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Significant Enhancement of Antimicrobial Activity in Oxygen-Deficient Zinc Oxide Nanowires

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ABSTRACT

The fabrication of antimicrobial surfaces that exhibit enhanced activity towards a large variety of microbial species is one of the major challenges of our time. In fact, the negative effects associated with both bacterial and fungal infections are enormous, especially considering that many microbial species are developing resistance to known antibiotics. In this work, we show how a combination of specific surface morphology and surface chemistry can create a surface that exhibits nearly 100% antimicrobial activity towards both Gram-negative and Gram-positive bacteria, and fungal cells. Arrays of vertically aligned, oxygen deficient zinc oxide (ZnO) nanowires grown on a substrate exhibit enhanced antimicrobial activity compared to surfaces containing either less defective nanowires, or highly oxygen-deficient flat films. This synergistic effect between physical activity (morphology) and chemical activity (surface composition) has been shown to be responsible for the outstanding antimicrobial activity of our surfaces, especially towards notoriously resilient bacterial or fungal species. These findings provide a series of design rules for tuning the activities of antibacterial and antifungal nanomaterials. These rules constitute an excellent platform for the development of next-generation antimicrobial surfaces.

INTRODUCTION

Pathogenic microbial surface colonization, commonly referred to as biofilm formation, represents a significant healthcare and industrial concern. This is particularly true for surfaces where sterilization is required, such as those present on medical implants, hospital surfaces, and other medical devices. This issue is further impacted by the current rise in antibiotic resistance amongst microbial species, which has caused a significant increase in post-operative infections and related deaths in recent years.^{1,2,3,4} Economic projections have estimated that bacterial infections could be responsible for upwards of 10 million deaths per annum by 2050 if new antibacterial therapies are not developed.⁵ In addition to bacteria, fungal cells are also estimated to infect 1.7 billion people annually, resulting in ~1.5 million deaths per annum, with these figures also increasing in recent years.^{6,7} Despite these staggering figures, the contribution of fungal infections to the global burden of disease remains poorly recognized.

In response to these issues, considerable research has focused on the development of surfaces that are capable of mitigating both bacterial and fungal biofilm formation. The two main mitigation strategies rely on either the addition of antimicrobial or inhibitory agents – including nanoparticles,⁸ antibiotics,⁹ and polymers^{10,11} – to the outer surface of biocompatible materials (chemical activity), or to the micro/nanostructuring of surfaces in order to achieve mechanical rupturing of the cell membranes when coming into contact with the nanofeatures, implying a physical kill-activity.^{12,13} Pure chemical approaches have become less viable as a long-term, antibacterial solution due to instances of increasing antibiotic resistance,¹⁴ toxicity concerns about nanomaterials,¹⁵ and dosage complications.¹⁶ Antibacterial nanostructures that are effective as a result of the physical effects of the surface are widely found in nature, such as the nanopillar arrays

native to cicada wings, and the hierarchically structured, self-cleaning lotus leaves. These natural surfaces have led to the development of numerous biomimetic, synthetic counterparts.^{13,17,18} However, they are intrinsically non-selective and their activity is heavily dependent on both the type of microorganism and the specific surface geometry present on the substrate surface.^{12,17} Combining both physical and chemical activity is therefore key to producing highly efficient antimicrobial surfaces, given the interplay of many factors, including bacterial cell size and physiology, the underlying topographical dimensions, specific bacterial adsorption processes, surface chemistry and the hydrophobicity/hydrophilicity of the surface.¹⁸

In this regard, chemical synthesis of (nano)materials is a versatile and powerful means for the development of materials and surfaces with the desired structure, morphology and chemical composition.^{19,20,21} As such, chemical methods have been extensively used to fabricate materials for applications such as optics, electronics, biology, catalysis, sensing, energy generation and storage.^{22,23,24,25} One of the main advantages of chemical methods for the synthesis of nanomaterials is the ability to precisely tune the composition of the target material. In fact, both doping and off-stoichiometric compositions in inorganic nanomaterials have been widely adopted in order to tune their properties and improve device performances.^{26,27,28,29} In particular, oxygen vacancies in metal oxides have been extensively studied and recently exploited to harness enhanced optical, electrical, electrochemical and catalytic properties in the oxygen-deficient materials compared to the stoichiometric oxide counterparts.^{21,30,31,32,33}

In this work, we show the combined effect of surface morphology and oxygen deficiency in the antimicrobial activity of zinc oxide (ZnO) nanostructures against both bacterial and fungal species. Highly textured ZnO nanowire “forests” provide an ideal surface morphology that physically limits the proliferation of microorganisms (physical activity). Their performance is greatly

enhanced, however, when these oxide surfaces are made highly oxygen deficient (chemical activity). Importantly, highly oxygen deficient flat ZnO surfaces show reduced activity, confirming the synergistic effect between chemical and physical microbiocidal activity. The results reported herein afford further insights into the role of both chemical and physical contributions in nanostructured surfaces for inducing microbial cell death. The findings of this research highlight the importance of non-stoichiometry in the antimicrobial activity of metal oxide nanostructures and could potentially be extended to other oxide materials of interest. All this combined will aid in the development of next-generation surfaces that can prevent surface microbial colonization.

