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Reduced *Staphylococcus aureus* proliferation and biofilm formation on zinc oxide nanoparticle PVC composite surfaces

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ABSTRACT

Conventional particulate zinc oxide (ZnO) is a known antibacterial agent. Studies have shown that reducing the size of ZnO particles to nanoscale dimensions further enhances their antibacterial properties. Polymers, like all biomaterials, run the risk of harboring bacteria which may produce an antibiotic-resistant biofilm. The addition of ZnO nanoparticles to form a polymer composite material may thus reduce undesirable bacteria activity. The purpose of the present in vitro study was to investigate the antibacterial properties of ZnO nanoparticles when incorporated into a traditional polymeric biomaterial. For this purpose, *Staphylococcus aureus* were seeded at a known cell density onto coverslips coated with a film of polyvinyl chloride (PVC) with varying concentrations of ZnO nanoparticles. Samples were cultured for 24 or 72 h. Methods of analysis, including optical density readings and crystal violet staining, indicated a reduced presence of a biofilm on ZnO nanoparticle polymer composites compared to pure polymer controls. Live/dead bacteria assays provided images to confirm the reduced presence of active bacteria on samples with zinc oxide nanoparticles. Conditioning of the cell culture medium by the composites was also investigated by measuring concentrations of elemental zinc (Zn^{2+}) and bacteria growth in the presence of conditioned medium. This study demonstrated that the development of ZnO polymer composites may improve biomaterial effectiveness for numerous applications, such as endotracheal tubes, catheterp and implanted biomaterials, which are prone to bacterial infection.

1. Introduction

Bacterial infection is one of the most common and universal complications associated with implanted medical devices. The high rate of infection of biomaterials inserted into or in contact with the body is of perpetual concern to the medical device community. For example, orthopedic implant failure due to infection occurs in approximately 1.5–2.5% of all implants (3000–6000 incidents per year) [1]. These infections on average increase hospital costs by $50,000 per episode, with significantly more lost in patient pain, discomfort and lost work time. As another example, ventilator-associated pneumonia (VAP), a hospital-acquired respiratory infection that frequently develops in patients who receive mechanical ventilation via endotracheal tubes for extended periods of time, develops at a rate of 8–28% in intubated patients [2]. The mortality rate of VAP is estimated to be 24–50% [3], and its positive diagnosis increases treatment costs by an average of $40,000 per episode [4].

Orthopedic implants and endotracheal tube material surfaces can harbor bacterial infections in the form of biofilm resistance to antibiotic treatment. Infections associated with endotracheal tubes are most often attributed to *Pseudomonas aeruginosa* (present in 41.7% of patients diagnosed with VAP) and *Staphylococcus aureus* (36.7%) [5]. An increase in infection attributed to multi-drug-resistant *S. aureus* (MRSA) has led to a growing interest in identifying novel ways to reduce bacteria activity without the use of antibiotics. The Center for Disease Control and Prevention reported an increase in the incidence of MRSA infection from 127,000 infections and 11,000 associated deaths in 1999 to 278,000 infections and 17,000 associated deaths in 2005.

Zinc oxide has been shown to naturally reduce the activity of a wide range of (mostly Gram-positive) bacteria strains without the use of antibiotics [6]. Implementation of nanotechnology, or the use of materials with nanoscale dimensions, has further enhanced the antibacterial properties of ZnO [7–9]. This is partially because the specific surface area of a dose of ZnO particles increases as the particle size decreases, allowing for greater material interactions with the surrounding environment. To support such claims, Reddy et al. [10] reported complete inhibition of planktonic *S. aureus* growth at ZnO nanoparticle (diameter $\sim$ 13 nm) concentrations of $\geqslant$ 1 mM in overnight cultures. Jones et al. [11] reported a 95% inhibition of *S. aureus* growth in the presence of 1 mM of 8 nm ZnO nanoparticles after 10 h and a 40–50% inhibition of the same *S. aureus* in the presence of 5 mM of 50–70 nm ZnO nanoparticles

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after 10 h. While highlighting the discrepancies in the antibacterial effect among ZnO nanoparticles of varying size, the authors also commented on the potentially important role of ambient ultraviolet (UV) light in providing antibacterial properties to ZnO. When comparable experiments were performed in the dark, high (5 mM) concentrations of 8 nm ZnO nanoparticles resulted in only a slight inhibition of bacterial growth after 10 h. However, increased doses of UV light in addition to the ambient light of the laboratory did not enhance the antibacterial effect beyond ambient light conditions.

Furthermore, Nair et al. [12] observed an increased antibacterial effects on *S. aureus* and *Escherichia coli* viability when exposed to ZnO nanoparticles of decreasing size, though a concentration of 5 mM was necessary to reduce bacteria viability at 24 h even with 40 nm diameter particles, the smallest diameter ZnO nanoparticle tested; irregularities in cell membranes of bacteria exposed to ZnO nanoparticles were also noted. Padmavathy and Vijayaraghavan [13] also found an increased antimicrobial effect of ZnO nanoparticles on *E. coli* at the 18 h time point as the particle diameter was reduced from 2 μm to 45 nm and again to 12 nm, and attributed the enhanced effect to the greater surface area to volume ratios and mechanical damage caused to the cells due to increased abrasiveness of the smaller nanoparticles.

McCarthy et al. [14,15] observed antibacterial effects of zinc ions (Zn^{2+}) and identified minimum inhibitory concentrations of 1917, 9 and 39 μg ml^{-1} at 48 h for *P. aeruginosa*, *S. aureus* and *Candida albicans*, respectively. Compared to *P. aeruginosa*, *S. aureus* was found to be far more sensitive to low concentrations of zinc ions.

