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Dynamics of the TiO₂ nanofilms bactericidal activity

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Abstract

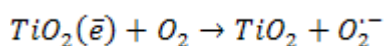
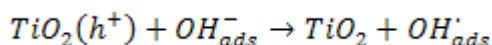
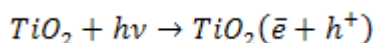
TiO₂ (Titanium dioxide) nanofilms are perspective nanomaterials for the purification of air and surfaces in the hospitals, which can reduce number of nosocomial infections. The bactericidal activity of the TiO₂ nanofilms induced by ultraviolet (UV) for a large number of Gram-positive and Gram-negative bacteria was investigated. The statistically significant decrease in viability of majority strains of bacteria was shown after incubation (15 min) on the TiO₂ nanofilms under UV (wavelength – 365 nm). Three strains (*Proteus vulgaris* 1212, *Klebsiella pneumoniae* 527 and *Klebsiella oxytoca* 525) do not lose the viability under these conditions. The incubation on the surface of TiO₂ nanofilms under UV during one hour leads to almost complete suppression of the bacteria viability. The dynamics of the viability reduce of the bacteria which are most often cause of nosocomial infections was studied. The leading role of reactive oxygen species (ROS) in the reduction of viability was demonstrated through the study of photocatalytic oxidation reactions of epinephrine on the TiO₂ nanofilm surface.

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INTRODUCTION

Despite increasing the knowledge about bacteria and development of new antibiotics, these organisms continue to cause significant morbidity and mortality, especially in the health care setting [1]. The problems of bacteria dissemination and forming of antibiotics-resistant organisms are facilitated by person-to-person transmission due to inconsistent application of basic infection control practices by hospital personnel [2] and contamination of surface which can be reservoirs of bacteria implicated in a wide variety of hospital acquired infections [3]. Environmental screening can identify pathogens on a variety of hospital surfaces [4-6]. These include general surfaces such as shelves and ledges; curtains, linen and clothes; furniture and all items of clinical equipment [7]. Therefore an important aim in recent years is the creation of specialized self sterilizing surfaces and their implementation in hospitals. Such a surface can destroy antibiotic-resistant strains in the case of their contamination and can not be reservoir of hospital acquired infections.

Semiconductors can be used for creation self sterilizing surface. Many semiconductor materials have been tested for creation of antibacterial surface but it is generally accepted that TiO₂ (Titanium dioxide), due to its low cost and high activity and stability under irradiation, is the most reliable material [8]. The following steps occur when a semiconductor is irradiated by energy higher or equal to the band gap (usually for this purpose UV used), causing the formation of a hole (h⁺) in the valence band and an electron (ē) in the conduction band:



So TiO₂ is the source of reactive oxygen species (ROS), including highly active superoxide anion radical. ROS attacks the cell wall of bacteria adhered on surface or contact with surface and as a result of oxidation the bacterial components the microorganisms

are destroyed. Self sterilizing activity of TiO₂ films toward different microorganisms was studied in many works [9-14].

The main purpose of this study was to determine the antibacterial activity of TiO₂ nanofilms toward bacteria, which cause the most common nosocomial infections, the identification of strains of the most and the least sensitive to the ROS which are generated by UV on the TiO₂ nanofilms surface, as well as the determination of the dynamics of the bactericidal effect after increasing exposure time.

MATERIALS AND METHODS

The strains a cause of hospital acquired infections were used for testing on the TiO₂ nanofilms surface. Among them Gram-positive: *Staphylococcus aureus* 956; *Staphylococcus aureus* 455; *Staphylococcus epidermidis* 1061; *Micrococcus spp.* 5028; *Enterococcus faecium* 2381; *Enterococcus faecalis* 971 and Gram-negative: *Escherichia coli* 321; *Pseudomonas aeruginosa* 969; *Klebsiella oxytoca* 525; *Klebsiella pneumoniae* 527; *Proteus vulgaris* 1212 are studied. Cultures were isolated in the bacterial laboratory of Infectious Hospital №2 of Nizhny Novgorod.

The production of ROS by TiO₂-nanofilms was examined by studying the change in the transmission spectrum of oxidized epinephrine hydrochloride (Adrenalin, 4(1-Hydroxy-2 [methylamino]etyl) -1,2-benzenediol hydrochloride, Sigma, USA).

