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The contribution of zinc ions to the antimicrobial activity of zinc oxide

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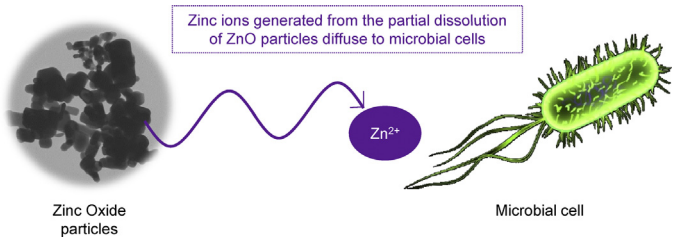
HIGHLIGHTS

- Proteins contained in a broth medium increase the solubility of ZnO particles.
- The respective activities of ZnO and Zn^{2+} show specificity with respect to the microorganisms.
- The contribution of Zn^{2+} to the antimicrobial activity of ZnO depends on the strain.
- The dissolution process depends on time, ZnO concentration, and type of ZnO powder.
- The combination of the three antimicrobial mechanisms of ZnO is beneficial.

GRAPHICAL ABSTRACT



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ABSTRACT

Zinc ions (Zn^{2+}) exhibit antimicrobial activity against various bacterial and fungal strains. The partial dissolution of zinc oxide (ZnO) particles releases Zn^{2+} ions in aqueous suspension that contributes to the antimicrobial activity of ZnO. In addition to the activity of the soluble zinc species that is common with water-soluble zinc salts, ZnO combines two additional mechanisms of antimicrobial activity that supplement its activity as preservative in topical formulations: generation of reactive oxygen species and by direct contact to the cells walls. The present study aims at the evaluation of the contribution of the soluble zinc species to the antimicrobial activity of ZnO on microbial cultures in broth medium and the investigation of the dissolution of zinc from ZnO suspensions. The antimicrobial activities against the five microorganisms of the Challenge Tests were measured for suspensions of three ZnO grades in broth, and for the isolated liquid phase of the suspensions containing soluble zinc species. The Zn^{2+} released in the broth brought about a significant contribution to the overall antimicrobial activity of ZnO. The complexation of Zn^{2+} ions by the components of the broth increased the solubility of the zinc in the liquid medium. The respective activities of the soluble zinc species and ZnO particles showed specificity with respect to the microbial strains. Dissolution was faster for high concentrations of ZnO and for ZnO powders of larger specific area. Such conditions led to a better antimicrobial efficacy of ZnO powders. ZnO appears an advantageous alternative to soluble zinc salts such as zinc gluconate.
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1. Introduction

Zinc oxide (ZnO) is an efficient antimicrobial agent that acts by means of several mechanisms involving different chemical species. Three distinct mechanisms of action have been put forwards in the literature: (i) the production of reactive oxygen species (ROS) because of the semiconductive properties of ZnO [1,2,3], (ii) the destabilization of microbial membranes upon direct contact of ZnO particles to the cell walls [4–6], and (iii) the intrinsic antimicrobial properties of Zn^{2+} ions released by ZnO in aqueous medium [7–9]. The present study aims at investigating the contribution of the soluble species released by ZnO, especially Zn^{2+}.

Zinc is an essential element for microorganisms and higher organisms because it is involved in many vital cellular reactions at its low endogenous concentrations [10–13]. The concentration of zinc is 10^{-4} M in blood [14]. Optimal rates of this cofactor are needed for catalytic and structural activities [14,15]. Zinc concentration is regulated under physiological conditions by several transporters [15–17], so that Zn^{2+} ions are essentially nontoxic to higher organisms [3]. Homeostasis regulates zinc uptake by cells, but it does not control zinc adsorption to cell membranes however. Increase of Zn^{2+} concentrationsabove optimal levels (typically between 10^{-7} M and 10^{-5} M depending on the microbial strain [18]) perturbs Zn^{2+} homeostasis and allows entry of Zn^{2+} inside cells, so that zinc starts being cytotoxic to prokaryotes above a concentration of $\sim 10^{-4}$ M [18,19]. Therefore, Zn^{2+} displays an antimicrobial activity and could act as either antibacterial or antifungal agent. The antimicrobial properties of Zn^{2+} have been known since a long time, both against bacterial [20–22] and fungal strains [23].

According to several reports on water-soluble zinc salts, the antimicrobial activity of Zn^{2+} depends on its concentration and contact duration. Zinc chloride acts in a dose-dependent manner against *Escherichia coli* [21,22]. Zinc acetate exhibits an antibacterial activity on *Pseudomonas aeruginosa* and *Staphylococcus aureus* for zinc concentrations above 11 mmol L^{-1} [20]. Moreover, a prolonged contact of zinc (at 100 $\mu g\, g^{-1}$) to *Aspergillus brasiliensis* spores inhibits their germination by 25% [23]. These antimicrobial activities were explained by two mechanisms, both leading to cell death: (i) a direct interaction with microbial membranes leading to membrane destabilization and enhanced permeability [24]; (ii) an interaction with nucleic acids and deactivation of enzymes of the respiratory system [25].

In dermatological products, zinc ions are interesting biocides and/or antimicrobial preservatives provided that high enough concentrations of Zn^{2+} are generated. The previously mentioned zinc salts can be simply dissolved in the aqueous medium. An alternative is solid powder such as ZnO particles that release Zn^{2+} in the aqueous medium. It is indeed recognized that part of the antimicrobial activity of ZnO particles originates from their ability to partially dissolve in aqueous media [26,27]. Release of Zn^{2+} would contribute to the global antimicrobial properties of this inorganic powder [7,29]. Nevertheless, the contribution of Zn^{2+} to the antimicrobial activity of ZnO is still unclear. Since ZnO particles exhibit two additional antimicrobial mechanisms with respect to zinc salts (ROS and direct contact) the combination of these three types of action broadens the antimicrobial spectrum of ZnO compared to zinc salts.

The present study has been focused on the contribution of zinc cations generated from the partial dissolution of ZnO particles in aqueous media to the global antimicrobial action. Even though the antimicrobial mechanism of Zn^{2+} has been disclosed, the antimicrobial activity of ZnO *via* a contribution of Zn^{2+} is still under debate because of the complexity of the underlying phenomena. According to Sawai [28] and Jiang et al. [6], the contribution of Zn^{2+} to the antimicrobial efficacy of ZnO particles would be minor because too low concentrations of soluble zinc species are released from the dissolution of ZnO particles. In other instances reported in the field of dentistry applications, the contribution of Zn^{2+} is predominant [30,31]. The aim of the present work was the assessment of the contribution of soluble ionic species of zinc to the antimicrobial efficacy of ZnO powders, as well as the factors that influence the dissolution of the particles. Indeed, it has been reported that the dissolution phenomenon was influenced by numerous parameters sorted into two types:

- the chemistry of the environmental media such as the pH [32,33], the duration of exposure [34–36], UV irradiation [32,37], the presence of other substances [33,38] or microorganisms [39–41];
- the physicochemical properties of the particles such as the elementary particle size [35,42,43], their porosity [44], their shape [35], their concentration [45].

The impact of all these parameters is not fully understood.

In order to use ZnO particles as an efficient antimicrobial preservative in cosmetic and dermopharmaceutical products, it was firstly aimed at discriminating the contribution of zinc cations generated by ZnO particles from the overall antimicrobial activity, and secondly identifying the parameters which directly impact the dissolution phenomenon and would enhance this mechanism. The study was performed on the five microbial strains used for Challenge Tests for checking the safety of pharmaceutical and cosmetic products. Microbiological tests on solid agar plate and in broth culture were performed to evaluate the antimicrobial efficacy of both ZnO particles and Zn^{2+}. This work was performed taking into consideration the dissolution of ZnO particles in aqueous media depending on the environmental conditions and on the physicochemical characteristics of ZnO powders using three different ZnO grades.

2. Materials and methods

2.1. Materials

The following ZnO powders of pharmaceutical grade were studied: ZnO-1 from Rockwood Pigments (Beltsville, US); ZnO-2 from SILOX (Engis, Belgium); ZnO-3 from Zinc Corporation of America (Pittsburgh, US). Zinc gluconate (ZnG) was supplied by Seppic (Castres, France).

