Possible Solutions to the Associated Cytotoxicity of Silver Nanoparticles

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ABSTRACT
Nanotechnology is the emerging field because of its antimicrobial and antibacterial activities. Because of these activities they can be used in different industries now-a-days. Because of this reason silver nanoparticles can be used in the drugs which may treat many fungal, bacterial and microbial diseases in human beings. Besides of these application, its concern related to the biological impacts of silver nanoparticles are also studied. Also their emerging risks and their problems in environment and living organisms are also studied. Many studies have been published in which their cytotoxicity effects on human health and on environment are investigated. An important factor related to the cytotoxicity of nanoparticles is that they may also increase the damage of genetic material as they can cross the cell membrane and nucleus because of their small size. So, there is an interest in studying the cytotoxicity of silver nanoparticles and also gives its possible solution to reduce its toxicity which may harm the human body or environment. For this reason, difference sizes of nanoparticles were used and synthesized to check their effect on environment and human body. Many studies are required for the solutions of the toxicity related to silver nanoparticles. This review is related to the recent works and publications and researches on the solutions of the toxicity related to silver nanoparticles and also about the more and safe application of silver nanoparticles. This study tells us that the silver nanoparticles which are naturally occurring are not toxic to the human body and environment instead of the silver nanoparticles which are artificially synthesized. This review also tells that that the human cells shows resistivity towards the cytotoxicity of silver nanoparticles while the silver nanoparticles toxicity may affect other organs very much. Also it concludes that how we can resist the toxicity of the chemically synthesized silver nanoparticles.

Key words: Silver Nanoparticles, cytotoxicity, characterization methods, physiological sensing**.**

INTRODUCTION:

Nanotechnology is a wide branch which can be defined at different levels which may include atomic, molecular and macromolecular levels (Roco, 2011). The basic blocks of nanotechnology are nanoparticles. Nanoparticles fall in the category of particles which have at least one dimension of <100nm. Nanoparticles comprise of oxides, organic and carbons and due to their composition they have different properties from random materials and particles which are present in the bulk (Hulla *et al*., 2015).

Nanoparticles have features like have large surface area, high stability and chemical reactivity and high mechanical power. Some nanoparticles are naturally occurring, some are fabricated and some are produces as a by- product. Nanoparticles are very important and can be used in biological systems. Viral capsids, peacock feather details, spider and spider mite silk are some examples of nanoparticles in biological structures (Sharma *et al*., 2015). Some biological colloids such as blood and milk are nanoscale. Horny structures which may include feathers, nails, hairs, beaks, claws and horns are also nanoparticles. Paper, cotton ever our own matrix consists of nanoparticles (Zänker& Schierz, 2012). According to the properties, some of the nanoparticles can be synthesized or fabricated commercially or in labs, e.g. carbon black and TiO_2 particles (Shang *et al*., 2014). Metal catalysts used alone are subjected to poisoning which reduces their efficiency. Pt catalyst used in fuel cells is subject to poisoning due to agglomeration of reactants on its surface reducing its surface activity. Besides this, Pt is very rare and expensive to use (Grillo *et al*., 2015). Some nanoparticles are formed as by-products and are called as incidental nanoparticles. For example, burning of carbon based fuel produces fullerenes along with carbon dioxide and water vapors

(Nogueira *et al*., 2014). Atmospheric nanoparticles are referred to as ultrafine particles which may because of air pollutions. These ultrafine particles may be of dust, lead or sulphur (Haihua Wu *et al*., 2011).

Nanoparticles are classified into organic, inorganic and carbon based and commonly known organic nanoparticles are Dendrimers and Liposome. They possess excellent properties such as environmental and non- toxic (Margulis-Goshen & Magdassi, 2012). Nano capsules show sensitivity to the heat and electromagnetic rays. These are used in biomedical fields due to remarkable properties, nanoparticles are used in drug delivery (Zhang *et al*., 2014).

All particles which are not made up of carbon are called as inorganic nanoparticles. It is further categorized into two classes including metal based nanoparticles and metal oxides based nanoparticles (Altavilla& Ciliberto, 2017). All particles which are made up of metals are called as metal based nanoparticles (Tourinho *et al*., 2012). All metal particles can be made into nanoparticles through various methods. For example, the metals which can be used to make their nanoparticles are gold and silver (Sweet *et al*., 2012). The oxides based nanoparticles are used to enhance the properties of the respective metal in Nano range (Solanki *et al*., 2011). The efficiency and the increased chemical reactivity is the main reason to synthesize the metal oxide based nanoparticles. Commonly synthesized metal oxide nanoparticles are iron oxide (Fe_2O_3), Aluminium oxide (Al_2O_2) and silver oxide (Ag_2O) (Miller *et al*., 2014).The particles which are only made up of carbon atoms are called as carbon based nanoparticles (S. Chen *et al*., 2019). These are further categorized into Graphene, Fullerenes, Carbon Nanotubes (CNT), Carbon Nanofibers, Carbon black.(Jiang *et al*., 2013).

It is made up of carbon atoms of sp^2 hybridization. Its shape is spherical. Fullerenes can be single layered or multilayered (Brinkmann *et al*., 2012). Carbon nanotube is a graphene Nano foil. The diameter of CNT is 0.7 for single layer and 100nm for multilayered (De Volder *et al*., 2013). It can varies in length from micrometers to several millimeters. The shape of CNT is hollow. CNT has remarkable mechanical strength (Rahmandoust & Ayatollahi, 2016). It is also made up of graphene but it is designed to cone or cup shaped (Feng *et al*., 2014). It's an amorphous material composed of carbon. Its shape is spherical and diameter is 20 to 70 nm (Donnet, 2018). It is formed due to strong interaction between the carbon atoms based nanoparticles (Vicentini *et al*., 2016). In 2007, a new scheme of classification of nanoparticles was described on the fact that how many dimensions of a nanoparticle fall in nanoscale. (Bhatia, 2016) In other words, it can be explained by the movement of electron. Number of dimensions in which electrons is allowed to move is referred as dimensions of nanoparticles like 0D, 1D, 2D and 3D (Purbia & Paria, 2015). In such nanoparticles, electrons are entrapped in a dimensionless entity which is structurally and electrostatically isolated from the bulk (Barnakov *et al*., 2019). Quantum dots are common example of such nanoparticles which consist of 100 to few thousands atoms in which electrons are confined. Quantum dots are categorized in planar QD, vertical QD or self-assembled QD based on the shape of electron confinement. Examples: Fullerenes, Quantum dots (Meffre *et al*., 2011). Different approaches are adopted for the fabrication of nanoparticles like biological methods, physical methods and chemical methods. Most commonly used and facile synthesis methods include hydrothermal process, sol-Gel method, solvothermal method and co- precipitation method (Rane *et al*., 2018).

Hydrothermal process is opted for the synthesis of materials whose constituent's solubility in water differs at high temperature (Lu *et al*., 2011). A heterogeneous reaction occurs in aqueous media above 100 ºC and 1 bar. Reaction is performed in Teflon lined steel bottles which can bear such high ranges of temperatures. Temperature can be as high as 300 ºC. At these elevated temperatures counter flow of reactants (by convictive process) occurs due to change in solubility level at high vapor pressures of solutions (R. Chen *et al*., 2014). Sol- Gel method is wet technique for crystal growth of many materials especially metal oxide nanoparticles of silicon and titanium. This technique is used to synthesize polymer based nanoparticles or nano-composites (Lemine *et al*., 2012). As the name refers, in this technique two solutions are made ―SOL‖ and ―GEL‖.

Sol is defined as a colloid or heterogeneous solution in which solid particles are suspended among continuous liquid phase which is usually prepared by dissolving the materials in suitable solvent.

Different kinds of precursor materials are dissolved to form sol like halides, nitride, alkaloids and metal oxides (Samat & Nor, 2013). Gel is jelly like material which consists of interlinked network of fibers which do not flow in their steady state. This ranges from soft to hard and tough. Due to three dimensionally cross linked fibers they become tough. They can be referred to as solution of liquid in solids giving them their characteristic strength (Khan *et al*., 2016). This bottom up chemical approach is widely employed due to its simplicity. Mixing of sol and gel leads to formation of hard product followed by their calculations. Reaction between Sol and Gel occurs in three stages from the hydrolysis of precursor, Condensation Crystal Growth and Agglomeration and Drying (Dwivedi *et al*., 2011).

