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Electron Microscopy Documents the Microorganisms' Biodestructive Action on Polyurethane and the Production, Internalization and Vesicular Trafficking of Nanoparticles

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Authors' contributions

This work was carried out in collaboration between all authors. Authors LVD, GAA and NVS designed the study, wrote the protocol and the first draft of the manuscript. Authors MM, RC and AE managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Prostheses in the oral cavity are constantly attacked by microorganisms. Bacteria and fungi colonize these surfaces concurring in the material's biodestruction; the corrosive action generates debris of different size, with particles ranging from few micrometers to nanometers. Transmission Electron Microscope (TEM), Scanning Transmission Electron Microscope (STEM) and Focused Ion Beam/Scanning Electron Microscope (FIB/SEM) used in this study show that bacteria

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(*Staphylococcus aureus*) and fungi (*Candida albicans*) are able to adhere to the prostheses' surfaces (polyurethane) and operate a biodestructive process. Electron images document the damages on the polymeric surfaces and the formation of debris. Polyurethane nanoparticles can be detected not only outside the bacterial cells but even in cells, surrounded by membrane vesicles; this work ascertains that the uptake process occurs through endocytosis, and outlines that the cytoskeleton is implicated both in the nanoparticles internalization and in the vesicular trafficking within the bacterial cell. Polyurethane nanoparticles we studied are not engineered, have unexpected characteristics and reactivity; moreover being surrounded by vesicles within bacterial cells they raise a new problem in toxicology, since this represents a new way through which nanoparticles may gain access to the body (driven by bacteria-host cells interactions), elude the immune system reaction to xenobiotic elements and provoke pathologies.

Keywords: Staphylococcus aureus; nanoparticles; polyurethane; endocytosis; cytoskeleton; membrane vesicles; (nano)toxicology; electron microscopy.

ABBREVIATIONS

PU: polyurethane; NP: nanoparticle; MV: membrane vesicle; TEM: transmission electron microscope; STEM: scanning transmission electron microscope; FIB/SEM: focused ion beam/scanning electron microscope; HFW: horizontal field width.

1. INTRODUCTION

The risk of infection associated with polymeric materials used in most medical fields, as stomatological and orthopaedical prostheses or catheters, is common whenever biomaterials are attacked by microorganisms [1-4]. Our focus in this work is on bacteria (*Staphylococcus aureus*) and fungi (*Candida albicans*) permanently present in the oral cavity [5] that colonize plastic surfaces such as polyurethane (PU) prostheses, form a biofilm and concur to the polymeric material's destruction (biodestruction) [6-10].

When cultures of microorganisms and polymeric material are incubated for a long time (more than six weeks), the critical augmentation of the bacterial or fungal mass causes nutrients depletion [11-13], therefore microbes are forced to use plastic materials as an alternative source of nourishment [7,14,15]. This is the starting point of the formation of a biofilm, a mucoid matrix with several physiochemical microenvironments in which bacteria and fungi act as a community [9,16].

The initial interactions that take place between bacteria or fungi and the plastic material' surfaces are nonspecific and driven by hydrophobic, electrostatic and van der Waals forces. Despite the many existing models trying to describe the bacterial adhesion, the degree of hydrophobicity of the staphylococcal cell surface and that of the matching material' surface are generally considered highly important for the first attachment [12,17-22].

After this step cells aggregate, accumulate in multiple layers and form microcolonies. It follows biofilm maturation and afterwards planktonic cells can detach from the biofilm, disperse and start forming a new biofilm elsewhere (Fig. 1), likelihood in bacterial target zones [23]. In fact microbial biofilms are a breeding-ground for pathogen agents, acting as "niduses" of acute infection, and provide a huge metabolic advantage over the planktonic mode of growth [9,24,25].

Bacterial or fungal biofilms have a corrosive effect on plastic surfaces, indeed microbes manage to eat parts of the polymer away and the plastic surface appears seriously damaged, almost lacy (Fig. 2). The microorganisms' action on PU generates debris of different size, with particles ranging from few micrometers to nanometers [7,8].

Current literature mainly talk about engineered nanoparticles (NPs) in cell cultures or NPs entering organisms via inhalation, ingestion or via skin absorption [26-28]; moreover functionalized NPs injection for drug delivery in medical applications is a field rapidly developing [29]. In this work we face a new type of "non-engineered" NPs generated from the prostheses' biodestruction. Our interest focuses on debris of about 20 nm, which are more readily absorbed than bigger particles [30]. At these sizes NPs

present peculiar features: being able to cross biological membranes they are more likely to react with cells and enter bacteria (and fungi). The NPs biocompatible surface properties depend both on the charges carried by the NPs themselves [26], and on the possible shielding from the elements dispersed in the medium (bio-molecules) [31]. Nanoscaled materials can display unexpected and unusual toxicity as compared with bulk material [26,28,30,32-35].

In this work we prove that Transmission Electron Microscope (TEM), Scanning Transmission Electron Microscope (STEM) and Focused Ion Beam/Scanning Electron Microscope (FIB/SEM) are useful techniques to document the formation of bacterial and fungal biofilm on plastic materials (Fig. 1), the damages that biofilms provoke on PU, the consequent biodestruction of the polymer, resulting in the generation of NPs (Fig. 2), the presence of NPs outside and inside the bacterial cells, many of them being included in membrane vesicles (MVs), and the transitory lysis of the bacterial membrane; the observed dynamics of NPs (which electrochemical characteristics are still unknown) to approach and enter bacterial cells suggest how the uptake process works and highlight the NPs/MVs spatial organization.

Combining our *in vitro* results with knowledge from literature, we hypothesize what could happen *in vivo*, in the event of a bacterial biofilm infection of a dental prosthesis (PU). Nomadic cells detached from a mature biofilm to start a new cycle of biofilm elsewhere represent a high risk since they have PU NPs within themselves. Nomadic cells, guided by their bacterial infectiousness, could transport the PU NPs to target zones, causing biomaterial-centered infections [36] in the human body. Eventually the nanoscaled material would be more likely to travel from bacteria to their target human cells, and the NPs' ways and chances to enter various organs would be increased, constituting an augmented toxicological risk [26,28,32-35,37].

