COMPARISON OF MICROBIAL INTERACTIONS OF ZINC OXIDE NANOMATERIALS IN VARIOUS SIZE AND SHAPE

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Abstract

Zinc oxide nanoparticles (ZnO NP) have shown great potential as a novel antibacterial material at a time when resistance towards conventional antibiotics is becoming more prevalent. We report bacteria inactivation by ZnO NP with novel hedgehog-like morphology using model gram-negative (*E. coli*) and gram-positive (*S. aureus*) bacteria. *E. coli* exposed to the novel ZnO hedgehog NP during growth resulted in 4 orders of magnitude reduction in viable cell concentration after 24 h, which is more than 2 orders higher reduction compared to commercially available ZnO NPs with nominal sizes from 50 nm to 20 um. There was a positive correlation between hedgehog NP concentration and bacteria cell concentration reduction within the range tested 0.1 - 1.0 mg/mL. *S. aureus* was less sensitive to ZnO NP exposure and inactivation effect of various ZnO NP, was comparable. The effect can be thus attributed to direct mechanical damage of the bacterial mebrane that is the most effective for the novel hedgehog ZnO NP. This conclusion was corroborated also by disk diffusion assays.

Keywords: Nanotechnology, microbiology, zinc oxide

1. INTRODUCTION

Materials at the nanoscale possess different physicochemical properties than bulk material due to increased surface area enhancing reactivity, and one such example is zinc oxide (ZnO). A large body of literature exists for ZnO nanoparticles (ZnO NP) particularly in photovoltaics due to the materials semi-conductive properties, wide band gap and ultra-violet light absorption capability [1]. ZnO NP is also known to possess antibacterial properties and the mechanism of inactivation is thought to be an interplay between a number of different processes: nanoparticle morphology and surface charge interaction, physical contact between the nanoparticle surface and bacteria cell surface leading to permeabilisation, and reactive oxygen species (ROS) and zinc ion (Zn^{2+}) generation and subsequent internalisation that damages intracellular biomolecules such as DNA [2]. Reports suggest that ZnO NP treatment can even re-sensitize multi-drug resistant (MDR) bacteria to antibiotics that were previously ineffective [3]. Other promising antibacterial applications of ZnO NP include wound treatment [4], cancer therapy [5,6] and as an additive in food packaging [7]. The precise mechanism of interaction between ZnO NP and biological cells is dependent on the unique physicochemical properties derived from the synthesis process.

ZnO NP can be synthesized via a number of different techniques: physical (e.g. gas plasma [8]), chemical (e.g. hydrothermal growth [9] or sol-gel technique [10]) or biological (extraction from plant tissue [11]). Each technique produces nanoparticles with unique morphologies with differing antibacterial potentials. ZnO NP characterization using microscopic and spectroscopic methods supplemented with standardized antimicrobial susceptibility testing is needed in order to compare the antibacterial potential of different ZnO NP due to the

vast array of possible particle morphologies e.g. spherical, rod-like, wire-like, flower-shaped and tetrapod. Morphologies composed of rough surfaces and/or sharp, pointed projections could be well suited to bacteria cell inactivation through irreversible physical damage to the outer cell envelope. Functional groups on the ZnO NP surface result in ROS generation, accompanied by the release of zinc ions (Zn^{2+}) and internalisation further contributes to the inactivation mechanism.

The aim of this research was to investigate the antibacterial potential of ZnO NP synthesized with hedgehog-like morphology (i.e. many long, thin tubules branching from a single nucleation point). Bacteria were exposed to different concentrations of ZnO in liquid during growth, and the number of viable cells after certain timepoints over 24h were recorded.

2. METHODOLOGY

2.1. Bacteria Culture

Gram-negative *Escherichia coli* (*E. coli*) and gram-positive *Staphylococcus aureus* (*S. aureus*) microorganisms were reconstituted using 200 mL Mueller Hinton broth (MHB, Oxoid) and grown overnight at 37 °C in an orbital shaker (150 rpm, Biosan). 2 mL of this stock culture was added to 1 mL sterile glycerol and stored at -20°C until required. A fresh culture was prepared for each experiment by inoculating 2 x 1 mL of the thawed bacteria suspension onto 2 Mueller Hinton agar (MHA, Oxoid) plates and placed in incubator overnight at 37 °C. Resultant growth was removed from one plate using a sterile loop and mixed with 5 mL MHB (relative 10^0 dilution). Three decimal serial dilutions using MHB were performed and 2 mL of the final dilution was transferred to a sterile conical tube (Sterilin) and mixed with equal volumes of ZnO solutions. For the positive experimental control, 2 mL sterile deionized water (dH_2O, conductivity (σ) = 0.1 µS/cm) was added to bacteria instead of ZnO solution.

