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Toxicity Study of Two Different Synthesized Silver Nanoparticles on Bacteria *Vibrio Fischeri*

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***Abstract*—**A comparative evaluation of acute toxicity of synthesized nano silvers using two different procedures (biological and chemical reduction methods) and silver ions on bacteria *Vibrio fischeri* was investigated. The bacterial light inhibition test as a toxicological endpoint was used by applying of a homemade luminometer. To compare the toxicity effects as a quantitative parameter, a nominal effective concentrations (EC) of chemicals and a susceptibility constant (Z-value) of bacteria, after 5 min and 30 min exposure times, were calculated. After 5 and 30 min contact times, the EC_{50} values of two silver nanoparticles and the EC_{20} values were about similar. It demonstrates that toxicity of silvers was independent of their procedure. The EC values of nanoparticles were larger than those of the silver ions. The susceptibilities(Z- Values) of *V.fischeri* (L/mg) to the silver ions were greater than those of the nano silvers. According to the EC and Z values, the toxicity of silvers decreased in the following order: Silver ions >> silver nanoparticles from chemical reduction method ~ silver nanoparticles from biological method.

***Keywords*—**Bioluminescence, Luminometer, silver nano particles, Toxicity, *Vibrio fischeri*

I. Introduction

NANOTECHNOLOGY plays an important role in many key technologies of the new millennium. The application of nanoscale and nano-structure materials within range of 1 to 100 nanometers is an emerging area of nanoscience and nanotechnology. Nanomaterials may provide solutions to technological and environmental challenges in the areas of solar energy conversion, catalysis, medicine and water treatment [1]-[2]. The noble metals especially gold and silver due to their innumerable applications in different branches such as catalysis, photonics, photography and more importantly in the field of medicine as antimicrobial factors have drawn much attentions to themselves[3]-[4]. In addition, colloidal silver is of particular interest because of distinctive properties, such as good conductivity, chemical stability, catalytic and antibacterial activity [5]. Silver nanoparticles have many important applications which include, spectrally selective coating for solar energy absorption and intercalation material for electrical batteries, as optical receptors, polarizing filters, catalysts in chemical reaction, biolabelling and as antimicrobial agents[6].

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Silver nanoparticles and different silver-based materials containing ionic silver or metallic silver are already being commercialized for their antimicrobial activity [7]-[8]. This antibacterial property was used in many interesting applications such as coatings on medical apparatus [9], assisting in fabrics [10]. Such toxicity is useful for these applications; however, it also propose eco-toxicological problems upon release [11]. Then it is very important to understand the grade of danger respecting silver-based nanoparticles to the environment. Several mechanisms for toxic effects of silver colloid particles have been hypothesized. The main mechanism of toxicity of silver nanoparticles, results from the release of Ag ions from nanoparticles which lead to oxidation stress (OS), damage of lipids, carbohydrates, proteins and DNA[12]-[13]. Some studies were conducted about the potential eco-toxicity of nano sized silver with crustaceans such as *Daphnia magna* and various bacterial strains. *D. magna, Daphnia pulex*, and *Ceriodaphnia dubia* are approved test models for effluent release into the environment by the Environmental Protection Agency and other regulatory agencies [14] because of their sensitivity towards potential pollutants, such as metal ion species. Toxicity study of Ag and Au nanoparticles on *Daphnia magna* were conducted by Li et al. The toxicity of all nanoparticles tested was found to be dose and composition depend. The concentration of Au nanoparticles that killed 50% of the test organisms (LC50) ranged from 65– 75 mg/L. In addition, three different sized Ag nanoparticles were studied to analyze the toxicological effects of particle size on *D. magna*. Changing the ratio between silver nitrate and sodium citrate during Ag NP synthesis led to an increase in the average particle size produced; however, no difference in toxicity was observed once the overall [Ag] was corrected using flame atomic absorption spectrometry results. Toxicity ranged from 3–4 μg/L for all three sets of Ag nanoparticles tested[15]. The antibacterial effect of silver nanoparticles on *E.coli* was investigated by Sondi and SalopekSondi [16]. The test results based on the TEM analysis and proteomic research, shown that the silver nanoparticles interact with elements of the bacterial membrane causing structural change, dissipation of the proton motive force, and eventually cell death [17]. Different from silver nanoparticles, studies about silver ions were performed for a long time and have been shown to interact with cytoplasmic components and nucleic acids, to inhibit chain enzymes of respiratory, and to interfere with penetrability of membrane [18]. The antimicrobial activities of silver nanopowder on three microorganisms was reported [19].

