IMAGING STAPHYLOCOCCUS AUREUS BY AFM AND SEM

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Analysis of biological samples is an important and complex endeavor that often times needs the utilization of advanced characterization methods. The aim of the present study is to highlight the advantages but also some of the limitations of using Atomic Force Microscopy (AFM) and Scanning Electron Microscopy (SEM) in bacterial research.

Staphylococccus aureus (ATCC 6538), purchased from Microbiologics, was reanimated according with manufacturer recommendations and cultivated in freshly tryptic soy broth (TSB) from Merck, over night 35-37°C and stirred at 200 rpm. After the incubation time portions of culture were centrifuged and washed twice with phosphate buffer saline and resuspended. The suspension was adjusted at approximately 107 CFU / ml [1, 2].

Preparation of disinfectants solutions In this study the influence of alcoholic solutions Ethanol (EMPROVE® Merck) and Isopropanol (EMSURE® Merck) sodium hypochloride (Merck), and bis(aminopropyl)laurylamine) (Isorapid Floor®, OCC Switzerland) were evaluated. Sterilization of the products was performed by filtration, using filter units Millex GP with 22 nm pores size (Millipore) and kept in airtight sterile vials, in the dark, at room temperature, until they were use. First, for each studied substances Minimal Inhibitori Concentration (MIC) was determined by broth microdilution method. The microorganism was grown with or without studied disinfectants at concentrations bellow MIC according to Wu and Liu .

Determination of MIC. For establishing the concentration of stress factors (decontamination agents) to be used in this study we have determinated the MIC (Minimal Inhibitory concentration) for each substance. MIC represents the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in the tubes or microdilution wells as detected by the unaided eye [3]. The MICs for each stress factor studied were determined by broth microdilution method in 96-well polypropylene microtiter plates (Costar no. 3790). Two-fold serial dilution of each stress factor into TSB medium were performed in triplicate. Negative and positive controls were associate [4]. After establishing MIC for each biocide substance, 30 ml portions of S. aureus, prepared as previously described, in fresh liquid culture media, were distributed in sterile Falcon tubes and grew in presence of the studied disinfectants at 1/2 x MIC values continuous agitation [5, 6]. After the incubation period, the suspensions were washed twice, by centrifugation, the bacterial cells were prepared for AFM and SEM imaging.

Sample preparation. Portion of S. aureus suspensions cultivated in the presence of biocides at ½ MIC were fixed with glutaraldehyde 2% (final concentration) for 24 h and after, susbsenquely, the samples were dehydrated by immersing them for 10 min in the solutions of ethanol with increasing concentrations (30-100%), then dried for 24 h at 50 °C [7].

Atomic force microscopy (AFM). Imaging of the cell surface was performed in Tapping Mode™ in air using a MultiMode NanoScope IIIa Controller (Digital Instruments Veeco Metrology Group, USA). Standard cantilever was used at a scan speed of 0.5 Hz and a resolution of 512 by 512 pixels. Images were recorded in both height and phase modes. Images were processed using 531r1 NanoScope AFM software.

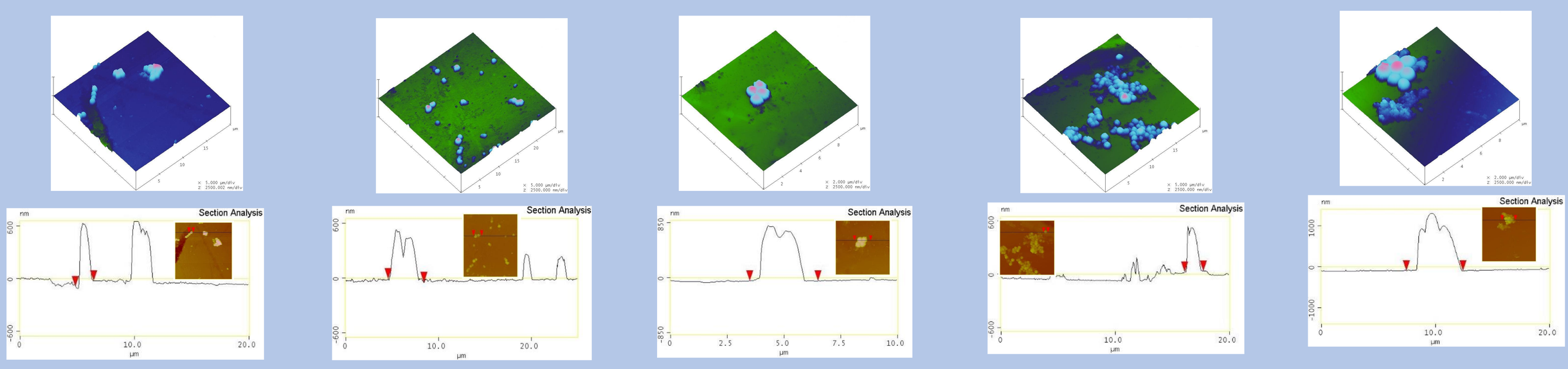

Fig. 1. AFM 3D/section analysis high resolution images of untreated S. aureus and S.aureus under different treatments: sodium hypochloride, *ethanol; bis(aminopropyl) laurylamine ; isopropanol*

Scanning Electron Microscopy (SEM). The samples were analyzed using a Zeiss EVO MA15 scanning electron microscope under high vacuum with the following parameters: a 10kV accelerating voltage, a 100 pA probe current at a working distance (WD) of approximately 5.5 mm. All samples were analyzed as a single batch under the exact same conditions. In order to avoid surface charging and to improve the image quality and resolution the samples were coated with a layer of gold (Au) 3-4 nm thick.

