Antibacterial activity of ZnO nanoparticles: dependence on particle size, dispersion media and storage time

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Abstract. Zinc oxide nanoparticles have a high potential as novel antimicrobial products. In this study ZnO nanoparticles of various sizes: 20-100 nm (ZnO-1) and 50-300 nm (ZnO-2) have been examined. This paper presents the results of an integrated study of the influence of physicochemical properties such as particle size and concentration of the freshly prepared and 24-hour suspensions of ZnO nanoparticles (0. 001…1000 mg/L) in distilled water and in normal saline upon their toxicity against bacteria \textit{E. coli}. Ionic solutions of ZnCl\textsubscript{2} were used for comparison study. Fresh aqueous solutions of nanoparticles displayed the highest antibacterial effect (\textit{E. coli} survival under 5–25%). Storage for 24 hours reduced the toxicity of all the samples. In normal saline solutions the toxicity of the studied nanoparticles decreased at concentrations below 10 g/L. In all cases ZnO-2 suspensions displayed lower toxicity levels, especially after ageing for 24 hours. Stability analysis showed that the highest antibacterial effect was produced by less stable solutions containing the largest aggregates. Thus, it has been shown that antibacterial effects of zinc oxide nanoparticles are affected by the size of particles and their aggregates in colloidal solutions, chemical composition of the media and the suspensions storage time. The obtained results can be used for creating new types of antibacterial products including phytoprotectors based on nano-sized zinc oxide particles as well as for development of methods to assess and predict toxicity of zinc-containing suspensions.

1. Introduction

Nowadays, the production and use of various nanomaterials such as zinc, silicon, titanium, iron, aluminium oxide nanoparticles, metallic nanoparticles of iron, copper, cobalt, nickel, aluminium, silver as well as fullerenes, carbon nanotubes, etc. are growing at a fast rate. These materials get released into the environment and consequently accumulated in biota components and abiotic environments with a further possibility of being transferred to humans \cite{1}

Zinc oxide is mostly employed for its antibacterial and photocatalytic properties that promotes its wide use in chemical, pharmaceutical and cosmetics industries. Antiseptic properties of ZnO make it a promising water purification agent \cite{2, 3}. Antibacterial properties of zinc oxide nanoparticles are well studied \cite{4-9}, in this case development of oxidative stress connected with photocatalytic activity of the particles is considered as the major factor in toxicity \cite{10, 11}.
Nevertheless, the influence of the dispersion medium on ZnO nanoparticles toxicity level lacks clarification though this parameter can be of importance when nanoparticles get released into the environment. Thus, the present study assesses the impact of the particle size, dispersion medium and the solutions storage time on the toxic activity of ZnO nanoparticles against *E. coli*.

2. Materials and methods

This paper examines zinc oxide nanopowders ZnO-1 manufactured by Nanostructured & Amorphous Materials, Inc. (USA), with the following stated specifications: mean particle size of 20 nm, specific surface 20 m²/g and ZnO-2 nanopowders manufactured by the chemical vapor deposition method at the Department of Functional Systems & High-Temperature Materials of NUST MISIS. The electron micrographs and elemental analyses have been obtained employing a transmission electron microscope JEM-1400 (TEM, Jeol, Japan) and scanning electron microscope JSM-6610lv (SEM, Jeol, Japan) with an INCA SDD X-MAX energy dispersive micro-analyzer (Oxford Instruments, UK).

ZnO solutions toxicity was measured by bioluminescence technique used for microbiological and molecular genetic evaluation of the nanomaterials influence on microbiocenosis species. The test is similar to a widely used naturally luminous Vibrio fischeri (Microtox) test [12 - 15].

The method measures inhibition of bioluminescence intensity of the genetically modified photobacteria strain *E. coli* affected by the nanoparticles present in the studied sample as compared to the control sample. Alteration in the bioluminescence intensity of the tested object in the analyzed sample as compared to the control sample containing no toxic agents was taken as the effect criterion. Bioluminescence intensity reduces in proportion to toxic effect. Toxic effect of the studied nanomaterial sample upon bacteria is determined by their bioluminescence inhibition after 30-minute exposure period. The quantitative test-reaction parameter assessment is expressed as a toxicity index T which is a nondimensional quantity determined from the formula

\[ T = 100 \left( \frac{I_0 - I}{I_0} \right) \]

where \( I_0 \) and \( I \) are luminous intensities of the control and tested samples accordingly, while the exposition time of the examined sample with the test-object is fixed.