As an initial step in investigating, the potential eco-toxicity to the environment and biological species a quantitative parameter was applied to compare the susceptibilities of three microbes and morphological changes were analyzed. In general, the susceptibilities decreased in the following order:
E.coli > *B. subtilis* > *S. cerevisiae*

In this paper, the toxicity effects of two type of synthesized nano silvers and silver ions were tested using Gram-negative bioluminescence bacteria *Vibrio fischeri*. Toxicity effecxts of silver nanoparticles and silver ions to *V.fischeri* were compared with application of two numerical values (Z and EC).

II. Materials And Method

A. Materials

Vibrio fischeri strain (PTCC 1693) and *E.coli* strain (PTCC 1763) were bought from Iranian Research Organization for Science and Technology (IROST). All components of culture medium containing sodium chloride, potassium chloride, sodium bicarbonate, calsium chloride dehydrate, agar, peptone, magnesium sulphate hepta hydrate, magnesium sulphate, magnesium chloride hexa hydrate, calsium chloride, magnesium chloride and sodium hydroxide, hydrochloric acid, Silver nitrate, 3-Aminophthalahydrazide(luminol), hydrogen peroxide, copper sulphate penta hydrate and sodium borohydride were purchased from E. Merck (Germany).

B. Preparation of Bacteria Vibrio Fischeri

To ensure the best quality of luminescent bacteria with maintainable viability, the bacteria can be inoculated and maintained in culture medium. Although different cultures can be used, the following culture mediums allow greatest luminescence, growth and stability that are practical for the tests. In this way, two basic growth media were examined:

1. Sea water agar (twin pack).
2. Sea water.

The first media was used for solid cultures and the second one for liquid cultures. The bioluminescence of *Vibrio fischeri* in sea water agar culture (solid media) and liquid culture has been shown in Fig. 1. Solid cultures were retained in incubator at 18°C. After inoculation of liquid medium by luminous *V. fischeri* from solid culture, the liquid culture was incubated for 48 h at 18°C in an orbital shaker at 120 rpm [20].

C. Preparation of Bacteria E.Coli

Culture medium of Muller-Hinton-Broth was prepared according to the conditions provided from the factory. 15 gr Muller-Hinton-Broth's medium was dissolved in 500 mL Erlenmeyer flasks containing 500 mL deionized water and stirred forcefully. pH 7.3 ± 0.2 was controlled by the addition of 1M NaOH or 1M HCl. After 20 minutes autoclave, bacteria *E. coli* was cultivated for a period of 1 day in Muller-Hinton-Broth's medium at temperature of 37°C in incubator. After 1 day, bacterium suspension was prepared and was made ready for subsequent experiments.

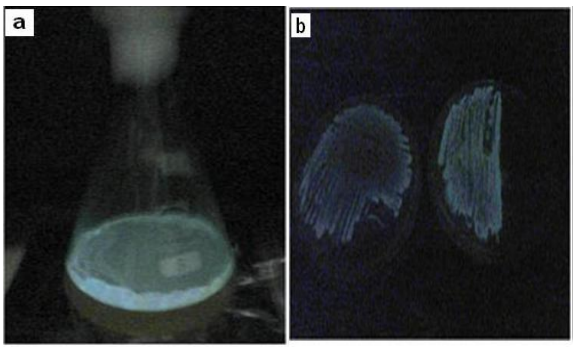

Fig. 1 Bioluminescence of bacteria *Vibrio fischeri* in liquid culture(a) and solid culture(b)

D. Synthesize of Silver Nanoparticles By Chemical Reduction Method

Aqueous solutions of $AgNO_3$ (1 mM) and $NaBH_4$ (2 mM) were prepared with deionized distilled water. $NaBH_4$ solution (15 mL) was ice cooled and stirred vigorously with the help of a magnetic stirrer. $AgNO_3$ solution (5 mL) was then added dropwise. The color of the solution slowly turned yellowish, indicating the reduction of the silver ions. The prepared suspension was stored at 5°C. The quality of nanoparticles was found to stable for several weeks (12-16 weeks). Transmission electron micrographs (TEM) at 200 kV and UV–VIS spectra were then recorded to characterize the prepared Ag NPs. The wavelength of maximum peak (λ_{max}) is about 400 nm, which indicates the presence of silver nanoparticles.

