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Antibacterial and antifungal activity of photoactivated ZnO nanoparticles in suspension

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ABSTRACT

Antibacterial activity of photoactivated zinc oxide nanoparticles (ZnO NPs) against human pathogens *Escherichia coli* O157:H7, *Listeria monocytogenes* $ATC_{L3}C$ 7644 and plant pathogen *Botrytis cinerea* was investigated. Data indicate that photoactivated ($\lambda = 400$ nm) ZnO NPs at concentration 1×10^{-3} M and incubation time 60 min reduced population of both bacteria by 7 log (CFU/ml). Clear dependence of antimicrobial properties of ZnO NPs on used concentration and incubation time was found. Scanning electron microscopy (SEM) images of treated bacteria indicate that treatment induced cell wall disintegration and lysis. Results obtained on examination of antifungal activity of ZnO NPs reveal that significant photoinactivation (58%) of *B. cinerea* was observed at NPs concentration 5×10^{-3} M and incubation time of 24 h. SEM analysis confirmed that substantial morphological changes occur in the microfungus after treatment.

The data suggest that ZnO NPs in the presence of visible light exhibit strong antibacterial and antifungal activity. Such ZnO NPs properties obviously could be used for the development of effective fungicides in agriculture or innovative physical antibacterial agents, so important in medicine and food microbial control.

1. Introduction

In recent years, a rapid development of nanotechnology opens up whole universe of new possibilities for biomedicine, industry and agriculture [1].

It is well documented that most of the harmful and pathogenic microorganisms are able to develop high resistance to many conventional chemical fungicides and disinfectants. Moreover, currently chemical sanitizers are suspected to be environmentally unsound, as they are associated with occupational and operational hazards. In addition, these compounds are potentially harmful for humans [2]. Thus, during the past decade the emphasis in microbial decontamination has shifted from using chemicals to various alternative techniques including physical antimicrobials. To this end, the development of novel nano-sized antifungal and antibacterial agents seems promising.

Recently many various nanoparticles have been synthesized and tested for their antimicrobial activity against different pathogenic microorganisms. The vast majority of these nano-sized agents are metal nanoparticles such as zinc oxide, titanium dioxide, magnesium oxide or silver nanoparticles. They are especially attractive due to their small size, large surface to volume ratio, chemically alterable physical properties, unique electrical, thermal and mechanical features [3]. Moreover, metal oxides such as zinc oxide and titanium dioxide are not only stable and have a longer life than organic-based disinfectants, but also are generally regarded as safe to human beings [4].

It is important to note, that nano-sized ZnO particles exhibit some advantages in comparison with other nanoparticles due to their lower cost, white appearance and UV blocking properties [5]. Moreover, some studies indicated high specific toxicity of zinc oxide nanoparticles (ZnO NPs) against bacteria and only minimal effects were observed on human cells. It opens new prospects of their application in agriculture and biomedicine [6–8].

A great deal of work has been done to investigate antimicrobial properties of ZnO NPs. Most of these studies examinated the ability of ZnO to inactivate foodborne bacteria [4,9–13]. Several studies revealed that ZnO NPs could be used as an effective fungicide against such plant pathogens as *Botrytis cinerea* and *Penicillium expansum* [14].

Although antimicrobial properties of ZnO NPs have been widely studied, just few articles have been published on their antimicrobial efficiency after photoactivation with UV light [15]. The main disadvantage of UV light is that it destroys not just microbes, but other materials and surfaces as well. Recently photoactivation of ZnO with visible light has proved to be more specific [13]. As a role, such photoactivation induces following production of reactive

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oxygen species (ROS), which ultimately damage cellular membrane and eventually kill bacteria [16].

Therefore, the aim of this study was to evaluate for the first time the antimicrobial efficiency of ZnO nanoparticles in suspension after photoactivation with visible light against pathogenic bacteria and harmful fungi in vitro. Thus, for this purpose susceptibility of Gram-negative bacteria *Escherichiacoli*, Gram-positive bacteria *Listeriamonocytogenes*, and fungus *B. cinerea* to photoactivated ZnO NPs was examined.

2. Materials and methods

2.1. Preparation and characterization of ZnO NPs

50% colloidal dispersion of ZnO NPs in H_2O with nonionic dispersant was purchased from Alfa Aesar (NanoShield, Germany). Stock solution of ZnO NPs was prepared in 0.9% NaCl and used immediately. Appropriate final concentrations of NPs were prepared by further dilution of a stock solution in 0.9% NaCl.

SEM images and size distribution of (a) 1×10^{-4} and (b) 5×10^{-4} M ZnO nanoparticles in H_2O and in NaCl were examined. Thus an Apollo 300 (CamScan, UK) scanning electron microscope at an accelerating voltage of 20 kV and Zetasizer Nano-S (Malvern instruments, England) were used. Absorption spectrum of ZnO NPs was recorded in a 10.01 mm cuvette using Heλios Gamma spectrophotometer (ThermoSpectronic, USA). The fluorescence spectrum of ZnO NPs was recorded with Perkin-Elmer model LS-55 fluorescence spectrophotometer (Beaconsfield, UK). Scan range parameters were as follows: Excitation wavelength – 375 nm, emission – 400–700 nm, excitation slit – 10 nm, emission slit – 20 nm, scan speed (nm/min) – 200.

