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Evaluation of Antibacterial Efficiency of Zinc Oxide Thin Films Nanoparticles against Nosocomial Bacterial Strains

Rania Dadi[a], Eloïse Kerignard[a], Mamadou Traoré[b], Christine Mielcareck[a], Andrei Kanaev[b], Rabah Azouani[a*]

[a]Ecole de Biologie Industrielle, 49 avenue des Genottes, CS 90009 – F95895 Cergy Cedex
[b]Laboratoire des Sciences des Procédés et des Matériaux, Université Paris 13, Sorbonne Paris Cité, 99 Avenue Jean Baptiste Clément, 93430 Villetaneuse, France
r.azouani@hubebi.com

The development of thins layers with antimicrobial priorities has been a key factor for inhibiting the growth of microorganisms and preventing nosocomial infections. This research work focuses on the synthesis and characterization of thin layers of functional metal oxides based on ZnO nanoparticles for applications to surfaces and coatings on various supports (paints, coatings …). In our work we developped as antibacterial agents, nanoparticles of zinc oxide.
The synthesis of nanoparticles is performed with sol-gel method, we obtained nanoparticles around 3 nm of diameter. The antibacterial activity has been studied on strains from the European pharmacopoeia; *Staphylococcus aureus* (ATCC 6538) and *Escherichia Coli* (ATCC 8739). The test consists in artificial contamination by inoculum of microorganisms. We developed specific methodology for thin layer antibacterial tests. Results show an inhibition of bacterial growth by reduction greater than 5 $\log_{10}$ of the initial bacterial population.

1. Introduction

Even though the existence of nanotechnologies is still recent, this infinitesimal world is already part of our daily lives. Nanomaterials - which by definition are materials containing at least 50% nanoparticles - are also widely used, like carbon nanotubes used in electronics, textiles and construction.
Health and medicine are no exception to this dynamic: human biology, governed by molecular phenomena, is one of the most promising fields of application of nanotechnology. They make it possible to structure molecular assemblies intended to interact, treat or reconstitute a particular tissue or organ in the human body. These tools also make it possible to miniaturize devices which will be unprecedented aids for in or ex vivo diagnosis.
In view of bacteria resistance to antibiotics (Lowy, 1998 and Komolafe, 2003) new materials with antibacterial property are in increasing interest. Metal oxide nanoparticles are becoming increasingly important in both industry and biomedical research (Azam et al, 2012). Biomedical use of metal oxide nanoparticles gaining increasing interest of researchers duo to their high antibacterial activities at low concentrations (Project on Emerging Nanotechnologies). This property is related to the great surface/volume ratio, which makes them more active on the surfaces of bacteria. In this work, a great interest was focused on Zinc oxide (ZnO).
Zinc is a transition metal, when he takes ZnO form some of its properties are increased. Biologically, zinc is also an essential element involved in many biological processes. It enters in composition of many enzymes where it plays a catalytic, structural or regulatory role. Zinc deficiency can induce to pathologies or dysfunction, but an excess of zinc gives way to pathologies that disrupt cellular homeostasis (Mueller et al, 2010). It can cause significant cellular damage, which gives it antibacterial and antifungal properties (Hackenberg et al, 2011).
The mechanism of action of nanoparticles remains complicated to explain but suggest that bacteria are unlikely to develop resistance. This would require that the bacteria acquire several mutations to escape the action mechanisms of the nanoparticles (Mueller et al, 2010, Dadi et al 2019).

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The objective of this work is the elaboration of zinc oxide nanoparticles ZnO in thin layer, in order to measure and optimize their biocide activity, especially for the decontamination of surfaces. Nanomaterials are synthesizing by sol-gel process, not expensive method compared to conventional ones and allows the production of materials with high purity and homogeneity (Haussonne, 1993 and Klein, 1994).

2. Experimental

2.1 Materials

Specifications of chemical reagents used in these experiments are summarized in Table 1.

