ЭКСПЕРИМЕНТАЛЬНЫЕ ИССЛЕДОВАНИЯ
EXPERIMENTAL RESEARCHES

ANTIBACTERIAL ACTIVITY OF PHOTOCATALYTIC TIO_2 SPHERICAL PARTICLES WITH 100–200 NM SIZES SYNTHESIZED BY THE PEROXO METHOD

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RESUME

Background. *The development of photocatalysts with antibacterial properties seems to be relevant for combating multiresistant microorganisms in medical institutions. Recently, the peroxo method has been used to synthesize semiconducting metal dioxide TiO_2 in the form of spherical particles (SPs) with size of 100–200 nm; its antibacterial properties have not been studied.*

The aim *is evaluation of the survival and morphology of Escherichia coli and Staphylococcus aureus after exposure of the TiO_2 SPs, and estimation their toxicity in bioluminescence test.*

Methods. *TiO_2 at 0.5–2 g/L concentrations after 10-120 min UV irradiation were added to E. coli or S. aureus suspension. Survival, microscopic examination (SEM, ASM), and toxicity bioluminescence test were made after 60–90 min contact.*

Results. *The antibacterial effect of TiO_2 SPs was maintained after UV irradiation was stopped. TiO_2 at 0.5–2 g/L with UV pre-irradiation (120 min) decreased the viability of both E. coli and S. aureus. According to the bioluminescence test, EC50 for TiO_2 SPs was 7.46, 2.61, 1.87 g/L after 10, 60, 120 min UV pre-irradiation, respectively. The electron microscopic observations suggested that TiO_2 SPs have adhesion and adherence to E. coli and S. aureus cells after UV pre-irradiation.*

Conclusion. *Understanding TiO_2 SPs interaction with bacteria allows development of new photocatalysts with antibacterial properties.*

Key words: *titanium dioxide, spherical particle, E. coli, S. aureus, toxicity, antibacterial property*

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АНТИБАКТЕРИАЛЬНАЯ АКТИВНОСТЬ ФОТОКАТАЛИТИЧЕСКИХ СФЕРИЧЕСКИХ ЧАСТИЦ TIO_2 РАЗМЕРОМ 100-200 НМ, СИНТЕЗИРОВАННЫХ ПЕРОКСОМЕТОДОМ

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РЕЗЮМЕ

Обоснование. *Разработка фотокатализаторов с антибактериальными свойствами представляется актуальной для борьбы с полирезистентными микроорганизмами в медицинских учреждениях. Недавно пероксометодом синтезирован полупроводниковый оксид металла TiO_2 в виде сферических частиц (СЧ) с размерами 100-200 нм, его антибактериальные свойства не изучены.*

Целью *является оценка выживаемости и морфологии Escherichia coli и Staphylococcus aureus под воздействием частиц TiO_2, оценка их токсичности в биолюминесцентном тесте.*

Методы. *TiO_2 в концентрациях 0,5–2 г/л после 10–120 мин УФ-облучения добавляли к суспензии E. coli или S. aureus. Выживаемость, микроскопическое исследование (SEM, ASM) и биолюминесцентный тест токсичности проводились после 60–90 мин контакта.*

Результаты. *Антибактериальный эффект TiO_2 СЧ сохранялся после прекращения УФ-облучения. TiO_2 в концентрации 0,5–2 г/л, предварительно активированный УФ (120 мин), снижал жизнеспособность как E. coli, так и S. aureus. Согласно биолюминесцентному тесту, EC50 для наночастиц TiO_2 составила 7,46, 2,61, 1,87 г/л после 10, 60, 120 мин предварительной УФ-активации, соответственно. Наблюдения с помощью электронного микроскопа показали, что наночастицы TiO_2 с предварительной УФ-активацией обладают адгезией и прочно прикрепляются к клеткам E. coli и S. aureus.*

Заключение*. Понимание механизма взаимодействия TiO_2 СЧ с бактериями позволит разработать новый фотокатализатор с антибактериальными свойствами.*

Ключевые слова: *сферические частицы, диоксид титана, E. coli, S. aureus, токсичность, антибактериальные свойства*

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INTRODUCTION

Infectious diseases pose a serious threat to public health worldwide, especially with the emergence of resistant bacterial strains to antibiotic and disinfectants [1]. Advances in technology are contributing to the development of new antibacterial agents used in the environment purification, disinfection and sterilization [2, 3].