RESULTS AND DISCUSSION

ZnO nanowires (NWs) supported on glass and silicon substrates were grown using a modified chemical bath deposition (CBD) approach as described previously.³⁰ Full experimental details are reported in the Methods section. Briefly, after depositing a ZnO seed layer using conventional sol-gel chemistry, the seeded substrates were dipped in a solution containing zinc nitrate and ammonia and heated to 80 °C to trigger ZnO growth. This method enables the growth of uniform arrays of ZnO NWs, highly oriented along the *c*-axis of the hexagonal wurtzite cell. To obtain flat ZnO surfaces, ammonium citrate was added. Citrate anions are known to bind preferentially to polar surfaces of ZnO crystals, either Zn-terminated (0001) or O-terminated (000-1) surface, therefore limiting the growth along the <001> crystallographic orientation. This promotes “lateral” over “longitudinal” growth of the nanowires, resulting in dense films composed of columnar grains oriented along the *c*-axis.³⁴ These dense ZnO films serve as a control surface for microbial testing, providing a surface with identical chemical composition, but with a rather different morphology.

Figure 1. Morphological and structural characterization of the ZnO surfaces. SEM images in top view (45 degree tilt) and in cross section of ZnO NW arrays (A,B) and dense films (D,E). Scale bars are 500 nm. The respective XRD patterns and the comparison with the predicted peak positions for bulk ZnO (ICDD No. 36-1451) are shown in panels (C) and (F). AFM profiles of ZnO NW arrays (G) and dense films (H). Scale bars are 1 μm . I) Schematic representation of the ZnO wurtzite crystalline cell.

Scanning electron microscopy (SEM) images of the resulting surfaces are shown in Figure 1 and Figure S1. Highly oriented ZnO wires can be clearly seen both in the top view and cross-sectional images. Notably, the simple addition of citrate causes a dramatic change in morphology, with the production of much larger wires that eventually merge together, resulting in a dense film.

The crystallographic orientation of these films has been demonstrated through X-ray diffraction (XRD), as shown in Figure 1C and 1F. All samples show prominent (002) diffraction peaks (at $2\theta = 34.5^\circ$), and almost complete absence of any other peaks, with the exception of the higher order diffraction from the same family of lattice planes (*i.e.* (004) at $2\theta = 72.6^\circ$). This evidence confirms the formation of highly textured ZnO crystals along the *c*-axis of the wurtzite cell. Atomic force

microscopy (AFM) measurements were also employed to better determine the nano-architectures present on the substrate surfaces (see Figure 1G, and 1H). The topographical parameters of fabricated nanowire arrays and the flat ZnO films evaluated from SEM and AFM images are given in Table S1.

Importantly, this modified CBD enables the fabrication of non-stoichiometric ZnO, and specifically highly oxygen deficient at the surface. This is verified with XPS through a detailed analysis of the O 1s core-level peak. It is well established that the O 1s peak can be fitted with three main components, corresponding to (from lower to higher binding energies) O^{2-} ions within the ZnO lattice (O_I component), oxygen atoms residing in an oxygen-deficient environment or surface hydroxides (O_{II} component) and to adsorbed oxygen-containing organic species (O_{III} component).³⁵ The ZnO nanowires synthesized in this study show an intense O_{II} component, with an integrated O_I/O_{II} signal of ~ 1.2 , suggesting highly oxygen deficient nanowires (Figure 2A). Similarly, the dense films are also highly oxygen deficient (Figure S2). This enables the comparison of morphological effects (wires vs. dense films) whilst keeping the chemical activity similar. However, if the nanowires are annealed in air, the oxygen vacancies are progressively filled, resulting in more stoichiometric ZnO (Figure 2B). Notably, the morphology of the annealed nanowires is identical to that of the as-deposited materials (see Figure S3). This enables a direct comparison of the chemical effect (oxygen vacancies) in coatings possessing the same morphology. It is important to highlight that the Zn 2p signals of the samples are identical, confirming that the only change in the chemical nature of the ZnO surface is the presence of oxygen vacancies (see Figure S4).

Figure 2. Optical and electronic characterization of ZnO NWs. X-ray photoelectron spectra of the O1s region for (A) as-deposited and (B) annealed ZnO NWs showing the fitting with the three components. C) Transmittance spectra of as-deposited and annealed ZnO NWs. The inset shows the respective Tauc plot and band gap estimation. D) Valence band XPS for as-deposited and annealed ZnO NWs. The inset shows simplified band structures highlighting the downward shift in Fermi energy following annealing

The oxygen deficient nature of these ZnO was further proved through optical and electrical properties. The samples show a distinctive UV absorption onset at around 3.3 eV typical of ZnO. Close inspection of this region shows a small red shift of the band gap of 50-100 meV following annealing (Figure 2C). Given that the ZnO crystals are far beyond the quantum confinement regime, this shift could be ascribed to the Burstein-Moss effect. In fact, as-deposited ZnO is naturally oxygen deficient, and therefore has excess free electrons, similar to what is observed in aliovalently doped ZnO.^{36,37} After annealing, the oxygen vacancies are at least partially filled,

causing a reduction of the charge carrier concentration, and a reversal of the Burstein-Moss effect. Valence band (VB) XPS is used to evaluate the position of the Fermi energy (E_F) with respect to the VB. Compared to as-deposited samples, the Fermi energy in annealed sample is located further (~ 0.3 eV) from the conduction band edge (Figure 2D). This is an indication of the reduced *n*-type character of annealed samples that can again be ascribed to a filling of the oxygen vacancies. This is further verified by electrical measurements on the dense films, which provide a sheet resistance of ~ 20 k $\Omega/\square$ for unannealed ZnO, and ~ 700 k $\Omega/\square$ after annealing. The increase in resistance by over one order of magnitude confirms the reduction in charge carrier concentration subsequent to the filling of oxygen vacancies.

