

Investigation on Toxicity of Manufactured Nanoparticles to Bioluminescence Bacteria *Vibrio fischeri*

E. Binaeian and SH. Soroushnia

Abstract—Acute toxicity of nano SiO₂, ZnO, MCM-41 (Meso pore silica), Cu, Multi Wall Carbon Nano Tube (MWCNT), Single Wall Carbon Nano Tube (SWCNT), Fe (Coated) to bacteria *Vibrio fischeri* using a homemade luminometer, was evaluated. The values of the nominal effective concentrations (EC), causing 20% and 50% inhibition of bioluminescence, using two mathematical models at two times of 5 and 30 minutes were calculated. Luminometer was designed with Photomultiplier (PMT) detector. Luminol chemiluminescence reaction was carried out for the calibration graph. In the linear calibration range, the correlation coefficients and coefficient of Variation (CV) were 0.988 and 3.21% respectively which demonstrate the accuracy and reproducibility of the instrument that are suitable. The important part of this research depends on how to optimize the best condition for maximum bioluminescence. The culture of *Vibrio fischeri* with optimal conditions in liquid media, were stirring at 120 rpm at a temperature of 15°C to 18°C and were incubated for 24 to 72 hours while solid medium was held at 18°C and for 48 hours. Suspension of nanoparticles ZnO, after 30 min contact time to bacteria *Vibrio fischeri*, showed the highest toxicity while SiO₂ nanoparticles showed the lowest toxicity. After 5 min exposure time, the toxicity of ZnO was the strongest and MCM-41 was the weakest toxicant component.

Keywords—Bioluminescence, effective concentration, nanomaterials, toxicity, *Vibrio fischeri*.

I. INTRODUCTION

THE applications of nanotechnology in the form of nanomaterials have increased, so research on the effects of exposure to nanomaterials and their toxicity is very important, especially the domestic and industrial waste water is likely to spread there. Diagnostic tests for toxicity in recent years have grown continuously, and they are useful tools for evaluating the spread of toxic substances into the environment. A lot of analytical methods that are used to check the pollution of the environment, require expensive equipments and sampling from environment which are time consuming. These problems are solved by one of these methods using the bacteria, as a biosensor. This method is sensitive (responding to the very low concentrations of particles), low-cost and easily reproducible and takes 5 to 30 minutes to predict toxicity. *Vibrio fischeri* is a luminescent

bacteria can be used in a toxicity test. The use of *Vibrio fischeri* bacteria in the bioluminescence inhibition test has the advantages that mentioned above, also sometimes, it can solve the ethical problems arising from the use of animals (fish, mice, etc). A biosensor is an analytical device that combines a biological sensing element with a transducer to produce a signal proportional to the analyte concentration [1]. Biosensors have been extensively applied in clinical, food and environmental areas due to the advantages of fast detection speed, high selectivity and sensitivity [2]. Risk hazards of nano particles are different because they do not behave as predicted. Nanoparticles offer unique, physical, chemical, electrical and optical properties while are generating toxins, cancer and allergies. The main mechanism of toxicity of nanoparticles resulting from oxidation stress (OS) that lipids, carbohydrates, proteins and DNA damage [3]. ZnO and CuO nanoparticles are used as an anti-bacterial protection in dentistry and as the construction of wood and anti-bacterial cloth, respectively [4]. Preparation of ZnO nanoparticles used in cosmetics and sunscreens is increasing day by day, because they reflect Ultra-violet better than larger particles [5]. The preparation of nanoparticles for self-cleaning coating is also used [6]. nano ZnO, in preparing the catalysts, ceramics and colored materials is used. Silica nano particles (silicon dioxide SiO₂) have importance in fabrication of catalyst support, electrical and thermal insulators and also are used in the coating process, creating Molecular Sieve adsorbents and filler materials[7]. In medicine and pharmacy are used as drug carriers and also for gene delivery [8]. Mesoporous silicates, such as MCM-41 (the most common mesoporous silicates), are porous silicates with huge surface areas (normally $\geq 1000\text{m}^2/\text{g}$), large pore sizes ($2\text{ nm} \leq \text{size} \leq 20\text{ nm}$) and ordered arrays of cylindrical mesopores with very regular pore morphology.

