The fight against multidrug-resistant organisms: The role of ZnO crystalline defects

Eva de Lucas-Gil^{1,2,*}, Adolfo Del Campo¹, Laura Pascual³, Mercedes Monte², Javier Menéndez², José F. Fernández¹ and Fernando Rubio-Marcos¹

¹*Electroceramic Department, Instituto de Cerámica y Vidrio, CSIC, Kelsen 5, 28049 Madrid, Spain*

²*NanoBioMatters Bactiblock, NanoBioMatters Industries S.L., Louis Pasteur 11, 46980 Paterna, Valencia, Spain*

³*Instituto de Catálisis y Petroleoquímica, CSIC, Marie Curie 2, 28049 Madrid, Spain*

*Correspondence and requests for materials should be addressed to E. L-G. (Email: elucas@icv.csic.es)

ABSTRACT

This article reports the excellent antimicrobial response of nanoparticulate ZnO against multidrug-resistant organisms (MDROs). We demonstrate that the enhanced antimicrobial activity against MDROs depends on the crystalline defects of ZnO. Hence, this work provides insights on the ZnO-microorganism interactions, and we pose combined physico-chemical action mechanisms against resistant bacteria.

Highlights

- Synthesis of ZnO nanoparticles with antimicrobial response against multidrug-resistant organisms.
- A high crystal defect concentration leads to a high surface reactivity and they improve the antibacterial response
- A combined action of surface reactivity is mandatory to obtain this inorganic antimicrobial agent.

KEYWORDS: *nanoparticles, ZnO, antimicrobial properties, MDROs, action mechanisms*

Introduction

The number of multidrug-resistant organisms (MDROs) has increased considerably over the past few years, as a result of an inadequate use of antimicrobial agents. The main causes of antimicrobial resistance are overuse of antibiotics, inappropriate antibiotic use in selecting resistant organisms, and the ability of resistant organisms to spread in the hospital setting [1]. The excessive exposure of microorganisms to drugs increases their ability to develop survival mechanisms, causing an emerging threat and a health challenge [2]. These microorganisms have developed the capacity to neutralize the action of the effects of the drugs [3,4]. For example, it is estimated that in Europe 25,000 people die each year as a result of multidrug-resistant bacterial infections [5]. For these reasons, the search and development of new antimicrobial agents capable to fight the increasing number of MDROs are being promoted by the World Health Organization [6].

Nanomaterials are being incorporated in a wide range of applications because of functional and structural properties are different from the bulk [7]. Anticipating the evolution of resistant microorganisms and taking into account the advantages of nanoparticles, an incipient use of nanomaterials has begun to generate alternative antimicrobial agents [8,9]. Specifically, inorganic nanomaterials are being presented as an alternative to classic antimicrobial agents. There are several reasons to propose inorganic nanomaterials as substitute antimicrobial agent for MDROs, from their less widespread use to their great stability [10]. One of the most researched and used inorganic materials for health care with antimicrobial properties are silver nanoparticles (Ag NPs) [11,12]. Ag NPs show a high antimicrobial response against bacteria, but a study about its behaviour against other microorganisms, such as fungi, has not been carried out. In addition, the obtaining cost of the nanoparticles and the short lifetime of antimicrobial action are other negative factors. Recently, *Panáček et al.* have discovered that bacteria repeatedly exposed to silver nanoparticles can rapidly develop resistance to their antibiotic activity [13]. With respect to gold nanoparticles (Au NPs), their antimicrobial activity against bacteria and fungi shows positive results but mainly in short times of action [14]. The functionalization capacity of Au NPs gives them a greater interest. For example, *Li et al.* have designed an antimicrobial strategy using functional Au NPs to combat resistant bacteria [15]. However, the high production costs of functional nanoparticles make the search of other inorganic antimicrobial agents necessary. In the field of metal oxides, the most commonly used is titanium oxide, TiO_2 . The antimicrobial activity of TiO_2 is improved with nanometric size, anatase structure and under ultraviolet (UV) irradiation [16,17]. However, the use of TiO_2 NPs under UV light is restricted due to the genetic damage in cells and human tissues [18,19]. Therefore, the search for an inorganic agent that can be used without endangering human health is necessary. Another metal oxide, which is increasingly relevant, is ZnO. Its versatility and a broad-spectrum of action against microorganisms place the ZnO as a promising antimicrobial agent [20–27]. Recently, we demonstrated that nanostructured ZnO microparticles shown excellent antimicrobial properties based on the electric charges accumulated in the Schottky barriers [28,29]. Many researchers correlate the antimicrobial activity of ZnO with the size of the constituent particles, being more effective the nanometric size [30–32]. However, the role of ZnO nanomaterial properties in antimicrobial activity is still not adequately understood.

Herein, we aim to synthesize ZnO nanoparticles for the antimicrobial applications. In order to study the antimicrobial response of nanoparticulate ZnO, different kind of bacteria has been tested. Common bacteria such as *E. coli* and *S. aureus* decrease their growth in presence of synthesized ZnO. In addition, three MDROs as *K. pneumoniae*, *Methicillin-resistant Staphylococcus aureus* (MRSA) and *Vancomycin-resistant Enterococcus* (VRE) are tested after the addition of nanoparticulate ZnO. We show the excellent antimicrobial activity at low-doses of synthesized ZnO against MDROs. Hence, these promising antimicrobial results of synthesized ZnO have been discussed and we pose an action mechanism against resistant bacteria. Ultimately, our study provides a new path to inorganic antimicrobial agents as a replacement of used common antimicrobial agents to fight against MDROs.

