

Inactivation of Bacteria in Biofilms by Shock Waves

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Abstract. The processes of destruction of biofilms and inactivation of bacteria under the influence of shock acoustic waves and photodynamic treatment were studied. Shock waves were generated by locally heating the medium with nanosecond pulses of focused laser radiation. The radiation energy was converted into heat in the process of nonradiative relaxation of the excited states of organic dye molecules dissolved in the medium. The processes of biofilm removal and destruction of the extracellular matrix by shock waves are discussed. The low efficiency of inactivation of bacteria in biofilms by shock acoustic waves with peak pressure commensurate with the threshold of tensile strength of the cell wall material has been experimentally proven. © 2024 Journal of Biomedical Photonics & Engineering.

Keywords: bacterial films; shock waves; inactivation of microorganisms.

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1 Introduction

The high survival rate of microbes in human and animal organisms, in reservoirs, in soil and other natural environments, is largely due to their ability to unite into communities called biofilms. The principles of formation and the main stages of development of bacterial films were formulated several decades ago [1], and it is now reliably established that this is the main form of existence of microorganisms [2]. The development of pathologies in organisms and the spread of many dangerous infectious diseases are directly related to the emergence and growth of biofilms [3–4].

In the middle of the last century, antibiotics appeared in drug therapy, the use of which dramatically increased the effectiveness of treatment. Antibiotics have been and remain the main and in-demand means of fighting bacteria, and their potential is far from being exhausted. At the same time, every year there are reports of an increase in the number of strains that develop immunity to antibiotic therapy. Most researchers associate the phenomenon of antibiotic resistance precisely with the formation of biofilms [5–7]. Therefore, it is important to have in your arsenal a variety of ways and techniques to combat pathogens in biofilms, to which they will not be able to develop resistance.

In Refs. [8–9], using the example of planktonic microorganisms, the possibility of effective destruction of bacteria by shock waves or by the combined action of shock waves with photodynamic treatment was demonstrated. Depending on the conditions of wave

generation and media treatment modes, it is possible to inactivate pathogenic microorganisms with almost 100% effectiveness. The purpose of this work is to apply methods of inactivation of planktonic microorganisms for bacteria in biofilms and to compare the effectiveness of bacterial damage in solutions and in biofilms.

2 Methods

2.1 Biofilms

The object of the research was biofilms of the Gram-negative rod-shaped bacterium *Pseudomonas aeruginosa*, known for its high resistance to antibiotics. To obtain monomicrobial biofilms of *P. aeruginosa*, a suspension of cells was prepared according to the McFarland turbidity standard 0.5, which in a volume of 1 ml was mixed in a sterile Petri dish with 20 ml of cetrinide broth and cetrinide agar (Russia), taken in a ratio of 1:1, and incubated for 24 h at a temperature of 37 °C. The quality of the biofilm was assessed by the evenly distributed blue-green staining of the nutrient medium with a pigment produced by *P. aeruginosa*. Using a 5 mm diameter punch, identical blocks were cut out of a jelly-like but sufficiently dense agar substrate with a biofilm formed on its surface.

A biofilm block on an agar substrate was placed in a saline solution with erythrosine concentration of 0.5 mM, positioned with a surface with cells frontally in relation to the influencing factor (shock waves, light) and the survival of bacteria after the action of shock waves and

photodynamic treatment was studied. Depending on the mode of mechanical action or irradiation of biofilms, a certain number of planktonic bacteria appeared in solutions. To estimate the number of such bacteria, an aliquot of a solution with a volume of 100 μl was selected, diluted 10^4 times, and followed by seeding and counting of individual colonies. *P. aeruginosa* cells were seeded on cetrimide agar in Petri dishes in at least 3 repetitions, followed by incubation for 24 h at a temperature of 37 °C.

To assess the survival of bacteria in biofilms after exposure to shock waves or radiation, the block was placed in 1 ml of saline solution after treatment, pipetted for 1 min and indexed (1 min). The resulting suspension of cells was diluted 10^7 times, seeded on cetrimide agar and colony-forming units (CFU) were counted after incubation at a temperature of 37 °C for 24 h in at least 3 repetitions.