The large surface areas of these solids increase the probability that a reactant molecule in solution will come into contact with the catalyst surface and react. The large pore size and ordered pore morphology allow one to be sure that the reactant molecules are small enough to diffuse into the pores. Today, mesoporous silica nanoparticles have many applications in catalysis, drug delivery and imaging [9]. In a study was conducted in 2009 by K.Kasemets [10], toxic effects of nanoparticles ZnO, CuO and TiO₂ on the single-cell eukaryotic organisms *Saccharomyces cerevisiae* were evaluated. The effect of metal oxide nanoparticles, the bulk

E.Binaeian is with the Department of Chemical Engineering, Qaemshahr Branch, Islamic Azad University, Qaemshahr, IRAN (Phone: +98-123-2145046; fax: +98-123-2145046; (E-mail: ehsan.binaeian@yahoo.com).

Sh.Soroushnia is with the Baran Sazan Novin Zanjan Company, Zanjan, IRAN, Tel:+98-241-3239288; fax:+98-241-3229289; (E-mail: shsoroushnia@yahoo.com).

and ion formation were compared. Both formulation of ZnO showed the same toxicity. Nano CuO was 60 times more toxic than bulk CuO. The reason of increase of toxicity (nano and bulk CuO) after 24 hour exposure time than 8 hour, was increase of copper ion dissolution in excess times.

The study was conducted in 2007 by Zhang et al.[11] for Nano ZnO, the mechanisms of membrane destruction and OS(oxidation stress) as an anti-bacterial agents on the bacteria *Escherichia coli* has been shown. Toxicity of nanoparticles and bulk ZnO, TiO₂, CuO to bacteria *Vibrio fischeri* and *Daphnia magna* and *Thamnocephalus platyurus* species of crustaceans, were analyzed by Margit Heinlaan et al. [12]. ZnO components (nano and bulk) and ZnSO₄ on three species were highly toxic. Unlike zinc, copper compounds on three species showed different toxicity provided copper ions were more toxic than bulk and nano copper oxide. Wei Jiang et al. [13] investigated on toxicity of nanoparticles SiO₂, Al₂O₃, TiO₂ and ZnO to bacteria *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas fluorescens* and compared the results with the toxicity of compounds in Bulk formulation. All nano particles except TiO₂ were more toxic than their bulk formulation. ZnO nano particles were more toxic than three other nano particles and 100% of the bacteria were destroyed. SiO₂ nano particles killed 40% of bacteria *B. subtilis*, 58% of *E.coli* and 70% of *P. fluorescens*. Flash assay which is performed in microplate can be used as a method with high efficiency, low cost and quick to measure the toxicity of nano particles (anti-bacterial properties) to the bacteria *Vibrio fischeri*. In the study, 11 materials with different properties in two groups of particles (metallic and organic) and metal salts in cuvette and microplate in flash assay were examined. EC₅₀ values after 30 min exposure time to nano scale organic cationic polymers, were between 215 to 775 mg/l. for metal oxides, EC₅₀ values after 30 min ,were about 4mg/l ,100 mg/l and 4000 mg/l for ZnO (bulk and nano formulation), nano CuO and bulk CuO, respectively[14]. Size dependent properties of nano materials such as difference in toxicity have been proved. Therefore, particular properties of nano materials (large specific area) may produce different biological effects than materials in micro size. Arthritis, tuberculosis and chronic renal disease are the phenomenon of contact to micro sized silica [15]. Cytotoxicity effect of SiO₂ nano particles (15 and 46 nm) in human bronchoalveolar carcinoma-derived cells and the oxidative stress mechanisms which is caused by nano silica (15nm) was evaluated by Weisheng Lin [16]. Silica nano particles with different concentrations were dispersed in the medium. By changing the concentrations (10 to 100 mg ml) and exposure time (24, 48 and 72 h) of both size of silica, cell viability decreased. Carbon nanotubes have thermal, chemical, electrical and mechanical properties uniquely. Reforms and chemical changes in carbon nanotubes for solubility in water, length, diameter, aspect ratio of nanotubes, their type and impurities are the effective parameters of the nanotubes that produce toxicity. In a research, suspensions of carbon nanotubes (single wall and