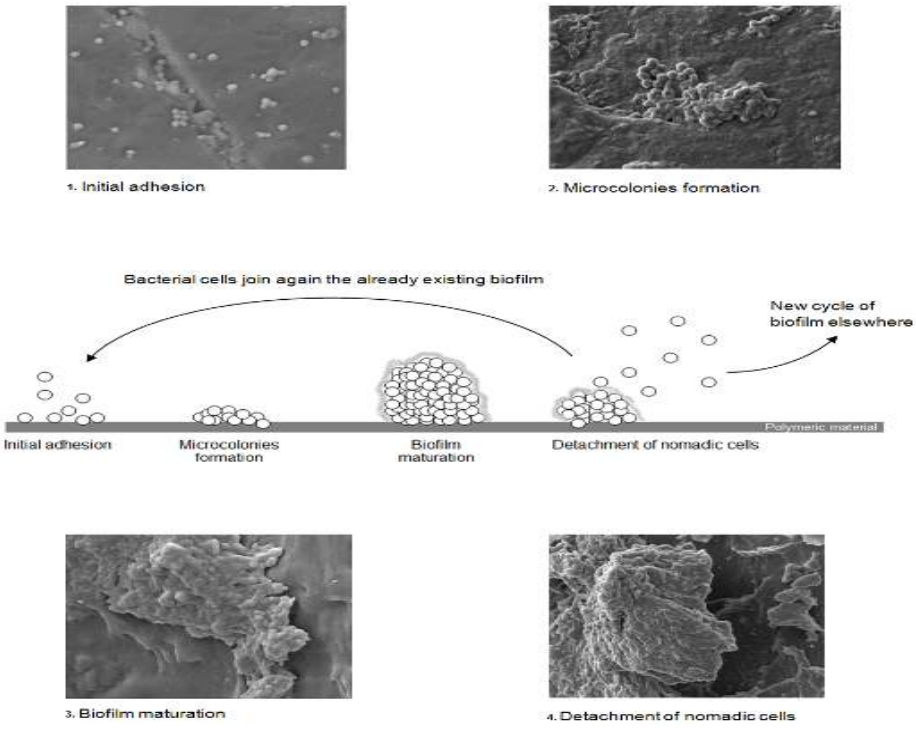

Fig. 1. SEM documentation of the four-step process of a staphylococcal biofilm formation on a polymeric surface [7,8]

1) Initial adhesion of S. aureus cells to the PU surface (HFW=25.4 µm), 2) Formation of microcolonies (HFW=23.7 µm), 3) Biofilm maturation with the presence of the exopolysaccharide (EPS) matrix (HFW=14.9 µm),4) Detachment of nomadic cells that either will join again the already existing biofilm or will initiate a new cycle of biofilm elsewhere, likelihood in bacterial target zones (HFW=28.6 µm)

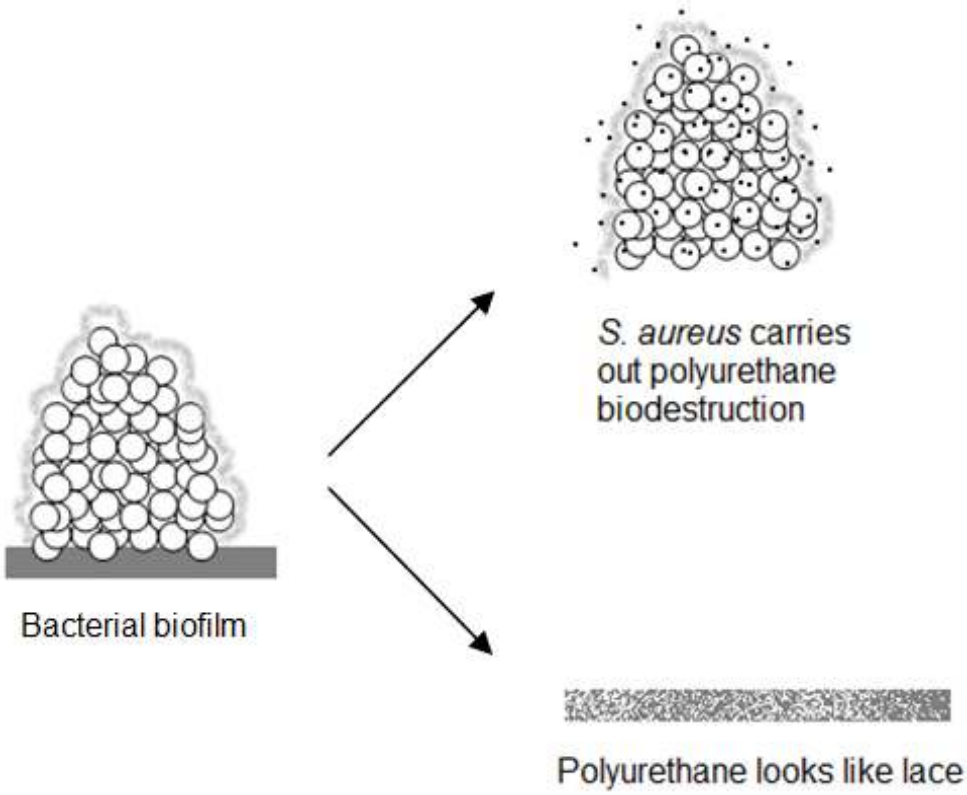

Fig. 2. Scheme of the biodestruction process carried out by bacterial cells (*S. aureus*). Bacteria, with their biodestructive action, corrode the PU surface, making the polymeric material lacy. In the same time PU NPs are visible both around and inside the bacteria [7,8]

2. MATERIALS AND METHODS

Samples of PU used for oral prostheses (Dentalur Russia, Russian Federation) with various types of surfaces (smooth, rough) were analyzed. As described in refs. 7 and 8 the culture of *Staphylococcus aureus* was isolated from a patient with a periodontal disease and was incubated with PU slices of different roughness. Control samples were a PU slice with a non-sawed surface in broth and a broth with *S. aureus* with no PU.

PU samples and bacteria were incubated from 1 to 45 days at 37℃. After centrifugation (6000 rpm for 10 mins) the bacterial pellet was placed on a thin silicon substrate for the FIB/SEM observation or prepared for the TEM/STEM analysis. Samples were prepared either for conventional SEM or TEM. Each sample was processed according to the following fixation procedures: aldehyde primary fixation, osmium postfixation and staining with lead citrate.

More precisely:

- Primary fixation: 1% glutaraldehyde and 0.4% paraformaldehyde in 0.1% M sodium cacodylate buffer (pH 7.4) for 2.5 hours;
- Osmium postfixation: 1% osmium tetroxide in distilled water (1 hour);
- Washing in distilled water (3x10min);
- Dehydration in a graded series of ethanol (from 30% to absolute ethanol) each step 10 mins and rinsing in acetone (3x10 min).

At this point the samples used for the FIB/SEM observation were dried, mounted on an aluminium stub and coated with a 5-nm-thick gold layer in a SPI-Module Sputter/Carbon Coater System (SPI Inc., USA).

