ROMANIAN ACADEMY

INSTITUTE OF BIOLOGY BUCHAREST

Ph.D. THESIS

SUMMARY

INTERACTION OF GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA WITH NANO AND MICRO SEMICONDUCTOR MATERIALS: FUNDAMENTAL AND APPLICATIVE ASPECTS

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CONTENTS

KEYWORDS:

ANTIBACTERIAL

TITANIUM DIOXIDE

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NANOMATERIALS

MICROMATERIALS

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Virgibacillus halodenitrificans

INTRODUCTION

Nanomaterials have become part of daily life, being used in an impressive number of products, such as cosmetics and personal care products, pharmaceuticals, pigments in paints, food, paper, biosensors, etc. Their effect on the environment has captured the attention of the researchers in the field, and the studies in this regard are continually increasing. Moreover, it is necessary to evaluate the physicochemical properties of nanomaterials, such as size, shape, solubility, crystalline structure, surface chemistry, etc., and to establish the role of each character in manifesting their toxicity (Gatoo și colab., 2014).

Currently, a particular category of inorganic nanomaterials, that of semiconductors, is under study. When the size of the materials is reduced at the nanometric scale, their physicochemical properties change radically compared to their conventional counterparts in the macroscopic form (Tran et al., 2011). The specific surface area and the surface/volume ratio increase significantly as the size of the material decreases. Parameters, such as size, shape, or surface characteristics, can be modified depending on the application potential (Seil et al., 2013).

Thus, these new properties of semiconductor nanomaterials are of significant interest for both scientific and technological research, with applications in nanophotonics, nanoelectronics, sensors, imaging devices, catalysis, solar cells, etc. (Suresh et al., 2013).

Due to their unique properties, some of these nanomaterials exhibit antibacterial properties, acting on a broad spectrum of Gram-positive and Gram-negative bacteria. Nanomaterials can be successfully used in the fight against antibiotic-resistant bacteria (Martinez et al., 2019). On the other hand, bacteria are essential organisms for life, due to the significant role they play in the biogeochemical circuit of the elements. As a result, there is a "duality" in bionanotechnological research. The first aspect is the applicative potential of these manufactured nanomaterials, and the second is to understand and limit their impact on the environment.

Purpose and objectives of the thesis

The purpose of the work was to identify micro and nano semiconductor materials with antibacterial properties to select the structures that showed an increased inhibitory effect on some strains of Gram-positive and Gram-negative bacteria and to establish the antibacterial mechanisms. The studies also aimed to identify the applicative potential of some nanomaterials based on sodium titanate to use them to obtain prototype mortars for restoration interventions.

The main objectives of the experimental research

1. Evaluation of the antibacterial activity of some micro and nano semiconductor materials based on zinc selenide (ZnSe) and titanium dioxide (TiO_2)

2. Studies on the identification of the antibacterial mechanisms of semiconductor materials

3. Testing the bacteriostatic/bactericidal potential of titanium dioxide nanotubes on some halophilic/halotolerant bacterial strains isolated from the mural painting in Humor Monastery (Suceava County) and Hurezi (Vâlcea County), involved in the biodeterioration process of the monuments.

Structures of the Ph.D. thesis

The doctoral thesis is organized in two main parts, followed by the general conclusions, bibliography and the list of publications on the topic of the Ph.D. thesis.

The first part of the paper (Chapters 1, 2 and 3) includes a bibliographic study in which generalities are presented about the micro and nano materials with antibacterial properties, the role of their physicochemical properties in the manifestation of the antibacterial character, as well as information about the antibacterial mechanisms.

The second part of the paper (Chapters 4, 5, and 6) contains the results of the experimental researches, their interpretation, as well as the conclusions obtained from the studies carried out.

Chapter 4 included data concerning the evaluation of the antibacterial activity of some zinc selenide materials, synthesized by the hydrothermal method, and also the commercial Aeroxide P25 titanium dioxide powder, after stopping the 1 hour UV irradiation. Chapter 5 presents experimental studies on the identification of the antibacterial mechanisms of zinc selenide nanomaterials. The last section refers to a study dedicated to the application potential of some nanotubes based on sodium titanate, by testing the antibacterial effect of the oxidic structure on some halophilic/halotolerant bacterial strains isolated from the mural painting from Humor (Suceava County) and Hurezi (Vâlcea County) Monasteries, involved in the process of biodeterioration of historical monuments.

ORIGINAL CONTRIBUTIONS

1. Evaluation of the antimicrobial activity of zinc selenide (ZnSe) based materials and titanium dioxide (TiO_2) nanomaterials

1.1. Materials and methods

New zinc selenide based materials were used for testing. Their synthesis and characterization were carried out within the Institute of Physical Chemistry "Ilie Murgulescu" of the Romanian Academy. A number of 4 samples were included in the tests and were scored as follows:

- Sample 1- ZnSe with spherical morphology
- Sample 2- ZnSe with flower type morphology
- Sample 3- Pd-ZnSe with flower type morphology (doped structure of ZnSe)
- Sample 4- TiO_2-ZnSe with flower type morphology (doped structure of ZnSe).

The method of obtaining the new structures based on ZnSe was the hydrothermal one, the synthesis being carried out at 120℃, using a mixture, in an equimolar ratio, of sodium selenite (Na_2SeO_3, 99% min, Alpha Aesar) and zinc sulfate ($ZnSO_4x7H_2O$, 99.5%, Roth). Hydrazine ($N_2H_4xH_2O$ 98%, Alpha Aesar) was used as a reducing agent.

Depending on the pH value, but also the reaction time, the materials with the proposed morphology were obtained (Neagu et al., 2019).

For the experimental study, two reference bacterial strains, represented by *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923, were used to observe, in comparison, the response of the bacterial cells after the treatment with the samples material. Also, the Gram-positive *Virgibacillus halodenitrificans* strain (IBB collection), isolated from the surface of a salt crystal from the Unirea mine, Slănic Prahova, was selected for experiments.

The concentrations of zinc selenide materials applied for the experiments were 0.05 mg/mL and 0.5 mg/mL, for samples 1, 2 and 4, and 0.05 mg/mL for sample 3. Culture media containing bacterial cells treated with material samples were incubated under shaking conditions (150 rpm) for 24 hours at 37°C (*E. coli* and *S. aureus*) and 48 hours at 28°C. (*V. halodenitrificans*). The control sample was the untreated bacterial culture. The effect of inorganic structures on *E. coli* and *S. aureus* strains was followed after 4, 6, and 24 hours after treatment.

The susceptibility testing of the halotolerant *V. halodenitrificans* bacterium to the action of the materials was performed after 24 and 48 hours after inoculation. In the case of titanium dioxide, 1 g of titanium dioxide was weighed and transferred to a sterile Petri dish and subjected to irradiation at 365 nm, at a distance of 5 mm, for 1h, using the portable UV lamp VILBER LOURMAT VL- 4.LC 230V / 50/60 Hz. Immediately after irradiation, titanium dioxide was mixed with 20 mL of culture medium, and the resulting suspension was sonicated in a water bath, followed by bacterial inoculation. The treated and untreated bacterial cells were incubated under stirring conditions (150 rpm), in the presence of titanium dioxide, at the appropriate growth temperatures and specified times. The luminous intensity inside the incubator was 4.6 lux.