There are contradictory data on the antibacterial activity of TiO_2, depending on concentration, sizes of particles, presence and time of photocatalytic irradiation. Some studies with *Escherichia coli* show that in the absence of UV irradiation TiO_2 (d = 6 nm, 0.002–0.2 g/L) may influence on the colony-forming capacity, but under acidic conditions [4]; may have bacteriostatic effect (d = 15 nm, 1 g/L; d = 100 nm, 0.3–0.6 g/L) [5, 6]; has no toxicity (d = 20 nm, 0.025–1 g/L; d = 90 nm, 0–319 g/L) [7; 8]. *Staphylococcus aureus* is not influenced by TiO_2 without UV (d = 20 nm; 1 g/L) [9]. Photocatalytic activation of TiO_2 by UV (simultaneous action) reduce the survival of *E. coli* and *S. aureus* (d = 20-30 nm, 0.025–1 g/L) [7, 9, 10, 11]. However it has been shown that TiO_2 NPs (10–30 nm, 0.00001–0.01 g/L) were not toxic to the bacteria (*E. coli, Bacillus subtilis*) after preliminary UV irradiation (20 min) [12].

Mechanism of TiO_2 action is associated with the destabilization of cell membrane integrity, the direct mechanical destruction of the cell wall, disruption of substances transport, and oxidative stress, which affect the bacteria activity and growth [13]. The antibacterial effect of photocatalytic materials includes the formation of reactive oxygen species (ROS: short-lived hydroxyl radicals and long-lived singlet oxygen, hydrogen peroxide), and the release of metal ions [14, 15]. TiO_2 has become a promising material due to its excellent optical and electronic properties, high photocatalytic activity, chemical stability, and low cost [16, 17].

Recent study has shown that monodispersed TiO_2 spherical particles (SPs) with diameters in the range of 100–200 nm provides a higher photovoltaic conversion efficiency, and advantages in electron transport compared to the typical 20 nm-sized TiO_2 nanoparticle (NP20) or conventional 250 nm-sized SPs (SP250) [18]. We suggest that titanium dioxide, synthesized by the peroxo method with particle size of 100-200 nm [19] may have the antibacterial effect after preliminary UV irradiation. This TiO_2 has high pore volume and large specific surface area, which contributes to the formation of structure and porous coating [19].

The purpose of this work is evaluation of the survival and morphology of *E. coli* and *S. aureus* under exposure of the photocatalytic TiO_2 SPs with size 100-200 nm, synthesized by peroxo method; estimation its toxicity in bioluminescence test.

MATERIALS AND METHODS

Titanium oxysulfate containing 33 % TiO_2 (Alfa Aesar, USA), propanol-1 (LenReactiv, Russia), aqueous ammonia (LenReactiv, Russia), hydrogen peroxide (Chimmed, Russia), and hydrochloric acid (Reachim, Russia) were used for the experiment. All the chemicals were analytically pure. TiO_2 microspheres were obtained by peroxo method [19]. The calcination temperature of 400 °C considered to be optimal as it allows obtaining a crystalline material. The TiO_2 microspheres were perfectly spherical and 100–200 nm in size. No irregular particles were detected. The microspheres were formed according to the solvent exchange method [19].

Low temperature nitrogen adsorption analysis showed that the SPs contained both micropores and mesopores. An XRD study shows that the material is crystalline. Its phase is 100 % anatase, known as the most photocatalytically active polymorphic modification of TiO_2. The crystal size was determined using the Scherrer equation based on the ratio of total width to half height for the most intense peak at 25 °C and 93 Å. No less photocatalytically active TiO_2 phases in the form of rutile or brookite were detected [19].

