Photodynamic Inactivation of Pigmented and Non-Pigmented *Pseudomonas Aeruginosa* in Biofilms

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Abstract. This study presents a method to improve the efficacy of photodynamic inactivation against both pigmented and non-pigmented *Pseudomonas aeruginosa* strains in biofilms through pre-treatment with acoustic shock waves. Biofilms were exposed to photosensitizers in solution, and shock waves were generated using laser pulses (10 ns duration, 5–10 MW/cm^2 power density). Pre-treatment with shock waves significantly enhanced photodynamic bacterial damage, increasing inactivation efficiency by twofold for the non-pigmented strain and sixfold for the pigmented strain. The observed improvement is likely due to shock wave-induced permeabilization of the biofilm matrix and bacterial cell membranes, facilitating greater uptake of photosensitizers and oxygen.

Keywords: biofilms; pyoverdine; shock waves; photodynamic inactivation.

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1 Introduction

The development of resistance by pathogenic bacteria to the action of traditional antibiotics stimulates the search for alternative methods of microbial inactivation. One promising approach is antibacterial photodynamic therapy (aPDT), which combines photosensitizers (PS), light, and oxygen to kill microorganisms [1−4]. aPDT offers several advantages: it is safe for in vivo use (no chemically toxic reagents, high temperatures), selectivity of action (bacterial damage only in areas where PS and light are delivered), and a low probability of bacteria developing photodynamic immunity. The main disadvantage of aPDT is its low efficiency in suppressing bacteria in the inner layers of tissues and biofilms, particularly against Gram-negative bacteria.

Biofilms are a special form of existence of a group of microorganisms, when bacteria produce a special matrix of polymers (DNA, proteins, polysaccharides) and then multiply within this environment. In biofilms, bacteria become significantly more resistant to antibiotics, disinfectants, and the host's immune response. The matrix limits the penetration of antimicrobial agents and creates a gradient of nutrients and oxygen, which slows down the metabolism of some cells but increases their survival [5−7].

In this work, the efficiency of aPDT of *Pseudomonas aeruginosa* bacteria in biofilms is assessed. *P. aeruginosa* bacteria were chosen because they are one of the main pathogens of hospital-acquired infections, including pneumonia, sepsis, urinary tract infections, skin lesions and soft tissues [8−10]. In addition to the ability to form biofilms, these bacteria produce a number of virulence factors, including pigments (pyocyanin, fluorescein) and exotoxins, which help them colonize tissues and suppress the host immune response. However, some bacterial strains do not produce pigments, which can be caused by random mutations (fixed as an adaptation to stress or immune response), as well as a lack of oxygen in the environment and excess iron [11−12]. Pyoverdin exhibits antioxidant and antibiotic properties and is a siderophore used for iron uptake by the cell [13]. Pyocyanin has its own antibiotic [14] and photodynamic activity [15], these properties can be used to inactivate pigmented strains.

A promising method for increasing the efficiency of aPDT is pre-treatment of biofilms with shock waves propagating in an elastic medium. Shock waves can cause mechanical damage in biofilms, increase the permeability of the biofilm matrix as a whole and the walls of individual bacterial cells for photodynamic agents (PSs and oxygen), increasing their sensitivity to photodynamic treatment. Various authors reported on the successful use of shock waves in the inactivation of biofilms in stents [16], in urinary catheters [17] and other medical instruments [18–19].

In Ref. [20], it was proposed to form shock waves directly in a solution with PS. For this, it is necessary to

use molecules with a high quantum yield in the triplet state as PS in order to simultaneously ensure efficient production of singlet oxygen and the generation of shock waves as a result of local heating of the medium after excitation by pulses of focused laser radiation. The aim of this work is to evaluate the efficiency of photodynamic inactivation of *P. aeruginosa* bacteria in biofilms after exposure to shock waves and to determine the role of the bacterial pigment in this process.

2 Materials and Methods

2.1 Biofilm Preparation

The work involved *P. aeruginosa* strains that produce the blue-green pigment characteristic of these bacteria (which primarily includes pyocyanin and pyoverdin), as well as those that do not produce such a pigment. In what follows, we will refer to such strains as pigmented and non-pigmented. To obtain multiple identical biofilm samples, the technique described in Ref. [21] was used. A combination of cetrimide both and cetrimide agar (Microgen, Russia) in a 3:2 ratio was used as a culture medium. It has a liquid consistency at an incubation temperature of 37 °C, but solidifies at room temperature. To obtain standard samples, a sterile metal punch with a diameter of 8 mm was used, which allows cutting out biofilm fragments of identical volume (cylindrical blocks) with minimal damage to the structure. The variation between fragments in bacterial cell density did not exceed 5%, which was confirmed in a series of control experiments with seeding of destroyed blocks and subsequent counting of colony-forming units (CFU).

