi drug resistant (MDR) infections and tumors (4, 18). Iron nanoparticles (IONPs) are being explored for contrast agents in magnetic resonance imaging (MRI) for tumor localization and pharmacokinetics of nanoparticles (19). Diagnostic applications of gold, silver and iron based nanoparticles are due to their ability to respond to a wide variety of external stimuli such as infra-red radiation, magnetic field and ultrasonic waves (20-22). These nanoparticles also offer opportunity of "clickable" release of encapsulated drugs when they reach target site (11). In addition, nanoparticles of gold, iron and silver are increasingly used for dual function nanoparticles that can help in diagnosis and treatments of different disease after single administration of such nanoparticles. One example is image guided therapy in which nanoparticles can locate and kill malignant cancer cells with loaded drug or burn it by heat produced after alternating photothermal exposure (23, 24). On the other hand, metallic nanoparticles also possess some detrimental effects like genotoxicity, inflammation, oxidative stress and interference with intracellular signaling (25-27). Such toxicity problems are encountered with these relatively inert materials when they are used at nanoscale. Toxicity of nanomaterials is usually described to be dose dependent which is further associated with size and surface engineering (28).

We summarized in this review, the aspects which are important from the perspective of drug delivery and toxicity of three commonly used

metallic nanoparticles i.e. gold, silver and iron. This review covers recent literature to elucidate underlying mechanisms of toxicity, so far established, in different conditions that are encountered *in vivo*. Attempts were also made to highlight currently formulated strategies to reduce toxicity of these nanomaterials and future prospects in the light of some studies already performed in this regard.

GOLD NANOPARTICLES

Gold is one of the most widely used material which is directly in contact with human body. Aurotherapy or Chrysotherapy is use of gold in medicine and use of raw gold for medicinal purpose can be traced back as far as the Chinese in 2500 B.C. In Medieval ages, Au was considered as heavenly precious glittering substance; which upon using against diseases can produce some relief. The methodological research about pharmacology of gold started in 1890, when Koch found its bactericidal activity against *tuberculosis bacilli*. The research about gold medicine gains its peak upon unrevealing of favorable results in arthritis leading to the development of anti-rheumatic agents like auranofin and disodium aurothiomalate (30). The toxicological symptoms in patients and unclear mode of action of gold in arthritis/inflammation imparted some gaps in pharmacology of aurhotherapy. AuNPs of different sizes with engineered biocompatible surface is currently acting as a bridge to reveal the pharmacodynamics and kinetics of gold in therapeutics (31). Current exploration on unveiling the biological applications of AuNPs include capping with biofunctional moieties like peptides and carbohydrates and looking for cellular insult or control of cellular processes (32, 33). Similarly drug delivery and photothermal therapy of cancer is another direction being debated for biomedical applications of AuNPs. Moreover, gold nanoparticles can enhance therapeutic efficacy of co-administered drugs (18). The surface plasmon resonance (SPR) and light reflecting ability of AuNPs have made them a good tool as a diagnostic agent (9). Surface functionalization and ability of AuNPs to bind with thiols and amine groups have been used for making nanoparticles as a vector for drug and DNA (34, 35).