2.2. ZnO particles characterization studies

The physicochemical properties of the three ZnO grades were investigated as previously reported [46]. The characteristics of the powders were assessed in a dry state by determining their specific area and the porosity by nitrogen adsorption measurements using a Tristar 3000 Micromeritics BET instrument. The specific area was determined by the Brunauer–Emmett–Teller (BET) multipoint method and the pore volume was analyzed by the Barrett–Joyner–Halenda (BJH) method. The crystal structures were established by X-ray diffraction measurements performed at the 'Centre Henri Longchambon' facility (University of Lyon) using a Bruker AXS D8 Advance X-ray diffractometer operating with the Cu Kα1 line at 1.54 Å wavelength. The crystallite size was estimated from the width at half height of the Bragg peaks using the Debye–Scherer equation. The apparent density was studied following an adapted protocol of the European Pharmacopeia. Size and shape of the elementary particles were studied by transmission electron microscopy (TEM) performed at the 'Centre Technologique des Microstructures' facility (University of Lyon) on a Philips CM120 microscope operating at 80 kV acceleration. A dilute aqueous suspension (0.1%) was spread on Formvar/carbon grids and dried before observation.

The behavior of the aggregates of ZnO particles was also evaluated in liquid media by measuring the isoelectric point by electrophoretic light scattering measurements using a Zetasizer Nano ZS (Malvern Instruments, UK). The sedimentation phenomenon was followed measuring the turbidity of the samples using a Carry 50 UV–vis absorption spectrophotometer (Varian). The turbidity τ (cm^{-1}) was deduced from the absorbance A_λ measured at a wavelength of 380 nm in a cell of optical path $l = 1$ cm according to the following equation: $\tau = 2.303 A_\lambda / l$. Finally, the diameters of ZnO aggregates in water and in nutrient broth used in antimicrobial tests were measured by low-angle laser light scattering using a Malvern Mastersizer 2000 instrument. Agglomerate size distributions were calculated according to Mie theory using the refractive indices of water (1.33) and ZnO (2.008). The refractive index of the Mueller Hinton culture broth used for antimicrobial tests measured using a PAL-1 refractometer (Atago, France) was 1.336. The full granulometric distribution was retained as a characteristic parameter.

2.3. Evaluation of antimicrobial activity

The microbiological experiments were carried out in triplicate on the five microbial strains used for Challenge Tests of the European 7.0 and US Pharmacopeias 35 supplied by the culture collection of the Institut Pasteur (France): *Escherichia coli* CIP® 53.126 (equivalent strain ATCC® 8739); *Staphylococcus aureus* CIP® 4.83 (ATCC® 6538); *Pseudomonas aeruginosa* CIP® 82.118 (ATCC® 9027); *Candida albicans* IP 48.72 (ATCC® 10231); *Aspergillus brasiliensis* IP 1431.83 (previously named as *Aspergillus niger* ATCC® 16404). Cell cultures and incubations were performed in a thermostated chamber in the dark.

2.3.1. Preliminary experiments by the disc diffusion test

Disc diffusion susceptibility tests on Mueller-Hinton (MH2) solid agar plate (BioMérieux, France) were performed. Sterile standard discs of cellulose of 6 mm diameter (BioMérieux, France) were impregnated with antimicrobial materials and deposited on a microbial culture in agar medium. This standard method has been used for solutions of ZnG and was adapted to the particulate nature of the present materials as follows. The cellulose discs were impregnated with sterile aqueous suspensions of ZnO at 10, 50 and 100 mg g^{-1} concentrations by soaking the discs into the suspensions for 5 s. The impregnated discs were placed onto the agar surface previously inoculated by spreading the microbial suspension on the surface of the agar. After overnight incubation in the dark at 32.5 °C for the bacterial strains and 48 h at 22.5 °C for the fungal strains, zones of inhibition around each disc were observed.

2.3.2. Measurements of antimicrobial activity in liquid broth

The antimicrobial efficacy of either ZnO particles or soluble zinc species was evaluated in Mueller Hinton (MH) broth (AES Chemunex, France), a sterile medium intended for microorganisms growth. Solutions of soluble zinc species consisted of either solutions of ZnG in MH broth, or the supernatant of ZnO suspensions in MH broth obtained by centrifugation for 10 min at 5000 rpm. Suspensions of ZnO of various concentrations (from 1.25 to 80 mg g^{-1}) were prepared by dispersing the ZnO powder in MH broth for 1 h in an ultrasonic bath. A preliminary study has validated the conditions of this dispersion step [46]; in particular it has shown that dispersion by ultrasounds did not alter the intrinsic antimicrobial properties of ZnO. Each sample was inoculated with the microbial strains and incubated at the appropriate temperature (32.5 °C for bacterial strains; 22.5 °C for fungal strains). Aliquots were collected at different incubation times (24 h for bacterial strains and 48 h for fungal strains), diluted in tryptone salt broth (AES Chemunex) before seeding the suitable dilution on TSA agar plate (BioMérieux) for incubation in the dark (24 h for bacterial strains, 48 h for *C. albicans* and 72 h for *A. brasiliensis*) and counting the colonies. A 'positive control' experiment consisted of inoculation of the microbial strains in pure zinc-free MH broth.

Additional experiments on ZnO suspensions and soluble zinc species were conducted in water in place of MH broth in order to assess the influence of zinc solubilization by MH broth. These experiments were conducted according to the same method as for those in MH broth.

2.4. ZnO dissolution experiments

The dissolution of ZnO particles under different experimental conditions was examined trough separate experiments, each performed in triplicate. ZnO suspensions were prepared and homogenized in hemolysis tubes of 5 mL. For each sample, ZnO suspensions were vortexed for 10 s and a 10 min centrifugation step at 5000 rpm was applied. The supernatant was collected, filtered through a 0.20 μm regenerated cellulose syringe-filter (Uptidisc®, Interchim, Montluçon, France) and introduced in a polypropylene 1 mL vial for analysis of zinc. Every chemical was of analytical grade and solutions/suspensions were prepared with water for HPLC (resistivity of 18 MΩ cm).

2.5. Zinc ions analysis

Zinc content released from the dissolution of ZnO particles was quantified using a high-performance liquid chromatography (Waters) coupled to a detection using a post-column reaction with the 4-(2-pyridylazo)resorcinol reagent [47]. This method allows a chromatographic separation of ions that provides the selectivity of the analysis and a sensitive detection through the post-column formation of a colored complex [48]; it has already been applied to the analysis of Zn^{2+} ions in protein media [47]. Firstly, zinc ions in the supernatant were separated on a non-polar stationary phase (column X Terra MSC18-5 μm-3.9 × 150 mm, Waters) with an acid mobile phase composed of sodium octane sulfonate (0.2 mM), tartaric acid (25 mM) and acetonitrile (2%). The pH of the mobile phase was adjusted at 3.5 adding sodium hydroxide. Then, a post-column reaction took place at 50 °C with 4-(2-pyridylazo) resorcinol (PAR reagent, Sigma-Aldrich) in ammonia solution. Complexation of Zn^{2+} by the PAR reagent yielded colored complex species that were detected by means of their absorbance in the visible domain. The detection of the complex PAR-Zn^{2+} was realized with a diode array detector at a detection wavelength of 505 nm corresponding to the maximum of the absorption spectra of the red–orange complex. Acquisition and data processing were done with the software Empower Photodiode Array (PDA, Waters). The quantitative analysis of Zn^{2+} concentration was performed with help of an external calibration with zinc gluconate solution in acidic conditions ensuring a total dissociation of the zinc salt as free zinc ions.

