

## Toxicity Study of Nanosilver (Nanocid®) on Osteoblast Cancer Cell Line

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Nanotechnology presents countless opportunities to develop new and improved consumer products for the benefit of society. Despite the wide application of nanomaterials, there is a serious lack of information concerning their impact on human health. The purpose of this study was to assess the biological assay of nanosilver (Nanocid®) on osteoblast (G292) cell line. The effect of nanosilver on these cells was evaluated by light microscopy, and by cell proliferation and standard cytotoxicity assays. The results demonstrate a concentration-dependent toxicity for the cell tested, and IC<sub>50</sub> was determined 3.42 µg/mL, suggest that the product is more toxic to cancerous cell comparing to other heavy metal ions.

**Keywords:** Nanosilver, Nanocid, Toxicity test, Osteoblast (G292)

### Introduction

Nanotechnology is the most promising field for generating new applications in medicine. However, only few nanoproducts are currently in use for medical purposes. The most prominent nanoproduct is nanosilver. Nanosilver particles are generally smaller than 100 nm and contain 20–15,000 silver atoms. At nanoscale, silver exhibits remarkably unusual physical, chemical and biological Properties [1]. Furthermore, nanosilver is used for treatment of wounds and burns as well as a contraceptive and marketed as water disinfectant and room spray [2-4]. Nano materials have unique antimicrobial properties. The toxic level of

nanosilver, with the commercial name of Nanocid was studied in Rainbow trout (*Oncorhynchus mykiss*) with the median weight of 1.049 g. The 48, 72 and 96-h median lethal concentrations (LC50) of nanosilver tests were obtained at 3.5, 3 and 2.3 mg/L, respectively, indicating moderate toxicity of nanocid in rainbow trout fish [5].

Many attempts have been made to use silver nanoparticles as an anti-cancer agent and they have all turned up positive [6]. The role of silver nanoparticles as an anti-cancer agent should open new doors in the field of medicine. The design of smart multifunctional nanosystems for intracellular imaging and targeted therapeutic applications requires a thorough understanding of the mechanisms of nanoparticles entering and leaving the cells. For biological and clinical applications, the ability to control and manipulate the accumulation of nanoparticles for an extended period of time inside a cell can lead to

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improvements in diagnostic sensitivity and therapeutic efficiency. This when revealed completely would eliminate the use of expensive drugs for cancer treatment. In general, silver nanoparticles should serve as one of the best ways of treating diseases that involve cell proliferation and cell death [6].

The present study aims to target osteoblast G292 to determine the cytotoxic threshold of nanosilver in this cancerous cell line.

## Experimental

### Nanoparticle production

Silver nanoparticles used in the present study were synthesized by a novel process that involves photoassisted reduction of  $\text{Ag}^+$  to metallic nanoparticles and their biostabilization based on undisclosed US-patent (United State Patent application under No. US/2009/ 0013825) [7]. Briefly, the 4.5 g LABS (Linear alkyl benzene sulfonate) was dissolved in 95 mL distilled water and then was added into a solution containing 0.32 g of silver nitrate. After mixing thoroughly, 0.2 g of hydrazine solution (0.03 M) was added in to the solution, a yellowish silver colloidal solution was formed. The UV-Vis spectrum of the reaction solution revealed an absorption bond at about 415 nm that is the characteristic absorption bond of the nano silver particle. Dynamic light scattering resulted of the reaction solution, show the average particle size is 4.5 nm. The produced nanosilver nominated as Nanocid<sup>®</sup>.

### Cell culture

Osteoblast G292 cell line obtained from Cell Bank of Institute Pasteur of Iran. Cancerous cells were seeded in flask with RPMI medium. The medium used was adjusted to contain 5-10% fetal bovine serum (FBS). The cells were incubated at 37 °C in a 5%  $\text{CO}_2$  atmosphere. After 24-48 h incubation period the attached cells were trypsinized for 3-5 min and centrifugated (1,400 rpm, 5 min). The cells counted and distributed in 96 well ELISA plate with 10,000 cells in each well. The plate was incubated 24-48 h at 37 °C in a 5%  $\text{CO}_2$  atmosphere to allow the cells attach to the bottom of the well [8].

### Cell treatment with nanosilver

The amount of different concentrations of Nanocid was added to each well in duplicates. Different silver nanoparticle concentrations (1, 3, 5, 10, 15, 25, 50, 100 ug/mL ) were inoculated into as-grown cell ( $1 \times 10^4$  cells/well) and the cell population was determined by optical microscopy at 24 and 48 hours [8].