2.2. Bacterial cultures and growth conditions

Two food-borne bacterial cultures were used for experiments: *L. monocytogenes* $ATC_{L3}C$ 7644 and *E. coli* O157:H7. All bacteria were maintained at 37 °C for 24 h onto Luria–Bertani Agar (LBA; Liofilchem, Roseto degli Abruzzi, Italy). *Listeria* and *Escherichia* cultures were grown overnight (~16 h) at 37 °C in 20 ml of tryptone soya medium supplemented with 0.6% yeast extract (TSYE) (Liofilchem) and in 20 ml of Luria–Bertani medium (LB; Liofilchem), respectively, with agitation of 120 rev/min (Environmental Shaker – Incubator ES – 20; Biosan, Riga, Latvia). Afterwards, *Listeria* and *Escherichia* bacterial cultures were diluted 20 times by the fresh TSYE and LB medium (OD_{540} = 0.164), respectively, and grown at 37 °C in a shaker (120 rev/min) to the mid-log phase (~1.16×10^9 colony-forming units (CFU)/ml), OD_{540} = 0.9 for *Listeria*; ~1×10^8 CFU/ml, OD_{540} = 0.9 for *Escherichia*). Bacterial optical density was determined in a 10.01 mm cuvette at λ = 540 nm using a Heλios Gamma spectrophotometer. Cells were then harvested by centrifugation (10 min, 3420×*g*) (Mikro 200, Hettich zentrifugen, Germany), resuspended in 0.9% NaCl to ~1×10^7 CFU/ml and used for the further experiments.

2.3. LED-based light source device for photoactivation of ZnO nanoparticles

LED based light source for the photoactivation of ZnO NPs was constructed at the Institute of Applied Research of Vilnius University. The emission maximum of the light source was 400 nm and the light intensity at surface of samples (at 6 cm from the light source) reached 96 W/m^2. Light dose was calculated as light intensity multiplied on illumination time. Light intensity was measured by *3 Sigma* power and energy meter "Coherent" equipped with a piroelectrical detector.

2.4. Inactivation of L. monocytogenes $ATC_{L3}C$ 7644 and E. coli O157:H7 by photoactivated ZnO NPs

Aliquots (20 ml) of bacterial suspension (1×10^7 CFU/ml in 0.9% NaCl) with appropriate concentration of ZnO NPs (1×10^{-5}–1×10^{-3} M) (pH 7.2) were incubated in the dark at 37 °C. For the following experiments, the cells were incubated in the shaker (130 rev/min) for various periods (10–60 min). Afterwards, 150 μl aliquots of bacterial suspension were withdrawn, placed into sterile flat bottom wells and exposed to light for 30 min.

Antibacterial effect of photoactivated and not-photoactivated ZnO NPs on bacterial cells was evaluated by the spread plate method. Thus, 100 μl of appropriate dilutions of bacterial test culture after treatment was surface inoculated on the LBA plates. Afterwards, the bacteria were kept in the thermostat for 24 h at 37 °C. The surviving cell population was enumerated and expressed by $\log_{10}$ (CFU/ml).

2.5. Inactivation of B. cinerea by photoactivated ZnO NPs

The culture of *B. cinerea* was isolated from strawberries. This fungus was grown on potato dextrose agar (PDA) for further experimentation.

The tested concentrations of ZnO NPs were obtained by adding the appropriate amount of stock solution to autoclaved PDA medium that had been cooled to about 50 °C prior to plating in petri dish (90 × 15 mm). The control samples contained PDA only. Each dish was inoculated in the center with an agar disk (diameter 5 mm) bearing mycelium growth from a 4-day-old *B. cinerea* culture. After 24 h incubation the tested plates containing ZnO NPs were exposed to 345.6 kJ/m^2 400 nm light dose. Afterwards both treated and control dishes were sealed with parafilm to avoid the evaporation of volatile compounds and incubated at 24 °C until the control plates were completely covered with mycelium. Each treatment (all concentrations for each treatment) was repeated 3 times.

The radial growth of fungal mycelium was recorded and the radial inhibition was calculated when growth of mycelia in the control plate reached the edge of the petri dish. The following formula was used for calculation of the inhibition rate (%).

$$\text{Inhibition rate } (\%) = R - r/R \times 100,$$

where *R* is the radial growth of fungal mycelia on the control plate and *r* is the radial growth of fungal mycelia on the plate treated with ZnO NPs.

2.6. Scanning electron microscopy (SEM)

The effect of photoactivated ZnO NPs on the morphology of *Listeria* and *Escherichia* was examined by SEM. Bacterial suspensions (1×10^7 CFU/ml) were incubated in the dark for 30 min at 37 °C with 5×10^{-4} M ZnO NPs and afterwards illuminated with light of 400 nm wavelength. In the next step, the samples consisting 20 μl of bacterial suspension were withdrawn, transferred to aluminum stubs, air-dried and sputter coated with 15-nm gold layer using Q150T ES sputter coater (Quorum Technologies, England). The treated samples of *B. cinerea* mycelium (3 days after treatment) as well as not treated ones were transferred to aluminum stubs, air-dried, and sputter coated. The scanning was performed with an Apollo 300 (CamScan, UK) scanning electron microscope at an accelerating voltage of 20 kV.

2.7. Statistics

The experiments were done in triplicate (from different inocula) for each set of exposure. Standard error was estimated for every