Production of TiO₂ nanofilms. TiO₂ coatings were brought chemically on a glass surface using solutions of hydrolyzed compounds by sol-gel technology. 5% tetra-butyl-oxy-titan in isopropyl Ti(OC₄H₉)₄ was used as a film forming agent. Hydrochloric acid was used as a catalyst and stabilizer. The solution was applied on the surface of Petri dish with spin coating techniques. A transparent homogeneous layer was developed on a glass surface and it was titanate acid polymers. Further temperature treatment (450°C, 5 h) resulted in the completion of decomposition reactions of intermediate hydrolysis products and it completes to remove of the solvent. Evaporation provides to create a transparent TiO₂ nanofilm strongly attached to the glass surface [15].

Bacterial suspension production. The strains were grown on beef-extract agar (Pharmacotherapy, Russia) at 37°C for 20 h. Each slant agar was washed off with a sterile physiological saline solution (PSS) (Biochemist, Russia) and twice washed with PSS (pH 7,2); the transmission of the bacterial suspension was brought to 0,269 using a spectrophotometer (Russia) (wavelength – 670 nm). It corresponds to 10 ME of turbidity standard. A series of dilutions was prepared from the

suspension so that approximately 200 CFU per Petri dish could grow. Each dilution was evaluated on the TiO₂ nanofilm surface.

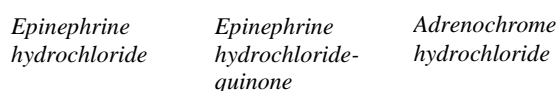
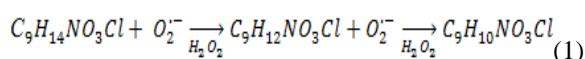
Evaluation of the bactericidal activity onto TiO₂ nanofilms. In the experiments, bacterial suspensions were brought onto TiO₂ nanofilm surfaces and they were irradiated by UV (wavelength – 365 nm) during 15 min. The density of UV-lamp light power (BIO-2, Ukraine) was measured by a detector of radiation energy (IMO-2N, Russia), it was 4,5 mW/cm². A filter UFS-6 was used to remove short UV-wavelengths (λ < 365 nm), since light at this wavelength has own bactericidal activity. As a control, a bacterial suspension deposited onto a sterile glass surface was UV-illuminated under the same experimental conditions. Further, a decrease in bacterial viability on a TiO₂ surface was compared against this control. After 15 min of UV-illumination 0,05 ml of bacterial suspension was carried onto an agar and evenly spread with a spatula. The cultures were incubated at 37°C for 20 h, after that CFU were counted. UV-irradiation time was increased to 30, 45 and 60 minutes for studying the dynamics of viability reducing of bacteria on the surface of TiO₂ nanofilms. The formation of ROS was demonstrated in the series of experiment with oxidation epinephrine hydrochloride. In this cause the solution of epinephrine hydrochloride was brought onto TiO₂ nanofilms and time of incubation was increased to 3 h. All studies were made and analyzed in the period 2009 - 2012 years

Statistical analysis was performed by using Origin 7.0 Server package software. Mean values and standard deviations were determined. Significant difference between two values (control and experiment) evaluated by the Student's test.

RESULTS

The production of ROS was showed by change in the transmission spectrum of epinephrine hydrochloride (Fig. 1).

Epinephrine hydrochloride under the influence of the superoxide anion radical is oxidized according to reaction (1) [16]:



The sensitive temperature sensor did not record differences in temperature during the incubation solution of epinephrine hydrochloride on the TiO₂ nanofilm, so the change of the spectrum is conditioned by ROS completely.