At the time intervals indicated in the protocol, samples were taken to determine the effect of titanium dioxide on bacterial cells.

Classic methods of microbiology, optical, and biochemical analysis have been used to determine the efficiency of micro and nano semiconductor materials.

1.2. Results and discussions

1.2.1. Characterization of semiconductor materials

1.2.1.1 Zinc selenide

ZnSe structures with spherical morphology (Fig. 1 a) are characterized by micrometric dimensions, while the materials in simple and doped form, with flower-like morphology (Fig. 1 b, c), have nanometric dimensions (thickness <10 nm, width of 500 nm and length in the micrometric domain (1-2 μm).

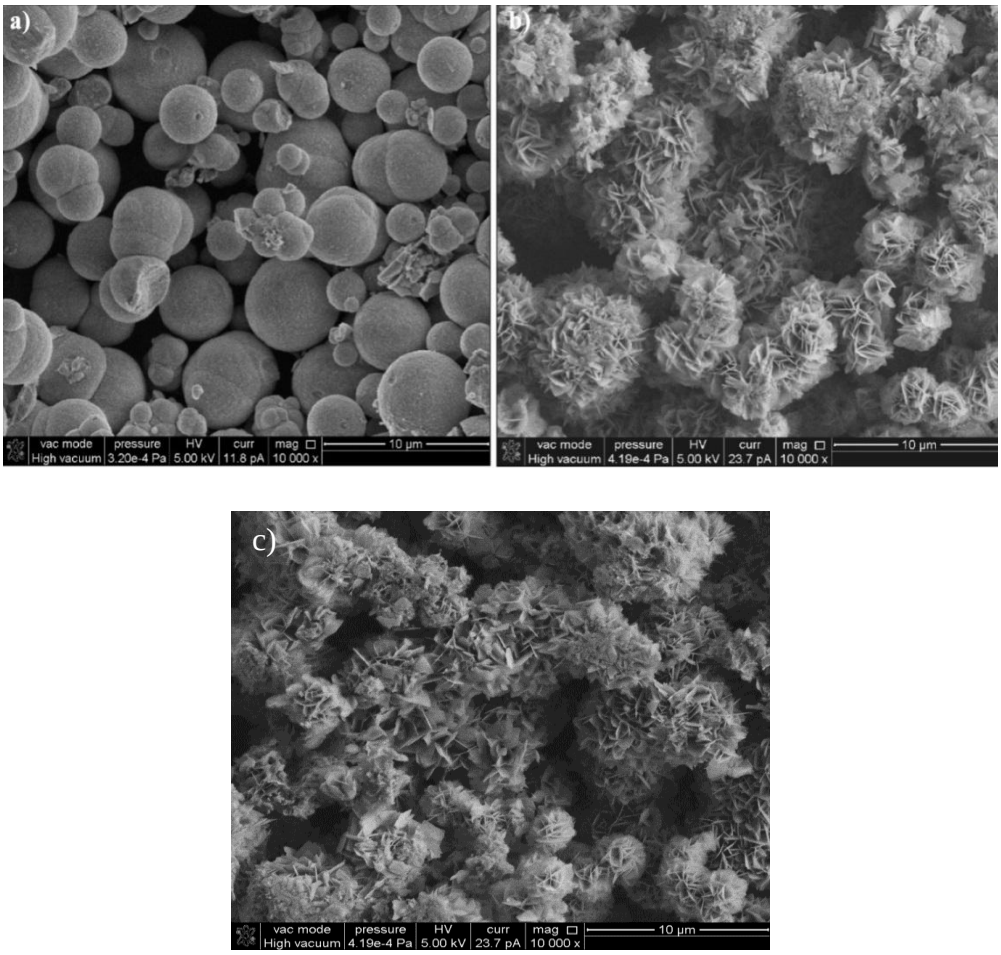


Fig. 1 SEM micrographs of synthesized ZnSe based materials:
a) ZnSe spherical morphology; b) ZnSe flower type morphology; c) TiO_2-ZnSe flower type morphology (Neagu et al., 2019)

1.2.1.2 Titanium dioxide

The Aeroxide P25 powder is a mixture of rutile and anatase, in a ratio of about 1: 4. According to the specifications described by the manufacturer, titanium dioxide has the following characteristics: spherical morphology, 21 nm, specific surface area (35-65 m^2/g), density (4.26 g/mL at 25℃).

1.2.2. Antibacterial activity of Aeroxide P25 titanium dioxide

From the results presented in Table 1 (a, b) was observed the inhibitory action of non-irradiated TiO_2 and after UV irradiation, on *E. coli* cells. It was found that the effect of the two forms of nanopowders is bacteriostatic.

For the *S. aureus* reference strain, a percentage of 100% colonies reduction was obtained after 4 and 6 hours of exposure to the cells to non-irradiated TiO_2 P25, compared with the untreated control sample (Table 2).

In the case of *V. halodenitrificans* strain, an increased inhibitory effect was observed for the two titanium dioxide samples, which increased with the exposure time (Table 3 a, b). However, there was a slight resistance of the Gram-positive bacterium to the action of the nanopowders, in both forms tested, compared to the two reference strains. Also, differences were observed regarding the appearance of the colonies that grew on the culture medium, these being of much smaller size compared to the control sample. The morphological changes of colonies can be attributed to titanium dioxide, which acted as a stress factor, the bacteria developing an adaptation mechanism after contact with the material.

Table 1. (a) Viable cells count (cfu/mL) of ***E. coli*** grown on LB culture medium after 4, 6 and 24h of incubation; (b) Percentage of bacterial reduction.

a)

	cfu/mL			
	T_0	T_{4h}	T_{6h}	T_{24h}
TiO_2 p25 with 1h of irradiation	$4.8x10^8$	$1x10^8$	$9x10^8$	$9.5x10^{10}$
TiO_2 p25 non-irradiated	$5.0x10^8$	$2x10^8$	$2x10^9$	$5.1x10^{10}$
E. coli (control)	**$4.9x10^8$**	**$3.5x10^9$**	**$9.3x10^{10}$**	**$11.6x10^{10}$**

(b)

Bacterial reduction (%)	**T_{4h}**	**T_{6h}**	**T_{24h}**
TiO_2 **p25 with 1h of irradiation**	97.14	99.03	18.1
TiO_2 **p25 non-irradiated**	94.28	97.84	56.03

Table 2. (a) Viable cells count (cfu/mL) of ***S. aureus*** grown on LB culture medium after 4, 6 and 24h of incubation; (b) Percentage of bacterial reduction.

	cfu/mL			
	T_0	**T_{4h}**	**T_{6h}**	**T_{24h}**
TiO_2 **p25 1h of irradiation**	$5.0x10^8$	$1.1x10^5$	0	$8x10^8$
TiO_2 **p25 non-irradiated**	$5.1x10^8$	$4x10^4$	0	0
S. aureus (control)	**$5.1x10^8$**	**nd**	**nd**	**nd**

nd-the colonies could not be counted

Table 3. (a) Viable cells count (cfu/mL) of ***V. halodenitrificans*** grown on MH culture medium after 24 and 48h of incubation; (b) Percentage of bacterial reduction.