Table 1: Chemicals, their purities and their origin

Chemical reagents	Purity	Provider
Isopropanol CH_3OCH_2-CH_2-OH	99.5 %	Acros Organics
Monoethanolamine (MEA) H_2N-CH_2-CH_2-OH	99%	Merck Millipore
Sulfuric acid H_2SO_4	96%	Panreac
Zinc acetate dehydrate Zn $(CH_3$-$COO)_2$, $2H_2O$	99%	Merck Millipore
Plate count agar	100%	Biomérieux
Staphylococcus aureus ATCC 6538	100%	Pasteur France
Escherichia Coli ATCC 8739	100%	Pasteur France

2.2 Thin films preparation

ZnO thin films were produced using four main steps according to the sol-gel process as shown in the flowchart below:

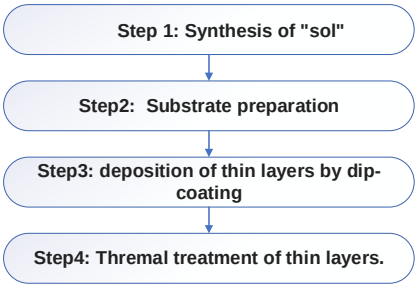

Figure 1: Flowchart of sol gel process

Stepe1 Sol synthesis: this stage involves two types of reactions; hydrolysis and condensation, the solution is prepared inside a glove box from "zinc acetate dihydrate" precursor dissolved in a mixture of isopropanol and monoethanolamine, respectively solvent and stabilizer. Magnetic stirring was carried out for 2 hours at 60°C, after 24 hours of rest, this solution was filtered using paper filter. The molar ratio [MEA] / [ZnO] = 1 and the concentration is 1.5 mol / l. All experimental details about this synthesis are reported in our previous work (Dadi et al, 2019).

Step 2 Substrate preparation: The substrate chosen are glass slides (76 mm x 26 mm, thickness = 1.1 mm) with refraction index of 1.5 at 633 nm, which are commonly used for their advantages of -OH groups allowing covalent adhesion of NPs. The preparation of surface is a very important step to avoid chemical contamination by halogenated residues. For this, the slides are firstly washed with soap and introduced into a beaker containing sulfuric acid with 96% purity for one hour. Then, the slides are rinsed with distilled water until a neutral pH and placed in an oven for 24 hours at 80°C before any deposit.

Stee 3 Deposition of thin layers: Several techniques are developed for the deposition of thin layers with this substrate. Dip-coating was performed on dip-coater KSV Mina KN 4001, which is fast and easy to implement (Brinker et al, 1992 and Brinker et al, 1994).

The clean support is immersed in the ZnO colloidal solution and then slowly withdraws at constant speed to form a uniform thin layer. This fixation takes place through chemical reactions with the active -OH sites on the support surface. Thin layers were obtained with the formation of a chemical bonds with -OH groups of the substrate, allowing high mechanical stability comparing to physical adhesion. This operation was repeated until obtaining desired thicknesses. After each layer, slides are dried in the oven at 80 ° C for 10 min (figure 2).

Step 4 Thermal treatment of thin films: The thin layers, once deposited, undergo a heat treatment. First, each layer is heat-treated at 80°C in the oven for 15 minutes to evaporate the solvent and at 250°C for 10 minutes to remove organic residues. After, the ZnO sample was crystallized at 500°C in an electric oven for one hour, this thermal treatment is an important process to obtain a desired crystalline structure of the deposit. The heating rate used is 5 °C/min.

