The Effects of Silica Nanostructures on Halotolerant Microorganisms Isolated from Rock Salt Crystal

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Abstract. This paper deals with the evidentiation of the potential effect both bactericidal and bacteriostatic of some oxidic silica nanostructures towards the halotolerant microorganisms *Bacillus subtilis* and *Virgibacillus halodenitrificans*, isolated from subterranean salt crystal. The studies were focused on the testing of these activities under relatively similar conditions to those observed in several environments, namely highly polluted areas or with nanobiotechnological potential. The as-prepared SiO_2 based microtubes showed a slight inhibitory effect for *Bacillus subtilis* but, on the other hand acted to stimulate the growth of *Virgibacillus halodenitrificans*. In opposite, the thermal treated microtubes at 400^0C for one hour stimulated the growth of *Bacillus subtilis* and diminished the growth rate of *Virgibacillus halodenitrificans*. The silica nanostructures with spherical shape and platinum doped silica microtubes had a positive effect on the growth of both tested strains.

Introduction

One of the most important research approaches in the modern sciences is represented by nanobiotechnologies (Klabunde, 2001), an area located at the

frontiers between several sciences with a relatively high interdisciplinary degree. The nanoscience could be considered as the science of the small particles of materials. These small particles are very important since all material properties (*i.e.* melting point, electrical and optical properties) are modified when the size of constitutive particles is reduced to nanoscale. The new obtained properties resulted in new opportunities for technological development and novel biotechnologies (Kohli and Martin, 2005).

Some of the applications of the micro and nanoparticles in biotechnologies are related to the enzyme's encapsulation (Chang and Prakash, 2001), DNA transformation (Kneuer *et al*., 2000; Koltover *et al*., 1998; Radler *et al.*, 1997) biosensors (Cao *et al.*, 2002; Demers *et al*., 2002; Park *et al*., 2002) and others like drug delivery (Ulrich *et al*., 1999; Lee *et al*., 2002; Murthy *et al.*, 2002).

Nanoparticles with functionalized surfaces could be used for the release of genetic material in viable cell by transformation processes. Silica nanospheres labeled with ammonium groups could be used for binding and transporting DNA in specific cells. The identification of the protein synthesized in the cell following codification of the introduced DNA by silica nanosphere could confirm the successful transformation of the genetic material (Kneuer *et al*., 2000). Other studies revealed that also nanospheres with a lipid nature (cations) and liposomes could play an important role in the transformation processes (Koltover *et al*., 1998; Radler *et al*., 1997).

Apparently, the spherical nanoparticles are easier to be obtained than microtubes and nanotubes. In comparison with the spherical nanoparticles, nanotubes are characterized by an inner space where chemical or biochemical materials with the same size as proteins or small molecules could be inserted (Mitchell *et al*., 2002; Lee *et al*., 2002). On the other hand, nanotubes have an internal and external surface which could be functionalized in different ways, either chemically or biochemically (Mitchell *et al*., 2002). In this way, it appears the possibility of functionalization of nanotubes inner side with biochemical compounds. This functionalization influences the chemical features of the outer side, making it compatible with some biological materials. Another particularity of nanotubes is represented by the presence of the two open ends which could be an advantage for the relatively easy functionalization of the internal surface.

This paper emphasizes the potential effect both bactericidal and bacteriostatic of some oxidic silica nanostructures towards the halotolerant microorganisms *Bacillus subtilis* and *Virgibacillus halodenitrificans*, isolated from underground salt crystal from Slănic Prahova area. The studies were focused on testing these activities in similar conditions as those observed in several environments, namely highly polluted areas or nanobiotechnology fields in which various nanostructures are used. Such kind of nanostructures could be applied in the technologies of obtaining some disinfectants based on photocatalysts, but also in other industrial areas. The bactericidal or bacteriostatic effect has been observed by monitoring the growth of

the halophilic bacterial cultures by recording the optical density and by evaluating the total dehydrogenase activity as an indicator of the biological activity, allowing in this way the monitoring of the biological activity of tested silica nanostructures.