Materials and Methods

Sample preparation. All the chemicals were directly used without further purification. First, a suspension of 6 wt.% micrometric zinc oxide (ZnO) in 3.6 mol of glycerol is prepared and homogenised. Subsequently, 3.6 mol of urea ($\text{CO}(\text{NH}_2)_2$) were added to the suspension under stirring at room temperature. The reaction was heated in a silicone bath at 120–140°C and stirred at 300 rpm for 2h. After naturally cooling, the precipitate was isolated by filtration and washed with water and ethanol several times to remove impurities. The white powder was dried at 80°C for 24h. At the end, the product was thermally treated at 500°C for a short time, 5 min, in the air.

Reference products. Two commercial references of ZnO were chosen based on their size to compare some properties of obtained ZnO. Micrometric ZnO (microZnO) was acquired from Asturiana de Cinc S.A. (Spain). Respect to nanometric ZnO (nanoZnO) was purchased from Evonik Industries (Germany). Both products were thermally treated at 500°C to keep annealing conditions of obtaining ZnO.

Structural characterization. Crystalline phases were characterized by X-ray diffraction (XRD, X'Pert PRO Theta/2theta of Panalytical, Cu K_α radiation, PANalytical, The Netherlands). The patterns were recorded over the angular range 20–70° (2 θ) with a step size of 0.0334° and a time per step of 100 seconds, using Cu K_α radiation ($\lambda = 0.154056$ nm) with working voltage of 40 kV and current of 100 mA. The surface composition was performed by an X-ray Photoelectron Spectrometer (XPS, K-Alpha, Thermo Scientific). Prior to analysis, the samples were cleaned by ion-bombardment with an Ar^+ beam (2 kV) for 5 min. A monochromated Al K_α (1486.6 eV) source running at a voltage of 12 KV with a pass energy of 40 eV was used for high-resolution region scans and 200 eV for survey scans. For charge correction, a 1 point scale with the C 1s peak shifted to 285.0 eV was used. The frequency modes of samples were characterized by Confocal Raman Microscopy (CRM, Witec alpha-300R). Raman spectra were obtained using a frequency-doubled Nd-YAG laser operating at 532 nm and an x100 objective lens (numerical aperture = 0.9). The incident laser power was 20mW.

Textural Characterization. The specific surface area was determined by the BET method (SSA, Monosorb Surface Area Analyzer MS-13, Quantachrome).

Electron Microscopy. The morphology of samples was evaluated using primary electrons images of field emission scanning electron microscopy (FE-SEM, Hitachi S-4700). An image processing and analysis program (Leica Qwin, Leica Microsystems Ltd, Cambridge, England) were performed to determine the average particle size from FE-SEM micrographs. Under conditions always is considered more than 200 particles in each measurement. In addition, a detailed morphology and crystal structure of the sample was evaluated using a JEOL 2100F transmission electron microscope (TEM/HR-TEM) operating at 200 KV and equipped with a field emission electron gun providing a point resolution of 0.19 nm. For TEM

sample preparation, the particles were carefully suspended in ethanol and dispersed using an ultra-sonication bath for 10 minutes. The suspension was dropped on a copper TEM grid with carbon film support. After evaporation of ethanol, the particles were kept at the grid.

UV Photocatalytic degradation. The ROS generation of samples was indirectly determined by photocatalytic activity. A degradation of a typical organic dye, methyl orange (MO, $C_{14}H_{14}N_3NaO_3S$), under UV light irradiation at an ambient temperature in air with magnetic stirring was performed. A 0.05 mM solution of MO was prepared in deionized water. Subsequently, 100 mg L⁻¹ of the sample was added to the MO solution. The mixture was magnetically stirred with a magnetic bar for 15 min in the dark, to allow adsorption equilibrium. After that, the suspension was irradiated with UV light (24 W) and aliquots are collected from the above suspension at different time intervals (from 10 to 300 min). The aliquots were centrifuged at high speed (6000 rpm) for 30 min. The obtained supernatant was then analysed using ultraviolet-visible spectroscopy (UV-Vis spectrophotometer, Pelkin Elmer-Lambda 950) by recording variations in absorbance at a wavelength of 463 nm. The degradation percentage, D(%), was calculated according to Beer-Lambert law as in Eq. 1:

$$D(\%) = \frac{A_0 - A}{A_0} \times 100 \quad (1)$$

where A_0 and A are the absorbances of samples at the beginning time (t_0) of irradiation and at time t , respectively.

Chemical Analysis. Zn²⁺ released was determined by inductively coupled plasma optical emission spectrometry (ICP-OES, Iris Advantage, Termo Jarrel Ash). For sample preparation, 10g L⁻¹ of samples was added in 25 mL of distilled water. The suspension was stirred under ultrasound 5 min and it was allowed to stand for 24 h. After that, the suspension was centrifuged and decanted obtaining a colourless supernatant.

Antibacterial activity test. Different kinds of bacteria were used for testing the antibacterial activity of samples: *Escherichia coli* (CECT 516, ATCC 8739), and *Staphylococcus aureus* (CECT 240, ATCC 6538P). In addition, three kinds of resistant bacteria were also employed: *Klebsiella pneumonia* (ATCC 4352), *Methicillin-resistant Staphylococcus aureus* (MRSA, CECT 5190) and *Vancomycin-resistant Enterococcus* (VRE, CECT 5253). The Macrodilution method from the National Committee of Clinical Laboratory Standards (NCCLS) was performed with some modifications. According to this methodology, 3 ppm of ZnO was introduced in test tubes containing buffered peptone water (PW) at pH 7.4 as a nutrient medium. Subsequently, tubes were inoculated with ca. 5×10^5 cells mL⁻¹ of bacteria in mid-exponential phase and incubated at 37°C for 24h. Bacterial counts were enumerated by sub-cultivation on tryptic soy agar (TSA) plates. All tests were performed in duplicate and the values were expressed in colony forming units (CFU) per mL of suspension. To know the efficacy of the product, the value of antimicrobial activity (R) was calculated (Eq. 2):

$$R = \log \left(\frac{C}{I} \right) - \log \left(\frac{P}{I} \right) = \log \left(\frac{C}{P} \right) \quad (2)$$

where I is the average of the number of viable cells of bacteria just incubated (t_0), C after 24h of incubation (control) and P after 24h of incubation treated with the product.

