

EXTRACELLULAR AND INTRACELLULAR SYNTHESIS OF SILVER NANOPARTICLES

PRASANNA SUBRAMANIAN*, KIRUBANANDAN SHANMUGAM

Former Graduate Student, Centre for Biotechnology, Anna University, Chennai, Tamil Nadu, India. Email: prasanarengarajan@gmail.com

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ABSTRACT

Objective: The cellular synthesis of nanoparticle is a green process and alternative for a conventional process for the preparation of silver nanoparticles. In our research, focus has been given to the development of an efficient and eco-friendly viable process for the synthesis of silver nanoparticles using cancer and non-cancerous cells, a cell culture that was isolated. The results of this investigation are observed that silver nanoparticles could be induced to synthesis intra- and extra-cellularly using mammalian cells such as cancerous and non-cancerous cells.

Methods: The silver nanoparticles are synthesized by the cancer and non-cancerous cells such as HeLa (*Homo sapiens*, human), SiHa, and human embryonic kidney-293 cell lines. The silver nanoparticles were characterized by ultraviolet (UV)-visible spectroscopy, transmission electron microscopy (TEM), and X-ray powder diffraction (XRD).

Results: The silver nanoparticles exhibited maximum absorbance at 415 nm in UV-visible spectroscopy. The XRD confirms the characteristic of the crystal lattice of silver nanoparticles by observing three peaks: Peak at 38 is due to reflection from (111), peak at 44 is due to reflection from (200), and peak at 65 is due to reflection from (220). TEM images showed the formation of stable silver nanoparticles in the cell lines.

Conclusion: The method of extraction of intracellular/extracellular synthesis of silver nanoparticles was inexpensive, simple, and effective in large scale with no need to use of complex process equipment. The cancer cell considered as a biological factory at nanoscale dimension which continued to grow after synthesis of silver nanoparticles. The silver reduction by these cancer cells has occurred through energy-dependent processes that lead to the high output of this reaction. Hence, this new approach of using a mammalian cell for the successful synthesis of nanosized silvers could be easily scaled up, which establishes its commercial viability and also useful in the drug delivery and drug targeting.

Keywords: Silver nanoparticles, Cancer cells, Biosynthesis and characteristics of silver nanoparticles.

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INTRODUCTION

The application of nanotechnology in life sciences, called as nanobiotechnology, is already having an impact on diagnostics and drug delivery/targeting. Now, researchers use nanotechnology in the field of drug discovery. Some of these are already established in research through well-known technologies such as biosensors and biochips. "Nanoparticles are still used extensively for developing diagnostics and some of the assays for drug discovery." Nanoscale assays can contribute significantly to cost saving in screening campaigns. "In addition, some nanosubstances could be potential drugs for the future. Although there might be some safety concerns with respect to the *in vivo* use of nanoparticles, studies are in place to determine the nature and extent of adverse effects. Future prospects for the application of nanotechnology in healthcare and for the development of personalized medicine appear to be excellent." For example, nanoparticulate technology could prove to be very useful in cancer therapy allowing for effective and targeted drug delivery by overcoming the many biological, biophysical, and biomedical barriers that the body stages against a standard intervention such as the administration of drugs or contrast agents. Among nanoparticles, in the biological sciences, many applications for metal nanoparticles are being explored, including biosensors [1], labels for cells and biomolecules [2], and cancer therapeutics [3].

Although a variety of chemical routes have been followed to synthesis of nanoparticles, natural routes are always desired. It has been known for a long time that in nature, a variety of nanomaterials are synthesized by biological processes. "For example, the magnetotactic bacteria synthesize intracellular magnetite or granitic nanocrystallites [4]. Similarly, certain yeasts synthesize intracellular CdS Nanocrystallites through a mechanism of detoxification when growing in the presence of toxic metals such as cadmium [5]." Bacteria involved in silver leaching have been reported to accumulate silver sulfide within their

membrane [6], and natural biofilms of sulfate-reducing bacteria were shown to deposit nanocrystalline sphalerite, i.e., ZnS [7]. Owing to the reported particle size uniformity in such "biosynthetic" processes and their inherent environment-friendly nature, there is a renewed interest in biological routes of nanoparticle synthesis [8-10]. "The interest also extends to the assembly of nanoparticles, using biological templates such as DNA or proteins" [1].