2.3 Nanoparticles characterization

To perform nanoparticles characterization TEM and SEM methods was used.
The transmission microscope used in this work is a 2011 JEOL equipped with a GIF filter 200. GATAN. Observations were made with an acceleration voltage of 200 keV. The samples were ground and prepared in suspension in a solvent (isopropanol) by ultrasound before being deposited on the surface of a covered copper observation grida film of polymer and carbon.
The SEM technique produces images on surface of a sample using the principle of electron-matter interactions. The analysis of microscope images is a reference method in the morphological characterization of nanoparticles. The scanning electron microscopy images presented in this work were carried out on a Zeiss Supra 40VP and Leica 440 type microscope with varying acceleration voltage between 1 and 30 kV. For glass plate substrates that are not conductive, 10nm Au / Pd or 3nm thick carbon deposits were made before shift to imagery. The resolving power under normal conditions of use is of 2 nm for the SEM FEG (scanning electron microscope equipped with an effect gun field) and 25 nm for the conventional SEM.
Also a X-ray Dispersive Energy was carried out. It is both a routine control technique and a powerful method of investigation, the Scanning Electron Microscopy (SEM) HITACHI TM 3000 is an extremely versatile for the study of surfaces and fine structures associated with microanalysis by X-ray Dispersive Energy (EDX) SwiftE d 3000 which allows local or global analysis of the most diverse materials. In addition, this EDX technique allows performing an analysis quantitative both on massive samples of a few square centimeters as well as on fragments, particles or residues of a few tenths of a millimeter. The sample to study is placed under vacuum, an electron beam is scanned across the surface and an image of the surface is produced, in addition, an elementary chemical microanalysis of the surface of the object by the EDX method.

2.4 study of the antibacterial activity with microbiological tests

A new method of bacterial detaching from the slides was developed based on the French standard NF EN 14561. Most bacteria have the ability to adhere to any surface via an adhesion mechanism by secreting a network of polysaccharide fibers. In order to improve the counting of bacterial viability, it is necessary to "snatch" the bacteria by cleavage of the polysaccharide chains. To do this, the method adopted includes two stall techniques: physical method (vortex agitation) and chemical method (detergents and chelators).
The principle of this method is based on spreading a bacterial suspension at 10^5 CFU / mL on a glass germ carrier. After drying in an oven at 37 ° C for 60 min, the germ carrier is immersed in a chemical agent solution which will allow the bacteria to detach from the surface of the germ carrier, followed by an abrasive physical treatment (glass beads + vortex agitation) to complete the stall operation. The solution then undergoes two successive dilutions, the germ carrier is also placed in a petri dish with supercooled agar and then incubated at 37 ° C. for 24 hours. After incubation, viable and cultivable microorganisms are then counted.

3. Results

3.1 TEM analysis

Analysis by SEM scanning electron microscopy showed that these films are homogeneous, dense and formed of spherical particles. The figure below presents SEM images showing deposits formed of particles of ZnO with a diameter close to 30-40 nm.

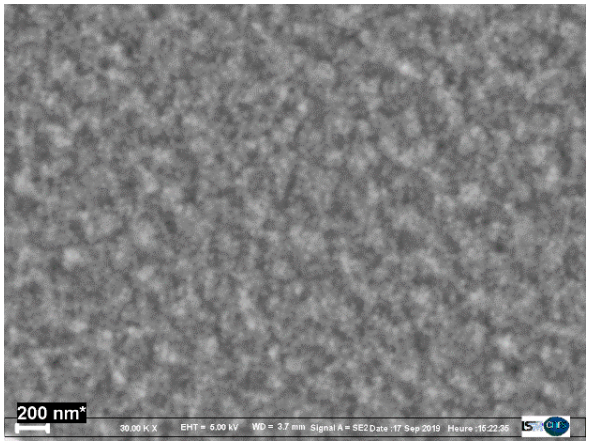

Figure 2: TEM images of ZnO nanoparticles and ([MEA] / [Zn2 +]) = 1 [Zn2 +] = 1.5 mol / L, 5 layers, 500°C

Figure 2 shows weakly dispersed and fairly agglomerated ZnO nanoparticles, with an average size of 37 nm. Oxides prepared from acetate precursors produce fairly well dispersed spherical shaped particles whose size is within the nanometer range. The film thickness increases with the number of layers deposited.

