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Cytotoxic evaluation of nanostructured zinc oxide (ZnO) thin films and leachates ☆

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ABSTRACT

Nanostructured ZnO films have potential use as coatings on medical devices and food packaging due to their antimicrobial and UV-protection properties. However, their influence on mammalian cells during clinical use is not fully understood. This study investigated the potential cytotoxicity of ZnO thin films in RAW 264.7 macrophages. ZnO thin films (~96 nm thick with a 50 nm grain) were deposited onto silicon wafers using pulsed laser deposition. Cells grown directly on ZnO thin film coatings exhibited less toxicity than cells exposed to extracts of the coatings. Cells on ZnO thin films exhibited a 43% and 68% decrease in cell viability using the MTT and 7-AAD/Annexin V flow cytometry assays, respectively, after a 24-h exposure as compared to controls. Undiluted 100% 24- and 48-h extracts decreased viability by 89%, increased cell death by LDH release to 76% 24 h after treatment, and increased ROS after 5–24 h of exposure. In contrast, no cytotoxicity or ROS were observed for 25% and 50% extracts, indicating a tolerable concentration. Roughly 24 and 34 μg/m^2 Zn leached off the surfaces after 24 and 48 h of incubation, respectively. ZnO coatings may produce gradual ion release which becomes toxic after a certain level and should be evaluated using both direct exposure and extraction methods.

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1. Introduction

Zinc oxide (ZnO) is a versatile material with a number of consumer applications due to its antimicrobial and UV-protection properties (Abd El-Hady et al., 2013; Skoog et al., 2012). Such properties can be imparted to device and product surfaces using high-precision nanoscale coating techniques. Nanometer film fabrication allows for a previously unattainable control of surface uniformity and particulate grain size and has potential to optimize antimicrobial efficacy and mitigate cell toxicity for materials such as ZnO. The PLD approach uses a high-powered laser plume that ablates purified ZnO powder and deposits it onto the desired surface. Physical properties of the resulting layer are customizable by tuning several parameters, such as the number of laser pulses, chamber pressure, temperature and others. Nanoscale ZnO thin films have several applications in the semiconductor industry (Gershon et al., 2012; Sonawane et al., 2011); yet represent novel coatings for medical applications. For medical device coatings and biological applications, nanoscale ZnO can be fabricated in a variety of nanoparticles and complex nanostructures, such as rods and flowers. For example, ZnO nanorods (Lee et al., 2008) are shown to modulate cell adhesion and macrophage responses, (Zaveri et al., 2010) whereas ZnO nanoflowers may improve bone tissue ingrowth (Park et al., 2010). Such physical properties combined with improved antimicrobial activity (Jansson et al., 2012) make nanostructured ZnO a desirable and promising implant coating. With the innovative applications of metal oxide coatings used for medical device coatings, namely those with nanometer scale patterning and thus increased surface area, concerns arise related to potential adverse cellular responses resulting from particulates and metal leaching at the implant site.

Bulk ZnO is a widely used food additive and is listed on the FDA's Generally Recognized as Safe (GRAS) database as a dietary supplement (21 CFR 182.5991), nutrient (21 CFR 182.5991), and a resinous/polymeric coating (21 CFR 175.300). ZnO can be used as an antimicrobial coating for both food and medical materials; for example, it has been shown to increase shelf life of orange juice

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(Emamifar et al., 2010) and is known to improve healing when coated on medical tape (Hughes and McLean, 1988). Additionally, it may serve as an effective UV blocker (Yadav et al., 2006) and antimicrobial (Rajendran et al., 2010) when incorporated in textiles. ZnO is also an attractive alternative or complement to widely used silver (Ag) products when stronger antifungal properties are additionally desired (Reddy et al., 2007). Zn is an essential nutrient which can cause toxicity through both deficiency (usually defined as a serum concentration below 0.7 μg/mL) (Atasoy and Ulusoy, 2012; Zago and L., 2010) and at higher than homeostatic concentrations (Tezvergil-Mutluay et al., 2010). The cytotoxicity of both bulk and particulate Zn has been described in several cell culture models (Moos et al., 2010; Daniels et al., 2004; Sun et al., 2011) and inhalation studies (Fuortes and Schenck, 2000). Studies which evaluate the cytotoxicity of nanoscale ZnO coatings and surfaces, as opposed to discrete nanoparticles in mammalian cells, however, are scant.

ZnO has a high likelihood of unintended metal ion release into its environment (Haider and Munroe, 2011; Savarino et al., 1999); however, in certain cases, ion release may be desired. By coating a precise thickness and by optimizing coating parameters, the rate of dissolution and the total amount of Zn that can leach out may be controlled. Zinc leaching has immunomodulatory properties that may be suited for special applications that require suppressed immune function. For example, ionic zinc has been shown to modulate the immune response from monocytes by suppressing TNF-alpha transcription and release from monocytes (von Bülow et al., 2007). This gives it potential for limiting aseptic loosening in orthopedic implants and possibly increasing their overall lifespan. Polymorphonuclear cells (PMNs) are known to be activated by hydroxyapatite (HA) wear debris present in inflamed regions; this event may, accordingly, be modulated to an extent by adding zinc to the scaffold material (Grandjean-Laquerrier et al., 2006). More specifically, levels of the pro-inflammatory mediator interleukin-8 (IL-8) and the matrix metallo-proteinase-9 in PMNs have been shown to decrease with a 5% Zn-substitution of calcium ions in HA powder (Velard et al., 2010).

