Biodestruction of polyurethane by *Staphylococcus aureus* (an investigation by SEM, TEM and FIB)


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An experiment is presented and discussed whose aim is to assess biodestruction of polyurethane, a material commonly used for prosthesis in odontostomatology under the influence of *Staphylococcus aureus*. Electron microscopy (scanning and transmission) and FIB were used in the investigation. It is established that bacteria realize the adhesion to polyurethane surface by means of extracellular polymeric substances of polysaccharidic nature, they form microcolonies and the permanent bonding result in a biofilm on a surface of the polymer. Electron and ion microscopy provide detailed information about biofilm characteristics and about the whole process leading to polymer destruction.

**Keywords** FIB; SEM; TEM; polyurethane; biodestruction; *S. aureus*; odontostomatology; biofilm; endocytosis; interaction of nanoparticles with bacterial cells, correlative microscopy

**Introduction**

The present paper discusses a series of experiments on the interaction between bacteria and plastic materials and proves the necessity of interdisciplinary investigation by researchers active in different fields showing that in this case top electron microscopy techniques must be cross-fertilized by a correct problem identification and by the resulting images interpretation.

It is accepted to call process of destruction of natural and artificial materials under the influence of microorganism’s biocorrosion and biodestruction. Biocorrosion is the process that takes place at metal surfaces associated with microorganisms, or with the products of their metabolic activities including enzymes, exopolymers, organic and inorganic acids, as well as volatile compounds such as ammonia or hydrogen sulfide. Biodestruction is the respective for polymeric materials.

Biocorrosion and biodestruction have negative effects. These processes can destroy cultural heritage, various industrial products, including space and sea ships and so on [1]. From the other side, biocorrosion and biodestruction have positive value. Without these processes our planet will be filled up with garbage.

One of the least studied problems in medicine is the problem of biodestruction of the polymeric materials applied in medicine, in particular, in orthopedic stomatology.

Different types of polymers are widely used in orthopaedic stomatology for the manufacture of artificial prostheses, specifically polyurethane (a synthetic elastomer) occupies a special position because it is bio-compatible, non-toxic, hypoallergenic, has high durability, elasticity and resistance to chemical-physical factors [2,3]. Prosthetic devices are placed in the oral cavity and they are constantly being attacked by microorganisms, therefore when manufacturing and using them it must be taken into account the nature of the relation between the materials composing the prosthesis and the population of microorganisms living in the oral cavity.

The colonization by microorganisms very often results in the destruction of the artificial material. It is obvious that biodegradation of dental materials adversely affects their strength and durability and it usually originates allergenic substances which are toxic to the human body. [4,2,5].

The study of the structural changes of artificial materials following the colonization by microbes have been conducted with different methods, including microscopic techniques which are of particular importance. The technique of scanning electron microscopy (SEM) is used to evaluate what, on morphological basis, can damage the artificial materials in the different stages of interaction with micro-organisms (adherence, formation of microcolonies and of biofilm).

At the moment we don’t have any information on which of the microorganisms found in the oral cavity have the greatest capacity for biodegradation of plastics materials or polymers used in orthopedic stomatology. The research on plastic materials and their properties of resistance to biodegradation is still a highly topical issue.

Currently, *Staphylococcus aureus*, a permanent component of the microbial population present in the oral cavity, is considered a potential pathogen agent with biodestruction capacity. Some studies show that staphylococci can adhere to the surfaces of titanium and polymeric materials (polyethylene) and form a mucilaginous matrix [6,7].
Coagulase-negative staphylococci are active plastics colonizers and represent a real threat as a source of endogenous infection which in literature is indicated with the term “plastic infection” [7].

Staphylococci cause acute and chronic inflammation of soft tissues of the oral cavity (periodontal disease, sialodenity, gingivitis, etc) and also influence on development of caries, [8]. It is necessary to consider the possibility of subsequent bacterial growth at prosthetics of patients with diseases of soft tissues in oral cavity of a staphylococcal etiology, as staphylococci are able to colonize artificial prostheses surfaces [9]. On one hand, this may exacerbate chronic infectious processes, or it can lead to the destruction of the prosthesis materials, because staphylococci may have a biodegradation potential. In the presence of negative processes for the survival of the bacteria, such as the depletion of nutrients in the environment, the threat of drying, the impact of chemical and physical factors in the external environment, the bacteria can form a biofilm [10].

The increase of the biodegradation capacity is directly related to ability of bacteria to form biofilms [1].