An aqueous solution of methylene blue (MB) dye (Biopharm, USA) was prepared at a concentration of 10 mg/L. TiO_2 photocatalyst (10 mg) was placed in a UV-transparent quartz glass with 30 ml of the MB solution. The degradation process was activated by UV irradiation. Prior to irradiation, an experiment on the absorption of TiO_2-based MB was carried out for 1–3 h. A control experiment was carried out to determine the role of TiO_2. During this experiment, the MB solution was exposed to UV irradiation for 1–3 h without the addition of the TiO_2 photocatalyst. The concentration of the MB solution was estimated from the peak value at 665 nm.

In the antibacterial test TiO_2 SPs were suspended in 0.9 % NaCl, sonicated for 1 h (37 kHz, Elma 30S, Germany), and used at concentrations of 0.5, 1, and 2 g/L. UV irradiation of TiO_2 SPs was performed using a 30 W DB-30-1 arc bactericidal lamp for 10, 60, and 120 min at a distance of 1 m from the irradiated plates. The wavelength of 253.7 nm corresponded to the UV-C spectrum.

The cultures of *Escherichia coli* ATCC®25922 and *Staphylococcus aureus* ATCC®25923 (Federal State Budgetary Institution Scientific Centre for Expert Evaluation of Medicinal Products of the Russian Ministry of Health, Moscow) were used as test objects for antibacterial analysis. Bacterial cultures were grown in LB medium (Amresco, USA) at 37 °C for 24 h, then diluted with 0.9 % NaCl to concentration of 1×10^5 cell/mL. TiO_2 (100 µL; 0.5; 1; 2 g/L) was added to 96-well plates and the photocatalyst was exposed to UV irradiation for 10, 60, and 120 min. NaCl (0.9 %) was used as a control. The bacterial suspension of *E. coli* or *S. aureus* (100 µL) was then added to 96-well plates with / without TiO_2. The 96-well plates were incubated at 37 °C for 1 h, then 10-fold dilutions of the cultures were plated on the LB agar, and the number of viable cells (CFU/mL) was evaluated after 24 h. The logarithm of the reduction, i.e., $\log(C/C_0)$, where C_0 is the concentration of control live bacteria without TiO_2 in the dark, expressed in CFU/ml, and C is the concentration of live bacteria for other conditions, expressed in CFU/ml, was determined.

Samples of *E. coli* and *S. aureus* for scanning electron microscopy (SEM) and atomic force microscopy

(AFM) were prepared as indicated at the viability assessment assay. For morphometric and quantitative analysis samples of *E. coli* and *S. aureus* were separated from TiO_2 by centrifugation at 300 × g, supernatants containing bacteria were removed to tubes, and glycerol (15 %) was added before freezing. The samples were thawed, placed on glass, and dried. The dried preparations were examined using a JEOL JSM 7001F SSA SEM and SMM-2000 AFM (PROTON-MIET, Russia) with MSCT-AUNM cantilevers (Veeco Instruments Inc., USA) with a beam stiffness of 0.01 nm. Twenty-five cells were examined for statistical manipulations.

For toxicity test of the photocatalyst by bioluminescence the lyophilized culture of the *E. coli* K12 TG1 strain with the full lux operon of *Photorhabdus luminescence* was used [20]. The lyophilized *E. coli* K12 TG1 was rehydrated for 30 min in 1 mL of 0.9 % NaCl at 4 °C and then for 30 min at 20 °C at the concentration of 10^6 cells/mL. TiO_2 suspension (100 µL) at concentrations of 0.5, 1, and 2 g/L were irradiated with UV for 0, 10, 60, 120 min in a 96-well white plate, and then the suspension of *E. coli* K12 TG1 was added in a ratio of 1:1. $ZnSO_4$ (Zn^{2+}: 0.5 or 1 mg/L) was used as positive control. Luminescence was estimated for 90 min using a Synergy™ H1 multiplate reader in relative light units (RLU) (BioTec, USA). The index toxicity (T) was calculated by the formula:

$$T = \frac{Ik - Io}{Ik} \cdot 100\%$$

where I_k, I_o are the luminescence of the control and samples after 30 min contact, respectively. The TiO_2 SPs concentrations were classified into three groups according to their toxicity level: T < 20, non-toxic; T > 20 < 50, toxic; T > 50, very toxic [21].

