Spectrally-selective mid-IR laser-induced inactivation of pathogenic bacteria

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Abstract: Micrometer-thick layers of *Pseudomonas aeruginosa* bacteria were prepared on fluorite substrates and scanned by focused mid-IR femtosecond laser radiation that was spectrally tuned to achieve the selective excitation of either the stretching C–H vibrations (3 μm), or stretching C=O, C–N vibrations (6 μm) of the amide groups in the bacteria. The enhanced biocidal efficiency of the latter selective excitation, comparing to the more uniform 3-μm laser excitation, was demonstrated by performing viability assays of laser-treated bacterial layers. The bacterial inactivation by the 6-μm ultrashort laser pulses is attributed to dissociative denaturation of lipids and proteins in the cell membranes and intra-cell nucleic acids.

1. Introduction

Exposure to ultraviolet (UV) radiation is the established method used to inactivate pathogenic microorganisms [1]. In general, UV radiation can be classified as short-wavelength (UVC, 200–280 nm), medium-wavelength (UVB, 280–315 nm), and long-wavelength (UVA, 315–380 nm) emission [1]. Regarding the disinfecting properties of UV radiation, pathological changes in deoxyribonucleic acid (DNA) molecules are caused by its photochemical reactions. The mutagenic effects of UVA and UVB are well known; UVA destroys DNA through oxidative stress, UVB causes direct DNA damage through the formation of cyclobutane-pyrimidine dimers and pyrimidine-(6-4)-pyrimidone photoproducts [2-4]. It is believed that such microorganism inactivation occurs through the UVC absorption-induced formation of cyclobutane dimers or pyrimidine photoproducts in the DNA chains. Exposure to small amounts of UV radiation causes double bonds in pyrimidine nitrogenous base molecules to break and instigates the formation of covalent bonds between adjacent nucleotides. It blocks DNA replication in cells [5]. As a result, UV irradiation renders microorganisms inactive rather than killing them outright [6, 7]. However, some species of UV-resistant bacteria are capable of DNA repair by expressing the DNA photolyase enzyme, which removes photoproducts and pyrimidine-pyrimidine dimers [8]. Photolyases are responsible for repairing various photoproducts and are found in most organisms; however, they are inactive or absent in mammals [6, 7]. The effects of absorbing UV radiation on human cells are well known: DNA is usually destroyed, leading to premature aging as well as the formation of mutations and burns, and, ultimately, can result in the development of melanoma. Hence, the use of UV light is limited.

Previously, Tsen et al. developed a method for inactivating bacteria and viruses based on the use of femtosecond laser radiation at visible and near-IR wavelengths [9]. The authors demonstrated that this method is effective regardless of the structure or mutational status of the pathogen. With regard to viruses, inactivation is achieved by exciting mechanical vibrations of the virus capsid, thereby causing bonds (specifically hydrogen bonds or hydrophobic contacts) in the protein envelope of the virus to weaken and break, which inhibits the virus. The mechanism underlying this process is known as impulsive stimulated Raman scattering and involves the laser-induced excitation of low-frequency acoustic vibrations of the virus capsid. For bacteria, relaxation occurs via the supercoiling of supercoiled DNA, which kills the bacteria. This method is selective, chemical-free, and has minimal side effects. The therapeutic window of laser radiation with power densities ranging from 1–10 GW/cm^2 enables the inactivation of most pathogens without damaging mammalian cells. This method has shown promise regarding the processing of blood products, pharmaceuticals, and vaccines [9]. Nevertheless, it also requires long exposure times and complex equipment, while its effectiveness has been refuted in [10].