3. Results and discussion

3.1. Antimicrobial activity of ZnO and Zn^{2+}

Preliminary experiments performed in agar medium revealed inhibition zones around ZnO impregnated discs (Fig. 1). Since ZnO particles were unable to diffuse out of the cellulose discs, antimicrobial species have necessarily been released from the ZnO impregnated discs to the agar medium. This indicated that ZnO particles were able to exhibit an antimicrobial effect by diffusion of soluble species into the agar medium. This test gave a first qualitative estimate of the sensitivity of microbial strains to ZnO particles through the contribution of released Zn^{2+}. *E. coli* and *S. aureus* were

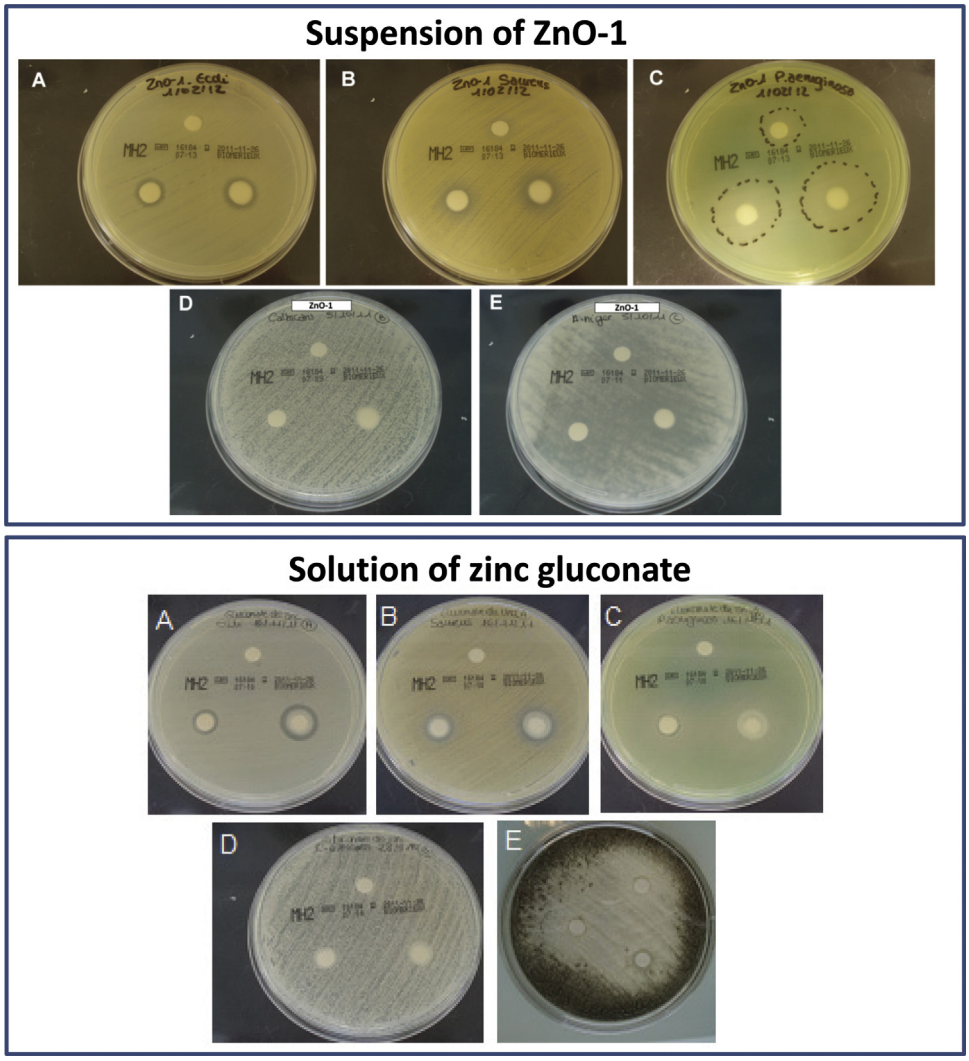


Fig. 1. Disc diffusion tests for the evaluation of antimicrobial activity of ZnO-1 (10, 50 and 100 mg g^{-1}) (top) and zinc gluconate (10, 50 and 100 mg g^{-1}) (bottom). (A) *E. coli*; (B) *S. aureus*; (C) *P. aeruginosa*; (D) *C. albicans*; (E) *A. brasiliensis*.

sensitive to soluble species since clear inhibition zones of the bacterial growth around the discs could be observed. These two bacteria were more sensitive than *P. aeruginosa* for which only a large pale green colored halo was observed. Similar results were obtained with the soluble ZnG, showing that the antimicrobial activities of the soluble zinc species were indeed at the origin of the inhibition zones. The disc diffusion test did not reveal any growth inhibition of the yeast *C. albicans*, neither by ZnO nor by ZnG. Conversely, the germination of the spores of *A. brasiliensis* was inhibited by ZnO and ZnG, the activity of ZnG being much higher. For the microbial strains on which an efficacy was proven with this test, a concentration-dependence has been noticed: the inhibition zones were larger for the highest concentrations of ZnG and ZnO. This latter result points out the impact of the dissolution phenomenon of ZnO particles into the culture broth medium.

Quantitative assessment of antimicrobial activities was done by experiments in liquid broth medium and in pure water. Various efficacy indexes were determined: the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) evaluated the efficacy on bacteria; an 'inhibition rate' was defined as the ratio of the population densities of the sample for 80 mg g^{-1} of ZnO to that of the positive control measured 48 h after inoculation to quantify the activity on *C. albicans*; and a visual MIC estimated as the lowest concentration of ZnO that prevented the development of mycelium 48 h after inoculation was used to measure the efficacy of ZnO on *A. brasiliensis*. Thanks to these values, ZnO grades were sorted with respect to their efficacy (Table 1): ZnO-1 exhibited the highest efficacy, whereas ZnO-3 was the less effective and ZnO-2 displayed an intermediate activity closer to ZnO-1 than ZnO-3 [46].

The antimicrobial effects of the three types of Zn-containing samples were comparatively tested in broth medium: ZnO suspensions, their supernatants and ZnG solutions. ZnG was used as a reference for soluble zinc species although zinc is not present as totally free Zn^{2+} in the solution. The use of ZnG allowed the study of high concentrations of soluble zinc that fully dissociated zinc salts such as zinc chloride or zinc acetate would not reach because of their instantaneous precipitation in the neutral or basic media. Zinc ions of ZnG are partially complexed by gluconate and complexing species contained in the MH broth. High enough concentrations of free Zn^{2+} would require an acidic pH, but the slightly basic pH of the culture broth must be retained for an optimal microbial growth. The results reported in Fig. 2 for ZnO-1 show a reduction of the microbial populations in the presence of ZnO-1suspensions, ZnO-1 supernatant, or ZnG. The comparison between ZnO suspensions and supernatant revealed that the antibacterial activity was significantly less for the soluble species alone than for ZnO-1 suspensions. The difference between the two gave the contributions

Table 1
Characteristics of the antimicrobial efficacy of ZnO particles and zinc gluconate on the five microorganisms.

Microbial strain	Raw material	ZnO-1	ZnO-2	ZnO-3	Zinc gluconate
E. coli	MIC (mg g^{-1})	1.2	2.5	12.8	1.7
	MBC (mg g^{-1})	14.4	17.2	18.1	8.4
S. aureus	MIC (mg g^{-1})	1.8	3.0	46.8	4.6
	MBC (mg g^{-1})	15.6	31.8	75.2	>80
P. aeruginosa	MIC (mg g^{-1})	23.0	34.0	57.0	33.6
	MBC (mg g^{-1})	64.0	68.1	>80	>80
C. albicans	Inhibition rate (%)	33.6	35.9	24.7	47.3
A. brasiliensis	Visual MIC (mg g^{-1})	10	20	80	<10

of the mechanisms that required the presence of ZnO particles *in situ*. The antimicrobial activity by direct contact of ZnO particles to the cell walls was absent in the experiments with the supernatant. There might be ROS present in the supernatant because they have been generated before its separation by centrifugation and remained in the state of the stable hydrogen peroxide species (H_2O_2). However, *in situ* photo-generation of the most active ROS by ZnO particles is not operative in the supernatant. As a whole, the antimicrobial activity of the supernatant mainly comes from the soluble zinc species; a slight supplementary activity may arise from residual ROS. The contribution of ROS to the antimicrobial activity of ZnO is a complex topic that deserves a full paper to be correctly addressed. A specific study will be published soon on this subject. Beyond the suppression of the direct action by close contact, the removal of ZnO powder from the media stopped any further dissolution of soluble zinc species. This part of the study demonstrated that soluble Zn^{2+} released by partial dissolution of ZnO had a definite contribution to the antimicrobial activity of ZnO. The quantification of the contribution of the soluble zinc species is difficult because the shape of the plots of Log(CFU mL^{-1}) with respect to [ZnO] (Fig. 2) are not similar for the full ZnO suspensions and their supernatants. A rough estimate was made from the slopes of the curves d(Log(CFU mL^{-1}))/d[ZnO]. For *E. coli*, the contribution of soluble zinc species was approximately 15% of the full antimicrobial activity of ZnO; it was 71% for *S. aureus*; and it finally reached almost 100% for *C. albicans* (the soluble zinc species were responsible for the full activity of ZnO). There was a definite specificity of the antimicrobial activity of the soluble zinc species with regards to the microbial strains. The behavior was more complex for *P. aeruginosa*: almost 100% of the antibacterial activity was caused by the soluble zinc species for [ZnO] < 10 mg g^{-1} and the contribution of soluble zinc species turned small for [ZnO] > 10 mg g^{-1}, because the antibacterial activity of ZnO became high above this ZnO concentration whereas the activity of the soluble zinc species did not increase significantly.