multi wall) and C60 were prepared by Sonication Process. Then they were examined by UV radiation in order to inactivate the bacteria *Vibrio fischeri* and production of reactive oxygen species (ROS) [17]. This study showed that ROS production and increase of toxicity are indicating the size reduction and contrary. Xiaoshan Zhu et al. studied on acute toxicity of SWCNT and MWCNT to species *Daphnia magna* after 48 hours exposure time [18]. This assessment was based on inactivity and mortality as endpoints of toxicology. This research exhibited that SWCNT was toxic than MWCNT. Applications of copper nano particles are in the manufacture of ceramics, films, polymers, oils, lubricants, coatings and electronic components [19]. The copper nano particles are used in pharmaceutical and as antibacterial substances. Toxicity of copper nano particles to bacteria *E.coli* and *Bacillus subtilis* using the agar plate test, were studied by Yoon et al. [20]. The results demonstrated that antimicrobial properties of copper nano particles to both types of bacteria. In the research was conducted in 2006, the toxicity of nano particles copper/sepiolite on *E.coli* and *S.aureus* were evaluated and was observed that the growth of bacteria was limited to 99/99% by nano particles[21]. Fred Rispoli et al. studied about the effects of aeration, concentration of nano particles, pH, concentration of bacteria and temperature on toxicity of nano copper based on *E.coli* test [22]. The magnetic and iron nano particles are used in biological separation and detection of biological (cells, proteins, bacteria, viruses, enzymes, nucleic acid), clinical diagnoses (MRI (magnetic resonance image)) and Drug delivery [23].

In this study, we calculated the effective concentration (EC) of nano materials causing 20% and 50% inhibition of bioluminescence to *Vibrio fischeri* using the homemade luminometer by two models: The gamma model and the Weibull distribution model, also the optimized conditions of culture for maximum bioluminescence of *V. fischeri* were determined.

II. MATERIALS AND METHOD

A. Materials

All nanosized materials were purchased from Nanotechnology Research Center, Research Institute of Petroleum Industry, Iran (RIPI) with particle sizes of 10-50 nm for nano ZnO, 60-100 nm for SiO₂, 60-150 nm for MCM-41, 10 - 20 nm diameter and mean 10 μ m length for MWCNT ,2-3 nm diameter and mean 10 μ m length for SWCNT and 5-25 nm for coated nano iron. The purity of materials were 95% for both type of carbon nano tubes, 24% for nano iron and 99.5% for other nano particles. Sodium dodecyl sulphate (SDS), luminol, CuSO₄.5H₂O and hydrogen peroxide (H₂O₂ 35%) were purchased from Fulka Chemical Company (Buchs, Switzerland). The SDS solution in its critical micelle concentration (CMC) was prepared (7-10 mM) and the stock suspensions of nano materials in SDS were sonicated for 30 min and stored in dimness at refrigerator. The stock concentrations were 6000,6000,6000, 1200,300,300,300 ppm

for nano Fe (coated), SiO₂, MCM-41, Cu, MWCNT, ZnO, SWCNT respectively. Before toxicity experiments, stocks were vortexed.

B. Organism and nutrient media

Vibrio fischeri strain (PTCC 1693) was bought from Iranian Research Organization for Science and Technology (IROST). 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 cultures medium allow greatest luminescence, growth and solidity that are practical for the mentioned procedure. In this way, three basic growth media were examined:

1. Bacto Marine Broth (DIFCO 2216)
2. Sea water agar (twin pack)
3. Sea water

The first media was used for reviving; the second one was used for solid cultures and the third one for liquid cultures. The bioluminescence of *Vibrio fischeri* in sea water agar culture (solid media) and liquid medium have been shown in Fig. 1. Solid cultures were retained in incubator at 18°C. After inoculation with luminous *V. fischeri* from solid culture, liquid cultures were incubated for 48 h at 18°C in an orbital shaker at 120 rpm [24].