2.2. Zinc oxide material synthesis & preparation

The synthesis procedure and extensive characterization of the hedgehog ZnO NP used in this study have been reported previously [9]. Briefly, crystalline ZnO NP were produced by adding equimolar volumes (25 mM) of zinc nitrate hexahydrate ($Zn(NO_3)2{\cdot}6H_2O$) and hexamethylenetetramine (HMTA) at 90 °C for 3 h. Precursor salt residue was removed from the sample by washing 3-times with dH_2O followed by centrifugation at 18,000 rpm (RCF: 23542g) for 20 min. Spherical ZnO (Sigma) with an average size of <50nm, and micron-sized ZnO (20 µm) were used for comparison with the hedgehog ZnO NP. Stock solutions of 2 mg/mL were prepared in glass vessels using 10 mL dH_2O, which was subsequently autoclaved then sonicated (30 min, Sonorex Digitec, Bandelin) immediately before use.

2.3. Antibacterial assessment techniques

The bacteria-ZnO suspensions were placed upon an orbital shaker (150 rpm) located inside an incubator and samples were taken at various timepoints, up to 24h. Decimal serial dilutions were performed using sterile 0.9% sodium chloride solution (NaCl, Penta) and 1 mL of the dilutions were added to MHA plates in duplicate and placed in incubator. Images of the plates after 18-24h incubation were taken and the number of colonies on each plate was determined using ImageJ.

3. RESULTS & DISCUSSION

3.1. Viable cell concentration

The number bacteria that remained viable and formed a colony on MHA after exposure to ZnO over an incubation time of 24 h can be viewed in **Figures 1** and **2**. Bacteria not exposed to ZnO act as a control sample.

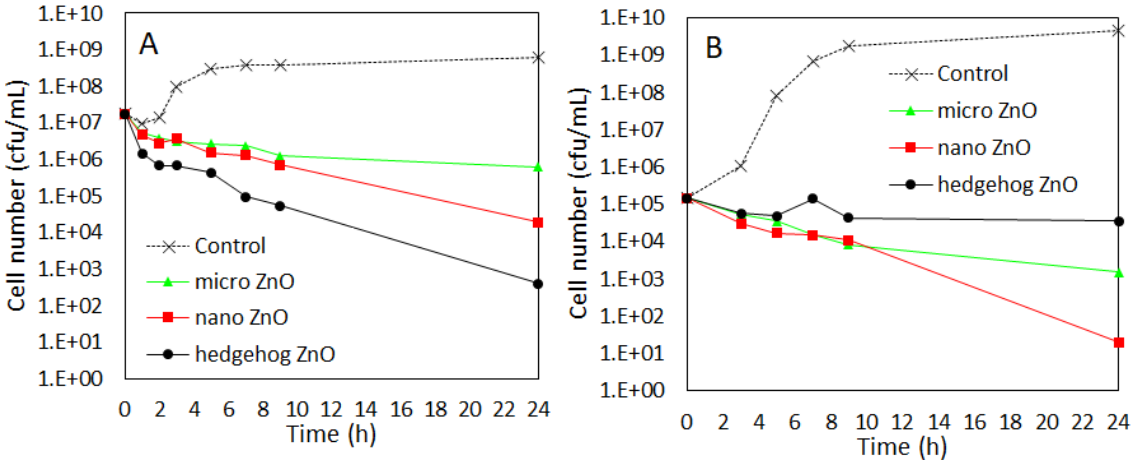

Figure 1 Number of viable *E. coli* cells after exposure to 3 types of ZnO, micron-sized powder (micro ZnO), nanopowder (nano ZnO) and synthesised nanoparticles (hedgehog ZnO) at 1 mg/mL (A) and 0.1 mg/mL (B).