Instead the samples observed with the TEM underwent:

- Embedment in plastic (Agar);
- Cutting with ultramicrotome;

Chemical-physical methods of drying were not applied in accordance with the traditional techniques, because standard drying operations led to structural changes of both the biofilm and the cells [38].

- Contrasting with copper sulphate and Alcian Blue in order to have a better view of the exopolysaccharide (EPS) matrix of the biofilm [39].

All preparation steps were done at room temperature.

Generally TEM is the most appropriate technique to investigate the characteristics of nanoscaled particles [40]; in our type of investigation, instead, the combined use of TEM, STEM and FIB/SEM is essential, since the user is allowed to freely search the surface of interest and to obtain images with a large range of magnification.

TEM and STEM images were obtained with a Tecnai F20 X-TWIN microscope (FEI Company, USA) equipped with a 200 kV FEG column and a CCD detector. Bright Field (BF), Dark Field (DF) and High Angle Annular Dark Field (HAADF) images have been collected to improve contrast and resolution [41], and to gain more information about PU NPs' size and their relative position compared to cell structures.

A set of detectors collected the transmitted electrons, according to the scattering angle influenced by density, thickness and atomic mass. STEM BF images contain information from all the electrons passed through the sample; STEM DF images are formed from specific diffracted beams that give orientation contrast [42]. Subtle features hidden in BF images are visible in high-contrasted and less intense DF images.

A third detector collected the electrons scattered at angles typically higher than 50 mrad (electrons derived from thermal diffuse scattering) to form HAADF images. With this last method the specimens' denser/heavier areas appear brighter allowing the extraction of information from what was usually interpreted as "background intensity" [41].

Comparing transmission techniques, STEM images had several advantages over the TEM ones. First of all the resolution in STEM is a $\sqrt{2}$ factor better than in TEM; moreover in conventional TEM imaging an objective aperture is used to select the transmitted or scattered beam in order to form BF or DF images respectively. In STEM imaging the transmitted or scattered beam is selected in an equivalent way using on-axis or annular detectors rather than apertures, since an annular detector collects more electrons than an aperture. Finally, as no lenses are used to form STEM DF images, they are less noisy than TEM DF ones.

Dual beam FIB/SEM Quanta 200 3D (FEI Company, USA) was used to observe specimens in both high and low vacuum, mostly at 5 kV electron beam acceleration. The beam penetration is related to the acceleration of the electrons: at 5 kV electron beam acceleration it is possible to get better images because of the lower signal contribution of the sample's sub-surface. The operator has less problems concerning the charge, and images have better details of superficial and subsuperficial parts [38,43-48].

3. RESULTS

Smooth and rough surfaces of PU were incubated with *Staphylococcus aureus*. After a 24 hours-long incubation on the smooth PU surface there were just single bacterial cells and/or microcolonies, whereas the processes of colonization and adhesion on the rough surface progressed much faster [7,8]. In Fig. 3 it is shown the process of biofilm formation after a 7-days-long incubation. After 45 days of incubation all the PU surfaces (both smooth and rough) were thoroughly covered by biofilm (Fig. 4).

Due to the coccal biodestructive action, damages appeared on the PU (Fig. 5), the plastic surfaces looked like lace [7,8], and micro- and nano-debris detached from the polymer were detected on the exopolysaccharide (EPS) matrix (Fig. 6).

Samples were observed with transmission microscopy, and TEM and STEM images were acquired. TEM BF image (Fig. 7) shows a *S. aureus* cell with PU NPs inside the cell within vesicles, on the cell wall not surrounded by membranous structures, and in the external environment.

The same sample of Fig. 7 was observed in the same transmission microscope using the STEM technique, and in Fig. 8 it is shown the resultant HAADF image. PU NPs have higher electron density than the cell biological components, so they appear darker than the surrounding medium in BF images (Fig. 7) and brighter in HAADF ones (Fig. 8).

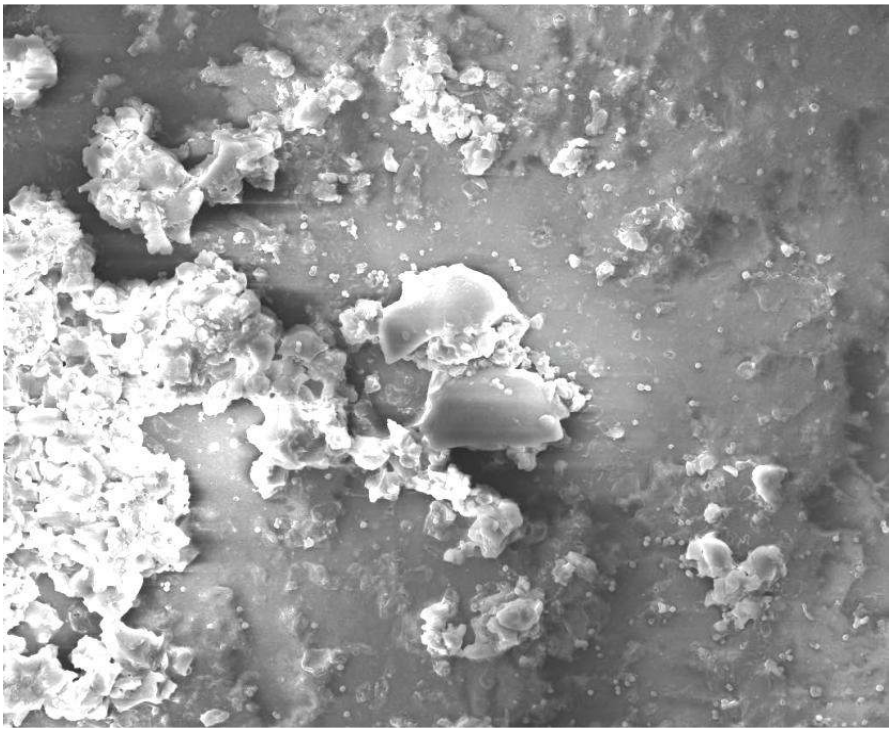

Fig. 3. SEM image of *S. aureus* cells after a 7-days-long incubation with PU (HFW=115 µm)