### MTT assay

Cell viability was evaluated by the MTT colorimetric technique [8]. Briefly, 100  $\mu\text{l}$  of the yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) without phenol red, are yellowish in color (Sigma) solution (5 mg/mL in PBS) was added to each well. The plates were incubated for 3-4 h at 37 °C, for reduction of MTT by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan solubilized the MTT crystals by adding and quantified by spectrophotometric mean and then the supernatants were removed. For solubilization of the MTT crystals, 100  $\mu\text{L}$  of isopropanol or DMSO was added to the wells. The plates were placed on a shaker for 15 min for complete solubilization of crystals and then the optical density of each well was determined. The quantity of formazan product as measured by the amount of 545 nm absorbance is directly proportional to the number of living cells in culture. Each experiment was done in duplicate. The relative cell viability (%) related to control wells containing cell culture medium without nanoparticles as a vehicle was calculated by  $[A]_{\text{test}}/[A]_{\text{control}} \times 100$ . Where  $[A]_{\text{test}}$  is the absorbance of the test sample and  $[A]_{\text{control}}$  is the absorbance of control sample [8].

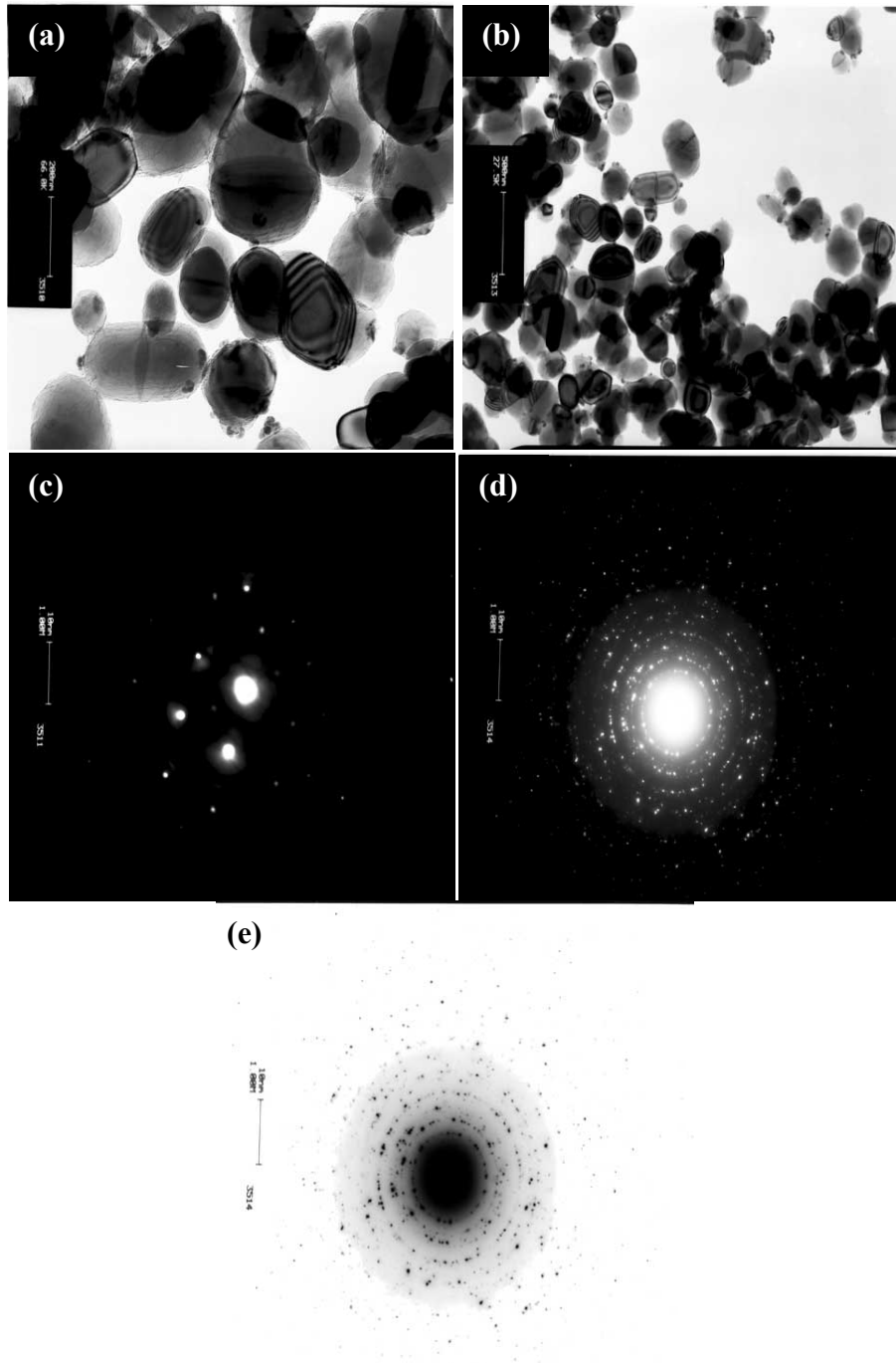
### Statistical Analysis

All experiments were done in duplicate, and the results were presented as mean  $\pm$  standard deviation. The experimental data were analyzed by using SPSS. Statistical significance was accepted at a level of  $p < 0.05$ . To calculate  $\text{IC}_{50}$  values, concentration–response curves were graphed using Graph-pad instat software. The  $\text{IC}_{50}$  of the curves