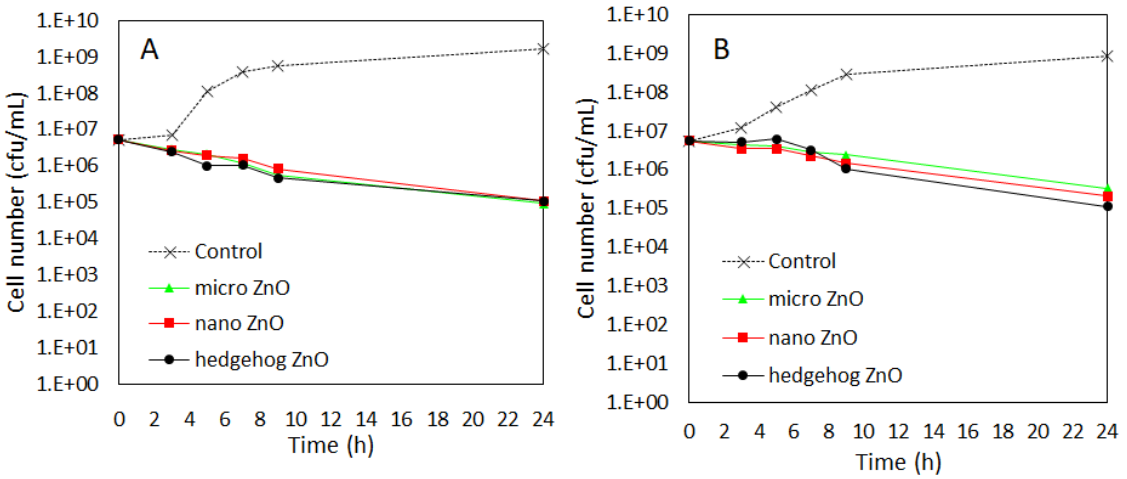

Figure 2 Number of viable *S. aureus* cells after exposure to 3 types of ZnO, micron-sized powder (micro ZnO), nanopowder (nano ZnO) and synthesized nanoparticles (hedgehog ZnO) at 1 mg/mL (A) and 0.1 mg/mL (B).

For a concentration of 1 mg/mL ZnO, gram-negative *E. coli* growth was inhibited to a greater extent than gram-positive *S. aureus* (**Figure 1 A** and **Figure 2 A**). In the case of *E. coli*, hedgehog ZnO NP showed the greatest antibacterial effect, resulting in approx. 4 orders reduction in viable cell concentration after 24h compared to the untreated bacteria. The nano-sized reference material (nano ZnO) inhibited growth by approx. 2.5 orders, and the micron-sized material (micro ZnO) was the least effective at inhibiting *E. coli* growth. Interestingly, when a lower concentration of 0.1 mg/mL ZnO was tested the hedgehog NP was the least effective compared to the other ZnO materials (**Figure 1B**).

There was no significant difference in the antibacterial potential of the ZnO materials tested against *S. aureus.* The viable cell concentration was reduced by approx. 1.5 orders of magnitude compared to the untreated bacteria after 24 h for all ZnO material independent of concentration (**Figures 2A** and **2B**). Thus, ZnO NP had generally a greater inhibitory effect against *E. coli* than against *S. aureus*.

The observed difference in bacterial inhibition efficacy of ZnO between the two bacteria types could be due to the fundamentally different outer cell structures. Gram-positive bacteria have a thick peptidoglycan layer located outside the phospholipid bilayer that enhances cell rigidity and makes cell death due to physical

damage less likely. Gram-negative bacteria contain a much thinner peptidoglycan layer, as well as an additional phospholipid bilayer, resulting in a more fluid cell membrane which could be more prone to damage as a result of physical impact. This would explain the lower final cell concentration of *E. coli* cells compared with *S. aureus* after 24 h (**Figures 1A** and **2A**). ZnO NP synthesised via the reaction between zinc nitrate and potassium hydroxide in ethanol achieved complete growth inhbition (8 orders reduction) of *E. coli* growth at a concentration of 200 µg/mL, yet under the same experimental conditions *S. aureus* inhibition was not complete (4 orders reduction [12]). However, flow cytometric analysis of live and dead cells after ZnO NP exposure revealed little difference between the bacteria strains. In our study, the size and morphology of ZnO particulates had a greater inhibitory effect on *E. coli* than *S. aureus*, where hedgehog-like NP caused the largest reduction in viable cells after 24 h compared to the untreated cells, followed by spherical nano-sized material and then micron-sized particles (**Figure 1A**). There was little difference in *S. aureus* inhibition due to ZnO particle morphology (**Figures 2A** and **2B**), again possibly due to the more rigid outer cell structure providing greater resistance to damage by physical impact. A study that used similar sized ZnO reference materials alongside synthesised ZnO NP, albeit at a much lower concentration, reduced the viable cell concentration by 2 orders of magnitude regardless of particle size [13]. The synthesized NP resulted in a further 1 order of magnitude reduction in viable cells, and was equally effective against *E. coli* and *S. aureus* [13]. The nano-sized reference material had a structure with high crystalinity, whereas the synthesised NP had irregular shaped edges which indicated low surface crystalinity and increased surface defects attributed to oxygen or zinc vacancies in the crystal lattice structure [13]. The authors reported increased Zn^{2+} concentration using the synthesised NP compared with either reference material and proposed a mechanism of inactivation dependent on Zn^{2+} release from the NP surface due to the observed defects rather than ROS generation or physical NP-bacteria interaction [13].