E. Synthesize of Silver Nanoparticles By Biological Method

Prepared *E.coli* bacterial suspension was filtered by means of a 0.22 m filter. 1 mL obtained filtrate was added to container containing 100 mL $AgNO_3$ (1mM). After a few minutes (about 20 min), colourless solution of silver nitrate in container turns into brown colour. This colour change indicates possibility of silver nanoparticles production. In order to prove existence of nanoparticles, product efficiency and their size, UV-VIS spectroscopy, transmission electron microscopy (TEM) were used. The wavelength of maximum peak (λ_{max}) at 5^0C and pH of 5.5 is about 415 nm which indicates the presence of silver nanoparticles.

F. Atomic Absorption

To measure the amounts of ionic silvers in silver nanoparticle suspensions, an atomic absorption spectrophotometer (Thermo Spectra AA M5) was used. In order to separate the Ag NP, the nanoparticle suspensions were centrifuged (Eppendorf, Minispin). The separation was done by centrifuging 2.2 mL of silver colloid solution, running at 12K rpm for 15 min [15].

Then 2 mL of supernatant was gathered. The supernatant was then analyzed by AA and compared to the original silver colloid samples. For calibration, fresh silver standard solutions were generated in the concentration of 20 ppm. The

stock solution was serially diluted to produce 10, 5.0, 1.0, 0.5, 0.1 mg/L Ag ion solutions.

G.Assay Procedure and Data Analysis

Flash assay is a test that inhibits *V.fischeri* luminescence and was done by homemade luminometer. A 1 ml volume of *V.fischeri* suspension was palced into 4.5 ml cuvette and 1 ml volume of silver nanoparticle or silver nitrate solution at various concentrations were added. When 1 mL of bacterial suspension was mixed with 1 mL of silver nanoparticles or silver ions, the resulting concentration was half of the initial concentration in the final 2 mL of mixed solution. A 100 ppm silver ions stock solution was prepared by dissolving 15.74 mg of $AgNO_3$ powder in 100 mL deionized distillated water. The concentration of both type of silver nanoparticles in stock suspensions were 120 ppm. The decrease of bacterial luminescence (INH%) due to addition of test samples was calculated after 5 and 30 min , as follows:

$INH\% = 100 - (IT_T / (IT_0 \times KF)) \times 100$ with $KF = IC_T / IC_0$

Where, KF is the correction factor based on control, IC_0 and IT_0 are the initial luminescene intensities of control and test samples. IC_T and IT_T are the luminescence intensities of the control and test samples after 'T' minute contact time. EC_{50} and EC_{20} values are the concentrations of nanoparticles or silver ions (mg/L) causing 50% and 20% decrease in bioluminescence after 'T' minute exposure time. Various exposure times have been used (e.g., 5 min, 15 min, 30 min and 45 min) [21]-[22]-[23]-[24]-[25]-[26]. In this study, 5 and 30 min were seclected as contact times. Three independent assays were carried out in each test. The data for percentage inhibition obtained in each experiment were converted to gamma values where:

Gamma = % inhibition / (100 -% inhibition)

Gamma values were plotted against their corresponding chemical concentrations, after first converting all data to natural logs (Ln), to generate Ln gamma / Ln concentration curves for each chemical. Values falling within the 1-99% inhibition range were used to fit a straight line to the Ln-transformed data by linear regression and the resulting equations used to calculate the EC_{20} and EC_{50}.

H.Apparatus

Bioluminescence detection was carried out by a homemade luminometer equipped with a model R-446 photomultiplier (PMT) (Hamamatsu,Japan). The luminometer connected to a personal computer via a suitable interface (Micropars, Tehran, Iran) as shown in Fig. 2. Experiments were done in cuvette of 100 mm^2 cross sectional area and 45 mm height at 25°C. Intensity of bioluminescence was recorded as a function of time, the time resolution of the luminometer was 0.01 s. Calibration of device was performed by the luminol chemiluminescence reaction [27].

The luminol chemiluminescence reaction is one of the most effective systems that generates chemiluminescence. The linear range of concentrations and responses (calibration graph), have been shown in Fig.3.