Infrared radiation can denature functional proteins in bacterial cells by destroying the hydrogen bonds responsible for the stabilization of secondary and tertiary structures. Proteins are essential components of all living organisms and perform functions important for cell proliferation, with these functions realized through the activation of biocatalytic reactions, electron transfer, and conformational transformations. In turn, secondary and tertiary structures are fundamental for normal protein functions [11–13]. The migration and relaxation of vibrational excitation energies in proteins have been studied in real time, using ultrafast spectroscopy and mid-IR multiphoton vibrational excitation [14,15]. The effect of IR radiation on the inactivation of pathogenic microorganisms has been studied previously [17,18]. For example, Hamanaka et al. [17] investigated the effect of using thermal IR sources with different wavelengths on the inactivation of bacterial spores with varying levels of water activity, revealing that the maximum inactivation efficiency was achieved when using a source with a wavelength of 950 nm. Elsewhere, Oduola et al. [18] demonstrated that the number of colony-forming units of mold spores was reduced more effectively by using selective IR irradiation as opposed to broadband IR irradiation. In addition, the authors reported that stationary IR treatment had quite significant inactivation effect in the 6-μm region, but was far less effective in the 3- and 4.5-μm regions.

Our previous studies have shown the strong perturbation of hydrogen bonds in the cells of bacterial cultures of *Staphylococcus aureus* and *Pseudomonas aeruginosa* when exposed to low-intensity (~ 0.1–10 GW/cm^2) ultrashort pulses in the mid-IR range (5–6.6 μm) in the region of their characteristic absorption bands of proteins and lipids [19]. Specifically, our studies of self-transmission of ultrashort laser pulses through a layer of separate bacteria cultivated on a silicon substrate showed a relative bleaching in the region of these bands and their blue shift, apparently indicating the breaking of hydrogen bonds. In the present work, we explicitly evaluate the inactivation influence of mid-IR femtosecond laser radiation in the spectral ranges of ~ 3 and 6 μm on a micrometer-thick layer of a culture of pathogenic bacteria *Pseudomonas aeruginosa*, followed by microbiological viability examination of the samples. Inactivation of *Pseudomonas aeruginosa*, the Gram-negative pathogen and cause a wide range of human diseases, is challenging because of the high resistance of the bacterium to antibiotics [20, 21].

2. Methods

2.1 Experimental procedure

A *Pseudomonas aeruginosa* culture was obtained from the Gamaleya National Research Center for Epidemiology and Microbiology. The daily broth culture of 1 ml was centrifuged and the supernatant was removed. Next, 1-ml volume of distilled water was added to the sediment and shaken intensively. The resulting suspension was diluted by serial decimal dilutions up to 10^5 CFU/ml. The bacterial culture was dripped onto the CaF_2 substrates in a volume of 100 μl and

dried for 10-15 minutes. Micrometer-thick (average thickness ≈1.5 μm measured in a contact mode, using an atomic force microscope Certus Standart) layers of the *P. aeruginosa* culture, consisting of sub-micron-wide and a few-micron long bacteria [22], were prepared on 2-mm-thick CaF_2 plates, which have 90% transmittance in the 0.15–9.0 μm range.

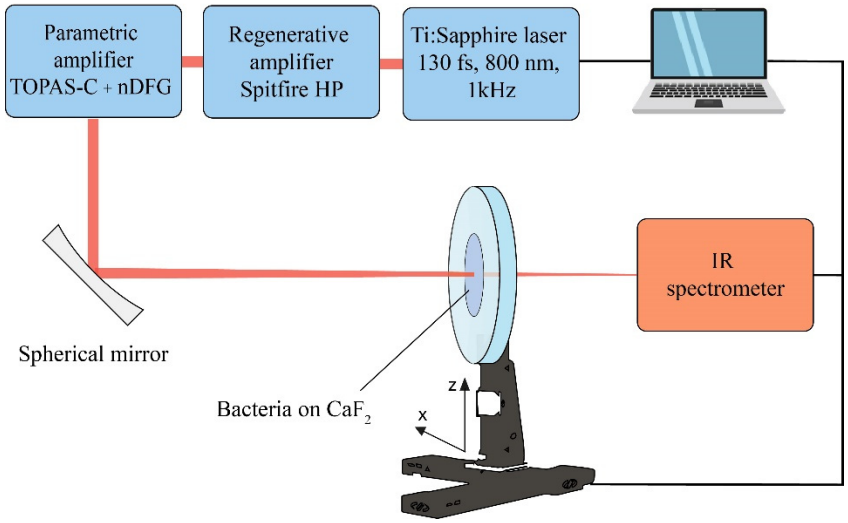

Fig. 1. Schematic diagram of the experimental set-up.