(a)

	cfu/mL		
	T_0	**T_{24h}**	**T_{48h}**
TiO_2 p25 1h of irradiation	$5.12x10^8$	$1.6x10^8$	$1.2x10^8$
TiO_2 p25 non-irradiated	$5.1x10^8$	$7.9x10^7$	$1.5x10^7$
V. halodenitrificans (control)	**$5.18x10^8$**	**$6.9x10^8$**	**$9.2x10^8$**

(b)

Bacterial reduction (%)	**T_{24h}**	**T_{48h}**
TiO_2 p25 1h of irradiation	76.8	86.9
TiO_2 p25 non-irradiated	88.5	98.4

1.2.3. Antibacterial activity of zinc selenide based materials

The results obtained after applying the direct method of estimating the growth of the *E. coli* bacterium, in the presence of zinc selenide materials, showed that the nanoflowers, at a concentration of 0.5 mg/mL, had an inhibitory effect on it. The sensitivity of the strain to the action of the zinc selenide materials in the doped form with titanium dioxide was observed, with a percentage reduction of the number of colonies, after 4 and 6 hours of incubation at 37°C, of 98.8% and 99.6%, respectively, compared with the control sample (Table 4).

Table 4. (a) Viable cells count (cfu/mL) of ***E. coli*** grown on LB agar plates, after 4, 6 and 24h of incubation; (b) Percentage of bacterial reduction.

(a)

Strain	**Sample**	**ZnSe (mg/mL)**	**cfu/mL**			
			T0	**T4h**	**T6h**	**T24h**
E. coli	1	0.05	$1.9x10^6$	$1.9x10^{10}$	$5x10^{10}$	$8.9x10^{10}$
		0.5	$1.5x10^6$	$2.6x10^{10}$	$5.1x10^{10}$	$8.7x10^{10}$
	2	0.05	$1.9x10^6$	$2.4x10^{10}$	$4.9x10^{10}$	$8.9x10^{10}$
		0.5	$1.5x10^6$	$1.7x10^{10}$	$3.6x10^{10}$	$1.3x10^{10}$
	3	0.05	$1.3x10^6$	$2.7x10^{10}$	$4.8x10^{10}$	$8.8x10^{10}$
	4	0.05	$1.2x10^6$	$2.6x10^{10}$	$4.9x10^{10}$	$8.4x10^{10}$
		0.5	$1.7x10^6$	$3x10^8$	$2.1x10^8$	$1.1x10^8$
	E. coli (control)		**$1.5x10^6$**	**$2.4x10^{10}$**	**$4.8x10^{10}$**	**$8.7x10^{10}$**

(b)

Strain	**Sample**	**ZnSe (mg/mL)**	**Bacterial reduction** (%)		
			4h	**6h**	**24h**
	1	0.05	20.8	0	0
		0.5	0	0	0

E. coli	2	0.05	0	0	0
		0.5	25	27.1	85.1
	3	0.05	0	0	0
	4	0.05	0	0	0
		0.5	98.8	99.6	99.8

The data obtained, after the growth of *S. aureus* strain, in the presence of the oxidic structures, for 4, 6 and 24 hours in the LB culture medium, also demonstrated the sensitivity of the strain to the action of the nanoflowers, both in doped form, as well as in simple form. Therefore, non-doped zinc selenide, at a concentration of 0.5 mg/mL, showed an increased inhibitory effect on the bacterium, resulting in a reduction of colonies, after 4, 6, and 24 hours of treatment, of 98.9%, 98.63% and 99.1% respectively (Table 5).

Table 5. (a) Viable cells count (cfu/mL) of ***S. aureus*** grown on LB agar plates, after 4, 6 and 24h of incubation; (b) Percentage of bacterial reduction.

(a)

Strain	**Sample**	**ZnSe (mg/mL)**	**cfu/mL**			
			T0	**T4h**	**T6h**	**T24h**
S. aureus	1	0.05	$2.3x10^{6}$	$1.4x10^{10}$	$4.5x10^{10}$	$2.2x10^{11}$
		0.5	$2.7x10^{6}$	$1.8x10^{10}$	$4.8x10^{10}$	$2.3x10^{11}$
	2	0.05	$2.7x10^{6}$	$2x10^{10}$	$4.4x10^{10}$	$2.3x10^{11}$
		0.5	$2.3x10^{6}$	$2x10^{8}$	$6x10^{8}$	$2x10^{9}$
	3	0.05	$2.5x10^{6}$	$1.4x10^{10}$	$5.4x10^{10}$	$2.2x10^{11}$
	4	0.05	$2.7x10^{6}$	$9x10^{9}$	$4.3x10^{10}$	$2.1x10^{11}$
		0.5	$2.4x10^{6}$	$1.9x10^{9}$	$8.1x10^{9}$	$5.9x10^{9}$
	S. aureus (control)		**$3.1x10^{6}$**	**$1.9x10^{10}$**	**$4.4x10^{10}$**	**$2.2x10^{11}$**

(b)

Strain	Sample	ZnSe (mg/mL)	Bacterial reduction (%)		
			4h	6h	24h
S. aureus	1	0.05	26.3	0	0
	1	0.5	5.2	0	0
	2	0.05	0	0	0
	2	0.5	98.9	98.63	99.1
	3	0.05	10.5	0	0
	4	0.05	52.6	2.3	4.5
	4	0.5	90	81.6	97.3

The tested materials, at a concentration of 0.05%, showed a low inhibitory effect on *E. coli* and *S. aureus* reference strains, with a decrease of the number of colonies after 24 hours only for the *V. halodenitrificans* strain. The sensitivity of the Gram-positive *V. halodenitrificans* strain to the action of all tested materials was noted, regardless of the concentration used (Table 6).

Table 6. (a) Viable cells count (cfu/mL) of ***V. halodenitrificans*** grown on MH culture medium after 24 and 48h of incubation; (b) Percentage of bacterial reduction.