Statistical processing was carried out using the standard Microsoft Office XP Excel and STATISTICA 6.0 software suite. All indicators were presented as the mean and standard deviation (M±m). The significance of differences was evaluated by Student's *t*-test. Differences between the data were considered significant at $p \leq 0.05$. Correlation analysis was performed using the Pearson's correlation coefficient.

RESULTS AND DISCUSSION

According to our data, the MB concentration decreased in suspension with the TiO_2 SPs by 44 % in the dark regardless of the exposure time, which positively correlated with the high surface area of the photocatalyst (Table 1), thus indicating the good adsorption properties of this material. After the adsorption process was completed, UV light was turned on to perform photocatalysis experiments. The MB solution with the photocatalyst was subjected to UV irradiation for 1 h, which completely degraded the dye and reduced its concentration to 0.5 mg/l. The 90 % of MB was degraded via adsorption and photo-catalysis synergy, which indicates high photocatalytic activity of TiO_2 SPs under these conditions and potential antibacterial effect. It indicated that TiO_2 SPs contained photocatalytically active sites. According to recently published data these sites contribute to the generation of electron–hole pairs and radicals [16].

To access the antibacterial property of TiO_2 SPs, the viability of *E. coli* and *S. aureus* after contact with photocatalytically active TiO_2 SPs was evaluated after cessation of UV irradiation (Fig. 1). It was found that there was no complete bactericidal effect at the studied concentrations of TiO_2 SPs after all UV pre-irradiation periods. However, the antimicrobial effect in term of CFU/mL was detected on both *E. coli* (log C/C_0: -(0.79–1.25)) and *S. aureus* (log C/C_0: -(0.31–0.51)) after UV pre-irradiation of TiO_2 SPs for 120 min at all concentrations; the number of bacteria decreased by 65.8 % for *S. aureus* and by 93.4 % for *E. coli*. The viability of *E. coli* was also less by 40 % and *S. aureus* by 83 % after UV

TABLE 1

PHOTOCATALYTIC DEGRADATION OF MB BY TIO_2 SPs

Experimental stage	MB concentration (C(MB)), mg/l	$C(MB)/C_0(MB)$	Degradation efficiency, %
Initial MB solution (C_0(MB))	5	-	0
1 h irradiation, without TiO_2	4.7	0.94	6
2 h irradiation, without TiO_2	4.5	0.90	10
3 h irradiation, without TiO_2	4.3	0.86	14
1 h absorption on TiO_2, without irradiation	2.9	0.58	42
2 h absorption on TiO_2, without irradiation	2.8	0.56	44
3 h absorption on TiO_2, without irradiation	2.8	0.56	44
3 h absorption on TiO_2, 1 h irradiation	0.5	0.10	90

Note. C_0(MB) – initial concentration, C(MB) – final concentration of methylene blue.

pre-irradiation for 60 min of TiO_2 SPs at 2 g/L. No antibacterial effect was found when TiO_2 SPs were added to bacteria without UV pre-irradiation (0.5–2.0 g/L), after 10 min (0.5–2.0 g/L) and 60 min (0.5–1.0 g/L) of UV pre-activation. The latter was in agreement with the previous research, which showed that the TiO_2 NPs (10–30 nm, 0.00001–0.01 g/L) pre-activated by UV for 20 min were nontoxic for *E. coli* and *Bacillus subtilis* [12]. Increasing the preactivation time of SPs to 120 min by UV in our research resulted in the appearance of antibacterial properties. According to the literature, when TiO_2 affected bacteria together with UV (15 min), the decrease in viability of *Pseudomonas aeruginosa* and *S. epidermidis* compared to the control was 43 % and 45 %, respectively [22]. The mechanism of antibacterial action of titanium dioxide NPs may be associated with conformational changes in surface characteristics (hydrophilic / hydrophobic ratio) or / and dissociative adsorption of water on metal particles, the appearance of short-lived hydroxyl radicals and long-lived singlet oxygen and hydrogen peroxide in the media [15]. We assume the similar mechanism for small TiO_2 SPs in our research.