Having analyzed the morphology, composition and electronic structure of the ZnO coatings, we can now assess their antimicrobial performance. *Escherichia coli* (*E. coli*), methicillin-resistant *Staphylococcus aureus* (MRSA), and fluconazole-resistant *Cryptococcus neoformans* (*fluc*^R *C. neoformans*) cells were chosen as representative Gram-negative bacterium, Gram-positive bacterium, and yeast-like fungus, respectively. In this way different classes of single-celled pathogens could be tested, two of which (MRSA and *C. neoformans*) show resistance to conventional antibiotics. Representative confocal laser scanning microscopy (CLSM) images of the cells adsorbed on various ZnO surfaces, as well as on glass controls are shown in Figure 3a (see Materials and Methods section for details on the samples preparation). The number of live/dead cells were counted, evaluated as a percentage, and displayed as a bar chart. The corresponding values and their associated errors are also reported in the SI as Table S2 for easy comparison.

As-deposited ZnO NWs result in the highest amount of non-viable (dead) cells, specifically $95.3\% \pm 4.3\%$, $68.8\% \pm 10.5\%$ and $94.8\% \pm 3.5\%$ for Gram-negative *E. coli*, Gram-positive

MRSA, and *C. neoformans*, respectively. Physically identical surfaces, but with reduced oxygen vacancy concentration, are obtained after annealing the ZnO NWs in air as discussed earlier. These annealed surfaces show a largely reduced antimicrobial activity, inactivating only $32.0\% \pm 8.5\%$, $19.5\% \pm 1.9\%$ and $16.7\% \pm 4.5\%$ of the *E. coli*, MRSA, and *C. neoformans* cells, respectively. Similarly, highly oxygen deficient flat surfaces (obtained introducing citrate as ligand during growth of ZnO) also show a strongly reduced activity compared to the textured NW counterparts. In details, the inactivated cells on oxygen-deficient, flat ZnO for *E. coli*, MRSA, and *C. neoformans* are $22.3\% \pm 7.6\%$, $27.9\% \pm 10.8\%$, and $3.3\% \pm 1.2\%$, respectively. These numbers are slightly higher than the values obtained for our control surfaces (plain glass slides), which show $<10\%$ dead cells for all three species investigated.

Figure 3. Cell viability studies. A) CLSM images of control (glass slides), ZnO NWs, ZnO dense film and annealed ZnO NWs following immersion in *E. coli*, MRSA and *C. neoformans* for 24

hours. After staining, the cells appear green (live) or red (dead) in the CLSM images. B) Quantification of cell viability obtained from the CLSM images. The statistical significance of the analysis as calculated by t-tests against the data for the glass controls is reported as * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$).

These results highlight that the oxygen-deficient ZnO NWs exhibited a greater degree of lethality to all microbial species investigated, when compared to either the oxygen-deficient flat ZnO, or the more stoichiometric NWs. This indicates that ZnO-based materials have inherent antibacterial properties, but that this activity is greatly enhanced by the presence of high-aspect-ratio nanostructures. Similarly, the mechanical (physical) action of the ZnO NWs is less effective in isolation, than when combined with a chemically active surface. This is noteworthy for two reasons. First, it highlights how chemical (oxygen vacancies) and physical (nanostructuring) features have a synergistic effect when combined, and greatly enhance the antimicrobial properties of ZnO. Second, it demonstrates that ZnO NWs exhibit high activity for both bacterial and fungal cells. Since these cells have vastly different physiologies, this often precludes equivalent antimicrobial treatment. Indeed, fungal cells *C. neoformans* are typically more difficult to kill than *E. coli*, especially since this fungal species is resistant to the antifungal agent fluconazole.¹² In light of this, the oxygen-deficient ZnO NWs show great promise as antimicrobial surfaces for both bacteria and fungi.

Interestingly, the antibacterial action of the NW surfaces is slightly less effective towards Gram-positive MRSA cells. This effect has been shown previously and it has been ascribed to the different size and membrane rigidity of Gram-positive and Gram-negative bacteria.^{38,39} Specifically, MRSA cells are spherical, and on average smaller than *E. coli*, and therefore they

interact with fewer NWs. Moreover, their cell membrane is more rigid compared to that of *E. coli*, which makes them more resilient to membrane stretching and tearing upon adsorption on textured surfaces. Compared to bacterial cells, fungal cells are often reported to possess thicker, more rigid cell walls, resulting in an overall stiffer membrane.¹² However, due to their much larger size, they interact with a larger number of NWs, which explains the high activity of ZnO towards *C. neoformans* cells despite their resilient membrane and cell wall.

Figure 4. SEM characterization of the interaction between cells and ZnO nanowires. SEM images in top view (A-L) and cross section after FIB cutting (M-O) of cell interaction with control substrates (A,E,I) and ZnO NWs (all others). Scale bars for control samples are 2 μm (A, I) and 1 μm (E). Scale bars for top views of ZnO NWs are 500 nm for (B-D and F-H) and 2 μm for (J-L). Scale bars for cross sections are 200 nm. As a point of interest, the nucleal cavity of the fungal cell can be clearly observed.