(a)

Strain	Sample	ZnSe (mg/mL)	cfu/mL		
			T0	T24h	T48h
V. halodenitrificans	1	0.05	$2x10^8$	$2.3x10^9$	$1.9x10^9$
		0.5	$2.1x10^8$	$8.9x10^8$	$7.2x10^7$
	2	0.05	$2.2x10^8$	$1.8x10^9$	$8.4x10^8$
		0.5	$2.1x10^8$	$1.03x10^8$	$7.9x10^6$

	3	0.05	$2.1x10^8$	$2.1x10^9$	$1.9x10^9$
	4	0.05	$2.3x10^8$	$2.2x10^9$	$2.9x10^9$
		0.5	$2.4x10^8$	$5.1x10^8$	$4x10^8$
	V. halodenitrificans (control)		**$2.5x10^8$**	**$2.8x10^9$**	**$2.9x10^9$**

(b)

Strain	**Sample**	**ZnSe (mg/mL)**	**Bacterial reduction (%)**	
			24h	**48h**
V. halodenitrificans	1	0.05	17.85	34.5
		0.5	68.21	97.5
	2	0.05	35.71	71
		0.5	96.32	99.7
	3	0.05	25	34.5
	4	0.05	21.42	0
		0.5	81.78	86.2

The obtained results indicated a high degree of inhibition of the zinc selenide sample in the non-doped form, on Gram-positive bacterial strains, compared to the Gram-negative strain. The antibacterial activity of the four samples of ZnSe based materials of different concentrations was also determined using 2,3,5-triphenyl tetrazolium chloride (TTC) as a chromogenic indicator and was expressed as μg/mL triphenyl formazan.

The data obtained by the direct antibacterial analysis method, but also by the biochemical method for assessing cell viability, were confirmed by those obtained by the spectrophotometric analysis method. However, the method cannot be applied under any conditions, due to the errors that may occur, the disadvantage being that they contribute to the turbidity of the sample. In conclusion, the antibacterial capacity of the synthesized materials, especially for the ZnSe nanostructures with flower-like morphology (0.5 mg/mL), in the simple

form (sample 2) and doped with titanium dioxide (sample 4), can be attributed to its physicochemical properties, composition and morphology of the respective semiconductor, and to the microorganism with which it interacts. Also, the antibacterial activity of the tested materials was influenced by the contact time with the tested microorganisms and concentration of the material. It was observed that the number of colony-forming units (cfu/mL) decreasing as the concentration of material increases.

2. Contributions to the identification of the antibacterial mechanisms of zinc selenide nanomaterials

2.1. Materials and methods

Following the evaluation studies of the antibacterial activity of the commercial form of titanium dioxide, Aeroxide P25, and of the materials based on zinc selenide (ZnSe), the inorganic structures that showed the best antibacterial activity were selected for the following tests. Compared to nano-TiO_2, one of the most widely used, known and intensively studied semiconductor, mainly due to the photocatalytic properties, proving to be an excellent antibacterial agent, zinc selenide, at present, has increased interest in the synthesis methods and characterization and very few studies offer information on its antibacterial properties. The zinc selenide nanomaterials with flower-like morphology were selected, in the simple form, but also doped with titanium dioxide, in a concentration of 0.5 mg/mL to carry out studies for understanding the antibacterial mechanisms. *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were used for the experiments. To date, no such studies have been reported in the literature. Thus, after the interaction of the materials with the bacterial cells in the LB culture medium for 4 hours, under shaking conditions (150 rpm), we proposed:

1. Analysis of possible morphological changes that the materials could induce cells, using scanning electron microscopy (SEM);

2. Spectrofluorimetric analysis of detection of reactive oxygen species;

3. Polymerase chain reaction (PCR) of the 16S rRNA gene from DNA extracted from untreated and untreated cells;

4. Repetitive Sequence-Based PCR (Rep-PCR) analysis of DNA extracted from treated and untreated cells;

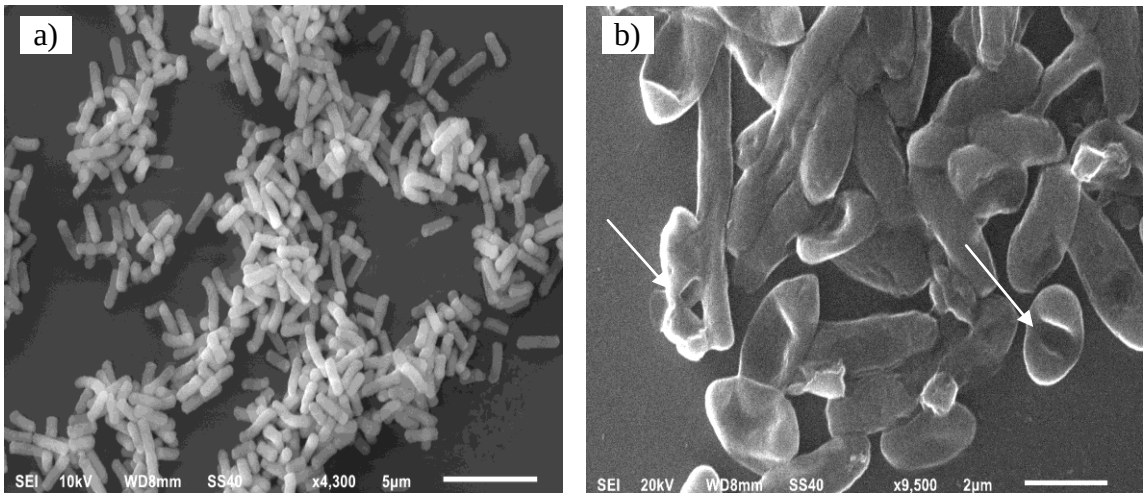
5. Spectrophotometric analysis to verify the membrane integrity;

6. Analysis of the protein profile of the treated and untreated cell lysate by the SDS-PAGE technique (electrophoresis in denaturing medium).

2.2. Results and discussions

2.2.1. Morphological analysis of samples

The SEM investigation of the effect of the zinc selenide materials on the two strains of selected bacteria demonstrated a different response of the cells to the action of the nanoflowers. The images are in accordance with the test results for the evaluation of the antibacterial activity of the materials. Thus, after 4 hours of incubation of the cells with the nanoflowers, significant changes of the *E. coli* cells were observed (cell deformations, appearance of "craters" on the surface of the membranes, release of the cellular content) (Fig. 2) compared to the *S. aureus* cells (Fig. 3), noting different mechanisms of action depending on the Gram character of the strains.



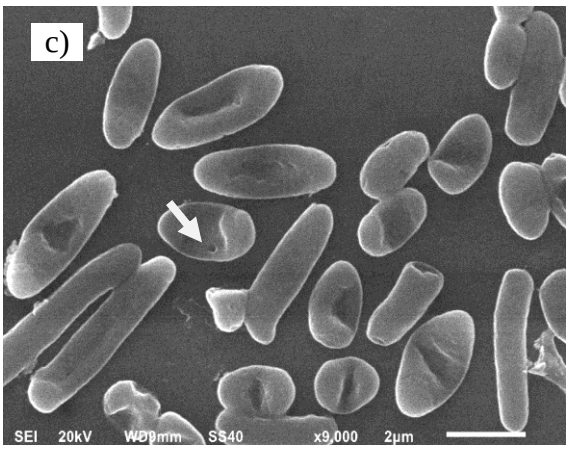


Fig. 2. SEM images of untreated *E. coli* cells (a) and treated with ZnSe with flower morphology (b) and TiO_2-ZnSe with flower morphology (c), after 4 hours of incubation.

