Effect of ZnO nanoparticle sizes and illumination on growth inhibition of *Escherichia coli* and *Staphylococcus aureus* bacteria in cultivation medium

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Abstract. We study antibacterial effects of the zinc oxide nanoparticles (ZnO NPs) in cultivation medium (Mueller-Hinton broth) on Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) bacteria models. We compare synthesized ZnO hedgehog NPs and commercial ZnO NPs (50 nm and 20 μm nominal size) in different concentrations (1 mg/mL and 0.1 mg/mL). Results show that *E. coli* are more sensitive to the ZnO presence in the cultivation media than *S. aureus*. We also characterize influence of visible and UV light on the ZnO NP effects.

1. Introduction

Zinc oxide (ZnO) is a material that attracts increasing attention due to its antibacterial abilities, especially as nanoparticles (NPs) [1]. The ZnO particles can be prepared in variety of sizes, shapes and surface modifications. The advantage of nanoscale particle sizes are larger specific surface areas and hence higher surface reactivity compared to larger particles. Small size of NPs enables them specific types of interaction with bacterial cells including penetration into the bacterium structure [2,3]. Special ZnO NP modifications are particles with hedgehog-like shape that showed for instance specific effect on the cultivation of osteosarcoma cells (SAOS-2) [4]. In addition, ZnO is non-toxic to human cells and has a good biocompatibility [5]. This opens the way for usage in the food applications like packaging and bacteria inhibition on food surfaces [6].

In this study we investigate the bacterial response of *E. coli* and *S. aureus* to the ZnO NPs in the Mueller-Hinton (MH) broth. The usage of broth was chosen so that the bacteria and their response would be studied in bacterial natural environment. Three types of ZnO NPs were compared – hedgehog-like ZnO particles and ZnO powder with 50 nm or 20 μm nominal particle sizes. We also studied the effect of illumination on bacterial growth. There was a light source present during the incubation process in broth. The first tested source was a UV lamp and after that we tested also a flatbed visible light source.
2. Materials and methods

Our study involves the use of three different ZnO samples. The samples of nano and micro sizes were obtained from commercial suppliers. The nano sample was purchased from Sigma Aldrich company, declared particle size 50 nm, purity >97%. The micro sample was purchased from the Nanografi nanotechnology company, declared particle size 20 μm, purity >99.9%. The hedgehog-like ZnO needle-clusters were prepared from zinc nitrate hexahydrate (Zn(NO$_3$)$_2$·6H$_2$O) by the hydrothermal growth method. More detailed preparation description is in [4].

All ZnO NPs used in the experiments were suspended in demineralized water to prepare the stock solutions with concentration of 2 mg/mL or 0.2 mg/mL. We sterilized these suspensions by autoclave for 30 min. at 120°C. Note that final concentration was 1 mg/mL or 0.1 mg/mL after 1:1 mixing with bacterial suspension. We also tested even lower concentration of the ZnO – 0.03 mg/mL, but this concentration was too low to observe any measurable effects. Typical SEM morphology of the employed ZnO nanoparticles is shown in figure 1. The samples were drop-casted onto silicon substrates. The images show us that the 50 nm NPs and 20μm NPs have similar crystalline structure, but the crystals are different in size. The structure of hedgehog-like ZnO shows predominantly clusters of needle-like shapes with sizes of few micrometres.

![Figure 1. SEM morphology of the employed ZnO samples: ZnO 50 nm (a), ZnO 20 μm (b) and the ZnO hedgehogs (c).](image)

Dynamic light scattering (DLS) and zeta potential were measured on colloidal suspensions of ZnO particles in the MH broth at 25 °C using Nano-ZS (Malvern, UK) equipped with a He-Ne laser. The ZnO samples were not ultrasonicated to prevent damage of microstructures. The samples were mixed with the MH broth by vortex to achieve the final concentration of 1 mg/mL.