2.2 Generation of Shock Waves

Erythrosine (tetraiodofluorescein, Sigma-Aldrich, Switzerland) was used as a sensitizer. The dye is quite photostable, an effective generator of singlet oxygen $^1O_2$, which allows it to be used both as a photodynamic agent and as a source of shock waves. Erythrosine is known to be used as a photodynamic agent in antimicrobial therapy [22−23]. To generate shock waves and subsequent photodynamic irradiation of biofilms, an experimental setup was used, schematically shown in Fig. 1.

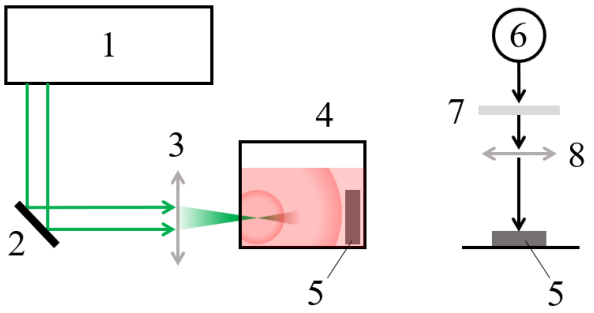

Fig. 1 Experimental setup for generating shock waves and photodynamic irradiation of biofilms. 1 – YAG:Nd^{3+} laser, 2 – mirror, 3 – cylindrical lens, 4 – cuvette with samples, 5 – biofilm on an agar substrate, 6 – KGM-150 lamp, 7 – light filter, 8 – collecting lens.

Excitation of erythrosine molecules in the solution was produced by the second harmonic radiation $\lambda = 532$ nm of a pulsed solid-state YAG:Nd^{3+} laser (Solar LQ-215, Belarus). The laser radiation was focused by a cylindrical lens 3 in a volume of 3×0.3×0.3 mm^3 inside a cuvette 4 near its end wall. Block 5 with the biofilm was placed on the opposite side of the cuvette at a distance of 15 mm from the focus of the lens. At a power density of 10 MW/cm^2 and a dye concentration of 0.5 mM, the excitation radiation penetrated into the solution to a distance of less than 10 mm, thus eliminating photodynamic or thermal effects in the biofilm. The energy of the excitation pulses was measured by a Field Master GS power meter with an LM-10HTD measuring head (Coherent, Inc., USA).

2.3 Photodynamic Treatment

For photodynamic treatment of biofilms, the radiation of a KGM-150 incandescent lamp (Institute of Light Sources named after A. N. Lodygin, Russia) was used (indicated by number 6 in Fig. 1). The biofilm treated with shock waves was removed from the cuvette and placed in a well of the plate with the FS solution so that the thickness of the liquid layer above the biofilm surface did not exceed 1 mm. With this arrangement, the oxygen concentration above the biofilm surface is the highest. Using lens 8, the radiation was focused on the biofilm (light spot diameter 8 mm). Radiation coinciding with the spectral absorption region of erythrosine and bacterial pigments was isolated from the continuous spectrum of the lamp by light filter 7 of the SZS-22 type (PhotonTechSystem, Russia). The power density of the light radiation was 0.8 W/cm^2.

3 Results and Discussion

3.1 Intrinsic Photodynamic Activity of Bacterial Pigment

After centrifugation and filtration through a bacterial filter of the enrichment culture of the pigmented strain *P. aeruginosa* in the cetrimide broth, a solution with the pigment pyoverdine was isolated [24]. The absorption spectrum of the solution shows a characteristic maximum in the region of 370 ÷ 380 nm, coinciding with the absorption maximum of pyoverdine. The maximum fluorescence of the solution was recorded at a wavelength of 470 nm (Fig. 2).

The photodynamic activity of the pigment was estimated experimentally. *P. aeruginosa* biofilms were placed in wells of a plate filled with water or a solution with the isolated pigment so that the thickness of the liquid layer above the biofilm surface did not exceed 1 mm (shown schematically in Fig. 3 under the histograms). There were three groups of samples in the experiment:

– non-pigmented biofilms;
– pigmented biofilms;
– non-pigmented biofilms immersed in a solution with a pigment.