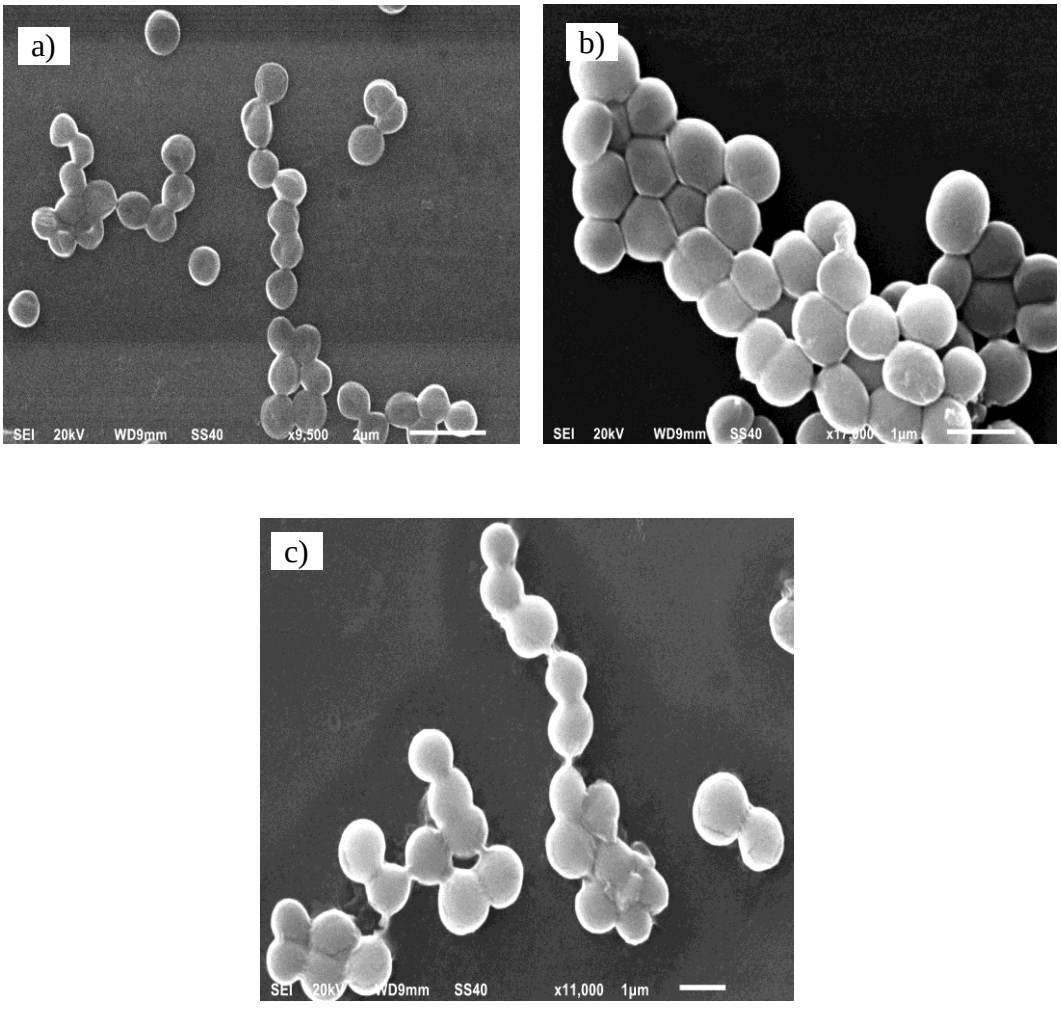


Fig. 3. SEM images of untreated ***S. aureus*** cells (a) and treated with flower type morphology ZnSe (b) and TiO_2-ZnSe with flower morphology (c), after 4 hours of incubation.

2.2.2. Spectrofluorimetric analysis of detection of reactive oxygen species

Spectrofluorimetric analysis of the detection of reactive oxygen species indicated an increased level of reactive oxygen species after 4 hours of treatment of cells with both forms of zinc selenide nanoflowers, as compared to the control samples, suggesting the significant presence of oxidative stress. The highest fluorescence intensity was obtained for the cells treated with *S. aureus*, after 4 hours of incubation (Fig. 4 c, d).