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was calculated by non-linear regression analysis and interpolation according to the method of

Alexander *et al.* (1999) [9].



**Fig. 1.** Nanoparticles were prepared by solvent displacement method. Results of particle size analysis by scanning electron micrograph (TEM) of nanosilver in dioxide titanium composite (a), and dioxide silver composite (b) particles, and also attached to dioxide titanium (c, d, e). The original magnification of the electron micrograph was 13 500× and the scale bar represents a distance of 1.0 μm.

## Results and discussion

### Characterization of Nanosilver

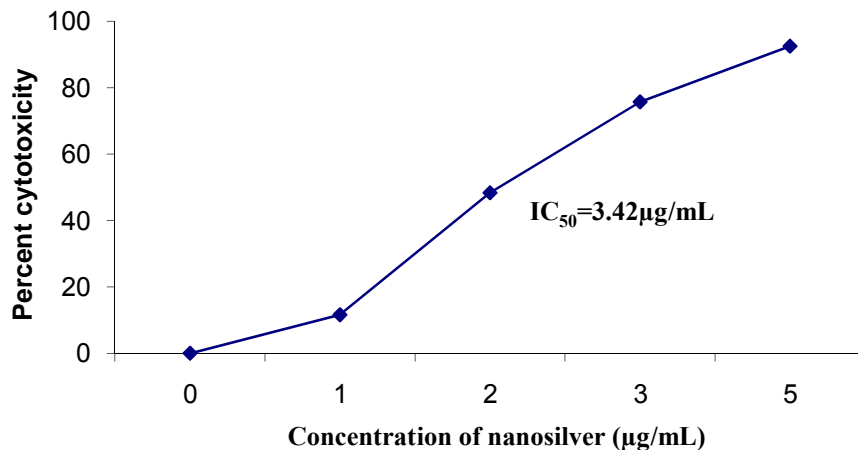
The morphology and microstructure of Nano-Ag as examined by transmission electron microscopy by high resolution (TEM) exhibiting characteristic surface plasmon peak at 436 nm was shown in Fig. 1. The images revealed that the uniform spherical nanoparticles (N90% particles in the size range of 7–20 nm) were obtained (Fig. 1a,b). The size distribution and average diameter of Nano-Ag in aqueous solution were further characterized as shown in (Fig. 1c,d). The data on particle size distribution measured by dynamic light scattering (Zetasizer, Malvern Instruments, UK) revealed the presence of particles in size range of 6.5 to 43.8 nm, with average size 16.6 nm. (Fig. 1e). They were used as colloidal aqueous suspension and were found to retain their stability in the culture media.

### Cytotoxic effects of Nano-Ag cancerous cell line

The *in vitro* cytotoxic effects of Nano-Ag were screened against cancer cell lines by means of MTT assay. As shown in Fig. 2, Nano-Ag exhibited a

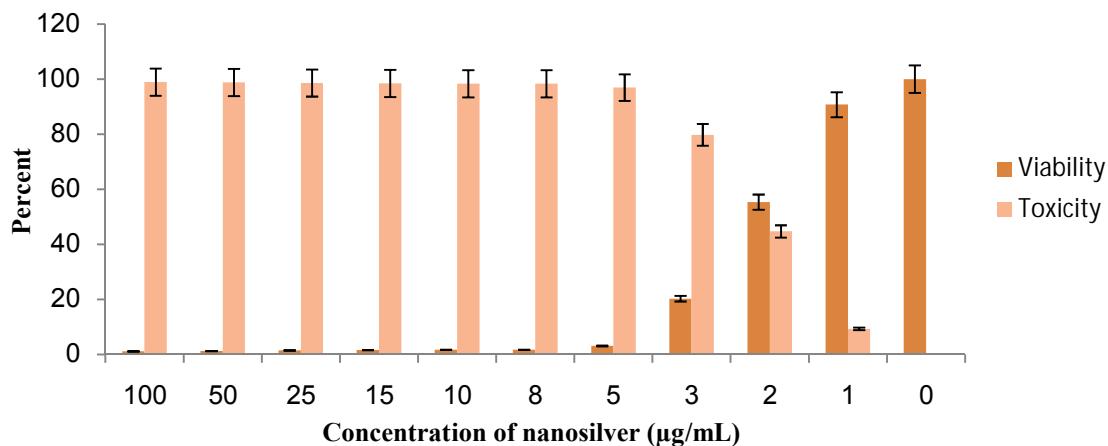
broad spectrum of inhibition against G292 cancer cell with  $IC_{50}$  value of 3.42  $\mu\text{g}/\text{mL}$  (Fig. 3). The result suggested that Nano-Ag possesses great selectivity to cancer cell and can display potential application in cancer chemoprevention and chemotherapy.