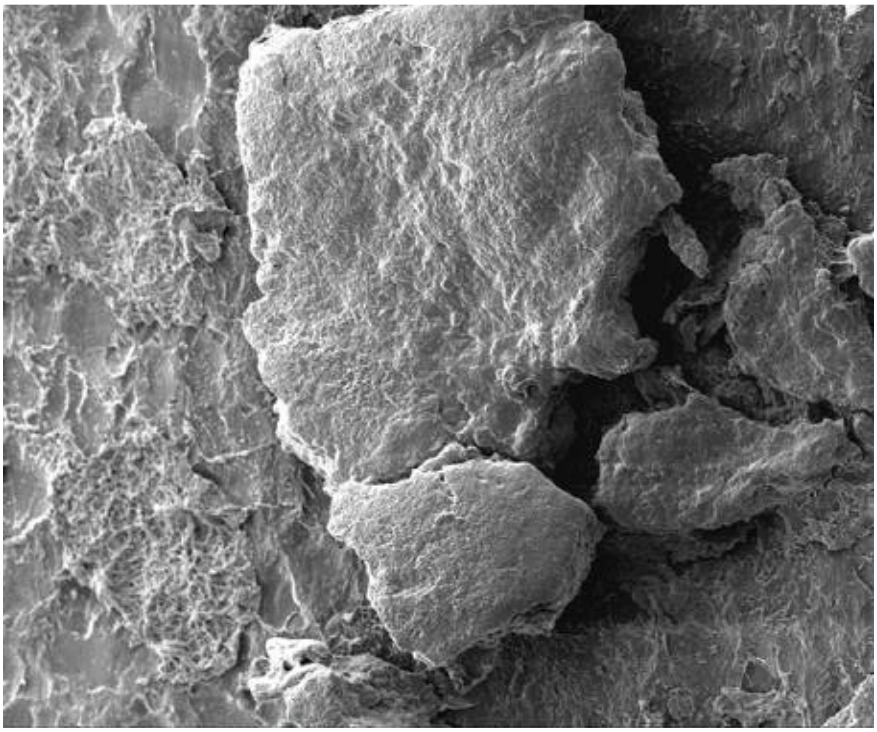

Fig. 4. SEM image of a fragment of a mature staphylococcal biofilm on PU after a 45-days-long incubation (HFW=249 µm)

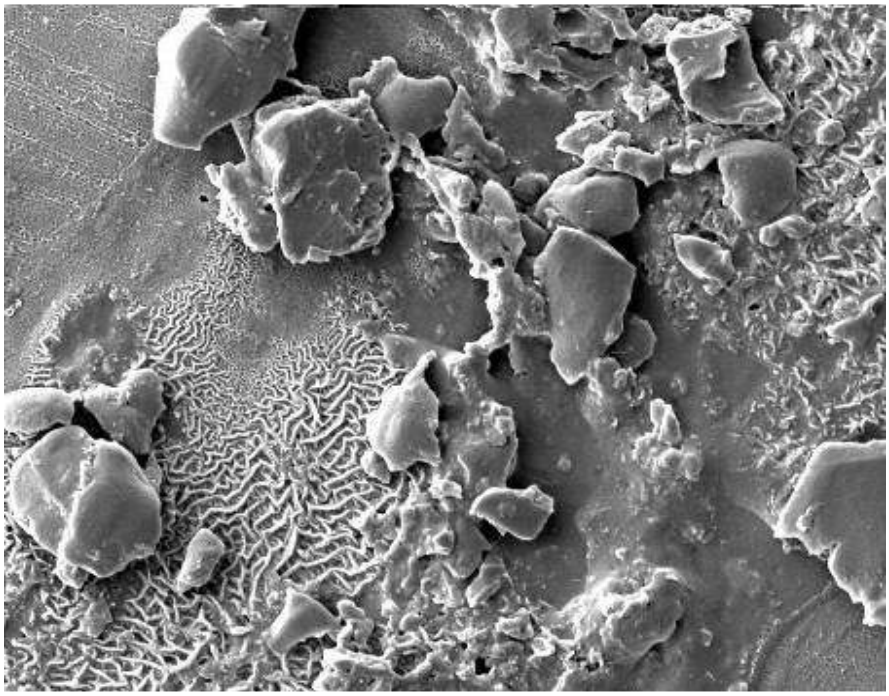

Fig. 5. SEM image of the evolution of the damage on the PU surface due to the staphylococcal biofilm after 3.5 months of incubation. (HFW=51.8 µm)

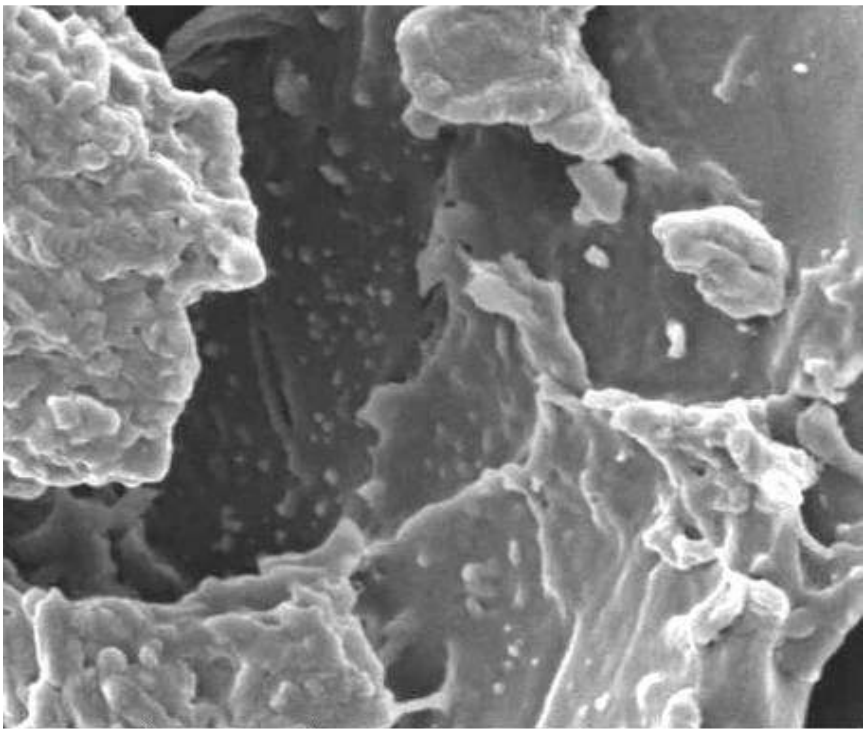

Fig. 6. SEM image of micro- and nano-debris detached from the PU surface, detected on the exopolysaccharide (EPS) matrix (HFW=19.5 µm)