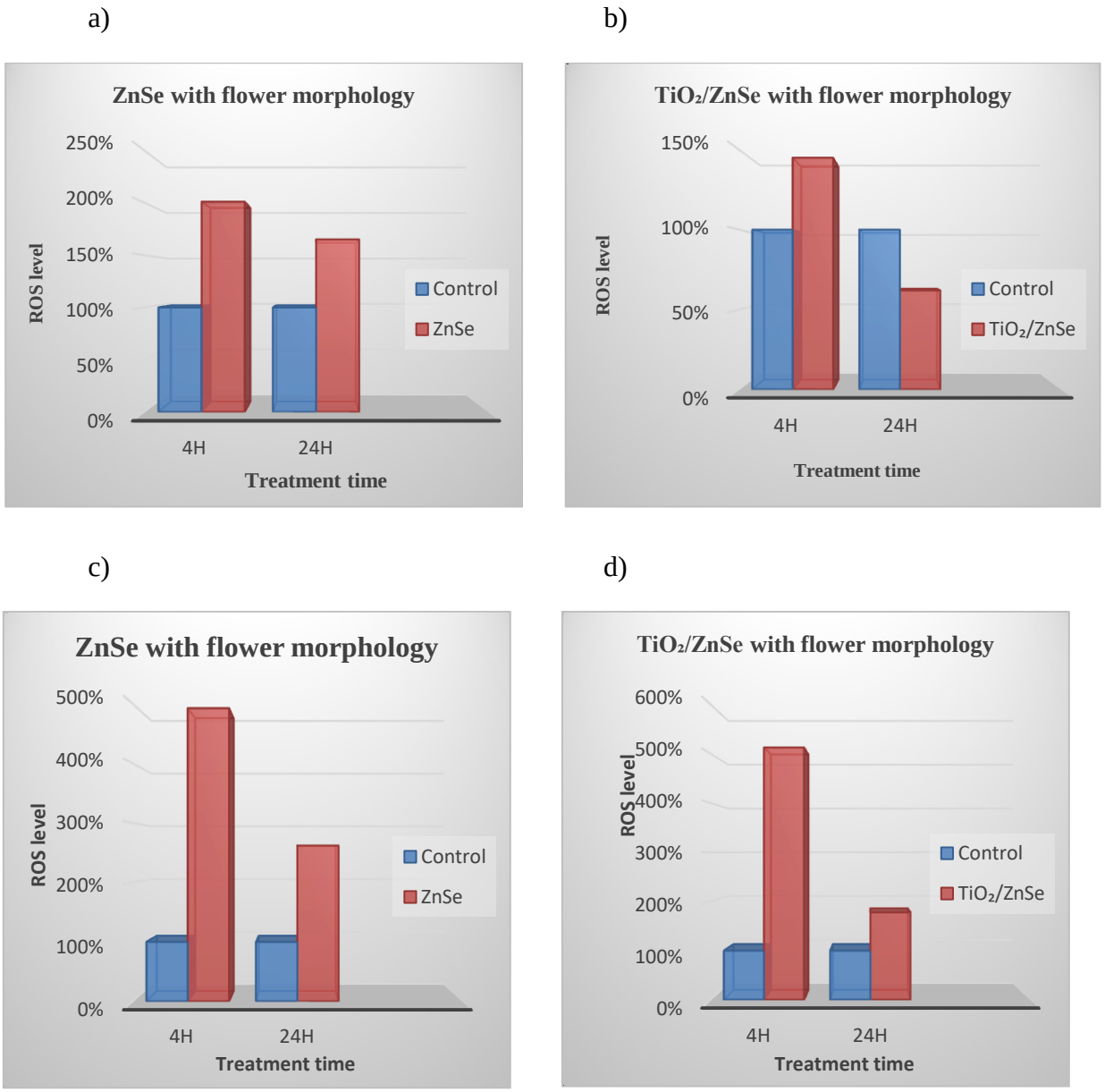


Fig. 4. The effect of ZnSe with flower morphology on the production of reactive oxygen species in *E. coli* (a, b) and *S. aureus* (c, d) cells.

2.2.3. PCR amplification of the gene for16S rRNA from DNA extracted from treated and untreated cells

PCR analysis for the 16S RNA gene indicates possible changes in the DNA in *E. coli* cells treated with the doped form of zinc selenium (Fig. 5 a-line 3). In *S. aureus* cells, no changes were found in the 16S rRNA gene, compared to the control sample, obtaining fragments of the same length, 1.5 kb (Fig. 5 b).

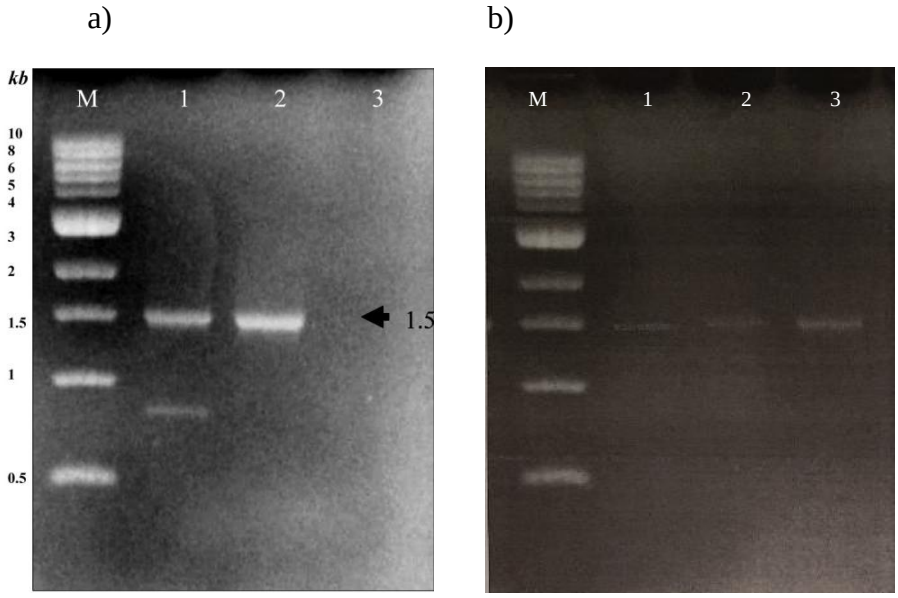


Fig. 5. PCR amplification of the 16S rRNA gene from DNA extracted from:
a) ***E. coli*** ATCC 25922. Line 1: untreated *E. coli* cells. Line 2: *E. coli* treated with ZnSe with flower morphology. Line 3. *E. coli* treated with TiO_2-ZnSe with flower morphology.
b) ***S. aureus*** ATCC 25923. Line 1: untreated *S. aureus* cells. Line 2: *S. aureus* treated with ZnSe with flower morphology. Line 3. *S. aureus* treated with TiO_2-ZnSe with flower type morphology. M: 1kb molecular weight marker (BioLabs).

2.2.4. Rep-PCR analysis of DNA extracted from treated and untreated cells

The results obtained from the rep-PCR analysis showed that the exposure of the two bacterial strains to the synthesized zinc selenide nanoflowers did not result in changes in the repetitive sequences in the genome structure (Fig. 6 a, b).

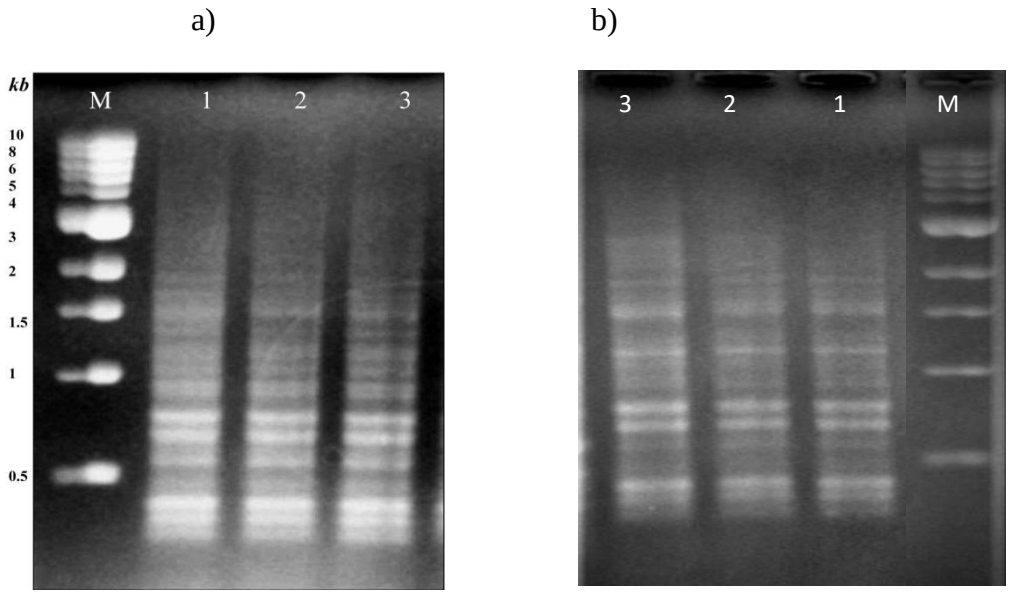
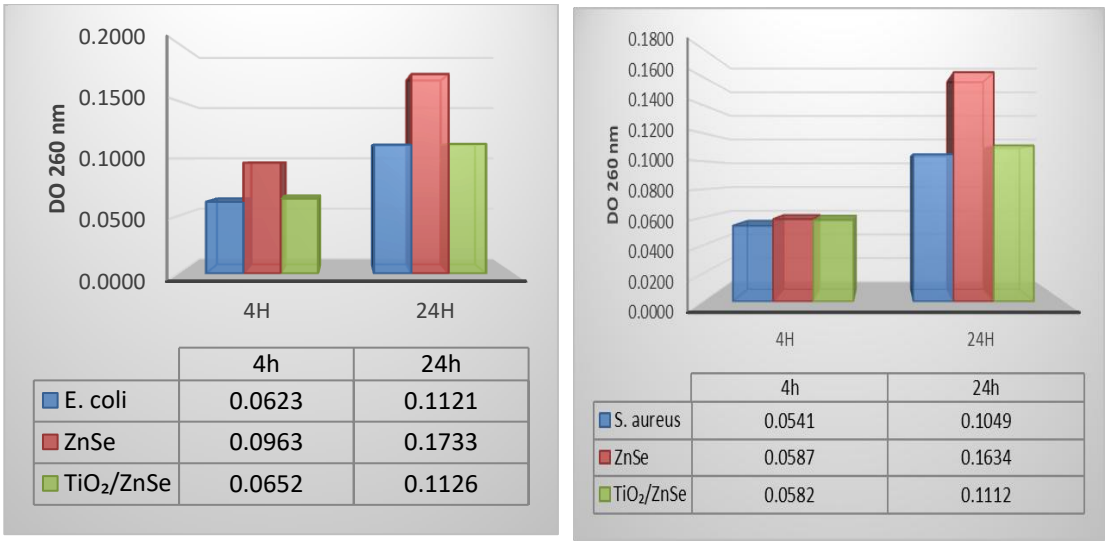


