Interaction of Bacteria With Nanostructured Zinc-oxide Thin Films

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***Abstract* – The effect of nanostructured ZnO thin films on one Gram positive and one Gram-negative bacterium is studied. The films are prepared by different methods: (i) RF magnetron sputtering of ZnO target in atmosphere of Ar (0.5 Pa) or Ar (0.5 Pa)+H_2 (0.1Pa), (ii) Sol-gel – glass substrate is dip coated in a colloidal sol prepared from zinc acetate, dried and then fired at high temperature in order to get thin ZnO films; (iii) Chemical deposition – seeds of ZnO are first casted on a glass substrate and then ZnO nanorods are repeatedly grown on them via deposition from a chemical bath. The ZnO films structures are studied by XRD, SEM and AFM. All patterns have a polycrystalline structure with preferential (002) crystallographic orientation and c-axis perpendicular to the substrate surface. The influence of the as-prepared films on *Bacillus cereus* and *Pseudomonas putida* is studied by two different methods - optical density measurement and classic cultivation (rich and poor medium). Periodic cultures of bacteria are investigated in a 24-hours experiment for sensitivity to the ZnO thin films immersed in the bacterial suspension. Our experiments prove that ZnO films made by wet colloidal methods (sol-gel or chemical bath) are toxic to the studied bacteria. The ZnO thin films obtained by r.f. magnetron sputtering activate the rate of cell division and increase the percentage of live cells in comparison with the control experiment (without ZnO film). The observed difference can be due to the release of zinc species from the colloid-made films.**

***Index Terms – Bacillus cereus, Pseudomonas putida*, nanostructured ZnO thin film**

I. INTRODUCTION

The effect of a new synthesized chemicals and materials on environment is often determined by bioassays. They are quantitative measure of the total toxic potential of a sample and show possible synergistic or antagonistic effects of contaminants and media, in which they occur. Microorganisms and cell cultures are preferred in toxicity studies of objects, because of their short test duration, low cost, environmental friendliness and large amount of the investigated organisms. *Pseudomonas putida* ATCC11778 and *Bacillus cereus* ATCC12633 tests are widely used for toxicity assessment of water and new synthesized chemical compounds [1,2,3]. Some investigations of ZnO nanoparticles have shown their toxicity to bacterial cells [4,5,6]. Yamamoto [7] has reported about increased toxicity of nanoparticles (of diameters smaller than 100 nm) on bacteria with decreasing of the nanoparticle size. Li et al. [8] have studied the toxicity of ZnO nanoparticles to *Pseudomonas putida* and other bacteria and found a dependence on the concentration of the dissolved Zn ions. Sapsford et al. [9] has suggested that the high temperature treatment of ZnO nanoparticles leads to lower antibacterial activity.

Summarizing the results, ZnO nanoparticles have in general a toxic effect to various bacterial populations in the nanoparticle suspension in water. Despite the vast number of papers on the ZnO nanoparticles, there are not enough studies on the interaction between bacteria and ZnO thin films [5,10].

In our study for the first time prokaryotic tests with *Pseudomonas putida ATCC11778* and *Bacillus cereus ATCC12633* are used to determine the influence of zinc oxide thin films prepared by three different methods: (i) magnetron sputtering [11], (ii) sol-gel dip coating [12] and (iii) chemical deposition [13]. Three different methods are used to determine the quantity of total, damaged and active bacterial cells: optical density measurements, classic cultivation in a rich solid medium and fluorescent microscopy. In the latter, *Bac*Light Bacterial Viability Kit is used to differentiate and count live and dead cells. We examine the sensitivity and the damages of different bacteria during their exposure on the surface of nano-structured ZnO thin films immersed in the bacterial suspension [14].

II. MATERIAL AND METHODS

II.1. Preparation of ZnO nanostructured thin films

(i) Magnetron sputtering [11]. Two sets of ZnOsamples were prepared - pure ZnO and ZnO doped with hydrogen (ZnO:H). The thin films were deposited by r.f. magnetron sputtering of a ZnO ceramic target (100 mm disc) in atmospheres of Ar (0.5 Pa) or Ar (0.5 Pa) + H_2 (0.1 Pa) at substrate temperatures 500°C and 400°C, respectively, and r.f. power of 180 W. The thickness of the deposited films is about 600 nm. The XRD spectra were collected using DRON 3 spectrometer with CuKα radiation (λ=1.5406 Å). SEM pictures are obtained by JSM-840A JEOL with $LaBa_6$ cathode.