2.2 Generation and Registration of Shock Waves

A saline solution with erythrosine was poured into a rectangular quartz cuvette measuring $5 \times 20 \times 30$ mm and irradiated through its end wall. The source of excitation was a pulsed YAG:Nd laser (SolarLS, Belarus). Using a cylindrical lens, the laser radiation was focused in solution into an extended area of 3.0×0.3 mm directly along the boundary of the end wall. Parameters of the exciting pulse: $\lambda = 532$ nm, pulse duration 10 ns, power density was adjusted within 0.1–20 MW/cm^2 . Depending on the concentration of the dye and the pumping energy of the laser, the exciting radiation diverging after focusing penetrated deep into the solution at a distance of no more than 10 mm. Shock waves appeared in the area of the lens constriction as a result of a rapid local increase in the temperature of the medium during nonradiative relaxation of the excited electronic states of erythrosine molecules.

A probing beam of a low-power He-Ne laser (632 nm) was used to register the emerging waves. The probing beam crossed the cuvette in the transverse direction at an adjustable distance from the end wall (Fig. 1), fell into the entrance slit of the monochromator with a width of 0.5–0.8 mm and further into the photodetector (PMT). At the moment when the probing beam is crossed by a shock wave, refraction occurs and the amount of light entering the photodetector changes. The wave profile is recorded on the monitor screen, reflecting the change in the density of the medium during the propagation of the disturbance. The equipment for generating and studying shock waves is described in detail in Ref. [8].

Knowing the distance from the lens constriction to the probing beam, the wave propagation velocity was determined, which at a temperature of 22 °C turned out to be equal to 1495 ± 30 m/s, which coincides with the speed of sound in saline solution. By the angle of deflection of the beam at the moment of intersection with an acoustic wave, using the empirical Eq. [10]

$$n = 1.332 + 0.322(\rho - 1)$$

for the relationship of the refractive index with the density of a liquid, the change in the density of the medium during the passage of the wave was estimated and the peak pressure was determined. At an excitation power density of 15 MW/cm^2 , the peak pressure was approximately 25–30 MPa, which is commensurate with the tensile strength σ_{ts} of the cell wall material in wet $\sigma_{ts} \sim 20$ MPa [11], which has a polymer structure. In all experiments, the results of which are presented below, the peak wave pressure was 25–30 MPa.

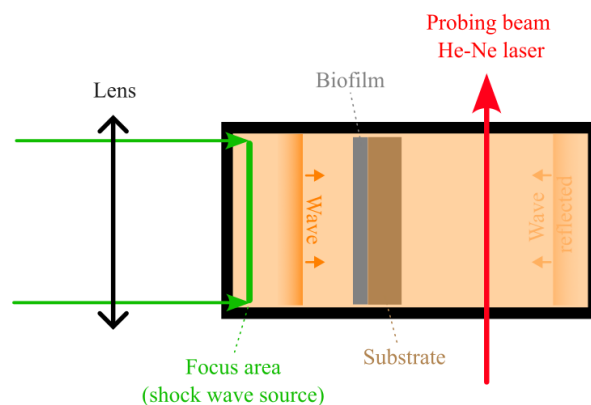


Fig. 1 Experimental registration of shock waves (a cuvette with a sensitizer solution and a biofilm is shown, top view). The focus area of the YAG:Nd laser radiation is located at the left end wall. Shock waves move from left to right. In their path there is a biofilm on a substrate, and then a probing beam of a He-Ne laser, under refraction of which registers the wave.

