RESEARCH LETTER

Antibacterial activity of ZnO nanoparticle suspensions on a broad spectrum of microorganisms

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Abstract

Nanoparticle metal oxides represent a new class of important materials that are increasingly being developed for use in research and health-related applications. Highly ionic metal oxides are interesting not only for their wide variety of physical and chemical properties but also for their antibacterial activity. Although the *in vitro* antibacterial activity and efficacy of regular zinc oxides have been investigated, little is known about the antibacterial activity of nanoparticles of ZnO. Preliminary growth analysis data suggest that nanoparticles of ZnO have significantly higher antibacterial effects on *Staphylococcus aureus* than do five other metal oxide nanoparticles. In addition, studies have clearly demonstrated that ZnO nanoparticles have a wide range of antibacterial effects on a number of other microorganisms. The antibacterial activity of ZnO may be dependent on the size and the presence of normal visible light. The data suggest that ZnO nanoparticles have a potential application as a bacteriostatic agent in visible light and may have future applications in the development of derivative agents to control the spread and infection of a variety of bacterial strains.

Introduction

The re-emergence of infectious diseases and the continuous development of antibiotic resistance among a variety of disease-causing bacteria pose a serious threat to public health worldwide (Desselberger, 2000). Among these pathogenic microorganisms, *Enterococcus, Staphylococcus* and *Streptococcus* are common closely related species that cause a wide variety of infections and diseases (Boyce, 1997; Lowy, 1998; Hancook & Gilmore, 2000). Despite antimicrobial therapy, morbidity and mortality associated with these bacterial infections remain high, partially as a result of the ability of these organisms to develop resistance to virtually all antibiotics. New strategies are therefore needed to identify and develop the next generation of drugs or agents to control bacterial infections.

Since the 1980s, methicillin-resistant *Staphylococcus aureus* (MRSA) has been commonly linked with hospital-associated (HA) infections, but recently a new kind of MRSA, a community-acquired (CA) strain, has emerged (Vandenesch *et al.*, 2003). CA-MRSA affects an entirely new population, for example healthy children and adults who have none of the risk factors found with HA-MRSA infections. The factors that facilitate the spread of CA- or HA-MRSA bacteria are crowding, skin-to-skin contact, sharing personal items that could be contaminated with wound drainage, such as towels, poor personal cleanliness and hygiene, and limited access to health care (Zaoutis *et al.*, 2006). Therefore, the key points in preventing staphylococcal and related bacterial infections are good standards of hygiene, avoidance of skin trauma, and the use of antibacterial ointments or surface-coating agents with potential antibacterial properties.

Recent advances in the field of nanotechnology, particularly the ability to prepare highly ordered nanoparticulates of any size and shape, have led to the development of new biocidal agents. Several studies have indicated that nanoparticulate formulations can be used as effective bactericidal materials (Tiller *et al.*, 2001; Lin *et al.*, 2002; Stoimenov *et al.*, 2002; Kuhn *et al.*, 2003; Sawai, 2003; Sunada *et al.*, 2003; Sondi & Salopak-Sondi, 2004; Lewis & Klibanov, 2005; Rosi & Mirkin, 2005; Ma *et al.*, 2006; Thill *et al.*, 2006). It has

been shown that illuminated suspensions containing TiO_2 are effective at killing *Escherichia coli*, a finding that spurred research towards the development of photocatalytic methods for the killing of bacteria and viruses using TiO_2 in aqueous media (Sunada *et al.*, 2003). The disadvantage of using TiO_2 is that UV light is required to activate the photocatalyst and initiate the killing of the bacteria and viruses. In recent years, visible light absorbing photocatalysts with $Ag/AgBr/TiO_2$ has proved to be successful at killing *S. aureus* and *E. coli* (Hu *et al.*, 2006). Klabunde *et al.* reported that aerogel-derived halogenated (Cl_2 and Br_2) adducts of nanocrystalline metal oxides such as MgO and CaO are effective for killing *E. coli, Bacillus cereus* and *Bacillus globigii* as well as for decontaminating MS2 bacteriophage in dry powder form or as an aqueous slurry (Koper *et al.*, 2002; Sawai, 2003; Sondi & Salopak-Sondi, 2004). The possibility that antibacterial formulations based on nanoparticles themselves may be effective in controlling the outbreak of new resistant strains of bacteria such as MRSA has also been reported (Tiller *et al.*, 2001). However, there is a need to develop new nanoparticulate-based formulations that are stable, robust and durable, and that are effective in the destruction or elimination of bacteria and viruses. Preliminary studies have indicated that ZnO nanoparticles at a concentration of between 3 and 10 mM caused 100% inhibition of bacterial growth as a result of the intracellular accumulation of nanoparticles (Brayner *et al.*, 2006).