The technique allows for three threshold levels of the toxicity index:

1. acceptable degree when toxicity index \( T \) is in the range from 0 to 20;
2. medium degree when toxicity index \( T \) is in the range from 20 to 50;
3. high degree when toxicity index \( T \) equals or is higher than 50.

In the process of toxicity evaluation simultaneous measurements of the control and studied samples were carried out. For higher data reliability the number of repeat sample tests was increased up to 5 measurements. The toxic properties were analyzed in freshly prepared (stored for no more than 1 hour) suspensions and 24 hour suspensions. The measurements were carried out using Biotox-10 specialized luminometer (Russian Federation).

For the present study dispersions of nanoparticles were prepared in distilled water (pH 7.2 ± 0.2) and in normal saline (0.9% NaCl, pH 7.2 ± 0.2). Quantities of ZnO nanoparticles (0.1 g) were poured into the prepared dispersion medium (100 ml) and stirred with a glass rod. All the initial dispersions had particle concentration of 1 g/L. After stirring the suspensions were processed in Ultrasonic Cleaner CD-4800 (Codyson, China) for 60 seconds (70 W, 44 Hz, volume 1.4 l). The initial solutions were then diluted with distilled water or normal saline in order to prepare suspensions with zinc concentrations of 0.1…0.000001 g/l.

For the analysis of Zn²⁺ ions efficacy zinc chloride solutions were used at concentrations of 1000…0.001 mg/L in terms of the equivalent amount of Zn.

The analysis of ZnO nanoparticles size distribution in the suspensions was performed by the dynamic light scattering method (DLS) using Malvern Zetasizer Nano (GB). Zeta potential measurements by means of laser-Doppler microelectrophoresis were also carried out using Malvern Zetasizer Nano.
3. Results and discussion

From SEM images one can observe that the particles in both samples are aggregated into large agglomerates (figure 1). For the ZnO-1 sample the agglomerates of about 30 μm consist, in their turn, of smaller particle clusters with the size of 0.5 – 1 μm. Evidently, the size of the particles is under 100 nm. In the sample of ZnO-2 powder the agglomerates were smaller in size - 5 μm or less, but when viewed at higher magnification, revealed individual particles larger than 100 nm.

![SEM images of ZnO nanopowders: a, c – ZnO-1; b, d- ZnO-2](image_url)

**Figure 1.** SEM images of ZnO nanopowders: a, c – ZnO-1; b, d- ZnO-2

From the presented microphotographs one can observe that the particles in both samples are aggregated into large agglomerates (figure 1). For the ZnO-1 sample the agglomerates of about 30 μm consist, in their turn, of smaller particle clusters with the size of 0.5 – 1 μm. Evidently, the size of the particles is under 100 nm. In the sample of ZnO-2 powder the agglomerates were smaller in size - 5 μm or less, but when viewed at higher magnification, revealed individual particles larger than 100 nm.

Transmission electron microscopy was used to determine the particles size. Figure 2 represents microphotographs and electron diffraction patterns of ZnO nanoparticles from the ZnO-1 and ZnO-2 samples.
The majority of the ZnO-1 sample consisted of particles under 100 nm with equiaxial dimensions. The particles had a crystalline structure. In the ZnO-2 sample the particles varied in morphology. The sample contained some amount of isomorphic particles with the sizes ranging from 50 to 300 nm in diameter as well as some rod-shaped particles with the lengths up to 500 nm. A number of plate-shaped particles with diameter of 3 μm and below has also been detected. The electron diffraction pattern revealed the clear crystalline structure of the particles.

The chemical composition of the studied samples was determined by X-ray microspectroscopy analysis. Figure 3 represents microphotographs and the data from X-ray microspectroscopy analysis of ZnO nanoparticles from the ZnO-1 and ZnO-2 samples.
From the above data it can be concluded that the samples consist mostly of zinc and oxygen, though a small amount (1 wt.% of sulfur has also been detected.

The study of toxicity of the freshly prepared ZnO-1 suspensions in distilled water (figure 4a) has shown high antibacterial efficacy at every studied concentration with the survival rate of 5...25%. After 24-hour storage of the suspensions the survival rate increased up to 77...92% at a concentration of 0.001...0.1 mg/L, though at a higher concentration of 1...1000 mg/L the change in the survival rate was negligible. The analysis of the particle size distribution and zeta potential (figure 4c, 4e) in aqueous suspensions revealed no evident correlation between these characteristics and the toxicity rate.