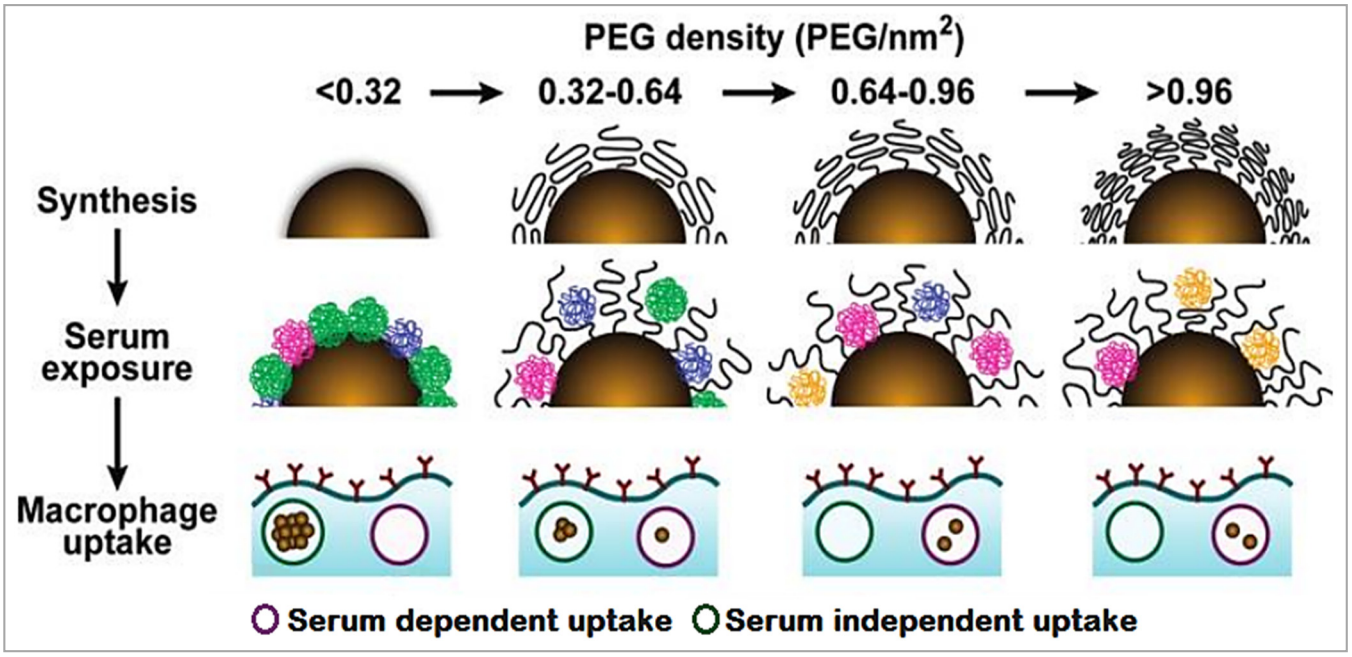

Figure 1: Schematic representation of influence by varying the density of PEG coating on nanoparticles: interaction with blood components and subsequent uptake by macrophages. The upper panel shows density of PEG on nanoparticles surface. Middle panel shows how type and amount of blood proteins interaction with nanoparticles varies with PEG density. Similarly, lower panel shows that serum dependent uptake by macrophage decreases as PEG density increases whereas serum independent uptake increases; (taken from (29) with permission)

The enhanced permeability and retention of small sized nanoparticles have given them an added advantage for use against diseases like cancer where the permeation of tumor vasculature has been exploited to selectively deliver the nanoparticles to tumor site (36). Enhanced permeability and retention, surface functionalization, SPR and photo thermal tumor ablation approach have been collectively exploited in search of theranostic applications of gold based nanomaterials (37). Despite of dramatic utilities of AuNPs there are some toxicological threats associated with their use as theranostics. AuNPs are considered as the safest metallic nanoparticles however toxicity is always subjected to considerable debate (38, 39) and in some studies toxicity is also documented (4, 5, 18, 40). The reported damages are particularly important due to their genetic, hepatic and renal toxicity nature even at lower doses (41). Toxicity of AuNPs is also reported on reproductive cells which may lead to anomalies in offspring (42). The critical analysis of the conditions reporting toxicity of AuNPs indicates that they shows sign of toxicity only specific conditions. If we could control those underlying parameters, the plethora of advantages of AuNPs may be availed without much of their side effects. The tools to control the side effect of these particles are discussed in the following sections.

Size and Shape

The size of AuNPs exerts dramatic effects on the interaction of these particles with macromolecules of living system. In 2007 Pan et al. performed experiments to evaluate toxicity of AuNPs in the range of 0.5 nm to 15 nm. They found that particles in the range of 1 - 2 nm are more cytotoxic than particles either smaller or larger than those. The results were very interesting in the sense that one cannot draw conclusion as to the safe size range. They also found that AuNPs of 1.2 nm lead to apoptosis whereas AuNPs of 1.4 nm produced necrosis (43). The cytotoxicity and associated necrosis is further confirmed by cytotoxicity against cell line of melanoma, macrophages, and fibroblasts. A logical explanation of this abnormal behavior is the fitting of the nanoparticles in the pockets of DNA coil or 3D quaternary structure of proteins (44). This pocket-fitting model is also supported by DNA or protein mediated synthesis of Au nanoclusters (NCs) where reduced Au atoms are grouped inside disulfide pockets in proteins or in folding of DNA duplex (45-49). Later in 2009, Pan et al. explained that toxicity of AuNPs as small as 1.4 nm is due to oxidative stress and damage to mitochondrial integrity (50). In another work done by (51) the same sized (1.4 nm) AuNPs have shown the ability to catalyze the reaction in conversion of ring shaped protein "trp RNA-binding Attenuation Protein" to capsid shaped protein. These result suggest that particles in the size range of 1-2 nm have intrinsic ability to bind with biological macromolecules leading to protein configuration conversion. This intrinsic activity may be an unwanted pharmacological effect where inert AuNPs are desirable. Similarly, teratogenic effects of AuNPs can be attributed to size dependent passage of nanoparticles from maternal blood to fetus, usually controlled by transport channels and endocytic or diffusive processes (52). AuNPs in 4-20 nm showed ligand dependent toxicity, which interns upon further investigation, revealed due to free ligation or Au precursor salts while nanoparticles showed not cellular toxicity (53). Overall, the shape of nanoparticles is also important in addition to size in a single dimension. Cellular uptake seems to be inversely related to size (in range of 30-90 nm) but directly related to roundness of AuNPs (54). Rod shaped AuNPs appeared to have less cellular uptake efficiency in comparison to spherical one. Usually, rod shaped AuNPs show lower internalization than spherical particles albeit toxicity profiles don't differ significantly (55). Although no hard and fast rules exist to serve as starting point, screening of size of nanoparticles along with appropriate surface group is necessary to assure the safety of nanoparticles before using them in clinical practice. Table 1 cites selected major reports that explain the size dependent toxicity of AuNPs *in vitro* and *in vivo*.