Materials and Methods

Halophilic baterial strains

The halophilic bacterial strains used in this study were isolated from underground salt crystal taken from Slănic Prahova area and identified in a previous investigation as being *Virgibacillus halodenitrificans* and *Bacillus subtilis.* The strains were cultivated on MH media with the following composition (g/L): yeast extract (10), proteose peptone (5), glucose (1), NaCl (100), $MgCl_2 \times 6H_2O$ (7), $MgSO_4 \times 7H_2O$ (9.6), $CaCl_2 \times 2H_2O$ (0.36), KCl (2), $NaHCO_3$ (0.06), NaBr (0,026) (Ventosa *et al.*, 1989). The medium pH was 7.0–7.2 before autoclaving. When necessary, during the experiments, the medium was supplemented with seven milligrams of each tested nanostructure (spheres, nanotubes, etc. – Table 1). For the solidified form of the medium, agar was added (20 g/L).

Table 1. The description of the silica microtubes and nanospheres used in the experiments

Sample	Description of the sample
A	As-prepared SiO_2 microtubes prepared accordingly to sol-gel method in the presence of *DL*-tartaric acid
B	Previous sample thermally treated at 400^oC, 1 hour
C	SiO_2 nanospheres prepared in similar conditions with sample A, but in the presence of *meso*-tartaric acid
D	Sample A doped with Pt by impregnation with H_2PtCl_6 and reduced in H_2, 300^0C (Pt/SiO_2)

Testing of antimicrobial properties–The effect of investigated nanostructures on the growth of the tested halotolerant strains has been estimated as follows: approximately seven milligrams of each tested nanostructure were added in the culture medium in the presence of microbial cells and the resulted mixture was incubated at 28^0C for 24 hours. The microbial growth was monitored for 24 hours by measuring the optical density at 660 nm.

The bactericidal or bacteriostatic effect of the investigated nanostructures has been evaluated by the following experiment: the investigated nanostructures were put in contact with a known amount of microbial cells in phosphate buffer (0.06 M, pH 6.1) and the resulted mixture was incubated for 48 hours at 28^0C. In the next

step, one milliliter of the mixture was spread in a Petri dish and incorporated in 25–30 ml of molten agar (cooled at around 55^0C). The total number of colony forming units was counted after 24 hours of incubation at 28^0C. This number has been correlated with the amount of microbial cells from the mixture.

Tested nanostructures – were SiO_2 microtubes and nanospheres obtained by sol-gel method in the presence of *DL or meso*-tartaric acid template. The experimental conditions and some characteristics are summarized in Table 1.

Dehydrogenase activity – as indicator of total biological activity has been evaluated following the Casida method (Casida *et al.*, 1964). Briefly, 0.5 ml 3% TTC (triphenyl-tetrazolium chloride) were added to three milliliters of bacterial cultures and the mixture was incubated at 37^0C for 24 hours. The dehydrogenase activity was expressed as mg formazan % (reaction product resulted from transformation of colorless TCC to red formazan).

Results and Discussions

Tested nanostructures

The nanostructures used in this study were SiO_2 tubes and particles obtained by sol-gel method using *DL or meso*-tartaric acid as template (Anastasescu *et al.*, 2010). Following synthesis, several post reaction treatments were performed and the resulted nanostructures were analyzed by transmission electron microscopy. The investigation revealed that samples A, B and D (see Table 1) had a microtubular structure, while sample C consisted of spherical nanostructures (Fig. 1).

The effect of nanostructures on the tested halotolerant strains

The presence of the investigated nanostructures in the culture medium of the used halotolerant strains revealed a different effect for each tested microbial strain (Fig. 2). Thus, the as-prepared SiO_2 microtubes (sample A) showed a slight inhibitory effect on the growth of *Bacillus subtilis*. On the other hand, the growth of *Virgibacillus halodenitrificans* appeared to be slightly stimulated by the presence of these microtubes. In the presence of sample B (thermally treated microtubes), the growth of *B. subtilis* was also slightly stimulated, an opposite effect if compared with sample A. The strain of *V. halodenitrificans* has a diminished growth rate in the presence of sample B, which appears to be an opposite effect if compared with sample A (as-prepared SiO_2 microtubes). On the other hand, in the presence of sample C (nanospheres) a positive effect for both tested strains is recorded (growth was stimulated). A similar behavior was also registered also in the presence of the platinum doped sample (D).

This variable response could be due either to the structure and/or morphology of the investigated nanostructures. The dehydrogenase activity recorded at the end of incubation period (24 hours) as indicator of total biological activity supported this

variable response (Table 2) mentioned before and revealed either a slight inhibitory effect on the microbial growth or a growth stimulation.