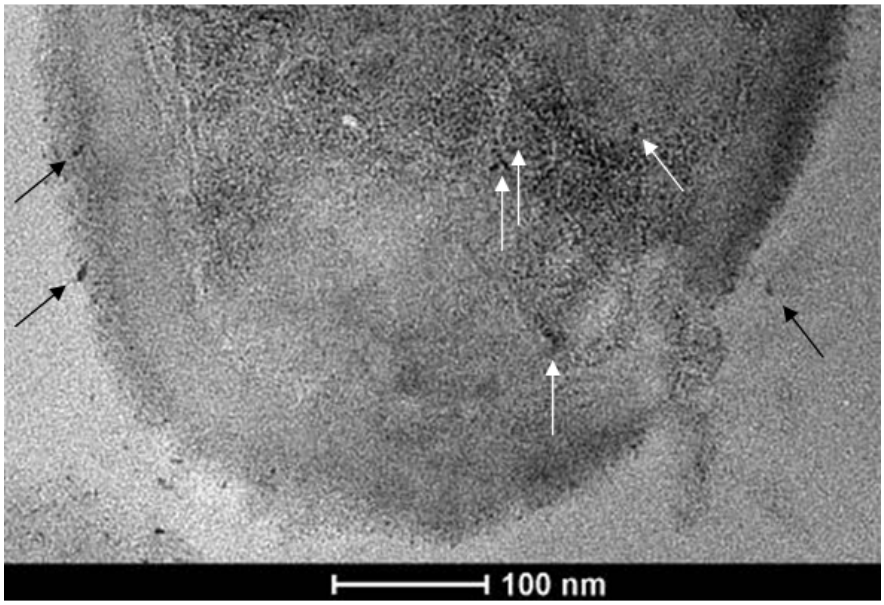

Fig. 7. TEM BF image of a *S. aureus* cell with PU particles (dark particles) detectable in the proximity of the cell wall, on the cell wall, and inside the bacterium within vesicles (arrows). Details of the ruffle and lysis of the plasma membrane are visible

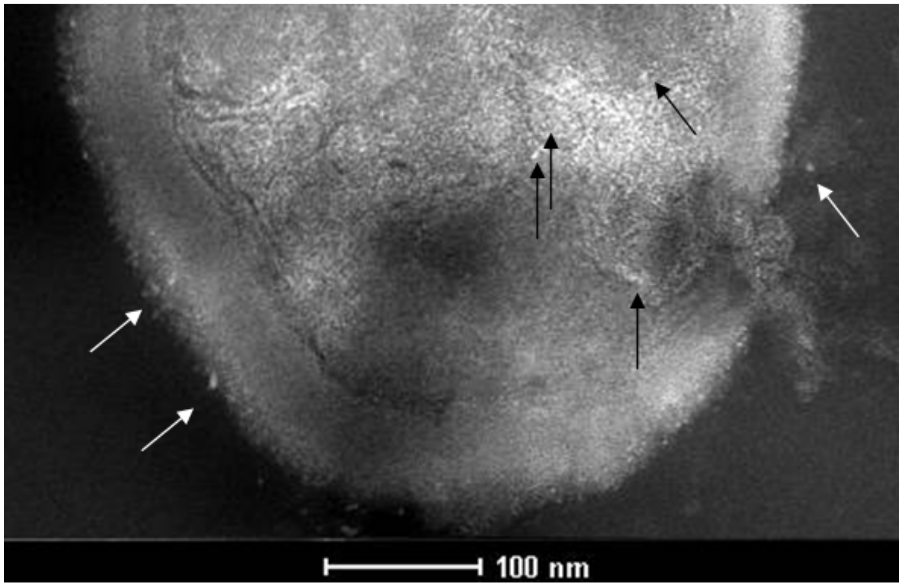

Fig. 8. STEM HAADF image of the same sample of Fig. 7. This microscopy technique gives a better view of PU particles (arrows), since they appear brighter than the surrounding medium

Analyzing Figs. 7 and 8 we recreated a scheme of the visible vesicles (Fig. 9) and their position within the bacterial cell. It is evident that vesicles follow a pre-established linear route that runs across the bacterium. In the next section it will be discussed the relation between this result and the existence of bacterial cytoskeletal structures, important not only as support for the cell and during the division phases of the cell cycle, but even as dynamic structures for vesicular trafficking.

From Figs. 7 and 8 it is also possible to see that *S. aureus* is invaded by vesicles carrying one or

more NPs (a magnification of a detail of Fig. 7 is provided in Fig. 10). Moreover PU NPs do not aggregate during the uptake process and maintain their individuality even when more than one NP is inside the vesicle. Electron images can be of help in the toxicological investigations, since we can have an idea of the dose of NPs taken up by a single cell in an *in vitro* assay. This situation, represented even in Fig. 9, opens discussions about NPs' physical features and debates about whether the biodestruction-generated nature of NPs could have any relations with the non-aggregated state on PU NPs [49].

In order to verify our findings, we analyzed the upper part of the same *S. aureus* cell as in Figs. 7 and 8. We obtained the same run down:

- PU NPs detectable in the proximity of the cell wall, on the cell wall, and inside the bacterium within MVs (Fig. 11);
- A course of PU NPs running across the bacterium (Fig. 12);
- A gathering of PU NPs in the centre of the bacterial cell (Figs. 11 and 12).