Surface chemistry

The surface chemistry is a key factor governing interaction of AuNPs with the living system (44). The first and foremost consideration in selecting surface coating materials is that they should be biocompatible and non-toxic. For example, phosphine-stabilized AuNPs having size of 1.4 nm have been prepared but failed electrophysiology-based safety testing in human embryonic kidney cells, a safety test prescribed in FDA guideline (56). In addition, leaching of such surface coating materials may be problematic such as toxicity caused by cetyltrimethyl ammonium bromide CTAB, a famous stabilizing agent for AuNPs (57). The behavior of AuNPs may also be explained on the basis of corona of functional groups attached on their

surface. The toxicity of gold nanorods and nanospheres on human hepatocellular carcinoma cell line has been proven to be reduced by encapsulating the nanomaterials in silica core (58). In another work, the intracellular accumulation of AuNPs in macrophages was shown to occur in chronic exposure only. AuNPs coated with polyethylene glycol (PEG) show decreased cytotoxicity along with low intracellular accumulation (54). This was further supported by another study which demonstrated inverse relation between PEG MW on surface and cellular uptake of AuNPs (54). PEGylated nanoparticles exhibited reduced interaction with intracellular proteins which ultimately resulted in rapid expulsion of these nanoparticles from the cells (59). In another study, different AuNPs coated with ethanediamine, glucosamine, hydroxypropylamine, taurine, and PEG were prepared and internalization of these particles in the primary culture of human endothelial cells was investigated. It was found that the particles coated with ethanediamine were internalized to very high extent indicating that these particles can be studied for chronic toxicity on human endothelial cells (60).

The density of PEG coating on AuNPs has also shown to affect the binding of serum protein on the nanoparticle surface, a property which actually affects the uptake by macrophages (Figure 1). At high PEG/nm of AuNPs, the serum protein adsorption on the surface of nanoparticles decreased while at low density of PEG/nm, the adsorption of serum protein increased supporting a competitive ligand displacement mechanism for serum protein adsorption on AuNPs. High density of PEG on the surface of AuNPs resulted in increased uptake of the nanoparticles by macrophages (61). Surface coating material has also been proven to modify other toxicity parameters. Aspartate, citrate and bovine serum albumin, when used as capping material, AuNPs appeared to be non-toxic in-vitro against human fibroblast cells (MRC-5). They found that all the three types of AuNPs were proven to be non-cytotoxic in *in vitro* experiments against human fibroblast cell line (MRC-5) but the in-vivo studies in murine models showed the citrate capped AuNPs were hepatotoxic while aspartic acid capped AuNPs were hepatotoxic as well as nephrotoxic (62). Liver contains diverse families of enzymes that can catalyze many types of materials. This can explain altered biodistribution of nanoparticles *in-vivo* (63). Surface modification can also play role in colloidal stability. Agglomeration of nanoparticles as imparted by coating material may lead to reduction in internalization, reduced renal clearance and blockade of blood vessels. Aggregation of AuNPs is mainly dependent on zeta potential imparted by ligands. Particles with positive surface charge are less prone to aggregate and presents longer stability in-vitro as compared to negative charge AuNPs (64). However this is inverted in-vivo where negatively charged proteins are electrostatically adsorbed on positive surface of AuNPs, phenomenon of opsonization, and results in aggregation-responsible for toxicity profiles and accumulation in glomerular filtration assembly and probably for nephrotoxic profile (18, 65-67).

Special Case: Oxidation Stress

Reactive oxygen species are free radicals formed inside that cells which have the potential to redox damage several intracellular processes. This mechanism is well reported for gold nanoparticles and many researchers claim it to be the key molecular event for its pharmacological activity (68). At the same time, gold nanoparticles have shown mutagenicity, genotoxicity and cytotoxicity in a number of studies and the mechanism evaluated for the cause of damage is increased reactive oxygen species (ROS). Production of ROS leads to damage in DNA resulting in genotoxicity, mutagenicity and cytotoxicity (41, 69, 70). Although some research work have also reported the toxicity of AuNPs independent of ROS production (71, 72). However, none of these studies could neglect the role of ROS. Thus, it can be stated that ROS production may not be the sole player of DNA damage and some other mechanism may also be responsible for its toxicity like leaching of Au ions from nanoparticles and complexing effect with surrounding bio-macromolecules (65, 73). Antioxidants have the ability to destroy the ROS and a large number of antioxidants have been reported which have been extensively used in the clinical practice. Ascorbic acid, glutathione and N-acetyl cysteine (a precursor of glutathione) are known antioxidants which have been known in clinical practice. If the glutathione production is genetically suppressed, the genotoxicity and cytotoxicity of AuNPs is reported to be increased (74).

Research work also supports the fact that use of antioxidants prior to or along with AuNPs has resulted in decreased ROS production and cytotoxicity. Triphenylphosphine monosulfonate,

glutathione and N-acetyl cysteine were used as antioxidants and pretreatment with these agents decreased AuNPs associated ROS production (50). In another study, the decreased level of ROS caused by dimethyl sulfoxide (DMSO) resulted in overall increased wound healing by combination therapy of therapeutic pulsed ultrasound, AuNPs and DMSO (75). In some cases, antioxidants have been beneficial in reducing the genotoxicity induced by the oxidative stress generation (35).