When normal saline was used as a dispersion medium (figure 4b), the freshly prepared suspensions produced no toxic effect at 0.001...0.1 mg/L concentrations and at 1 mg/L the effect was much weaker than in freshly-prepared aqueous suspensions. However, at 10...1000 mg/L high toxicity was observed with the bacterial survival rate below 5%. Also the experiment revealed almost complete absence of correlation between the antibacterial properties and storage time of the solutions. Comparison of the obtained data with the results of stability and particle size distribution (figure 4c 4,e) showed that less stable suspensions (zeta potential 9...0 mV) with the largest aggregates (600... 1600 nm for freshly prepared suspensions and 650 - 2900 nm for 24-hour ones) had the highest antibacterial efficacy. The impact of particle aggregation in colloidal systems on toxicity rate has been described for various types of nanoparticles in a number of publications [16, 17], including ZnO nanoparticles [18].

**Figure 3.** The data from X-ray microspectroscopy analysis of ZnO nanoparticles from the ZnO-1 (a, b) and ZnO-2 (c, d) samples.
Figure 4. Toxic properties of ZnO-1 suspensions in aqueous medium (a) and in normal saline (b); zeta potential of ZnO-1 nanoparticles in aqueous medium (c) and in normal saline (d); size distribution of ZnO-1 nanoparticles in aqueous medium (e) and in normal saline (f).

The study of toxicity of ZnO-2 suspensions in distilled water (figure 5) revealed the effects similar to those observed in the case of ZnO-1, i.e. the freshly prepared suspensions had high antibacterial effect at every concentration. Although the efficacy of 24-hour solutions was by 45-25% lower than that displayed by ZnO-1. Zeta potential analysis (figure 5c) showed that the dispersions with nanoparticle concentration of 0.001…1 mg/L were more stable with the zeta potential value of 33 – 40 mV indicative of the system stability. Further increase in concentration reduced the zeta potential values, this effect is associated with large aggregates formation (figure 5e). Comparison of the toxicology and stability tests revealed a clear correlation between the antibacterial effect and the
particle size in the suspension, i.e. the suspensions with the largest aggregates had the maximal toxicity.

![Graphs and images showing toxic properties and size distribution of ZnO-2 suspensions.](image)

**Figure 5.** Toxic properties of ZnO-2 suspensions in aqueous medium (a) and in normal saline (b); zeta potential of ZnO-2 nanoparticles in aqueous medium (c) and in normal saline (d); size distribution of ZnO-2 nanoparticles in aqueous medium (e) and in normal saline (f).

In normal saline freshly prepared suspensions with low concentrations displayed lower toxicity, similar to the results observed for ZnO-1, although at the concentrations of 10…1000 mg/L the toxic effect was very high with the bacterial survival rate below 1%. In 24-hour preparations a decrease in antibacterial effect was observed, the efficacy dropped to 80 units, i.e. the bacterial survival rate was
about 20%. As in the cases above, the least stable solutions with the largest aggregates displayed the highest antibacterial activity (figure 5d, 5f).

It is worth noting that smaller ZnO-1 particles displayed higher toxicity than larger particles in the ZnO-2 sample. It is known that antimicrobial activity of ZnO increases with decreasing particle size. This is attributed to the larger surface-to-volume ratio which results in a more efficient means for antibacterial activity [19]. At the same time, our data indicating higher toxicity of colloids with large aggregates do not contradict the results of the previous studies as in order to display their toxic properties zinc nanoparticles do not necessarily have to be accumulated on the surface or penetrate into the bacterial cells as happens in cases of smaller particle aggregates. Nanoparticles of ZnO have an impact on the microenvironment around the bacteria which is sufficient for killing them [20]. Presumably, individual ZnO particles retain sufficient surface activity after aggregation.

In literature the following mechanisms of ZnO nanoparticles toxic effect are considered: induced oxidative stress [21, 22], Zn$^{2+}$ ions release [23] or direct contact of nanoparticles with the cell membrane [20]. Besides, the idea of ZnO nanoparticles photocatalytic activity being responsible for their antibacterial activity has been advanced by a number of researchers [24].