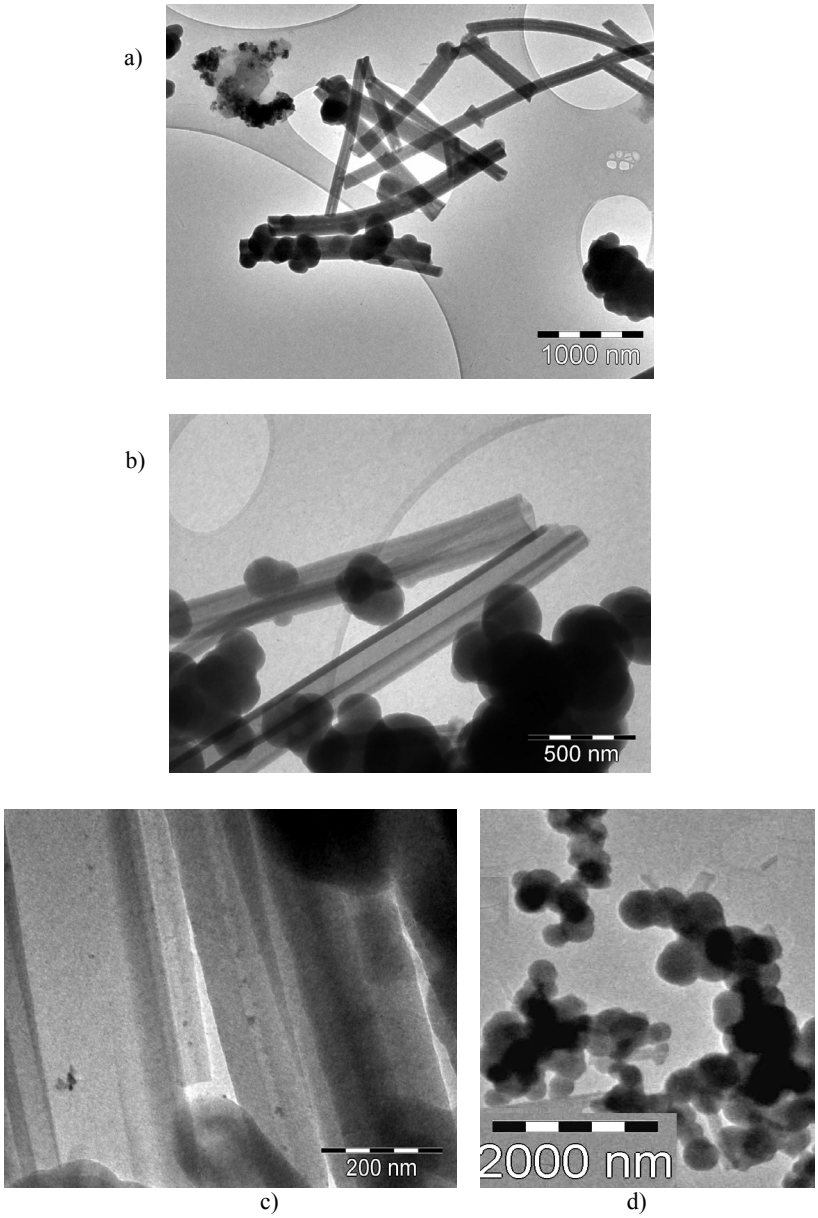

Fig. 1. The microtubular shape of the sample A (a), B (b), D (c) and spherical shape of sample C (d).

Table 2. Dehydrogenase activity
1 = *Virgibacillus halodenitrificans*; 2 = *Bacillus subtilis;* M = blank (bacterial culture without nanostructures); A, B, C, D = investigated nanostructures described in Table 1; mg formazan growth = dehydrogenase activity in growth of culture as data recorded in Fig. 2; mg formazan viability T_0 = dehydrogenase activity recorded at start of the experiment *The bactericidal or bacteriostatic effect* as described in Materials and Methods section; mg formazan viability 24h = dehydrogenase activity recorded at the and (24 hours) of the experiment *The bactericidal or bacteriostatic effect* as described in Materials and Methods section

Sample	**mg formazan growth**		**mg formazan viability T_0**		**mg formazan viability 24 h**	
	1	2	1	2	1	2
M	*3.89*	*0.23*	**0.20**	**0.17**	0.11	0.09
A	*0.47*	*0.21*	**0.12**	**0.51**	0.09	0.01
B	*0.29*	*0.22*	**0.10**	**0.25**	0.13	0.10
C	*0.52*	*0.22*	**0.13**	**0.23**	0.09	0.10
D	*0.36*	*0.22*	**0.11**	**0.15**	0.10	0.10