The purpose of this study is to analyze, by means of SEM, FIB and TEM, the characteristics and dynamics of the interaction of S. aureus with polyurethane in relation to the incubation time and to assess the characteristics of damages caused by bacteria as a function of substrate macroscopic properties and machining procedures

2. Materials and Methods

Objects of the research were samples of polyurethane (Dentalur Russia). Various types of plastic surfaces (smooth, rough, surfaces resulting from sawing and splitting operations) were investigated. The clinical culture of S. aureus was isolated from a patient with a periodontal disease. Control samples were a polyurethane non-sawed surface in broth and a broth with S. aureus without polyurethane samples. pH was checked after sample incubation.

The samples of polyurethane were put into a tube with nutrient broth. The culture of S. aureus (concentration: 10^6/ml) was added to this tube too. Polyurethane samples (size 0.5x0.5 cm²) and bacteria were incubated for 24, 48 hours, 7, 14, 30, 45 days at 37°C.

After incubation the polyurethanes samples were fixed in 1% glutaraldehyde solution buffered to pH 7.4, and subsequently covered with gold (thickness of about 5 nm). Samples were analyzed in dual beam electron microscope Quanta 200 3D (FEI Company, USA) under conditions of both high and low vacuum mainly at an acceleration of the electron beam of 5 kV.

The focused ion beam (FIB)/scanning electron microscope (SEM) is a scanning microscope with an electron column and an ion column embedded in the same specimen chamber; both beams are aiming at the same point on the specimen surface[11]. The FIB, generated by a Ga Liquid Metal Ion Source (LMIS), impacts the sample normal to the surface and can be focused to a spot as small as few nanometres. The FIB can be rastered in an user defined pattern over large areas to selectively sputter and mill away the surface. By flooding the exposed surface with specific gases, during ion or electron bombardment new material can be deposited or some specific face can be removed faster (enhanced etching). The combination of both unselective ion milling and selective etching using reactive species creates a very powerful sample preparation tool.

The focused ion beam operated at low beam currents is used for imaging, and high beam currents are used for site specific in situ sputtering or milling. The signal from the sputtered secondary ions or secondary electrons is collected to form an image. The FIB/SEM investigation can be applied on bulk samples, prepared for conventional SEM or on bulk resin-embedded specimens prepared for conventional TEM, at any chosen site. The focused ion beam system for microscopy and nanomachining is widely utilized in semiconductor technology. Up to now, the FIB/SEM was applied on a variety of biological samples, however there are still many questions left opened which have to be answered before FIB/SEM will be widely applied in structural research in life sciences.

The bacterial pellet recovered by centrifugation (6000 rev / min for 10 min).was placed on a silicon substrate and observed both in High and Low vacuum.

The preparation of biological specimens for scanning or transmission electron microscopy starts with primary fixation, which is followed by washing, secondary fixation and dehydration. For SEM, it is continued by drying, mounting on a metal specimen stub, and coating a specimen with a thin, electrically conductive layer. For TEM, it is proceeded by infiltration of specimen with transitional solvent, infiltration with resin, embedding, curing and cutting. Note that in the process of sample preparation for the observation in the electron microscope chemical-physical methods of dehydration were not applied in accordance to the traditional preparation for scanning electron microscopy. When using the standard methods of sample preparation for scanning electron microscopy, drying operations presumably leads to structural changes of biological objects, in particular of the biofilm; that are structures with mucoid matrix and are made up of 90% of water.

For polyurethane control sample the surface analysis was carried out before and after the incubation in nutrient broth. Examples of the application of FIB/SEM to yeast cells and an epithelial tissue are discussed in refs.12-18.

The advantages of FIB/SEM over conventional SEM or TEM are discussed and some future perspectives of FIB/SEM for biological samples are exposed.

We start to develop a method for the evaluation of the area of fouling of a polyurethane sample. For this aim we stained samples by copper sulfate for identification of polysaccharide matrix of biofilm.
3. Results

The polished surface of the control sample is relatively smooth, with individual fragments of small sizes; traces of mechanical damage resulting from polishing take on the appearance of shallow scratches (Fig. 1a). The rough surface of the control is still rough, with ridges and many fragments (Fig. 1b). This internal surface of prosthesis should be rough not to damage soft tissues. The splitting-machined surface had almost smooth surface within levels of different height (Fig. 1c). The sawing-machined surface consists of parallel packed fibrous structure (Fig. 1d). After incubation in the nutrient broth, an amorphous layer of thin film can be seen on the surface of samples.

On the shortest incubation time (24 hours) separate bacteria adhered to a polyurethane surface, thus the most intensive attachment of bacteria occurred in sites of mechanical defects (Fig. 2a).