The interaction of nanoparticles with microorganisms and biomolecules is an expanding field of research, which as yet is largely unexplored. In this report, preliminary results suggest that, among six nanoparticles tested, ZnO nanoparticles with relatively small particle size show a wide range of antibacterial effects on various microorganisms, including major pathogens, under normal visible light conditions.

Materials and methods

Bacterial strains, media and materials

The following bacterial strains were used in this study: Gram-positive methicillin-sensitive *S. aureus* (MSSA), a routinely used strain RN6390 and clinical isolate UAMS-1; sequenced MRSA strains COL and N315; CA-MRSA strain USA400; *Staphylococcus epidermidis* 1487 and sequenced RP62A strains; *Streptococcus pyogenes* NZ315; *Enterococcus faecalis*; *Bacillus subtilis* 168; and Gram-negative *E. coli* K12. All these strains were grown in tryptic soy broth (TSB) except for *E. coli* and *B. subtilis* strains, which were grown in Luria–Bertani (LB) medium. All strains were grown aerobically at 37 °C, in 10 mL of medium in 18 mm × 150 mm borosilicate glass culture tubes (Fisher Scientific) with shaking at 200 r.p.m. under normal laboratory lighting conditions unless specified.

All metal oxide nanoparticles (MgO, TiO_2, Al_2O_3, CuO, CeO_2, and ZnO) were purchased from Nanoscale Materials Inc. (KS), except for zinc oxide powder (<1 μm particle size) and nanopowder or nanoparticles (50 − 70 nm particle diameter), which were obtained from Sigma-Aldrich. All these nanoparticle metal oxides are more than 99% pure. All metal oxide nanoparticles were resuspended in sterile double-distilled water and briefly sonicated so that they dispersed and formed a uniform colloidal suspension. All the experiments were performed from a freshly prepared colloidal suspension.

Determination of the antibacterial activity of the metal oxide nanoparticles

In order to examine the antibacterial activity of the metal oxide nanoparticles on various microorganisms we used culture turbidity as a qualitative measure of cell growth, and also performed plating assays to compare viability. To examine the bacterial growth rate and to determine growth behaviour in the presence of the considered nanoparticles, various microorganisms were grown in liquid medium supplemented with nanoparticle colloidal suspensions. Cultures of nanoparticle-free medium under the same growth conditions were used as a control. To avoid potential optical interference during optical measurements of the growing cultures caused by the light-scattering properties of the nanoparticles, the same liquid medium without microorganisms, but containing the same concentration of nanoparticles cultured under the same conditions as blank controls.

The overnight culture (50 mL) was inoculated into 10 mL of the respective growth medium, which was equivalent to an initial cell $OD_{600\,nm}$ of 0.045–0.06 as measured by a spectrophotometer (Spectronic 20D, Thermo Electron) for the tested strains. Following inoculation, the OD of the cultures was serially monitored every hour or every 2 h up to 10–12 h, with a final reading at 24 h. Cultures from some selective concentrations and growth points were plated onto Tryptic soy agar (TSA) plates to determine the viable cell count. The numbers of colonies were counted after overnight incubation at 37 °C. The minimal inhibitory concentration (MIC) was calculated in TSB as well as in Mueller–Hilton media as the concentration of ZnO nanoparticles leading to a more than 95% reduction in growth. All assays were repeated three times in duplicate, and similar results were obtained.

