You shall not pass: titanium and silver nanospikes-based flow-through filter for liquid sterilization

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Abstract. Antibacterial nanostructures were written on the surface of a silver (Ag) film and titanium (Ti) wafer using femtosecond laser ablation. The bactericidal properties were tested on *S. aureus* and *P. aeruginosa* bacterial strains. The resulting nanostructures were characterized by scanning electron microscopy (SEM). A flow-through filter, based on Ag plates with nanospikes, used as a sterilization of the liquid, led to the almost complete death of bacteria. It is assumed that the antibacterial properties are due to mechanical damage caused by the sharp nanoscale relief.

1. Introduction
The widespread use of antibiotics has contributed to the emergence of pathogens resistant to them, including strains with multidrug resistance [1]. Today this problem is circumvented by using micro- and nanostructures with protrusions (nanospikes) similar to protrusions on plants and wings of insects (for example, cicada, dragonfly [2]), which are responsible for keeping these surfaces free from microorganisms [3].

Micro- and nanostructures were fabricated by laser ablation. It is a simple, inexpensive, fast and effective method for precise surface treatment that allows obtaining unique structures with the required morphology and properties [4]. The main purpose of this work was to determine the most effective antibacterial surface, which was subsequently used to create a flow-through filter for sterilizing liquids. Such filters based on silicon and titanium nanostructures with sharp-edged morphology have shown their antibacterial efficacy against *P. aeruginosa* and *S. aureus* [5, 6].

2. Materials and methods.
To obtain an Ag film, the silica glass surface was cleaned in an ultrasonic bath, after which the film was deposited on both sides by magnetron sputtering (SC7620 Mini Sputter Coater) in argon atmosphere. Femtosecond laser ablation of the resulting Ag film (thickness 500 nm) in ambient air was implemented using an ytterbium-doped fiber laser system “Satsuma”, Amplitude Systems (center wavelength $\lambda = 1030$ nm, pulse width at half maximum $\tau = 300$ fs, TEM$_{00}$) (Fig. 1). The laser beam was focused into a
spot with a radius of ~ 10 μm using an f-theta objective (focal length 100 mm) on an Ag plate. The modification of the Ag surface was carried out with the following parameters of laser radiation: maximum pulse energy $E_{\text{max}} = 7 \mu \text{J}$, repetition rate $f = 2 \text{ kHz}$, scanning speed of the laser beam $v_{\text{scan}} = 100 \text{ mm/s}$ with a constant filling of the treated surface 30 lines/mm. In order to select the surface with the most effective bactericidal activity, nanostructures on the Ti surface were also fabricated. In this case, the laser energy density equaled to $F = 2.8 \text{ J/cm}^2$, repetition rate $f = 2 \text{ kHz}$, scanning speed of the laser beam $v_{\text{scan}} = 50 \text{ mm/s}$ with a constant filling 100 lines/mm.

Figure 1. Experimental setup for laser-ablative micromachining of Ag surface

Microorganisms attach to the surface and form biofilms. Attachment is a complex process governed by a variety of characteristics of the nutrient medium, substrate and cell surface [7]. In our case, biofilm formation was carried out as follows. The cultures of $S. aureus$ and $P. aeruginosa$ grown on a nutrient medium were each diluted with broth in a ratio of 1:100 and nanostructured samples with 2 ml of culture were placed in Petri dishes (diameter 9 cm). At a constant temperature of 37 °C, the incubation time of the strains was 18 hours. Live and dead bacteria were visualized using the Live/Dead Biofilm Viability Kit. Fluorescent dyes SYTO®9 (3 μl) and propidium iodide (3 μl) were diluted in 1 ml of distilled water and used as markers of functional and dead cells. To visualize live/dead bacteria, a Nikon H600L fluorescence microscope with a 40× magnification lens and an instrument magnification of 600× was used.

The base of the flow-through filter consisted of 8 glass plates coated with silver on both sides (1 cm × 2 cm each). Then, plates with Ag films were laser treated on both sides and attached to the inner walls of the cuvette. Thereafter, 30 ml of the $S. aureus$ strain suspension was passed through a filter at a constant speed of 13 rpm (speed 0.32 ml/min) through a cross-sectional area of 0.8 mm² using a BT 100-2J DG-2 precision peristaltic pump (LongerPump) for 1 hour. This fluid flow with the $S. aureus$ strain ensured maximum contact of bacteria with the nanostructured surface, after which the resulting solution was analyzed for bacterial viability.

3. Experimental results and discussion

The structured Ag film and the modified titanium surface were analysed using a scanning electron microscope (SEM, JSM 7001F, JEOL). From the SEM characteristics of the Ag film, it can be seen that the surface is an array of spots, the average distance between which equals to $\approx 9 \mu \text{m}$ (Fig. 2a), the average spot size was $\approx 7 \mu \text{m}$ (Fig. 2b), and the region inside the spot was filled with nanospikes (Fig. 2c). The modification of the Ti surface also led to the formation of nanospikes (Fig. 2d). The formation of nanospikes during femtosecond laser ablation is often observed in the spallative mode, accompanied by the formation of subsurface voids, their agglomeration, breaking of the spallated film, and nano-foam solidification, which leads to the formation of nanojets (nanojet peaks) [8, 9].
After processing the samples, live/dead tests were performed and the results were interpreted for maximum efficacy using red-green-blue (RGB) analysis. Blue was excluded from color histograms due to its absence in photomicrographs obtained with a fluorescence microscope. The created filters (Fig. 3) with Ag and Ti nanospikes were tested on S. aureus and P. aeruginosa strains, respectively. From the resulting 30 ml of the culture of the overnight broth, 1 ml was immediately taken to determine the number of colony-forming units (CFU) in the original sample (control). Then, in a filter with nanostructured plates, the bacteria suspension was run several times for more than 3 hours. After 24 hours of incubation (after titration of the filtrate and plating on Petri dishes with solid nutrient medium), the number of CFU was determined. The study showed that the use of a filter with nanostructured Ag plates almost completely destroys the bacterial population (Fig. 4). In turn, the filter based on nanostructured Ti plates reduced the bacterial population by 2 orders of magnitude (from $10^6$ to $10^4$).

Although it is generally accepted that cell death on nanostructured surfaces is of mechanical nature, a clear understanding of the main mechanisms has not yet been determined and several different mechanisms have been proposed in [10-17] such as reactive oxygen species abundance etc. Variety of proposed mechanisms can be explained by the complex adaptive nature of microbial cells, which creates a variety of interactions between cells and the surface.

4. Conclusion
In our work, Ag and Ti nanostructures were tested as potential elements for a sterilization liquid filter. These structures were characterized using SEM, after which their antibacterial properties were tested.
The most effective types of nanostructures turned out to be Ag nanostructures, which were subsequently used to create a flow-through filter. A flow-through filter based on nanostructured Ag plates almost completely destroyed the S. aureus bacteria population.

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