Nanoscale zinc topography may also be beneficial in other orthopedic applications. A comparison study of nanophase and microphase ZnO surfaces revealed that the nm grain surfaces had better bone tissue response and decreased *Staphylococcus epidermidis* colony forming units than microphase surfaces (Colon et al., 2006). We previously evaluated the antimicrobial activity (Skoog et al., 2012) of a uniform atomic layer deposited ZnO coating on a porous substrate and showed that it may also be cytotoxic to macrophages (Petrochenko et al., 2013). The present study aimed to examine the cytotoxicity and dissolution of nanotextured ZnO. A granular textured layer was deposited using a pulsed laser method onto an inert substrate to isolate the effect of ZnO. Mouse RAW 264.7 macrophages were selected to evaluate the biological responses because: (1) they are an important cell type involved in immune and inflammatory responses and wound healing, and (2) to make inter-laboratory comparisons across different studies using this cell type. Therefore, the goals of the study: (1) develop a "test chip" consisting of a silicon wafer substrate coated with a highly uniform ZnO coating with nanometer scale thickness and grain size, (2) assess the cytotoxicity of ZnO thin film coatings which have previously exhibited considerable antimicrobial activity (Gittard et al., 2009) and (3) determine whether leaching of Zn ions from the surface coating plays a role in cytotoxicity.

2. Methods

2.1. ZnO pulsed laser deposition on Si

A high-purity ZnO powder (Alfa Aesar, Ward Hill, MA) was pressed into round 2-in. diameter pellets. The pellets were subsequently sintered in an oxygen atmosphere at 1000 °C for 12 h. Wafers are highly pure, defect-free single crystalline Si polished slices. A krypton fluoride excimer laser (l = 248 nm, repetition rate = 10 Hz) was used to ablate the high-purity ZnO target. The thin films were grown under O_2 partial pressure of 5×10^{-5} Torr at room temperature for 5 min. The ZnO thin film deposition rate using a KrF excimer laser was on the order of 0.01 nm/pulse (He et al., 2007). The materials were then cut into approximately 0.5 × 0.5 cm (7 cm diagonal) squares to fit a 48-well plate with a diameter of 11 mm.

2.2. Surface characterization

Atomic force microscopy (AFM) was performed on the ZnO-coated Si substrates and the smooth Si substrates (control) using a Digital Instruments (now Bruker AXS) Dimension 3000 AFM and an Olympus AC160TS tip (resonant frequency of 330 kHz, force constant of 40 N/m). Topographical analysis was done using a scan size of 5 μm and 1 μm with a scan rate of 1 Hz. A roughness analysis was also conducted using a scan size of 5 μm for mean roughness (Ra) and 1 μm for root mean square (RMS) roughness and maximum height (Rmax) to exclude outliers due to dust particles on the surface. 3D reconstruction was performed using the SurfaceJ plugin for ImageJ software (NIH, USA).

Scanning electron microscopy (SEM) was performed using a JEOL 6400 Cold Field Emission Scanning Electron microscope (JEOL, Tokyo, Japan). Energy Dispersive X-ray Spectroscopy (EDX) was performed in order to confirm the presence of Zn on the surface. The SEM was equipped with an energy dispersive X-ray spectrometer attachment with a Link Pentafet detector (Link Analytical, Redwood City, CA) and a 4Pi Universal Spectral Engine pulse processor (4Pi Analytical, Hillsborough, NC). An accelerated voltage of 20 keV and 5 keV was used.

2.3. Cell culture

RAW 264.7 macrophage cells (TIB-71, ATCC, Manassas, VA) were cultured until confluent and plated directly onto 48-well plates containing uncoated, smooth Si wafers or the ZnO-coated Si wafers. Cells were plated at a density of 2×10^5 cells/mL and maintained in DMEM with L-glutamine (GIBCO® DMEM, Invitrogen, Carlsbad, CA), 10% Fetal Bovine Serum (HyClone FBS, Thermo Scientific, Rockford, IL) and 1% Penicillin–Streptomycin (P/S) solution (Sigma, St. Louis, MO). Coated and uncoated wafers were transferred to new wells prior to performing assays to ensure only cells growing on the wafers were evaluated (see individual assay description for details).

2.4. Cell imaging

To investigate and confirm cell attachment with the test surfaces, macrophages were plated at a density of 2×10^5 cells/mL directly on the ZnO-coated and smooth wafers and incubated for 24 h. Media was removed, cells were washed with warm PBS, fixed with 3.7% formaldehyde, washed with PBS again, and permeabilized using 0.2% Triton X-100. Cells were then washed again and incubated with Molecular Probes® DAPI and Alexa Fluor® 488 (Life Technologies, Grand Island, NY) for 1 h, then washed with PBS 3 times and allowed to dry in the dark. Samples were sealed with ProLong® mounting medium (Life Technologies, Grand Island, NY) and a cover slip. Images were acquired using a confocal microscope (Leica, Buffalo Grove, IL).