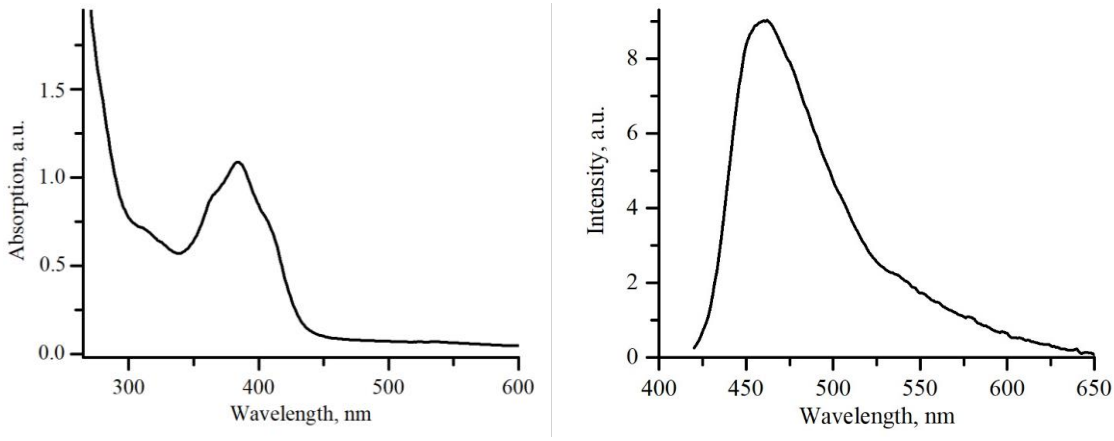

Fig. 2 (a) Absorption and (b) fluorescence spectra of pigment isolated from bacterial suspension.

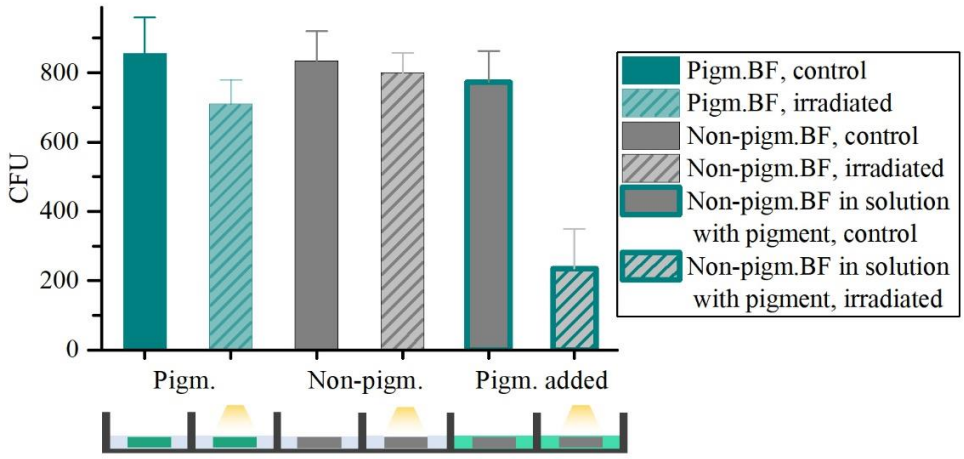

Fig. 3 Histograms of *P. aeruginosa* CFU after photodynamic treatment of pigmented and non-pigmented biofilms, as well as non-pigmented biofilms with added pigment. Non-shaded columns are the control, shaded columns are the experimental samples.

Photodynamic exposure was carried out using a KGM-150 lamp with a SZS-22 light filter for 15 min to obtain a dose of 50 J/cm^2. Each biofilm was then crushed, the resulting suspension was diluted and plated on a nutrient medium, as described above. The CFU histograms for each of the three groups are shown in Fig. 3 in comparison with their dark controls.

Irradiation of native biofilms (non-pigmented and pigmented) does not affect cell viability. The number of CFU from non-pigmented biofilms immersed in a pigment solution and in water is practically the same without a light dose. This indicates the absence of a chemical (dark) effect of the pigment on the cells. And only in the case of irradiation of non-pigmented biofilms "colored" with pigment, the number of CFU decreases almost fourfold, which confirms the photodynamic activity of the pigment.

