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**Article** · September 2013

DOI: 10.5897/JTEHS2013.0271

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Vol. 5(9), pp. 172-177, September, 2013
DOI 10.5897/JTEHS2013.0271
ISSN 2006-9820 ©2013 Academic Journals
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Journal of Toxicology and Environmental Health Sciences

*Full Length Research Paper*

# Estimation of toxic effects of chemically and biologically synthesized silver nanoparticles on human gut microflora containing *Bacillus subtilis*

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Accepted 30 July, 2013

**Biological silver nanoparticles were successfully synthesized from a simple green and natural route using the extract of *Allium cepa* (onion) with the use of silver nitrate as precursor and chemically synthesized using silver nitrate and tri sodium citrate. Nanoparticle synthesis was proven under UV-Visible absorption spectroscopy. Toxicity of sliver nanoparticles was tested using ToxTrak test, in which, fresh overnight broths of *Bacillus subtilis* and resazurin dye were used to calculate percentage inhibition (PI). PI is only a relative measure and since there is toxic substances that increase respiration, to give result to a negative number. The PI of both chemically and biologically synthesized silver nanoparticles was compared in order to evaluate toxic effect value. The toxic effect value, PI of chemically synthesized silver nanoparticles is much greater (85.45%) than the biologically synthesized sliver nanoparticles from onion (51.39%). These observation shows that the bacteria *B. subtilis* killed by chemically synthesized silver nanoparticles are more as compare to biologically synthesized sliver nanoparticle.**

**Key words:** Silver nanoparticles, *Allium cepa*, ToxTrak toxicity test, resazurin dye, ultraviolet spectroscopy, *Bacillus subtilis* and percentage inhibition (PI).

## INTRODUCTION

Nanoparticles are generally classified based on their dimensionality, morphology, composition, uniformity, and agglomeration. An important additional distinction should be made between nanostructured thin films or other fixed nanometer-scale objects (such as the circuits within computer microprocessors) and free nanoparticles. (Tyagi et al., 2011). The motion of free nanoparticles is not constrained, and they can easily be released into the environment leading to human exposure that may pose a serious health risk. In contrast, are the many objects containing nanostructured elements that are firmly attached to a larger object, where the fixed nanoparticles should pose no health risk when properly handled? An example of this important distinction is the material asbestos, which is perfectly safe in its primary state (basically a type of solid rock), but is a significant health hazard when mined or worked in such a way as to produce the carcinogenic nanometer-scale fibrous particles that become airborne (aerosol) and are therefore readily absorbed in the lungs.

It is also very important to recognize that not all nanoparticles are toxic; toxicity depends on at least chemical composition and shape in addition to simply size and particle ageing. In fact, many types of nanoparticles seem to be non-toxic (Connor et al., 2005; Goodman et al., 2004), others can be rendered non-toxic (Derfus et al., 2004), while others appear to have beneficial health effects (Bosi et al., 2003; Schubert et al.,

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2006). An important lesson we can learn from nano-science is that simple classifications of physical behavior (and therefore toxicity) are overly limiting and that toxicology of each material and morphology must be studied, in addition to particle ageing, to obtain accurate information to inform policy and regulatory processes.

Nanotoxicology was proposed as a new branch of toxicology to address the adverse health effects caused by nanoparticles (Donaldson et al., 2004). Despite the suggestions that nanotoxicology should only address the toxic effects of engineered nanoparticles and structures (Oberdörster et al., 2005), it is recommended that nanotoxicology should also encompass the toxic effects of atmospheric particles, as well as the fundamentals of virology and bacteriology. While significant differences exist between the health effects of non biological particles and viruses and bacteria, there are significant common aspects of intrusion and translocation. Toxicity is the degree to which a substance can damage an organism. Toxicity can refer to the effect on a whole organism, such as an animal, bacterium, or plant, as well as the effect on a substructure of the organism, such as a cell (Cytotoxicity) or an organ such as the liver (Hepato-toxicity). Toxicity is mainly dose dependent thus drugs should have more bioavailability and reduced toxic effects on cellular environment and normal micro flora inside the human body.