For longer incubation times the formation of microcolonies was the main tendency of behavior of bacterial population. Microcolonies were merging with each other and were being covered with mucoid (exopolysaccharide) matrix (Fig. 2b).
We have analyzed the changes of polyurethane surfaces under the influence of *S. aureus* at different stages of interaction.

On the polished surface of the polyurethane only single bacterial cells and/or microcolonies are present, while the processes of colonization and adhesion on the rough surface progresses much faster. After 45 days an incubation with staphylococci practically all the polyurethane surface (both rough and smooth) is covered by a biofilm (Fig. 3a), as a part of it was present strongly pronounced exopolysaccharide matrix in which contours of actually bacterial cells were observed (Fig. 3a,b,c).

Small particles of polyurethane were detected on the surfaces of the exopolysaccharidic matrix of biofilm and on bacterial cells (Fig. 3 d, e)
The pellet of planktonic cells of staphylococci, after an incubation with a polyurethane (45 days), generally consisted of mass of cells on which surface it was possible to see parts of a polyurethane in the form of slices and fine grains (Fig. 4a). Also there were amorphous masses with inclusions of fine particles of polyurethane (Fig. 4b).

The adhesion of certain bacteria in the early stages of the formation of microcolonies does not entail significant changes to the surface of polyurethane. When the surface of polyurethane is covered by a rigid biofilm with a well
developed exopolysaccharidic matrix, we can observe pieces and particles of polyurethane with clear visible changes and defects of the surface of the polyurethane in the form of cracks and hollows. (Fig. 5 a, b, c).

Figs. 5. a) Spalling of the samples of polyurethane ↑; b) Cracks of biofilm and polyurethane ↑; c) Hollows in polyurethane ↑

Summarizing: 6 stages were detected of interaction between S. aureus and polyurethane samples.

i. Formation of film without bacteria was the first stage. It is a very important stage. This stage facilitates the process of bacterial adhesion.

ii. Bacterial adhesion was the second stage. Bacteria preferred sites with mechanical traces.

iii. Then bacteria start to breed and to form microcolonies.

iv. As time pass by during incubation of samples, there is environment exhaustion of the main sources of nutrient substances for bacteria. The result of this – bacteria start to produce exopolysaccharidic matrix; the overproduction of the matrix is the main feature of biofilm formation.

v. In a mature biofilm nomads are formed and there is cracking.

vi. Process of bacterial dissemination happens thanks to nomads and cracking.

Image processing

We used the Scandium software to evaluate the dynamics of changes of the area of bacterial fouling of samples, depending on incubation time. We start to develop a method for the evaluation of the area of fouling of a polyurethane sample.

The changes of surfaces are not evident in the first stage of a microbic film formation. Bacterial adhesion occurred in sites of mechanical defects of a surface formed after all stages of processing of materials and also on surface obtained by sawing.

It is important to mention that ideally smooth surface was not colonized by bacteria at early stages (until 48 hours).

FIB analysis of surface machining

To assess the changes (surface and subsurface) of the polyurethane incubated with staphylococci the ion beam was used to create sections both of the incubated sample and the control one. In samples incubated with staphylococci areas were selected covered with a thick layer of biofilm. In control samples of polyurethane, after ion beam drilling, a smooth surface appeared, while the surface of the drilled samples (after 45 days of incubation with staphylococci) appears "lacy": the thickness of this layer was estimated to be about 2-3 microns. (Figs. 6 a, b).
Transmission electron microscopy analysis

We prepared a sample of *S. aureus* taken from the culture broth from which ultrafine sections were obtained, the usual procedures were followed in accordance with the preparation standard methodology for electron microscopy, (described previously) [19], and the sections were analyzed in the electron microscope JEM B-100 (Jeol Japan) with an acceleration of 80 kV.

It was also been observed a sample of the culture broth of *S. aureus*, with the same duration and same incubation temperature, without the addition of the polyurethane samples. Control samples of polyurethane were also observed in the presence of only the culture broth without the addition of any culture of *S. aureus*.

During the incubation of the *S. aureus* culture with polyurethane the culture broth was buffered by a buffer in an environment initially neutral, pH: 7.4 and then acid at pH 5.5 Staphylococci had typical structure in control samples [20].

In these samples the number of destroying cells and protoplasts was significantly lower than the ones observed in the tubes with *S. aureus* incubated for growth without polyurethane. Most of the cells were larger in size than the control. Most of the cells had desquamated cell wall (Figs. 7 a,b).

