Bactericidal effect of zinc oxide nanoparticles on Gram-positive and Gram-negative strains in reverse spin bioreactor

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Abstract. We present an experimental study investigating the efficacy of zinc oxide nanoparticle use for bacterial decontamination of water using reverse spin technology. The number of viable bacteria reduced with treatment time and the rate of inactivation was dependent on zinc oxide concentration. Gram-positive S. aureus bacteria were more resistant to zinc oxide nanoparticles compared to Gram-negative E. coli, however both strains were completely eradicated after 4 hours using a concentration of 10 µg/mL. Nanoparticles did not inhibit growth of bacteria when added to an agar surface, neither when in liquid before bacteria inoculation nor when loaded onto filter paper disks after bacteria inoculation. These results emphasise the importance of efficient reactor mixing to enhance interaction capability for ZnO use in water decontamination.

1. Introduction

Water-borne diseases which arise from consumption of water of poor quality contribute to over 80% of reported illnesses in the developing world and it’s estimated that around 4 billion people worldwide lack access to sanitized water [1]. The presence of pathogenic microorganisms, such as bacteria and viruses, are thought to be the main causative agents of water-borne diseases and disinfection strategies are necessary to provide people with an adequate supply of drinkable water. The ever-growing human population puts increasing pressure on a finite water resource and there is a need for innovative solutions for water decontamination that are efficient, low-cost and environmentally friendly. There are already many established methods such as chlorination, (electro)oxidation, illumination by ultraviolet light, or photocatalytic decomposition. All these methods have some limitations or drawbacks.

Nanotechnology has shown great potential for inactivating bacteria in water through the application of semi-conductive photocatalytic materials with low toxicity such as titanium dioxide and zinc oxide. The fundamental mechanism entails the formation of electron-hole pairs upon photon absorption which results in the initiation of a complex cascade of chemical reactions and the formation of reactive oxygen species (ROS) e.g. OH, O₂⁻, H₂O₂ [2]. These radicals can rapidly oxidize biomolecules such as DNA, protein and lipid to cause irreversible cell damage and lead to cell death [3]. Recently, nanomaterials with low toxicity that retain a strong antibacterial effect without the need for additional illumination have become a viable alternative to conventional materials used in photocatalytic disinfection of water by eliminating the requirement for additional light source thereby reducing energy consumption without any adverse effect on decontamination efficacy [4].
Efficient mixing of the contaminated water with nanoparticles increases treatment efficacy. Reverse spin technology is a mechanically-driven mixing technique that rotates the cell-nanoparticle suspension about its axis with a change of direction of rotation motion [5]. This non-invasive technique has low energy consumption and can operate with or without additional illumination, making the apparatus ideal for investigating the antibacterial effects of nanoparticles.

2. Materials and Methods

2.1. Zinc oxide nanoparticle preparation and characterisation
A stock suspension of 0.2 mg/mL zinc oxide nanoparticles (ZnO NP, 50 nm nominal size, Sigma) was prepared using deionised water (dH$_2$O), autoclaved (121 °C for 15 min) then sonicated before use (37 kHz, 100 W, 30 min). Any dilutions made from the stock solutions were made using dH$_2$O. Material was characterized using SEM (Tescan MAIA3) and UV-vis spectroscopy (Biotek Epoch 2 96-well microplate spectrophotometer).

2.2. Bacteria preparation
Bacteria strains were purchased from Czech collection of microorganisms, one representative of Gram-positive bacteria (S. aureus) and one representative of Gram-negative bacteria (E. coli). Frozen bacteria stock (in Mueller Hinton broth, MHB, and glycerol at -20 °C) were allowed to thaw at +4 °C then 100 μL was added to MH agar and spread evenly across the surface and incubated for 18-24 h at 37 °C. From the resultant growth, one colony was removed and added to 10 mL MHB and placed on an orbital shaker (200 rpm) positioned inside an incubator set at 37 °C for 18-24 h. The bacteria suspension was adjusted to 0.5 McFarlands density in dH$_2$O, then subsequently diluted by 1:1000 to achieve an approximate initial cell concentration of 1×10$^5$ colony forming units per millilitre (cfu/mL).