TiO_2 anatase NPs are more prone to attach to bacterial surfaces than rutile nanoparticles, and larger nanoparticles interact less with cells than smaller nanoparticles [23]. As the particle size decreases, the surface area to mass ratio increases and the physicochemical properties (e.g., reactivity of surface atoms, electronic and optical properties) of the nanoparticles change. Therefore, smaller particles tend to agglomerate more, which can further affect their reactivity and binding properties [23]. Small TiO_2 NPs can penetrate into cells and then induce a potential photocatalytic process inside, as well as adsorb and deactivate biomolecules, such as proteins [24, 25]. The SEM images (Fig. 2) obtained in our research showed that bacterial morphology was also significantly altered after incubation with both TiO_2 SPs and TiO_2 SPs pre-activated by UV. However, these changes depended on the bacteria and the conditions of exposure. TiO_2 SPs adhered well to *S. aureus* cells and damaged the cell wall. The greater contact with TiO_2, the greater the adhesion of TiO_2 SP to bacteria cells (Fig. 2 a, b). However, the adhesion of TiO_2 SPs to *S. aureus* cells did not affect their viability (Fig. 1 b, without pre-UV irradiation). It was shown that TiO_2 SPs with UV pre-activation (120 min) damaged the cell wall by pore formation (Fig. 2 c). This is confirmed by an increase in inhibition of bacterial growth (Fig. 1 b, pre-UV irradiation 120 min, 2 g/L).

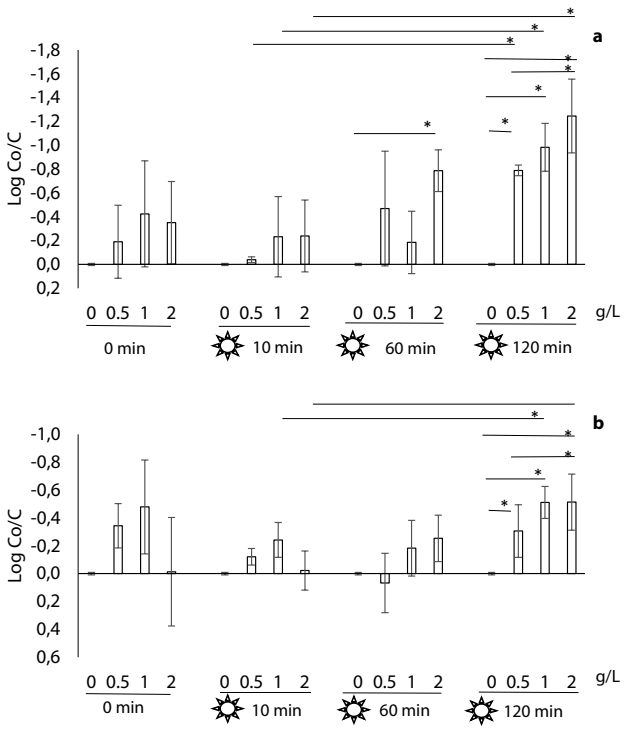

FIG. 1.