Toxicological study of zinc chloride (ZnCl$_2$) aqueous solutions has revealed their inhibiting effect against *E. coli* though their antibacterial efficacy is weaker than that of nanoparticle-based suspensions with the bacterial survival rate ranging from 75% at the lowest concentration to under 10% at the highest concentration (figure 6a).

![Figure 6. Toxic properties of zinc chloride in aqueous medium (a) and in normal saline (b).](image)

When normal saline was used as a medium, toxicity of the solutions decreased and at concentrations of 0.001...1 mg/L no effect was detected at all, although at high concentrations the antibacterial efficacy remained high with the bacterial survival rate under 5%. Further tests showed that a 24-hour storage period had no significant effect on the solutions toxicity values. Other authors have also indicated the important role that chemical composition of the aqueous media plays in the antibacterial efficacy of preparations based on zinc oxide nanoparticles [25]. It has been noted that increase in the medium salt content decreased ZnO nanoparticles toxicity against the harpacticoid copepods *Tigriopus japonicus* [26] and the marine diatom *Thalassiosira pseudonana* [27], which correlates well with the results of our study performed on *E. coli*.

4. Conclusion

Freshly prepared aqueous dispersions of nanoparticles displayed the highest toxicity, their considerable antibacterial efficacy has been revealed at all the studied concentrations with the bacterial survival rate ranging between 5 and 25%. Toxic effects of solutions were less pronounced for all the concentrations below 1 mg/L. The study of 24-hour preparations revealed decrease in the toxicity level for all the samples with concentrations of 0.001...0.1 mg/L, the inhibiting effect was reduced to zero
at the lowest concentration. It is also worth noting that the aged ZnO-2-based dispersions had the lowest toxic effect with the bacterial survival rate more than 20% lower compared ZnO-1 dispersions. When normal saline was substituted for distilled water in fresh dispersions it lead to decrease in the antibacterial efficacy right down to zero toxicity at concentrations below 1 mg/L. In the aged solutions the observed toxicity remained almost equal to that of the fresh ones except in ZnO-2 nanoparticle suspensions were, similar to the aqueous medium, the toxicity of saline-based suspensions with concentrations 10…1000 mg/L was reduced by 20%. The study of the colloidal system stability has revealed higher antibacterial effect of less stable preparations with the largest aggregate sizes. Although, as a whole, the larger ZnO-2 particles was more toxic than the smaller ZnO-1 ones.

The obtained results can be used for creating new types of antibacterial products including phytoprotectors based on nano-sized zinc oxide particles as well as for development of methods to assess and predict toxicity of zinc-containing suspensions.

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References

[1] Crane M, Handy RD, Garrod J and Owen R 2008 Ecotoxicity test methods and environmental hazard assessment for engineered nanoparticles Ecotoxicology 17(5) 421
[2] Serpone N, Dondi D and Albini A 2007 Inorganic and organic UV filters: their role and efficacy in sunscreens and sunscreen products Inorg. Chim. Act. 360 794
[3] Dastjerdi R, Montazer M 2010 A review on the application of inorganic nano-structured materials in the modification of textiles: focus on anti-microbial properties. Colloid Surf B 79(1) 5
[4] Yamamoto O 2001 Influence of particle size on the antibacterial activity of zinc oxide. Int. J. Inorg. Mater. 3 643
[5] Stoimenov PK, Klinger RL, Marchin GL and Klabunde KJ 2002 Metal oxide nanoparticles as bactericidal agents Langmuir 18 6679
[6] Jin T, Sun D, Su JY, Zhang H and Sue HJ 2009 Antimicrobial efficacy of zinc oxide quantum dots against Listeria monocytogenes, Salmonella enteritidis, and Escherichia coli O157:H J. Food. Sci. 74(1) 46
[7] Lingling Z, Yunhong J, Yulong D, Malcolm P and David Y 2006 Investigation into the antibacterial behavior of suspensions of ZnO nanoparticles (ZnO nanofluids) J. Nanoparticle Res. 9(3) 479
[8] Reddy KM, Kevin F, Jason B, Denise GW, Cory H and Alex P 2007 Selective toxicity of zinc oxide nanoparticles to prokaryotic and eukaryotic systems J. Appl. Phys. Lett. 90(21) 1
[9] Rizwan W, Amrita M, Soon-Il Y, Young-Soon K and Hyung-Shik Sh 2010 Antibacterial activity of ZnO nanoparticles prepared via nonhydrolytic solution route J. Appl. Microbial. Biotechnol. 87(5) 1917
[10] Sawai J, Shinobu S, Igarashi H, Atsushi H, Takao K, Masaru S and Hiromitsu K 1998 Hydrogen peroxide as an antibacterial factor in zinc oxide powder slurry J. Ferment. Bioeng. 86 521.
[11] Baranwal A, Srivastava A, Kumar P, Bajpai VK, Maurya PK and Chandra P 2018 prospects of nanostructure materials and their composites as antimicrobial agents Front. Microbiol. 9 422
[12] Lopes I, Ribeiro R, Antunes FE, Rocha-Santos TA, Rasteiro MG, Soares AM, Gonçalves F and Pereira R 2012 Toxicity and genotoxicity of organic and inorganic nanoparticles to the bacteria Vibrio fischeri and Salmonella typhimurium Ecotoxicology 21(3) 637
[13] Garcia A, Recillas S, Sánchez A and Font X 2012 The luminescent bacteria test to determine the acute toxicity of nanoparticle suspensions Methods in Molecular Biology 926 255
[14] Mogiľ'naia OA, Puzyr' AP and Bondar' VS 2010 Growth and bioluminescence of luminous
bacteria under the action of aflatoxin B1 before and after its treatment with nanodiamonds.