In a study developed to elucidate the mechanism of the antibacterial effect of ZnO particles, Sawai et al. [16] measured the production of hydrogen peroxide, a reactive oxygen species attributed to bacteriocidal activity, from a variety of ceramic powder chemistries. ZnO produced the greatest amount of hydrogen peroxide and the antibacterial effect of ZnO powders was attributed to this phenomenon. Hydrogen peroxide production was not detected from CaO and MgO powders.

Nevertheless, the mechanism of antibacterial activity of ZnO nanoparticles is complex and still not fully understood. Zinc ions are known to inhibit multiple activities in bacterial, such as glycolysis, transmembrane proton translocation and acid tolerance [17]. In contrast, the presence of zinc ions is likely to only inhibit proliferation, or be bacteriostatic, rather than be capable of killing bacteria, or be bacteriocidal. The production of active oxygen species and the disruption of cell membranes caused by ZnO nanoparticles may actually be bacteriocidal. For bacteria grown in suspension in vitro, existing literature suggests that smaller diameter particles are more effective at reducing bacteria activity than larger particles with identical chemistry [11,13].

In spite of the many studies which have tested the antibacterial effects of ZnO nanoparticles, few studies have examined the use of ZnO nanomaterial surfaces. Ghule et al. [18] developed a method of synthesizing ZnO nanostructures on cellulose fibers to produce a ZnO nanoparticle coated paper. The material exhibited a significant resistance to *E. coli* growth, particularly in the presence of UV or fluorescent light, which enhanced antimicrobial hydrogen peroxide generation. Colon et al. [19] compared compacts composed of ZnO nanoparticles to compacts composed of micron-scale ZnO particles. The number of colony forming units of *Staphylococcus epidermidis* observed on compacts of ZnO nanoparticles with a diameter of 23 nm after a 1 h adhesion experiment was fewer than on micron-scale ZnO compacts or titanium controls. These studies investigated cell activity in the presence of ZnO nanoparticles but not specifically biofilm formation, or when incorporated into polymers currently used in medical devices.

The present study continued the exploration of the inherent antibacterial properties of ZnO nanoparticles by incorporating them into films of polyvinyl chloride (PVC), an FDA-approved polymer frequently used to fabricate medical devices including endotracheal tubes and catheters. Here, the growth of *S. aureus* was measured in the presence of ZnO nanoparticles and PVC composites as measured by optical density readings, crystal violet staining and live/dead staining for up to 72 h. The conditioning of bacteria cell culture medium by incubating the medium in the presence of ZnO nanoparticle polymer composites was also tested for antibacterial properties. Results provided evidence that *S. aureus* proliferation, biofilm formation and viability were reduced in the presence of ZnO nanoparticle PVC composites.

2. Materials and methods

2.1. Bacteria culture

Staphylococcus aureus bacteria were obtained in freeze-dried form (# 25923, ATCC, Manassas, VA). The cell pellet was rehydrated in Luria broth (10 g of tryptone, 5 g of yeast extract and 5 g of NaCl per liter of distilled water; Sigma–Aldrich, St. Louis, MO). Cells were propagated at 37 °C until they reached a stationary phase (18 h) and were then passed into a tube of 6 ml tryptic soy broth (TSB; Sigma–Aldrich, St. Louis, MO) at a 1:200 ratio. When the culture reached stationary phase, it was frozen in 1:1 TSB and glycerol (Sigma–Aldrich, St. Louis, MO) and used for all experiments.

2.2. Composite preparation

ZnO nanoparticles (~60 nm diameter; Nanophase Technologies, Romeoville, IL) were added to tetrahydrofuran-dissolved PVC endotracheal tubes (Hudson RCI, Research Triangle Park, NC) at weight percentages of 0%, 2%, 10%, 25% and 50%. Solutions were sonicated to promote nanoparticle distribution, pipetted onto 12 mm diameter glass coverslips (Fisher Scientific, Pittsburgh, PA), and dried in a vacuum oven. For cell culture experiments, samples were cleaned by immersing the composites in ethanol and drying three times. The surface morphology of the samples was analyzed at magnifications of ×6000 and ×30,000 with an accelerating voltage of 7 kV, using a scanning electron microscope (SEM; Hitachi 2700, Hitachi High-Technologies, Berkshire, UK) and image analysis software (Quartz PCI, Quartz Imaging Corporation, Vancouver, Canada). Carbon tape was used to fix samples to aluminum stubs (Electron Microscopy Sciences, Hatfield, PA) and the samples were sputter coated (Emitech K-550, Quorum Technologies, East Sussex, UK) for 2 min at 20 mA from a height of 45 mm to produce a 15 nm AuPd coating.

2.3. Bacteria cell counts determined by optical density

Tubes of TSB were inoculated with frozen bacteria stock and incubated for 18 h until the stationary phase was reached. The initial density of *S. aureus* was determined with optical density readings on a microplate reader (Spectra Max 340PC, Molecular Devices, Sunnyvale, CA) at 560 nm and diluted to obtain an optical density of 0.12. Population density was determined by serially diluting and plating samples with a range of optical densities, and counting colony forming units on the plates. Composite samples were seeded at 3×10^6 colony forming units per well in a 24-well plate (Fisher Scientific, Pittsburgh, PA), with one composite in each well.

After 24 h of bacteria culture incubation at 37 °C, bacteria suspensions were aspirated from the wells and the optical density was measured at 550 nm with a microplate reader (Spectra Max