*Influence of photocatalytic TiO2 SPs on E. coli (a) and S. aureus (b) cell viability after UV pre-irradiation. Bars indicate standard deviations, ☼ – time of UV pre-irradiation. * ($p < 0.05$), indicate the significance of the difference between sample using Student's t-test. The contact time is 1 h*

Recent studies have shown [26-28] that the antibacterial mechanism of NPs involves the destruction of the bacterial cell membrane. Liou, et al. (2011) showed by AFM that the antibacterial properties of visible light-sensitive photocatalysis were associated with hole-like structures after 1–5 min of treatment [29]. In this work, we observe

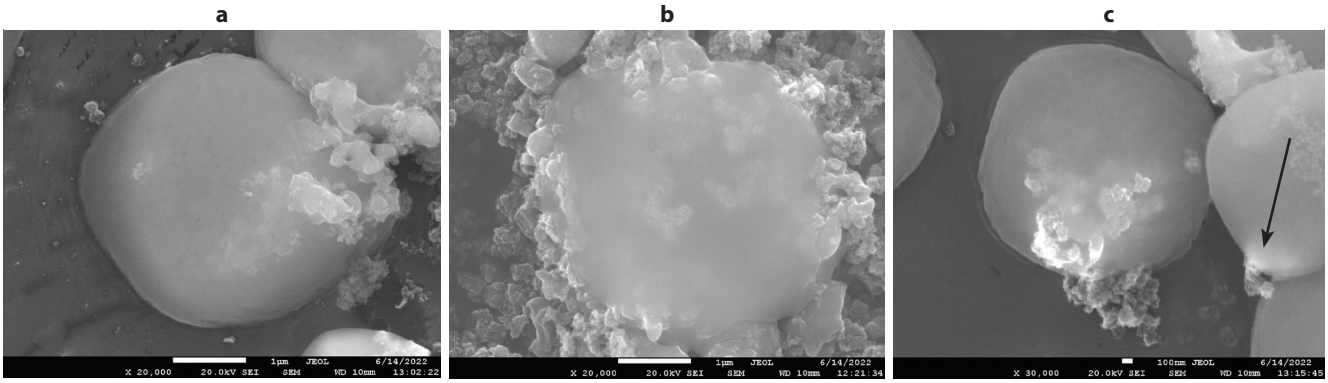

FIG. 2.

SEM images of S. aureus exposed to 2 g/L *TiO_2: a – TiO_2; contact 60 min, b – TiO_2; contact 180 min, c – TiO_2 with pre-UV irradiation (120 min); contact 60 min. Arrow indicates pore*

that mesoporous TiO_2 SPs synthesized by the peroxo method have excellent adhesion and also adhere tightly to bacterial cells too, thus deforming the cell and damaging the membrane of bacterial cells.

Morphometric studies showed that the diameter of *S. aureus* varied from 0.66 μm to 1.28 μm, with an average of 1.05 ± 0.13 μm (Table 2). Upon contact with TiO_2 SPs, the diameter of *S. aureus* increased significantly, correlating with the time of UV pre-exposure. (Table 2, Fig. 2 a, b). The area and volume of *S. aureus* changed in a similar manner. *S. aureus* cells exposed to TiO_2 SPs became less rounded and increased in volume. This can be considered as an adaptive mechanism of the cell upon contact with SP TiO_2. However, as the diameter of the cell increases, its surface area to volume ratio (S/V) decreases, which is accompanied by a disproportion between the intensity of cell metabolism (V) and the transport of substances (S) across the bacterial membrane. Moreover, an absolute increase in the surface area of the bacteria contact with TiO_2 can make an additional contribution to the disruption of transport. The decrease in the surface/volume ratio was observed after a 1-hour TiO_2 SPs contact with/without UV pre-activation (2 h): the analyzed indicators were 1.4 times lover than the control value.

The shape of the gram-negative microorganism, *E. coli* bacteria, was slightly distorted (cells became rounded) already after contact with TiO_2 SPs even without UV (data not shown). The morphology of the *E. coli* cells changed significantly from rod-shaped to spherical or biconcave disk. Similar observations were made in [29], which showed that *E. coli* cells undergo significant morphological changes after an hour of UV incubation in a TiO_2 film, namely, becoming oval or round with small protrusions.