It was revealed that in the case of noble-metal nanocrystals, the electromagnetic, optical, and catalytic properties are highly influenced by shape and size [11-13]. Among noble-metal nanomaterials, silver nanoparticles have received considerable attention due to their attractive physicochemical properties. "The surface Plasmon resonance and large effective scattering cross section of individual silver nanoparticles make them ideal candidates for molecular labeling [14] where phenomena such as surface-enhanced Raman scattering can be exploited. In addition, the strong toxicity that silver exhibits in various chemical forms to a wide range of microorganisms is very well known [15-17], and silver nanoparticles have recently been shown to be a promising antimicrobial material [18]. It was found that silver nanoparticles inhibit the virus from binding to host cells as revealed *in vitro* [19]."

Different approaches of synthesis of silver nanoparticles have been carried out previously [20-22]. Silver nanoparticles were synthesized biologically using fungus and other microorganisms [23,24]. Silver nanoparticles with different sizes were synthesized by electrochemical reduction [25]. Biologically synthesized silver nanoparticles could have many applications, such as spectrally selective coatings for solar energy absorption and intercalation material for electrical batteries [9], optical receptors [14], catalysts in chemical reactions, and bio-labeling [26]. In line with this trend, we have done a novel work in synthesizing silver nanoparticle using human cells. The cells chosen were cancerous and

non-cancerous cell lines. These synthesized silver nanoparticles have potential application in drug discovery and drug delivery/targeting.

EXPERIMENTAL DETAILS

Preparation of silver nitrate solution

Silver ion was prepared in the concentration of 10^{-3} M. The solution was prepared in HEPES (21 mM) buffer. Sodium nitrate (137 mM) and sodium hydroxide were added to adjust the pH to physiological pH.

Preparation of cell culture

The cultures are viewed to assess the degree of confluency, and the absence of bacterial and fungal contaminants is confirmed. The spent medium is removed. The cell monolayer is washed with HEPES using a volume equivalent to half the volume of culture medium. The wash step if the cells are known to adhere strongly is repeated. The trypsin/ethylenediaminetetraacetic acid onto the washed cell monolayer using 1 ml per 25 cm² of surface area is pipetted. The flask is rotated to cover the monolayer with trypsin. The excess trypsin is removed by decantation. The flask is returned to the incubator and left for 2-10 minutes. The cells are examined by an inverted microscope to ensure that all the cells are detached and floating. The side of the flasks may be gently tapped to release any remaining attached cell. The cells are resuspended in a small volume of fresh medium containing medium (1 ml of 10% serum containing Dulbecco's Modified Eagles Medium [DMEM]) to inactivate the trypsin. The solution is centrifuged at 3000 rpm for 5 minutes. The cells are resuspended in 1 ml of 10% serum containing DMEM. The cells are resuspended in 1 ml of 10% [10]. The cell count is performed (the wells are seeded with same initial value of 3×10^5 cells per well for each kind of cell line). The required number of cells is transferred to a new labeled flask containing pre-warmed medium. It is incubated in 37°C, 5% CO₂ environment for duration of 48 hrs before ionic solution can be added.

Addition of silver ion solution to cell lines

Three cell lines (cancerous as well as non-cancerous) were grown to 70% confluency level after seeding in a 6-well plate (9 cm², tetraphenylporphyrin with oxygen). The cell lines, which we have used for the study, are human embryonic kidney cell (HEK-293), HeLa cell, and SiHa Cell. During the growth period, culture medium containing DMEM is used. Just before the addition of silver ions to the well, the culture medium is removed. The cells were washed twice with HEPES. Then, silver ion solution in HEPES is filter sterilized using MILLEX® GV Durapore® PVDF Membrane filter (pore size: 0.22 µm) and is added to the well. To each well, 2 ml of the solution is added. The cells are then kept in a 37°C, 5% CO₂ incubator. The biotransformation was observed by periodic sampling of aliquots (200 µl diluted to 1 ml of the aqueous component and measuring the ultraviolet (UV)-visible spectrum of the solution. The absorbance values are compared over a period of 4-day. On the last day, the cells are scrapped off the well surface and sample preparation is done for transmission electron microscopy (TEM).

Sample preparation for TEM

The Ag²⁺ HEPES solution is removed from well-containing cells. It is washed with HEPES and discards this HEPES (repeat twice). Trypsin (2 ml) is added to the well. The cells are incubated for 5 minutes and 37°C and 5% CO₂. The medium is added with serum (2 ml) to the cells. The liquid from well is transferred to a centrifuge tube and carry out centrifugation for 5 minutes at 3000 rpm. The pellet is collected after centrifugation. The pellet is resuspended in HEPES. It is centrifuged again under same conditions. The pellet is collected and washed with HEPES twice. This final pellet is collected and resuspended in 1% glutaraldehyde. It is stored at 4°C.