Solvothermal process is more or less similar to hydrothermal method. In hydrothermal method water is used as a solvent while in solvothermal process any other solvent like alcohol or any other solvent of organic or inorganic nature can be used. Process is carried out in Teflon lined steel vessel in autoclave because high temperature and pressure are required just like hydrothermal method. So solvothermal method is a technique by which precursor solution are transformed into desired products by using solvent other than water and heating it above boiling point and atmospheric pressure. These conditions are known as hydrothermal conditions. Variety of metal oxides, semiconductors, ceramics, polymers and nano-composites are synthesized by this method. Zinc oxide is prepared by solvothermal method which exhibits quantum dots effects (Hongyan Wu *et al*., 2012).

Low solubility of a compound in a specific solvent is the basic concept behind co-precipitation method. Two or more components which are formed from precursor solution can be precipitated out due solubility differences. That is to say precursor is soluble in a specific solvent at certain pH when pH is raised or decreased insoluble entities formed precipitate out (Mascolo *et al*., 2013). In this method a precipitation agent can be used. Most commonly metals hydroxides are insoluble in water. When an alkali (precipitating agent) is added in a precursor solution of metal (which may sulfates, chlorides or nitrates of respective metal) which is soluble, hydroxide newly form precipitate out immediately. In this way, many metal

hydroxides can be precipitated. Reaction behind this phenomenon is: $\boldsymbol{A^{A^+}} + \boldsymbol{AAA} \rightarrow (\boldsymbol{AA})$ (Petcharoen & Sirivat, 2012).
Graphene is an allotrope of carbon having sp2 hybridization forming sheets of attached carbon atoms via continuous hexagonal rings. Each carbon atom has 4 electrons to make bond. Three of the electrons are involved in making bond with neighboring carbon atoms in the same plane while forth electrons is perpendicular to the plane of Nano sheet which forms pi bond between the sheet holding the sheets. Usual thickness of graphene layer is 1nm (Bai *et al*., 2010). Graphene is known as zero overlap semimetal which contains both holes and free electrons to conduct electricity and heat in one dimension. This electronic flow is due to pi electron which reside perpendicular to the plane of the other three bonds. Electronic properties of graphene are assigned due to bonding and anti-bonding (conduction band) of these pi electrons. Electronic mobility of above 15000 cm^2/Vs. reported up till now (Soldano *et al*., 2010).
The most used nanoparticles are silver nanoparticles. They have chemical and physical properties of their own kinds. Due to this, they can be used in different fields such as, medical, food, health care, consumer and industrial purposes. These nanoparticles are unique and they can change their physical, chemical and biological properties depending upon their surface to volume ratio, that's the reason why these nanoparticles are used in various fields. Different methods are used for the synthesis of AgNP's. Generally, the physical and chemical methods used for the synthesis of AgNP's are very expensive and dangerous. So, mostly biological methods are used for the synthesis of AgNP's. Due to these methods, there is high yield, solubility and high stability of AgNPs. So, biological methods are simple, non- toxic and cheap for the synthesis of AgNPs. There are two approaches to synthesize nanoparticles.

- Approach based on bottom up strategy.
- Approach based on top down strategy (Mulfinger *et al*., 2007).

In this approach, nanoparticles are formed from simple substance and this is known as the building up approach. Bottom up approach includes the Sol Gel and synthesis of nanoparticles by biochemical synthesis (Haes & Van Duyne, 2002). In top down methods, destructive approach is adopted. It starts from the larger molecules which through different methods are breakdown into smaller and simpler units and these units are further transformed into the desired nanoparticles. This method include the grinding of the bulk, CVD, vapor disposition based on physical properties and other techniques such as electro-explosion and laser ablation (Tiwari *et al*., 2008) Silver and its compounds also have many antimicrobial activities. In earlier 19^{th} century a German scientist uses $AgNO_3$ in the treatment of many microbial diseases such as *ophthalmic neonatorum.* The silver usage was disappeared when first antibiotic i.e. penicillin was invented. But in present era, these antibiotics show resistance too many diseases so, use of silver again increased. The major risk of using the silver is that they can be inactivated by complexion and precipitation (Laliwala *et al*., 2014).
So it's alternative come in the form of silver nanoparticles which are zero valent and very valuable. It is harmless and safe antimicrobial agent and also safe for human body. Beside to it, it also gained some antimicrobial activities which may include antifungal, anti-inflammatory and antiviral activities. These can be applied in therapies. It also has some apoptotic activities as hydroxyl ions can show their interest in apoptotic deaths. Several studies also describe the greater and strong activity of AgNPs with other compounds. When amoxicillin was combined with the AgNPs it showed greater and strong activity against the bacteria i.e. *E. coli* rather than when they were used separately. Polymyxin B when interacts with Ag NPs, they show strong effectiveness against gram negative bacteria (Jain *et al*., 2019).
Some bacteria also show resistance to antibiotics and multiply in a great number in the presence of antibiotic. Biofilm is same to this resistivity. Biofilm matrix is 3D, gel- like environment having lots of water in it and lots of charges in it. Bacteria stick with the surface through this environment and may also affect the entire organ. Additionally, ongoing articles showed that deadly dosages of bactericidal anti- toxins make hereditary and many biochemical changes and advances the arrangement of profoundly unfavorable oxidative extremist species. For example, the various classes of bactericidal anti- infection agents actuate hydroxyl revolutionary development, while the other various classes of bacteriostatic medications don't produce hydroxyl revolutionaries. But when AgNPs interact with the antibiotics then it can affect the biofilm and also affect the bacteria and viruses making them ineffective to human beings (Owaid, 2019). The most important and used nanoparticles are sliver nanoparticles because of their antimicrobial activities. On contrary to that, there are some problems which may include the toxicity of Ag NPs. They are toxic to the cell membranes as they result in the breakdown of cell membrane, damage of proteins or DNA and formation of ROS. Some plants can also exposed to Ag NPs through the water which may contain contaminants during irrigation, by using bio-solids which are contaminated by Ag NPs or discharge of water which may contain wastes from industries or houses. There are many studies related to the toxicity of the Ag NPs which may harm many bacteria, fish and freshwater alga. Some scientists indicate that Ag NPs may affect the photosystem II of freshwater alga. Some scientists indicate that Ag NPs may also affect the plant growth by affecting its cells of cortical of roots, epidermis and root tips (Borase *et al*., 2014).

Nanotechnology is a field of science which is increasing day by day. Their uses and applications become so valuable and important for daily life. So to compensate with this increasing demand of nanoparticles green synthesis method is used. As population is increasing day by day, waste is also increasing. So to remove these waste materials from the globe, the chemistry of green processes and the chemical processes related to this chemistry are also used in science and industry. So for using green synthesis process, there are 12 important processes which should be kept in mind.
These principles are used in making the chemical processes work well and also give the logic about toxic materials (Emam *et al*., 2016).
Silver is very toxic when it is mixed with water and it is very harmful for living organisms. It becomes less toxic when it is converted into silver nanoparticles where it acts as an agent which can kill the microbes. So in many cases, it can also mix

with aquatic water as it can go to the water by several pathways. It has positive as well as negative effect in the environment. In case of negative effect in the environment, it can cause harmful effect in the growth of plants due to which ecosystem can't change regularly and cause irreversible damages. The research work on effects of NPs on plant is very limited. Also it was seen that Al nanoparticles could diminished the growth of roots and shoots (Tripathi *et al*., 2017). As the size of nanoparticles is very small which is 1 and 100 nm and sue to this it has many basic and unique properties. But it is to be said that nanoparticles may have serious effects on the environment and on the life of human beings. As it has strikingly higher surface to volume proportion of NPs upgrades their surface properties in this manner expanding the connection with serum, saliva, bodily fluids or lungs lining liquid segments and makes NPs conceivably more receptive than bigger particles. As there are a number of studies which are related to toxicity of NPs and but no appropriate conclusion is made because the work was not performed under experimentations (Y. S. Kim *et al*., 2010).

AgNPs have antimicrobial activities and they are widely used now-a-days all over the world. These are also used in many medicinal products such as bandages and other items. As it is very much beneficial but is can also cause toxicity in many species. If silver is exposed for a long time it can cause a disease which is called as argyria or argyrosis in human beings. The toxicology of AgNPs has additionally been appeared by various in vitro contemplates. Toxicological examinations of NPs infer that size, shape, solubility and capacity to tie have an impact on the toxicology of NPs.