![Figure 2. DLS measurement of ZnO samples mixed in MH broth.](image)
Figure 2 shows that the size of nominally 20 μm ZnO sample is actually in units of μm while the size of nominally 50 nm ZnO sample is actually between 100-300 nm with some clusters of size around 1 μm. The ZnO hedgehogs have micron size with some fragments of 100-200 nm size. Another important parameter for interaction with cells is zeta potential. The zeta potential is important for the stability of nanoparticles in suspension. It depends on the surface charge and is also the major factor in the initial adsorption of nanoparticles onto the cell membrane. After adsorption, the endocytotic uptake rate depends on the particle size. It was measured in the same DLS apparatus using the same ZnO samples in MH broth. The resulting values are listed in the table 1. All ZnO samples exhibit negative zeta potential. Its value is relatively small, between -12 to -18 mV, still the colloidal solutions are reasonably stable as shown also by the DLS measurements.

| Sample     | Hedgehog ZnO | ZnO 50 nm | ZnO 20μm |
|------------|--------------|-----------|----------|
| Zeta potential [mV] | -11.7        | -17.5     | -15.5    |

The bacterial solutions were prepared in the following manner. We spread 1 ml of concentrated *E. coli* or *S. aureus* solution from the freezer after 15 min melting on the 9 cm Petri dish with the MH agar and let it grow overnight in the thermostat at the temperature 37 °C. Then all the bacteria from the Petri dish were wiped off and put into 5 ml of the MH broth. The distance between two pairs was exposed to the light. Only one ZnO sample was chosen for simplicity represented by a light panel Reflecta L230 T8, which had the spectral maximum at the wavelength of 250 nm. Then we tested a source presence around 15 cm in both cases.

After that we calculated the CFU number of reference samples in triplicates. The dishes were put into the incubator and let grow for 24 hours. After that we counted the number of bacterial colonies on Petri dishes and we calculated CFU concentrations for each sample. Finally, we calculated the R-value, the logarithmic ratio between the CFU number of reference and the CFU number of evaluated sample.

As an additional experiment we also measured the bacterial response to the ZnO NPs with a light source present during the incubation of the tubes. The first source was a UV lamp Philips TUV 15W/G15 T8, which had the spectral maximum at the wavelength of 250 nm. Then we tested a visible light source represented by a light panel Reflecta L230 generating fluorescent light. For these experiments we used two pairs of reference and ZnO samples, when one pair of tubes was packed in aluminium foil and the second pair was exposed to the light. Only one ZnO sample was chosen for simplicity – the hedgehog. The distance between the tubes and light source was around 15 cm in both cases.

### 3. Results and discussion

The main results of our experiments are depicted in figure 3. There are growth curves for both types of used bacteria in both used concentrations of the three types of ZnO particles. The results show that the *E. coli* bacteria are more sensitive to higher concentrations (1 mg/mL) of the ZnO structures than *S. aureus*. The best antibacterial activity against the *E. coli* was observed for the hedgehog NPs, where...
we achieved the highest ratio of the reference CFU number compared to measured sample, which was six orders here in the stationary phase. The response of *S. Aureus* to higher ZnO concentration was similar for all types of ZnO structures, resulting in a four order difference between the CFU number of reference and ZnO samples.

![Growth curves](image)

**Figure 3.** Growth curves of the *E. coli* with 1 mg/mL ZnO (a), *S. aureus* with 1 mg/mL ZnO (b), *E. coli* with 0.1 mg/mL ZnO (c) and *S. aureus* with 0.1 mg/mL ZnO (d).

The response to lower concentration (0.1 mg/mL) of the ZnO particles was different. While *S. aureus* had similar response resulting in almost four orders ratio compared to the reference, the effect on *E. coli* was lower, only up to 2.5 orders during the exponential phase and only one order in the stationary phase.

The results were also interpreted in the form or R-value, where the R-value was calculated according to the formula $R = \log(C/E)$, where $C$ is the CFU number of the reference sample and $E$ is the CFU number of the measured sample. The R values for the *E. coli* and *S. aureus* measurements for both concentrations of ZnO are summarized in the bar graphs in figure 4. The sampling times for the bar graphs were selected so that they characterize both exponential phase of the growth curve (5 hours) and stationary phase of the curve (24 hours).

We can see from the bar graphs that the response of *S. aureus* is more uniform in both exponential and stationary phase, achieving the R-value up to the value of 4 for the high ZnO concentration. The response of the *E. coli* is more ZnO type dependent where the hedgehog shows the best antibacterial
results with the R-value up to the 6 for higher ZnO concentration. There is also notable that lower ZnO concentration against *E. coli* is the only configuration, where the R-value drops from the exponential phase towards the stationary one and the bacteria are able to partially recover.