Physical features (membrane rigidity, size) alone cannot explain the antimicrobial activity of the oxygen-deficient ZnO NWs, because annealed NWs show a drastically reduced activity, although still well above control levels. This is where the interplay between chemical and physical activity is vital. The chemical action of the ZnO surfaces is well-known, as ZnO-based nanomaterials have been previously investigated as microbial inhibitory agents against both bacterial⁴⁰ and fungal cells.⁴¹ Importantly, it has also been demonstrated that ZnO-based nanomaterials are capable of producing reactive oxygen species (ROS such as $\bullet\text{OH}$, $\bullet\text{O}_2^-$, and H_2O_2) as a consequence of surface defects.^{41,42,43} These ROS are capable of inducing cell lysis, via membrane and intercellular damage through both cell-surface interaction and general cell oxidation.^{44,45} Considering the highly oxygen deficient (defective) nature of the as-deposited ZnO NWs, it is therefore reasonable to assume that they can generate ROS more effectively, and consequently it is not surprising that their ability to cause cell lysis is greater compared to less defective, although morphologically identical surfaces. We also note that a change in stoichiometry might cause a change in surface charge between different surfaces, and this might affect cell adhesion and interaction with the membrane. Importantly, leaching of zinc cations following dissolution of ZnO in the nutrient broth could potentially explain the antimicrobial activity of our ZnO. To test this hypothesis, we measured the amount of zinc ions released from the different ZnO surfaces following 24 hours of immersion in the nutrient broth (Table S3). Zinc ions are detected in the broth for all three surfaces, in slightly larger amount for the NWs samples, consistent with their higher surface area with respect to flat ZnO. However, no difference in leaching between pristine and annealed NWs could be observed. Therefore, considering the dramatic reduction in antimicrobial activity of the annealed NWs, we can confidently exclude a major role of zinc cations in the antimicrobial activity of our surfaces. SEM was used to analyze the cell/surface interaction: from the top-down images

on control glass surfaces (Figure 4) we can clearly observe the three cell types investigated, each with their distinctive morphologies: elongated *E. coli*, spherical MRSA and larger spherical *C. neoformans*. The remaining top view SEM images presented in Figure 4 show cell rupture and eventually cell collapse (moving left to right in the images) for all microbial species interacting with oxygen deficient ZnO NWs. Compared to healthy control cells, the cells attached to the ZnO NWs show a stretched and deflated (flattened) membrane, a clear indication of damage that led (or will lead) to cell death (see also Figure S5). Importantly, in the cross-sectional images (Figure 4, right panels) the ZnO NWs can be observed to have entered the intracellular space of all microbial species. This indicates that during the cellular adsorption, the nanowires are capable of “puncturing” the pathogenic cells, via a combined physical/chemical mechanism as described earlier.

Figure 5. ZnO antibacterial properties under UV illumination. A) CLSM images of control (glass slides) and ZnO NWs following immersion in MRSA for 24 hours and exposed to UVA light for an increasing amount of time. B) Quantification of MRSA viability obtained from the CLSM images. The statistical significance of the analysis as calculated by t-tests against the samples without UV exposure (0 min) is reported as * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$). C-

E) SEM images of MRSA cells on ZnO NWs (C,D) and on glass (E) following exposure to UV light (30 min). Scale bars are 2 μm in (C) and 1 μm in (D, E).

In order to improve the activity of our ZnO NWs against the most resilient of the cell types tested (MRSA), we assessed their performance under UV irradiation. It is known that ZnO can be photodoped using ultraviolet light, which causes the photo-oxidation of adsorbates (including oxygen, water, carbon dioxide and organic contaminants) on the surface of ZnO.⁴⁶ We postulate that the activation of the surface of our ZnO NWs could enhance their antimicrobial properties through the photo-induced oxidation of the adsorbed bacteria. To verify this, ZnO surfaces were exposed to low-energy UVA light using a commercial LED (365 nm, 3 mW/cm²) after 24 hours of MRSA incubation and compared the results with glass controls which have been subjected to the same UV treatment. Illumination was done from the bottom of a Petri dish, therefore through the back of the substrate (glass). The power density of the UV light was measured on top of the glass slides through the Petri dish using a calibrated photodetector, and therefore is representative of the amount of UV light reaching the active surface. A progressive increase in activity for ZnO NWs with increasing UV exposure time is observed, which culminates with a striking 98% MRSA cell death after 30 min of UV exposure (Figure 5). The values and associated errors are reported in the SI as Table S3. This data shows that the as-deposited ZnO NWs are highly antimicrobial even for MRSA, a notoriously resilient species, and these results provide a design rule for further improvements in efficacy for other antimicrobial surfaces and materials. We note that UV exposure slightly increases the antibacterial efficacy of other ZnO morphologies (flat films, Figure S6), however the results achieved by the ZnO NWs are still unparalleled.

Importantly, only a small increase in the non-viable cell count is observed for the control glass slides after UV illumination. This increase might be due to a direct effect of UV light in damaging the MRSA cells. However, in the case of the ZnO NWs (as the samples are illuminated from the back side), over 90% of UV radiation is absorbed by ZnO before reaching the top surface and therefore the MRSA cells (see Figure S7). As a consequence, only less than 10% ($\sim 0.3 \text{ mW/cm}^2$, as measured using the photodetector) reaches the MRSA cells adsorbed on the ZnO surfaces, while the full UV power (3 mW/cm^2) is incident upon the cells in the glass control samples. Considering this, we can confidently conclude that it is the photodoping of ZnO, and not the direct UV exposure, that causes the enhanced activity and close to 100% cell death in ZnO NWs samples. These results are extremely impressive as MRSA is notoriously very difficult to eradicate with high kill percentages.