C. Assay Procedure and Data Analysis

Flash assay is a test that inhibits *V. fischeri* luminescence and was done by homemade luminometer that will be illustrated in the following section. A 1 ml volume of bacterial suspension was contacted with 1 ml volume of suspension of nano materials. All suspensions were diluted in SDS solution. The decrease in bacterial luminescence (INH%) due to addition of test samples was calculated as follows [12]:

$$\text{INH}\% = 100 - \left(\frac{IT_T}{(IT_0 \times KF)} \right) \times 100 \quad \text{with } KF = \frac{IC_T}{IC_0}$$

where, KF is the correction factor based on control, IC₀ and IT₀ are the initial luminescence intensities of control and test samples. IC_T and IT_T are the luminescence intensities of the control and test samples after 'T' minutes contact time. EC50 and EC20 values are the concentrations of toxicants (mg/L) causing 50% and 20% decrease in bioluminescence after 'T' minutes exposure time, respectively. up to now, various exposure times have been used, e.g., 5 min, 15 min, 30 min [12]-[14]-[25].

In this study, 5 min and 30 min were selected as operational times. Three independent assays were carried out. The data for percentage inhibition obtained in each experiment were converted to gamma values according to first model [25], where:

$$\text{Gamma} = \% \text{ inhibition} / (100 - \% \text{ inhibition})$$

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 in this model. values falling within the 10-90% inhibition range were used to fit a straight line to the Ln-transformed data by linear regression and the resulting

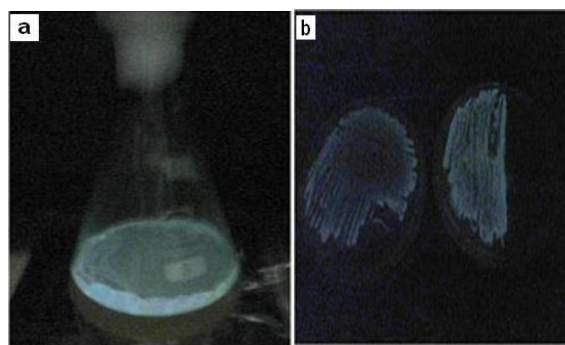


Fig. 1 Bioluminescence of bacteria *Vibrio fischeri* in (a) liquid culture and (b) solid culture

equations used to calculate the EC20 and EC50. For each compound, the EC20 and EC50 values have been calculated from linear regression equations of dose/response curves of the form:

$$\text{Ln}y = m\text{Ln}x + c$$

where, Ln is the natural log, y is the value for gamma, x is the dose (ppm), m and c are the slope and intercept, respectively. In the second model, the concentration and response (INH) obtained from each experiment, are fitted by two parametric Weibull distribution equation [26]:

$$F(C) = (1 - \exp(-\exp(k_1 + k_2 \log_{10}(C))))$$

In this equation, F(C) represents the amounts of INH, C is the concentration (mg/l), k₁ and k₂ are the location and the slope parameters, respectively. With linear regression of Weibull equation, the plotting ln(-ln(1-F(C))) values as the y-axis versus log₁₀(C) values as the axis of x, k₁ and k₂ are calculated. With an equation as follows, EC20 and EC50 values are calculated with the INH = 0.2 and INH = 0.5

$$y = k_1 + k_2 x$$

where x, y are log₁₀(C) and ln(-ln(1-F(C))), respectively.

D. Apparatus

Bioluminescence detection was carried out by a homemade luminometer supplied 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 double layer cuvettes of 49 mm² internal cross sectional area, 100 mm² external cross sectional area and 45 mm altitude at 25°C.

bioluminescence intensity was recorded as function of time, the time resolution of the luminometer was 0.01s. Calibration of device was performed by the luminol luminescence reaction [27] (see Fig. 3). The luminol luminescence reaction is one of the most effective non-biological system producing light or chemiluminescence. For evaluating the precision, accuracy, reproducibility of device, some quantities like LOD (Limit of detection), LOQ (Limit of quantity), LLR (Limit of linear range), LDR (Linear dynamic range), C.V (Coefficient of variation), should be calculated [28]. The values of these quantities are listed in Table I.