(ii) Sol-gel dip coating [12]. The precursors are zinc acetate, 2-methoxyethanol and monoethanolamine (MEA). The film deposition was carried out on glass substrates (ISO-

LAB-Germany). Each deposited layer was dried up in air at 60°C for 30 min. Total five layers were deposited. The final annealing was carried out at 500°C for 1 hour and the film thickness is about 1 μm. The films were investigated by SEM - JSM-5510 JEOL operating at accelerating voltage of 10 kV. XRD spectra were recorded by apparatus Siemens D500 with CuKα radiation.

(iii) Chemical bath deposition [13]. A wet chemical method was used to obtain ZnO films in two steps: deposition of seeds and growth of nanorods on them. Zinc acetate dissolved in ethanol was coated onto a glass substrate for four cycles. The coated substrates were then rinsed with water and dried at room temperature. After that they were annealed in air at 320 °C for 20 min. The above procedure was repeated twice before the final growth of ZnO nanowires. The seeded substrates were then placed in aqueous solution of zinc nitrate and methenamine and heated up in a closed vial at 87 °C for 3 h. The samples were then removed from the solution, rinsed with distilled water, and placed in a new batch of precursor solution. The growth process was repeated eight times and finally the samples were dried in air. The film structure is studied by SEM (JSM-5510 JEOL operating at accelerating voltage of 10 kV) and XRD (Siemens D500 with CuKα radiation).

II.2. Toxicity tests on bacteria

Two types of bacteria were used in our tests to study the influence of nanostructured ZnO films: *Pseudomonas putida ATCC12633* (Gram-negative) [15] and *Bacillus cereus ATCC11778* (Gram-positive) [16].

To study the effect of the as-obtained ZnO thin films and the bacterial survival of *Pseudomonas putida*, two types of nutrient media were used: a rich medium ISO 10712 for maintaining and a poor synthetic medium ISO 10712 for testing the toxicity. The ZnO thin films were sterilised by ethanol inflammation and put in the nutrient medium. The bacterial inoculum is prepared in solid rich medium and after that adapted to poor mineral medium, by three consecutive sub cultivations (ISO 10712). The experiment was conducted in aerated dark and light conditions by orbital bench top shaker Certomat® at 150-170 rpm, 25˚C for *Pseudomonas putida* and 30˚C for *Bacillus cereus*. The suspensions were cultivated in 100-ml Erlenmeyer glass flasks with 20-ml nutrient medium. The ratio of the suspension volume to the surface area of the ZnO film was 10:1 and 2,5:1. The samples of bacterial cultures with ZnO thin films and the control experiment were collected at 3, 6, 9, 12 and 24 hours. The lighting conditions were provided by illumination with a tungsten lamp (100 W) and energy saving lamp Ecoline Eco 32 (20WE27 warm light at 2700 K) placed at a distance of 30 cm from the suspension.

The number of survived cells in the suspension was determined by the most probable number method in a rich solid ISO medium for *Pseudomonas* and other medium for *Bacillus cereus* [3]. Second, the total cell number (live plus dead) is considered by spectrophotometer measurements (λ=600 nm) of the optical density of the sample in a poor medium used as the control. All samples were taken in 3 replicas.

For SEM observations, the ZnO thin film was taken sterile from the bacterial suspension after the experiment and put in a sterile Petri dish. Then it was dried at room temperature in a closed dish before the observation with SEM. Golden thin film coating was made on the film before observation.

III. RESULTS AND DISCUSSION

SEM pictures of the cross section of the thin films deposited by RF magnetron sputtering are presented in Fig. 1a (ZnO) and Fig. 1b (ZnO:H). They show columnar structure of the films. The XRD spectra in Fig. 2 show a polycrystalline structure of the films with preferential crystallographic orientation (002) and *c*-axis perpendicular to the substrate surface. The estimated grain size according to Debye-Scherrer equation is about 25 nm in the ZnO films and about 17 nm in ZnO:H films.

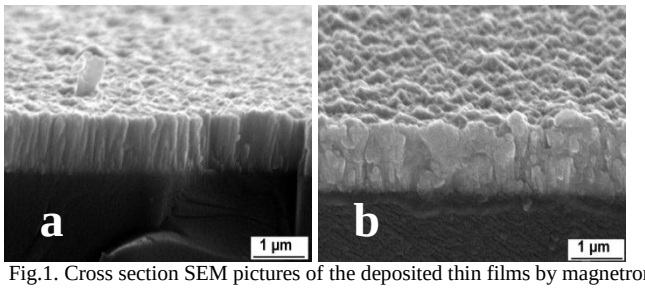


Fig.1. Cross section SEM pictures of the deposited thin films by magnetron sputtering - ZnO (a) and ZnO:H (b).