Table 1. *in vitro* and *in vivo* toxicity of gold nanoparticles depending upon size

Size (nm)	Ligand*	Cell line	Dose	Toxicological effect	Reference
		in vitro studies in cell line			
0.8, 1.2, 1.4, 1.8, 15	TPPMS, TPPTS	HeLa	250 µM	AuNPs of 1.2 nm lead to apoptosis whereas AuNPs of 1.4 nm produced necrosis. 1.4 nm AuNPs are most toxic of these	(43)
2	Quaternary ammonium	COS-1 mammalian cells	0.38-3 µM	Cationic nanoparticles are toxic	(76)
3.5	Lysine/polylysine	RAW 264.7 mouse macrophage	10-100 µM	Non-toxic and Non-immunogenic	(77)
3.7	PEG	HeLa	100 µM	No toxicity	(78)
<10	–	A549 cells	5 µg/ml	Nanoparticles prepared in water are non-toxic whereas those prepared in acetone induce apoptosis	(79)
13.1	Citrate	Human dermal fibroblast	4 mM	Decreased cell proliferation	(80)
15	Citrate	Huma lveolar macrophage (A549) cells	2000 µM	No toxicity	*(81)*
15, 50, 100 nm	–	Caco-2 cells	5 µg/ml	Cytotoxic, larger particles are more toxic to mitochondria	*(82)*
18	Citrate	HeLa	2 nM	Non-toxic	(83)
16, 26, 40, 58	(10-Mercapto-decyl)-trimethyl-ammonium bromide (TMA) for positive charge and 11-Mercaptoundecanoic acid (MUA) for negative charge	RAW 264.7 and non-phagocytic HepG2 cells	Au concentration of 10 mg/L	Positive nanoparticles show higher cytotoxicity against HepG2 cells. Negative nanoparticles show higher cytotoxicity against RAW 264.7 cells	(84)
33	CTAB and citrate	BHK21 cells of hamster kidney	120 nM	Not toxic	(85)
50	Citrate	Blood		Non toxic	(86)
90		Human prostate carcinoma (PC-3) cell	34 nM	Non-Toxic	*(87)*

Table 1 Continued…

100	PCL	Human ublical vein endothelial (ECV 304) cells		Non-toxic	(88)
		in vivo studies in animal models			
20	Arabic gum	Pigs	1.8 mg/kg	No toxicity	(89)
4, 10, 28, 58	Citrate	Mice (BALB/c)	200 mg/kg	No toxicity	(90)
3, 5, 8, 12, 17, 37, 50, 100	Citrate	Mice (BALB/c)	8 mg/kg	8-37 nm show lethality	(91)
3, 10, 50, 100	Citrate	Zebra fish	Up to 250 μM	No toxicity	(92)
15, 50, 100, 200	Citrate	Mice (ddy)	1000 mg/kg	15 and 50 nm were found in liver, kidney, heart and brain. No toxicity reported.	(93)
13	PEG	Mice (BALB/c)	4.26 mg/kg	Inflammation and apoptosis in liver	(86)
13.5	Citrate	Mice	2.2 mg/kg	Reduction in body weight and RBC count	*(94)*
17, 37	–	Mice (BALB/C)	8 mg/kg/week	Produce fever and altered dopamine and serotonin secretion; 17 nm particles impair learning and memory	(91)
20, 40, 80	PEG	Mice (BALB/c)	2010 mg/kg	No toxicity	(95)
18.6, 67.5	Tannic acid	Polymorphonuclear neutrophil cells	100 μM	Induction of apoptosis associated with degradation of cytoplasmic proteins and endoplasmic reticulum stress	(96)
2, 10, 25 nm and their aggregate	PVP	HeLa	0.83 nM	2 nm don't but 10 and 25 nM cause cytotoxicity. However, larger aggregates promote cell growth.	(97)

* Ligand are not toxic at this concentration when administered alone.

SILVER NANOPARTICLES

AgNPs are widely used due to their unique properties like catalysis and sensing (98, 99). Recently, AgNPs have become focus of biomedical research due to their antimicrobial properties (100-105). AgNPs are bactericidal in nature due to multimodal pathways. Although molecular basis of bactericidal action is unknown, different studies show that AgNPs can cause lysis by arresting different processes such as cell wall synthesis, arrest mitochondrial system, ribosomes inhibition or damage to nucleic material *i.e.* DNA (106-108). In addition, they can also synergize efficacy of many antibiotics and has opened new horizon to treat MDR infections (109). Now, various orthopedic devices and wound dressings are available in market with AgNPs coatings to prevent against microbial contamination of underlying wound (110). The toxicity of AgNPs is also studied and a body of literature is available in this direction. In some studies the genotoxicity of nanoparticles is well documented while in some studies contradictory effects are also reported.

AgNPs are supposed to damage mammalian cells in the same way as they do the bacterial and fungal cells.