The surface of high region of metal based NPs increment the potential that metal particles are delivered from these NPs. Although, the highest toxicity of the silver particles are known, estimation of the measure of silver particles inside examined AgNPs suspension has been seldom done and along these lines information about how much silver particles add to the toxicity of AgNPs is as yet restricted (Beer *et al*., 2012).

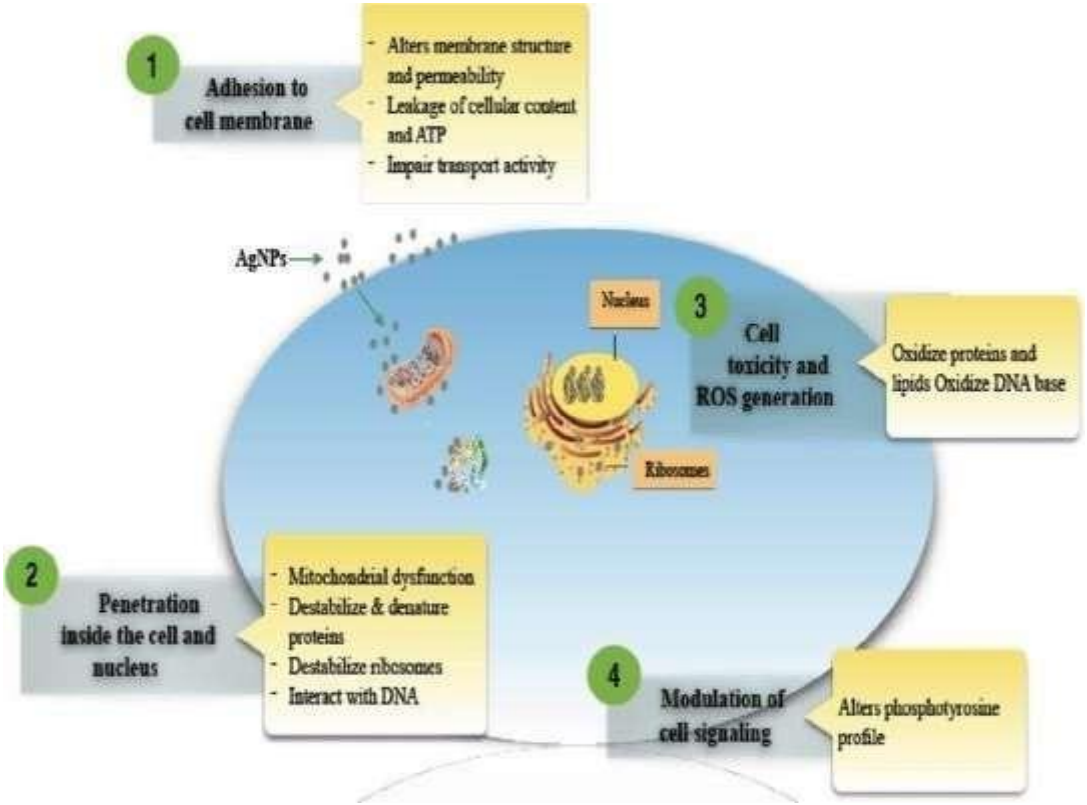

Figure 1: Shows the entry of AgNPs in the cell

This diagram shows that how AgNPs can enter the cell. In the first step, AgNPs adhere with the cell membrane due to which there is alteration in the membrane structure which affects its permeability. Also the cellular content and ATP leakage takes place due to which there is impaired transport activity. In second step, AgNPs penetrate inside the cell and nucleus. It interacts with the mitochondria and other organelles and destabilizes and denatured the proteins. It also destabilizes the ribosomes and interacts with the DNA. In third step, it forms toxicity in the cell and produce ROS in large amount by oxidizing the proteins, lipids and DNA bases. In last step, there is modulation of cell signaling where there is alteration of phosphotyrosine profile and ultimately cell death occurs.

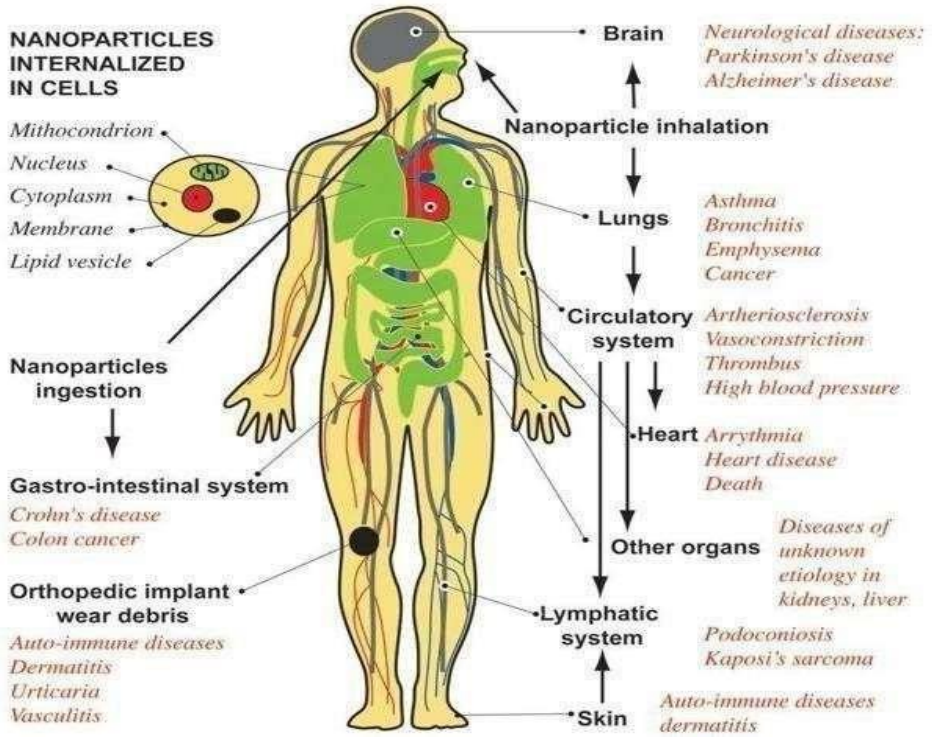

Figure 2: Shows the effect of AgNPs on body organs

This picture shows the entry of AgNPs in the human body and how it affects the human organs. The AgNPs are present in the environment and can cause the environmental pollution. When we inhale the AgNPs in the body, it passes through the air pathways and can affect the lungs and other body parts which may cause severe health consequences. It also affects circulatory system and lymphatic system and through olfactory system it can affect the brain and can cause severe Neurological diseases such as Alzheimer's disease and Parkinson's disease. It also affects other organs such as liver and kidney and can cause liver and renal failure. It also affect gastrointestinal tract and cause some severe diseases such as Crohn's disease and colon cancer and also affects skin by causing different diseases such as autoimmune diseases and dermatitis. In this way AgNPs can affect the body organs.

In vitro cytotoxicity of silver nanoparticles on human periodontal fibroblasts

Juan Francisco Hernández-Sierra and his colleagues demonstrated that silver nanoparticles are widely utilized in a large number of antimicrobial activities in clinical examination. Their adequacy has been exhibited against streptococcus freaks, which is related with dental caries. Be that as it may, their cytotoxic impacts on human periodontal tissue are not totally perceived (Braydich-Stolle *et al*., 2005). The goal of this study was to assess the cytotoxicity impact of silver nanoparticles on an in vitro model of essential fibroblasts culture disengaged from human periodontal tissue. For this, silver solution was used with a reducing agent and their ratio was greater than 1. An agent was also used to stable the size of the nanoparticles. Three different sizes of nanoparticles were used: less than 10 nm, 15-20 nm and 80- 100 nm. Their pH was maintained by using nitric acid.

After this human periodontal fibroblast primary cultures were made (Alt *et al*., 2004). These were extracted from the healthy donor. After extraction they were placed in Hank's saline solution. Then they were put in petri dishes containing Dulbecco's altered Eagle medium enhanced with 100.5U/mL penicillin and 100.5µg/mL of streptomycin. Periodontal tissue was painstakingly taken out from the coronal area and $1mm^2$ explants were brooded with 2.5mg/mL collagenase F, and 0.25% trypsin in a supported saline arrangement for 2hrs under controlled conditions. After this the cells were centrifuged at high speed for 7mins. After this the cells were grown in 96-well cultured microplates for experiments. Then cytotoxicity assay were made to check he cytotoxicity effect of silver nanoparticles on human periodontal fibroblasts monolayer (Braydich-Stolle *et al*., 2005).