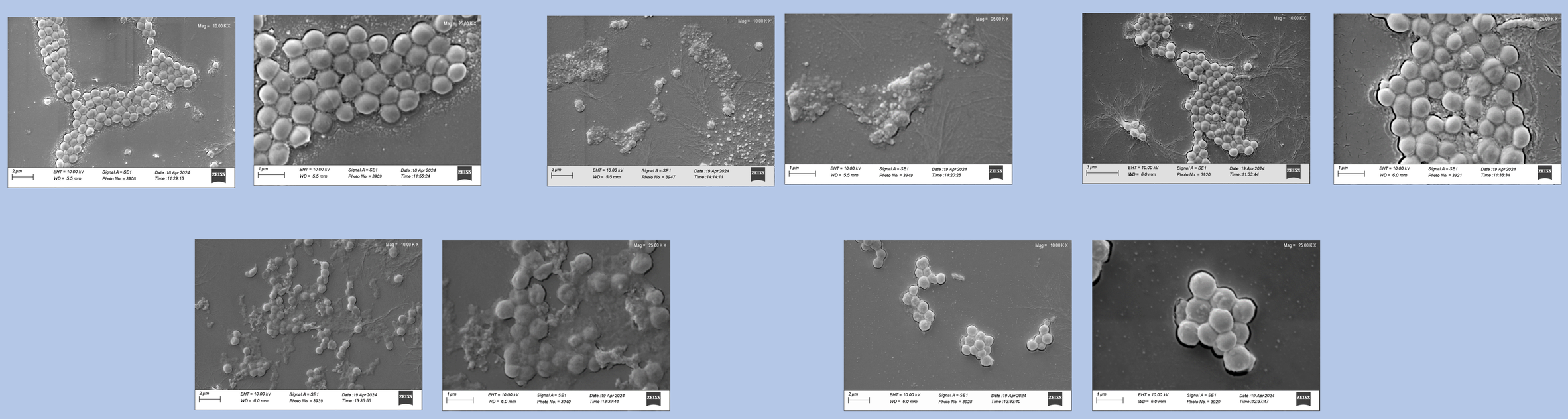

Fig.2. SEM images of untreated S.aureus; S.aureus with hypochlorite; S.aureus with ethanol; S.aureus with bis(aminopropyl) laurylamine; ; S.aureus with isopropanol.

Discussions

In the case of positive control samples, in the image (CP_25kx) it can be observed a characteristic *Staphylococcus aureus* cells structure: spherical cells, in different phases of cellular cycle, grouped in cluster.
S. aureus exposed to ethanol solution the cells maintain the shape and the cluster structure. In the images (E_25kx)

It can be observed the fact that cells have similar dimensions and are in different phases of cellular cycle, similar with the ones from positive control. Isopropanol exposure had determined a hypertrophy of the cells. The mean of the cells is almost 21% larger comparing with the control mean. In exchange, the other two treatments (Isorapid and Domenstos) determined cells reduction of diameter with ~21%, respectively ~31% comparing with the control.

S. aureus response at hypochlorite solution (active substance in Domestos) have the most important variations. This variations can be observed in the images and also in the cells measurements. These variations are consistent with the larger differences between cells diameter (Min / Max) and greatest Standard deviation value (0.164)
The surface of the untreated *S.aureus* clearly showed typically round shape cells attached individually or in groups across the mica substrate, fig1.

All images exhibit several differences when compared with the untreated sample in terms of shape and height. An increase in height (from 400 nm **control sample** to 500 nm **sodium hypochloride**, 700 nm **ethanol**; 500 nm **bis(aminopropyl) laurylamine** ; 800 nm **isopropanol**) is observed after the treatments used (section analysis, figure 1).

Table 1. *Staphylococcus aureus* – cells dimensions after exposure to disinfectants at ½ MIC

Treatments	Positive Control (µm)	Ethanol (µm)	Isopropanol	Isorapid	Domestos
Mean	0.717	0.701	0.868	0.57	0.501
SD	0.088	0.135	0.112	0.089	0.164
Min	0.547	0.477	0.631	0.414	0.239
Max	0.933	0.997	1.04	0.757	0.742

AFM reveal high resolution details of the bacterial cells with little sample preparation while for **SEM** investigation a complex preparation of the sample should be done. Even these two techniques appear very different they have some similarities which give a complete representation of a sample down to the nanometer scale.

References

1.Cui, Y., et al., *AFM study of the differential inhibitory effects of the green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) against Gram-positive and Gram-negative bacteria.* Food Microbiol, 2012. **29**(1): p. 80-7.

2.Meincken, M., D.L. Holroyd, and M. Rautenbach, *Atomic force microscopy study of the effect of antimicrobial peptides on the cell envelope of Escherichia coli.* Antimicrob Agents Chemother, 2005. **49**(10): p. 4085-92.

3.CLSI M07-A9 Vol. 32 No. 2

4.Wiegand, I., K. Hilpert, and R.E. Hancock, *Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances.* Nat Protoc, 2008. **3**(2): p. 163-75.

5.Bolshakova, A.V., et al., *Comparative studies of bacteria with an atomic force microscopy operating in different modes.* Ultramicroscopy, 2001. **86**(1-2): p. 121-8.

6. Wu, G.F. and X.H. Liu, *Characterization of predominant bacteria isolates from clean rooms in a pharmaceutical production unit.* J Zhejiang Univ Sci B, 2007. **8**(9): p. 666-672.

1.Czerwińska-Główka D, Krukiewicz K. Guidelines for a Morphometric Analysis of Prokaryotic and Eukaryotic Cells by Scanning Electron Microscopy. Cells. 2021 Nov 25;10(12):3304. doi: 10.3390/cells10123304.

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