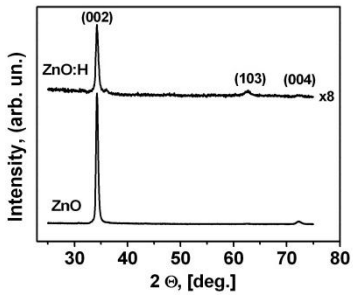


Fig. 2. XRD spectra of thin films ZnO (T_s= 500^{0}C) and ZnO:H (T_s= 400^{0}C) prepared by magnetron sputtering.

SEM picture of ZnO thin films prepared by sol-gel dip coating is shown in Fig. 3a and by chemical bath deposition in Fig. 3b.

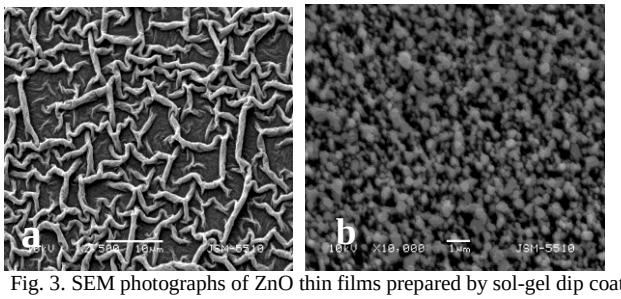


Fig. 3. SEM photographs of ZnO thin films prepared by sol-gel dip coating (a) and chemical bath deposition (b).

The mean grain size of ZnO thin film prepared by sol-gel dip coating (Fig. 3a) is between 20-32 nm [12]. The top view of SEM image of ZnO thin film prepared by chemical bath deposition (Fig. 3b) shows nanorods with hexagonal cross-section of typical size 100-200 nm and surface density ~1-3×10^9 cm^{-2}. The nanorod length is about 3-3.5 μm [20]. The XRD pattern of ZnO thin films prepared by sol-gel dip coating (Fig. 4a) and chemical bath deposition (Fig.4b) show mostly (002) diffraction peak.

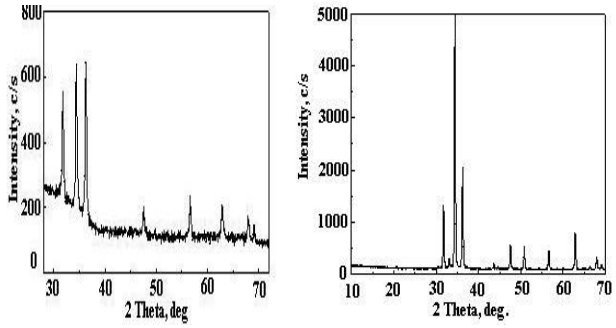


Fig. 4. XRD spectra of ZnO thin films prepared by sol-gel dip coating (a) and chemical bath deposition (b).

The results from the control bacterial growth in rich and poor medium and at dark and light conditions are presented in Fig. 5.

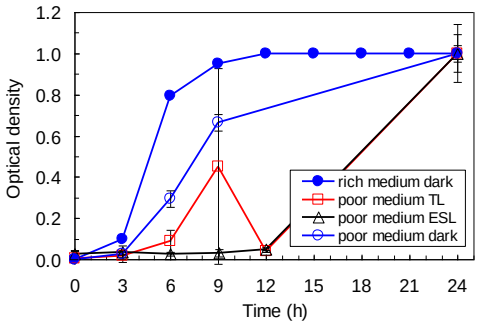


Fig. 5. Bacterial growth of *Pseudomonas putida* determined by optical density at different experimental conditions.

There is significant difference between the quantities of cells in the control samples grown in rich and poor medium. The thin ZnO films obtained by magnetron sputtering have a rather smooth surface and the cells are not damaged in a contact to it. Figure 6 shows the optical density of *Pseudomonas putida* (total number of cells) versus time of incubation in the presence of ZnO films. It is established that in all experiments the optical density of the suspensions treated with nanostructured ZnO thin films, obtained by magnetron sputtering, follows clearly the trend from Fig. 5. It increases moreover faster than the one in the control experiment and the cells division of *Pseudomanas putida* is similar to that in rich medium. In the case of cultivation with ZnO:H films, also obtained by magnetron sputtering (MSD), the optical density is very close to the control sample though a bit lower at the initial stage (within the experimental error). If the bacteria are cultivated with nanorods ZnO films obtained by chemical deposition method there is a strong inhibition of the cells division till the 12th hour (Fig. 6).