For this cells were grown in microplates and were heated with three different sizes of nanoparticles. Nanoparticles with size less than 10nm are incubated for 24 hours and NPs with sizes 15-20 nm were incubated for 72hrs and that of the NPs with sizes 80-100 nm were incubated for 7 days. Then 25mL of MTS/PMS were added to each cell type well and were heated for 1hr at 37 ºC in dark. 3 times the experiments were designed. The cytotoxic effect of less than 10 nm NPs were checked as they were heated with 1 -1000 µm NPs for 24hrs, 72hrs, and 7 days. It was shown that less than 10nm sized NPs treated fibroblasts with 250, 500 and 1000 µm were decreased continuously. Same results were shown by 72hrs of incubation. But after 7 days an increase in cell viability of fibroblasts was shown at concentrations 250, 500 and 1000 µm.

After this, the nanoparticles of size 15- 20 nm were presented to human fibroblasts of concentration 10- 1000 µm for 24- 7 days. A reduction in cell viability was seen. Then there was no any decrease in cell of viability of 80-100 nm sized nanoparticles which were heated with human periodontal fibroblasts cells. So, from this study, it was concluded that the NNPs of size 80-100nm had no any cytotoxic effect on the human periodontal cells with fibroblasts but an increase in cell

viability could be seen with respect to time and concentrations of the fibroblast cells (Keiser *et al*., 2000).

Controllable synthesis of mono-dispersed silver nanoparticles as standards for quantitative assessment of their cytotoxicity

Yan Zhang and his team demonstrated that now-a-days Ag NPs care used as antimicrobial and antibacterial agents. Besides this, Ag NPs has some toxicity. In this work, a surfactant polyvinyl pyrrolidone (PVP) is used to get the different sizes of Ag NPs are obtained which are: 25, 35, 45, 60 and 70 nm respectively. For determining the size and characterization of size of Ag NPs, time, concentration of surfactant and reactants play an important role (Wang *et al*., 2011). For this purpose, DMEM medium is used to culture the HLF cells which was characterized by 12% of fetal bovine serum, 2.5mM of L-glutamine and 1.5% of penicillin streptomycin. Humidified incubator with controlled condition was used for the growth of the cells. After this cell viability assays were used. By using MTT assay, the viability of HLF cells which was treated with Ag NPs, was measured. This is a colorimetric assay which is used to detect the change of color from blue formazan to the purple color when MTT assay reduce its activity by using enzymes which showed the viability and proliferation of cells. In 96-well microplate, the HLF cells were placed and for overnight, they were incubated (Shi *et al*., 2006). After this, the supernatant cultures were replaced by different sizes of Ag NPs of various concentration 255, 120, 62 and 31.25 µg/mL for 72 hours. Then staining techniques were used. For this purpose, Annexin V stain was used to clear the difference between necrosis and apoptosis which was due to Ag NPs. Phosphatidylserine concentration was high in this staining agent. The second staining agent which was used to distinguish between the necrosis and apoptosis through the damaged and live cells of cell membrane was PI stain. Synchronous staining of cells with FITC- Annexin V (green fluorescence) and the non-imperative color PI (red fluorescence) permits the separation of flawless cells, early apoptotic or necrotic cell death. At 250 µg/mL concentrations of different sized Ag NPs were used for the test.

LDH Production

After this, the LDH production was detected (Teodoro *et al*., 2011). Cell death may occur due the necrosis or the apoptosis. Necrosis is due to the mitochondrial expanding and expanded plasma film penetrability, while apoptosis is due to the verbalized breakdown of the cell into film bound apoptotic bodies. LDH is dissolvable cytosolic chemical that discharged in culture medium after loss of layer uprightness coming about because of the apoptosis or necrosis. LDH action, thusly, can be utilized as the pointer of cell layer uprightness and filled as an overall way to survey the cytotoxicity coming about because of substance compounds or natural poisonous variables. The different sized Ag NPs of concentrations 250 µg/mL were used for experiment. After this, the ROS production was detected (Lubick, 2008). For this purpose, fluorescence of 2, 7-dicholorofluorescein was used for the measurement of ROS. When DCFH-DA passed through the cells, the esterase's which is present inside the cell separate out its diacetate group. Due to this, non- fluorescent dichlorodihydrofluorescein converted into exponentially green 2, 7- dichlorofluorescein. After 72hrs of incubation period of different sized Ag NPs of concentrations of 250 µg/mL with HLF cells, ROS was measured.

Absorbance measurement by using UV-Vis spectroscopy

TEM was used to get the particle sizes and the shapes of the Ag NPs at a speed voltage of 200 kV. To measure the absorbance of Ag NPs, UV- Vis spectroscopy technique was used as shown in Fig 3.

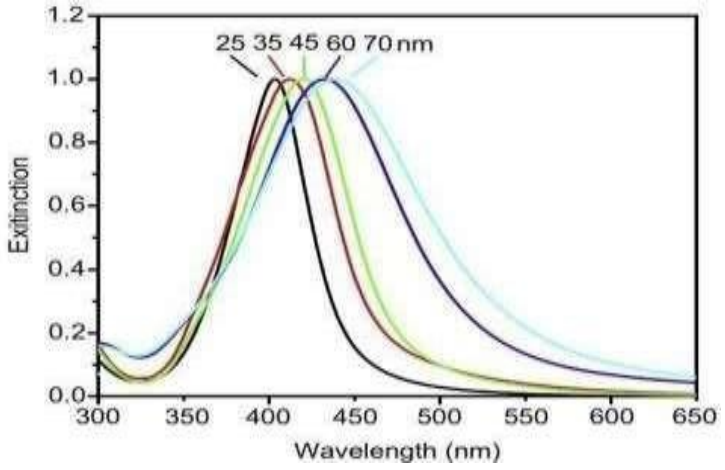

Figure 3: UV- Vis spectra of different sized Ag NPs

MTT assay test were detected by using ELISA reader. For cytotoxic study, the five distinct sizes of Ag NPs which was characterized by the PVP were used to check the cell viability, apoptosis, necrosis and LDH production. To check the cell viability, five sized Ag NPs characterized by PVP were used and there were increase in the size of particle and increase in cell viability.

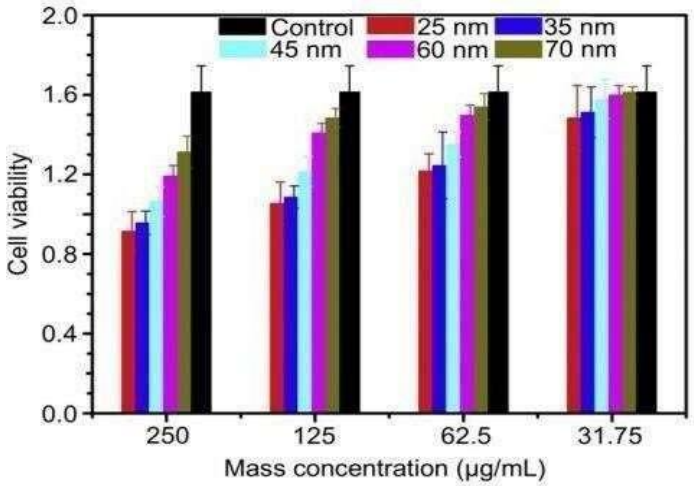

Figure 4: Increase in cell viability and particle size

This shows that the cell subordinate cytotoxicity diminished with the expanding of the Ag NPs sizes. It was predicted that the sizes of Ag NPs 60 nm- 70 nm have a slight cytotoxicity at high doses. On the other hand, the Ag NPs of sizes 25, 35 and 45 nm at high doses shows higher cytotoxicity and 50% death rate of the cells was recorded. But at lower doses, all five sized Ag NPs showed the slight cytotoxicity. This was predicted that the dose and size show a significant role in the cytotoxicity process (Samuel & Guggenbichler, 2004). Different staining techniques were used to differentiate between necrosis and apoptosis. It was shown that the necrotic and apoptosis death increased when the particle size decreased from 70- 25 nm of concentration 250 µg/mL as shown in figure 5. Here again particle size plays an important role.