Protocol for cell lysis

The solution is aspirated. The cells are scraped from the surface of the well using a cell scraper. The solution contained in the well is transferred into a centrifuge tube. The cells are sonicated at duty cycle value of 50% and in output mode and 3 pulses are given for 10 seconds,

each pulse separated by 20 seconds of cooling in ice. It is carried out centrifugation at 5000 rpm for 5 minutes at 4°C.

Ultra-microtome and TEM

Fixation

Primary fixation was done with 1% glutaraldehyde (as described above). After primary fixation, the cells were washed repeatedly with phosphate buffer and were subjected to secondary fixation with 1% osmium tetroxide for 1 hr followed by repeated washing with phosphate buffer.

Dehydration

Before proceeding for dehydration, the cells were suspended in molten agar (2%). Small blocks of solidified agar (1 mm²) were cut and passed through series of 30%, 50%, and 100% ethanol v/v for 15 minutes each. The lower grade ethanol was prepared in DD water.

Embedding

The dehydrated agar blocks were suspended in propylene oxide for 20 minutes at 4°C (2 changes), followed by treatment with 1:1 mixture of propylene oxide and Araldite A for 1 hr at 60°C.

Araldite A: Araldite (resin) Cy-212: 10 ml

Dodecyl succinic anhydride (hardener): 12 ml

Dibutyl-phthalate (plasticizer): 1 ml

The agar blocks containing cells were then incubated in Araldite A for 1 h at 60°C followed by overnight incubation at room temperature. The next day Araldite B was prepared freshly as follows:

- Araldite A: 23 ml
- Trimethyl aminomethyl phenol: 0.4 ml (DMP-30 accelerator)
- Araldite A was poured out and freshly prepared Araldite B was added to the agar blocks. This was followed by incubation for 2 hrs at 60°C for infiltration. The blocks were then finally removed and placed in refined beam capsule containing Araldite B and incubated for polymerization for 48-72 hrs, at 60°C.

Block trimming and sectioning

The resin blocks were carefully trimmed to expose the underlying agar blocks. Sections of various thicknesses (200 nm, 300 nm, 500 nm, and 1000 nm) were cut using Leica Ultra cut UCT microtome and transferred to 300 mesh copper grids.

Uranyl acetate staining

10% alcoholic solution of uranyl acetate was prepared and centrifuged to remove any precipitate there in. The sections on copper grids were stained for 1 hr with uranyl acetate in dark at room temperature and then washed with distilled water thoroughly.

TEM

Sections on grids were observed with a TEM working at 120 keV.

Instruments used

The optical spectroscopic studies were done on a Perkin Elmer Lambda 25 UV-visible spectrophotometer, and scanning was done from 1100 nm to 200 nm. The bright-field TEM images were taken using a JEOL 200 keV TEM. The samples for TEM were prepared by dropping a dispersion of the particles on copper grid supported Formvar films. Microtome was done on selected samples as described. X-ray powder diffractograms (XRD) were measured with a Shimadzu diffractometer with Cu K α radiation.

RESULTS

Nanoparticle characterization

Fig. 1 shows the UV-visible spectrum of silver nanoparticles synthesized by chemical process. The spectrum shows the characteristic peak of silver nanoparticles at 415 nm.

Time bound characterization of silver ion solution taken from the cell well

The process of Ag^{2+} reduction on exposure to human cells can easily be tracked by monitoring of UV-visible spectroscopy measurements. We used the silver ions in hepes. This medium was found to be stable to the cells. Sodium nitrate was used to maintain ionic balance. Subsequently, pH was adjusted to 7.2 using sodium hydroxide. The UV-visible spectrum of the Ag^{2+} in HEPES is taken for 24 and 96 hrs which shows the absence of peak corresponding to silver nanoparticle (Fig. 2). The UV-visible spectrum of the Ag^{2+} sample after 24, 55, 72, and 96 hrs of exposure to HEK-293, HeLa, and SiHa (Figs. 3-8) showed a well-defined surface plasmon band centered around 420-435 nm characteristic of colloidal silver. Further, the steady increase in the peak absorbance value for all experimental samples over a period of 96-hrs suggest that reduction process is slow and is a biological process. The solution at this stage becomes yellow in color. The cells due to over stressful condition detach itself from the well. The intracellular reduction of cell samples is also easily tracked by UV-visible spectroscopy measurements. The cells attached to the wells were detached by trypsinization and were subsequently lysed following experimental protocol for cell lysis. The UV-visible spectra of lysed samples were taken after 24, 55, 72, and 96 hrs of exposure to Ag^{2+} solution. The peak value is subsequently higher than the corresponding spectra got by extracellular reduction at different time intervals.