Zinc concentration has been measured in the supernatant separated by centrifugation, thus allowing the comparison of antimicrobial activities of the soluble zinc species released by ZnO particles in the MH broth and that of ZnG. Such comparison is presented in Fig. 3. The antimicrobial activity of soluble zinc species and ZnG appeared of comparable magnitude for most strains except *E. coli*. A noticeable feature of the antimicrobial activity of soluble zinc species is the weak activity at high concentrations. There were two regimes (Fig. 3): the microbial populations decreased significantly with respect to the concentration of zinc for low Zn^{2+} concentrations, and remained approximately at the same level whatever the concentration of Zn^{2+} above 1 mg g^{-1}. ZnG had a higher antimicrobial activity against *E. coli* than a similar Zn^{2+} concentration released from ZnO in the broth. Such an enhancement of antimicrobial activity by the gluconate suggests that *E. coli* is

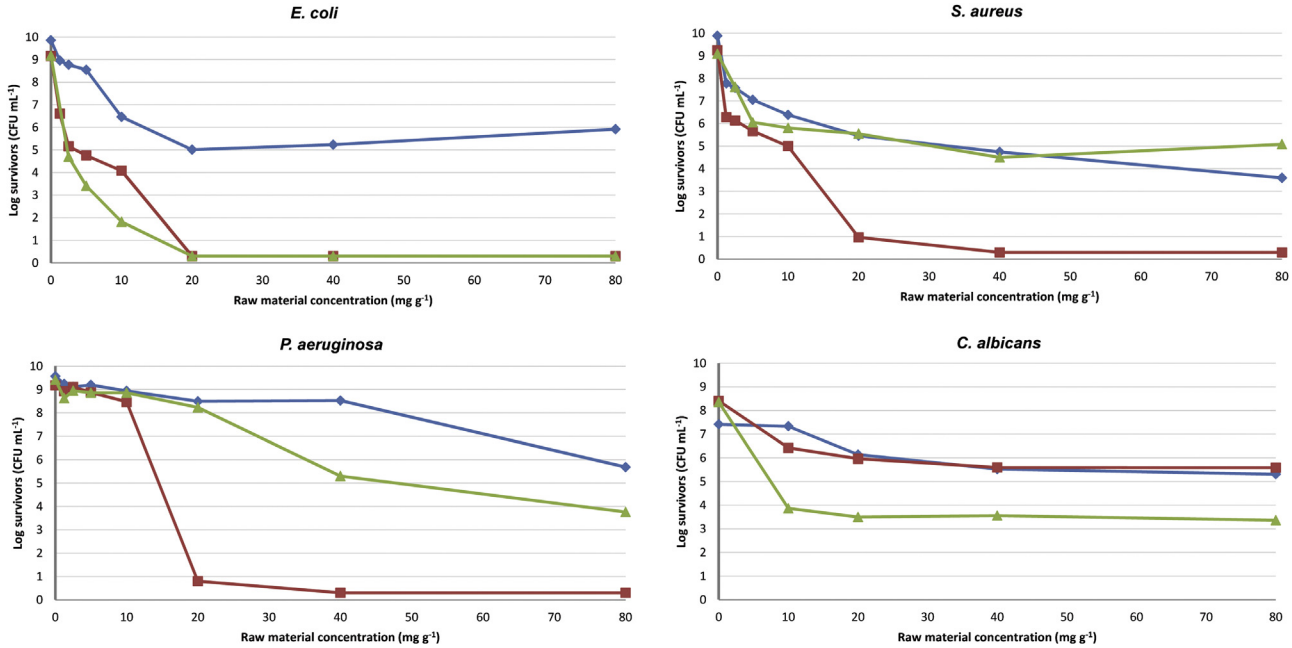


Fig. 2. Decrease of microbial populations with respect to zinc concentration for ZnO-1 suspension (red squares), its supernatant (blue diamonds), and zinc gluconate solution (green triangles). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

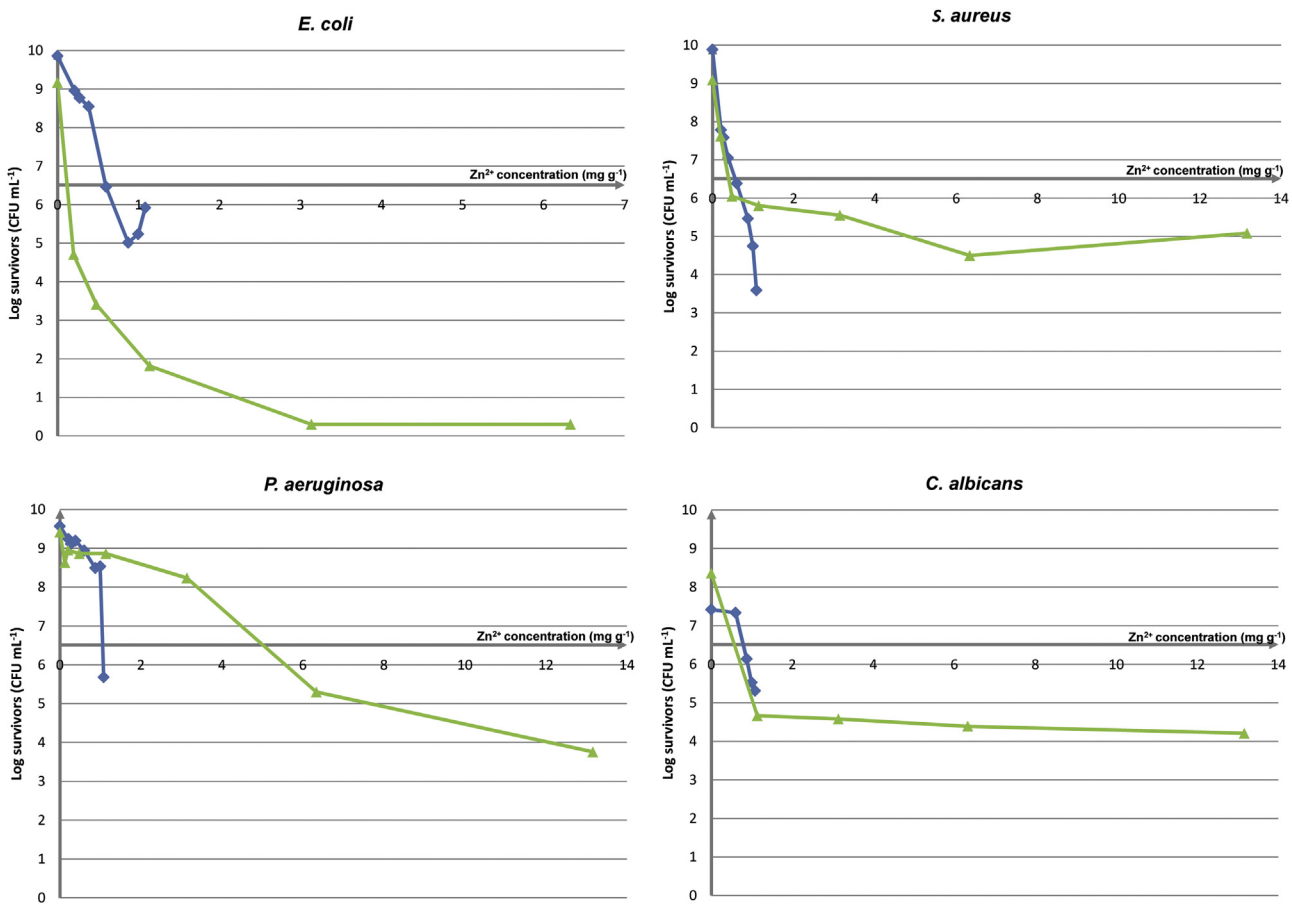


Fig. 3. Microbial populations as a function of zinc ions concentration as analyzed in the supernatant of ZnO suspensions (blue diamonds) and in zinc gluconate solutions in MH broth (green triangles). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

also sensitive to the ZnG complex. On the contrary, the antimicrobial activity of ZnG against *S. aureus* and *P. aeruginosa* was less than for the supernatant of ZnO suspensions. The type of bacterial membrane does not allow sorting antibacterial activities since the two gram negative strains *E. coli* and *P. aeruginosa* showed different sensitivities to ZnG and soluble zinc species from ZnO. The antimicrobial activities of soluble zinc species appeared strain-specific. Conversely, the effect of ZnO-1 particles and ZnG are almost similar for the bacterial strain *E. coli*; their MIC and MBC were of similar magnitude, showing that the contributions of the presence of ZnO particles *in situ* compensated the difference of activities between ZnG and the ZnO supernatants. This result proved that the combination of the three antimicrobial mechanisms is of major importance to exert a significant effect on these microorganisms compared to a zinc salt.