2.3 Photo- and Thermal Sensitizer

The choice of erythrosine as a photo- and heat sensitizer is due to its physical and chemical characteristics. In erythrosine molecules, the quantum yield of ϕ_T in the triplet state in aqueous solutions is close to unity [12], therefore it is characterized by high photodynamic activity [13–14]. In erythrosine solutions, the energy of laser radiation is very efficiently converted into heat, which is also associated with a high value of the ϕ_T value. Indeed, with a quantum yield of $\phi_T \sim 0.99$ and a rate constant [12] of the intersystem crossing $S_1 \rightarrow T_1$, almost every molecule after excitation in the baseband $S_0 \rightarrow S_1$ passes into a triplet T_1 state in about 1 ns. If the power density of the exciting radiation is $P \geq 1$ MW/cm^2 , and the dye concentration is 0.5 mM, then after 1 ns all molecules in the excitation zone turn out to be in a metastable triplet T_1 state. The absorption spectra of $S_0 \rightarrow S_1$ and $T_1 \rightarrow T_m$ in erythrosine strongly overlap; therefore, exciting radiation is absorbed in the $T_1 \rightarrow T_m$ band. Relaxation of high triplet T_m states mainly occurs nonradiatively, which ensures local heating of the medium. The lifetime of high triplet T_m states does not exceed several picoseconds [15–16], therefore, during the exciting pulse ($\tau = 10$ ns), each molecule can repeatedly absorb exciting quanta, performing cyclic

$T_1 \leftrightarrow T_m$ or $T_1 \rightarrow T_m \rightarrow S_1 \rightarrow T_1$ transitions, each of which is accompanied by heat release.

Erythrosine is highly soluble in water and saline, has an absorption maximum in the region of 532 nm and a large molar extinction coefficient ($\varepsilon \sim 10^5 \text{ M}^{-1}\text{sm}^{-1}$). Note that instead of erythrosine, any other compounds can be used that effectively absorb exciting radiation, are photodynamic active and provide rapid local heating of the medium.

3 Results and Discussions

3.1 Impact of Shock Waves on Biofilms

The results of an experimental study of the impact of shock waves on bacteria in biofilms grown on a jelly-like agar substrate are shown in Fig. 2. First, the shock wave was fixed in erythrosine saline solution without biofilm at a distance of about 9 mm from the lens constriction. Then, at a distance of about 7 mm from the front wall (from the focus of the lens), a biofilm on a substrate (a fragment measuring $5 \times 30 \text{ mm}$) was placed in a cuvette and the shock wave was again recorded under the same excitation and registration conditions. The profile of the shock wave at a distance of 9 mm from the lens band is shown in Fig. 2a. It can be seen that after installing the biofilm, the area under the curve reflecting the wave profile decreases by about 25%.

After exposure to an incident shock wave, the biofilm together with the jelly-like substrate become a source of secondary waves. Fig. 2b shows direct and reflected waves from the back wall of the cuvette in saline solution with dye before (blue line) and after (red line) placing the biofilm in the cuvette. In the recorded wave profile, in addition to changing the amplitude, a structure appears due to the generation of secondary waves by the biomaterial after exposure to direct and reflected acoustic waves. The presented data serve as a convincing illustration of the effects of direct and reflected shock waves on biofilm.

A shock wave at high peak pressure and a short front, acting on the biofilm-agar substrate, causes significant changes in the latter – from ablation of the biomaterial to bacterial damage in the biofilm. Ablation should be understood as the separation of a fragment of biofilm, in some cases together with the substrate, at the time of direct impact of the shock wave. At the same time, it must be borne in mind that shock waves are nonlinear waves of finite amplitude, and the current arising behind such a wave cannot be neglected. According to the wave profile in Fig. 2 it can be seen that it consists of a compression zone and a dilution zone.

Individual fragments of biofilms formed as a result of ablation have macroscopic dimensions of $\sim 0.5 \text{ mm}$ and are visually observed. The ablation mechanism is determined by the parameters of the shock wave (laser pumping energy, pulse duration, shock wave front and peak pressure, etc.), as well as the properties of the biofilm-agar substrate material (composition, structure, morphology, nature of interaction with the shock wave,

distribution of absorbed energy, etc.). Establishing a specific ablation mechanism is a separate task, beyond the scope of this work. Here, it is important to reliably establish the fact that the threshold for destruction of the structure under the influence of a shock wave and destruction of the object under study is exceeded, regardless of the initiated physical processes. The formation of biofilm fragments significantly affects the result of bacterial inactivation under the action of shock waves and photodynamic treatment.