Size and Shape

Some studies have also shown that the smaller sized AgNPs causes increased toxicity (111). This observation is supported by the fact that smaller sized particles can easily enter into the cells. However, some studies report contrasting results and role of silver nanoparticle size in toxicity is not well supported (28). Lee et al. evaluated the biodistribution of AgNPs of around 10 and 25 nm size in brain and testicles, and found it to be independent of size. However on the other hand, when Park et al. scanned biodistribution of AgNPs with significant size differences (22, 42, 71 and 323 nm), the accumulation of AgNPs in different tissues including brain and testicles was inversely related to size. The AgNPs of 323 nm were not able to diffuse in any organ (112, 113). Similarly, Hendrickson et al. have recently reported the affinity of 12 nm AgNPs *i.e.* smaller size range having more affinity to liver and kidney (114). These studies revealed size to be important factor in controlling the biodistribution of AgNPs but results are only visible when size is significantly varied. There is possibility of achieving a balance between size and pharmacology of AgNPs and minimizing its tissue accumulation which subsequently results in toxicity. Although size of AgNPs can alter biodistribution, it association with toxicity is not established. Moreover, shape of nanoparticle may also not significantly affect toxicity of AgNPs (115).

Surface chemistry

The antibiotic effects of AgNPs, although well documented, prerequisite its efficacy and clinical use to specific bacterial cells *i.e.* it should kill only bacterial cells without harming the host cells. Considerable efforts have been made in order to make AgNPs host specific. One possibility is to treat host cells with anti-oxidants e.g. N acetyl cysteine or reduced glutathione (GSH) potentiating their ability to detoxify ROS species. Cytotoxicity and genotoxicity of Ag nanoparticles is observed at concentrations much higher than MIC of AgNPs (88). Dose dependent toxicity is also observed in zebrafish models even using albumin as capping agent (116). In this way, it has been proved that the cytotoxicity and genotoxicity of host cell may be prevented by using appropriate dose of AgNPs. As toxicity of AgNPs is mainly due to leaked Ag ions (117), organic coating is more capable to prevent leaking of Ag ions as compared to inorganic coating (118).

Just as discussed for AuNPs, intrinsic toxicity of surface coating material can also participate in overall toxicity of nanoparticles. Lu et al. conducted a comprehensive study to evaluate toxicity of AgNPs with different coating properties. They found that polyvinylpyrrolidone (PVP) coated AgNPs are more biocompatible than citrate coated nanoparticles. Interestingly, they also observed that citrate coating may undergo chemical changes during drying process rendering them more cytotoxic, genotoxic and phototoxic (115). Protein ligations on the surface of AgNPs enhanced their cellular biocompatibility and kept them dispersed in cytoplasm of fibroblast cells (119). Yang et al. determined toxicity of AgNPs with different ligands in order of Gum Arabic>PVP>citrate (28). Gum Arabic is of natural and biocompatible origin than citrate and PVP but presented greater cytotoxicity which is explained on the basis of ability of ligand to tightly cap the underlying AgNPs. Citrate has more ability to tightly ligate the Ag in nanoparticles thus poses lesser risk of leaking out of Ag ions and thus less toxicity.

Zeta potential or surface charge of AgNPs plays important role in anti-microbial potential of nanoparticles. The presence of carboxyl, amino and phosphate groups in cell wall of bacteria, gives it a net negative charge which repels negatively charges AgNPs like citrate (-40 mV) and PVP (-12 mV). AgNPs capped with branched polyethyleneimine (+39 mV) showed greater bacterial interaction (120). AgNPs with near to zero *i.e.* neutral zeta potential appeared to be less toxic as compared to negatively charges AgNPs. Park et al. synthesized AgNPs with 0.91 mV zeta potential and they showed EC20 (concentration for 20 % cell death and 80 % viable) of 1.6 µg/ml against 5000 cells/ml of RAW264.7 (121). While Park et al. synthesized AgNPs with -47 mV zeta potential and its EC20 was around 0.18 µg/ml against 3×10^6 cells/ml of RAW264.7 (122). It can be deduced from these two independent studies that more negative zeta potential greatly enhance the cytotoxicity potential of AgNPs, excluding the effect of size and ligands.

Special Case: Ionization of Silver

Genotoxic potential is reported in many studies (26, 87, 121, 123) and the mechanism is supposed to be ionization of AgNPs by Trojan horse type

mechanism leading to production of Ag^{+2} and ROS (117). Stored AgNPs formulation was proven to be more toxic than freshly formulated preparation. Reason for this increased toxicity is the erosion of surface coating or dissolution/leaking of silver to Ag^{+} ions (124). If these particles are to be marketed for clinical use for therapeutic application, it is very difficult to make fresh AgNPs for use as antibacterial agent. Similarly, no study is present to investigate the dissolution of AgNPs when it is absorbed in the systemic circulation. If the dissolution of AgNPs takes place *in vivo*, it would cause systemic toxicity which may over weigh the beneficial effect. This ROS produces oxidative stress which ultimately leads to genotoxicity and cytotoxicity of the cell. In some studies, oxidative stress is associated with reduced amount of glutathione present in the cell (125). But some contradictory studies are also documented as in one experiment; AgNPs were injected in group of mice for 28 days and any mutation on the cells of bone were investigated by hematological studies and no significant damage to the blood cells were documented (126). Similarly, nanoparticles were administered in mice by inhalation for twenty eight days and no significant change in blood chemistry was reported (127).

Over all conclusion, from so far reports on toxicity of AgNPs is to control the dissolution and leaking of Ag ions from AgNPs by size or surface ligation. More efficient surface ligation with tightly packed ligands on nanoparticles surface will prevent the Ag ions leaking, enhancing its colloidal stability and thus reducing toxicity.