CONCLUSION

In conclusion, we have shown the outstanding antimicrobial properties of oxygen-deficient ZnO nanowires towards bacteria (both Gram-positive and Gram-negative) and fungal cells. The work demonstrated that a combination of surface texturing and surface chemical environment is necessary to achieve the highest activity. Specifically, the antimicrobial activity of our optimized ZnO NW surfaces is reduced when either the texturing is lost, or the NWs are made less oxygen deficient. With our ZnO surfaces we achieved $\sim 95\%$ antimicrobial activity for both *E. coli* (Gram-negative bacterium) and *C. neoformans* (fluconazole-resistant fungus). The activity was slightly lower (60-70%) towards MRSA (Gram-positive bacterium), however it could be greatly improved to over 95% through a low intensity UVA exposure. These results highlight the synergistic effect between surface morphology and surface chemistry in order to achieve effective antimicrobial

activity. Therefore, this study provides the foundation for further investigations into the combined physical and chemical activity of various (nano)materials and surfaces.

METHODS

Materials

Zinc acetate dihydrate (99%), ethanolamine (99%), 2-methoxyethanol (99%), zinc nitrate hexahydrate (98%), ammonium citrate dibasic (98%) were purchased from Sigma Aldrich. Ammonium hydroxide solution (25% in water) was purchased from Chem Supply. Acetone (99.5%) and isopropanol (99.5%) were supplied by Univar. Milli-Q water was used for all syntheses, and deionized water was used for all rinsing and washing steps. All chemicals were used without further purification. Standard microscope glass slides (soda-lime glass) were purchased from Livingstone. Silicon wafers (Boron doped, p-type, <100> oriented, 10-20 Ωcm) were supplied by Silicon Materials.

ZnO synthesis

ZnO nanowires and films were synthesized using a modified chemical bath deposition (CBD) similar to previous reports.³⁰ Glass and silicon substrates were cleaned via ultrasonication in acetone and isopropanol (IPA) for 10 minutes each, thoroughly rinsed with IPA and stored under IPA until needed. First, a ZnO sol-gel seed layer was deposited on glass and silicon substrates using a 0.4 M solution of zinc acetate in 2-methoxyethanol, with a 1:1 molar ratio between zinc and ethanolamine. Films were deposited by spin coating at 2500 rpm and annealed at 200 °C for 5 minutes. This procedure was repeated once more, with a final annealing at 200 °C for 30 minutes. Seeded substrates were then dipped in a 20 mM zinc nitrate solution prepared in a ~1% ammonium

hydroxide solution and heated to 80 °C for 1 hour. Samples were positioned vertically, with the seeded surface facing slightly downwards. To obtain dense films, ammonium citrate was added to the growth solution with an overall concentration of 2 mM right before dipping the seeded substrates. After 1 hour at 80 °C, samples were removed from the bath, rinsed thoroughly with water and dried with a nitrogen stream. The backside of the samples was wiped cleaned first with water, and then gently with a cotton bud dipped into a ~0.1 M HCl solution to remove traces of precipitates that might have settled during deposition. Eventually samples were rinsed with water multiple times, dried thoroughly and stored under inert atmosphere until needed.

Bacterial and fungal strains, growth conditions, and sample preparation

Escherichia coli DH52 and methicillin-resistant *Staphylococcus aureus* ATCC1698 were obtained from the American Type Culture Collection (ATCC). Fluconazole-resistant *Cryptococcus neoformans* strains were obtained from a fluconazole resistant isolate derived from strain KN99 α which originally produced by Nielsen *et al.*⁴⁷ For the bacterial experiments, bacteria cultures were grown on Luria-Bertani (LB) agar overnight at 37 °C. For fungal experiments, fungal culture was grown on yeast peptone dextrose (YPD) agar supplemented with 10% of glucose at 25 °C. Bacterial and fungal cells were collected from the culture via an inoculation loop and suspended in LB and YPD (supplemented with 10% glucose) broths. The density of the bacterial and fungal suspensions was then adjusted to OD_{600nm} = 0.1.

Characterizations

X-ray diffraction (XRD) patterns of ZnO nanowires and films deposited on glass were collected using a Bruker D4 Endeavor diffractometer equipped with a Cu-K α radiation source and operated at 40 kV and 35 mA. X-ray photoelectron spectroscopy (XPS) analyses were conducted on a

Thermo Scientific K-Alpha XPS equipped with a Monochromated Al K α X-ray source (1486.7 eV) and a concentric hemispherical analyzer operated with a pass energy of 50 eV. Optical transmission spectra of ZnO films and nanowires deposited on glass substrates were obtained on an Agilent Cary 7000 UV-Vis-NIR spectrophotometer. Scanning electron microscopy (SEM) images of the ZnO prior to antimicrobial studies were acquired on a FEI Verios 460L SEM operated at 3-5 kV and 25 pA. For top-view cellular imaging, all samples were affixed using formaldehyde/glutaraldehyde 3% solution in 0.1 M sodium cacodylate buffer (pH = 7.4), rinsed with PBS (10 mM, pH = 7.4), dehydrated using a series of 30%, 50%, 75%, 90% and 100% ethanol solutions, then air dried prior to coating with a thin film of gold and imaged using the same parameters described above. Cross sectional SEM of the ZnO-cell interaction was performed using FEI Scios Dualbeam FIBSEM. The cells were first coated with platinum (Pt) using an ion gun at 16 kV/0.15 nA to prevent further damage followed by sequential ion slicing at 16 kV/0.15 nA. The imaging was then carried out at 2 kV and 0.1 nA. Atomic force microscopy images were obtained using an Oxford Instrument Cypher ES AFM under ambient conditions. Olympus Micro Cantilevers (OMCL-AC240TS) with nominal spring constant $k_c = 2.0$ N/m) were used for all measurements, and imaging forces were minimized via a setpoint ratio (Imaging Amplitude (A)/free amplitude (A₀)) of >0.7. Zinc leaching experiment was conducted by immersing ZnO samples in the nutrient broth for 24 hours and the broth was then analyzed with microwave plasma atomic emission spectroscopy (MP-AES) using an Agilent 4200 MP-AES instrument. Confocal scanning laser microscopy (CSLM) was performed on a Olympus Fluoview FV1200 inverted microscope and a ZEISS LSM 880 Airyscan upright microscope. Bacterial and fungal cells were dyed using a LIVE/DEAD BacLight Bacterial Viability Kit (including SYTO 9 and propidium iodide) and a LIVE/DEAD FungaLight Yeast Viability Kit, respectively (purchased from