Results and Discussion

The new ZnO material, thereafter nanoparticulate ZnO (NP-ZnO), is obtained by a soft chemistry method. The ZnO synthesis is more detailed in Material and Methods, see the *Electronic Supplementary Information*. At the end, the as-prepared material is thermally treated at 500°C for a short time, 5 min, in the air. The NP-ZnO antimicrobial activity is evaluated by the macrodilution method against two common and extended bacteria:

Escherichia coli (CECT 516) and *Staphylococcus aureus* (CECT 240). In order to compare the results, two commercial references of ZnO of different size are used: one micrometric (microZnO) and another nanometric (nanoZnO). It is worth to remark that the ZnO concentration used in this study is as low as 3 ppm. **Fig. 1a** shows the behaviour of NP-ZnO antimicrobial response against *E. coli*. The control represents the bacteria population incubated after 24 hours without the ZnO addition. After the ZnO addition, the number of *E. coli* colonies is reduced. Both commercial references of ZnO (microZnO and nanoZnO) reach R values close to 2.1, demonstrating a moderate bactericidal behaviour. However, the antimicrobial response of NP-ZnO against *E. coli* is more effective with an R value of 3.4, in spite of the low ZnO concentration. With respect to *S. aureus*, the behaviour of references micro and nanoZnO is similar to *E. coli*. **Fig. 1b** displays that both commercial samples reach values ca. 2.2. Once more, NP-ZnO shows a better R value against *S. aureus* (R=3.3) than commercial references. In this case, the bacteria growth decreases up to 99.95% when NP-ZnO is added to the medium. Therefore, NP-ZnO is a more effective antimicrobial agent than reference samples.

Figure 1 | Comparative antibacterial activity of NP-ZnO and commercial references (micro and nanoZnO) against *E. coli* (a) and *S. aureus* (b). The bacterial population is expressed as colony forming units per mL (CFU mL⁻¹). The initial bacteria count (control) is represented by red colour for *E. coli* and orange colour for *S. aureus*. The bacteria population after incubation with ZnO commercial references is indicated as grey colour and after incubation with NP-ZnO is symbolized by blue colour. The antibacterial activity is shown as antimicrobial activity value (R). Each data represents an average of duplicate measurements for a concentration of 3 ppm ZnO and the error bars correspond to the standard deviation of the measurements.

After the excellent NP-ZnO response against common bacteria (*E. coli* and *S. aureus*), the next step is to evaluate the antimicrobial activity against common multidrug-resistant organisms (MDROs). In this context, three resistant bacteria were selected for antimicrobial tests: *Klebsiella pneumoniae*, Methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-resistant *Enterococcus* (VRE). The MDROs antimicrobial test keeps the same conditions as *E. coli* and *S. aureus*. Again, the bacteria colonies after 24 hours without NP-ZnO are represented as a control. **Fig. 2a** shows the decrease of *K. pneumoniae* population after the bacteria incubation with 3 ppm of NP-ZnO. Surprisingly, the R value obtained is about 3.7, which means ~99.98% of bacteria population reduction. The excellent antibacterial response against *S. aureus* is also reflected against MRSA (**Fig. 2b**). The addition of NP-ZnO reduces the colony forming units a 99.85% with respect to control. This percentage expressed as antimicrobial activity value corresponds to a R=2.9. Regarding VRE, **Fig. 2c** displays a decrease of bacteria population about 99.88% after the incubation with 3 ppm of NP-ZnO, which means an R value of 2.9.

As a result, the NP-ZnO shows an incredible antibacterial response against common bacteria and MDROs even for concentrations as low as 3 ppm.

Figure 2 | Antimicrobial response of 3 ppm NP-ZnO against MDROs: *K pneumoniae* (a), Methicillin-resistant *S. aureus* (b) and Vancomycin-resistant *Enterococcus* (c). The bacterial population is expressed as colony forming units per mL (CFU mL⁻¹) and the antibacterial activity is shown as antimicrobial activity value (R). Each data represents an average of duplicate measurements and the error bars correspond to the standard deviation of the measurements.

According to identify the possible action mechanism of NP-ZnO against “superbugs”, different assays are performed. First, XRD spectra (**Fig. S1**) shows a unique crystalline structure. The position and intensity of the diffraction peaks are adjusted with the wurtzite hexagonal structure, typical of ZnO (JCPDS Card No. 36-1451). The morphological characterization of NP-ZnO synthesized by FE-SEM (**Fig. 3a**) shows nanoparticles’ agglomerates with a heterogeneous distribution and irregular forms. The image analysis of the nanoparticles displays the average size to 56 ± 8 nm (**Fig. 3b**). Therefore, NP-ZnO is nanoparticles without a defined organization. Furthermore, the ZnO commercial references are also morphologically characterized. The micrometre reference, microZnO, is characterized as hexagonal rods with lengths of 1-2 μ m (**Fig. S2a**). On the other hand, the micrograph of commercial nanoZnO shows larger aggregates of primary ZnO nanoparticles with a diameter ca. 20 nm (**Fig. S2b**). Hence, NP-ZnO particles have a nanometric size with a larger diameter than commercial reference nanoZnO. The specific surface area of NP-ZnO is also compared with commercial references. NP-ZnO has an SSA ($27.6 \text{ m}^2\text{g}^{-1}$) just over commercial nanoZnO one ($23.9 \text{ m}^2\text{g}^{-1}$). The **Fig. 3c** displays the TEM micrograph of NP-ZnO particle. As shown, the distance between crystalline planes is $\sim 1.625 \text{ \AA}$, which agrees with indexation of the ZnO plane (103), according to JCPDS Card No. 79-0206. In addition, irregular particle edges without defined crystalline ordering and surface roughness are observed, which indicates a low surface crystallinity of NP-ZnO. A detail of lattice spacing (**Fig. 3d**) shows multiple crystalline defects such as ion “holes” and crystal dislocations, and differences in the crystallographic orientation. These defects are typically attributed to oxygen or zinc vacancies in the ZnO structure [33,34]. By contrast, TEM micrograph of nanoZnO (**Fig. 3e**) displays high crystalline particles having faceted edges. The distance between crystalline planes ($\sim 2.8 \text{ \AA}$) agrees with indexation of the ZnO plane (010). The **Fig. 3e** shows perfect faceted edges of 120° , in accordance with the hexagonal wurtzite structure. Regarding lattice spacing (**Fig. 3f**), nanoZnO has a high crystalline structure without defects. Comparing both ZnO structures (**Fig. 3d and 3f**), the disorder and multiple defects in the NP-ZnO particle are clearly evidenced.