2.3. Antibacterial tests
Standardised microbiological tests were used to assess the antibacterial capability of ZnO. Broth dilution assay [6] was employed to determine the number of bacteria that remained viable after exposure to ZnO. Suspensions of bacteria and ZnO NP in dH$_2$O were added (1:1) and subjected to reverse spin technology (200 rpm; 5 s per spin) and samples were removed every 2 h (see figure 1).

![Figure 1. Schematic representations of (a) reverse spin technology used to expose bacteria to ZnO NP and (b) the broth dilution technique [6] to determine the number viable bacteria cells from a sample.](image-url)
The disk diffusion assay [7] was also used to probe the antibacterial effect when bacteria were present on an agar surface. For comparison, ZnO NP were added to the agar surface prior to the inoculation of a known concentration of bacteria and the resulting colonies that grew were counted.

3. Results

The commercial ZnO used in this study was characterised using SEM and UV-vis spectroscopy. Material morphology was probed by SEM in both in-beam secondary electron and back-scattered electron modes (see figure 2(a) and 2(b), respectively). The images revealed that the primary particle shapes were grain-like, although the supplier stated the shape to be spherical. Also, we observed large agglomerates of primary particles up to 1 µm in diameter, which is approximately the same size as a bacterium. The bacteria-ZnO NP interaction was studied in ambient light conditions. Thus, UV-vis spectroscopy was applied to investigate the light absorbance capabilities of the material. Figure 2(c) shows the light absorbance spectral profile obtained from ZnO NP in dH₂O with an absorbance peak at 363 nm which is typical for ZnO.

We employed standardised microbiological techniques to characterize the antibacterial potential of commercially available ZnO NP. The first of these was the “broth dilution” technique (see the process scheme in figure 1(b)), which measured the number of bacteria that retained the ability to grow after exposure to ZnO NP using reverse spin technology. The results are shown in figure 3 for both bacteria strains and two concentrations of ZnO. The reference samples had only bacteria in water and no ZnO, and as expected there was no reduction observed under these conditions (see figure 3). It is clear from figure 3 that the antibacterial effect of ZnO is dependent on exposure time, ZnO concentration, and cell type. Complete eradication of both bacteria strains was achieved after 4 hours at a concentration of 10 µg/mL. The equivalent exposure time using 1 µg/mL only achieved between 1.5-2 orders of magnitude reduction in viable cells. At each sample point, the number of viable Gram-positive S. aureus was greater than the number of Gram-negative E. coli.

There was no antibacterial effect observed when ZnO NP was added to the agar surface prior to bacteria addition (see figure 4(b)). Similar results were observed when ZnO NP were added to filter paper disks for the concentration range used in this study (10 – 1000 µg/mL, see figure 4(c).

4. Discussion

Our observations indicate that only under certain conditions does ZnO exert an antibacterial effect. The constant mixing of the bacteria-nanoparticle suspension by reversing the direction of rotation enhanced the interaction capability and the antibacterial effect of ZnO NP. Yet, when ZnO NP were added to filter paper disks and placed upon bacteria, there was no observed antibacterial effect. This
Figure 3. Effect on viable cell concentration as a function of exposure to two concentrations of ZnO. Solid lines are reference samples and contain only bacteria in water i.e. no ZnO (red – *E. coli* and blue – *S. aureus*). Dashed lines represent bacteria exposed to ZnO with stated concentration.

Static type interaction is vastly different to the interaction mechanism involved in reverse spin technology. A second type of static interaction, ZnO NP added to agar before bacteria, also did not have any bactericidal effect. These observations confirm the importance of efficient sample mixing in the antibacterial effect of ZnO.