Nanotechnology, which deals with devices typically less than 100 nanometer in size, is expected to make a significant contribution to the fields of computer storage, semiconductors, biotechnology, manufacturing and energy. Nanomaterials can enter human tissues through several ports via the lungs after inhalation, through the digestive system and possibly through the skin [10,11]. Systemic distribution of nanoparticles has been demonstrated after inhalation and oral uptake [10,12], and nanoparticles have been found to cross the blood–brain barrier, reaching the olfactory bulb and the cerebellum [13]. Chen and colleagues also reported that nanoparticles can penetrate the blood–testis barrier [14]. Although organ- or cell-specific drug delivery through nanoparticles is a promising area of medicine, and nanoparticles might be used some days as sensors for intracellular mechanisms, few toxicology studies are available.



**Fig. 2.** Cytotoxicity measured by MTT assay on Osteoblast G292 with (1.0–5 $\mu\text{g}/\text{mL}$ ) for 24 h. A statistically significant difference compared to control ( $p < 0.05$ ).  $IC_{50}$  determined by using Graph-pad instat software.

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**Fig. 3.** Percent viability measured on osteoblast cell after treatment with Nanocid (1.0 to 100 µg/mL) for 24 h, by MTT assay. The data are expressed as mean±standard deviation (SD) of three independent experiments. An OD value of control cells (unexposed cells) was taken as 100% viability (0% cytotoxicity). A statistically significant difference compared to control ( $p < 0.05$ ).

Silver nanoparticles have shown to be effective in triggering the activation of Caspase-3 molecule and thus resulting in the mediation and amplification of the death signal. The activation of the Caspase cascade could be through any of the three possible ways which include; granzyme B mediated activation, death receptor mediated activation and the apoptosome mediated activation which includes the release of cytochrome c. The study on Caspase-3 alone gives a very transparent idea making the discussion very ambiguous and so examining the other caspases would be the next level of interest. There is yet another point of discussion which involves the evidence for the molecule leading to only programmed cell death and not necrosis, although both mechanisms result ultimately in cell death. The  $IC_{50}$  values would determine the level of toxicity to the cells and so for apoptosis to occur the toxicity effect must be less significant. The Caspase-3 activation makes it evident that it leads to cleavage of Caspase substrates, resulting in the fragmentation of the DNA. Thus, the triggering of the death inducing signal forms an important area of interest in the trafficking of the nano molecule [15]. Apoptosis is a tightly regulated and at the same time highly

efficient cell death program which requires the interplay of a multitude of factors. Apoptotic pathways involve the activation of the downstream of mitochondrial proapoptotic events. The cancer cells can be targeted using silver nanoparticle conjugated with a tissue specific ligand that can activate any of the VEGF receptors and express its effect on any of the downstream molecules [6].

Many of the artificially manufactured nanoparticles are made of nonbiodegradable pollutants, such as carbon black and metals. The long-term behavior of such substances is not known. Nanosilver (Nanocid®) which is a liquid product of Nano Nasb Pars Co., in Iran has been produced which is a potent antibacterial. There is no data concerning the toxicity effect of Nanocid in cancerous cell. In this study the effect of Nanocid on cell viability was estimated using MTT assay. The G292 osteoblast cell line used as cancerous cell line. The result showed that the cytotoxicity of Nanocid in cancerous cell is quite effective. Moreover, in a time-course study, we found that the monodispersed Nano-Ag can keep stable at least for 12 months in the soluble state (data not shown). This stabilization of Nanosilver is an important issue for its medical applications.

### Conclusion

Nanosilver (Nanocid®) which is a liquid product of Nano Nasb Pars Co., in Iran has been produced which is a potent antibacterial. The purpose of this study was to assess the biological assay of Nanocid® on cancerous cell line. The results demonstrated a concentration-dependent toxicity for the cells tested, and IC<sub>50</sub> was determined to 3.42 µg/mL in G292 osteoblast cancerous cell, suggest that the product is more toxic to cancerous cells comparing to other heavy metal ions.

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