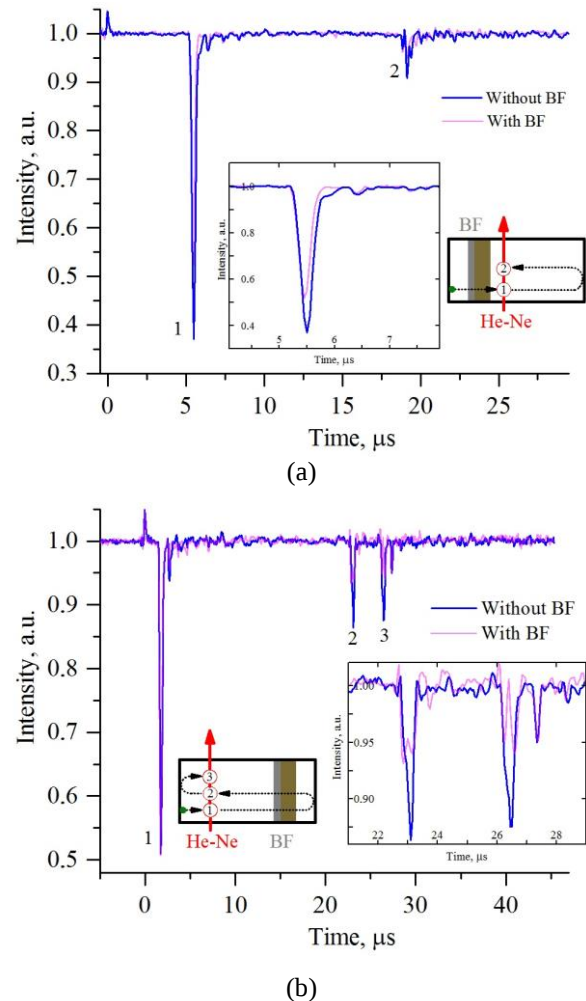


Fig. 2 The experimental scheme and the results of the interaction of shock waves with a biofilm. Direct and reflected waves are shown at different positions of the biofilm (BP) and the probing beam (He-Ne). (a) The change in the intensity of the shock wave after passing through the biofilm. (b) Direct (1) and reflected (2, 3) waves before (blue line) and after (red line) biofilm placement.

A shock wave with a high peak pressure and a short front, acting on a biofilm at an agar substrate, causes significant changes in the latter – from the ablation of biomaterial to damage to bacteria in the biofilm. Ablation should be understood as the separation of a biofilm fragment, in some cases together with the substrate, at the time of direct impact of the shock wave. Also, it must be borne in mind that shock waves are nonlinear waves of

finite amplitude, and the stream arising behind such a wave cannot be neglected. According to the wave profile shown in Fig. 2 it can be seen that it consists of a compression zone and a dilation zone.

Individual fragments of biofilms formed as a result of ablation have macroscopic dimensions of ~ 0.5 mm and are visible visually. The ablation mechanism is determined by the shock wave parameters (laser pumping energy, pulse duration, shock wave front and peak pressure, etc.), as well as the properties of the material biofilm-agar substrate (composition, structure, morphology, nature of interaction with the shock wave, distribution of absorbed energy, etc.). Elucidation of the specific ablation mechanism is a separate task beyond the scope of this work. Here it is important to reliably establish the fact that the threshold of destruction of the biofilm under the influence of a shock wave was exceeded, regardless of the initiated physical processes. The formation of biofilm fragments significantly affects the result of bacterial inactivation under the action of shock waves and photodynamic treatment.