It is noteworthy that in the case of a pigmented strain, 90% of cells survive after photodynamic irradiation, and in the case of a "colored" strain-less than 25% of cells. Apparently, the concentration of PS in samples with added pigment was significantly higher than in pigmented samples. An alternative assumption is that pigmented cells have greater tolerance to photodynamic action than cells grown without pigment.

3.2 Combined Action of Shock Waves and Photodynamic Treatment on Biofilms

In the experiment, CFU were counted for pigmented and non-pigmented strains for four groups (Fig. 4):

– samples without exposure (control);

– biofilm samples exposed only to shock waves (SW);

– biofilm samples after photodynamic (PD) treatment (dose 50 mJ/cm^2);

– biofilm samples exposed first to shock waves (1000 pulses), then to photodynamic treatment (dose 50 mJ/cm^2) (SW + PD).

All samples, including controls, were kept in erythrosine solution (0.5 mM). After shock wave treatment, biofilms were removed from the cuvette, placed in wells of the plate with PS solution and exposed to photodynamic treatment according to the method described above.

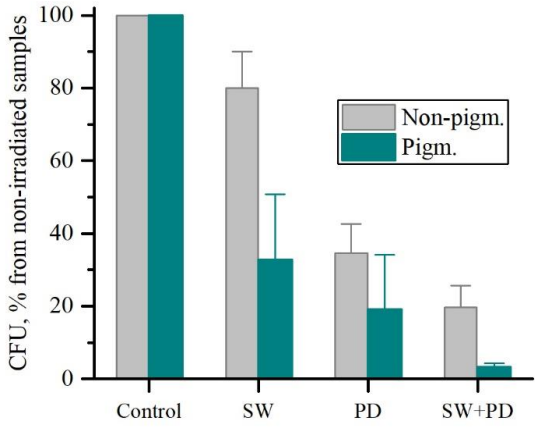

Fig. 4 Histograms of CFU of bacteria from *P. aeruginosa* biofilms exposed to 0 and 1000 pulses without subsequent photodynamic treatment, as well as 5 min of irradiation with a KGM-150 lamp with an energy density of 50 J/cm^2 for 15 min. Light columns are for the non-pigmented strain, dark columns are for the pigmented strain.

The action of shock waves generated by laser pulses in an erythrosine solution leads to a decrease in viable bacteria in the biofilm (approximately three times). Shock acoustic waves with a peak pressure close to the tensile strength threshold of the cell wall material are capable of destroying biofilms by the ablation mechanism, mechanically separating the resulting fragments into viable bacteria [21]. At the same time, the relatively low efficiency of damaging bacteria in biofilms by such shock waves has been experimentally proven. Probably, the elastic protective matrix of biofilms protects bacteria from mechanical damage. The photodynamic effect of erythrosine on gram-negative bacteria is low-effective. Figure 4 shows that with an accumulated energy of 50 J/cm^2, the number of viable cells in the biofilm remains virtually unchanged. However, after preliminary treatment with shock waves, the picture changes dramatically: the number of CFU decreases almost 20 times! Apparently, shock waves disrupt the structural integrity of the biofilm as a whole and increase the permeability of cell membranes for PS and oxygen.

For the pigmented strain, physical effects (both shock waves and photodynamic treatment) were more destructive than for the non-pigmented strain. The pigment has weak photodynamic activity in relation to the cells in which it is synthesized and does not harm *P. aeruginosa* bacteria. However, the pigment can suppress the growth of other competing microorganisms under conditions of food resource deficiency, including during biofilm formation. It is possible that under stressful effects on *P. aeruginosa* (shock waves, photodynamic action), the pigment under light irradiation makes an additional contribution to the inactivation of bacteria. This assumption is confirmed by the fact that pigmented *P. aeruginosa*, all other things being equal, survived somewhat worse than non-pigmented ones.

Thus, preliminary treatment of the medium with acoustic shock waves significantly increases the efficiency of photodynamic inactivation, which can find wide application in clinical practice. This synergistic effect is due to the disruption of the structural integrity of the biofilm matrix and permeabilization of cell walls, which improves the penetration of PS and molecular oxygen into the deep layers of the biofilm. The data obtained indicate the prospects for developing combined physical methods for combating biofilm infections, where mechanical destruction of the extracellular matrix enhances the efficiency of subsequent antimicrobial action.

Acknowledgments

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Conflicts of Interest

The authors declare that they have no conflicts of interest or competing financial interests related to this work.

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