Human skin, lungs, and the gastro-intestinal tract are in constant contact with the environment. While the skin is generally an effective barrier to foreign substances, the lungs and gastro-intestinal tract are more vulnerable. These three ways are the most likely points of entry for natural or anthropogenic nanoparticles. Injections and implants are other possible routes of exposure, primarily limited to engineered materials. Due to their small size, nanoparticles can translocate from these entry portals into the circulatory and lymphatic systems, and ultimately to body tissues and organs. Some nanoparticles, depending on their composition and size, can produce irreversible damage to cells by oxidative stress or/and organelle injury.

Silver nanoparticles have significant role in the field of diagnostic (Schultz et al., 2000), antimicrobial and therapeutics (Rai et al., 2009; Elechiguerra et al., 2005). The silver ion (Ag+) is bioactive and insufficient concentration readily kills bacteria *in vitro*. Silver also kills bacteria in external wounds in living tissue, so physicians use wound dressings containing silver sulfadiazine (Ag-SD) or silver nanomaterials to treat external infections (Qin et al., 2005; Hermans et al., 2006).

The disinfectant properties of silver are used in medical applications, such as urinary catheters and endotracheal breathing tubes in reducing incidences of catheter-related urinary tract infections and ventilator-associated pneu-monia, respectively (Saint et al., 1998; Kollef et al., 2008).Toxicity caused due to increased dosage of silver nanoparticles may cause accumulation of silver or silver sulfide particles in the hair, skin, kidneys, liver, heart and serious neurologic, renal, or hepatic complications, as well as headaches, stomach distress, fatigue, and skin irritation have been reported (Lansdown et al., 2006; Brandt et al., 2005; Stepien et al., 2009). Numerous methods concerning the fabrication of silver nanoparticles, as well as various silver-based compounds containing ionic silver ($Ag^+$) or metallic silver (Ag0), because of such a wide range of applications, have been developed (Lu et al., 2007; Willner et al., 2006; Mallikarjuna et al., 2007; Chen et al., 2006). In many synthetic methods, some toxic chemical are used as a reducing agent such as $NaBH_4$, citrate, or ascorbate for the preparation of silver nanoparticles (Lou et al., 2006; Kuo et al., 2003; Gardea-Torresdey et al., 2002). Consi-dering that such reducing agents may be associated with environmental toxicity or biological hazards, the development of a green synthesis for silver nanoparticles is desired. Biosynthesis of nanoparticles using plant extracts is the favourite method of green, eco-friendly production of nanoparticles and exploited to a vast extent because the plants are widely distributed, easily available, safe to handle and with a range of metabolites (Manish et al., 2009). The plant materials already used for biosynthesis of nanoparticles includes *Helianthus annus*, *Oryza sativa*, *Zea mays*, *Sorghum bicolor* (Arangasamy and Munusamy, 2008), *Eucalyptus hybrid* (Manish et al., 2006), *Artocarpus heterophyllus* (Thirumurugan et al., 2010), Cycas (Anal and Prasad, 2010) and many more.

The present study is based on evaluating the percentage toxicity of chemically synthesized silver nanoparticles and biologically synthesized silver nanoparticles from onion. Biologically synthesized silver nanoparticles are biocompatible and friendlier to the nominal human body micro flora and do not severely disturbs it during the ingestion of drugs containing silver nanoparticles. Normal gut micro flora of a human body contains *Bacillus subtilis* which is a Gram-positive, catalase-positive bacterium. It is mainly present in the normal gut flora of humans and used as a probiotic in healthy individuals which rarely causes food poisoning. It has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions, but still, silver nanoparticles prove to be toxic and killing most of them.

## MATERIALS AND METHODS

### Sample collection

All chemicals used in this experiment were of the highest purity and obtained from Sigma (Bangalore, India) and Merck (Mumbai, India). Onion (*Allium cepa*) was used for the biological synthesis of silver nanoparticles collected from local market of Meerut in the month of April, 2012. Silver nitrate ($AgNO_3$) and tri sodium citrate ($Na_3C_6H_5O_7.2H_2O$) of analytical grade were used for chemical synthesis of silver nanoparticles. A stock of 1 mM was prepared and stored in a brown bottle to avoid light disintegration of silver nitrate and a stock of 38 mM of tri sodium citrate was prepared.