On the surface of the cell wall and outside the cells was observed an identical polyurethane material (Figs. 7 c,d). The area of mesosomes was considerably higher than in control. An atypical division is observed in the cells.
Figs. 7 Polyurethane grains on cells surfaces and in cytoplasm↑ c) Magnification 90.000x and d) 70.000x respectively.

Analysis with Annular Dark field detector (Tecnai G2 F20 S-TWIN) is reported in Figs. 8 a-b

Figs. 8. a) vesicles can be seen in the bacterial cytoplasm: they may contain polyurethane possibly internalized by endocytosis; b): the process of division in S. aureus.

The organisms appeared to divide in three alternating perpendicular planes, with sister cells remaining attached to each other after division. This may explain the image obtained from TEM, in which there are 3 cells seemingly placed in orthogonal planes [21].

Images taken with bright field detector (Tecnai G2 F20 S-TWIN) give information about the internal organization of bacterial cells (Figs. 9).

Membranes are formed thanking invagination of cytoplasmic membrane, which localized under cell wall and surround bacterial cell.
Figs. 9. a) polyurethane nanoparticles can be seen behind the wall (or glycocalyx); b) bacterial wall and membrane glycocalyx are easily seen, together with vesicles inside the bacterium; b) *S. aureus*. After incubation with polyurethane (samples were prepared without additional staining; Uranil citrate and Osmium tetroxyde were added during traditional protocol of sample preparation for TEM) Small particles at cell wall surfaces, and inside bacterial cell into cytoplasm envioned by membranes ↑ are seen.
4. Discussion

The study shows structural evidence of the process of biological degradation of polyurethane by the \textit{S. aureus}.

The biodegradation of polyurethane by staphylococci can be summarized in the following main points. In the first place, staphylococci produce lactic acid from the enzymatic fission of sugar [22]. This study demonstrated the compensation of the acid value of pH with a long incubation of staphylococci with samples of plastic materials. The increased concentration of metabolic acids produced by staphylococci may have a negative impact on polymeric materials and thus represent the starting point of their destruction.

Secondly, with the longer duration of incubation in given conditions there was a critical augmentation of bacterial mass, causing a depletion in sources of important of nutrients. The depletion is the starting point of biofilm formation [10]. In this study the environmental conditions of nutrient depletion were created artificially by prolonged incubation without renewal of the environment. With the depletion of nutrients in a broth, the staphylococci can use the plastic materials as an alternative source of nutrients.

Formation of biofilm is accompanied by an increase of the sizes and depths of defects which were earlier on surfaces (result of machining) and formation of new defects in the form of large and fine particles of a polyurethane, cracks, ulcerations, caverns, up to a chopping of large particles from a sample.

True nature of damages of samples after an incubation with bacteria can be estimated after biofilm removal from a surface. Ultrasound were used to remove biofilm from surfaces. After this procedure, a clear picture of damage of surface appears.

Surface under biofilm looks like a lace.

The analysis of preparations of bacteria after incubation with polyurethane allowed to answer a question: why bacteria are capable to destroy polymers. Formation of small particles of polyurethane occurs under the influence of bacterial enzymes (esters). Particles of polyurethane get in a bacterium by endocytosis process.

The reason of this statement is based on data obtained in this study by scanning and trasmission electron microscopy. The absorption of the plastic material is clearly visible in the ultrafine slices sections of staphylococci, in the form of small grains included on the cell surface, in the periplasm and in the cytoplasm. This confirms the data of scanning electron microscopy, which allows viewing of the process of destruction of plastic materials with amorphous masses of bacteria (Staphylococci), which were clearly visible for the presence of particles of plastic materials and for the detection of surface defects of plastics. The destruction mechanism of plastics (polyurethane) comes from processes associated with the bacterial enzymes (enzyme catalysis), the breakdown of the polymeric connections occurs also because of the acid producing bacteria and their catalysis processes during non fermentative activities[4].

Cracks, scratches and pores reduce durability of dentures [22,23,24].

The fundamental role of the biofilm is the possibility to guarantee a long resistance to the infectious agent which in turn supports a chronic infection of the oral cavity in patients with removable dentures. In addition it should be noted that the plastic material of polyurethane have influenced the properties of staphylococcus from the morpho-functional standpoint. It is possible that the new properties may have undesirable effects on the human body, change the sensitivity of bacteria to antibiotics and biocid drugs, thus creating problems in the treatment of infections of the oral cavity.

The destruction of polymeric materials is important not only from the point of view of the duration of the prosthesis. In fact the destruction of the polymer materials is accompanied by the emission of substances which are likely to cause allergic and toxic reactions in patients.