IRON NANOPARTICLES

Iron is considered one of the most inert materials used in nanotechnology. It is a common component of many biological systems such as bio-imaging, blood circulation (128, 129), energy production (130), enzyme catalysis (131, 132) and immune system (133). Iron oxide nanoparticles (IONP) have drawn considerable attention in medical field. Just as discussed for gold and silver, iron showed improved properties at nanoscale. They are widely investigated for diagnostics, drug delivery and dual function modalities *i.e.* therapeutic and MRI diagnostic. This paramagnetic behavior can be exploited in MRI which use strong magnetic field for diagnosis of various lesions and pathological changes in the body. MRI uses a combination of magnetic field radiofrequency pulse to image body organs containing IONP. They improve contrast of image and ensure imaging of target organs with particular safety for pediatrics and geriatrics patients (134-138).

Therapeutic use of IONP is mostly related to its applications in drug delivery. It can load drug and target it to specific site under the influence of magnetic field. IONP are usually intravenously administered to patient for diagnosis and targeting. IONP can be targeted to a specific area by applying external magnetic field directly over tumor affected body part (Figure 2). IONP based targeting with magnetic field has been comprehensively reviewed elsewhere by (139) and readers interested in this aspect are encouraged to read their review article. This will localize nanoparticles in this region when they reach with blood circulation. Another strategy is to use targeting (140-142). IONP based hyperthermia is utilized for controlling the release of the drug from the temperature sensitive micelles due to phase change in micelles. When micelles are deformed, drug is released in the target tissue (11, 143). In another study the synergistic effects of the hyperthermia and chemotherapeutic drugs has been reported on various cell lines (144). These site specific retention characteristics of IONP is also utilized in hyperthermia mediated treatment of tumor. When IONP are exposed to alternating magnetic field, heat is generated. Heating tumor mass up to 45°C will lead to apoptosis whereas heating above it may cause necrosis. Recently, it has also been used for thermotherapy or thermos-ablation of tumor tissues (145). IONP are leading paradigm in theranostic nanoparticles due to above mentioned diagnostic and therapeutic uses. One example is image guided therapy in which IONP can locate cancer and kill them with loaded drug or burn them by heat produced after alternating magnetic field application (23, 24).

Thus, IONP will not only prevent the unwanted effects of the anti-cancer drugs but it will also augment the cytotoxicity of these drugs. Like Ag and AuNPs, the toxicity of IONP is subjected to considerable debate with results depending upon various factors. In the work of (146-149) the genotoxicity of IONP is reported. Among these results the effect of IONP on human skin and lung cell lines were of considerable importance as drug carrier in humans (146). Iron may exist as ferrous (+2) or ferric (+3) form in NPs and both oxidation states show similar physicochemical properties. Fe_2O_3 and Fe_3O_4 nanoparticles may present different level of interaction with biological tissues and

relative abundance of these materials may be of significant importance in in-vivo toxicity studies (147).

Size

IONP which are used for diagnostics are usually smaller than 10 nm because superparamagnetic properties can only be achieved at such small size. However, this size can lead to removal of IONP after they are settled in tumor mass via leaky vasculature. Thus, passive targeting by EPR effect may not be feasible for IONP. Their toxicity may not be related to its size as shown by many studies. Thus, size of IONP may not be of much significance except for its magnetic characteristics that are usually found to be safe (150).

Surface chemistry

IONP have been reported with various surface coatings materials. In addition to above discussed materials for biocompatibility or penetration enhancement, they have been combined with other metals to form novel structures with modified magnetic and heating properties (151). However, toxicity of IONP is dependent upon materials forming the shell and we have not discussed nanoparticles with non-iron metallic shells. IONP have been prepare with PEG and folic acid coating for enhancing their release only in cancerous cells. The results obtained in these experiments are of substantial importance as tumor mass was decreased up to 10 fold than control group. In another study, the IONP were coated with different materials and their toxicity was evaluated. Results clearly revealed dependence of toxicity on surface ligation. It was established that the surface of IONP could be manipulated to alter the endocytosis of nanoparticles and their subsequent toxicity (152). Similarly, coating of IONP nanoparticles with three very closely related carbohydrates *i.e.* glucose, lactose and maltose resulted in very different behavior in human cell line suggesting that the effect of surface coating will markedly affect nanoparticles fate *in vivo* (153).