Toxicity bioluminescent test showed that photocatalytically active TiO_2 SPs reduced the luminescence of *E. coli* K12 TG1 (*lux*$^+$). The addition of TiO_2 SPs without prior UV activation had no toxic effect on *E. coli* K12 TG1 *(lux*$^+$*)* cells (Fig. 3). When exposed to TiO_2 SPs without UV activation, the correlation coefficient between TiO_2 concentration and luminescence was -0,95. Similar effects have been described previously for bioluminescence under the influence of photocatalysts without UV activation [15] or under the combined influence of NPs and UV [30]. In our research when the culture was contacted with TiO_2 after UV pre-irradiation, the bioluminescence level of *E. coli* K12 TG1 decreased throughout the entire time and reached a minimum in the range of 60–90 min (Fig. 3).

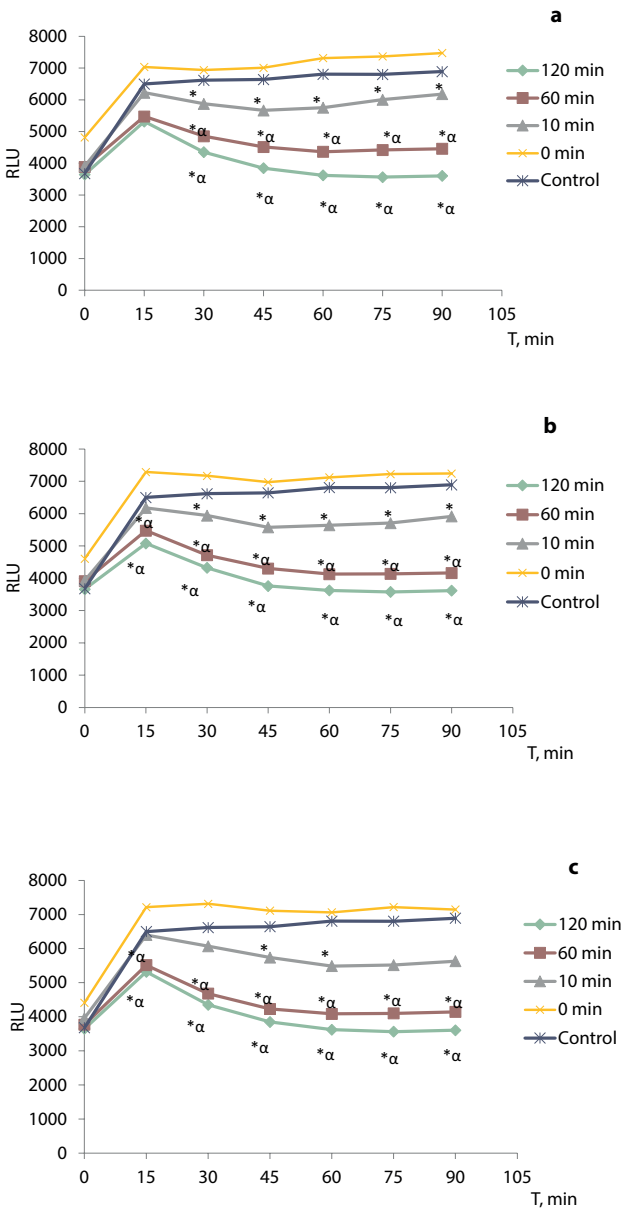

FIG. 3.
*Luminescence of E. coli K12 TG1 pF1 after UV pre-irradiation of TiO_2 SPs exposure at 0.5 (a), 1.0 (b), 2.0 (c) g/L. * – the difference between the luminescence level of the control (without TiO_2) and the variant with TiO_2 pre-irradiated by UV is statistically significant (t-test: $p \le 0.05$); a = the difference is significant from the luminescence level of the sensor strain after 10-minute UV pre-irradiation*