The antifungal activity against *C. albicans* was the same for the ZnO suspension and the supernatant. Such a remarkable behavior indicated that the antifungal activity of ZnO was almost fully caused by the soluble zinc species. The mechanism by which the microbial membrane was altered either upon contact with ZnO particles, or by oxidation by ROS did not operate to a significant extent. This behavior is probably the result of a stronger resistance of the yeast membrane, compared to that of bacteria. Antifungal activity requires that antifungal agents enter the cells and disturb physiological events of vital importance inside them. ZnG appeared more effective against *C. albicans* than ZnO particles and soluble species from ZnO dissolution. Indeed, a significant reduction of the initial population was observed after 48 h of contact with ZnG compared to ZnO suspensions and supernatant for which yeast populations remained at the same concentration with respect to contact time after inoculation. These results showed that ZnG is more efficient than the supernatant of ZnO-1 suspensions, suggesting that higher zinc concentrations would exhibit stronger antimicrobial activity. Therefore a better antifungal activity should be reached if Zn^{2+} could be generated from ZnO particles. The fungistatic behavior in MH broth is different of the behavior in the agar medium of the disc diffusion test where no inhibition of yeast growth was observed. Such a difference probably comes from a lesser Zn^{2+} solubilization capacity or from slower Zn^{2+} solubilization kinetics of agar than MH broth that caused a lesser release of soluble zinc species and a lower antimicrobial activity.

Finally, ZnO particles, soluble species and ZnG inhibited the germination of spores of the mold strain *A. brasiliensis* on agar plate and in liquid broth. ZnG was the most efficient between 10 mg g^{-1} and 80 mg g^{-1}. No development of mycelium was observed whereas germination of spores was observed at [ZnO-1] < 10 mg g^{-1} and at [ZnO-1] < 20 mg g^{-1} for their supernatant. These results confirmed that Zn^{2+} exhibited an activity on the spore germination, in accordance with the conclusions reached by Miller and McCallan [23].

The contribution of Zn^{2+} to the global antimicrobial activity of ZnO powders was definitely significant, especially against fungal strains. The direct contact between ZnO particles and microbial cells claimed by many authors as the main mechanism operating on bacteria is indeed probably the predominant contribution to the antibacterial activity. Owing to the combination of three antimicrobial mechanisms, the spectrum of antimicrobial activity is broad and ZnO appears as a suitable preservative of topical products compared to ZnG. Since the antimicrobial activity of Zn^{2+}

increased with respect to their concentration, the dissolution of ZnO particles should be enhanced again so as to reinforce the overall antimicrobial activity of ZnO.

3.2. Dissolution of ZnO

Our previous study [46] revealed that the antimicrobial efficacy of ZnO particles was dependent on the physicochemical parameters of these powders and the present study shows an important contribution of the released zinc ions to the antimicrobial activity. The present section reports studies aimed at identifying the most relevant parameters that influenced the dissolution process of ZnO in aqueous suspensions. Then, in order to optimize and maintain this mechanism over time, the influence of the environmental conditions was investigated.

The dissolution process was depending on whether the solvent was pure water or MH broth. The dissolution process of ZnO in these two media depended on time, ZnO concentration, and type of ZnO powder. The aqueous phases of ZnO suspensions prepared between 1.25 and 80 mg g^{-1} in distilled water and MH broth were analyzed for their concentration of Zn^{2+} in order to assess the dissolution of ZnO that took place during the microbiological tests.

The released Zn^{2+} concentrations immediately after immersion of the ZnO-1 powder in the liquid medium and after 24 h dissolution time under stirring were quite surprising (Fig. 4). The concentration of Zn^{2+} was strongly dependent on the concentration of solid ZnO; this feature suggesting that a slow dissolution persisted after 24 h of equilibration time. Additionally, Zn^{2+} concentration in pure water medium did not increase with respect to time and showed an odd dependence with respect to ZnO concentration.

3.2.1. Increased solubility of zinc in MH broth

A prominent feature of all solubility experiments was an increased solubility of zinc in MH broth. Indeed, Zn^{2+} concentrations in MH broth were two or three orders of magnitude higher than in water. Several authors have already reported very slow dissolution processes and an 'equilibrium concentration' in solution after a long equilibration time that depended on the type of liquid medium [45,49].

The solubility of ZnO in pure water strongly depends on the pH because there are several types of soluble zinc species. The pH of the pure water in which ZnO has been dissolved was between 6.9 and 7.0 at the beginning of the dissolution process and increased to 7.8 after a long equilibration time (14 days). The pH of the MH broth was 8.0. At such pH's, the predominant soluble zinc species are Zn^{2+} and $ZnOH^{+}$, so that the overall saturation concentration (solubility) of ZnO is controlled by the two following chemical equilibria and is given by Eq. (1) [50]:

$$Zn(OH)_2(solid) \leftrightarrows Zn^{2+}(aq) + 2OH^{-}(aq)(K_{SP})$$

$$Zn(OH)_2(solid) \leftrightarrows Zn(OH)^{+}(aq) + OH^{-}(aq)(K_1)$$

$$\text{solubility} = \frac{K_{SP}}{\left[OH^{-}\right]^2} + \frac{K_1}{\left[OH^{-}\right]} \quad (1)$$

with $Log(K_{SP}) = -16.76$ and $Log(K_1) = -10.60$. The solubility of ZnO is therefore 2×10^{-3} M at pH 7.0 and 4×10^{-5} M at pH 8.0, which correspond to 130 ppm and 2.8 ppm, respectively. The concentrations measured in pure water were equal or slightly below the solubility of ZnO, depending on time and concentration of ZnO. On the contrary, the concentrations measured in MH broth were much higher than the solubility in pure water at pH 8. Such behavior shows that chemical species contained in MH broth are able to increase the solubility of ZnO by the formation of complex species with Zn^{2+}. The components of MH broth are casein acid hydrolysate, meat extract and starch. The presence of proteins affects the ZnO dissolution as already reported. Studies carried out on human or bovine serum albumin (BSA) [33,38] showed that these proteins are able to enhance the dissolution of ZnO particles by binding their peptides to zinc [51], creating a complex of high solubility. These results were confirmed by Tantra et al. who showed a decrease of the ZnO particle size in the presence of BSA [52]. Zn^{2+} can bind to electron donors such as carboxylate and phosphate groups, and possibly with amino groups in basic medium. In the present case, casein acid hydrolysate and proteins of the meat extract might be the chemical species that were able to form complex with Zn^{2+} ions and enhance the dissolution of particles. The high solubility of ZnG in water, even in rather basic medium, is also due to the complexation of Zn^{2+} ions by the carboxylate group of the gluconate. This result points out the importance of the dispersion medium that influences the concentration of soluble zinc species, and consequently the antimicrobial activity of ZnO particles in complex formulations compared to pure water. In an example where ZnO is used as an antimicrobial preservative of cosmetic products, various peptides and proteins are usually used as active ingredients in such products: the combination of these molecules with ZnO could probably enhance the dissolution of the particles and thus the antimicrobial activity of ZnO. The protein–Zn complex probably displays an antimicrobial activity, as it is the case for ZnG.

The zinc concentrations measured in the liquid media are above the concentrations between 10^{-7} and 10^{-5} M required for an optimal microbial growth *in vitro* [18]. The zinc concentrations that prove toxic strongly depend on the type of microbial strain and the duration of exposure, so that it is difficult to tell a priori whether such concentration can show antimicrobial activity. It was claimed that zinc concentrations lower than 5 Mm (corresponding to 330 ppm) in broth media were not toxic to microorganisms [21]. The concentrations of Zn in the MH broth were above this threshold, so that an antimicrobial activity of the soluble zinc species was expected on this basis. An antimicrobial activity coming from the supernatant of ZnO dispersions in MH broth has indeed been observed. The concentrations of Zn^{2+} in pure water were below the limit, suggesting that the soluble zinc species did not contribute to the antimicrobial activity of ZnO.