Invitrogen Molecular Probes). Cell dying was performed following an incubation period of 24 hours. The amount of live and dead cells was quantified using Cell-C software (<https://sites.google.com/site/cellcsoftware/>). The statistical significance of the analyses was calculated by t-tests against the control data and reported as * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$).

ASSOCIATED CONTENT

Supporting Information. Additional characterizations and data. This material is available free of charge via the Internet at <http://pubs.acs.org>

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Author Contributions

AE and EDG conceived the idea, directed the work and analyzed most of the data. PW and EDG synthesized the ZnO and conducted SEM and optical characterization. AE, SC and VKT performed the antimicrobial studies, including SEM of the cell/surface interaction, with help from AEM and KJB. JK performed the XPS characterization. RJC, JC supervised the antimicrobial work. CFMcC and JvE helped with materials characterization and analysis. EDG and AE wrote the manuscript with inputs from all authors.

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REFERENCES

1. Boucher, H. W.; Talbot, G. H.; Bradley, J. S.; Edwards, J. E.; Gilbert, D.; Rice, L. B.; Scheld, M.; Spellberg, B.; Bartlett, J., Bad Bugs, No Drugs: No ESKAPE! An Update from the Infectious Diseases Society of America. *Clinical Infectious Diseases* **2009**, *48* (1), 1-12.
2. Levy, S. B.; Marshall, B., Antibacterial resistance worldwide: causes, challenges and responses. *Nature Medicine* **2004**, *10*, S122-S129.
3. Spellberg, B.; Guidos, R.; Gilbert, D.; Bradley, J.; Boucher, H. W.; Scheld, W. M.; Bartlett, J. G.; Edwards, J. J., The Epidemic of Antibiotic-Resistant Infections: A Call to Action for the Medical Community from the Infectious Diseases Society of America. *Clinical Infectious Diseases* **2008**, *46* (2), 155-164.
4. Rodvold, K. A.; McConeghy, K. W., Methicillin-Resistant *Staphylococcus aureus* Therapy: Past, Present, and Future. *Clinical Infectious Diseases* **2014**, *58* (suppl_1), S20-S27.
5. O'Neill, J., Tackling drug-resistant infections globally: Final report and recommendations. 2016. <https://amr-review.org/> **2018**.
6. Boyce, K.; Morrissey, O.; Idnurm, A.; Macreadie, I., Insights into the global emergence of antifungal drug resistance. *Microbiology Australia* **2019**, 87-91.
7. Brown, G. D.; Denning, D. W.; Gow, N. A.; Levitz, S. M.; Netea, M. G.; White, T. C., Hidden killers: human fungal infections. *Science translational medicine* **2012**, *4* (165), 165rv13.
8. Knetsch, M. L. W.; Koole, L. H., New Strategies in the Development of Antimicrobial Coatings: The Example of Increasing Usage of Silver and Silver Nanoparticles. *Polymers* **2011**, *3* (1), 340.
9. Källicke, T.; Schierholz, J.; Schlegel, U.; Frangen, T. M.; Köller, M.; Printzen, G.; Seybold, D.; Klöckner, S.; Muhr, G.; Arens, S., Effect on infection resistance of a local antiseptic and antibiotic coating on osteosynthesis implants: An in vitro and in vivo study. *Journal of Orthopaedic Research* **2006**, *24* (8), 1622-1640.
10. Nederberg, F.; Zhang, Y.; Tan, J. P. K.; Xu, K.; Wang, H.; Yang, C.; Gao, S.; Guo, X. D.; Fukushima, K.; Li, L.; Hedrick, J. L.; Yang, Y.-Y., Biodegradable nanostructures with selective lysis of microbial membranes. *Nature Chemistry* **2011**, *3* (5), 409-414.
11. Banerjee, I.; Pangule, R. C.; Kane, R. S., Antifouling Coatings: Recent Developments in the Design of Surfaces That Prevent Fouling by Proteins, Bacteria, and Marine Organisms. *Advanced Materials* **2011**, *23* (6), 690-718.
12. Elbourne, A.; Chapman, J.; Gelmi, A.; Cozzolino, D.; Crawford, R. J.; Khanh Truong, V., Bacterial-nanostructure interactions: the role of cell elasticity and adhesion forces. *Journal of Colloid and Interface Science* **2019**, *546*, 192-210.
13. Elbourne, A.; Truong, V. K.; Cheeseman, S.; Rajapaksha, P.; Gangadoo, S.; Chapman, J.; Crawford, R. J., The use of nanomaterials for the mitigation of pathogenic biofilm formation. In *Methods in Microbiology*, Gurtler, V.; Ball, A. S.; Soni, S., Eds. Academic Press: 2019; Vol. 46, pp 61-92.