Biofilm is the base for a long persistence of the infectious agent that in turn supports chronic infectious process in an oral cavity of patients with prostheses [25].

In addition it should be noted that the plastic material can influence on the morpho-functional properties of staphylococci.

It is possible that the new properties may have undesirable effects on the human organism, change the sensitivity of bacteria to antibiotics and biocide drugs, thus creating problems in the treatment of infections of the oral cavity.

Desquamation of particles with biofilm represents a threat for the metastasis of the infection.

For improvement of quality of the materials used in an orthopedic stomatology, it is expedient in the list of demands to include indicators of fastness them to a biodestruction.

Methods of a scanning electron microscopy are optimum for an assessment of processes of a biodestruction of artificial materials.

5. Conclusions and Perspectives

Results show that:

- Images obtained from electron microscopy FIB / SEM indicate that polyurethane undergoes bio-corrosion by \textit{S. aureus} (Fig. 12).
- Biocorrosion is a process that involves several steps ranging from the colonization of the material to the formation of biofilms (Fig 8, 9, 10, 11).
• *Staphylococcus aureus* is able to corrode surfaces of polyurethane generating a number of polyurethane particles (Fig 13), the size of about 1nm.

• TEM images show that *S. aureus* internalizes polyurethane particles (Fig 14).

• TEM images show the presence inside *S. aureus*, (after incubation with polyurethane), of rounded structures surrounded by a membrane, similar to vesicles (Fig 15 and 17).

These results raise some questions:

What is the fate of the nanoparticles inside the planktonic cells: whether they die a natural death, or after antibiotic administration.Polyurethane particles inside bacterial cells can provide energy supply to a bacterial cell in the conditions of nutrient deficiency. Ultrastructural data do not reveal traumatic situations that could lead to bacterial death; confirmation of this is cell fission that testifies cell viability. Moreover it may be that the conjugate of nanoparticles with antibiotics can provide the best way to uptake the complex into bacterial cells and the bacterial death will be the result.

Studies of nanoparticles [26] show that they can escape action from the immune system (phagocytosis by macrophages), enter the bloodstream and lymphatic system, deposit themselves on organs and tissues and cause inflammation. The chapter on nanoparticles and related nanopathologies is still open and under investigation. Yet the same polyurethane is used as an excipient for the synthesis of new anticancer drugs [27] resulting in a good vehicle for its solubility properties and low toxicity. The presence of vesicles inside *S. aureus* opens a new perspective in the study of endocytosis mechanism (Figs. 15a, 17b). The vesicles could have been formed by endocytosis, a mechanism that has been extensively studied in the eukaryotic domain, but has few experimental results in the bacteria domain. The endocytosis is a mechanism that allows the internalization of extracellular material through the invagination of the cytoplasmic membrane and the formation of vesicles containing foreign material.

In literature there is a lot of material regarding the mechanism of endocytosis in eukaryotic domain but, the road to true understanding of this mechanism is still a long way away. A recent study [28] shows for the first time the type of mechanism into the bacteria domain through the use of various techniques including transmission electron microscopy, it shows the presence of vesicles containing material of incubation (GFP). This study supports the hypothesis that the vesicles found in our samples of *S. aureus* contain nanoparticles of polyurethane and that the mechanism through which they enter the bacteria is the endocytosis. In both cases TEM proved to be the most suitable instrument for this type of investigation. Future prospects regard the dental health care sector; the study of the endocytosis mechanism of the nanoparticles and the use of electronic microscopy in forensic samples not prepared with standard protocols of preparation. In the dental care industry the attention is focused on planktonic cells: biofilms are characterized by the presence of planktonic cells that can detach from them, cross the digestive tract, deposit in the intestine and cause another infection outbreak (metastasis) leading to disease caused by material. Our work indicates that the use of TEM / STEM allows us to view vesicles located inside the bacteria: the preparative and the instruments used can be re-proposed to view vesicles and nanoparticles.

Electron microscopy [29] has proved an excellent tool of research for the study of bio-corrosion of polyurethane (SEM / FIB), for biofilm characterization [30] and for the study of details inside the bacteria (TEM / STEM) regarding the presence of nanoparticles and of endocytosis related processes.

Future prospects are aimed at its use in forensics, a science that involves comparing and analyzing known and unknown materials. There are some factors that could affect the credibility of the results: it is imperative to preserve the integrity and evidentiary value of the sample and the results. Electron microscopy allows investigation of the sample without any kind of preparation.

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