The fact that the presence of nanoparticles was showing up in the samples collected from the solution (external to the cell membrane) proves the presence of silver nanoparticles in the solution without any

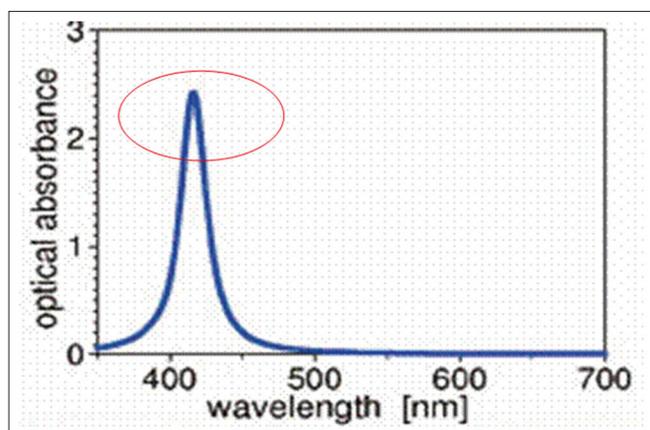


Fig. 1: Ultraviolet-visible spectrum of citrate capped silver nanoparticles

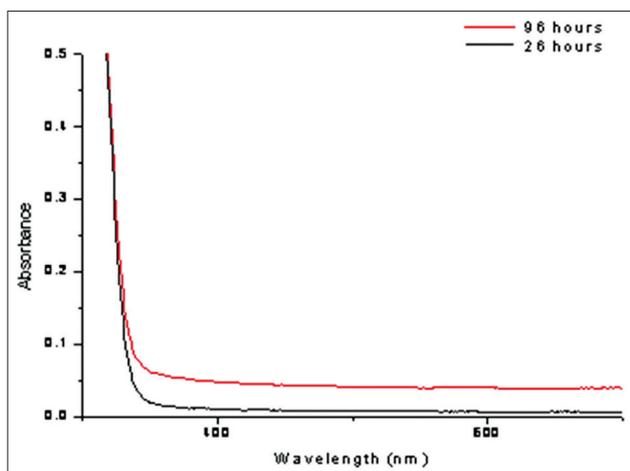


Fig. 2: Time bound ultraviolet-visible measurements for 0.001 M silver ions in HEPES

aggregation. In addition, the detached cells from the well after exposure to Ag^{2+} solution were intensely yellowish brown in color at all-time intervals. Before detaching, the cells present in the well were found to be yellowish in color after 96 hrs (Figs. 9-14).

Fig. 15 shows the XRD patterns for HEK samples obtained after lysing the cells and plating the lysate on a glass slide. It clearly shows the

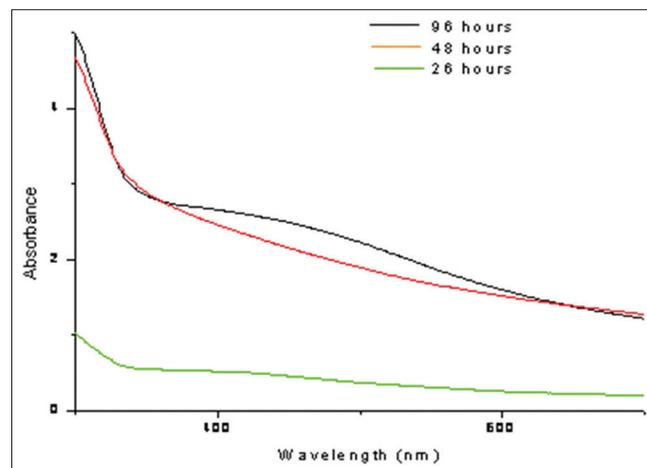


Fig. 3: Time bound ultraviolet-visible measurements for human embryonic kidney cells with 0.001 M silver ions in HEPES