Figure 3 | Morphological characterization of synthesized NP-ZnO compared with nanoZnO. (a) FE-SEM micrograph shows aggregates of primary NP-ZnO nanoparticle. (b) The size population distribution of nanoparticles shows a mean size of 56 nm. (c) TEM micrograph of a single NP-ZnO nanoparticle displays the irregular morphology of the nanoparticle surface and the crystal ordering of the nanoparticle core. (d) A detail of defects in the crystal structure in the core region of the nanoparticle. (e) TEM micrograph of a single nanoZnO particle displays the faceted edges and the crystal ordering of the nanoparticle core. (f) A detail of the perfect crystalline order of nanoZnO.

One of the main factors of ZnO action mechanism is the generation of reactive oxygen species (ROS) such as oxygen ions, free radicals, and peroxides, e. g. oxygen (O_2), hydrogen peroxide (H_2O_2), hydroxyl ion (OH^-), hydroxyl radicals ($OH^\bullet$), peroxide (O_2^{2-}) and anion superoxide ($O_2^{\bullet-}$) [35]. To identify the ROS generation in NP-ZnO, a photocatalytic degradation of a standard organic dye is carried out under UV light irradiation. The dye degradation was followed by UV-Vis spectroscopy. The organic dye used is methyl orange (MO). **Fig. 4a** shows the percentage of degradation of the dye after the ZnO addition. At short times (10-30 min) there is practically no degradation of the dye. When the time is extended to 300 min, the MO dye is degraded by about 82%. The results are compared with commercial references (microZnO and nanoZnO). The microZnO completely degrades the dye at 120 min, and nanoZnO needs 180 min to the full MO degradation. These data indicate a high ROS generation in commercial references and a decreased amount of ROS generated by NP-ZnO. Another important factor in the antimicrobial activity of ZnO is the amount of leaching Zn^{2+} cation [36]. In this work, the Zn^{2+} cation release is performed by inductively coupled plasma atomic emission spectrometry (ICP-AES). **Fig. 4b** shows the concentration of Zn^{2+} released in the medium for NP-ZnO and it compared with ZnO commercial references. The commercial microZnO leaches the lowest Zn^{2+} concentration (3.4 mg L^{-1}). The

nanoZnO reference has a similar behaviour to microZnO, which Zn^{2+} released concentration is 4.2 mg L^{-1} . By contrast, NP-ZnO releases a large concentration of Zn^{2+} (15.3 mg L^{-1}). Usually, two of the main factors that influence the Zn^{2+} release are particle size and surface area. In addition, the presence of crystalline defects shows a relevant effect on cation leaching.

Figure 4 | Results of ROS generation and leaching assays. (a) Photocatalytic degradation of MO under UV light irradiation in the presence of ZnO. The NP-ZnO results (blue) are compared with commercial references micro and nanoZnO (grey). (b) Chemical analysis of Zn^{2+} released in distilled water after 24h at 37°C.

In order to know what are the reasons for the great leaching of Zn^{2+} cations in NP-ZnO, a structural study is carried out compared with commercial references. X-ray photoelectron spectroscopy (XPS) is used to determine the surface composition of NP-ZnO from the two components of zinc oxide: zinc, Zn 2p, and oxygen, O 1s. In Zn 2p spectra (**Fig. 5a**), two peaks are observed at binding energies 1022.3 and 1045.4 eV. These peaks can be identified with the Zn $2p_{3/2}$ and Zn $2p_{1/2}$ lines, respectively. The binding energy difference between these two peaks is 23.1 eV. This fact confirms that the chemical valence of Zn element exists mainly in the +2 oxidation state on the sample surfaces [37]. **Fig. 5b** shows the XPS spectra of O 1s region. These spectra display an asymmetric peak, which can be fitted with three Gaussian curves of different binding energies. The lower binding energy at 529.9 eV is attributed to the O^{2-} ions which are surrounded by zinc atoms in the wurtzite structure [38]. At a binding energy of 531.0 eV, the second Gaussian curve corresponds to an oxygen-deficient region within the ZnO matrix [39]. The higher binding energy at 532.6 eV is attributed to chemisorbed oxygen [40]. According to the relative areas of each Gaussian curve (**Table 1**), it is possible to indicate a different behaviour between ZnO microparticles and nanoparticles. ZnO microparticles (microZnO) have the highest presence of oxygen in the wurtzite type structure and the lowest oxygen deficiency within the ZnO matrix. These results indicate that microZnO is more crystalline and with fewer surface defects than ZnO nanoparticles. On the other hand, both the commercial ZnO nanoparticles (nanoZnO) and the NP-ZnO synthesized have less oxygen surrounded by zinc atoms, being lower in the synthesized sample. The oxygen deficiency seems to be related to the structural defects observed by TEM (**Figs. 3c and d**). With respect to chemisorbed oxygen

contribution, the amorphous surface of the synthesized NP-ZnO allows a greater surface reactivity; and, therefore, a greater contribution of chemisorbed oxygen.