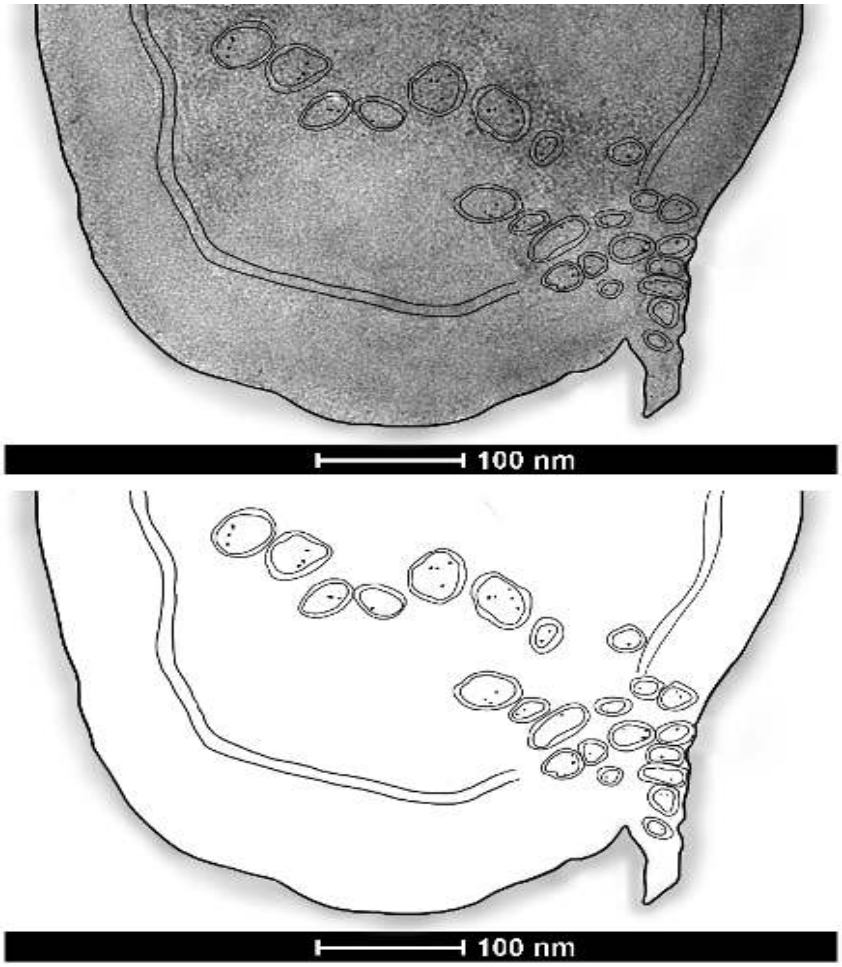

Fig. 9. Representation of the same sample of Figs. 7 and 8. Electron images (Figs. 7 and 8) could not be analyzed automatically using a software [50,51], so the analysis and the schematization were conducted integrating manual and automated procedures. This scheme highlights the vesicles' spatial distribution within the bacterial cell: vesicles seem to follow a linear route that runs across the bacterium. In this picture it is also schematized that not all the vesicles carry the equal number of NPs, in fact one or more NPs per vesicle are visible (a detail of a MV loaded with electron dense PU NPs is provided in Fig. 10)

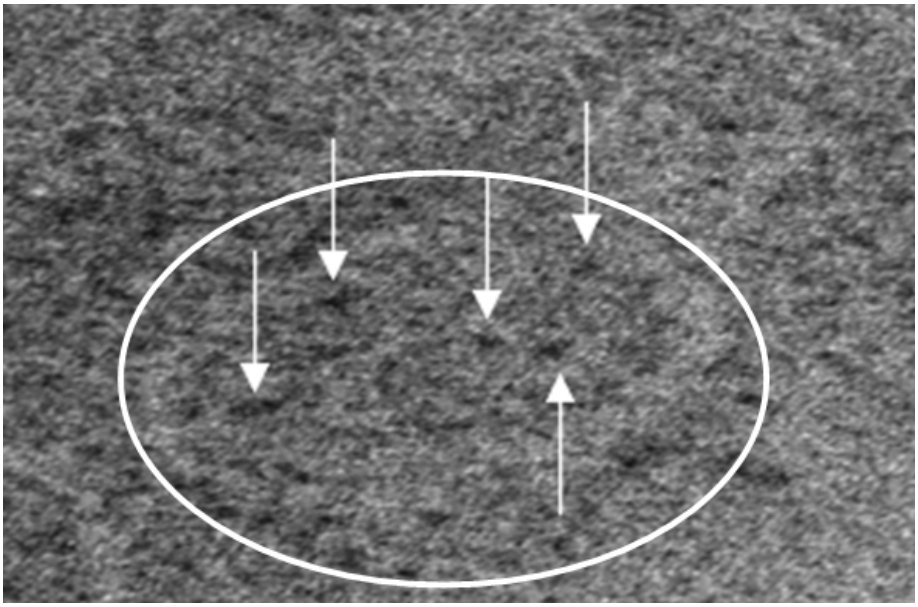

Fig. 10. Detail of Fig. 7. In this magnification it is possible to detect electron dense PU NPs (arrows) within a MV (oval) which diameter is about 30 nm

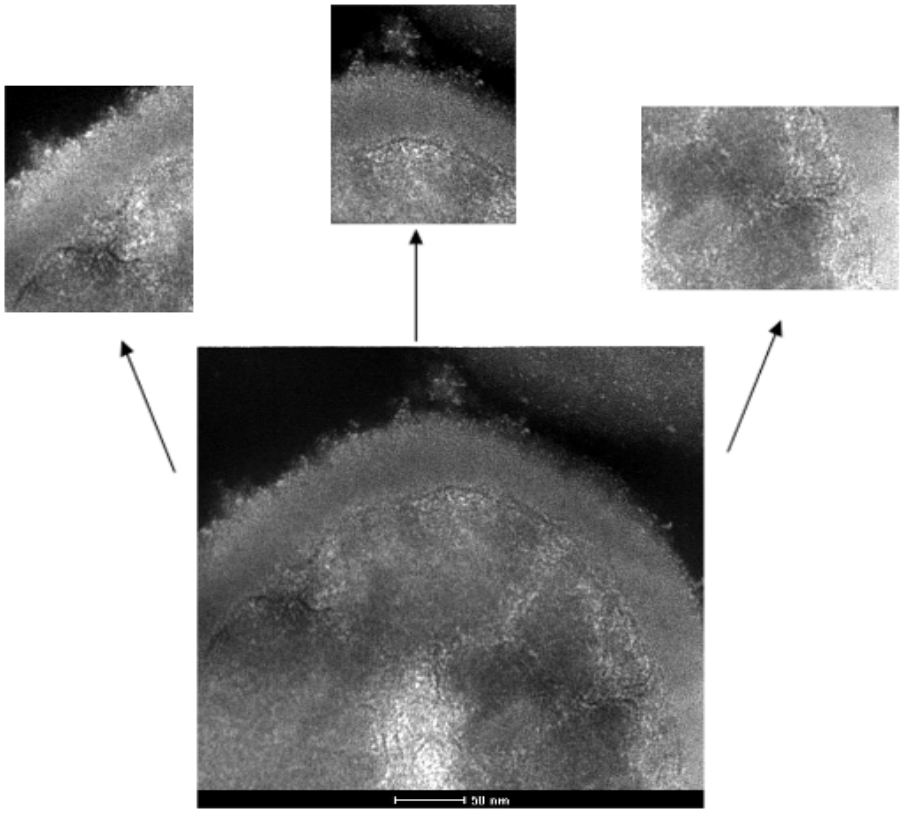

Fig. 11. STEM HAADF magnification of the upper portion of the same *S. aureus* cell as in Figs. 7 and 8. PU NPs (white particles) can be detected in the proximity of the cell wall, on the cell wall, and inside the bacterium within MVs. In the centre of the cell it is visible a gathering of PU NPs