When the interaction between bacteria and ZnO NP was enhanced using the reverse spin technology, the “broth dilution” assay revealed three distinct ways that ZnO acted upon bacteria to render them non-viable. The first was related to the exposure time, and the longer the cells were exposed to ZnO the greater the antibacterial effect. Commercial ZnO with an average particle size of 30 nm at a concentration of 10 mg/mL was able to reduce pathogenic *E. coli* (0157:H7) by 1 order of magnitude after 4 hours that increased to 2 orders of magnitude by 8 hours exposure time [8]. The interaction between bacteria and ZnO NP occurred in liquid, but it is not evident whether the

Figure 4. (a) Schematic diagram of the two antibacterial tests. (b) The concentration of viable cells after exposure to different concentrations of ZnO NP on agar surface. (c) Disk diffusion assay: Mueller Hinton agar plates with *E. coli* (top) and *S. aureus* (bottom). Numbers denote concentration of ZnO on disk in µg/mL. C = positive control (50 µL of 0.1 M H₂O₂).
suspension was mixed throughout exposure. We observed a 5 orders of magnitude reduction of non-pathogenic \textit{E. coli} with a considerably lower concentration of 10 µg/mL 50 nm ZnO.

The second way was associated with ZnO concentration, and the positive correlation with bactericidal effect as shown by greater reduction in viable cells observed with the higher concentration of ZnO. We achieved complete inactivation of both Gram-positive \textit{S. aureus} and Gram-negative \textit{E. coli} within 4 hours using 10 µg/mL, yet for the same treatment time viable cells remained after exposure to 1 µg/mL. Navale \textit{et al.}, incubated synthesised ZnO NP with \textit{S. aureus} and mixed the suspension at 200 rpm for 4 hours in one direction i.e. without reverse spin, and a complete loss of viability was achieved using a 10-fold greater ZnO NP concentration (100 µg/mL) [9].

Finally, there appeared to be differences in antibacterial effect dependant on the type of bacteria cell being exposed, since Gram-negative \textit{E. coli} were reduced to a greater extent than Gram-positive \textit{S. aureus} at every sample time point. This can be explained by differences in the outer cell wall structure of the two bacteria types. \textit{S. aureus} has a thick layer of peptidoglycan (~30 nm, [10]) which plays an important structural role and provides rigidity, whereas the peptidoglycan layer is much thinner in \textit{E. coli} (7-9 nm, [11]) and would be more susceptible to mechanical damage.

The results from our investigation lead us to believe that the primary mechanism by which ZnO NP exert an antibacterial effect using reverse spin technology involves direct interaction of ZnO NP with the bacteria cell surface which can cause damage and lead to cell death. The constant mixing prevents nanoparticle agglomeration and enhances the efficiency of ZnO NP. Other factors such as the chemical reactions induced by electron-hole pair formation on the nanoparticle surface would not be dominant due a lack of light irradiation since the bacteria-nanoparticle suspension was shielded during exposure. Whilst reactive oxygen species can be generated from zinc oxide surface without illumination [12], the concentrations produced would be too low to be responsible for the observed bactericidal effect [13].

5. Conclusion

We achieved complete eradication of both model Gram-positive and Gram-negative bacteria in water using commercially available nano-sized ZnO without additional illumination. When the bacteria-nanoparticle suspension was constantly mixed using a reverse spin bioreactor, no viable cells remained after only 4 hours treatment time using a relatively low concentration of ZnO. However, no antibacterial effect was observed when the bacteria and nanoparticle suspensions were not sufficiently mixed together, either when ZnO was present on the agar surface nor on filter paper disks even for much higher concentrations. The reduction in viable bacteria using ZnO and reverse spin technology was achieved without the need for additional illumination sources, therefore reducing the potential cost and energy consumption of the treatment compared to conventional photocatalytic reactors which use UV light and TiO\textsubscript{2}.

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