The data presented in Fig. 2 revealed that the thermal treatment of the SiO_2 microtubes (sample B) modifies the nanostructures effect on the tested microbial strains. In this way the antibacterial effects towards the strain of *V. halodenitrificans* could be observed, in opposition to the effect of growth stimulation registered in the case of *B. subtilis*. On the other hand, the thermal treatment of the samples C and D conducted to a stimulating growth effect also for *V. halodenitrificans*. This behavior could be also attributed to the mechanisms of interaction established between microbial cells and silica microtubes and nanosphers. The morphology of investigated nanostructures affected their activity on the tested halotolerant microbial cells. Thus, if the two microbial strains were grown in the presence of microtubules (sample A), the antimicrobial activity was observed only against *B. subtilis*, whereas in the case of cultivation in the presence of silica nanospheres (sample C) both tested strains were able to grow.

The data recorded in Table 3 showed that the viability of the tested strains was affected by the presence of the investigated nanostructures. The bactericidal effect was recorded only in the case of *Virgibacillus halodenitrificans*. The dehydrogenase activities (Table 2) are in accordance with the data recorded in Table 3, both for *V. halodenitrificans* and *B. subtilis*.

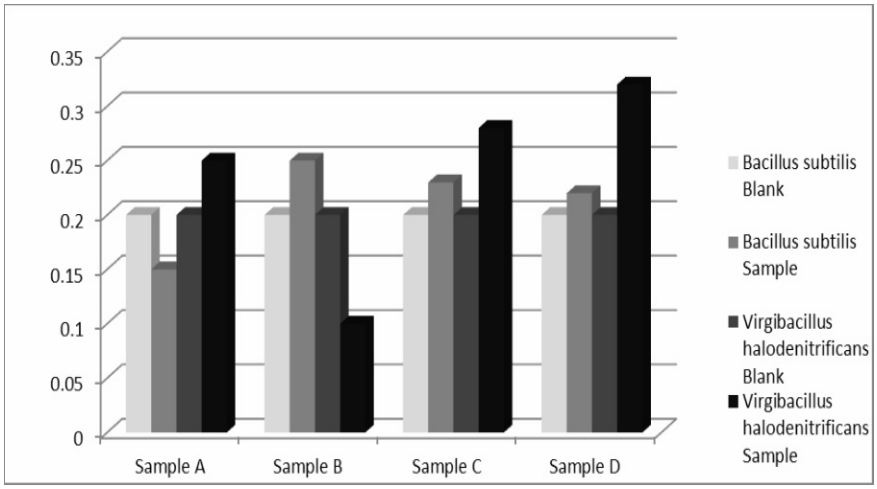

Fig. 2. The growth of tested halotolerant bacterial strains in the presence of nanostructures with various compositions and morphology (Tabel 1) after 24 hours incubation time; X axis representing D.O. at 660 nm.

Table 3. The bactericidal effect of investigated nanostructures on the microbial cells

	1	**2**
M	Infinite	1×10^6
A	99×10^6	34×10^6
B	126×10^6	32×10^6
C	163×10^6	22×10^6
D	89×10^6	2×10^6

1 = *Virgibacillus halodenitrificans*; 2 = *Bacillus subtilis*;
M = blank (bacterial culture without nanostructures);
A, B, C, D = investigated nanostructures described in Table 1

Concluding Remarks

The as-prepared SiO_2 microtubes (sample A) showed a slight inhibitory effect against *Bacillus subtilis* but, on the other hand, they stimulated the growth of *Virgibacillus halodenitrificans*. In opposition, the microtubes thermally treated at 400^0C for one hour (sample B) stimulated the growth of *Bacillus subtilis* and diminished the growth rate of *Virgibacillus halodenitrificans*. The silica nanostructures with spheric shape (sample C) and platinum doped silica microtubes (sample D) showed a positive effect on the growth of both tested strains.

The variable response of the tested halotolerant microbial strains could be attributed to the morphology and composition of the investigated nanostructures as resulted also from the dehydrogenase activity data and the viability data of the strains.

On the other hand, the thermal treatment of the nanostructures conducted to a positive effect towards *Virgibacillus halodenitrificans,* in the case of un-doped microtubes (sample A), silica nanospheres (sample C) and platinum doped microtubes (sample D). In the case of thermally treated microtubes (sample B) the effect towards *Virgibacillus halodenitrificans* was a negative one.

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