![Graphs of R-values](image)

**Figure 4.** R-value graphs of the *E. coli* with 1 mg/mL ZnO (a), *S. aureus* with 1 mg/mL ZnO (b), *E. coli* with 0.1 mg/mL ZnO (c), and *S. aureus* with 0.1 mg/mL ZnO (d).

The experiments with visible and UV light sources provided different results. We performed the experiments only with the hedgehog ZnO in concentration of 1 mg/mL, because they showed the best antibacterial effect. The visible light had no significant effect on bacterial growth, which is demonstrated by the growth curves in figure 5.

One can also notice good reproducibility of the experiments. Contrary to that, the UV illumination resulted in a very strong antibacterial effect. All bacteria in solutions that were not packed in the aluminium foil were killed, both in the ZnO and reference sample, so we could not distinguish between the effect of ZnO itself and additional UV light. The data is thus not plotted.

The antibacterial mechanisms of the ZnO particles are still a subject of discussion. There are several proposed mechanisms of such ability [1,3]. The first mechanism is the generation of reactive oxygen species (ROS), which can destroy cellular components like lipids, proteins etc. The generation of ROS is mostly photocatalytic. Our experiments were performed mostly in the dark. Illumination by visible light had also no noticeable effect. The ROS mechanism will be thus minor in our case.

The second mechanism is the release of zinc ions Zn$^{2+}$ that can affect the amino acid metabolism and enzyme system disruption. The ability of releasing zinc ions is surface dependent, hence the small ZnO NPs, that have larger surface area to volume ratio than larger particles, are supposed to have better antibacterial ability due to this mechanism. Comparison of our results of 50 nm and 20 μm particles seems to confirm that. However, the ZnO hedgehogs have smaller surface area yet superior bactericidal effect on *E. coli*. 
Figure 5. Growth curves for the additional visible light experiment. Dark means a sample packed in aluminium foil, light means a sample exposed to the visible light.

The third possible mechanism is electrostatic interaction. The surface of bacteria is negatively charged. The electrostatic forces can then lead to create an envelope of the ZnO particles around bacteria which could result in mechanical disruption of bacterial membranes. Similar effect has been observed for nanodiamond particles [7]. Smaller ZnO particles have smaller size and larger number per weight, thus they have better chance to create such envelope compared to large ones. This would again agree with the data for 50 nm and 20 μm particles. However, the ZnO hedgehogs have superior effect although they are large and less concentrated. Moreover, zeta potentials of all ZnO materials are negative and quite low (see table 1). The electrostatic interaction thus cannot make a predominant effect.

The fourth possible mechanism is a mechanical damage of bacterial cell walls caused by specific shape of the ZnO NPs [8]. The hedgehog needles have such specific shape, with numerous spikes in all directions. Relatively large size (micrometers) provides them also with higher kinetic energy during bacteria cultivation in the shaker. The ZnO hedgehogs thus may exert their antibacterial effect mainly by mechanical damage of bacteria. This is pronounced especially against the Gram-negative bacteria like *E. coli* that have thinner peptidoglycan layer (2-7 nm [9]) compared to Gram-positive *S. aureus* with thick peptidoglycan layer (20-30 nm [10]). This effect is observable when using higher concentration of ZnO structures, namely 1 mg/mL. As for lower concentration of 0.1 mg/mL the effect is much less pronounced for all types of ZnO, the mechanical disruption seems in all cases to be the dominant effect compared to other factors described above.

4. Conclusions
We found that the ZnO particles, when used in higher concentration of 1 mg/mL, had significant antibacterial effect suppressing the bacterial growth in MH broth by up to six orders of magnitude compared to the reference after 24 hours. Lower concentrations of 0.1 mg/mL had significant effect only on the *S. aureus* while the *E. coli* indicated only inhibited growth finishing one order down compared to the reference after 24 hours. Smaller 50 nm ZnO particles had better antibacterial effect against the *E. coli* compared to larger 20 μm particles. However, ZnO particles with hedgehog structure had the best antibacterial effect against the *E. coli* compared to the particles with crystallite structure. Visible light did not affect the bacteria growth or the ZnO antibacterial activity. The mechanical disruption thus seems to be the dominant effect compared to other possible antibacterial mechanisms. This is in
agreement with the observation that the antibacterial effect against Gram-positive *S. aureus* with robust membrane was similar for all employed types of ZnO particles.

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