### Collection of pathogens

The cultures of *B. subtilis* were used to demonstrate the toxic effects of silver nanoparticles collected from the microbiology laboratory in Biotechnology Department of Meerut Institute of Engineering and Technology, Meerut.

### Biologically synthesized silver nanoparticles

100 g of onion was ground to obtain the extract. Extract was filtered using Whatmann No. 1 filter paper and the filtrate was collected and centrifuged at 5000 rpm for 20 min. Silver nitrate was used as precursor for synthesis of silver nanoparticles. 5 ml of 1 mM silver nitrate aqueous solution was added to 100 ml of clear plant extract (Supernatant). Then, the conical flask containing the solution was put into a shaker (150 rpm) at 30°C for 72 h. In this process, the *A. cepa* (onion) extract acts as the reducing and stabilizing agent. Silver nanoparticles were obtained gradually by the erosion and chemical degradation of plant extract.

### Chemically synthesized silver nanoparticles

Thirty-eight millimeter tri sodium citrate ($Na_3C_6H_5O_7.2H_2O$) and 1 mm silver nitrate ($AgNO_3$) were used for chemically synthesized sliver nanoparticles. 50 ml aqueous silver nitrate was taken and boiled up to 70 to 80°C and was mix to 10 ml tri sodium citrate in a drop wise method. The solution was continuously stirred through magnetic stirrer for 4 to 5 min. After proper mixing, the solution was then incubated at 30°C for 45 min. Silver nanoparticles were obtained gradually by the erosion and chemical degradation.

### UV-VIS spectra analysis of sliver nanoparticle

The reduction of pure $Ag^+$ ions was monitored by measuring the UV-Vis spectrum of the reaction medium at 2 h after diluting a small aliquot of the sample into distilled water. The technique involves ultra-violet and visible spectroscopy. UV-Visible absorption spectra were measured using UV-Vis spectrophotometer operated at a resolution of 1 nm. The colour change in reaction mixture solution was recorded through visual observation which showed bioreduction of silver ions in aqueous solution. UV-Vis spectral analysis was done using UV-VIS spectrophotometer V-530 (JASCO).

### Resazurin dye

Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) is a blue dye, nonfluorescent until it is reduced to the pink colored and highly red fluorescentresorufin. It is used mainly as an oxidation-reduction indicator in cell viability assays for bacteria and mammalian cells. Early scientist used resazurin dye to quantify bacterial content in milk (Pesch and Simmert, 1929). It is also used as an indicator for cell viability in mammalian cell cultures (Anoopkumar et al., 2005). It was introduced commercially initially under Alamar Blue trademark (Trek Diagnostic Systems, Inc), and now also available under other names such as AB assay, Vybrant (Molecular Probes) and UptiBlue (Interchim). Usually, resazurin dye is used for bacterial toxicity in milk quality, but this experiment is its first use globally to estimate the toxicity of sliver nanoparticles.

### ToxTrak test

Three test tubes of broths for 48 h containing *B. subtilis* were used for Toxtrak test for determining toxicity in chemically and biologically synthesized silver nanoparticles on human gut microflora (*B. subtilis*). One test tube was marked as control and other two test tubes were incubated with 1 ml chemically synthesized silver nanoparticles and 1 ml biologically synthesized silver nanoparticles, respectively for 4 h. The concentration of the silver nanoparticles ranges from 25 to 50 µg/ml in both solutions. Resazurin dye is added in the volume of 40 µl per test tube and incubated from 0 to 4 h. The absorption was recorded just after adding the dye (0 h) in all the three test tube and then the absorption is recorded after every 1 h intervals for 4 h.

## RESULTS AND DISCUSSION

Reduction of Ag ion into silver particles during exposure to the plant extracts from onion supernatants could be followed by color change. The plant extract from onion supernatants were pale yellow before the addition of silver ions and this changed to a brownish color on completion of the reaction with ions. The result obtained in this investigation is very interesting in terms of identification of potential extract for synthesizing the sliver nanoparticles. UV Vis spectrograph of the colloidal solution of silver nanoparticles has been recorded as a function of time. A surface plasmon peak located at 420 nm (1.2823) was observed for the biological silver nanoparticles (Figure 1). Similar results of UV Vis spectrophotometer producing max peak 399 nm for onion have been made by Packia Lekshmi et al. (2012). In case of chemically synthesized silver nanoparticles, the solution turns transparent golden brownish which shows the presence of silver nanoparticles and on performing UV Vis spectrophotometry, the absorption peak is observed to be at 400 nm (Figure 2). Similar results of UV Vis spectrophotometer producing max peak 412 nm for chemically synthesized silver nanoparticles have been made by Maribel et al. (2009).