Table 1 | Gaussian peaks deconvoluted to O 1s XPS spectra and the relative area corresponding to each peak.

NP-ZnO			
	O _{1s} (1)	O _{1s} (2)	O _{1s} (3)
Peak (eV)	529.87	531.09	532.65
Area (%)	3.13	70.44	26.43

nanoZnO			
	O _{1s} (1)	O _{1s} (2)	O _{1s} (3)
Peak (eV)	529.88	531.06	532.64
Area (%)	3.63	71.35	25.02

microZnO			
	O _{1s} (1)	O _{1s} (2)	O _{1s} (3)
Peak (eV)	529.98	531.02	532.60
Area (%)	9.23	67.95	22.83

A Raman study is performed to explore the relevance of the observed structural defects of the nanomaterial (Fig. 5c). The first Raman mode shown in the Raman spectra (99 cm^{-1}) corresponds to the low-frequency E_2 mode (E_2^{low}). This Raman mode is mainly associated with the zinc atom movements. At 203 cm^{-1} , the $2E_2^{\text{low}}$ mode is detected. The next Raman mode observed at 332 cm^{-1} is related to the difference between E_2^{high} and E_2^{low} modes, called as $E_2^{\text{high}} - E_2^{\text{low}}$ mode. The E_2^{high} mode that is located at 438 cm^{-1} is mainly involved in the oxygen atom movements [41]. Finally, the A_1 (LO) and E_1 (LO) modes are also observed at 574 cm^{-1} and 589 cm^{-1} , respectively. The A_1 (LO) mode corresponds to the local vibration modes associated with the intrinsic defects of the network [42]. More specifically, this A_1 (LO) mode is attributed to oxygen (V_O) and interstitial zinc (Zn_i) vacancies, primarily in the atomic movement along the axis c [41]. In addition to the expected Raman modes, the presence of a Raman 2LA mode is detected at 478 cm^{-1} , which corresponds to an overtone mode of the longitudinal acoustic branches (LA). Raman spectra of NP-ZnO compared to micro and nanoZnO shows changes in Raman A_1 (TO), E_1 (TO) and A_1 (LO) modes (Fig. 5c). The high intensity of these Raman modes in NP-ZnO indicates high polarizability changes of the ZnO crystal with respect to commercial references (micro and nanoZnO). In addition, a red-shift in the E_2^{high} Raman mode is observed for the synthesized NP-ZnO which correlates with a decrease in the strength constant of the bond favoured by the presence of oxygen vacancies. The enhancement of defect polarizability correlates with the decreasing in the short-range bond strengths of the nanoparticles, in particular, the dipolar surface interactions of the nanoparticles is modified as evidenced

by the different state of agglomeration of NP-ZnO when compared to the commercial nanomaterial. These results show that the synthesized NP-ZnO has defects in the crystal lattice that affect greatly their functional response. The appearance of these oxygen defects in the crystal structure can explain the high leaching of Zn^{2+} cations in the NP-ZnO. Moreover, the antimicrobial activity enhanced in spite of the nanoparticle size as a clue that crystal defect is relevant. However, the increase of Zn^{2+} cations into the medium is not enough by itself for the improvement of the antimicrobial activity. Moreover, the reduction of ROS production in NP-ZnO is related to a decreasing in the exciton formation from the UV photons. This ROS reduction could be related to the large diameter of the NP-ZnO, that exceed the quantum confinement diameter or by the defective crystal structure of their surface that limited the output of the charge carriers. In this sense, the higher reactivity of the NP-ZnO surface has a more relevant role in the antimicrobial activity than the previously expected one.

Figure 5 | Structural characterization of synthesized NP-ZnO. (a) XPS spectra of the Zn 2p core level region for NP-ZnO (blue) micro (dark grey) and nanoZnO (light grey). (b) XPS spectra of the O 1s core level region, fitted with three Gaussian peaks: lower (green), medium (purple) and high (orange) binding energies. (c) Assignment of different Raman modes of ZnO. The Raman spectrum display changes in main Raman modes for NP-ZnO (blue) with respect to micro (dark grey) and nanoZnO (light grey).

According to the results obtained, a mechanism based mostly on chemical interactions between NP-ZnO and microorganisms is posed (Fig. 6). When NP-ZnO is in the same medium as the bacteria, a Zn^{2+} cations release occurs (Fig. 6.1). Also, reactive oxygen species (ROS) are generated to a lesser extent. The ions released into the medium come into contact with bacteria (Fig. 6.2). Regarding Zn^{2+} cations, the penetration into bacteria is favoured because of bacterial membrane presents a negative charge [43], so there is an electrostatic attraction (Fig. 6.2.1). The effects of the toxic Zn^{2+} cation against the microorganism are: (i) destabilization of the membrane and greater permeability through a direct interaction with the bacterial membranes [36,44], (ii) penetration and interaction with nucleic acids causing a deactivation of the enzymes of the respiratory system [36], and (iii) release of excess Zn^{2+} ions to the cell can induce cytokine production and cytotoxic effect [45,46]. As shown in Fig. 4, the leaching of Zn^{2+} cations in NP-ZnO is larger than in nanoZnO. Taking into account the TEM micrographs of both materials (Fig. 3c-f), there is a notable difference in the crystallinity of their structures. The amorphous structure and oxygen deficient, in addition to the nanometric size of the synthesized NP-ZnO