Fig. 6. Rep-PCR profile of the strains of
a) *E. coli* ATCC 25922. Line 1: untreated cells of *E. coli*. Line 2: *E. coli* treated with ZnSe with flower morphology. Line 3. *E. coli* exposed to TiO_2-ZnSe with flower morphology.
b) *S. aureus* ATCC 25923. Line 1: untreated cells of *S. aureus*. Line 2: *S. aureus* treated with ZnSe with flower morphology. Line 3. *S. aureus* treated with TiO_2-ZnSe with flower type morphology. M: 1kb molecular weight marker (BioLabs).

2.2.5. Spectrophotometric analysis of the membrane integrity

Verification of the membrane integrity by the spectrophotometric method allowed us to observe differences between the absorbance values of the sample treated and not treated by *E. coli*, which demonstrates the release of nucleic acids in the extracellular environment (Fig.7a). In the case of *S. aureus*, after 4 hours of exposure to the tested nanomaterials, no differences in absorbance values were observed compared to the control sample.

a) b)



	4h	24h
E. coli	0.0623	0.1121
ZnSe	0.0963	0.1733
TiO_2/ZnSe	0.0652	0.1126

	4h	24h
S. aureus	0.0541	0.1049
ZnSe	0.0587	0.1634
TiO_2/ZnSe	0.0582	0.1112

Fig. 7. Optical density at 260 nm after 4 and 24 hours of incubation: a) *E. coli* and b) *S. aureus*, with zinc selenide materials.

2.2.6. Analysis of total protein profile by SDS-PAGE technique

Following the treatment of bacterial cells tested with zinc selenide nanomaterials with flower morphology, it was found that the protein profile of *S. aureus* and *E. coli* strains is identical to that of the control strains (Figs. 8, 9).

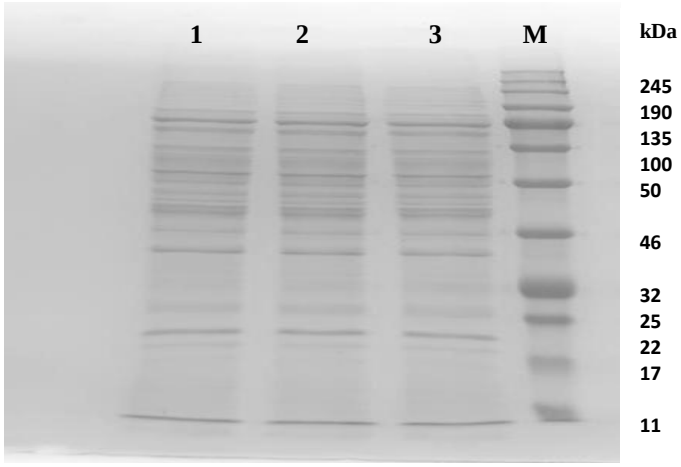


Fig. 8. Protein profile for *S. aureus*: Line 1. *S. aureus* untreated (control). Line 2. *S. aureus* treated with 0.5 mg/mL ZnSe with flower morphology. Line 3. *S. aureus* treated with TiO_2-ZnSe with flower type morphology. Line 4. BioLabs Protein weight marker.

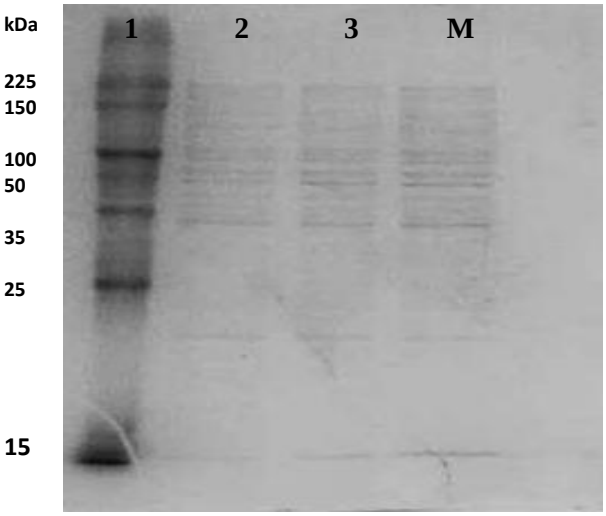


Fig. 9. Protein profile for *E. coli*: Line 1. Protein weight marker. Line 2. untreated *E. coli* (control). Line 3. *E. coli* treated with 0.5 mg/mL ZnSe. Line 4. *E. coli* treated with 0.5 mg/mL TiO_2-ZnSe.

3. The applicative potential of some nanomaterials based on sodium titanate

The last objective of the thesis was to follow the dynamics of growing strains of halophilic bacteria isolated from the mural painting of the Hurezi Monastery (*Garicola koreensis* and *Halobacillus naozhouensis*) and Humor Monastery (*Halobacillus hunanensis*), in the presence of sodium titanate nanotubes, in terms of identifying the applicative potential. Thus, the antibacterial nanomaterials could be successfully used for the manufacture of mortars for restoration interventions that prevent the colonization of their surface with biodeteriogens.

The nanotubes were synthesized within the Institute of Physical Chemistry "Ilie Murgulescu" of the Romanian Academy.