3.2.2. Acceleration of dissolution kinetics in MH broth

Since Zn^{2+} concentrations measured in the liquid phase of ZnO suspensions in water and MH broth were different immediately after dispersion of ZnO powder and after 24 h dissolution time, the kinetics of dissolution of ZnO appears very slow. The concentration of soluble zinc species was dependent on the concentration of solid ZnO, although the concentration in the liquid phase should have been kept constant and equal to the equilibrium solubility of ZnO. This later observation also strongly suggested that the concentration at saturation has not been reached after 24 h equilibration time. The slow rates of dissolution were measured by means of kinetic experiments of dissolution.

The kinetics of ZnO dissolution in pure water appeared quite complex. In all dissolution experiments, a burst release of Zn^{2+} ions took place immediately upon dispersion of ZnO powder. Zn^{2+} concentration decreased during the first day and started to increase after longer times. The equilibrium concentration was not reached after 48 h even for the most concentrated (80 mg g^{-1}) ZnO dispersion (Fig. 5). The kinetics of dissolution were slower for lower concentrations of ZnO since it have shown that the kinetics ran faster with respect to the concentration of ZnO (Fig. 4). The combination of complex kinetics (burst dissolution followed by re-precipitation, and finally dissolution again) together with the concentration-dependent dissolution rate was the origin of the apparently erratic behaviors shown in Figs. 4 and 5. The dissolution was much faster in MH broth. For the highest ZnO concentration

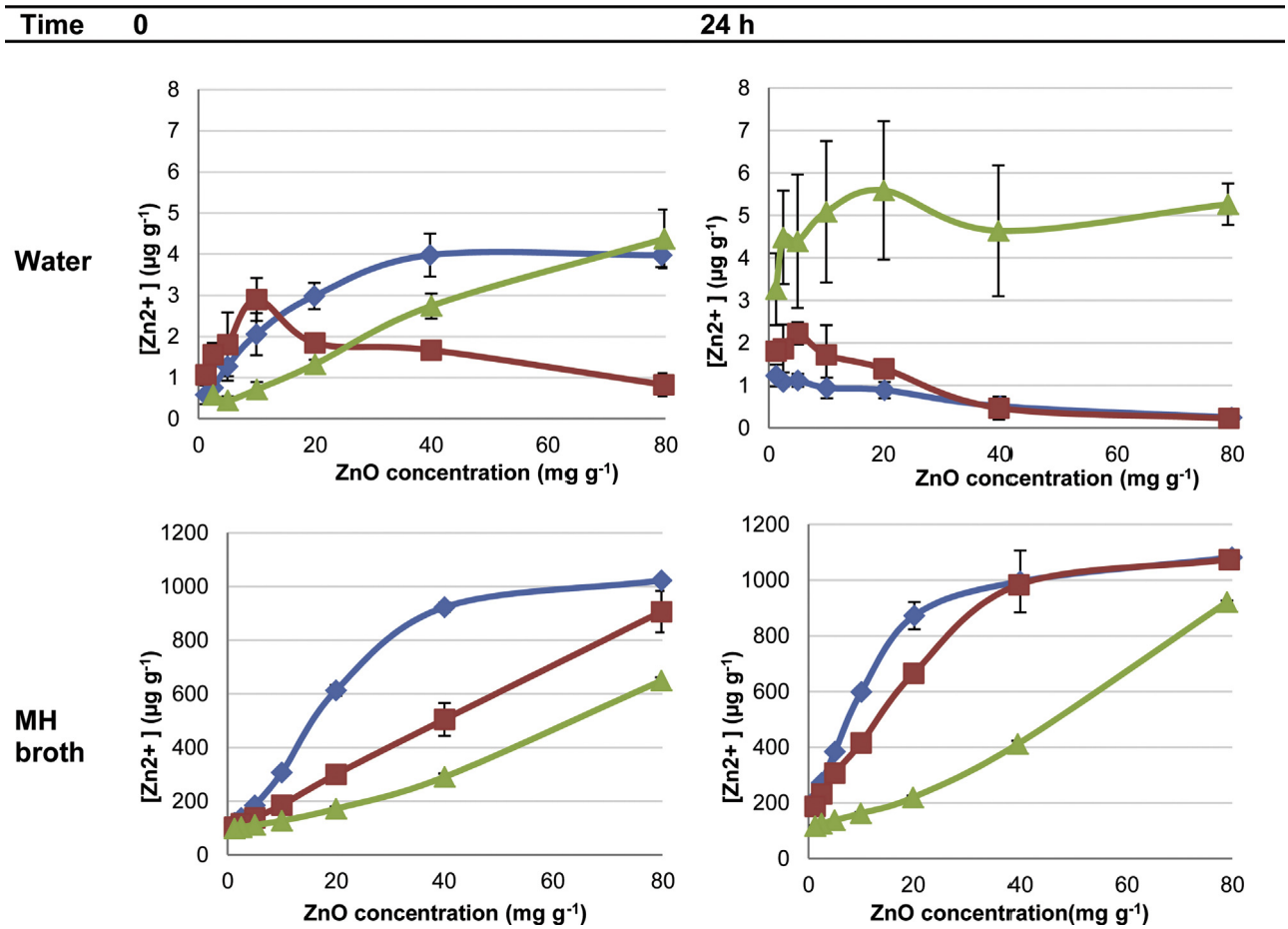


Fig. 4. Dissolution profiles of ZnO-1 (blue diamonds), ZnO-2 (red squares) and ZnO-3 (green triangles) in distilled water and Mueller Hinton broth at $t = 0$ and $t = 24$ h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tested (80 mg g^{-1}), a constant Zn^{2+} concentration of ~1000 ppm was reached within less than 6 h for ZnO-1 and ZnO-2; the dissolution of ZnO-3 took a longer time, approximately 24 h. This was a second beneficial effect of the MH broth to the dissolution process. Not only the MH broth increased the solubility of ZnO, it even made the dissolution faster than in pure water.

In order to confirm that the accelerated dissolution in MH broth was due to the complexation of Zn^{2+} ions by the components of the culture broth in relation to the concentration of the complexing species, comparative dissolution experiments in broth medium at two different concentrations were performed. The ZnO-1 powder was suspended in pure MH broth, a 1:5 dilution was applied after 24 h equilibration time, and the supernatant was collected by centrifugation and analyzed for Zn (sample A). Another sample was prepared by adding the ZnO-1 powder to the 1:5 pre-diluted MH broth (sample B). The concentration of Zn^{2+} was 1.7 times higher in sample A ($[Zn^{2+}] = 900 \pm 20$ µg g^{-1}) than in sample B ($[Zn^{2+}] = 530 \pm 5$ µg g^{-1}), showing that the contact with a high concentration of complexing species accelerated the dissolution of ZnO. Finally, the dissolution kinetics depended on the concentration of ZnO because the contact area between the solid ZnO and the liquid medium was higher when the amount of ZnO powder was larger.

A complex dissolution behavior was observed in water because the dissolution kinetics was slow. It is likely that the same behavior was also present in MH broth and could not be observed because

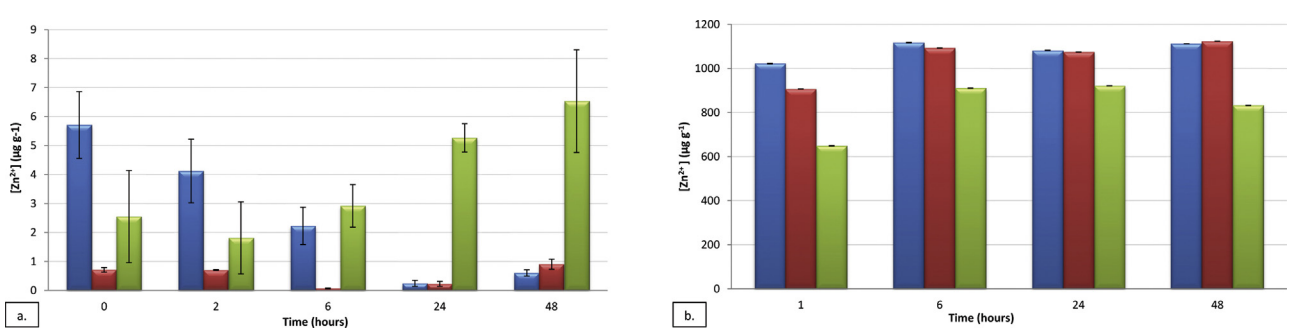


Fig. 5. Dissolution profiles from ZnO-1 (blue), ZnO-2 (red) and ZnO-3 (green) suspensions at 80 mg g^{-1} until 48 h in water (a) and in MH broth (b). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the kinetics was faster. The decrease of Zn^{2+} concentration after the initial burst dissolution of Zn^{2+} meant that a re-precipitation was taking place. One possible mechanism was the dissolution of Zn^{2+} and $ZnOH^{+}$ ions from ZnO and precipitation of the hydroxide $Zn(OH)_2$ onto the ZnO surface. The layer of $Zn(OH)_2$ at the surface of the ZnO particles made a barrier against the dissolution, so that the overall dissolution was very slow.