14. Bridier, A.; Briandet, R.; Thomas, V.; Dubois-Brissonnet, F., Resistance of bacterial biofilms to disinfectants: a review. *Biofouling* **2011**, *27* (9), 1017-1032.
15. Johnston, H. J.; Hutchison, G.; Christensen, F. M.; Peters, S.; Hankin, S.; Stone, V., A review of the in vivo and in vitro toxicity of silver and gold particulates: particle attributes and biological mechanisms responsible for the observed toxicity. *Critical reviews in toxicology* **2010**, *40* (4), 328-346.
16. Stigter, M.; Bezemer, J.; De Groot, K.; Layrolle, P., Incorporation of different antibiotics into carbonated hydroxyapatite coatings on titanium implants, release and antibiotic efficacy. *Journal of controlled release* **2004**, *99* (1), 127-137.
17. Elbourne, A.; Crawford, R. J.; Ivanova, E. P., Nano-structured antimicrobial surfaces: From nature to synthetic analogues. *Journal of Colloid and Interface Science* **2017**, *508* (Supplement C), 603-616.
18. Luong-Van, E.; Rodriguez, I.; Low, H. Y.; Elmouelhi, N.; Lowenhaupt, B.; Natarajan, S.; Lim, C. T.; Prajapati, R.; Vyakarnam, M.; Cooper, K., Review: Micro- and nanostructured surface engineering for biomedical applications. *Journal of Materials Research* **2012**, *28* (2), 165-174.
19. Arias, A. C.; MacKenzie, J. D.; McCulloch, I.; Rivnay, J.; Salleo, A., Materials and Applications for Large Area Electronics: Solution-Based Approaches. *Chemical Reviews* **2010**, *110* (1), 3-24.
20. Diodati, S.; Dolcet, P.; Casarin, M.; Gross, S., Pursuing the Crystallization of Mono- and Polymetallic Nanosized Crystalline Inorganic Compounds by Low-Temperature Wet-Chemistry and Colloidal Routes. *Chemical Reviews* **2015**, *115* (20), 11449-11502.
21. Kim, H.-S.; Cook, J. B.; Lin, H.; Ko, Jesse S.; Tolbert, Sarah H.; Ozolins, V.; Dunn, B., Oxygen vacancies enhance pseudocapacitive charge storage properties of MoO₃-x. *Nature Materials* **2016**, *16*, 454.
22. Elbourne, A.; Coyle, V. E.; Truong, V. K.; Sabri, Ylias M.; Kandjani, A. E.; Bhargava, S. K.; Ivanova, E. P.; Crawford, R. J., Multi-directional electrodeposited gold nanospikes for antibacterial surface applications. *Nanoscale Advances* **2019**, *1*, 203-212.
23. Della Gaspera, E.; Guglielmi, M.; Agnoli, S.; Granozzi, G.; Post, M. L.; Bello, V.; Mattei, G.; Martucci, A., Au Nanoparticles in Nanocrystalline TiO₂-NiO Films for SPR-Based, Selective H₂S Gas Sensing. *Chemistry of Materials* **2010**, *22* (11), 3407-3417.
24. Paglia, F.; Vak, D.; van Embden, J.; Chesman, A. S. R.; Martucci, A.; Jasieniak, J. J.; Della Gaspera, E., Photonic Sintering of Copper through the Controlled Reduction of Printed CuO Nanocrystals. *ACS Applied Materials & Interfaces* **2015**, *7* (45), 25473-25478.
25. van Embden, J.; Della Gaspera, E., Ultrathin Solar Absorber Layers of Silver Bismuth Sulfide from Molecular Precursors. *ACS Applied Materials & Interfaces* **2019**, *11* (18), 16674-16682.
26. Luther, J. M.; Jain, P. K.; Ewers, T.; Alivisatos, A. P., Localized surface plasmon resonances arising from free carriers in doped quantum dots. *Nature Materials* **2011**, *10*, 361.
27. Della Gaspera, E.; Chesman, A. S. R.; van Embden, J.; Jasieniak, J. J., Non-injection Synthesis of Doped Zinc Oxide Plasmonic Nanocrystals. *ACS Nano* **2014**, *8* (9), 9154-9163.
28. Mitzi, D. B.; Yuan, M.; Liu, W.; Kellock, A. J.; Chey, S. J.; Deline, V.; Schrott, A. G., A High-Efficiency Solution-Deposited Thin-Film Photovoltaic Device. *Advanced Materials* **2008**, *20* (19), 3657-3662.