TABLE 2

MORPHOMETRIC AND QUANTITATIVE INDICATORS OF STRUCTURAL CHANGES IN *S. AUREUS* AFTER TIO_2 SPs EXPOSURE

Variant	Diameter, μm	Area, μm²	Volume, μm³	Relative area (S/V, μ/m)
Control	1.05 ± 0.13	3.44 ± 0.07	0.60 ± 0.23	5.73
TiO_2 SPs	1.41 ± 0.23*	6.42 ± 2.16*	1.59 ± 0.83*	4.04
TiO_2 SPs with UV pre-activation (2 h)	1.51 ± 0.40*	7.13 ± 4.32*	1.79 ± 0.97*	3.98

Note. * – the difference is statistically significant in the control (t-test: p ≤ 0.0005). # – 2 g/L TiO_2 SPs, 1 h exposure.

According to the luminescence inhibition index (T), the toxicity of TiO_2 SPs was concentration-dependent and increased with the duration of UV pre-irradiation (Fig. 3, 4). Ten minute of UV pre-irradiation induced the toxicity of TiO_2 SPs at all concentrations, but the T values did not exceed 17.2 % (toxic effect). At all concentrations after 60, 120 min UV pre-irradiation TiO_2 SPs were very toxic (T > 20 %). The inhibition of luminescence was maximum (50.8 %) with TiO_2 SPs at 2 g/L after UV pre-irradiation for 120 min. The toxicity of TiO_2 SPs with 10 min UV pre-irradiation was less than that with 60 min UV pre-irradiation at all photocatalyst concentrations (*t*-test: $p < 0.05$). The toxicity of TiO_2 SPs with 60 and 120 min UV pre-irradiation was not statistically different. Notably, a significant difference between the action of TiO_2 at a concentration of 0.5 and 2.0 g/L was registered for all UV pre-irradiation periods (*t*-test: $p < 0.05$).

According bioluminescence, EC50 (concentration of TiO_2 SPs that cause 50 % inhibition of luminescence from the control) was 7.46, 2.61, 1.87 g/L after 10, 60, 120 min UV pre-irradiation, respectively.

The photocatalytic antibacterial properties of TiO_2 SPs that found in our research may have wide practical application in the production of various dual-action materials. Progress towards a more widespread use in clinical settings depends on the key outstanding issues such as antibacterial property of photocatalysts.

CONCLUSIONS

The creation of new compounds and structures with antimicrobial activity, including those based on metal nanoparticles, is a promising and intensively developing area in the physical chemistry of nanomaterials. TiO_2 is one of the most commonly applied photocatalysts.

This paper presents the results of characterization of photocatalytically active TiO_2 SPs synthesized by the peroxo method from an inorganic precursor. Their antimicrobial effect was shown on two representatives of clinically important bacterial species: *E. coli* and *S. aureus*. The data suggest that the use of TiO_2 SPs as biocidal components in coatings for medical organizations is promising. Further research will be aimed at studying the antibacterial activity of films containing TiO_2 SPs.

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Conflict of interest

The authors of this article report that there is no conflict of interest.

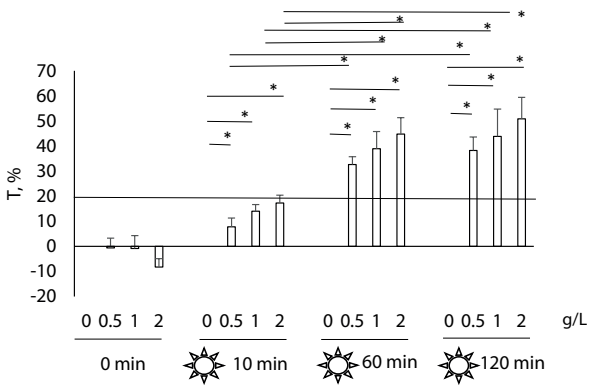

FIG. 4.

*Luminescence inhibition index of E. coli K12 TG1 after contact with pre-UV activated TiO_2. Bars indicate standard deviations, – UV pre-irradiation time. * ($p < 0.05$), indicate the significance of the difference between sample means, obtained using Student's t-test*

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