3.2.3. *Influence of physicochemical parameters of ZnO powders on the dissolution kinetics*

The three ZnO grades have been fully characterized for their elementary particle size and shape, aggregate size and related parameters such as specific area and density of powders. The dispersions in water and MH broth have also been characterized (Fig. 6). The ZnO-3 grade was quite different of the two others ZnO-1 and ZnO-2; it was characterized by a lower specific area, a larger crystal size and a rod-like shape of particles. ZnO-1 and ZnO-2 were very close considering their specific area and had both a platelet shape but they differed by their porosity. These two powders were mesoporous with pore sizes in the range 2-100 nm; the porosity of ZnO-1 was higher than ZnO-2. The powder density of ZnO-2 was the highest compared to the other grades. As a consequence, ZnO-2 aggregates settled faster than ZnO-1 and ZnO-3 (which behave similarly).

The dissolution behavior was different for the three ZnO grades. A rapid dissolution of ZnO particles during the first hours after the preparation of the suspension was observed for all grades, but was particularly remarkable with ZnO-1. Further dissolution up to the saturation concentration was very slow. Such burst initial dissolution followed by a slow dissolution that lasted over 72 h has also been noticed by several authors [30,34–36]. The different behaviors of the three ZnO grades revealed an influence of the

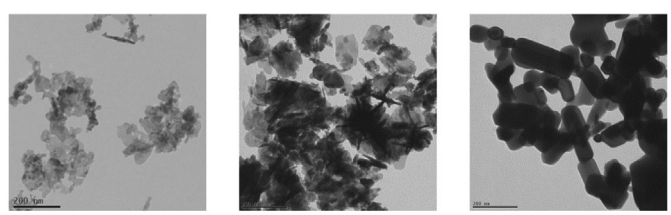
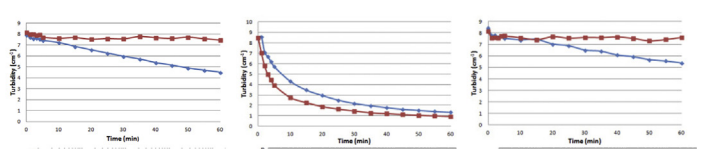
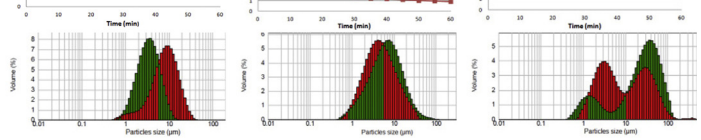
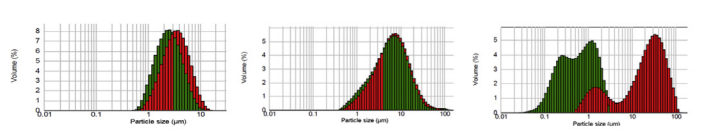
	ZnO grade	ZnO-1	ZnO-2	ZnO-3
CHARACTERISTICS OF ZnO PARTICLES				
Specific area ($m^2\ g^{-1}$)	BET method	39.1	46.9	8.25
Mean pore size (nm)	BJH method	17.3	11.6	10.6
Volume of pores between 1.7 and 300 nm ($cm^3\ g^{-1}$)	BJH method	0.18	0.16	0.02
Crystallite size (nm)	X-ray diffraction	14.7	17.5	76.2
Apparent density ($g\ cm^{-3}$)		0.38	0.89	0.56
Thickness of elementary particles: mean ± SD (nm)	TEM	17.2 ± 2.8	11.5 ± 4.2	/
Width of elementary particles: mean ± SD (nm)	TEM	41.7 ± 20.2	55.0 ± 27.4	85.7 ± 33.4
Length of elementary particles: mean ± SD (nm)	TEM	107.0 ± 40.2	92.6 ± 38.1	146.8 ± 51.7
Morphology of elementary particles	TEM	Platelet shape	Platelet shape	Rod-like shape
TEM images	TEM			
BEHAVIOR OF ZnO AGGREGATES IN AQUEOUS MEDIA				
Isoelectric point	Electrophoretic light scattering	9.75	9.6	9.9
Sedimentation against time (blue curve: before dispersion; red curve: after dispersion)	Turbidity from UV-visible absorbance at 380 nm			
Particle size distribution in water (red) and MH broth (green)	Small-angle light scattering			
Particle size distribution in MH broth (red: before dispersion; green: after dispersion)	Small-angle light scattering			

Fig. 6. Main physicochemical characteristics of the three tested ZnO grades.

physicochemical characteristics of the particles on their dissolution. The pH increased also as the dissolution of ZnO proceeded in accordance with previous observations by Chowdhury et al. [53]. Dissolution kinetics of ZnO-1 and ZnO-2 in MH broth were similar and it was significantly slower for ZnO-3. This behavior correlates nicely with the specific areas of these powders: the specific areas of ZnO-1 ($39\,m^2\,g^{-1}$) and ZnO-2 ($47\,m^2\,g^{-1}$) were similar, whereas ZnO-3 had a much lower specific area ($8.25\,m^2\,g^{-1}$) [46] that allowed a lower contact area with the liquid medium and a slower dissolution rate. Such a simple picture meets expectations based on common sense. Several authors claimed that dissolution was a size-dependent phenomenon [33,41,43,54], faster dissolution being for the smallest particles. This is in agreement with the present study in MH broth since surface area and size of elementary particles are related together for a given particle shape. Such a trend was not observed in pure water, in agreement with Franklin et al. who found similar dissolution profiles for nanoparticles and coarse powders of ZnO [34].

The behavior in pure water was much more complicated. The order of the initial burst release of Zn^{2+} ions was ZnO-1 > ZnO-3 > ZnO-2. It did not follow the order of the specific areas as in MH broth. Another independent parameter obviously contributed to the dissolution behavior. It was suggested that the shape of the elementary particles influenced the dissolution in agreement with Peng et al. [35] who reported that the dissolution of spherical ZnO particles was faster than that of rod-shaped particles. The state of dispersion of aqueous suspensions of ZnO appeared as an important parameter governing the dissolution phenomenon. The partial dissolution of ZnO particles was thus improved by breaking agglomerates. The shape of ZnO particles appeared a decisive parameter governing the dispersion ability (Fig. 6). Ultrasonic dispersion of ZnO-1 and ZnO-2 grades did not cause significant differences of aggregate diameters with respect to powders dispersed by gentle stirring with a magnetic bar. Conversely ZnO-3 aggregates could be efficiently dispersed into smaller aggregates; a population of particles of submicron sizes was measured after ultrasonic dispersion for ZnO-3. Aggregates of platelet shaped ZnO particles were harder to break under mechanical dispersion process than rod-shaped particles, probably because the adhesion forces operating between the flat faces of platelets were quite strong. Therefore, the influence of the shape of elementary particle on the dissolution kinetics might be an indirect consequence of its influence on the dispersion ability.

Re-precipitation was massive for ZnO-1 and ZnO-2suspensions in water, whereas it remained of limited extent for ZnO-3. The final dissolution rates for long times were in the order ZnO-3 > ZnO-2 > ZnO-1, ZnO-2 and ZnO-1 being close to each other. Such a complex pattern of dissolution behavior made difficult the search for a rationale. It was presumed that re-precipitation took place by either growth of ZnO particles or heterogeneous nucleation at the surface of already existing ZnO particles. Indeed heterogeneous nucleation is easier than homogeneous. The slow re-precipitation of ZnO-3 presumably came from different surface groups onto which $Zn(OH)_2$ could bind, causing particle growth or secondary nucleation. Indeed, the shape of elementary particles of ZnO-1 and ZnO-2 was platelet-like, whereas it was rod-like for ZnO-3. The crystallographic planes exposed to the external surface of the crystals were then different, which could have been the origin of the different behaviors regarding re-precipitation of $Zn(OH)_2$ onto the ZnO crystals.