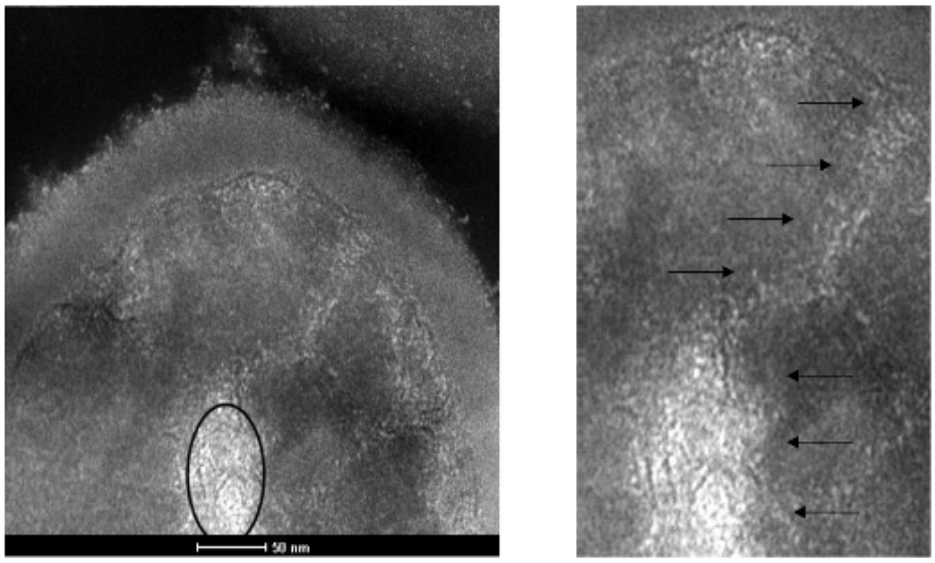

Fig. 12. STEM HAADF magnification of the upper portion of the same *S. aureus* cell as in Figs. 7 and 8. In the left image it is possible to detect PU NPs in the centre of the cell (circle) within MVs; in the right image it is shown the course of PU NPs running across the bacterium (from the outside to the centre of the cell)

4. DISCUSSION

Electron and ion correlated microscopies (FIB/SEM, TEM and STEM) are important to prove NPs generation after the plastic material's biodestruction carried out by bacteria (*Staphylococcus aureus)*; similar results can be expected also for fungi, since *Candida* biofilms are able to colonize polymeric surfaces [52,53], and can result in the biodestruction of the PU (Fig. 13).

Electron images prove the presence of bacterial biofilm on PU surfaces (Figs. 1, 3, 4 and 5). The FIB/SEM subsuperficial analysis of the PU surface allows viewing the increment of existing defects and the formation of new ones on the polymer. The plastic surface looks like lace [7], and PU particles (Fig. 6) ranging from micrometers to nanometers can be detected in the cellular environment, revealing the biodestructive potential of *S. aureus* [8].

In vitro experiments show that nanosized particles detached from the PU surface can be internalized into the bacterial cells (Figs. 7-12) [7,8,54]. It is remarkable that the presence of PU NPs does not compromise the bacterial viability; in fact ultrastructural data prove that the internalization of NPs does not prevent cell fission [7]. Bulk PU is non-toxic [10], but it is not possible to say the same thing about PU NPs. NPs behavior and effects depend on their morphology, size, surface characteristics, inner structure and reactivity. They can remain free or group together, according to the attractive or repulsive interaction forces between them [33,49,55-57]. The possible resulting aggregation strongly affects the NPs internalization dynamics. The ability of NPs to move inside the body sets a great threat when associated with the potential hazard of the nanomaterial. Further studies are necessary in order to understand whether the particles' size and/or their bioaccumulation potential are linked to any pathogenicity factor [32,58-60].

It is not known how NPs interact with the bacterium, but surely the NPs vicinity to the microorganism brings into play forces of electromagnetic origin. In literature researchers talk about a protein cover that could envelop NPs, called protein corona [61,62]; it has been shown that the NP itself, which can be positively or negatively charged as well as neutral, is able to affect the composition and maturation of the protein corona, attracting bio-molecules dispersed in the environment [31,63-66]. We are not able to see the effective presence of the corona in our images, but we must keep in mind that this is another element that modifies the NP charge [31,67-73] and its approach to the

bacterium. The bacterial membrane has a very high electric field up to 10^9 V/m [74-76]; therefore the closeness of PU NPs, with or without the protein corona, to the bacterial cell can induce electrical dipoles in NPs and consequently determine their movement [55,57].

From Figs. 7, 8, 11 and 12 it is evident that NPs in the proximity of bacterial cells do not aggregate. This could be related to the biodestruction-generated nature of the NPs that put them in a "non-engineered" class, but we cannot forget to take into account all the elements (protein corona) that likely can play a key role interfering with bacteria [49,77-79].

Polymeric NPs are internalized by the bacterial cell through endocytosis, a general term for uptake processes. Endocytosis, including pinocytosis and phagocytosis, describes the internalization of fluids, solutes, macromolecules and particles through the invagination of the plasma membrane and the scission of MVs which envelop the external material. Vesicles, freely moving or actively transported along or by cellular structures, can assume different names (early endosome, late endosome, lysosome, endolysosome, exosome) [80-83]. TEM and STEM images show that NPs within the bacterium are surrounded by a MV which diameter is about 30 nm (Figs. 7, 8, 10-12).

Endocytosis is a well known process in the eukaryotic domain [84], but debate is intense about prokaryotes [85]. Since the mechanisms of endocytosis and the roles of the different molecules involved are complicated, it is necessary a general view of how endocytosis works for eukaryotes and prokaryotes. The cytoskeleton has an important role in the endocytic process of both eukaryotes and bacteria [86], not only during the internalization of molecules and particles, but also in the vesicle trafficking within the cell. Cytoskeletal structures have been studied in the budding yeast *Saccharomyces cerevisiae*. Studies on the eukaryotic microorganism have been useful to clarify the complex structure of cytoskeleton, the role of molecular homologues of actin, tubulin and intermediate filament, the involvement of the actin homologue in internalization and post-internalization events, and the sorting of endocyted material into the recycling vacuolar pathways [87].

Bacteria were thought to lack cytoskeletal filaments, but over the past few years bacterial homologues of eukaryotic microtubules, actin filaments and intermediate filaments have been observed in prokaryotes [86, 88-94], and studies confirmed that bacteria not only possess cytoskeletal structures but even the cytoskeleton was a prokaryotic invention [91]. Bacterial homologues of tubulin (FtsZ) and actin (MreB, ParM and FtsA) not only resemble their eukaryotic counterparts structurally, but also show similar functional characteristics. As eukaryotes use endocytic processes mainly based on actin regulation, so bacteria have actin-like molecules responsible for the preliminary steps of endocytosis (membrane curvature, invagination and vescicles formation) [95-101] and for the internal trafficking of vesicles. The actin-like molecule found in *S. aureus* is FtsA; MreB genes are found exclusively in non-spherical cell types [97,98,100,102,103].