These observations are proven also by the classical cultivation method determining the live cells appeared as colonies in solid medium. The trend is an increase of bacterial number in the presence of nanostructured ZnO obtained by MSD. *Pseudomonas putida* cells have not lag-phase and the number of the live active cells is higher than in the control experiment especially after the 9th hour. The bacterial growth in the presence of nanostructured ZnO nanowires is inhibited and the bacterial quantity is all time lower than in the control experiment.

Three experiments are conducted in light conditions with energy saving lamp and ratio 2.5:1 of bacterial suspension and different ZnO nanofilms (Fig.7).

Cultivation of bacteria is conducted in 6-well plastic plates and the results of optical density are presented in Fig.7 versus the time.

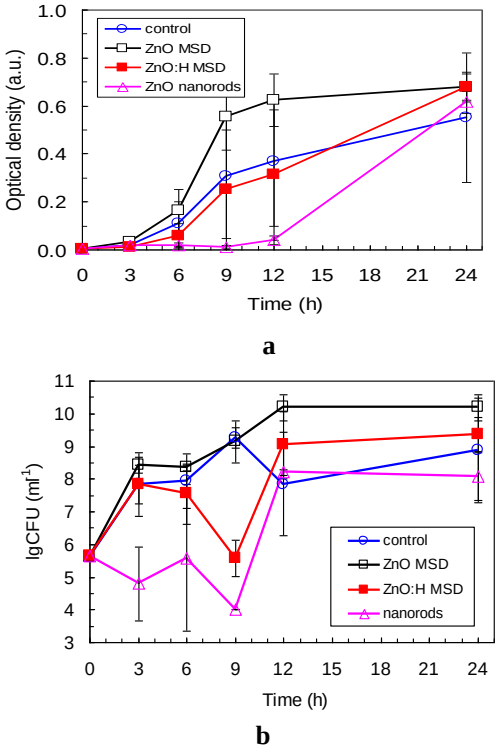


Fig. 6. Effect of ZnO thin films prepared by different deposition methods on the bacterial growth of *Pseudomonas putida* (a) - Optical density method, and *Bacillus cereus* (b) - CFU method, in poor nutrient medium.

The data are close to each other and there is strong inhibition effect of the films on bacteria until 9-12 hours. The data resemble those from Fig. 5 and especially the lag-phase for the ESL light illumination in poor nutrient medium. Only later (12-24 hours) there is an appreciable exponential growth.

The data are confirmed by the most-probable number method (CFU) in a solid medium – there are no significant deviations from the control variant and the increasing of the bacterial populations is generally stable with no inhibition effect of ZnO MSD thin films. The data for ZnO films obtained by sol-gel method show always inhibition effect, as proved by different methods.

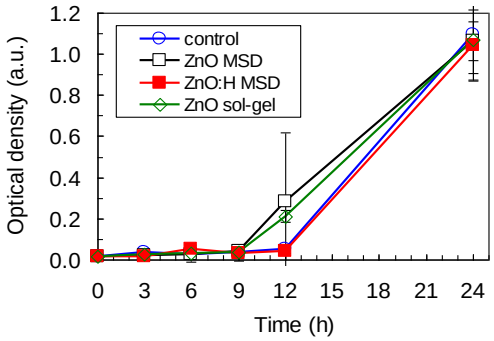


Fig. 7. Influence of differently deposited ZnO thin films on the growth of *Pseudomonas putida* in poor nutrient medium, plastic plates and illumination with an energy saving lamp.

The results for *Bacillus cereus* are different from those of *Pseudomonas putida* cells. *Bacillus cereus* cells sporulate at the 6th hour to form fewer colonies on the solid rich medium – about 10^3 CFU per millilitre. The spores after 48 hours of cultivation in rich solid medium appeared as visual colonies.

This is the reason to count very different number of *Bacillus cereus* colonies from the samples of the 6 and 9th hours cultivation at the 24th and 48th hours of Petri dish cultivation.

As could be seen from Fig. 6 and 7 both bacteria are more sensitive to the ZnO thin films with a ruffle structure deposited by sol-gel dip coating or to nanorods films obtained by chemical bath deposition. The tendency is the same till the 24th hour of cultivation (data are not presented). The results could be due to the bigger surface of ZnO ruffle thin films for interaction with bacteria or to the higher dissolving rate of Zn ions or nanoparticles. Our results are in accord with the report of Huang et al. [6]. In other papers [10, 5], ZnO seems more effective for the destruction of Gram-positive than for Gram-negative bacteria because they have simpler cell membrane structure. Our experiments prove the same difference that the films made by wet colloidal methods are toxic for the bacteria at least in the first several hours.