[15] Zarubina AP, Lukashev EP, Deev LI, Parkhomenko IM and Rubin AB 2009 Biotesting the biological effects of single-wall carbon nanotubes using bioluminescent bacteria test-system. 

[16] Römer I, White TA, Baalousha M, Chipman K, Viant MR and Lead JR 2011 Aggregation and dispersion of silver nanoparticles in exposure media for aquatic toxicity tests. J. Chromatogr. A. 8 4226

[17] Albanese A and Chan WC 2011 Effect of gold nanoparticle aggregation on cell uptake and toxicity ACS Nano. 5(7) 5478

[18] Tripathy N, Hong TK, Ha KT, Jeong HS and Hahn YB 2014 Effect of ZnO nanoparticles aggregation on the toxicity in RAW 264.7 murine macrophage J. Hazard. Mater. 15 110

[19] Baker C, Pradhan A, Pakstis L, Pochan DJ and Shah SI 2005 Synthesis and antibacterial properties of silver nanoparticles J. Nanosci. Nanotechnol. 5 244

[20] Heinlaan M, Ivask A, Blinova I, Dubourguier HC and Kahru A 2008 Toxicity of nanosized and bulk ZnO, CuO and TiO₂ to bacteria Vibrio fischeri and crustaceans Daphnia magna and Thamnocephalus platyurus Chemosphere 71 1308

[21] Vijayaraghavan R 2012 Zinc oxide based inorganic antimicrobial agents Int J Sci Res. 1 35

[22] Leung YH. et al. 2016 Toxicity of ZnO and TiO₂ to Escherichia coli cells Sci. Rep. 6 35243

[23] Li Q, Mahendra S, Lyon DY, Brunet L, Liga MV, Li D and Alvarez PJ 2008 Antimicrobial nanomaterials for water disinfection and microbial control: potential applications and implications Water Res. 42 4591

[24] Sirelkhatim A, Mahmud S, Seeni A, Kaus NHM, Ann LC, Bakhori SKM, Hasan H and Mohamad D 2015 Review on zinc oxide nanoparticles: antibacterial activity and toxicity mechanism Nano-Micro Lett. 7 219

[25] Dimapilis EAS Hsu CS, Mendoza RMO and Lu MC 2017 Zinc oxide nanoparticles for water disinfection Sust. Env. Res. 28 47

[26] Park J, Kim S, Yoo J, Lee JS, Park JW and Jung J 2014 Effect of salinity on acute copper and zinc toxicity to Tigriopus japonicus: the difference between metal ions and nanoparticles. Mar Pollut Bull. 85(2) 526

[27] Yung MMN, Kwok KWH, Djurišić AB, Giesy JP and Leung KMY 2017 Influences of temperature and salinity on physicochemical properties and toxicity of zinc oxide nanoparticles to the marine diatom Thalassiosira pseudonana Sci. Rep. 7(1) 3662