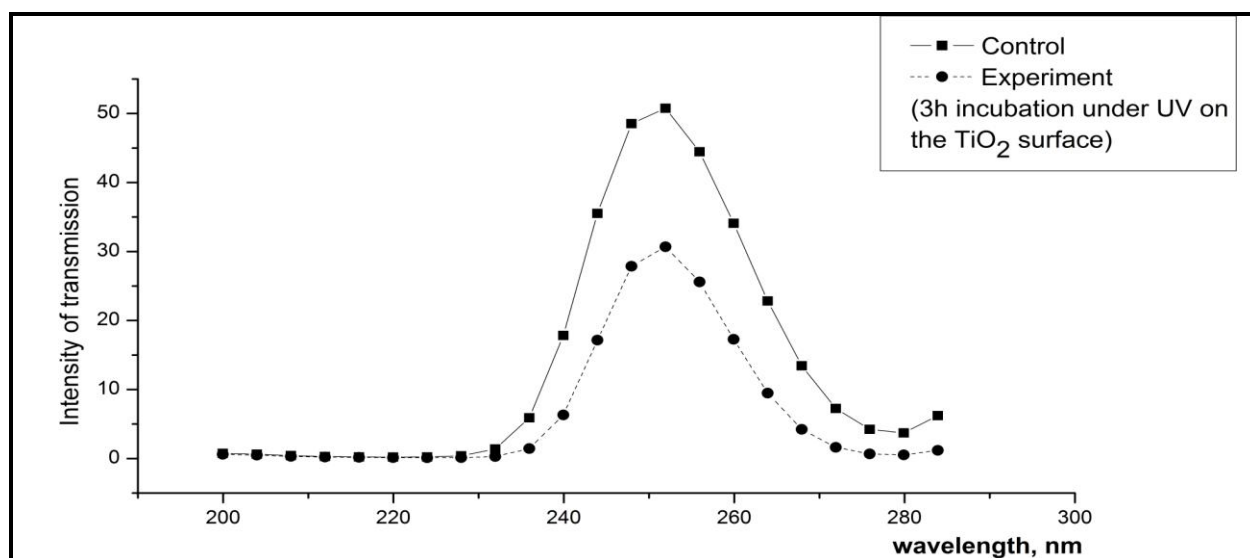


Fig. 1 Change of epinephrine hydrochloride transmission spectrum after 3 hours of irradiation by UV on the surface TiO_2 nanofilm. Transmission coefficient was reduced due to differences of epinephrine hydrochloride color as a result of oxidation by ROS.

Table 1. The reduction of bacterial viability on the surface of TiO_2 nanofilms, under UV (wavelength – 365 nm) during 15 min

Bacterial strain	Control (CFU on the glass surface under UV)	Experiment (CFU on the TiO_2 -nanofilms under UV)	Decreased viability relative to control (%)
Gram positive bacteria			
<i>E. faecalis</i> 971	64,91 ± 14,58	38,82 ± 7,74*	40
<i>E. faecium</i> 2381	175,72 ± 12,33	129,09 ± 16,39*	27
<i>S. aureus</i> 956	115,80 ± 10,70	82,50 ± 6,50*	29
<i>S. aureus</i> 455	100,57 ± 14,06	51,71 ± 6,21*	49
<i>S. epidermidis</i> 1061	152,30 ± 24,60	84,00 ± 17,00*	45
<i>Micrococcus spp.</i> 5028	71,27 ± 11,79	49,79 ± 10,80*	30
Gram negative bacteria			
<i>E. coli</i> 321	279,00 ± 34,50	148,30 ± 27,80*	47
<i>K. oxytoca</i> 525	105,30 ± 9,20	91,70 ± 9,60	-
<i>K. pneumoniae</i> 527	127,60 ± 12,00	107,40 ± 10,22	-
<i>P. vulgaris</i> 1212	103,33 ± 27,12	106,20 ± 23,64	-
<i>P. aeruginosa</i> 969	111,42 ± 3,38	64,42 ± 3,94*	43

* Differences between the control and experiment are significantly different ($p < 0.05$)

CFU – colony-forming units

UV – ultraviolet

The results of the viability reduction estimated by the number of colony forming units (CFU) are presented in Table 1. Induction of ROS was produced on the surface by UV; the bacterial suspensions on the surface of the TiO_2 nanofilms under UV were incubated during 15 min.

For the majority of pathogenic strains were sufficient to 15 min exposure onto the surface of TiO_2 nanofilms to significantly reduce the viability (Fig. 2), but three strains were not sensitive to the effects of ROS formed on the surface of the TiO_2 nanofilms. These are *Proteus vulgaris* 1212, *Klebsiella pneumoniae* 527 and *Klebsiella oxytoca* 525.

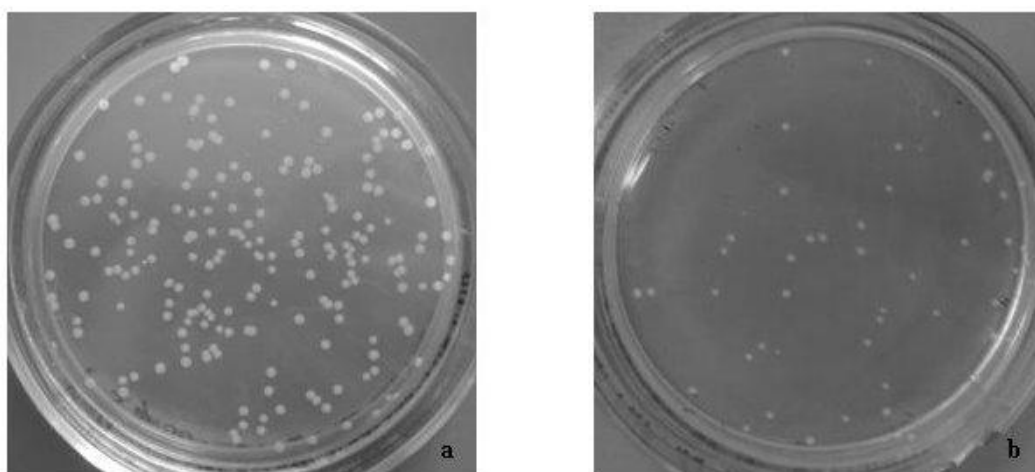


Fig. 2 Reduction of CFU *S. epidermidis* 1061 on a Petri dish: a. control – the suspension was incubated for 15 min on the surface of sterile glass under UV, b. experiment – the suspension was incubated for 15 minutes on the surface of TiO₂ nanofilms under UV.