particles is related to the greater release of Zn^{2+} . The reactive oxygen species (ROS) generated by NP-ZnO also try to penetrate the bacterial membrane (**Fig. 6.2.2**). Mainly, the negative charge of some ROS limits the entering through the microorganism's membrane having a similar charge. However, some ROS without negative charge such as hydrogen peroxide is able to penetrate the bacteria, while the other species remain in contact with the bacteria membrane [31]. The main role of ROS is to cause oxidative stress to the cells, but a massive presence of negatively charged ROS on the outer surface of the microorganisms can neutralize the efficacy of the Zn^{2+} cations. Since the NP-ZnO has a greater Zn^{2+} release and a lower ROS generation than the commercial references, microZnO, and nanoZnO, this neutralization is avoided. Therefore, the decrease in the generation of reactive species reduces the possibility of a higher concentration of ROS in the outer membrane of microorganisms. In addition, the great surface reactivity of NP-ZnO nanoparticles with a high polarizability of the defective crystal structure contributes to disturb the membrane surface and to debilitate the microorganism resistance. Moreover, NP-ZnO has surface irregularities which are able to cause abrasions in the membrane when it comes in direct contact with the bacteria (see **Fig. S3** of the *Supplementary data*). The main action of the Zn^{2+} cations, aided to a lesser extent by the ROS generation and nanoparticles' abrasions, finally causes the death of the microorganism (**Fig. 6.3**).

Figure 6 | Possible antibacterial mechanism of NP-ZnO. The mechanism consists of three steps. (1) The release of Zn^{2+} and the generation of ROS by ZnO nanoparticles. (2) The attraction between Zn^{2+} ions and bacteria, besides the presence of ROS. (2.1 and 2.2) The penetration of ions and molecules into the bacteria. (3) The collapse of the bacteria due to chemical damage.

Conclusions

In summary, we have developed a nanoparticulate ZnO with an excellent antimicrobial response by a soft chemistry process. The antimicrobial activity of NP-ZnO is tested against common bacteria, where the NP-ZnO shows a higher antibacterial activity as compared with two commercial references of ZnO. Outstanding, NP-ZnO also displays an exceptional antimicrobial activity against multidrug-resistant organisms. Here, interestingly, the great antibacterial response of NP-ZnO is obtained even at low concentrations. The synthesis of NP-ZnO with high crystal defect concentration allows a greater Zn^{2+} release and a high surface reactivity. Such defects are revealed by Raman spectroscopy, showing an important contribution of the 2LA mode in

correlation with the crystalline defects for NP-ZnO. The ROS reduction of NP-ZnO also helps to improve the effectiveness of Zn²⁺ cations. The high polarizability of the crystal defects favours the electrostatic attraction between ZnO nanoparticles and the negative bacterial membrane, that later favours the penetration of Zn²⁺ cations and without charge ROS. Therefore, the combined action of surface reactivity, Zn²⁺ and ROS presence leads to MDROs death. The promising results of NP-ZnO open a gate towards inorganic antimicrobial agents to fight against MDROs.

Conflicts of interest

There are no conflicts to declare.

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Supplementary data

Supplementary data to this article can be found online

Materials and Methods, X-Ray diffraction of NP-ZnO compared to ZnO pattern, Morphological characterization of ZnO commercial references, XPS spectra of the Zn 2p core level region and Gaussian peaks deconvoluted to O 1s XPS spectra and the relative area corresponding to each peak.

References

- [1] R.C. Moellering, NDM-1 — A Cause for Worldwide Concern, *N. Engl. J. Med.* 363 (2010) 2377–2379. doi:10.1056/NEJMp1011715.
- [2] T. Saga, K. Yamaguchi, History of antimicrobial agents and resistant bacteria, *Japan Med. Assoc. J.* 52 (2009) 103–108. http://www.med.or.jp/english/pdf/2009_02/103_108.pdf (accessed December 18, 2017).
- [3] S.B. Levy, M. Bonnie, Antibacterial resistance worldwide: Causes, challenges and responses, *Nat. Med.* 10 (2004) S122–S129. doi:10.1038/nm1145.
- [4] D.I. Andersson, D. Hughes, Antibiotic resistance and its cost: Is it possible to reverse resistance?, *Nat. Rev. Microbiol.* 8 (2010) 260–271. doi:10.1038/nrmicro2319.
- [5] J.M.A. Blair, M.A. Webber, A.J. Baylay, D.O. Ogbolu, L.J.V. Piddock, Molecular mechanisms of antibiotic resistance, *Nat. Rev. Microbiol.* 47 (2011) 4055–4061. doi:10.1039/c0cc05111j.
- [6] E. Tacconelli, N. Magrini, G. Kahlmeter, N. Singh, Global Priority List Of Antibiotic-Resistant Bacteria To Guide Research, Discovery, And Development Of New Antibiotics, *World Heal. Organ.* (2017) 1–7.
- [7] M. Sneha, K. Oluwatobi, Samuel Oluwafemi Nandakumar, S. Thomas, APPLICATIONS OF NANOMATERIALS : advances and key technologies., 2018. <https://www.sciencedirect.com/science/book/9780081019719> (accessed July 31, 2018).
- [8] N. Cioffi, M. Rai, *Nano-Antimicrobials*, Springer, Berlin, Heidelberg, 2012. doi:10.1007/978-3-642-24428-5.
- [9] K.P. Miller, L. Wang, B.C. Benicewicz, A.W. Decho, Inorganic nanoparticles engineered to attack bacteria, *Chem. Soc. Rev.* 44 (2015) 7787–7807. doi:10.1039/C5CS00041F.
- [10] J. Sawai, Quantitative evaluation of antibacterial activities of metallic oxide powders (ZnO, MgO and CaO) by conductimetric assay, *J. Microbiol. Methods.* 54 (2003) 177–182. doi:10.1016/S0167-7012(03)00037-X.
- [11] X. Chen, H.J. Schluesener, Nanosilver: A nanoproduct in medical application, *Toxicol. Lett.* 176 (2008) 1–12. doi:10.1016/j.toxlet.2007.10.004.
- [12] G.A. Sotiriou, S.E. Pratsinis, Antibacterial activity of nanosilver ions and particles, *Environ. Sci. Technol.* 44 (2010) 5649–5654. doi:10.1021/es101072s.
- [13] A. Panáček, L. Kvítek, M. Smékalová, R. Večeřová, M. Kolář, M. Röderová, F. Dyčka, M. Šebela, R. Prucek, O. Tomanec, R. Zboril, Bacterial resistance to silver nanoparticles and how to overcome it, *Nat. Nanotechnol.* 13