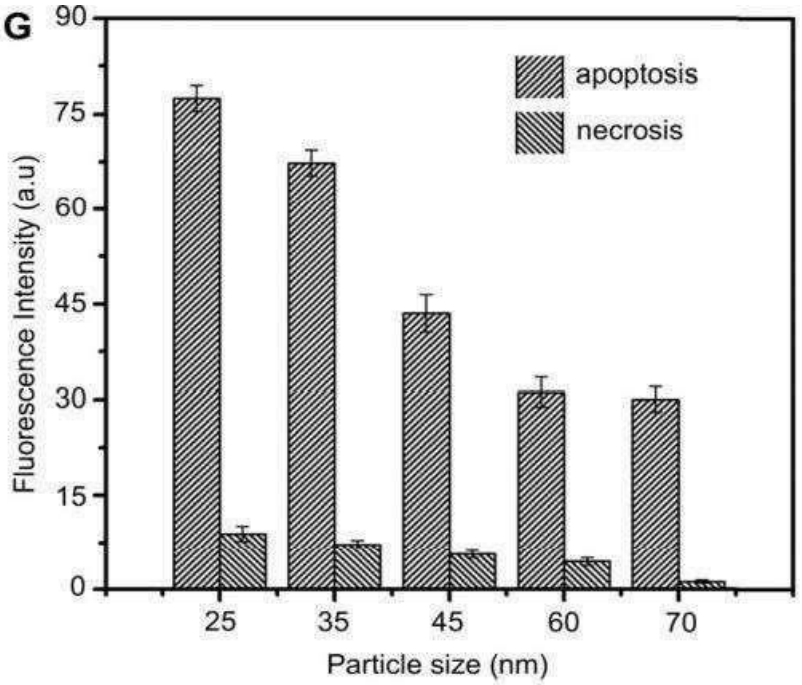

Figure 5: Annexin- PI staining of Ag NP characterized HLF cells

LDH assay was also observed. LDH leakage is due to the high rate of death of cells and increase in damage of plasma membrane. There is decrease in the rate LDH when the size of Ag NPs increases with 72 hours. This perception proposes that the smaller sized Ag NPs are adept to be taken up by the cells and hence brings about the harm of the plasma membrane as shown in figure 6.

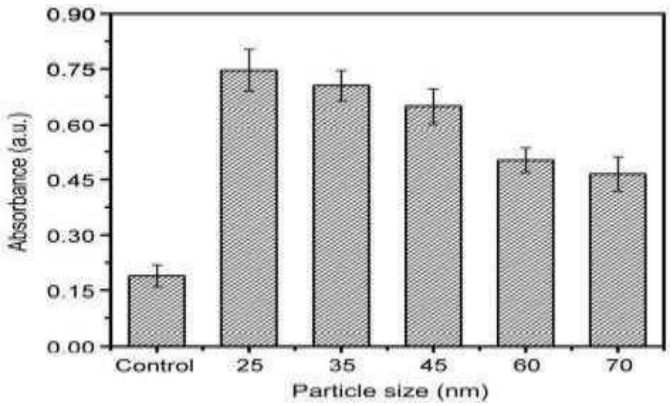

Figure 6: Production of LDH in Ag NPs treated HLF cells

So it was predicted that the cytotoxicity increase with the decrease of Ag NP size and increase of concentrations. Overall, these outcomes are ascribed to the creation of different sized Ag NPs and hence give information on Ag NP cytotoxicity and a reason for such risky appraisal. Additionally, it is basic that the organic applications utilizing Ag NPs ought to be given exceptional consideration other than accepting antimicrobial potential (Benn & Westerhoff, 2008).

Magnetic Nano-beads decorated with silver nanoparticles as cytotoxic agents and photo thermal probes Riccardo Di Corato and his colleagues, predicted an adaptable strategy for adorning magnetic Nano beans which were treated with 3-6 nm sized silver nanoparticles. Authority over the silver nanoparticles inclusion at Nano bead surface is accomplished by changing the response boundaries (Powers *et al*., 2011). For this purpose, a polymer matrix was used which was made up of poly (maleic anhydride-alt-1-octadecene) in which a number of MNPs were placed. Then Ag NPs were nucleated. The carboxylic group which was deprotonated by the process of hydrolysis of anhydrides could associate with silver ions at basic pH. Then reduction of silver ions occurred by using the sodium borohydride (Ott *et al*., 2007). It could be detected by the change of color from yellow to blue to brown as shown in figure 7.

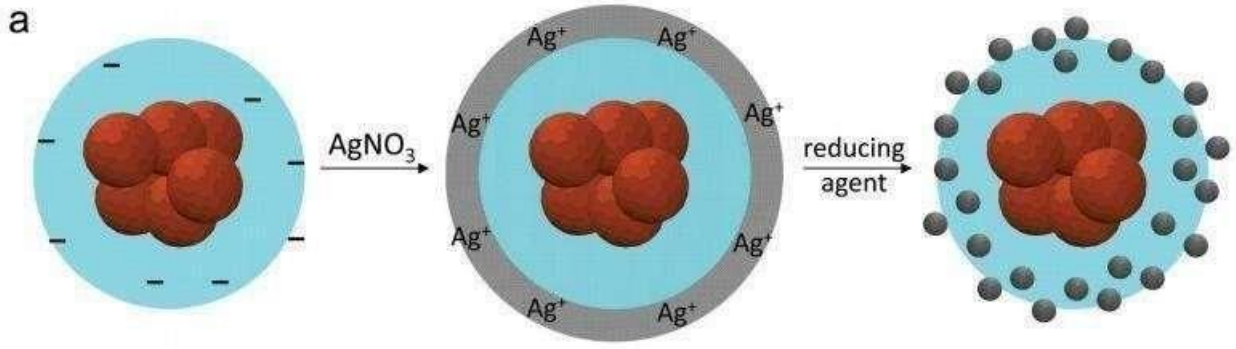

Figure 7: Color change when Ag NPs were nucleated

The cytotoxicity was checked with two different cancer cells HeLa and KB cells. Both cells were treated with the Ag- MNBs at regular intervals. A magnetic field was made by using the magnet placing it with the cell culture dish where time and dose dependent cytotoxicity was found. A relatable cytotoxicity was found in Ag-MNPs in both cell lines at 24hrs with IC_{50} doses at respective concentrations of 32 and 29 µg/ mL. Also the curves of these cells were found in the absence of magnet (Amstad *et al*., 2011). The proliferation effect of the HeLa cells without magnet was very little even at high doses. 80% cell viability of KB cells was shown after 6hrs and when time period increased (12 and 24hrs) the Ag-MNPs show high cytotoxicity at higher concentrations of 20 µg/mL. The samples which were treated with only MNPs which was further not treated with Ag NPs showed no toxicity as shown in figure 8.

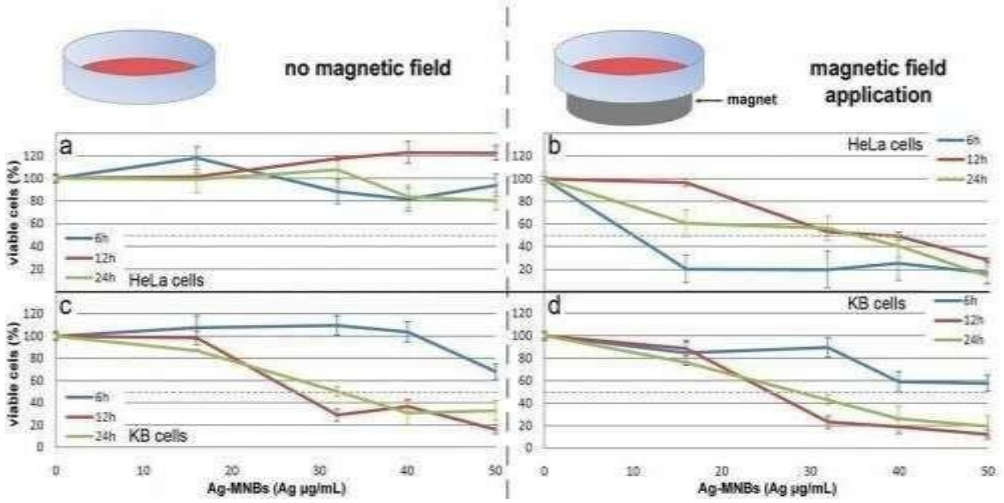

Figure 8: The cell viability of HeLa cells and KB cells in presence and absence of magnetic field

The curves showed that not only due the amount of Ag-MNPs but also due the dose of the Ag- MNPs which a cell took in. for this a concentration of 32 µg/ mL was measured. Figure 8 shows the that the cell took up a large amount Ag- MNBs due to magnetic field around both cell lines as compared to cells without magnetic field.