Results

Determination of the antibacterial activity of metal oxide nanoparticles

To identify the agents with potential antibacterial effects, six metal oxide nanoparticles (MgO, TiO_2, Al_2O_3, CuO, CeO_2

and ZnO) were selected for our preliminary studies in liquid broth culture with aeration. *Staphylococcus aureus* strain RN6390 was used in most of the experiments. Among the nanoparticles tested, four nanoparticles, namely MgO, TiO_2, CuO and CeO_2, did not show any significant growth inhibition up to 10 mM colloidal suspension, whereas Al_2O_3 (*c.* 50%) and ZnO ($\geq$50%) showed significant growth inhibition (data not shown). As Al_2O_3 is highly toxic to most living cells, further studies were not pursued. Interestingly, ZnO nanoparticles showed a significant growth inhibition compared with the control (Fig. 1a and b). Although antibacterial activities of other metal oxide nanoparticles, including MgO and CuO, have been reported with other bacteria, for example *E. coli* (Kuhn *et al.*, 2003; Sawai, 2003; Furno *et al.*, 2004; Rincon & Pulgarin, 2004; Cioffi *et al.*, 2005; Thill *et al.*, 2006), no significant growth inhibition was detected with the used experimental setting for *Staphylococcus aureus*. TiO_2 is also known to kill various bacteria, including *Staphylococcus aureus*, under photoactivation with UV light; however, about 20% of growth reduction in liquid growth conditions under normal laboratory lighting has been seen (data not shown). ZnO nanoparticles have thus been selected for further studies, as they showed a significant growth inhibition.

Effect of size of ZnO nanoparticles on bacterial growth inhibition

The functional activities (chemical, catalytic or biological) of nanoparticles are heavily influenced by the size of the particles (Lewis & Klibanov, 2005; Rosi & Mirkin, 2005). Therefore, to determine if the size of ZnO nanoparticles was also playing an important role in inhibiting bacterial growth, three different ZnO materials from two different sources were tested: ZnO ultrafine powder ($>1\,\mu m$), ZnO nanoparticles (mean particle size *c.* 8 nm; Fig. 1a), and ZnO nanopowder (mean particle size 50 – 70 nm; Fig. 1b) were used for growth inhibition assay of *Staphylococcus aureus*. For ZnO ultrafine powder (data not shown) and ZnO nanopowder (Fig. 1b), which have relatively large particle sizes, reduced growth rates (*c.* 50%) were observed. However, ZnO nanoparticles, with smaller particle size, were able to reduce *c.* 99% of growth colloidal suspension concentration of 2 mM (Fig. 1a). The data clearly suggest that nanoparticles with smaller particle sizes (e.g. *c.* 8-nm diameter) showed more than 95% growth inhibition at 1 mM concentration (0.008%), whereas 5 mM of ZnO nanoparticles with relatively larger particle sizes (e.g. 50 – 70 nm) showed only 40 – 50% growth inhibition as compared with the control. Similarly, there was no significant variation of the bacterial growth inhibition as measured by OD at 24 h as compared with that at 10 h. Growing cultures from various time-points of growth were also plated to count viable cells, and the viable cell numbers are consistent with the OD of the growing cultures. The minimum inhibitory concentration (MIC) for ZnO nanoparticles with smaller size was calculated to be 1 mM or 80 $\mu g\,mL^{-1}$ for *Staphylococcus aureus* in both TSB and Muller–Hilton medium, whereas that for larger particles of ZnO was calculated to be 15 mM or 1.2 $mg\,mL^{-1}$.

Photoactivation of ZnO nanoparticles

ZnO is a semiconductor and can be excited by UV light. A normal laboratory environment has fluorescent lighting, and conventional fluorescent lamps emit $\leq$4% UV light so