3.2 Bacterial Survival after Exposure to Shock Waves and Radiation

When the biofilm is immersed in a cuvette, some bacteria of the peripheral layer enter the saline solution and eventually enter the planktonic state. Before exposure to the biofilms to shock waves and photodynamic radiation, the samples were kept in solution for about 5 min to achieve dynamic equilibrium in the biofilm-saline system. After mixing, an aliquot in a volume of 100 μ l was taken from this suspension, which was dissolved in 1 mL of saline solution and sown in a volume of 20 μ l on a Petri dish with cetrimide agar as a control sample. Before each exposure to the next series of pulses, a new biofilm on an agar substrate was placed in the cuvette, the procedure was repeated and the number of bacteria in the control samples was determined. The number of CFU bacteria in saline solution above the surface of the biofilm was estimated before and after exposure to shock waves and light, as well as the number of surviving bacteria in biofilms.

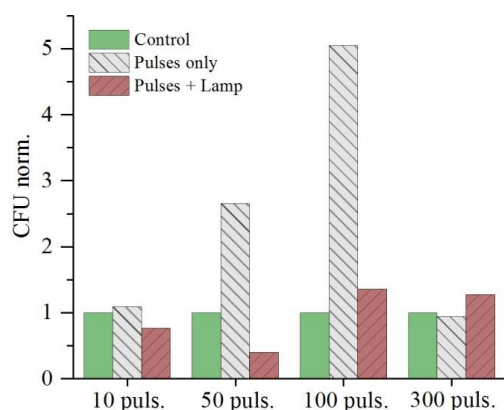


Fig. 3 Histograms of CFU bacteria in saline solution after exposure to shock waves and photodynamic treatment.

Histograms of CFU bacteria in saline solution after exposure to 10, 50, 100 and 300 shock waves and irradiation with visible light from a KGM-150 incandescent lamp through a SZS-22 light filter for 15 min are shown in Fig. 3. Unexpected was the increase in the number of viable bacteria in solution above the surface of the biofilm after exposure to 10, 50 and 100 shock waves. At the same time, the obtained result is in good agreement with the data of the work [17], where the dispersion of biofilms formed on medical catheters with the release of bacteria into the bacterial environment was observed. The increase in the number of bacteria in the solution seemed unexpected because earlier in Refs. [8–9] a decrease in the CFU of planktonic microorganisms was established after the effect of acoustic waves on bacteria in solutions.

The explanation of the obtained result is possible on the basis of modern ideas about the structure of biofilms. Microorganisms in a biofilm exist and are grouped differently from bacteria in a culture medium or saline solution. In biofilms, this is an interacting community of microorganisms, which are usually grouped into microcolonies surrounded by a protective matrix [18]. The process of interaction of shock waves with a biomaterial can be represented as follows. First, as a result of ablation, the most loosely bound biofilm fragments (sometimes together with an agar substrate) up to 1 mm in size are separated from the block. In the future, two competing processes are developing. On the one hand, shock waves destroy the binding matrix of the formed fragments (probably, protecting bacteria from mechanical damage is one of the important functions of the matrix) mechanically separating bacterial viable cells, which form new colonies after sowing. On the other hand, the waves damage the resulting planktonic microorganisms. According to the data presented in Fig. 3, if the number of shock waves is less than 300, the number of bacteria appearing in the solution as a result of the destruction of the matrix of biofilm fragments is greater than those affected by shock waves.

With an increase in the number of shock waves from 300 to 1000, the picture changes – the number of CFU decreases. Obviously, under the conditions of our experiments, after exposure to about 200–300 waves, biofilm fragments are almost completely destroyed, and subsequently the formed planktonic bacteria are damaged by shock waves, as demonstrated in Refs. [8–9].

At the same time, the permeability of bacterial membranes increases [19–21] and the probability of penetration of erythrosine (an effective anionic photosensitizer [22]) into cells increases. The basis for this assumption is a sharp decrease in CFU after irradiation of saline solution with bacteria with light with a wavelength that coincides with the absorption spectrum of erythrosine. There is a pronounced antibacterial photodynamic effect, a prerequisite for which is the penetration of a photosensitizer into cells [23–24]. Fig. 3 shows that the number of CFU bacteria of *P. aeruginosa* in the presence of erythrosine decreases significantly after photodynamic irradiation of samples treated with