Dynamics of viability reduction has been investigated for the strains, which are most common cause of hospital acquired infections. The incubation time for the TiO₂ nanofilms with bacterial suspension under UV was increased to 30, 45 and 60 min. The control of the bacteria viability was made by the following way: the suspension was carried out onto the glass – the incubation time was the same (30, 45, 60 min), the bacteria were incubated in an environment without any influence. The incubation under UV on the surface of sterile glass was used to assess the impact of UV on the bacteria viability. The results are presented in Figures 3 – 6.

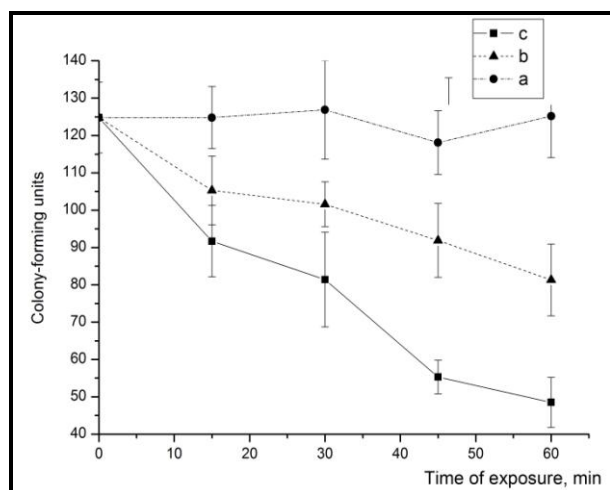


Fig. 3 Dynamics of viability reduction of *K. oxytoca* 525 during 60 min exposure: a. control – viability of the strain in the environment (without any influence), b. reduction in viability under the influence of UV, c. reduction in the viability under the combined effects of UV and ROS, generated by TiO₂ nanofilms.

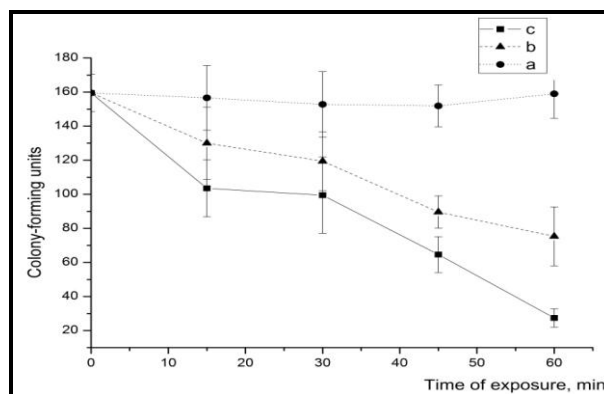


Fig. 4 Dynamics of viability reduction of *S. aureus* 455 during 60 min exposure: a. control – viability of the strain in the environment (without any influence), b. reduction in viability under the influence of UV, c. reduction in the viability under the combined effects of UV and ROS, generated by TiO₂ nanofilms.

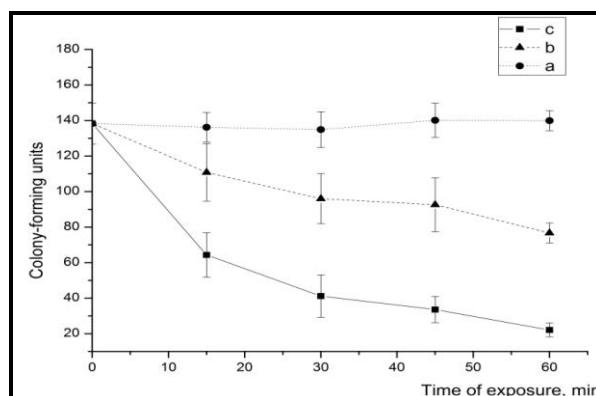


Fig. 5 Dynamics of viability reduction of *P. aeruginosa* 969 during 60 min exposure: a. control – viability of the strain in the environment (without any influence), b. reduction in viability under the influence of UV, c. reduction in the viability under the combined effects of UV and ROS, generated by TiO₂ nanofilms.