We do not establish any significant difference in the influence between ZnO and ZnO:H films obtained by MSD. The reason could be the smooth surface and the similar quantity of Zn^+ and nanoparticles dissolved into the suspension. The presence of zinc ions at low concentration (<0,2mM) in the poor medium increases the cell division rate. If their concentration is higher (>0.25 mM) [6], this effect is opposite, i.e. toxic. Obviously, the surface arrangement of ZnO thin films changes the toxicity of nanoparticles [18].

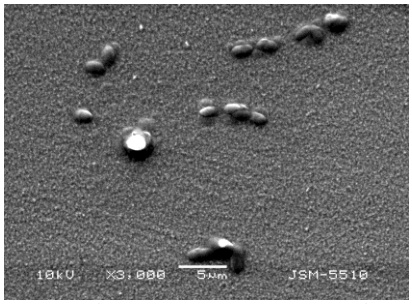
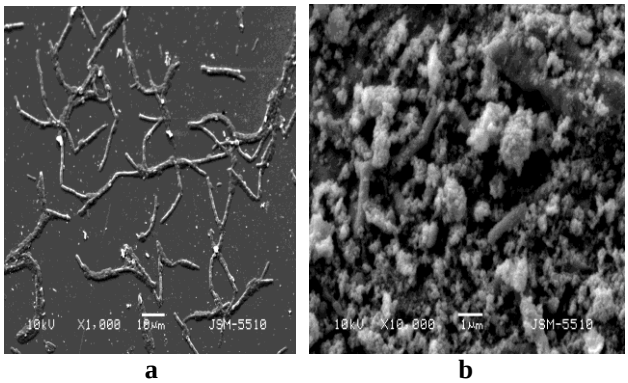


Fig. 8. SEM pictures of *Pseudomonas putida* on ZnO films obtained by magnetron sputtering - after 9 hours of cultivation.



a **b**

Fig. 9. SEM pictures of *Bacillus cereus* on ZnO thin films obtained by different methods - chains on magnetron sputtered ZnO (a) and bacteria after 24 hours of cultivation on ZnO thin film obtained by sol-gel method (b).

The SEM images show separate cells and cell colonies in Fig. 8 and 9a. The *Pseudomonas* cells are intact and have well preserved capsules (Fig. 8). There are micro colonies formed on the smooth surface of the thin films. The *Bacillus* cells also form a net structure on the ZnO thin films, deposited by magnetron sputtering, but they are more sensitive to the impact of ZnO nanoparticles and they are easily damaged (Fig. 9b) because of different cell wall structure (Gram-positive). This is especially true in the case of nanofilms with irregular surface relief (Fig. 9b) obtained by the sol-gel methods. Our results are in accord with the report of Greist et al. [15], who have received similar pictures by SEM. These results are quite encouraging as a first step in the development of ZnO based biosensor [19].

IV. CONCLUSIONS

The influence of nanostructured ZnO thin films on the cells division rate of bacteria *Pseudomonas putida* (Gram-negative) and *Bacillus cereus* (Gram-positive) was studied. The ZnO films are prepared on glass substrates by three different methods - RF magnetron sputtering, sol-gel and chemical bath. The structure of the films was studied by XRD, SEM and AFM. All patterns have a polycrystalline structure with preferential (002) crystallographic orientation and c-axis perpendicular to the substrate surface. The influence of the as-prepared films on *Bacillus cereus* and *Pseudomonas putida* was studied by two different methods - optical density measurements and the classic cultivation in rich and poor medium (most-probable number method - colony forming units.ml^{-1} (CFU). Periodic cultures of bacteria were investigated in a 24-hours experiment for sensitivity to the ZnO thin films immersed in the bacterial suspension. The films of peculiar ruffle-like surface structure have shown considerable inhibition effect at the first 9-12 h, especially for the Gram-positive bacteria. The thin films, obtained by magnetron sputtering show stimulation effect on the cells division. After the sporulation at the 6th h Gram positive *Bacillus cereus* cells also acquire resistance to the ZnO thin films. Our experiments proved that ZnO films made by wet colloidal methods (sol-gel or chemical bath) are toxic to the studied bacteria.

These results are quite encouraging as a first step in the development of ZnO based biosensor.

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