The micro-layer samples of the *P. aeruginosa* culture were exposed to femtosecond laser pulses at two wavelengths, corresponding to the characteristic vibrations of proteins and fatty acids in bacterial cells. As shown in Fig. 1, the samples were placed in front of the entrance slit of the IR-spectrometer (Solar TII MS2004) along the normal to the optical axis of radiation. The incident laser light was focused on the slit by a spherical mirror with a focal length of 150 mm. Mid-IR laser irradiation (central wavelength: 5.8 μm, FWHM: 0.6 μm; central wavelength: ≈ 3.4 μm, FWHM ≈ 0.4 μm) with the FWHM pulse duration τ≈130 fs, maximum pulse energies of 10 μJ (6 μm) and 30 μJ (3 μm), and the repetition rate of 1 kHz was obtained via parametric generation using a Ti:sapphire laser (Spitfire HP, Spectra-Physics, central wavelength: 800 nm, frequency: 1 kHz, FWHM: 50 fs) and an optical parametric amplifier (OPA) with a difference-frequency module (OPA TOPAS-C + nDFG, Light Conversion) [19].

These particular wavelengths were selected in accordance with the characteristic vibrations of the C–N and C=O bonds of amides (1510–1700 cm^{-1}, ≈6 μm), the C–H bond of fatty acids, and the N–H bond of amides (~3100–3500 cm^{-1}, ≈3 μm) (Fig. 2) [23]. The peak intensity ranges of the 6-μm and 3-μm ultrashort laser pulses are I_0 = 0.18–1.1 TW/cm^2 and 0.07–8 TW/cm^2, respectively. The peak intensity was changed by moving the samples along the optical axis to change the size of the irradiation area (defocusing). The beam diameter was measured using a scanning slit beam profiler. All beam diameters were measured at the FWHM and ranged from 60 to 640 μm and from 88 to 224 μm at the wavelengths of 3 μm and 6 μm, respectively. The samples were raster-scanned with 50% overlapping (N = 2 shots/spot), using a software-controlled motorized two-coordinate translation stage and making 3 series with new samples for each set of laser-irradiation conditions. Stationary spectra of the optical density (from 400–4000 cm^{-1}) were obtained using a Fourier-transform infrared (FT-IR) spectrometer (Vertex V-70, Bruker).