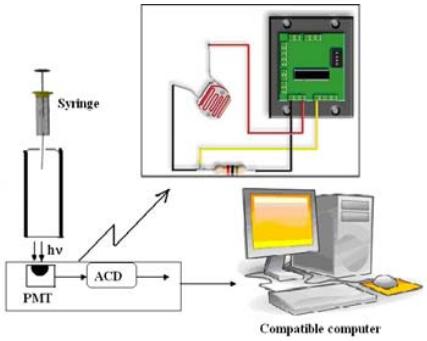

Fig. 2 Schematic representation of homemade luminometer for measuring of bioluminescence

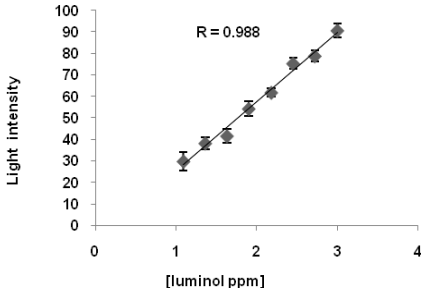

Fig.3 Correlation diagram(calibration diagram) for Chemiluminescence emission intensity as a function of luminol concentration. The all reagent concentratioins are: $CuSO_4.5H_2O$(6×10^{-3}M,0.1ml), Hydrogen peroxide (10%,0.1ml),water (1ml)and varying concentrations of luminol solution in NaOH(0.1 M):(1) 1.09 ppm ,(2) 1.36 ppm , (3) 1.63 ppm ,(4) 1.9 ppm ,(5) 2.18 ppm ,(6) 2.45 ppm ,(7) 2.72 ppm ,(8) 3 ppm

III. Results And Discussion

A. Characterization of Ag Nanoparticles

Ag NP suspentions, synthesized by two different procedure, were analyzed applying absorption spectroscopy (UV-Vis,Cecil CE5501) and transmission electron microscopy (TEM, JEOL JEM2010 electron microscopy, UIC). UV–vis absorbance spectrometry is one of the first methods for the analysis of silver colloids since they are plasmonic in nature [28]. The oscillation of the conduction electrons, i.e., the plasmon, is very sensitive to changes in particle size, dielectric constant of the medium, and aggregation state of the particles. Therefore, freshly synthesized Ag NPs were analyzed between the wavelengths of 390–450 nm. According to absorption spectroscopy of silver nanoparticles synthesized by chemical reduction method, the observed wavelength of maximum peak (λ_{max}) was about 400 nm which was indicative of the presence of silver nanoparticles(Fig. 4a).

TEM results for the Ag NP samples showed about 10 nm of nanoparticles size (Fig. 5a). The silver nanoparticles synthesized by biological method, were characterized by UV–vis absorbance spectrometry. The maximum peak (λ_{max}) of about 415 nm was recorded (Fig. 4b).

Transmission electron microscopy (TEM) shows and confirms that silver nanoparticles are at nano-size. TEM images of the produced nanoparticles are shown in Fig. 5b. Flame atomic absorption spectrometry (AAS) was used to determine whether excess silver ions (Ag^{+}) remained after reduction of silver nitrate. It was determined that for both type of the Ag NPs , very little silver was lost during synthesis. When NPs were separated out of the stock solutions by centrifugation and the supernatants measured using AAS, no [Ag] was detectable in the supernatant, suggesting that all of the bioavailable Ag was in a NP form and was not in a silver ion form.

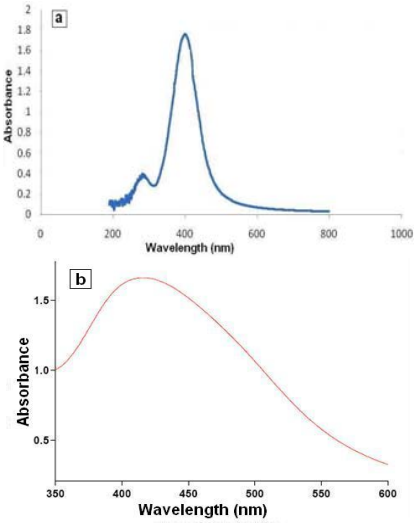

Fig. 4 Absorption spectra (UV-vis spectra) of silver nanoparticles synthesized by chemical reduction method (a) and biological method (b)