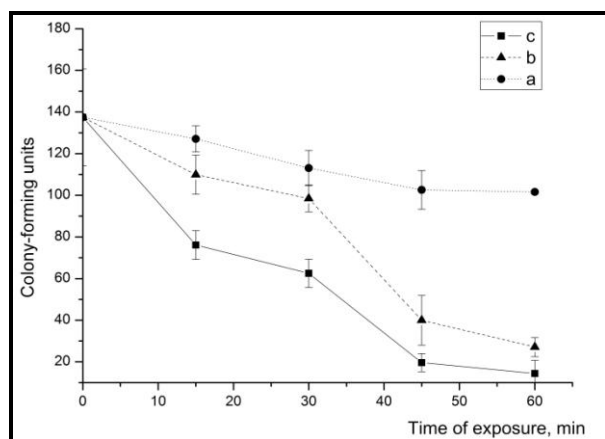


Fig. 6 Dynamics of viability reduction of *E. faecium* 2381 during 60 min exposure: a. control – viability of the strain in the environment (without any influence), b. reduction in viability under the influence of UV, c. reduction in the viability under the combined effects of UV and ROS, generated by TiO₂ nanofilms.

DISCUSSION

The results showed a significant reduction in the viability of the most of hospital acquired infections pathogens under influence of combination of two factors: UV and ROS produced by the surface of the TiO₂ nanofilms. Significant suppression of the bacteria viability was observed though even 15 minutes of exposure time and during this time the survival of bacteria decreases as compared with only UV treatment from 27 to 49%. It means that the treatment of surface with TiO₂ nanofilms can improve the efficiency of traditional sterilization by UV.

However, it was found that three strains of bacteria are not sensitive to the ROS produced by the semiconductor surface. These bacteria have some peculiarities of external structures which localizes on the cell wall. Indeed, the traditional staining revealed the presence of capsule which expressed in both species of *Klebsiella*. *Proteus vulgaris* 1212 strains indicate a significant phenomenon of swarming on agar surface which denotes the existence of a large number of flagella. As a result, the developed bacteria structures on the surface (capsule of *Klebsiella* and flagella of *Proteus*) can play the role of the peculiar "trap" of ROS. ROS oxidizes these external structures and their short lifetime makes impossible to oxidize the cell wall, as result the bacteria can survive.

This hypothesis is indirectly confirmed by the study of the reducing dynamics of the bacteria viability with increasing exposure time on the surface of TiO₂ nanofilms. The viability of *Klebsiella oxytoca* 525 emplaced onto the surface of TiO₂ nanofilms is different from the viability of the bacteria, which were irradiated by UV only significantly and after 45 min

reduced viability was 40%. Increasing to exposure time leads to the formation of more ROS, which is enough not only for the oxidation of the bacteria surface structures but also the oxidation of cell wall components.

The best results of bactericidal activity of TiO₂ nanofilms were obtained for strains – the leaders in the occurrence of nosocomial infections: *Staphylococcus aureus* 455, *Pseudomonas aeruginosa* 969 and *Enterococcus faecium* 2381. *Staphylococcus* and *Pseudomonas* were resistant in the environment and their viability was at a constant level within one hour of incubation (control), but the strain of *Enterococcus* was less stable and control showed that after 60 min of incubation there is a decrease in the viability of these bacteria was 24% (Fig. 4 – 6). The combination of two factors – the ROS formation by TiO₂ nanofilms and UV during 60 min on the surface of semiconductor resulted the following data: a decrease of viability *Staphylococcus aureus* 455 – 82,6 % (for UV only – 52,6%); a decrease of viability *Pseudomonas aeruginosa* 969 – 84% (for UV only – 44,4%); a decrease of viability *Enterococcus faecium* 2381 – 88,7% (for UV only – 79,7%). Thus, the presented results point out importance of contribution to the development of a common bactericidal system made of ROS and UV.

CONCLUSION

Thus, the work clearly demonstrates the existence of contact bactericidal activity for TiO₂ nanofilms. When the additional energy (in this cause it is energy of UV photon) taken up by these surfaces they produce reactive oxygen species which can destroy the vast majority of strains of nosocomial infections pathogens. The use of medical surface treated by semiconductors can significantly reduce the role of these surfaces as reservoirs of infection and reduce the number of hospital acquired infections. However, it must be remembered that it is important to select the exposure time, the optimum for the destruction of all bacteria kinds, since there are strains that are due to structural peculiarity of structures located under the cell wall are resistant to the combined effect of UV and ROS.

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