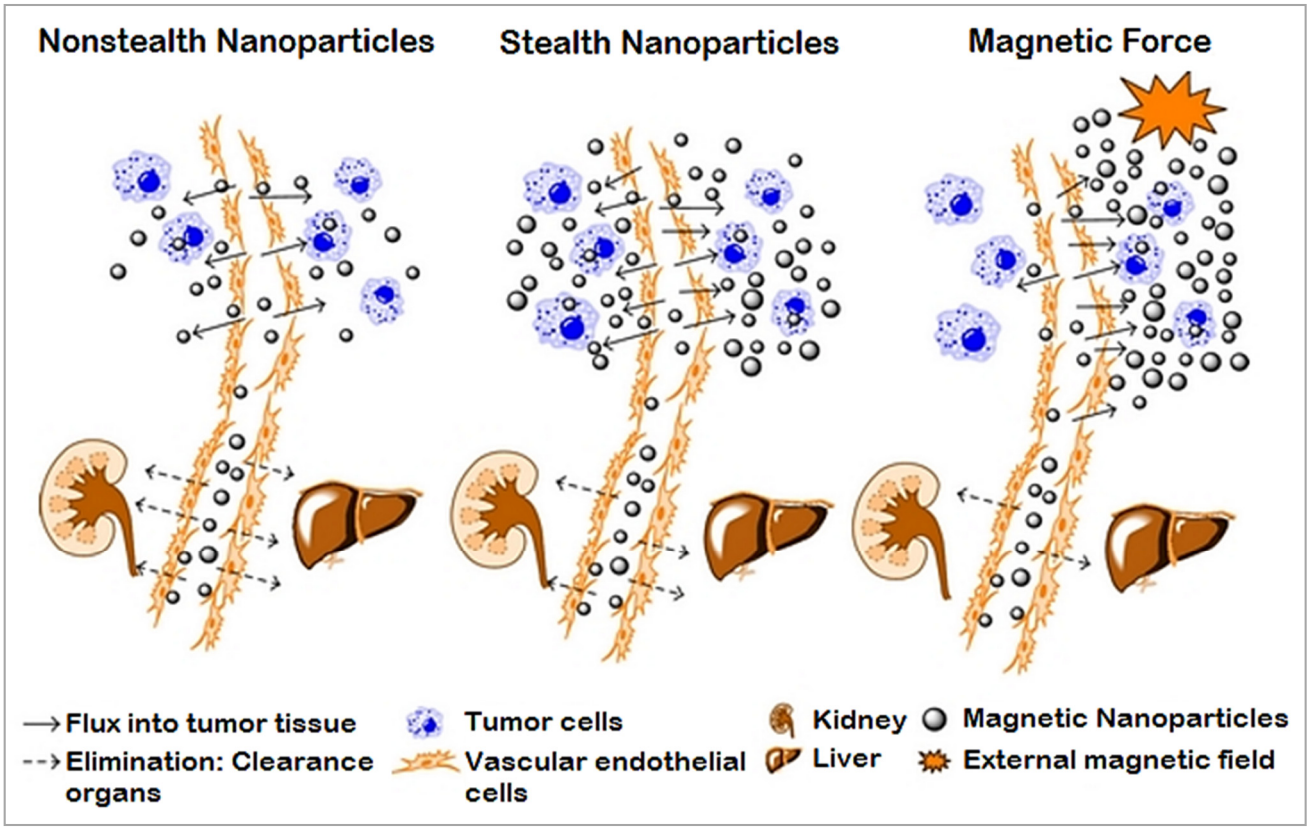

Figure 2. Enhanced permeability and retention (EPR) effect under magnetic field. Nanoparticles are accumulated in tumor tissues due to leaky vasculature. Non-stealth nanoparticles are rapidly cleared through kidney and lungs whereas stealth nanoparticles can escape this elimination step. Nanoparticles accumulation in tumor is further enhanced after external application of magnetic field (produced with permission from (138)).

Soensen et al. performed a series of studies to find interdependence of nanoparticles coating and toxicity. They found that toxicity may be observed with materials that are intrinsically toxic (154). Later, Sorensen et al. showed that cellular uptake of oxide nanoparticles is also influenced by surface coating. Thus, toxicity of IONP is mainly controlled by extent of internalization and number of nanoparticles per cells (155, 156).

When coating is not homogenous on nanoparticles, the resultant nanoparticles will present coated and uncoated surfaces that may pose different toxicity issues (157). In some studies, the mechanism seemed to be responsible for the genotoxicity was the ROS generation (158). In other studies, contradictory results were observed including peroxidase like activity of nanoparticles to reduce oxidative stress (159, 160). Thus, involvement of ROS in IONP toxicity remains controversial. In the case of IONP, we lack the data in which N acetyl cysteine or glutathione could be used for reduction of ROS generation and prevention of DNA damage could be achieved. Although the mechanism responsible for the toxicity of IONP is suggested to be ROS generation in (159) so we suggest that pretreatment with N acetyl cysteine if lead to decreased toxicity of IONP, will indicate ROS dependency of IONP for toxicity. We should also consider the factor of iron overload, when considering them for clinical application, the problem which is commonly encountered in the patients of thalassemia (161). The metal may dissolve inside the body and lead to hemosiderosis *i.e.* accumulation of iron in various body organs especially liver. If such condition appears, patient may be treated with iron chelator like desferrioxamine (162).

Special case: Interference with Intracellular Signaling

IONP have shown to inhibit differentiation of stem cells. These effects have been observed with dextran coated IONP when used during labelling of mesenchymal stem cells (163, 164). Another study found that IONP can suppress formation of new blood vessels from progenitor cells (165). These studies support the interference of IONP with different intracellular pathways leading to altered cell response to growth factors (157). However, these effects are also dependent upon intracellular concentration of IONP and many strategies aimed to reduce IONP dose may serve to overcome these problems (166). In magnetic field hyperthermia, tumor cells respond to applied hyperthermia by producing heat shock proteins. Although their function is to prevent cell damage resulting from heat, they are recognized by human immune system resulting in anti-tumoral immune response (167). However, no link has yet been found between these immunomodulatory effects and IONP and these vaccine like effects are attributed to hyperthermia (168).