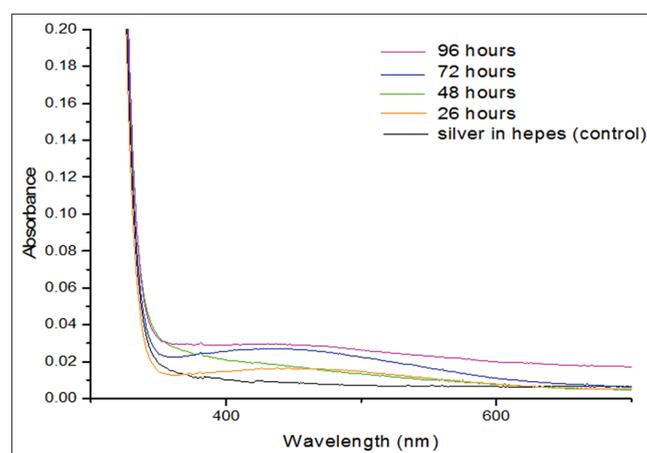


Fig. 4: Time bound ultraviolet-visible measurements for HeLa cells with 0.001 M silver ions in HEPES

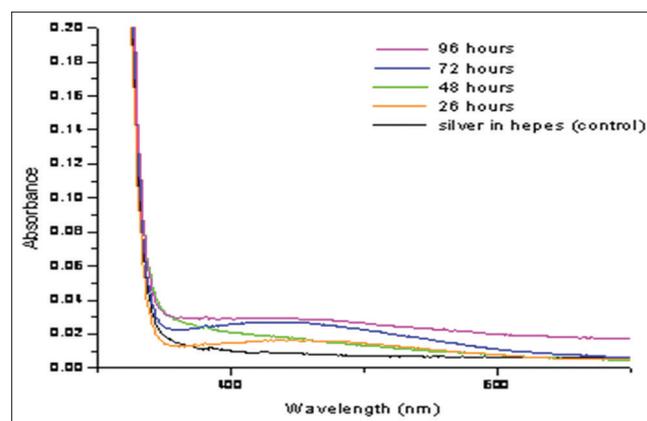


Fig. 5: Time bound ultraviolet-visible measurements for SiHa cells with 0.001 M silver ions in HEPES

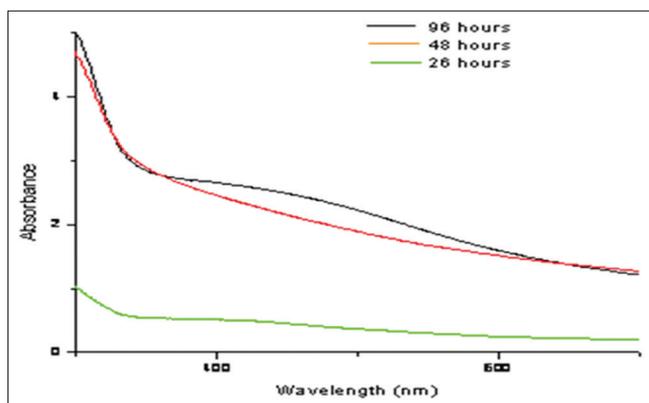


Fig. 6: Time bound ultraviolet-visible measurements for lysed human embryonic kidney cells treated with 0.001 M silver ions in HEPES

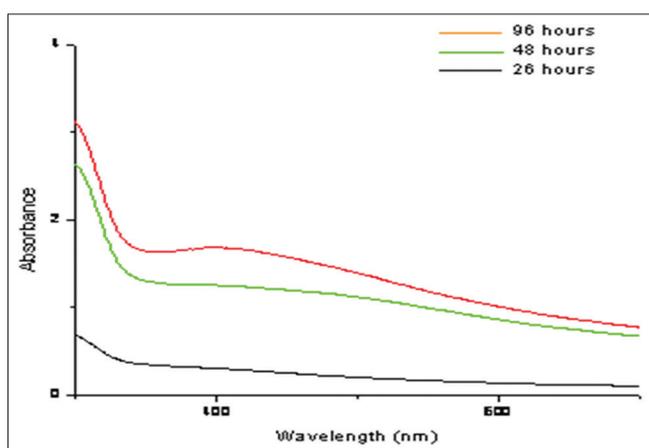


Fig. 7: Time bound ultraviolet-visible measurements for lysed HeLa cells treated with 0.001 M silver ions in HEPES