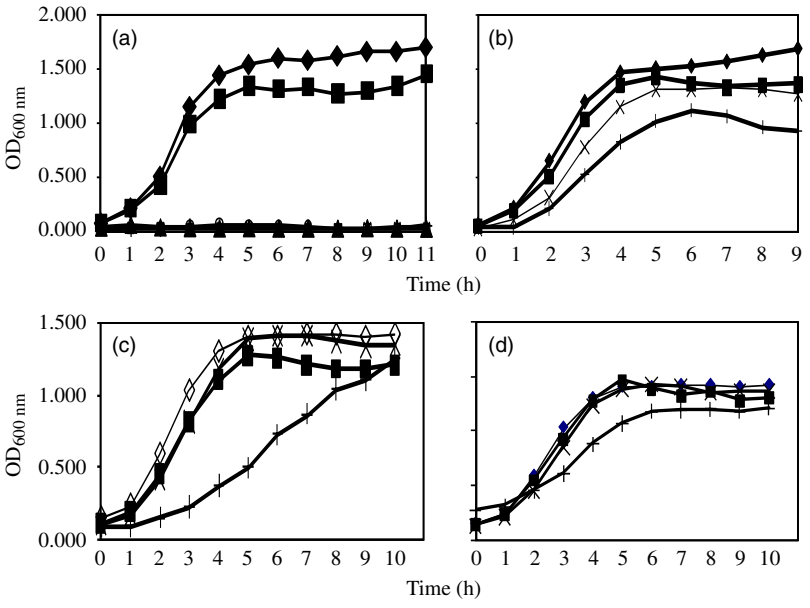

Fig. 1. The effect of ZnO nanoparticles on the growth of *Staphylococcus aureus* strain RN6390. Cultures were set up at an initial inoculum of 0.5% dilution from overnight cultures (equivalent to 0.04–0.06 OD) in nutrient broth containing various concentrations from 0 to 5 mM (0–0.04%; ◇, control 0 mM; ■, 0.5 mM; ×, 2 mM; +, 5 mM) of ZnO nanoparticles colloidal suspension from two different sources. Cultures were incubated at 37 °C with constant shaking at 220 r.p.m under normal ambient laboratory light conditions (a and b) and in the dark (c and d). (a and c) ZnO nanoparticles with average particle diameter *c.* 8 nm; (b and d) ZnO nanoparticles with average particle diameter *c.* 50 – 70 nm.

there is a possibility that ZnO is 'activated' by this small amount of UV component or by the visible light prevalent in the laboratory. In order to verify this possibility, the growth analysis was performed under dark conditions, using similar conditions to those described previously but with the suspensions prepared in the dark and the cultures grown in the dark by means of wrapping the glass culture tubes with aluminum foil. The data shown in Fig. 1c and d for both sizes of ZnO nanoparticles clearly suggest that the antibacterial activity of ZnO nanoparticles in the dark is less than that in ambient laboratory conditions. In the case of the nanoparticles with smaller particle sizes, only 30–50% of the antibacterial activity is retained at 5 mM concentration up to 10 h, and thereafter there was no significant difference in growth inhibition. Similarly, photoactivation of both ZnO nanoparticle suspensions was performed by exposure with UV light at 254 nm for 30 min in a UV transilluminator, and the growth analysis was performed with 2 and 5 mM concentrations using similar conditions to those described previously for the ambient laboratory conditions. Interestingly, the growth inhibition patterns did not differ significantly from the data presented in Fig. 1a and b (data not shown). Similarly, photoactivation of TiO_2 nanoparticles was performed under the same activation conditions as used for ZnO nanoparticles and used to determine antibacterial activity under normal growth conditions. A significant increase in growth inhibition was found (more than 60%) as compared with the nonphotoactivated form (20%) (data not shown). Therefore, the overall growth analyses suggest that the ambient laboratory conditions are sufficient for the optimal biocidal activity of the ZnO nanoparticles, which is probably dependent on the size of the nanoparticles.