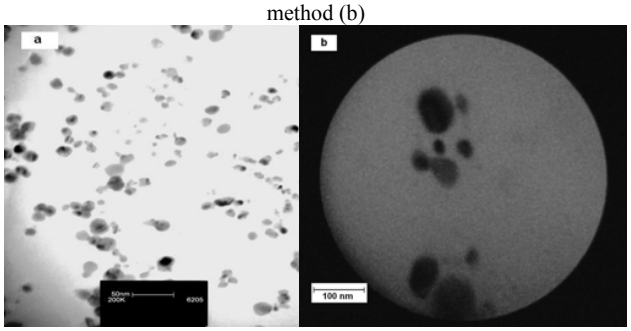

Fig. 5 TEM images of silver nanoparticles synthesized by chemical reduction method (a) and biological method (b)

B. Comparison of EC Values

The toxic effects of exposure to Ag nanoparticles were tested using bacteria *Vibrio fischeri*, after contact times of 5 and 30 min. The results are shown in Tables I and II. As we see in Tables I and II, after 5 and 30 min exposure times, the calculated EC values of nanoparticles are about similar. TEM images (Figs. 5a nad 5b) showed a differences in the size of synthesized nanoparticles.

Therefore, it can be said that in this study, toxicity of nanoparticles did not correlate to the procedures of production but particle size is dependent on the procedures.

As shown in Tables I and II, the concentrations of silver nanoparticles in contact with *V.fischeri* , killed more bacteria after 30 min than 5 min and led to higher light inhibition (INH%) and higher toxicity. In other word, they need a few times to diffuse to the cells and degrade lipids, carbohydrates, proteins and DNA. To determine the toxicity effects of the silver ions in comparison with silver nanoparticles, the toxicity of silver ions were tested. Silver ions strongly affected the viability of *V.fischeri* with 50% inhibition of bioluminescence after 5 and 30 min contact times at about 15.9 ppm and 1.85 ppm, respectively. Silver ions may affect *Vibrio fischeri* cells by inactivating proteins through combining with the -SH group [4] and interacting with respiratory chains, as believed to occur for heavy metals [29]. According to the Table I, the EC_{20} and EC_{50} values of silver ions after 5 min, were about 3.5 and 2.9-fold lower than those of both silver nanoparticles. The EC_{20} and EC_{50} values of silver ions after 30 min (Table II), in comparison with Ag nanoparticles, decreased about 5.7 and 17 times, respectively. These results indicate that Ag ions which obtained from $AgNO_3$ in water solution were always more toxic than that of both synthesized silver nanoparticles.

C. Comparison of Z- Values

To compare the toxicity effects of silver nanoparticles and silver ions on bacteria *Vibrio fischeri* as an another quantitative parameter, the susceptibility has been used in this work [30]. For the assessment of toxicity effects of silver materials, the susceptibility constant Z (L/mg) can be used and is defined by the following equation:

$$Z = - \ln (IT_t/IT_0)/C$$

Where, IT_t is the bioluminescence of *V.fischeri* after exposure to siver naanoparticles and silver ions, IT_0 is the bioluminescence of *V.fischeri* in the absence of silver materials, and C is the concentration of the silvers(ppm). Using the Z value, a given C value, and the exposure time, the survival fraction of bacteria can be predicted. A higher Z value shows that the microorganisms are more sensitive to the materials, signifying that the materials are more toxic to the bacteria. The calculated susceptibilities are shown in Table III. C^{*} and $C^{\dagger}$ represent the concentrations of silver nanoparticles in the suspensions and the concentration of silver ions in the silver nitrate solution, respectively. To estimate the Z values, a range of concentrations from 0.66 to 6.66 ppm for both C^{*} and $C^{\dagger}$ were used. As shown in Table III, the susceptibility increases with increasing of the exposure time from 5 to 30 min. Based on the C* concentrations and for exposure times of 5 and 30 min, the susceptibilities of *V.fischeri* were not much different, indicating that the toxic effects of two types of naoparticles on *V.fischeri* are comparable.For silver nanoparticles synthesized by chemical reduction method, the average Z value of *V.fischeri* increased to 0.1630 (600%) from 5 min to 30 min.