### ToxTrak test

In the presence of toxicity, the decreasing rate of degradation also decreases the reduction of resazurin dye and these changes are measured by the changes in absorbance of the sample as compared to a control sample. The absorbance test is carried out at a wavelength of 603 nm, which is specific for the blue color. The percentage inhibition (PI) is expressed equation is as follow:

$$PI = [1- (\Delta As / \Delta Ac)] \times 100$$

In this PI equation, the ΔAs and ΔAc represent the changes/differences (decrease) in absorbance for the sample and the control, respectively. In this case, Δ is the initial-final value. The PI is a relative measure only, in which the presence of toxic substances that increase the respiration and the results of PI equation were observed in a negative number.

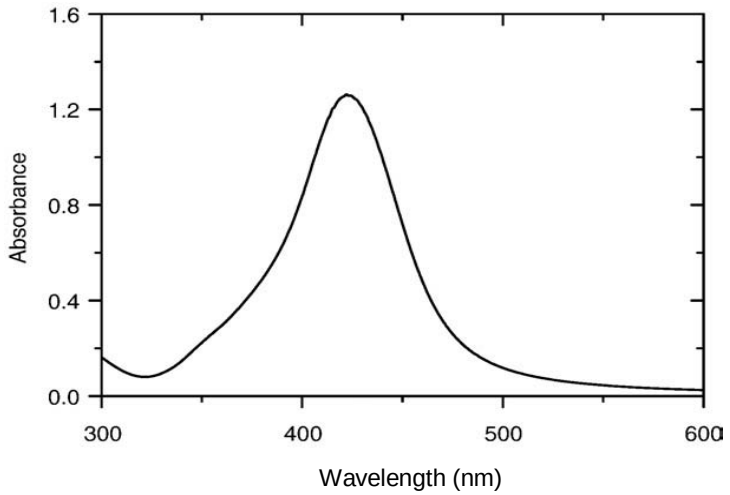


**Figure 1.** UV-Vis absorption spectra of biological biosynthesized silver nanoparticles from Onion extract.

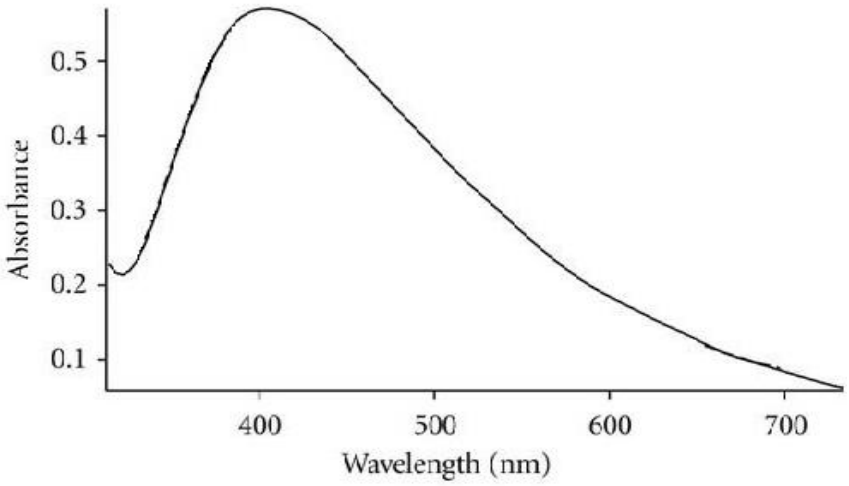


**Figure 2.** UV-Vis absorption spectra of chemically synthesized silver nanoparticles from sliver nitrates.

**Table 1.** Absorption value of control and various broths treated with chemically and biologically synthesized silver nanoparticles.