Antibacterial effects on other Staphylococci strains and microorganisms

Analysis of the antibacterial effects of two ZnO nanoparticles was extended to other microorganisms as mentioned in 'Materials and methods'. Among the *Staphylococcus aureus* strains tested, no significant difference in the growth inhibition was found from that seen in the case of *Staphylococcus aureus* strain RN6390. In the case of MSSA strain UAMS-1 the studies indicated about 60% inhibition of growth when compared with RN6390 (data not shown). Figure 2 shows the growth of (a) *Staphylococcus aureus* N315, (b) *Staphylococcus epidermidis* 1487, (c) *Streptococcus pyogenes* and (d) *B. subtilis* with various concentrations of various ZnO nanoparticles as indicated. It is clear that even a 5 mM (0.004%) colloidal suspension of ZnO nanoparticles with the smaller size could inhibit more than 95% of growth for most Gram-positive microorganisms tested in this study. In addition, ZnO nanoparticles with relatively larger diameter were also able to inhibit growth, but to a lesser extent (data not shown). Similar results were found for *Enterococcus faecalis*, another important Gram-positive bacterium closely related to *Staphylococcus* and *Streptococcus* pathogenic bacteria, and for the Gram-negative *E. coli*. Thus, it is clear from preliminary studies that nanoparticles of ZnO have significant antibacterial effects on a wide range of microorganisms. The size of the nanoparticles may have a greater impact on their activity, probably because of a greater accumulation of the nanoparticles inside the cell membrane and cytoplasm. However, much remains unknown about the function, specificity, toxicity and efficacy of nanoparticles – and these topics will be the subject of future research .

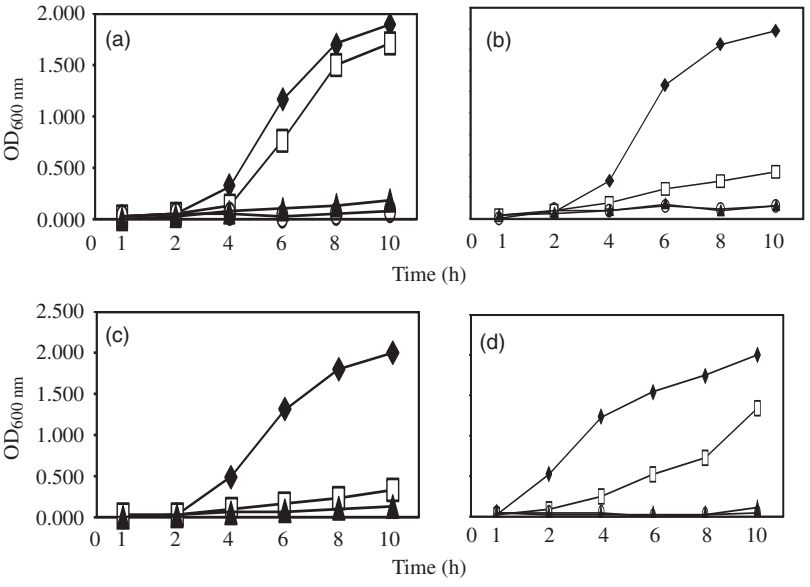

Fig. 2. Growth curves of various bacterial strains in tryptic soya broth (a, b and c) and LB (d) media in the presence of various concentrations of ZnO nanoparticles with smaller particle diameter. Cultures were set up and grown under the same conditions as described in Fig. 1a, expect that different concentrations were used (♦, control 0 mM; □, 2 mM; ▲, 5 mM; ○, 10 mM). (a) *Staphylococcus aureus* N315; (b) *Staphylococcus epidermidis* 1487; (c) *Streptococcus pyogenes* NZ131 and (d) *Bacillus subtilis* 168.

Discussion

The re-emergence of infectious diseases poses a serious threat to public health worldwide, and the increasing rate of the appearance of antibiotic-resistant strains in a short period of time within both Gram-positive and Gram-negative microorganisms is a major public health concern. Alternative therapeutics to control and prevent the spread of infections in both community and hospital environments are required (Lowy, 1998). Although various classes of antibiotics (penicillin and derivatives) were discovered in 1940s and 1950s, in the past 40 years only two antibiotics representing new chemical classes (namely linezolid and daptomycin) have reached the market to treat multiple antibiotic-resistant Gram-positive infections. Recent advances in the field of nanotechnology, particularly the ability to prepare highly ionic metal oxide nanoparticulates of any size and shape, may lead to the development of new antibacterial agents.