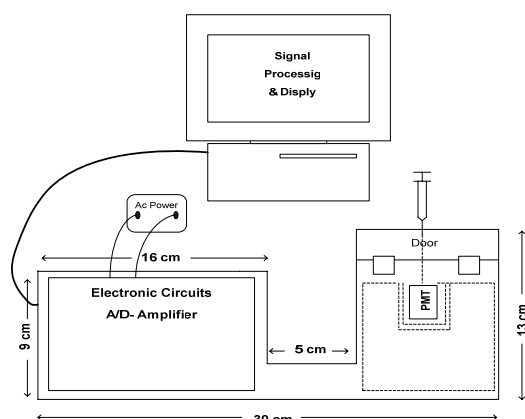


Fig. 2 Schematic representation of homemade luminometer for measuring of bioluminescence

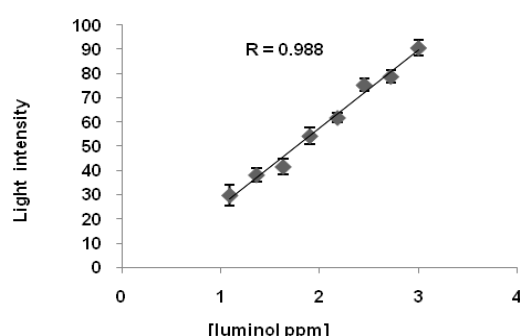


Fig. 3 Correlation diagram(calibration diagram) for Chemiluminescence emission intensity as a function of luminol concentration. The all reagent concentrations are: $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ($6 \times 10^{-3}\text{M}$, 0.1ml), Hydrogen peroxide (10%, 0.1ml), water (1ml) and varying concentrations of luminol solution in NaOH (0.1 M): (1) 1.09 ppm, (2) 1.36 ppm, (3) 1.63 ppm, (4) 1.90 ppm, (5) 2.18 ppm, (6) 2.45 ppm, (7) 2.72 ppm, (8) 3.00 ppm

TABLE I

THE FIGURE OF MERIT (PRECISENESS, ACCURACY AND REPEATABILITY) OF CHEMILUMINESCENCE TEST

Quantities	Amounts
LOD: Limit of detection	0.16 ppm
LOQ: Limit of quantity	0.53 ppm
LDR: Limit of dynamic range	2.47 ppm
LLR: limit of linear range	3.00 ppm
C.V: Coefficient variation	3.21%

III. RESULTS AND DISCUSSION

Toxicity of nano particles SiO_2 , ZnO, MCM-41 (Meso pore silica), Cu, MWCNT, SWCNT and Fe (Coated) to bacteria *Vibrio fischeri* were evaluated. The values of EC50 and EC20, at 5min and 30 min contact times were calculated using two mathematical models. At 30 min contact time, the suspension of SiO_2 showed the lowest toxicity, means EC50 values calculated from two models for SiO_2 were greater than that of the other reagents. In contrast, the suspensions of ZnO showed the highest toxicity (see Table II). EC50 values obtained for ZnO from two models are comparable with effective concentrations of ZnO suspensions obtained by Margit Heinllan (1.9ppm) [12] and M. Mortimer (4.8ppm) [14]. The test results and calculation of effective concentration (EC) values showed that MCM-41 and nano ZnO have the lowest and highest toxicity after 5 min exposure time to *V.fischeri* respectively (Table III). MCM-41 after 30 min contact time to *Vibrio fischeri*, was more toxic than nano SiO_2 . MCM-41s are listed to the latticed silica nano particles which are quite porous and have meso pore structure, while silica (SiO_2) is nonporous-spherical nanoparticle. SWCNT was a little more toxic than MWCNT after 30 min contact time. The reason of this matter is that the size of SWCNT is smaller than MWCNT [29]. EC50 values for SWCNT calculated by two models to *Vibro fischeri* (Table II), are similar to the values obtained by A.P.Roberts (20ppm to 100% mortality) [30], the species was *Daphnia magna*. Also, E.J.Petersen [31] for 51% mortality of the *Lumbriculus variegates*, reported EC50 value about 10 ppm. For Nano copper, effective concentrations after 5 min, were not calculable, but EC50 values obtained after 30 min (see Table II) are greater than the values obtained for the nano-CuO by Margit Heinllan (EC50 = 79ppm) [12] and M.mortimer (EC50 = 68.1ppm)[14]. It can be concluded that copper oxide nano particles are a little more toxic than copper nano particles. This toxicity may be because of greatest specific area of nano CuO and Cu ions. The required effective concentrations causing 20 % and 50% light reduction after 30 min are less than that of required effective concentrations after 5 min. As shown in Tables II and III, the concentrations of reagents in contact to *V.fischeri*, kill more bacteria after 30 min compared to 5 min and lead to higher light inhibition (INH%) and higher toxicity. There is enough time to diffuse to cells and ruining lipids, carbohydrates, proteins and DNA.