| Incubation period with dye (h) | Test tube 1:Control (ΔAc) | Test tube 2: Chemically synthesized AgNPs (ΔAcs) | Test tube 3: Biologically synthesized AgNPs ($\Delta A_{BS}$) |
|---|---|---|---|
| 0 | 2.9314 | 1.9914 | 2.6021 |
| 1 | 2.5528 | 1.8327 | 2.5686 |
| 2 | 2.0489 | 1.7375 | 2.2518 |
| 3 | 1.0041 | 1.6049 | 2.0757 |
| 4 | 0.2163 | 1.5962 | 1.2823 |

Table 1 contains 5 groups of values. It can calculate 4 PI for each sample. Absorptions of control and various broths samples were treated with chemically (Acs) and biologically ($A_{BS}$) synthesized silver nanoparticles. To determine PI value, first the changes/differences (decrease) in absorbance for the control (ΔAc), chemically synthesized sliver nanoparticle (ΔAcs) and biologically synthesized sliver nanoparticle ($\Delta A_{BS}$) value of decrease

were calculated. The value of decrease was substituted in PI Equation to finally get the toxicity percentage.

**Calculate changes/differences (decrease) in absorbance**

a) For control sample:

ΔAc = 0.2163-2.9314 = -2.7151

b) Nanoparticle sample:

ΔAcs = 1.5962 - 1.9914 = -0.3952

c) For biologically synthesized sliver nanoparticle sample:

ΔABS = 1.2823 - 2.6021 = -1.3198

**Calculate the toxicity percentage**

To estimate the sliver nanoparticles toxicity by putting the final value of changes/differences (decrease) of ΔAcs, $\Delta A_{BS}$ and ΔAc in PI Equation 1(PI = [1- (ΔAs / ΔAc)] × 100)

***Toxicity of chemically synthesized sliver nanoparticle sample***

This is to calculate the chemically synthesized sliver nanoparticle toxicity for putting the final changes/differences (decrease) of ΔAcs and ΔAc in PI Equation. Here, ΔAs is $\Delta A_{CS}$, so the PI Equation is:

$PI = [1- (\Delta A_{CS} / \Delta Ac)] \times 100$
PI = [1-(-0.3952 / -2.7151)] × 100
PI = 85.45%

***Toxicity of biologically synthesized sliver nanoparticle sample***

This is to calculate the biologically synthesized sliver nanoparticle toxicity for putting the final changes/differences (decrease) of $\Delta A_{BS}$ and ΔAc in PI Equation 1. Here, ΔAs is $\Delta A_{BS}$, so the PI Equation is:

$PI = [1- (\Delta A_{BS} / \Delta Ac)] \times 100$
PI = [1-(-1.3198/-2.7151)] × 100
PI = 51.39%

The aforementioned data clearly indicates that the toxic effect value PI of chemically synthesized silver nanoparticles is much greater (85.45%) than that of biologically synthesized sliver nanoparticles from onion (51.39%). These observation shows that the bacteria killed by chemically synthesized silver nanoparticles are more as compare to biologically synthesized sliver nanoparticle from onion and thus degradation of dye is less when compared with that biologically synthesized. Thus, silver nanoparticles that are synthesized using onion extracts are proven to be more biocompatible and less toxic to cellular microenvironment and normal gut microflora inside the human body. This study relates that the drugs using silver nanoparticles should utilize biosynthesized silver nanoparticles to reduce the risk of toxicity.

## Conclusion

This study describes a simple environmentally ecofriendly benign method of synthesis of silver nanoparticles from plants which is the best source. This method can be further used for industrial production of nanoparticles at room temperature and with a single step. This investigation provides evidence that plant extract-stabilized nanoparticles may be ideal candidates for future studies exploring their use in biomedical and pharmacy applications. This synthesis procedure offers a less cost-effective and green alternative to traditional protocols that may be readily scaled up for industry as a result of the low synthesis temperatures and time required. Since *A. cepa* (onion) is easily available throughout the nation and also is used in every house for cooking as a flavouring agent, the active nano compound from this can be prepared and used effectively in the field of diagnostic, antimicrobial and therapeutics.

## ACKNOWLEDGEMENTS

Authors are highly thankful to Chairman, Director General, Director, HOD and Faculty members of Department of Biotechnology, Meerut Institute of Engineering Technology for their continuous encouragement and problem solving assistance. Authors are also highly thankful to Editor and Revivers of Journal of Toxicology and Environmental Health Sciences for his valuable comments to strengthen this research article.

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