Preliminary studies as discussed in this report have demonstrated that ZnO among the six metal oxide nanoparticles analysed shows a significant growth inhibition under normal laboratory lighting conditions. ZnO powder has been used for a long time as an active ingredient for dermatological applications in creams, lotions and ointments on account of its antibacterial properties (Sawai, 2003). However, nanoparticles of ZnO are much more effective agents in controlling the growth of various microorganisms; furthermore, the preliminary studies show that the smaller the particle size, the greater the efficacy in inhibiting the growth of bacteria. Photoactivated (e.g. by UV light) TiO_2 is known to kill various bacteria including MRSA (Sunada *et al.*, 1998, 2003), but nonactivated TiO_2 was not able to inhibit significantly the growth of *Staphylococcus aureus*. Toxicological impact studies of ZnO nanoparticles on *E. coli* were performed by plate assays and transmission electron microscopy (TEM) analyses. The studies suggested that synthesized ZnO nanoparticles (average particles diameter *c.* 12 nm) are able to slow down the bacterial growth (100% with 3 mM) as a result of disorganization of the *E. coli* membranes, which increases the membrane permeability leading to the accumulation of nanoparticles in the bacterial membrane and cytoplasm regions of the cells (Brayner *et al.*, 2006).

Although metals and metal oxides such as ZnO are known to be toxic to host human cells at relatively high concentrations, they are not expected to be toxic at very low concentrations. In fact, it has been shown that ZnO protects against intestinal diseases by protecting intestinal cells from *E. coli* (ETEC) infection by inhibiting the adhesion and internalization of bacteria (Roselli *et al.*, 2003). Therefore, substantial bacterial growth at lower concentrations of ZnO suggest that ZnO nanoparticles may not be toxic for various tested microorganisms. This may be consistent with the prediction that *E. coli* can metabolize Zn^{2+} as an oligoelement (Roselli *et al.*, 2003). Similarly, metal-ion homeostasis is important for bacterial life because of their involvement in the regulation of a wide array of metabolic functions as coenzymes, cofactors and catalysts, and as structural stabilizers of enzymes and DNA-binding proteins (Gaballa & Helmann, 1998). However, excess metal or metal ions are toxic for bacterial cells. Therefore, certain bacteria have developed mechanisms to regulate the influx and efflux processes to maintain the steady intracellular concentration of metal ions, including the Zn^{2+} ion. The genes responsible for the transport of zinc ions have been characterized in several bacteria, including *Streptococcus pneumoniae, Haemophilus influenzae, Staphylococcus aureus, Synechococcus* sp. strain PCC6803, *Escherichia coli,* and *B. subtilis* (Gaballa & Helmann, 1998; Lindsay & Foster, 2001). In *Staphylococcus aureus*, *zntA* and *zntR* genes have been characterized, and it has been shown that ZntA, a transmembrane protein, is responsible for the efflux of zinc and cobalt ions and that ZntR encodes for a Zn-responsive regulatory protein (Xiong & Jayaswal, 1998).

Overall, the preliminary findings suggest that ZnO nanoparticles can be used externally to control the spreading of bacterial infections. It would be interesting to determine if any derivatives of ZnO nanoparticles with various chemical groups or bioagents are more effective at eliminating various microorganisms. In the prevention and control of bacterial spreading and infections, the main target is the cell wall structure. The cell wall of most pathogenic bacteria is composed of surface proteins for adhesion and colonization, and components such as polysaccharides and teichoic acid that protect against host defences and environmental conditions (Navarre & Schneewind, 1999). These components are charged macromolecules; therefore, specific interactions to disrupt their main function and location may be triggered by introducing specific groups on the surface of the nanoparticles. It has been reported that certain long-chain polycations coated onto surfaces can efficiently kill on contact both Gram-positive and Gram-negative bacteria (Tiller *et al.*, 2001; Berry *et al.*, 2005). These studies have indicated that families of unrelated hydrophobic groups are equally efficient at killing bacteria. Therefore, in the future, ZnO nanoparticle-containing formulations may be utilized for external uses as antibacterial agents in ointments, lotions, mouthwashes, and surface coatings on various substrates to prevent microorganisms from attaching, colonizing, spreading, and forming biofilms in indwelling medical devices.

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