3.2 SEM analysis

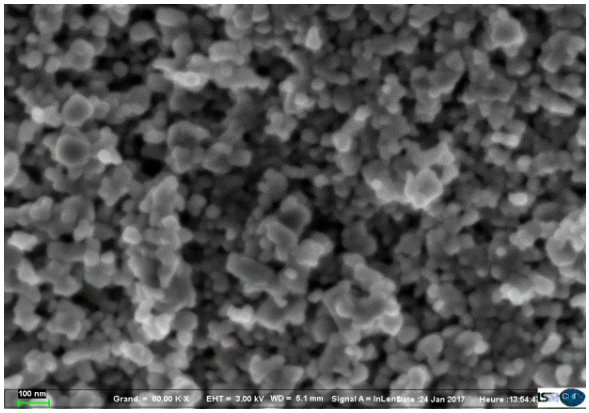

Figure 3 : SEM images of ZnO nanoparticles and ([MEA] / [Zn2 +]) = 1 [Zn2 +] = 1.5 mol / L, 5 layers, 500°C

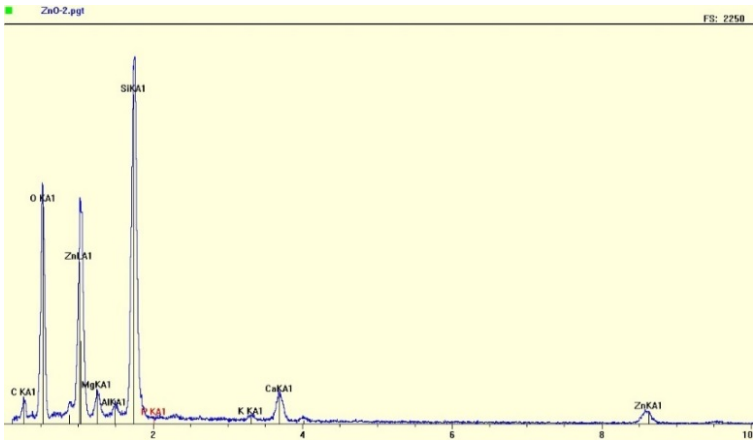

Figure 4 : ZnO thin films Spectrum (Concentration=0.75M, T = 500°C, 5 Layers)

The SEM scanning electron microscopy analysis was done on thin layer of ZnO, it showed that these films are homogeneous, dense, and formed with mostly spherical particles.
The Figure 4 shows ZnO thin films spectrum with concentration of 0.75M, treated at 500 ° C. We essentially seek to the most intense peaks of Zn, Si and other elements present in the form of traces. On the spectrum, we can identify around 1 KeV the characteristic line of Zn and the Si line around 1.8 KeV due to the use of the glass plate as substrate (an analysis of the blank plate was carried out to serve as a control and shows in addition to Si, the presence of calcium in small quantities). The presence of carbon is due to organic contamination. Thus, these results confirm the presence of a ZnO deposit on the glass plates.

3.3 Growth inhibition of *S. aureus* and *E. coli* by ZnO nanoparticles

The results obtained after incubation of the slides are presented in figure 5 and show a decrease in the viability of the strains used when they are in contact with thin films of ZnO NPs (figure 5 b, c). In fact, no bacterial growth is observed on the agars in which the slides of thin layers of NPs have been placed in comparison with the positive control (Figure 5 a).