In the electron images (Figs. 7, 8, 11 and 12) it is possible to observe a lot of vesicles, all loaded with one or more NPs, arranged along linear paths that connect the whole cell. These linear routes seem to indicate the actual existence of bacterial internal structures, important as support for the cell. Cytoskeleton and in particular microtubules are considered the dynamic spatio-temporal structures supporting vesicle trafficking [104]. Therefore electron microscopy, pointing out the vesicles' spatial distribution, provides us a direct proof of the presence of cytoskeletal structures within the bacterial cell. In literature papers support the existence of the cytoskeleton showing images of cytoskeletal structures obtained with fluorescence microscopy [95,99,105]; preparing samples using different preparation techniques and observing them with electron microscopes, one can see cytoskeletal structures in many different types of bacterial and eukaryotic cells [106-108].

From *in vitro* experiments it is shown that the microorganisms' biodestructive action leads to the generation of NPs from PU prostheses (Figs. 1-6) and snapshots of the internalization and vesicular trafficking of PU NPs within bacterial cells are provided (Figs. 7-12). Extrapolating *in vitro* results, this study suggests some hypotheses regarding NPs-cell interactions in *in vivo* dynamics and the implications in the toxicological field.

Since PU NPs we are taking into account derive from the PU biodestruction carried out by microbes and therefore are not engineered, the nanomaterial can present unexpected physical

and structural characteristics and follow unexplored pathways. Bacteria (*S. aureus*) and fungi (*Candida albicans*) studied in this work are microorganisms permanently present in the oral cavity as commensals [5]; sometimes they can be highly pathogenic, especially *S. aureus* have plenty of virulence factors, including a great ability to evade host immune defenses and to develop resistance against the most used antibiotics [109-112].

Driven by intrinsic infectious potential, bacteria loaded with PU NPs could infect numerous human host tissues provoking pathologies [113-115], releasing, in the same time, PU NPs in human host cells. Therefore NPs surrounded by vesicles within bacteria represent a new way through which PU NPs may gain access to human organs with the risk of bioaccumulation [32]. NPs could be discharged through exocytosis, and therefore exit the bacterial cell without the vesicle (little is known about the presence or the absences of the protein corona), or they could be released in consequence of the bacterial death and exit the bacterium enveloped in the vesicle [116]. Moreover TEM and STEM images show the presence of NPs stuck on the bacterial cell wall and not yet internalized (Figs. 7, 8, 11 and 12). Once the bacterium attacks the host cell, these NPs could access it with no vesicle at all [116].

The probability to find NPs surrounded or not by vesicles in human host cells [117] raises an important issues in the toxicological field since bacterial cells can act as targeted vectors of possible toxic material and could release in the body highly reactive PU NPs, eluding the immune system reaction to xenobiotic material [109-112,116,118].

Biofilm formation and PU biodestruction are not unique for *S. aureus*. In literature authors talk about the synergy of fungi and bacteria in the biofilm formation, and the clinical implications that these complex interactions would have in immunocompromised hosts [119]. For instance, denture-associated biofilms contain multiple microbial species and include *S. aureus*, *E. coli* and fungi in the genus *Candida*, most notably *C. albicans*. The aetiology of denture stomatitis is multifactorial with *Candida* infections, trauma and poor dental hygiene playing an important role; moreover plaque may serve as a protected reservoir for *C. albicans* [9].

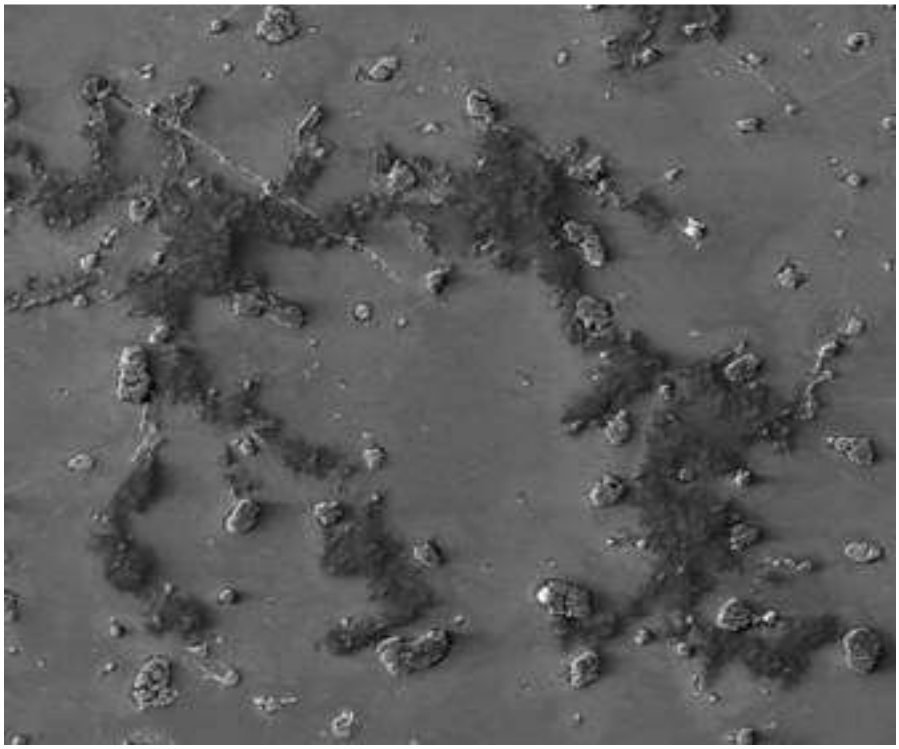

Fig. 13. SEM image of the colonization of a PU surface carried out by *C. albicans* (HFW=894 µm)

Interactions between *Candida* and *S. aureus* are involved in several functions such as coaggregation, growth stimulation or inhibition, and production of changes in candidal adherence [9].

Fig. 13 shows that *C. albicans* has an interaction with PU similar to that of *S. aureus*, and an increment of the biodestructive capacity is expected by the mutual work of both *S. aureus* and *C. albicans*.

5. CONCLUSION

Electron microscopy techniques are promising in the investigation of bacterial and fungal PU biodestruction, and in the observation of the consequently generated PU NPs. Although microscopy cannot cover all the investigating parameters, it is an effective method to study how NPs approach cells, which chemical or physical interactions lead to the plasma membrane invagination, and whether several materials with different electric characteristics carry to diversified interactions with cells, resulting in supravesicular spatial organization.

This study contributes to raise some important issues which need to be further investigated: the bacterial cytoskeletal activities, the role of the cytoskeleton in endo- and exo-cytic processes, and the transport of PU NPs within vesicles from the medium to bacteria and then to infected host cells, with related toxicological issues.

Transposing the situation seen *in vitro* to *in vivo* host organisms remains a challenge [120] and more research needs to be done in order to understand the NPs' behavior and their bioaccumulation potential in living systems at different structural levels (cells, tissues, organs), giving contributes to studies in the toxicological field [78].

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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