3.2. Disk diffusion assay

Disk diffusion assay assesses the ability of an antibacterial solution to diffuse through agar and inhibit growth without coming into direct contact with the bacteria. For a concentration of 1 mg/mL we determined a zone of growth inhibition using ZnO-loaded filter paper disks that were placed directly onto the agar surface containing bacteria. Limited inhibition was achieved against *S. aureus* yet none for *E. coli*. Spherical ZnO NP synthesised via a wet chemical method using zinc chloride and sodium hydroxide produced a larger zone of inhibition for *S. aureus* than *E. coli* [14]. By using different Zn-containing precursor molecules, the authors were able to synthesise nanorods and multi-faceted grain particles that also inhibited bacterial growth but to a lesser extent. Grain-like ZnO NP that were synthesized from plant extract also inhibited *S. aureus* growth more than *E. coli*, and a synergistic effect was observed when applied in conjunction with streptomycin [11]. These data suggest that NP size and morphology can affect the antibacterial effect of ZnO.

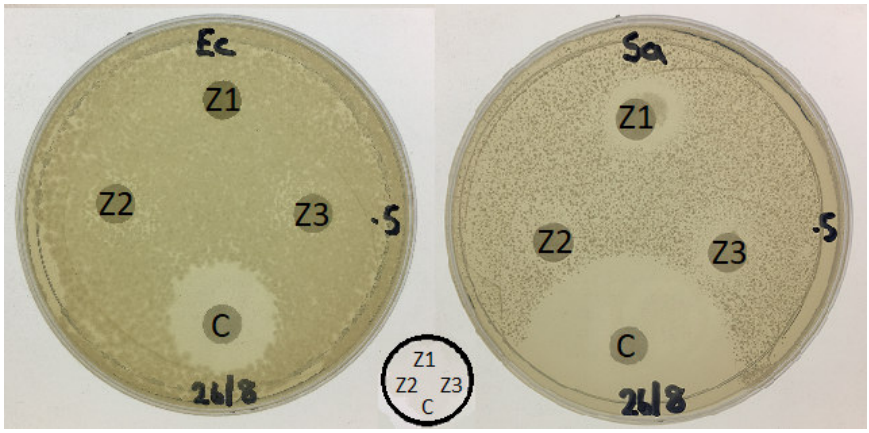

Figure 3 Images of bacteria on agar subjected to ZnO-loaded filter paper disks (a - *E. coli*; b - *S. aureus*). Bacteria concentration on agar = 1 x 10^5 cfu/mL. Inset plate layout displays positioning of disks and solutions: Z1 & Z2 are hedgehog ZnO NP, Z3 is nanosized reference material and C is 25µL of 0.1 M H_2O_2.

4. CONCLUSION

We studied antibacterial potential of ZnO NP with hedgehog-like morphology and comparison with commercially available nano-sized and micron-sized ZnO material. The hedgehog ZnO NP inhibited both *E. coli* and *S. aureus* growth. More pronounced effect was achieved using higher concentrations up to 1 mg/mL. Both nano and micron-sized ZnO inhibited bacterial growth but to a lesser extent. *E. coli* was more sensitive towards hedgehog NP exposure than *S. aureus* when the interaction occurred in liquid with agitation. Growth inhibition was only observed when the NP directly interacted with the bacteria cell, which implies physical damage rather than chemical processes as the dominant mechanism underlying the antibacterial potential of hedgehog NP. These promising results provide evidence that ZnO NP shape is an important property which governs the antibacterial potential and ZnO NP with complex geometries such as the hedgehog-like particles could be used as an effective antibacterial treatment.

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