29. Della Gaspera, E.; Griggs, J.; Ahmed, T.; Walia, S.; Mayes, E. L. H.; Calzolari, A.; Catellani, A.; van Embden, J., Augmented band gap tunability in indium-doped zinc sulfide nanocrystals. *Nanoscale* **2019**, *11* (7), 3154-3163.
30. Della Gaspera, E.; Kennedy, D. F.; van Embden, J.; Chesman, A. S. R.; Gengenbach, T. R.; Weber, K.; Jasieniak, J. J., Flash-Assisted Processing of Highly Conductive Zinc Oxide Electrodes from Water. *Advanced Functional Materials* **2015**, *25* (47), 7263-7271.
31. Guo, H.-L.; Zhu, Q.; Wu, X.-L.; Jiang, Y.-F.; Xie, X.; Xu, A.-W., Oxygen deficient ZnO_{1-x} nanosheets with high visible light photocatalytic activity. *Nanoscale* **2015**, *7* (16), 7216-7223.
32. Geng, Z.; Kong, X.; Chen, W.; Su, H.; Liu, Y.; Cai, F.; Wang, G.; Zeng, J., Oxygen Vacancies in ZnO Nanosheets Enhance CO₂ Electrochemical Reduction to CO. *Angewandte Chemie International Edition* **2018**, *57* (21), 6054-6059.
33. Singh, M.; Jampaiah, D.; Kandjani, A. E.; Sabri, Y. M.; Della Gaspera, E.; Reineck, P.; Judd, M.; Langley, J.; Cox, N.; van Embden, J.; Mayes, E. L. H.; Gibson, B. C.; Bhargava, S. K.; Ramanathan, R.; Bansal, V., Oxygen-deficient photostable Cu₂O for enhanced visible light photocatalytic activity. *Nanoscale* **2018**, *10* (13), 6039-6050.
34. Tian, Z. R.; Voigt, J. A.; Liu, J.; McKenzie, B.; McDermott, M. J.; Rodriguez, M. A.; Konishi, H.; Xu, H., Complex and oriented ZnO nanostructures. *Nature Materials* **2003**, *2* (12), 821-826.
35. Marrani, A. G.; Caprioli, F.; Boccia, A.; Zanoni, R.; Decker, F., Electrochemically deposited ZnO films: an XPS study on the evolution of their surface hydroxide and defect composition upon thermal annealing. *Journal of Solid State Electrochemistry* **2014**, *18* (2), 505-513.
36. Della Gaspera, E.; Duffy, N. W.; van Embden, J.; Waddington, L.; Bourgeois, L.; Jasieniak, J. J.; Chesman, A. S. R., Plasmonic Ge-doped ZnO nanocrystals. *Chemical Communications* **2015**, *51* (62), 12369-12372.
37. Della Gaspera, E.; Bersani, M.; Cittadini, M.; Guglielmi, M.; Pagani, D.; Noriega, R.; Mehra, S.; Salleo, A.; Martucci, A., Low-Temperature Processed Ga-Doped ZnO Coatings from Colloidal Inks. *Journal of the American Chemical Society* **2013**, *135* (9), 3439-3448.
38. Xue, F.; Liu, J.; Guo, L.; Zhang, L.; Li, Q., Theoretical study on the bactericidal nature of nanopatterned surfaces. *Journal of Theoretical Biology* **2015**, *385*, 1-7.
39. Pogodin, S.; Hasan, J.; Baulin, Vladimir A.; Webb, Hayden K.; Truong, Vi K.; Phong Nguyen, The H.; Boshkovikj, V.; Fluke, Christopher J.; Watson, Gregory S.; Watson, Jolanta A.; Crawford, Russell J.; Ivanova, Elena P., Biophysical Model of Bacterial Cell Interactions with Nanopatterned Cicada Wing Surfaces. *Biophysical Journal* **2013**, *104* (4), 835-840.
40. Jin, T.; Sun, D.; Su, J.; Zhang, H.; Sue, H. J., Antimicrobial efficacy of zinc oxide quantum dots against *Listeria monocytogenes*, *Salmonella enteritidis*, and *Escherichia coli* O157:H7. *Journal of food science* **2009**, *74* (1), M46-M52.
41. Lipovsky, A.; Nitzan, Y.; Gedanken, A.; Lubart, R., Antifungal activity of ZnO nanoparticles—the role of ROS mediated cell injury. *Nanotechnology* **2011**, *22* (10), 105101.
42. Dwivedi, S.; Wahab, R.; Khan, F.; Mishra, Y. K.; Musarrat, J.; Al-Khedhairy, A. A., Reactive Oxygen Species Mediated Bacterial Biofilm Inhibition via Zinc Oxide Nanoparticles and Their Statistical Determination. *PLOS ONE* **2014**, *9* (11), e111289.

43. Lakshmi Prasanna, V.; Vijayaraghavan, R., Insight into the Mechanism of Antibacterial Activity of ZnO: Surface Defects Mediated Reactive Oxygen Species Even in the Dark. *Langmuir* **2015**, *31* (33), 9155-9162.
44. Sirelkhatim, A.; Mahmud, S.; Seenii, A.; Kaus, N. H. M.; Ann, L. C.; Bakhori, S. K. M.; Hasan, H.; Mohamad, D., Review on Zinc Oxide Nanoparticles: Antibacterial Activity and Toxicity Mechanism. *Nano-Micro Letters* **2015**, *7* (3), 219-242.
45. Ranjan, S.; Ramalingam, C., Titanium dioxide nanoparticles induce bacterial membrane rupture by reactive oxygen species generation. *Environmental Chemistry Letters* **2016**, *14* (4), 487-494.
46. Morfa, A. J.; MacDonald, B. I.; Subbiah, J.; Jasieniak, J. J., Understanding the chemical origin of improved thin-film device performance from photodoped ZnO nanoparticles. *Solar Energy Materials and Solar Cells* **2014**, *124*, 211-216.
47. Nielsen, K.; Cox, G. M.; Wang, P.; Toffaletti, D. L.; Perfect, J. R.; Heitman, J., Sexual cycle of *Cryptococcus neoformans* var. *grubii* and virulence of congenic α and α isolates. *Infection and immunity* **2003**, *71* (9), 4831-4841.