Finally, the density of the powders appeared an important parameter. Indeed, the densest ZnO-2 grade generated the lowest concentrations of soluble Zn^{2+}. This parameter is related to the other ones previously discussed since high powder density was related to low dispersion ability. Another consequence of a high density of the aggregates was a fast sedimentation in water even after application of a powerful dispersion step (Fig. 6).

The dissolution kinetics gives a rationale to consider the contribution of the soluble zinc species to the antimicrobial activity of ZnO in MH broth. Zn^{2+} concentrations measured after 24 h of dissolution of ZnO-1 were higher than those of ZnO-3. The antimicrobial activity of the supernatants of the ZnO-1 and ZnO-3 correlated well with this observation since the ZnO-1 supernatant exhibited a better antimicrobial efficacy than ZnO-3 supernatant on all the

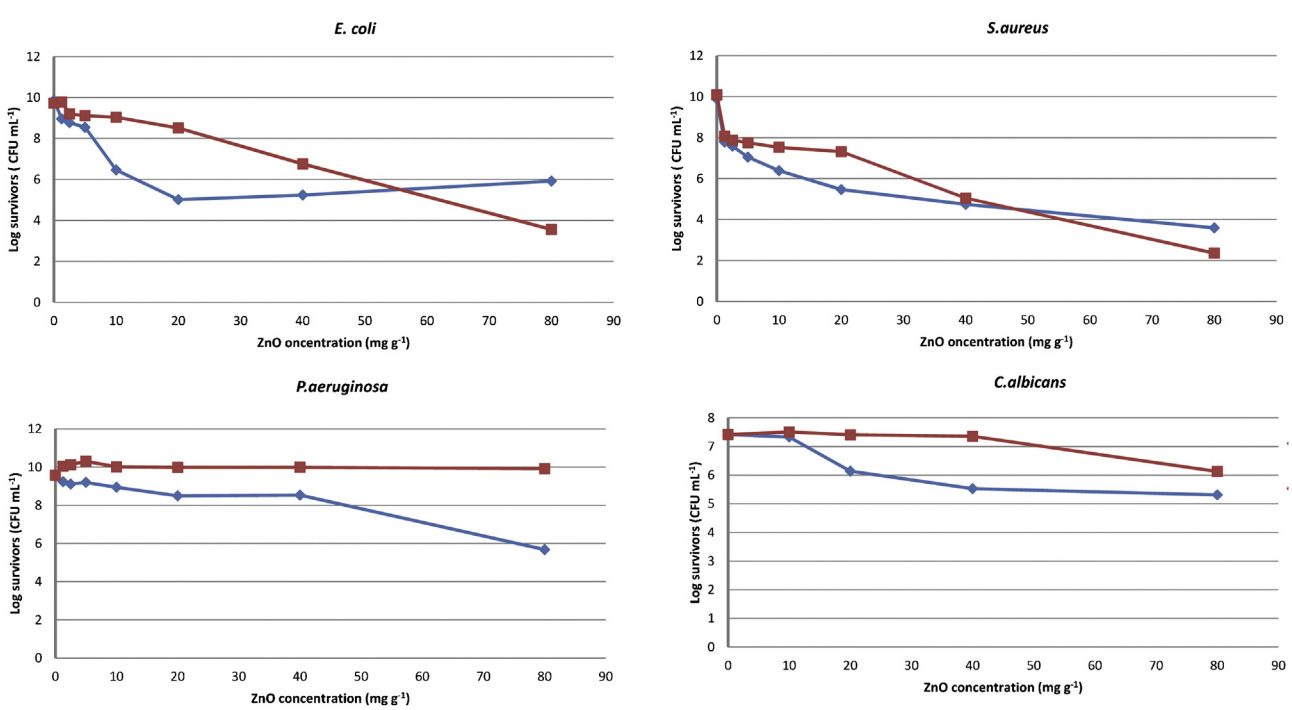


Fig. 7. Comparison of the antimicrobial effect of ZnO-1 (blue diamonds) and ZnO-3 (red squares) suspensions supernatants in Mueller Hinton broth after 24 h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

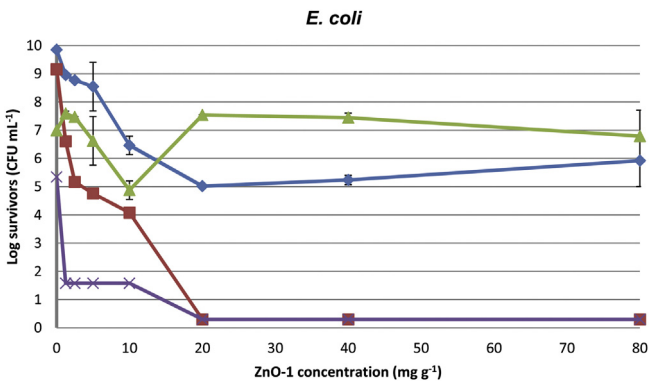


Fig. 8. *E. coli* population as a function of ZnO-1 concentrations (mg g^{-1}) after 24 h of contact to the ZnO-1 suspensions in water (purple cross) and MH broth (red squares) or their supernatant in water (green triangles) and MH broth (blue diamonds). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tested microbial strains (Fig. 7). These results show that the partial dissolution of ZnO is an important mechanism contributing to the global antimicrobial activity. It is worth noticing that the solubility enhancement by the complexing species contained in the MH broth increases the antimicrobial activity coming from soluble zinc species, but the kinetics of dissolution also appears as a crucial parameter.

3.3. *Antimicrobial activity of ZnO and released Zn^{2+} ions in pure water*

Since the complexing molecules present in the liquid medium look necessary in order to ensure a zinc concentration above the MIC or MBC of Zn^{2+} ions, the possible activity of Zn^{2+} ions in pure water remains an open issue. Indeed the concentrations of Zn^{2+} ions in the supernatants of ZnO dispersions in pure water were very low. The antimicrobial activity of ZnO dispersions and their supernatants were evaluated in pure water on *E. coli* (Fig. 8). Pure water is not a favorable medium for the growth of bacteria. Thus, *E. coli* inoculated at 10^6 CFU mL^{-1} in pure water grew up to 10^7 CFU mL^{-1} in 24 h, which was a moderate but significant growth. The antimicrobial activity was evaluated by comparison with the 'positive control' free of ZnO. The microbiological data confirmed the assumption of several authors who stated that the concentration of Zn^{2+} is too low in pure water. Indeed, population *E. coli* population grown in the supernatant of ZnO suspensions in pure water was the same whatever the concentration of ZnO. The positive control gave the same concentration of bacteria as in the supernatants of ZnO suspensions. On the contrary, the full ZnO suspensions showed a high antibacterial activity because of the presence of the ZnO particles. The same experiments carried out in MH broth allow the positive control to reach 10^{10} CFU mL^{-1} and showed the large antibacterial activity of both the full ZnO suspensions and its supernatant.

4. Conclusion

The partial dissolution of ZnO in aqueous media releases soluble zinc species that show antimicrobial activity. A high enough concentration of soluble zinc species requires the presence of complexing molecules in water that form water-soluble zinc complexes. In that way, the overall concentration of zinc is much larger than in pure water. The soluble zinc species are not free Zn^{2+} ions however, but it appeared that the zinc complexes had a high antimicrobial activity. ZnG is a well-known example of such an antimicrobial complex of zinc [55]. The contribution of the zinc release in the MH broth medium gives a significant contribution to the overall antimicrobial activity of ZnO suspensions.

The control of the concentration of soluble zinc species is a difficult matter because of the very slow dissolution of ZnO and the complex dissolution phenomena. Complexing molecules contained in a broth medium increase the solubility of zinc in the liquid medium. They also make the dissolution kinetics faster. The combination of the two phenomena definitely appears crucial. For an application of ZnO either for an antimicrobial topical formulation, or as a preservative of a cosmetic product, this study brought some keys to understand the dissolution phenomenon of ZnO. Since the spectrum of antimicrobial activities of ZnO particles and Zn^{2+} ions are different, the combination of several mechanisms broadens the spectrum of antimicrobial activity.

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