shock waves. Changes in the permeability of biofilms treated with shock waves were previously observed in the work of [25], which showed an increase in the efficiency of damage to *S. aureus* bacteria by antibiotics in biofilms on titanium discs. Authors [17] observed a significant increase in the ability of human neutrophils to kill *S. epidermidis* bacteria in the planktonic state and within biofilms on catheters after exposure to shock waves. It should be noted that without photodynamic treatment, an increase in the permeability of bacterial membranes after exposure to acoustic waves can also stimulate an increase in CFU of microorganisms [26]. The wave creates microvortices in the environment surrounding the cell, ensuring effective mixing of nutrients and changing their concentration in the cytoplasm, initiating the process of cell division [27].

The threshold for destruction of biofilms on a substrate under the action of shock waves depends on the growing conditions of the biomaterial and its structure. In some samples, the number of planktonic bacteria in solution above the biofilm surface increased up to 1000 shock waves (Fig. 4). But in all the samples studied, the most rapid decrease in the number of viable bacteria in biofilms occurred after exposure to about 200 pulses.

Fig. 4 shows graphs of comparative changes in the number of viable bacteria remaining in biofilms and in solution above the film surface after exposure to different numbers of shock waves. As you can see, the dependencies are not symmetrical. In the range from 10 to 200 waves, there is a rapid decrease in the number of viable bacteria in biofilms due to ablation of the biomaterial and the transition of the formed fragments into solution. In the future, up to 1000 pulses, the decrease in the number of bacteria in biofilms, if it occurs, is very slow. The relatively slow growth in the number of microorganisms in the solution is due not only to the specifics of crushing fragments of biomaterial, but also to the competition of the processes of release of bacteria and their destruction by shock waves.

A slow decrease in the number of viable bacteria in biofilms in the range of 200–1000 pulses indicates that shock waves with peak pressure commensurate with the strength threshold of the cell membrane are capable of damaging planktonic bacteria (Fig. 3), but practically have no effect on microbes in biofilms. There are known works [28] in which a clear dose-dependent antibacterial effect is described, for the manifestation of which at least 1000 pulses were required. In our experiments, such an effect was not detected; a statistically significant

decrease in the number of bacteria in the biofilm was observed with 200–250 pulses.

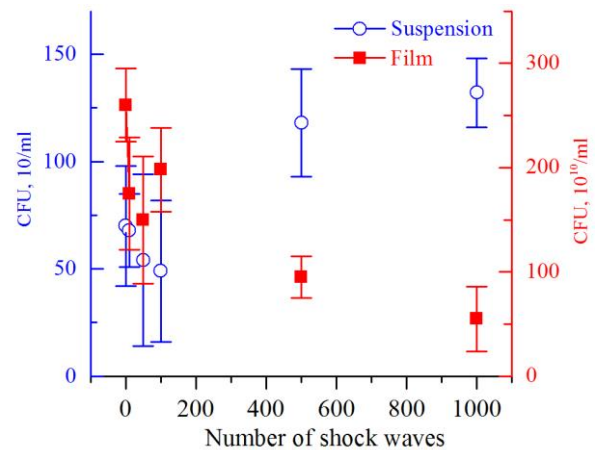


Fig. 4 CFU of bacteria in the biofilm after shock wave irradiation (red line), and bacteria in suspension beyond the biofilm (blue line).

4 Conclusion

The exact mechanisms of the impact of shock waves on biofilms remain to be elucidated. Our work shows that shock acoustic waves with a peak pressure close to the tensile strength threshold of the cell wall material are capable of destroying the peripheral layer of the biofilm by the ablation mechanism, mechanically separating the formed fragments into viable bacteria, and damaging planktonic microorganisms. At the same time, the relatively low efficiency of bacterial damage in biofilms by such shock waves has been experimentally illustrated. Probably, the elastic protective matrix of biofilms protects bacteria from mechanical damage. The manifestation of the antibacterial photodynamic effect suggests that after treatment with shock waves, the structure of the biofilm is disrupted and the penetration of the photosensitizer into deeper layers of the film is facilitated.

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Disclosures

The authors declare no conflicts of interest.

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