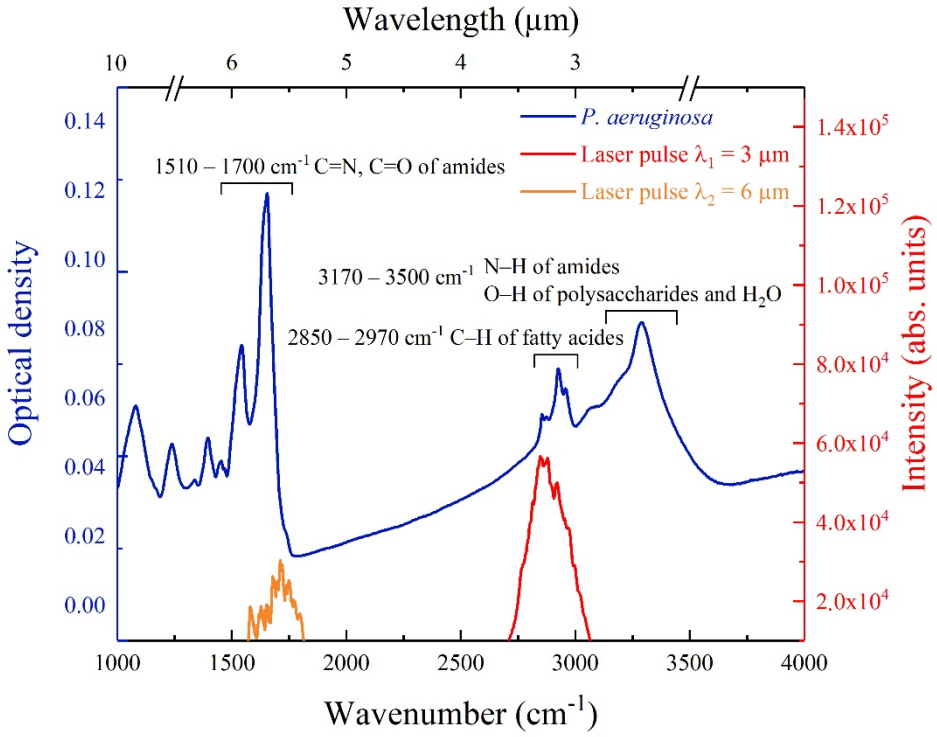

Fig. 2. FT-IR optical density spectrum of 1.5-micrometer thick layer of *P. aeruginosa* bacteria (left axis, spectral assignment after [24,25]) shown in relation to the intensity spectra of the 3-μm and 6-μm laser pulses (right axis).

2.2 Viability assays

After laser exposure, the entire laser-treated and control substrates were moved to individual sterile tubes with saline solution and shaken intensively for 30 minutes. The resulting suspension was sown on dense nutrient medium and placed in a thermostat for one day at 37 ° C. A day later, the bacterial colony was counted to determine the number of colony forming units (CFUs)and recalculated to CFU/ml values. The obtained *P. aeruginosa* values were compared with the control samples that were not exposed to the laser (Fig. 3). The decrease in the CFU/ml value for Pseudomonas aeruginosa by 2-3 or more orders of magnitude showed that the method has a pronounced antibacterial effect. In the future, such multi-parametric experiments will be performed for a wide range of microorganisms with multiple repetitions.

3. Results

3.1 Bacterial inactivation

The results of the viability assays showed the threshold-like, distinct and significant intensity-dependent reduction in the number of CFUs for the samples of the *P. aeruginosa* culture exposed by the 6-μm laser radiation (Fig. 3, right). The threshold value, where the CFU number reduction (*not total sterilization!*) becomes evident, was evaluated as 0.3±0.1 TW/cm^2. In contrast, for the *P. aeruginosa* samples exposed by the femtosecond laser radiation at the wavelength of 3 μm, changes in the number of CFUs were rather negligible until 1.4±0.3 TW/cm^2 (Fig. 3, left). It should be noted that previous CW studies on the mid-IR treatment of bacteria also showed efficient inactivation for the 6-μm region, but not in the 3- and 4.5-μm

spectral regions [26]. For both these 3-μm and 6-μm exposures, at the above-threshold laser intensities the bacterial abundance decreases by 4-5 orders of magnitude regarding the control, thus indicating the definite disinfection of the fluorite substrates. Potentially, such MIR-laser sterilization is possible too, but some technical difficulties, as scan line stitching and interline sub-threshold exposure should be solved, resulting in the artifact residual bacterial presence (~10 CFU/ml) at higher above-threshold intensities in Fig. 3.

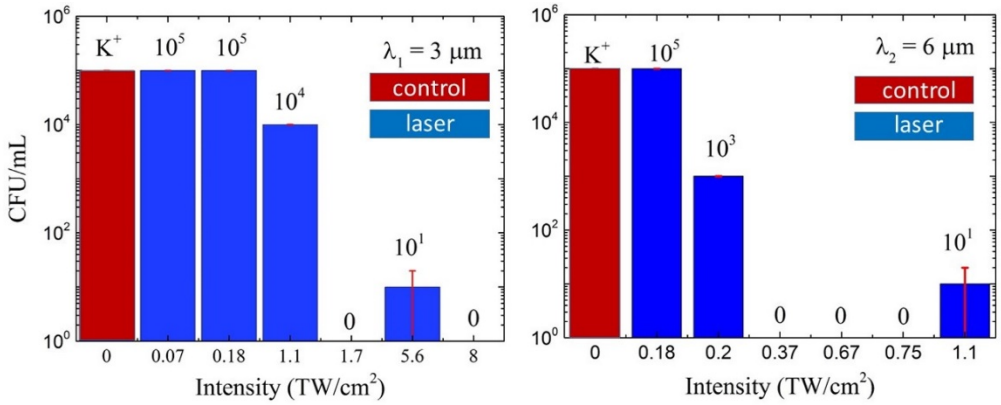

Fig. 3. CFU numbers versus laser intensity at the wavelengths of 3 μm (left) and 6 μm (right); K^+ is the control sample. The minor residual bacterial presence at higher intensities is the technical artifact of incomplete stitching of laser scan lines at the reduced focal size and the threshold-like bacteria inactivation.