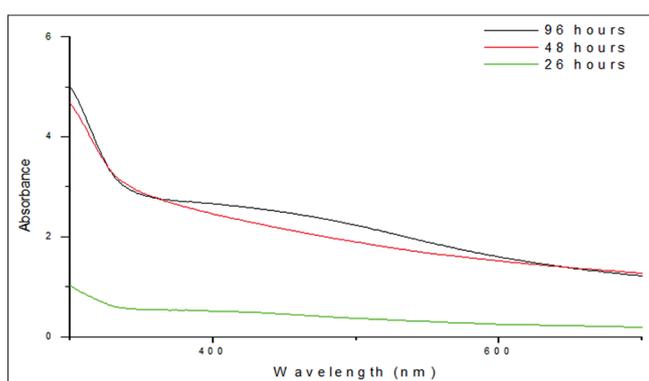


Fig. 8: Time bound ultraviolet-visible measurements for lysed SiHa cells treated with 0.001 M silver ions in HEPES

appearance of three prominent peaks which are characteristic of crystal lattice of silver nanoparticles. Peak at 38 is due to reflection from (111), peak at 44 is due to reflection from (200), and peak at 65 is due to reflection from (220).

Electron diffraction patterns of ultramicrotomed samples show the presence of silver crystals (Fig. 16). It is also well known that various kinds of proteins present inside the cytoplasm of cell have amine and/or thiol groups present in them. The presence of these two groups crates favorable binding interactions between proteins and nanoparticles



Fig. 9: Photograph of human embryonic kidney-293 cells with silver ions (Day 1, $\times 40$)



Fig. 10: Photograph of human embryonic kidney-293 cells with silver ions (Day 4, $\times 40$)

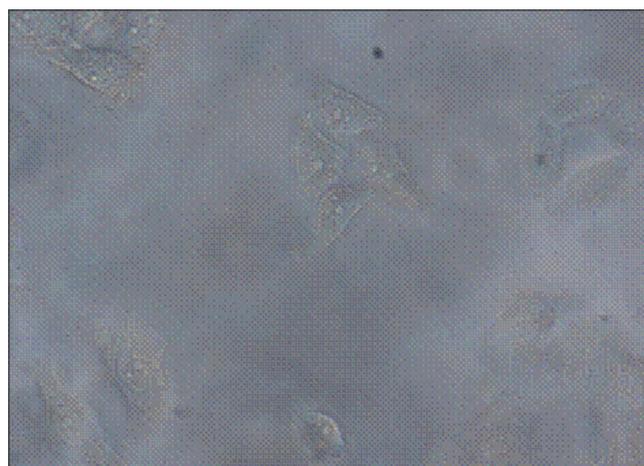


Fig. 11: Photograph of HeLa cells with silver ions (Day 1, $\times 40$)

surfaces. The presence of capping agent, in addition to causing size changes, also contributes to stabilization of nanoparticles and shift in characteristic peak value position in UV-visible spectrometry measurements. The appearance of peak at above 420 nm suggests that silver nanoparticles are capped with certain molecular species containing amine group or cysteine residues in protein, etc.

As observed from the images, the cells had become hugely stressed out (Figs. 9-14), due to absence of growth medium and their involvement in reduction of silver ions. There was no further growth observed in

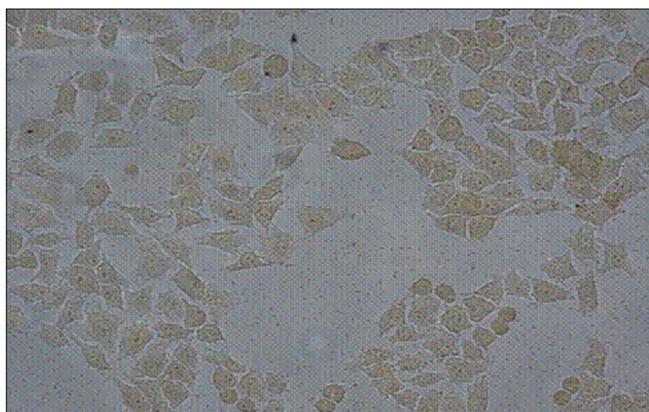


Fig. 12: Photograph of HeLa cells with silver ions (Day 4, ×40)



Fig. 13: Photograph of SiHa cells with silver ions (Day 1, ×40)