TABLE I
EFFECTIVE CONCENTRATIONS (EC) FOR THREE CHEMICALS THAT RESULT IN 20% AND 50% INHIBITION IN TEST SYSTEM THAT MEASURE BIOLUMINESCENCE OF *VIBRIO FISCHERI*. THE EC_{50} AND EC_{20} VALUES WERE CALCULATED AFTER 5 MIN EXPOSURE TIME AT 25°C. THE EC VALUES HAVE BEEN OBTAINED FROM LINEAR REGRESSION EQUATION OF CONCENTRATION/RESPONSE CURVES OF THE FORM : LN Y = M LN X + C , WHERE Y IS THE VALUE OF GAMMA (GAMMA= %INHIBITION/(100 - % INHIBITION)), X IS THE CONCENTRATION AND M AND C ARE THE SLOPE AND INTERCEPT, RESPECTIVELY. THE INDIVIDUAL VALUES FOR THE SLOPE AND INTERCEPT FOR EACH CHEMICAL MAY BE USED TO CALCULATE ANY DESIRED GAMMA VALUE AND THUS ANY REQUIRED EC

Chemicals	EC_{50} (ppm)	SD(±)	EC_{20}(ppm)	SD(±)	m	C	r
Nano Ag (Biologica methodl)	47.220	2.330	17.800	0.490	1.426	- 5.493	0.963
Nano Ag (Chemical reduction method)	45.077	2.18	15.310	0.740	1.284	- 4.889	0.971
Ag ions (Ag^+)	15.890	0.813	4.830	0.460	1.164	- 3.221	0.978

TABLE II
EFFECTIVE CONCENTRATIONS (EC) FOR THREE CHEMICALS THAT RESULT IN 20% AND 50% INHIBITION IN TEST SYSTEM THAT MEASURE BIOLUMINESCENCE OF *VIBRIO FISCHERI*. THE EC_{50} AND EC_{20} VALUES WERE CALCULATED AFTER 30 MIN EXPOSURE TIME AT 25°C

Chemicals	EC_{50} (ppm)	SD(±)	EC_{20}(ppm)	SD(±)	m	C	r
Nano Ag (Biological method)	34.550	5.670	5.410	1.550	0.765	-2.690	0.949
Nano Ag (Chemical reduction method)	29.320	2.220	3.970	0.860	0.693	-2.343	0.929
Ag ions (Ag^+)	1.856	0.007	0.824	0.005	1.712	-1.063	0.957

TABLE III
SUSCEPTIBILITIES (Z- VALUES) OF BACTERIA *VIBRIO FISCHERI*

		C_1^*	SD(±)	C_2^*	SD(±)	$C^\dagger$	SD(±)
Average Z - values (L/mg)	5 min	0.032	0.009	0.032	0.011	0.067	0.020
	30 min	0.192	0.050	0.182	0.056	0.430	0.120

$Z = - \ln (IT_i/IT_0)/C$
Concentration of both C^* and $C^\dagger$ ranging from 0.66 to 6.66 ppm were used for calculations.
C_1^* = concentration of silver nanoparticles from chemical reduction method (ppm).
C_2^* = concentration of silver nanoparticles from biological method (ppm).
$C^\dagger$ = concentration of silver ions not that of nitrate ions in $AgNO_3$ solution (ppm).

For biological synthesized silver nanoparticles, Z values increased to 0.1501 (570%). During 30 min contact time, the susceptibilities of *V.fischeri* to both silver nanoparticels increased. It may be possible that synthesized silver nanoparticles showed acute toxicity. As shown in Table III, based on the $C^{\dagger}$ concentration, the average Z value of *V.fischeri* increased to 0.363 (641.8%) from 5 min to 30 min. The susceptibilities of *V.fischeri* to silver ions were higher than two types of silver nanoparticles, indicating that *V.fischeri* was more sensitive to the silver ions. When comparing the results from the $C^{*}$and $C^{\dagger}$ concentrations, the susceptibilities of *V.fischeri* based on $C^{\dagger}$ were larger than those of C^{*}, then silver ions were more toxic than both type of synthesized silver nanoparticles. Diffusivity of silver ions to the bacterial cells are more than that of the Ag NPs.

IV. Conclusion

In this study, we have reported the toxicity of silver ions and silver nanoparticles on bacteria *Vibrio fischeri*. According to the EC and Z values, the toxicity decreased in the following order:

Silver ions >> silver nanoparticles from chemical reduction method ~ silver nanoparticles from biological method.

Toxicity of two silver nanoparticles were about similar that indicating procedure of production did not influence on toxicity. Whereas, the effective concentrations (EC_{50} and EC_{20}) of materials after 30 min exposure times were less than 5 min and but the susceptibility constants were greater at the vice versa direction. It can be concluded that the bacteria *V.fischeri* is so sensitive to toxicity effects of chemicals and can be used as a biosensor for rapid and low-cost detection of acute toxicity of nonmaterials.

Acknowledgment

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