As shown in Fig. 2 in the FT-IR spectrum of *P. aeruginosa* bacteria, the 6-μm laser pulses (wavenumbers in the range of 1650–1750 cm^{-1}) hit their characteristic vibrational bands, corresponding to 1) >C=O-bond in nucleic acids (1680–1715 cm^{-1}), 2) C=O stretching vibrations of ester functional groups from lipids and fatty acids (~ 1740 cm^{-1}), and 3) C=O stretching vibrations of amides associated with α- and β- protein structures (amide I band: 1650 cm^{-1}), through very intense local excitation. In contrast, the 3-μm laser pulses (wavenumber spectrum of 2700–3050 cm^{-1}, Fig. 2) can hit the only C-H asymmetric stretching vibration in -CH_2 and -CH_3 fragments of fatty acids and lipids in the bacterial cell wall in the range of 2800–3000 cm^{-1} [24, 25], providing rather homogeneous excitation of the bacteria, but requiring for the inactivation much higher laser intensities.

3.2 Local "effective" temperatures in bacteria

In the context of the strongly different MIR-laser bacterial inactivation thresholds at the 3-μm and 6-μm laser wavelength, we were tempted to investigate the underlying reasons. First, based on the pre- and post-irradiation FTIR spectroscopic optical density measurements (see, e.g., Fig. 4), as well as optical visual/microscopic inspections, we can exclude global ablation of the bacteria from the substrate (catapulting [27]) or local ablation of the walls. The internal laser ablation inside the cells could be considered as a dissociation process, while we can't distinguish non-equilibrium and equilibrium dissociation in non-thermalized and thermalized molecules, respectively, but can evaluate the driving "effective" local temperatures. Moreover, the condensed matter environment in the bacterial cells enables to distinguish thermalization and heat-conduction stages in the intracellular vibrational dynamics, where for the multi-micron focal spots we will neglect by the latter millisecond stage on the inactivation scale for the low thermal conductivity value of water, being the main component of the intracellular fluid.

First, we evaluated the local MIR-absorption effects in the *P. aeruginosa* bacteria at the 3-μm and 6-μm laser wavelengths. According to the FT-IR optical density spectrum in Fig. 2, the average extinction coefficient over the 1.5-micron thick bacterial layer measured by atomic

force microscopy, is $\kappa_6 \approx 8\times10^2$ cm^{-1} at the 6-μm wavelength for the amide groups of proteins and nucleic acids versus $\kappa_3 \approx 40$ cm^{-1} for the C-H groups at the 3-μm wavelength. Second, we accounted for the local distribution of the amide groups in the bacteria. For this purpose, we used energy-dispersive X-ray (EDX) analysis (Fig. 5) to measure the molar ratio C:N as the number of C-N (local absorption) and C-H (almost uniform absorption) vibrations in the bacterial cells, determined as 5 for their weight ratio 41.5:9.6 and the nearly even molar masses M_C= 12 g/mole and M_N = 14 g/mole (Fig. 5), respectively. Hence, the effective extinction coefficient of the amide groups at the 6-μm wavelength can be increased five-fold till $\kappa_6^* \approx 4\times10^3$ cm^{-1}, i.e., two orders of magnitude higher, than at the at the 3-μm wavelength. As a result, one can anticipate much stronger spectrally and spatially selective 6-μm absorption and vibrational excitation for the amide groups in the bacteria (Fig. 6), than the uniform 3-μm absorption by C-H bonds.

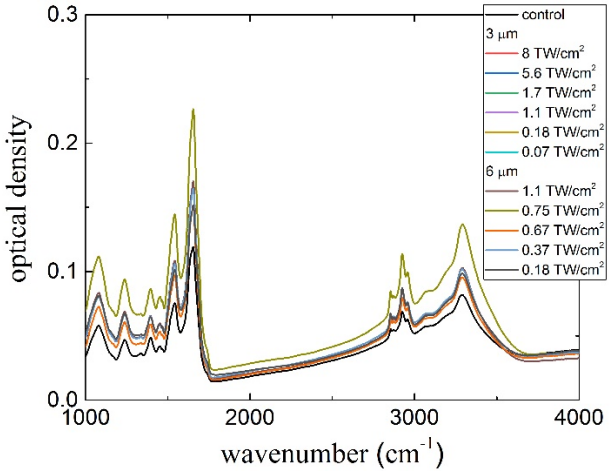

Fig. 4. FT-IR optical density spectra of 1.5-micrometer thick layer of *P. aeruginosa* bacteria upon 3-μm and 6-μm fs-laser exposures at different intensities, shown in the frame.