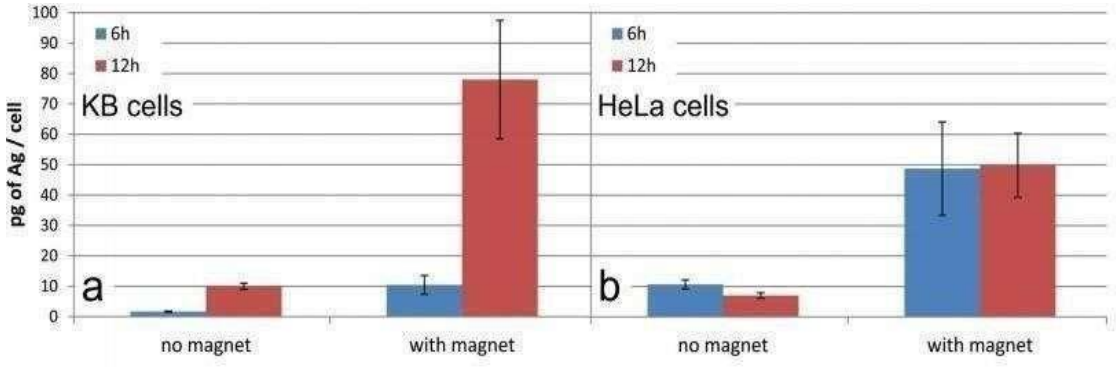

Figure 9: The intracellular uptake of the Ag-MNPs on two cell lineages in the presence or absence of magnetic field

Since toxicity identified with silver particles spillage from Ag-MNBs surface was recognized after giving an ensuring layer to Ag-MNBs; these mixture materials may find some uses for the attractive focusing on and controlled arrival of poisonous silver particles along with photo thermal actuation in disease treatment (Allione *et al*., 2011).

Synthesis and In Vitro cytotoxicity of Glycan- capped silver nanoparticles

Luciana Dini and her colleagues demonstrated in their work that silver nanostructures were effectively blended through a basic and green strategy utilizing sugars as diminishing and covering agents. TEM and UV-Vis spectroscopy were used to guarantee the nature of silver nanoparticles gotten: first and foremost, size and scattering. This work indicates the cytotoxicity of Ag NPs in sugar capping in HeLa cells (Kawata *et al*., 2009). By using positive and negative control the cell viability of HeLa cells were detected after 48 hours when cells were taken under controlled condition by increasing the number of Ag NPs cells. For this purpose, the chemicals used were: silver nitrate, sucrose and α-D- glucose. The filtered water without ionization was used with Milli- Q purified water system was used in whole experiment (Kashiwada, 2006). Then preparation of Ag NPs took place. For this purpose, 5.5 g of sucrose in 100ml of water and was boiled for several minutes then 2.8ml of silver nitrate was added. First of all, 2.2ml of aliquot of 2.8ml of silver nitrate was added to 100 ml of 0.2 M of α-D- glucose. Secondly, 2.2ml of aliquot of 2.8ml of silver nitrate was added to 100ml of 0.12 M of α-D- glucose. Then solutions are boiled for 30min with continuous mixing and color changed to pale yellow and this was the indication of presence of Ag NPs. With the help of spectrophotometer, the absorbance was recorded from 320 and 800 nm. Optical spectra were also detected from the prepared solutions. By using TEM, the images were recorded. The HeLa cells were placed in EMEM medium characterized by 11% fetal bovine serum, 2.5mM of L- Glutamine, 100.2 U/ml of penicillin and streptomycin solution and 10000 U/ml of nystatin which was placed under controlled conditions for 3-4 days. For detection of cytotoxicity, MTT assay which may include 3- (4, 5-dimethyldiazol-2-yl) - 2, 5- diphenyltetrazoliumbromide was used (J. S. Kim *et al*., 2006). The cells which were present with Ag NPs were washed with 0.2 M phosphate buffer which had a pH of 7, after 24 and 48 hours of incubation period. After this they were placed under controlled conditions with 1.2 mg/ml of MTT for 2 hours.

Cell viability measurement by using spectrophotometer

The cell viability could be seen with the help of spectrophotometer at a wavelength of 570nm. For internal visibility of HeLa cells, light inverted microscope was used. In controlled experiments, saccharide solution was used for placing in the HeLa cells. The UV-Vis absorbance spectra of Ag
NPs in α-D- Glucose solution and Ag NPs in α-D- Glucose- sucrose solution can be seen in figure 10. It shows the absorbance at 420 nm as the nanoparticles are spherical.

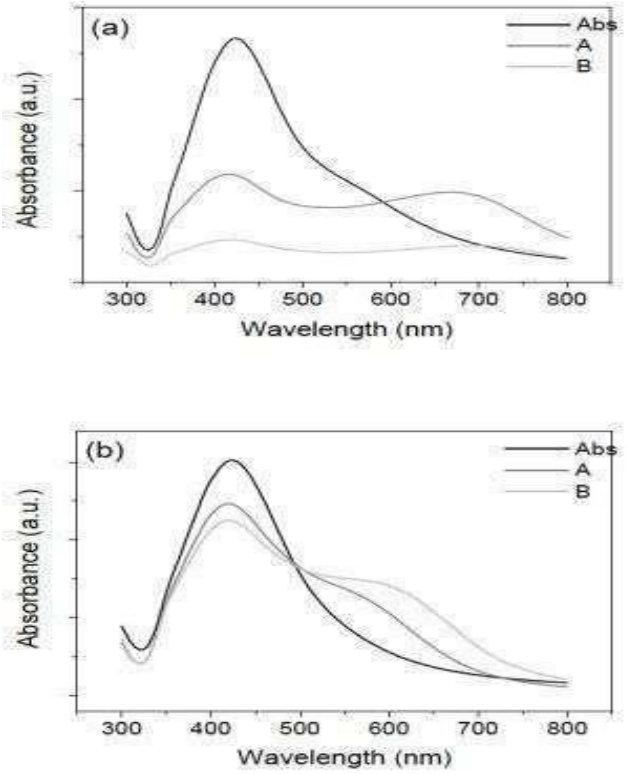

Figure 10: UV-Vis absorbance spectra of Ag NPs G and Ag NPs GS solutions

Images by using TEM

The images taken from the TEM are shown of Ag NPs G and Ag NPs GS solutions.

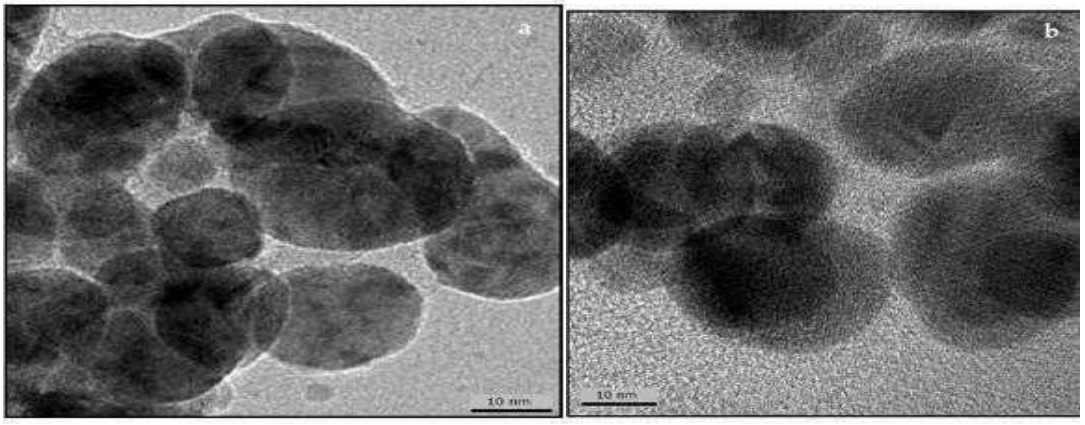

Figure 11: The images from TEM on Ag NPs G and Ag NPs GS solutions

So from above figures, it is evident that nanoparticles are spherical and spread all over. So for this reason, above 50 images are captured from the TEM by Image Pro-Plus Software. Both solutions have nanoparticles of size 5nm to 40 nm and average size is 30 nm with standard deviation of 5nm. For this reason histograms were made which show the size dispersion of the nanoparticles in Figure 12.