CONCLUSION

Relatively inert materials such as gold, silver and iron can show toxicity at nanoscale as the mechanism of nano-toxicity is dependent markedly upon size and surface chemistry, which intern, is further associated with degree of internalization in cells or leaking. Thus, enhancing the colloidal stability and purity of AuNPs, AgNPs and IONPs can lead to reduction in their toxicity, making their clinical application possible. Surface coating material may modulate nanoparticles toxicity either directly or by altering penetration in cell. After internalization, nanoparticles can interact in dose and colloidal stability dependent fashion with different intracellular systems such as mitochondria, ribosomes and chromosomes. AuNPs and AgNPs have shown to induce production of ROS that can arrest different cell processes. On the other hand, IONP have shown to modulate intracellular signaling pathways, thus altering different cell processes leading to cell death. Careful selection of coating materials and comprehensive characterization of surface coated nanoparticles is prerequisite for clinical applications. We further stress the need of uniform guidelines of test procedures that will aid in systematic analysis of toxicity of different nanomaterials.

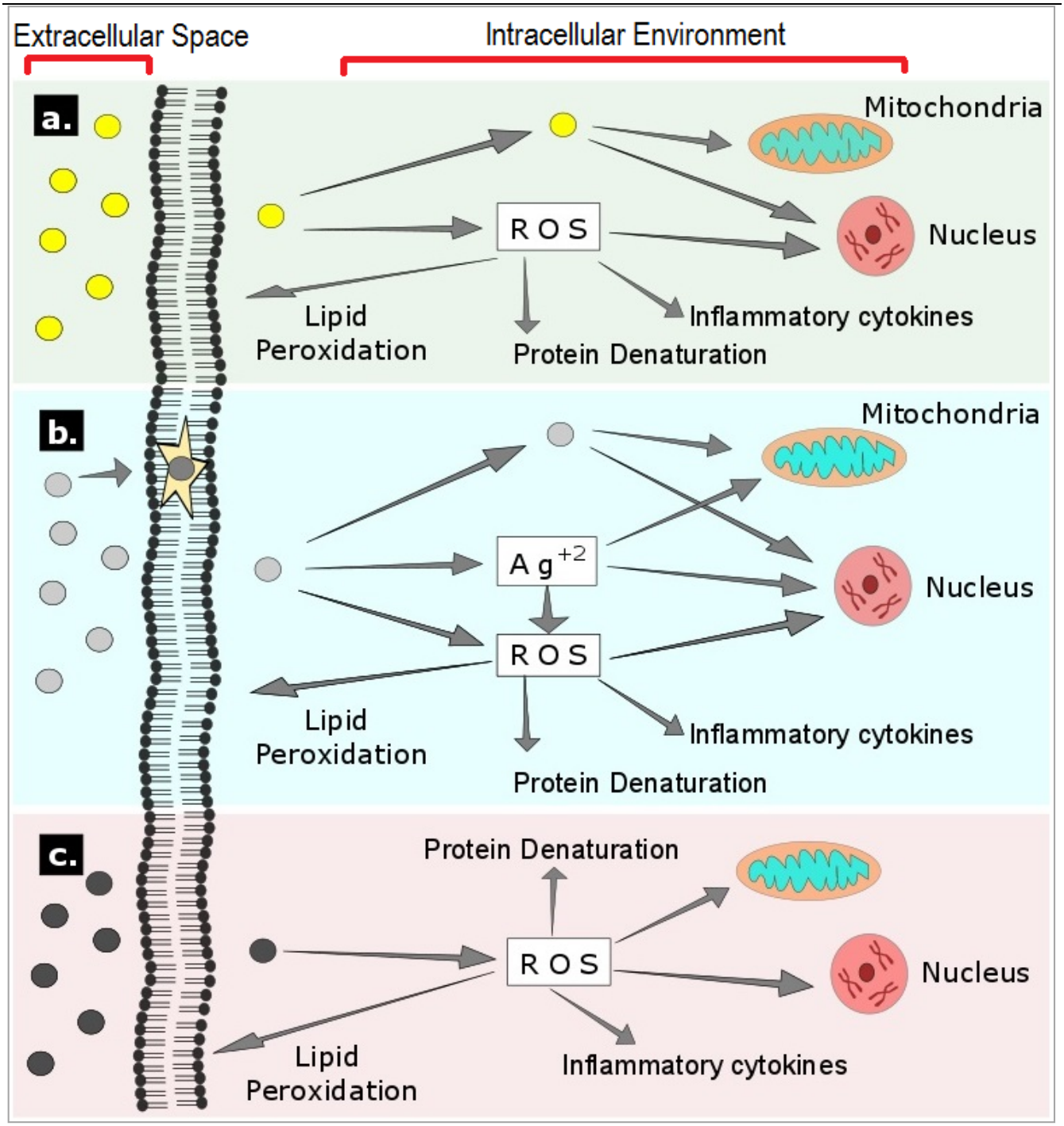

Figure 3. Mechanism of toxicity after internalization into cell, a) gold nanoparticles cause toxicity by physical interaction and ROS production, b) silver nanoparticles case toxicity by physical interactions, silver ions (Ag^{+2}) and ROS production and c) iron oxide nanoparticles intracellular toxicity by ROS mediated oxidative stress.

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