- (2018) 65–71. doi:10.1038/s41565-017-0013-y.
- [14] E. Lima, R. Guerra, V. Lara, A. Guzmán, Gold nanoparticles as efficient antimicrobial agents for *Escherichia coli* and *Salmonella typhi*, *Chem. Cent. J.* 7 (2013) 1. doi:10.1186/1752-153X-7-11.
- [15] X. Li, S.M. Robinson, A. Gupta, K. Saha, Z. Jiang, D.F. Moyano, A. Sahar, M.A. Riley, V.M. Rotello, Functional gold nanoparticles as potent antimicrobial agents against multi-drug-resistant bacteria, *ACS Nano*. 8 (2014) 10682–10686. doi:10.1021/nn5042625.
- [16] F. Chen, X. Yang, Q. Wu, Antifungal capability of TiO₂ coated film on moist wood, *Build. Environ.* 44 (2009) 1088–1093. doi:10.1016/j.buildenv.2008.07.018.
- [17] A.S. Roy, A. Parveen, A.R. Koppalkar, M.V.N.A. Prasad, Effect of Nano -Titanium Dioxide with Different Antibiotics against Methicillin-Resistant *Staphylococcus Aureus*, *J. Biomater. Nanobiotechnol.* 1 (2010) 37–41. doi:10.4236/jbnb.2010.11005.
- [18] S.M. Dizaj, F. Lotfipour, M. Barzegar-Jalali, M.H. Zarrintan, K. Adibkia, Antimicrobial activity of the metals and metal oxide nanoparticles, *Mater. Sci. Eng. C*. 44 (2014) 278–284. doi:10.1016/j.msec.2014.08.031.
- [19] A.M. Allahverdiyev, E.S. Abamor, M. Bagirova, M. Rafailovich, Antimicrobial effects of TiO₂ and Ag₂O nanoparticles against drug-resistant bacteria and leishmania parasites, *Future Microbiol.* 6 (2011) 933–940. doi:10.2217/fmb.11.78.
- [20] R. Brayner, R. Ferrari-Iliou, N. Brivois, S. Djediat, M.F. Benedetti, F. Fiévet, Toxicological impact studies based on *Escherichia coli* bacteria in ultrafine ZnO nanoparticles colloidal medium, *Nano Lett.* 6 (2006) 866–870. doi:10.1021/NL052326h.
- [21] N. Jones, B. Ray, K.T. Ranjit, A.C. Manna, Antibacterial activity of ZnO nanoparticle suspensions on a broad spectrum of microorganisms, *FEMS Microbiol. Lett.* 279 (2008) 71–76. <http://www.scopus.com/inward/record.url?eid=2-s2.0-37349033634&partnerID=40&md5=5e14cc89796defaf187bf870098dfd86>.
- [22] D. Sharma, J. Rajput, B.S. Kaith, M. Kaur, S. Sharma, Synthesis of ZnO nanoparticles and study of their antibacterial and antifungal properties, *Thin Solid Films*. 519 (2010) 1224–1229. <http://www.scopus.com/inward/record.url?eid=2-s2.0-78049274166&partnerID=40&md5=43f942fca02414b9e52d23e950d214b9>.
- [23] M. Li, L. Zhu, D. Lin, Toxicity of ZnO nanoparticles to *Escherichia Coli*: Mechanism and the influence of medium components, *Environ. Sci. Technol.* 45 (2011) 1977–1983. doi:10.1021/es102624t.
- [24] L. He, Y. Liu, A. Mustapha, M. Lin, Antifungal activity of zinc oxide nanoparticles against *Botrytis cinerea* and *Penicillium expansum*, *Microbiol. Res.* 166 (2011) 207–215. doi:10.1016/j.micres.2010.03.003.
- [25] Y. Jiang, L. Zhang, D. Wen, Y. Ding, Role of physical and chemical interactions in the antibacterial behavior of ZnO nanoparticles against *E. coli*, *Mater. Sci. Eng. C*. 69 (2016) 1361–1366. doi:10.1016/j.msec.2016.08.044.
- [26] Y. Wang, J. Ma, Q. Xu, J. Zhang, Fabrication of antibacterial casein-based ZnO nanocomposite for flexible coatings, *Mater. Des.* 113 (2017) 240–245. doi:10.1016/J.MATDES.2016.09.082.
- [27] A. Hui, J. Liu, J. Ma, Synthesis and morphology-dependent antimicrobial activity of cerium doped flower-shaped ZnO crystallites under visible light irradiation, *Physicochem. Eng. Asp.* 506 (2016) 519–525. doi:10.1016/j.colsurfa.2016.07.016.
- [28] E. de Lucas-Gil, J.J. Reinosá, K. Neuhaus, L. Vera-Londono, M. Martín-González, J.F. Fernández, F. Rubio-Marcos, Exploring New Mechanisms for Effective Antimicrobial Materials: Electric Contact-Killing Based on Multiple Schottky Barriers, *ACS Appl. Mater. Interfaces*. 9 (2017) 26219–26225. doi:10.1021/acsami.7b09695.
- [29] E. de Lucas-Gil, P. Leret, M. Monte-Serrano, J.J. Reinosá, E. Enríquez, A. Del Campo, M. Cañete, J. Menéndez, J.F. Fernández, F. Rubio-Marcos, ZnO Nanoporous Spheres with Broad-Spectrum Antimicrobial Activity by Physicochemical Interactions, *ACS Appl. Nano Mater.* (2018) acsanm.8b00402. doi:10.1021/acsanm.8b00402.
- [30] O. Yamamoto, Influence of particle size on the antibacterial activity of zinc oxide, *Int. J. Inorg. Mater.* 3 (2001) 643–646. doi:10.1016/S1466-6049(01)00197-0.
- [31] N. Padmavathy, R. Vijayaraghavan, Enhanced bioactivity of ZnO nanoparticles – an antimicrobial study, *Sci. Technol. Adv. Mater.* 9 (2008) 035004. doi:10.1088/1468-6996/9/3/035004.
- [32] K.R. Raghupathi, R.T. Koodali, A.C. Manna, Size-dependent bacterial growth inhibition and mechanism of antibacterial activity of zinc oxide nanoparticles, *Langmuir*. 27 (2011) 4020–4028. doi:10.1021/la104825u.
- [33] D. Chen, Z. Wang, T. Ren, H. Ding, W. Yao, R. Zong, Y. Zhu, Influence of Defects on the Photocatalytic Activity of ZnO, *J. Phys. Chem. C*. 118 (2014) 15300–15307. doi:10.1021/jp5033349.
- [34] Y. Lv, C. Pan, X. Ma, R. Zong, X. Bai, Y. Zhu, Production of visible activity and UV performance enhancement of ZnO photocatalyst via vacuum deoxidation, *Appl. Catal. B Environ.* 138–139 (2013) 26–32. doi:10.1016/j.apcatb.2013.02.011.
- [35] A. Sirelkhatim, S. Mahmud, A. Seeni, N.H.M. Kaus, L.C. Ann, S.K.M. Bakhori, H. Hasan, D. Mohamad, Review on zinc oxide nanoparticles: Antibacterial activity and toxicity mechanism, *Nano-Micro Lett.* 7 (2015) 219–242. doi:10.1007/s40820-015-0040-x.
- [36] J. Pasquet, Y. Chevalier, J. Pelletier, E. Couval, D. Bouvier, M.-A. Bolzinger, The contribution of zinc ions to the antimicrobial activity of zinc oxide, *Colloids Surfaces A Physicochem. Eng. Asp.* 457 (2014) 263–274. doi:10.1016/j.colsurfa.2014.05.057.
- [37] R. Al-Gaashani, S. Radiman, A.R. Daud, N. Tabet, Y. Al-Douri, XPS and optical studies of different morphologies of ZnO nanostructures prepared by microwave methods, *Ceram. Int.* 39 (2013) 2283–2292.