IV. CONCLUSION

The obtained information from calibration test of device and comparison the toxicity results obtained from two models, prove that toxicity test based on bioluminescence will produce similar and repeatable results using other standard distribution models. This research has produced a large amount data on optimization of the best condition for maximum bioluminescence to *Vibrio fischeri*, setting up, calibration of homemade luminometer and evaluation of toxicity of some toxicant and nano materials to *Vibrio fischeri* which had not been investigated up to now. We deduce that

there are some differences in the results acquired from two models and results of other investigations. Deviations are chiefly because of differences between laboratory protocols

and the method which chemicals are made ready. Procedure is reproducible and relatively low cost.

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 EC₅₀ AND EC₂₀ VALUES WERE CALCULATED AFTER 30 MIN EXPOSURE TIME AT 25 °C

Component	gamma model					Weibull model				
	EC 50	EC 20	m	c	r	EC 50	EC 20	K ₁	K ₂	r
Fe(coated)	238.19	51.06	0.90	-4.94	0.85	255.45	25.05	-3.07	1.12	0.90
MCM-41	319.68	26.80	0.55	-3.25	0.96	366.86	25.38	-2.84	0.96	0.97
Cu	86.31	12.64	0.72	-3.21	0.94	89.28	12.74	-2.97	1.34	0.95
SiO ₂	333.82	26.98	0.55	-3.20	0.92	381.27	27.27	-2.91	0.98	0.88
ZnO	10.24	2.14	0.88	-2.06	0.88	7.79	3.32	-3.11	3.08	0.90
MWCNT	13.87	1.52	0.62	-1.65	0.95	13.98	2.04	-2.19	1.60	0.94
SWCNT	12.13	3.73	1.17	-2.93	0.93	11.03	3.16	-2.54	2.09	0.97

TABLE III

EFFECTIVE CONCENTRATION(EC) FOR CHEMICALS IN 20% AND 50% INHIBITION IN TWO MODELS. THE EC 50 AND EC 20 VALUES (PPM) WERE OBTAINED AFTER 5 MIN EXPOSURE TIME AT 25 °C FROM GAMMA AND WEIBULL EQUATIONS

Component	gamma model					Weibull model				
	EC 50	EC 20	m	c	r	EC 50	EC 20	K ₁	K ₂	r
Fe(coated)	545.42	105.15	0.84	-5.32	0.81	653.75	76.88	-3.80	1.22	0.88
MCM-41	966.50	142.95	0.72	-4.98	0.96	959.37	157.75	-4.67	1.44	0.95
SiO ₂	664.15	33.03	0.46	-3.00	0.98	702.70	32.74	-2.78	0.85	0.97
ZnO	18.32	3.16	0.79	-2.29	0.94	16.44	2.87	-2.19	1.50	0.92
MWCNT	20.16	4.32	0.90	-2.70	0.97	18.43	5.18	-3.43	2.42	0.94
SWCNT	21.51	5.21	1.02	-3.13	0.90	17.13	4.37	-2.72	1.90	0.92

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