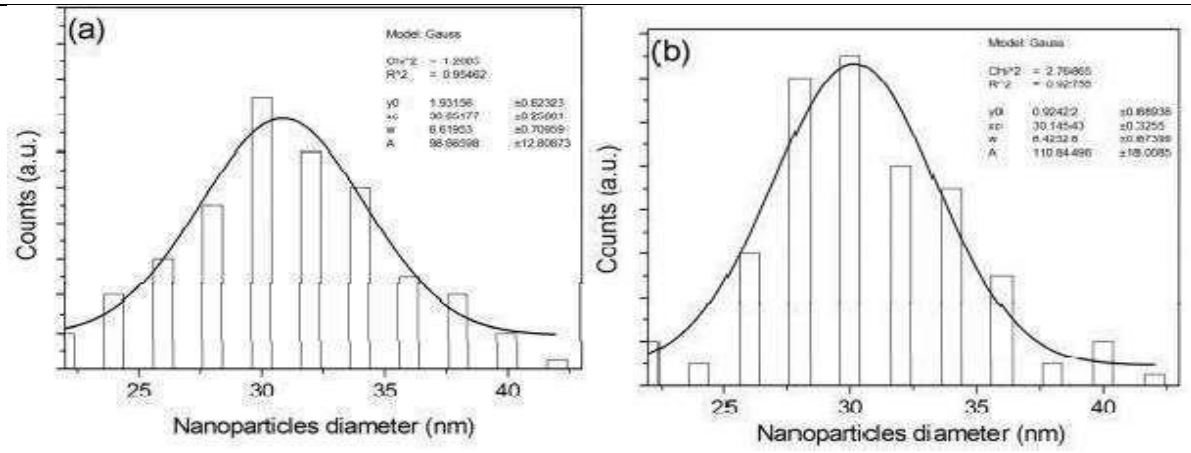

Figure 12: Size distribution of Ag NPs G and Ag NPs GS solution

MTT Assay

The two solutions shoed the cytotoxicity effect after 4 days, when HeLa cells were incubated at different time periods with different numbers of Ag NPs and different concentrations of saccharide solutions. This can be shown in figure 13. This was measured by using MTT assay technique (X. Chen & Schluesener, 2008).

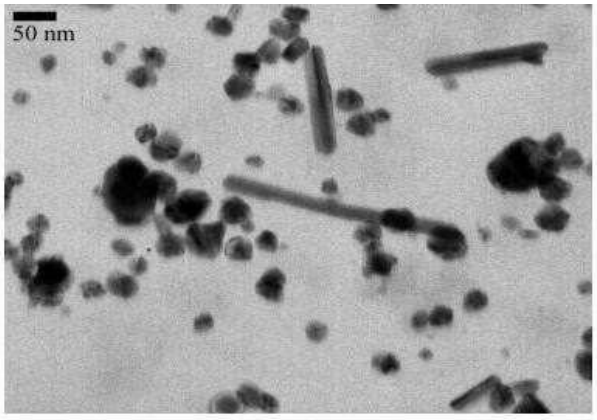

Figure 13: Image taken from TEM of Ag NPs solutions after 4 days

So, cytotoxicity increases with the increase in the number of Ag NPs and incubation time. It was shown that the Ag NPs G is more toxic than that of Ag NPs GS. But 20% of cell proliferation occurred when there are fewer amounts of Ag NPs GS. After 4 hours of incubation period with 13.7 µg/ ml of silver ions, there was death of all cells in the solution. Also cell viability showed no change in the presence of solutions i.e. Ag NPs G and Ag NPs GS at 24 and 48 hours. So this shows that cytotoxicity is only when nanoparticles are present. There is the death of cells when HeLa cells are present in the large concentration of Ag NPs G and Ag NPs GS within 1 hour. So Ag NPs also affect the cancer cells as they behave normal in the presence of Ag NPs. Also 20% of cell death is due the lowest concentrations of Ag NPs GS. So, the information for the communication of nanoparticles with cells plays an important role to synthesized materials from nanoparticles to get the biocompatibility. All this could be used in synthesis of new varieties of materials such drugs or medicines which are used to treat the cancer disease (Maynard *et al*., 2011).

Toxicity of silver nanoparticles in human macrophages: uptake, intracellular distribution and cellular responses

Andrea Haase and his colleagues demonstrated that silver nanoparticles are very important due to their commercial uses and can be used to synthesize large number products due to its antimicrobial properties. On the other hand, only few papers are published about the diverse effects of nanoparticles on human health. Due to different ranges of sizes and shapes, it is very difficult to link the published data with the biological data. In this article, silver nanoparticles are obtained by controlling its size and shape. For these purpose amino acids with L- isomers were used. Nanoparticles of two sizes of 20nm and 40 nm were prepared (Ji *et al*., 2007). For cell culture, THP-1 cell line was used. Cells were grown in RPME medium which was characterized by 10.2% fetal bovine serum, 2.5mM of L- glutamine, 10.2mM of HEPES, 1mM pyruvate, 100.1 U/ml penicillin and 0.5 mg/ml of streptomycin. WST-1 assay was used to check the cell viability. 96- Well plate was used to culture the cells which were incubated with nanoparticles. WST-1 reagent was mixed with the cultured cells after 24 and 48hrs, and the solution is centrifuged after change of color to remove the extra amount of nanoparticles in the mixture. Finally absorbance was detected by using spectrophotometric technique. For testing cytotoxicity of silver nanoparticles human monocytic leukemia cell line THP-1 was used.

Macrophages provide first- line of defense system in vivo working against unfamiliar agents, for instance, foundationally

circulated nanoparticles they are of high pertinence as model framework (Tang *et al*., 2009). Also they allow the synthesis and working of cytokines. In this, the WST-1 assay was used to observe the cytotoxicity related to cells. The cytotoxicity was checked when cells were treated with silver nanoparticles within 24 and 48 hours. In both sized nanoparticles cytotoxicity was checked which depends upon time and dose. The 20 nm sized silver nanoparticles show high toxicity as compared to 40 nm sized nanoparticles due to mass of the dose. After 24hrs the IC (50) value of 20nm and 40 nm sized silver nanoparticles were 110.5µg/ml and 140.5 µg/ml. after 48hrs, the IC (50) values for 20nm and 40 nm sized nanoparticles were decreased to 18.5 µg/ ml and 30.5 µg/ml. The viability curves of both sized nanoparticles were similar. Due to some reasons gold nanoparticles were chemically inactive. Also at higher amounts cytotoxicity was very little but 80% of cell viability was observed (Larese *et al*., 2009).

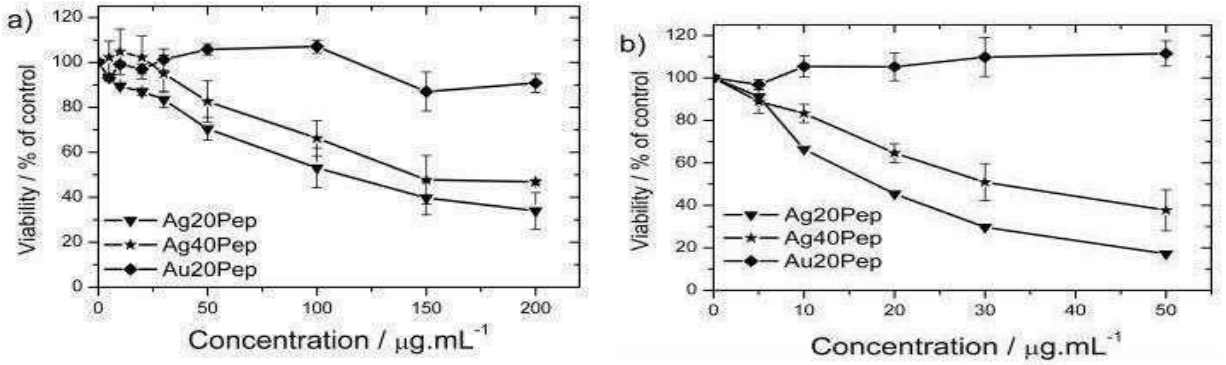

Figure 14: measurement of cell viability of THP-1 treatment with Ag20Pep, Ag400Pep or Au20Pep