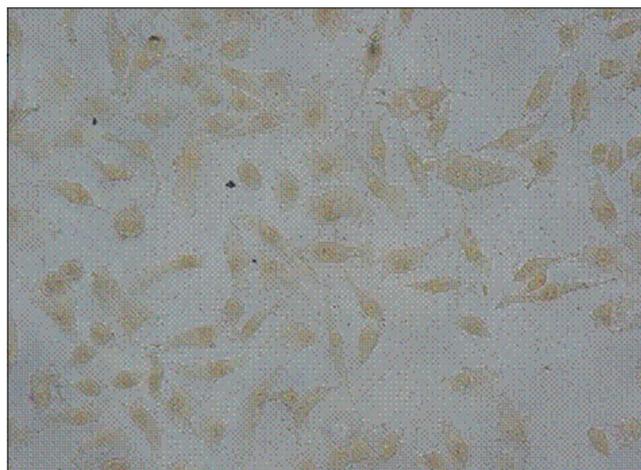


Fig. 14: Photograph of SiHa cells with silver ions (Day 4, ×20)

the cells after day 0 as nutrient medium removed. The changes in cell morphology were observable. TEM images show the additional data of presence of silver nanoparticles in the cell lines (Figs. 17-20).

DISCUSSION

It is reported that silver nanoparticles have found to be useful in the field of biomolecular detection and diagnostic and therapeutic agents and antimicrobials for drug delivery and targeting. Various strategies are involved in the preparation of silver nanoparticles by physical and

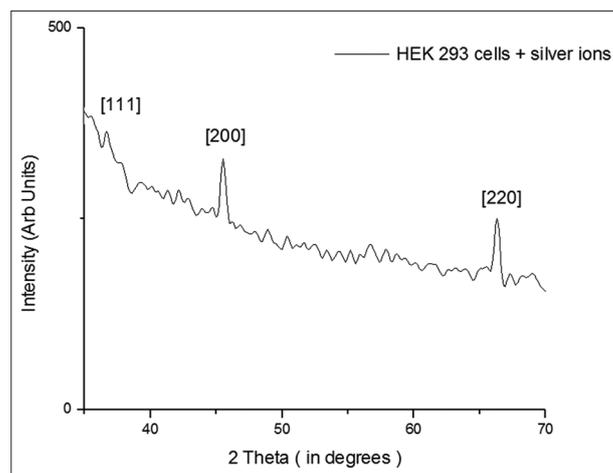


Fig. 15: X-ray diffractogram of human embryonic kidney-293 cells after treatment with silver ion solution (lysed)

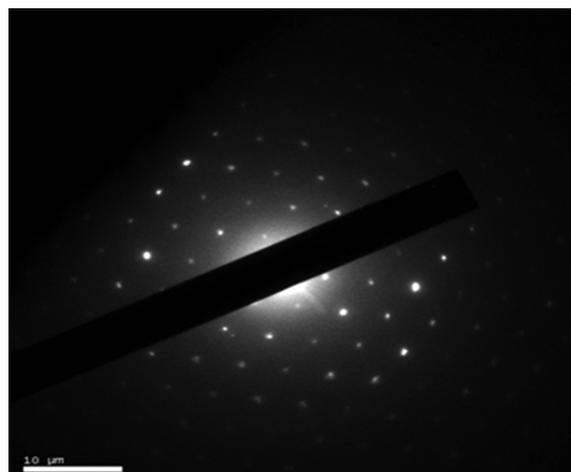


Fig. 16: Electron diffraction of human embryonic kidney-293 cells after treatment with silver ion solution (lysed)

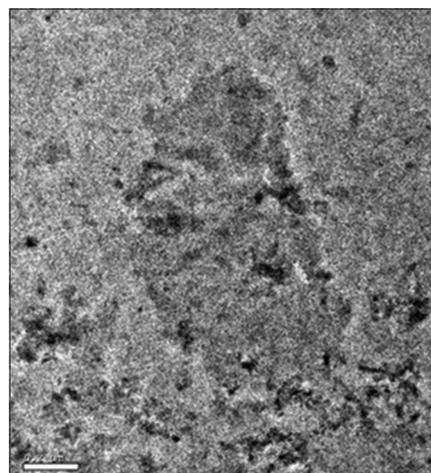


Fig. 17: Transmission electron microscopy image of HeLa cell line treated with silver ions for 96 hrs

chemical methods which are useful to produce nanoparticles with low yield and environmental issues such as usage of volatile solvents and high-temperature processes such as pyrolysis and high-attrition process. Biological process for preparation of silver nanoparticle is