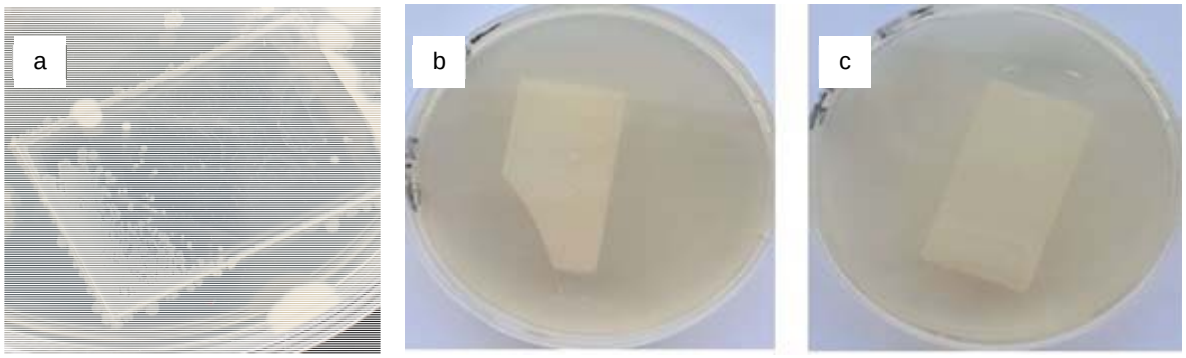

Figure 5 : Results after 24h of incubation at 37°C of slides in contact with bacteria (a) Positive control, (b) ZnO/ E.coli , (c) ZnO/ S.aureus

Growth inhibition of *S. aureus* and *E. coli* is analyzed by comparing petri dish growth of bacterial populations deposited on slides with (thin films) or without (positive control) concentration of ZnO nanoparticles 1.5 mol/L and then recovered by washing (NF EN 14561). Measurements were done in triplicate, the results obtained show a bacterial inhibition of *S. aureus* and *E.coli as shown in figure 6* . The technique used makes it possible to recover all the germs for *S. aureus and E.coli*. Indeed 4.0 10^5 CFU/ml are deposited on a glass slide and 3.9 10^5 and 4.2 10^5 CFU/ml are recovered for *S. aureus and E.coli* respectively. Compared to the positive control (Figure 5-a) there is a decrease of 5 $\log_{10}$ after a drying and contact time of 60 min at 37°C

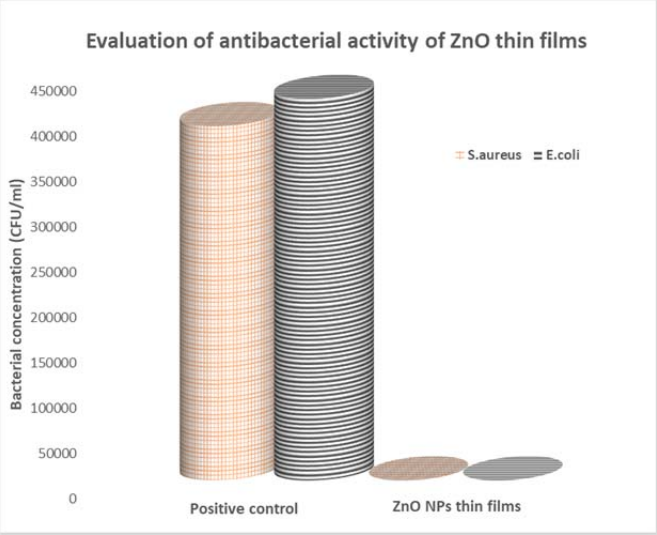

Figure 6: Evaluation of antibacterial activity of ZnO thin films after 24h of incubation at 37°C

4. Conclusions

In this study, we developed elaboration of nanodeposits ZnO thin layer with sol gel method on glass substrates, using colloidal solutions of nanoparticles at concentration of 1.5 mol/L. The colloidal solution was characterized by TEM and DLS, the results of these two characterizations lead to nanoparticles of average sizes of 3 nm. Whereas thin films have been characterized by SEM, the results converging towards the conclusion of the presence of a layer of zinc oxide. These nanomaterials based on ZnO nanoparticles have demonstrated an antibacterial power against *S. aureus* and *E. coli*. In fact, a 99.999% reduction from the initial bacterial population is observed. The challenge of this work is to produce stable formulations with these nanoparticles for applications as thin layers on surfaces. For this, we have to obtain products with good covering power, strong mechanical resistance and maintaining antibacterial activity.

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