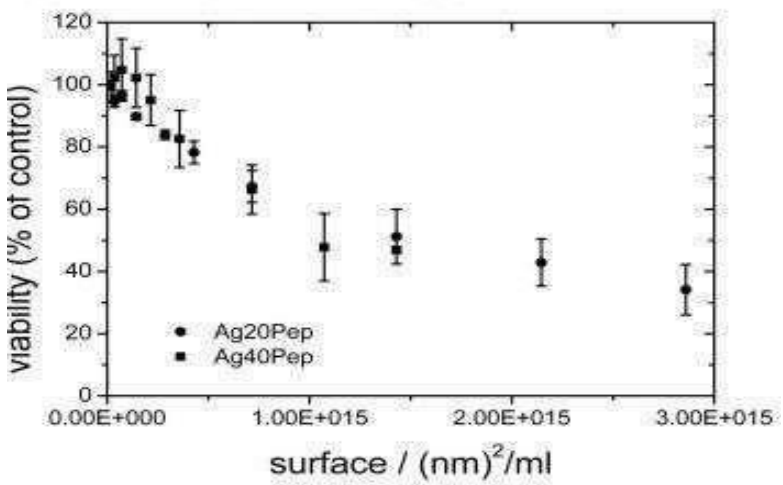

Figure 15: Calculation of cell viability after treatment with Ag20Pep, Ag40Pep or Au20Pep Images through TEM

TEM was used to take the images of macrophages which were mixed with different sized nanoparticles. Two sized silver nanoparticles were observed under TEM when they were ingested by macrophages. Some large pieces of nanoparticles could be seen in the cytoplasm. But a large number of silver nanoparticles could be seen which were small in size and were equally distributed within the cytoplasm and membrane envelop did not get them within it. Also they were present in nuclei but they could be seen deep in nucleoli and lysosome. Furthermore, they were not present in the mitochondria and ER. As they were seen in cell, it could be said that they were taken up freshly without any process of phagocytosis (Foldbjerg *et al*., 2009).

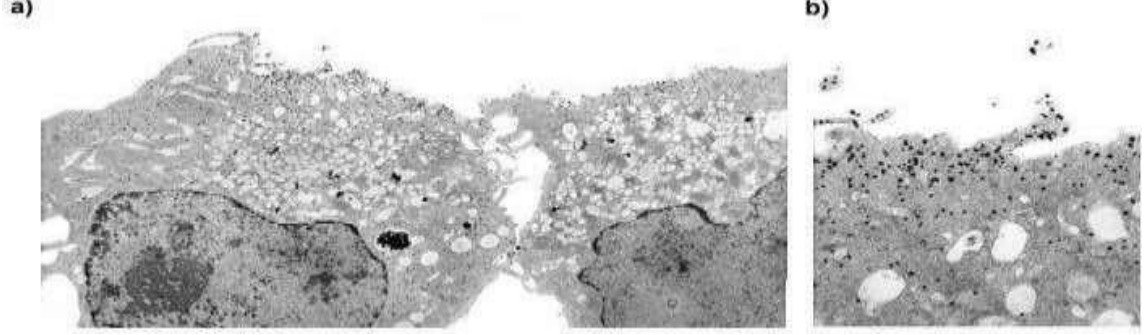

Figure 16: Image of nanoparticles taken from TEM

There are two ways that nanoparticles could be ingested by the macrophages.

1. Phagocytic pathway could be used to taken up the spotted nanoparticles
2. Nanoparticles which were without spots can be taken by the non- phagocytic ways.

Confocal Roman Microscopy

Also confocal Raman microscopy was used to observe the nanoparticles as shown in figure 17.

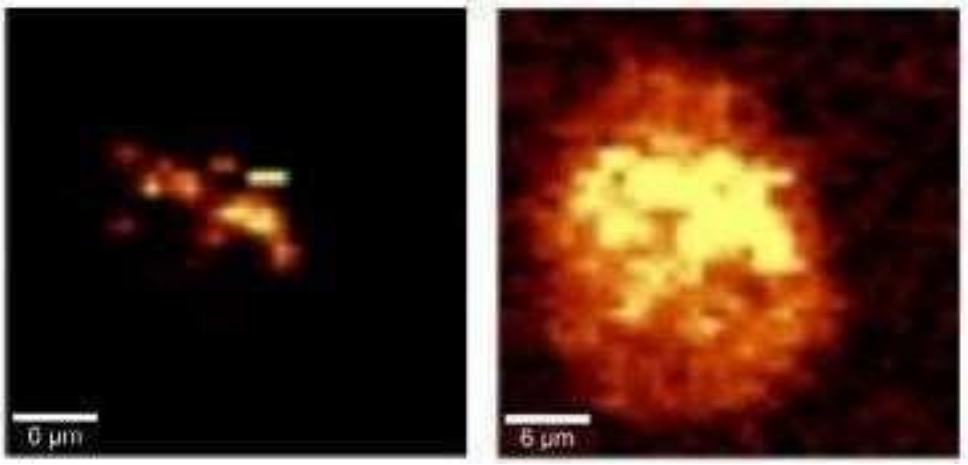

Figure 17: Image of nanoparticles taken from the confocal Raman Microscope

So, basic toxicity was observed with the nanoparticles which were characterized by the peptides coats. Also it was observed that the cytotoxicity was due to the time period and amount of dose. Also smaller sized nanoparticles were more toxic than that of larger ones. Also it was observed that the cytotoxicity was same if the amount of dose was calculated. Three tests were done in order to check the cytotoxicity. Also silver nanoparticles were present in different sizes and different shapes. So the toxicity of peptide- coated silver nanoparticles and other types of nanoparticles was observed. So tests of peptide-coated silver nanoparticles and citrate- coated nanoparticles were done. Both were of same size that is 20 nm. They were grown in 200.2 µg/ml of THP-1 cultures. So the cytotoxicity of peptide treated nanoparticles was more than that of the citrate- treated nanoparticles. So it was shown that small concentrations of nanoparticles could give adverse effects on macrophages (Arora *et al*., 2008).

Surface charge- dependent toxicity of silver nanoparticles

It is investigated that silver nanoparticles are used in a large number of commercial sites but they also mix with the air in the environment. Also their toxicity depends upon the size and shape. The most important factor is charge on the surface which plays an important role in the toxicity of Ag NPs. For this purpose, four Ag NPs were used. Uncoated H_2 Ag NPs, citrate coated- Ag NPs, polyvinyl-pyrroliodine coated Ag NPs and branched polye-thyleneimine coated Ag NPs. Here, the membrane used was 10kDa poly-ethersulfone (Choi *et al*., 2009). To check the impurities in solutions, conductivity method was used. Less amount of impurities are present when the conductivity method was slow. To remove extra Ag ions from the cell suspension, Milli-Q water was used. To get less than 10 µs cm^{-1}, the conductivity levels were checked continuously. The conductivity was checked for 3 weeks for observing the removal of Ag ions.

The Zetasizer Nano series was used to observe the HTT and zeta amounts of the Ag NPs. Before and after the purification, the measurements were taken within consecutive 3 weeks. The spectrophotometer technique was used to detect the concentrations of the solution (Carlson *et al*., 2008). To get the images of bacterial cells and the Ag NPs, TEM was used. The consumption of oxygen was detected by the biological oxygen demand. For making the BOD solution, 1 liter of Milli-Q water was used in which 1.5 ml of each of phosphate buffer, $MgSO_4$, $CaCl_2$ and $FeCl_3$ concentrations were added. For positive control glutamic acid and glucose were used. Impurities are present in Ag NPs and when they are purified, the impurities are removed. It not only contains the impurities but also some residual materials. When residual materials are removed they show some change in the toxicity of the Ag NPs. This reduction in toxicity is due the removal of Ag ions. So the Ag NPs characterized by the citrate, PVP and BPEI show not toxicity as a result of which the oxygen consumption did not lower down. After purification, the change of Ag NPs in Ag ions may cause toxicity. Also the conductivity was measured with continuous 3 weeks. It was also observed that the Ag NPs are spherical. Also Ag NPs were not spotted due to neutral pH and ionic strength is lesser of the BOD test media (Silver *et al*., 2006).

CONCLUSION

From this study, it is determined that there are number applications of silver nanoparticles which make Ag NPs an important product. Besides to its applications, there are many problems related to the silver nanoparticles. The most important is the cytotoxicity related to Ag NPs as they were exposed to the environment and the human body. For this reason different sized nanoparticles were synthesized. But it was seen that the chemically synthesized nanoparticles are more toxic than naturally occurring nanoparticles. Also it was investigated that cells show resistivity towards

nanoparticles but organs may affect from nanoparticles. But by using different chemicals and treatment we can inhibit the toxicity of silver nanoparticles so they can be used for long- term.

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