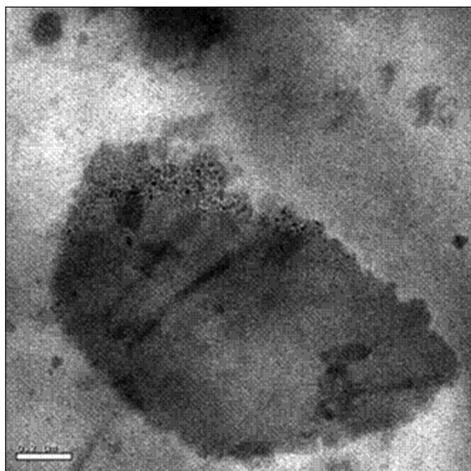


Fig. 18: Transmission electron microscopy image of HeLa cell line treated with silver ions for 96 hrs

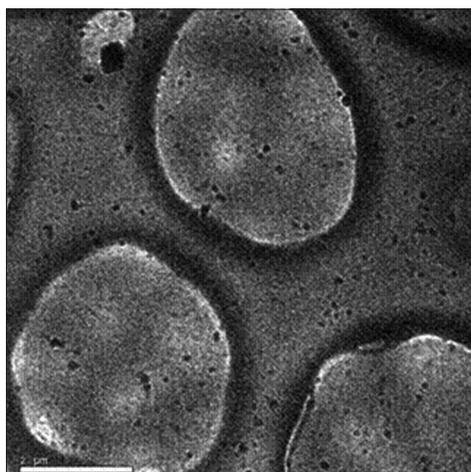


Fig. 19: Transmission electron microscopy image of human embryonic kidney cell line treated with silver ions for 96 hrs

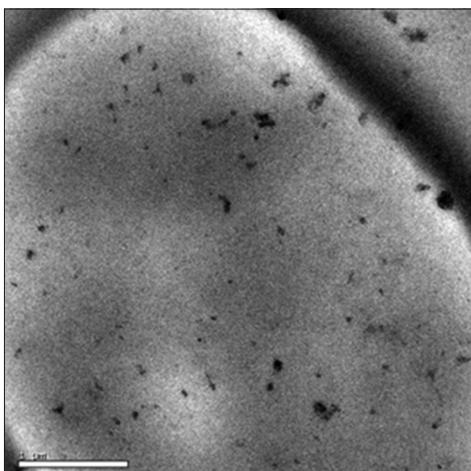


Fig. 20: Transmission electron microscopy image of human embryonic kidney cell line treated with silver ions for 96 hrs

an alternative to replace conventional method and it will be expected that the biological process could produce clean and high yield of silver nanoparticles. Biosynthesis approach utilizing living cells for production of silver nanoparticles has emerged as a simple, clean,

and viable alternative for conventional processes. It is testified that a number of biological systems including microbial and plant cells are used for synthesis of nanoparticles [27].

The utilization of mammalian cells used for biosynthesis of silver nanoparticles is a novel approach in this work. The cancer and non-cancerous cell lines are used for preparation of silver nanoparticles. The biosynthesized silver nanoparticles were characterized by optical absorption spectroscopy. When metal nanoparticles are exposed to electromagnetic waves, the oscillating electric field causes electron in the conduction band to oscillate. When the frequency of the oscillation of electrons becomes equal to the frequency of incident light, resonance takes place which results in the Plasmon absorption peak. This happens in the visible region of silver nanoparticles (Fig. 1).

The exact mechanism of silver ion reduction, transport into cells, and its subsequent nucleation is yet to be studied thoroughly. It is well known that sugars and enzymes present on the cell wall and within cytoplasm can accomplish the reduction process. The secreted proteins from the cells to the silver solution medium might also assist in the reduction process. Although the cells become dead, the subsequent increase in the Plasmon peaks of lysed samples at increasing time intervals suggests the cell membrane becoming porous. The transport of silver ions inside the cells may be facilitated by silver ions acting as biomimetic for transport through ionic channels. Furthermore, electrostatic interactions are created between membrane and cations due to negatively charged nature of membrane surface. This interaction may favorably increase the rate of ion transport inside the cells. Based on this investigation, we show that both intra- and extra-cellular synthesis of silver nanoparticles observed in all the three cell lines.

CONCLUSION

The synthesis of silver nanoparticles might be due to reduction process by the proteins or carbohydrate groups in the cell. The silver nanoparticles might be capped by the biomolecules stabilizing the nanoparticle. Several other studies are in progress to find the location inside the cell where the synthesis is occurring and to characterize the intra- and extra-cellular silver nanoparticles. This method could be novel process of synthesis of silver nanoparticles using cell culture and will be applicable in the area of drug targeting and delivery to the cancer cell [28].

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