- doi:10.1016/j.ceramint.2012.08.075.
- [38] J. Das, S.K. Pradhan, D.R. Sahu, D.K. Mishra, S.N. Sarangi, B.B. Nayak, S. Verma, B.K. Roul, Micro-Raman and XPS studies of pure ZnO ceramics, *Phys. B Condens. Matter* 405 (2010) 2492–2497. doi:10.1016/j.physb.2010.03.020.
 - [39] X.-J. Yang, X.-Y. Miao, X.-L. Xu, C.-M. Xu, J. Xu, H.-T. Liu, Structure, X-ray photoelectron spectroscopy and photoluminescence properties of highly ordered ZnO microrods, *Opt. Mater. (Amst)* 27 (2005) 1602–1605. doi:10.1016/j.optmat.2004.05.007.
 - [40] J. Lee, J. Chung, S. Lim, Improvement of optical properties of post-annealed ZnO nanorods, *Phys. E Low-Dimensional Syst. Nanostructures* 42 (2010) 2143–2146. doi:10.1016/j.physe.2010.04.013.
 - [41] R. Cuscó, E. Alarcón-Lladó, J. Ibáñez, L. Artús, J. Jiménez, B. Wang, M.J. Callahan, Temperature dependence of Raman scattering in ZnO, *Phys. Rev. B* 75 (2007) 165202. doi:10.1103/PhysRevB.75.165202.
 - [42] F. Rubio-Marcos, A. Quesada, M. a. García, M. a. Bañares, J.L.G. Fierro, M.S. Martín-Gonzalez, J.L. Costa-Krämer, J.F. Fernández, Some clues about the interphase reaction between ZnO and MnO₂ oxides, *J. Solid State Chem.* 182 (2009) 1211–1216. doi:10.1016/j.jssc.2009.02.009.
 - [43] M. Arakha, M. Saleem, B.C. Mallick, S. Jha, The effects of interfacial potential on antimicrobial propensity of ZnO nanoparticle, *Sci. Rep.* 5 (2015) 9578. doi:10.1038/srep09578.
 - [44] V. Stanić, S. Dimitrijević, J. Antić-Stanković, M. Mitrić, B. Jokić, I.B. Plećaš, S. Raičević, Synthesis, characterization and antimicrobial activity of copper and zinc-doped hydroxyapatite nanopowders, *Appl. Surf. Sci.* 256 (2010) 6083–6089. doi:10.1016/j.apsusc.2010.03.124.
 - [45] A.E. Nel, L. Mädler, D. Velegol, T. Xia, E.M. V Hoek, P. Somasundaran, F. Klaessig, V. Castranova, M. Thompson, Understanding biophysicochemical interactions at the nano-bio interface., *Nat. Mater.* 8 (2009) 543–557. doi:10.1038/nmat2442.
 - [46] T. Xia, M. Kovoichich, M. Liong, L. Mädler, B. Gilbert, H. Shi, J.I. Yeh, J.I. Zink, A.E. Nel, Comparison of the Mechanism of Toxicity of Zinc Oxide and Cerium Oxide Nanoparticles Based on Dissolution and Oxidative Stress Properties, *ACS Nano* 2 (2008) 2121–2134. doi:10.1021/nn800511k.