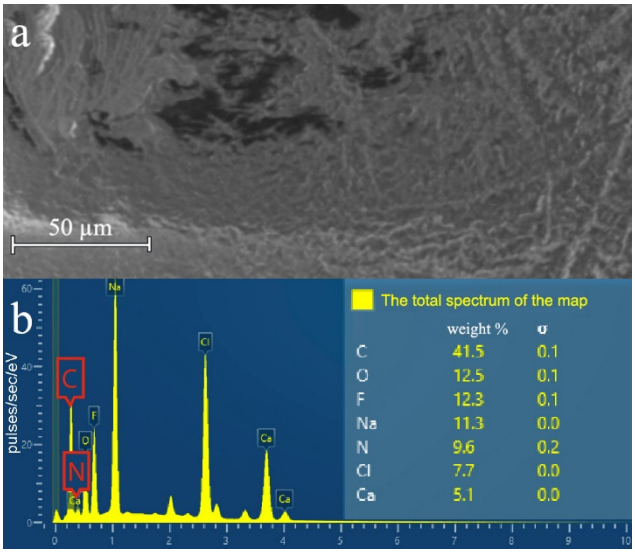

Fig. 5. (a) Top-view scanning electron microscope image of the micron-thick *P. aeruginosa* layer on the CaF_2 substrate and (b) the corresponding 10-keV EDX spectrum and datasheet, including also the contribution of the CaF_2 substrate.

Finally, the intensity dependence of the elevated bacterial temperature was calculated for the 2 pulse/spot exposures at the 1-kHz repetition rate (Fig. 7), using the experimental (I_0, τ, N=2) and derived (κ_3,κ_6*) parameters for the bacterial heat capacity, approximated by that one for water ($C_p \approx 1.3$ J/cm^{3}K), as follows

$$T(I_0,\lambda) = T_\infty + N\frac{\kappa_\lambda^* I_0 \tau}{C_p}. \quad (1)$$

The calculated dependences demonstrate the much stronger temperature rise for the more selective 6-μm heating, which could result in *P. aeruginosa* inactivation above the protein denaturation threshold temperature of ≈80 °C (350 K) [28], where for the most of proteins, denaturation is irreversible. The much higher effective absorption coefficient at the 6-μm wavelength anticipates the corresponding much lower threshold intensity for the bacterial inactivation (Fig. 3).

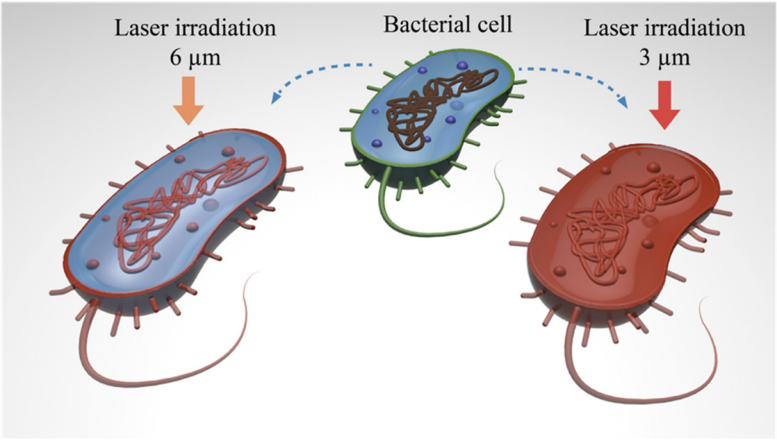

Fig. 6. Schematic representation of the local and global absorption/vibrational excitation/heating effects of laser radiation at the wavelengths of 3 μm and 6 μm, respectively, on the bacterial cells.