The nanomaterials were obtained by the hydrothermal synthesis method, starting from the commercial precursor Degussa P25 (Sigma-Aldrich) (Merciu et al., 2009). The synthesized nanotubes have a length of 50 nm and a diameter of about 8-9 nm and were used in a concentration of 0.025%. The evaluation of the antibacterial activity was performed by the spectrophotometric method. The growth of the bacterial strains in the presence and absence of the nanotubes was followed by measuring the turbidity of the samples at the wavelength of 660

nm at specified times. Also, the biochemical method of assessing cell viability was used by determining the total dehydrogenase activity (qualitative and quantitative methods), within 24 hours.

Preliminary results have shown their efficiency in bacterial strains, especially Gram-negative. The different response of bacterial cells can be attributed to the structural differences in the cell wall, but also the composition and morphology of the oxidic nanostructure. Also, another essential factor in the manifestation of the antibacterial character of the sodium titanate nanomaterials is the exposure time.

After a brief characterization of the material, to be able to associate a particular physicochemical property with the biological response and to ensure the reproducibility of the results, these fabricated nanostructures could be introduced into the various mortars, thus helping to prevent the biodeterioration process of the historical monuments.

CONCLUSIONS

This study was conducted to investigate the interaction of Gram-positive and Gram-negative bacteria with synthesized nano and micro materials, considering the dynamics of growing bacterial strains in the presence of these materials, selecting structures with antibacterial properties in order to identify the action mechanisms.

Based on the experimental data obtained, as well as the partial conclusions are drawn, the general conclusions will be presented below.

- The antibacterial effect of both titanium dioxide nanopowders Aeroxide P25 (non-irradiated and after stopping UV irradiation) and zinc selenide materials obtained by the hydrothermal method has been demonstrated

- *E. coli* ATCC 25922, *S. aureus* ATCC 25923 bacterial cells introduced in the experimental study, responded differently to the action of semiconductors

- The data obtained indicate different mechanisms of the materials used on the cells of Gram-positive and Gram-negative bacteria

- The bacteriostatic effect of the two forms of titanium dioxide nanopowders on *E. coli* ATCC 25922 cells was noted

- The bactericidal effect of nano-TiO2 on the Gram-positive *S. aureus* strain ATCC 25923 has been found

- The morphological changes were observed in *V. halodenitrificans* colonies and can be attributed to titanium dioxide, which acted as a stress factor, the bacterial strain developing an adaptation mechanism after the contact with the material

- The antibacterial effect of zinc selenide nanoflowers, in the doped forms with titanium dioxide and non-doped, at a concentration of 0.5 mg/mL has been demonstrated

- The sensitivity of the Gram-positive *V. halodenitrificans* strain to the action of all the tested materials was observed, regardless of the concentration used, but a strong bacterial effect was obtained for the zinc selenide nanoflowers (0.5 mg/mL)

- A higher degree of inhibition of the zinc selenide sample in doped form was found in Gram-positive strains (*S. aureus*, *V. halodenitrificans*), compared to Gram-negative strain (*E. coli*)

- It has been shown that the antibacterial effect of zinc selenide structures is due to the material properties, composition and morphology of the semiconductor, the exposure time, the concentration of the material, but also the type of bacteria with which it interacts

- The identification in small amounts of secondary phases for the zinc selenide sample in the non-doped form could be a factor that would contribute to reducing cell viability

- It has been shown that the main mode of action of zinc selenide materials, with flower morphology, on the two reference strains, *E. coli* ATCC 25922 and *S. aureus* ATCC 25923, is due to the generation of reactive oxygen species

- The result of the action of the materials on the *E. coli* cells consisted of the destruction of the membrane, with the release of the cellular content and possibly the DNA damage

- In *S. aureus* cells, we assume that reactive oxygen species have caused intracellular component damage, without observing the release of cellular content

- Sodium titanate nanotubes (0.025%), used to identify their antibacterial potential, have shown antimicrobial effect, acting in particular on Gram-negative bacterial strains. These can be a candidate in preventing the biodeterioration process of historical monuments.

Contributions to scientific knowledge

Following the scientific objectives of the doctoral work, the originality of the studies carried out was materialized by several elements of novelty, which bring scientific value to the studies performed. Thus, new zinc selenide based materials with flower morphology, obtained by the hydrothermal synthesis route, which have demonstrated antibacterial properties, have been used, with action on the *E. coli* ATCC 25922, *S. aureus* ATCC 25923, and *V. halodenitrificans* bacterial strains.

Also, the main mode of action of these nanostructures on the reference bacterial strains, represented by the generation of reactive oxygen species, has been demonstrated. To date, no such studies have been identified in the literature.

At present, the antimicrobial mechanisms of the various synthesized nanomaterials are not yet fully elucidated. For example, antibacterial activity may be attributed to oxidative stress, as demonstrated for the zinc selenide semiconductor, while for other materials, the antimicrobial mechanism may not be associated with the regulation of bacterial metabolism.

The lack of standards is one of the limitations of the existing studies on the antimicrobial mechanisms of the synthesized nanomaterials.

The diversity of bacterial strains, the action times, and the characteristics of the different nano or micro structures have been examined in various studies, which makes it difficult to compare the results obtained in terms of antibacterial activity. Moreover, a single method does not offer all the conditions for obtaining information about the antibacterial mechanisms of the manufactured materials.

As different types of nano and micro structures have different antibacterial effects. A comprehensive analysis is proposed to study the possible antibacterial mechanisms.

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List of scientific papers published in the subject of the doctoral thesis

ARTICLES

1. Neagu S., Anastasescu C., Balint I., Zaharescu M., Ardelean I., Enache M. (**2019)**. Răspunsul celulelor de *Escherichia coli* la acțiunea materialelor pe bază de ZnSe/ The response of *Escherichia coli* cells to the action of ZnSe based materials. Revista Română de Materiale/Romanian Journal of Materials, 49 (3): 322 – 330 **(0,661 IF)**.

2. Neagu S., Preda S., Zaharescu M., Kamekura M., Cojoc R., Enache M. (**2018)**. The effect of titanate nanotubes towards moderately halophilic bacteria. Romanian Biotechnological Letters, 23(4): 13814-13822 **(0,59 IF)**.

3. **Neagu S.**, Preda S., Anastasescu C., Zaharescu M., Enache M., Cojoc R. (**2014).** The functionalization of silica and titanate nanostructures with halotolerant proteases. Rev. Roum. Chim., 59, 97-103 (**0,411 IF**).

4. **Merciu S.**, Văcăroiu C., Filimon R., Popescu G., Preda S., Anastasescu C., Zaharescu M., Enache M. (**2009)**. Nanotubes biologically active in media with high salt concentration, Biotechnol. & Biotechnol. Eq. – special issue, 23(2): 827 – 831 (**1,097 IF**).

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BOOK CHAPTER

1. Enache M., **Neagu S.**, Anastasescu C., Cojoc R., Zaharescu M., **2014**. The effects of silica nanostructures on halotolerant microorganisms isolated from rock salt crystal. In New Applications of Nanomaterial, Series in "Micro and nanoengineering," A. Catrinel Ion, D. Dascălu, G. Cârjă, M.L. Ciurea eds., Ed. Academiei Române, 51-59, ISBN 978-973-27-2434-7.