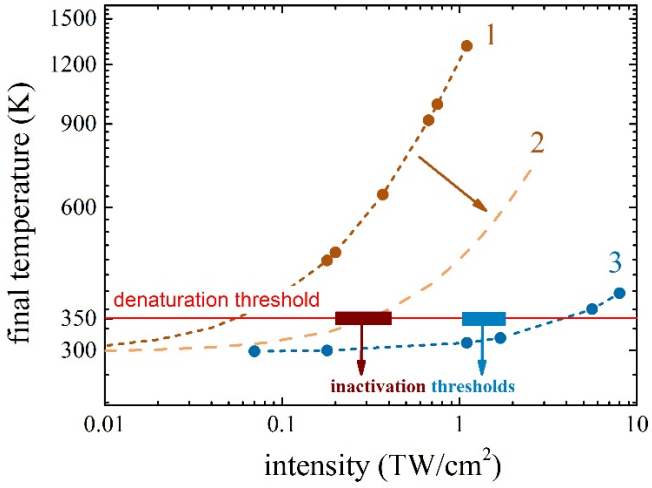

Fig. 7. Calculated dependences of effective bacterial temperature as a function of the femtosecond laser intensity at the wavelengths of 6 (curves 1,2) and 3 (curve 3) μm. The color symbols indicate the experimental intensity values, and the inactivation threshold intensities

(color rectangles with the arrows) corresponds to the onset of CFU reduction in Fig. 3. The curves 1 and 2 differ by the factor of intramolecular energy transfer (≈5.5), derived to fit the 6-µm inactivation threshold intensity to the denaturation threshold temperature for most of proteins ≈ 80 °C (350 K). Such intramolecular energy transfer factor in the case of uniform heating by the 3-m radiation is negligible.

However, the calculated denaturation threshold intensity at the 6-µm wavelength (≈0.06 TW/cm^2) exceeds almost five-fold the experimentally measured inactivation threshold of ≈0.3 TW/cm^2 (Fig. 3). This apparently indicates the considerable intramolecular (*but almost not inter-molecular!*) energy transfer around the local absorbing amide groups almost naturally down to the uniform heating (cfg., the EDX-derived factor of 5 above for the density of local C-N and highly-abundant C-H vibrations in the bacteria). As a result, the temperature dependence calculated for the 6-µm wavelength (curve 1) was scaled down in Fig. 7 by this factor (curve 2). Meanwhile, the large handicap in the effective absorption coefficient between the 3-µm and 6-µm wavelengths still results in much higher inactivation threshold, as predicted by our calculations and the resulting curve 3 in Fig. 7.

4. Conclusion

In this study, we investigated the efficiency of selective and non-selective femtosecond mid-IR (3 µm and 6 µm) laser irradiation with respect to the inactivation of the pathogenic *P. aeruginosa* bacteria. Microbiological viability studies demonstrated that rather uniform femtosecond laser-induced vibrational excitation of the bacteria at 3-µm wavelength corresponds to ≈five-fold higher intensity threshold for the efficient bacterial inactivation, comparing to the more spatially-selective excitation by femtosecond laser pulses at the 6-µm wavelength. In the latter case, the laser irradiation apparently causes hydrogen bonds in the secondary and tertiary structure of bacterial proteins to break and damages the DNA of the bacteria via non-equilibrium or equilibrium (thermal) dissociation. Owing to the strong absorption of 6-µm laser radiation by proteins and lipids, the anticipated effective temperature rise in the cells even after intense intramolecular energy transfer is significantly higher, than for the 3-µm irradiation, resulting in the irreversible denaturing of proteins and nucleic acids in the bacterial cells. The proposed approach appears to be promising for antibacterial treatment and, potentially, sterilization in medical and industrial food environments.

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Disclosures. The authors declare no conflicts of interest.

Data availability. The data underlying the results presented in this paper are available from the authors upon reasonable request.

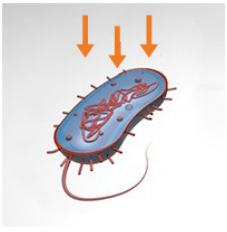

Fig. 2. Preview of thumbnail image display on the author submission page.

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