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## Electron Microscopy Furthers the Investigation of Bacteria-Nanoparticles Interactions Sub-Cellular Dynamics

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Analysis of the electron images can bring better comprehension of processes such as adhesion, in response to the reciprocal attraction between nanoparticles and cells, and endocytosis. Understanding the course of nanoparticles, we can suppose the existence of reversible mechanisms (exocytosis), and clear up how bacteria-host cells interactions work.

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Analysis of the electron images can bring better comprehension of processes such as adhesion, in response to the reciprocal attraction between nanoparticles and cells, and endocytosis. Understanding the course of nanoparticles, we can suppose the existence of reversible mechanisms (exocytosis), and clear up how bacteria-host cells interactions work.

Electron microscopy gives helpful answers for the research in toxicology and raises new questions about the relations between bacteria and polymeric materials.

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## 1. INTRODUCTION

Electron microscopy is a powerful mean through which it is possible to gain information at a cellular and sub-cellular level. The elements brought out by electron microscopy are not only about morphology, but regard the cellular dynamics and the roles of several structures as well. Images obtained on a Transmission Electron Microscope (TEM) can give valuable details about the interactions that occur at a cellular scale. Samples prepared and fixed, according to different protocols and procedures, are observed with the TEM, in TEM or STEM (Scanning Transmission Electron Microscope) mode. During the analysis different data collection techniques such as Bright Field (BF) and Dark Field (DF) are applied, each technique highlighting different details of the same sample's area.

After the acquisition of the images, the main purpose is to identify the distinct structures in an unambiguous and objective way, possibly automating the whole process. This could be obtained processing the micrographs with an image editing software, combining personal skills and software tools. It would mean being able to increase on a large scale the number of events examined in reasonable time, favouring the statistics which generally are complicated to get in transmission microscopy. Unfortunately this approach has severe limitations since most of the times the image background prevents the setting of parameters, key of an automatic recognition, so that all the work relies on personal abilities.

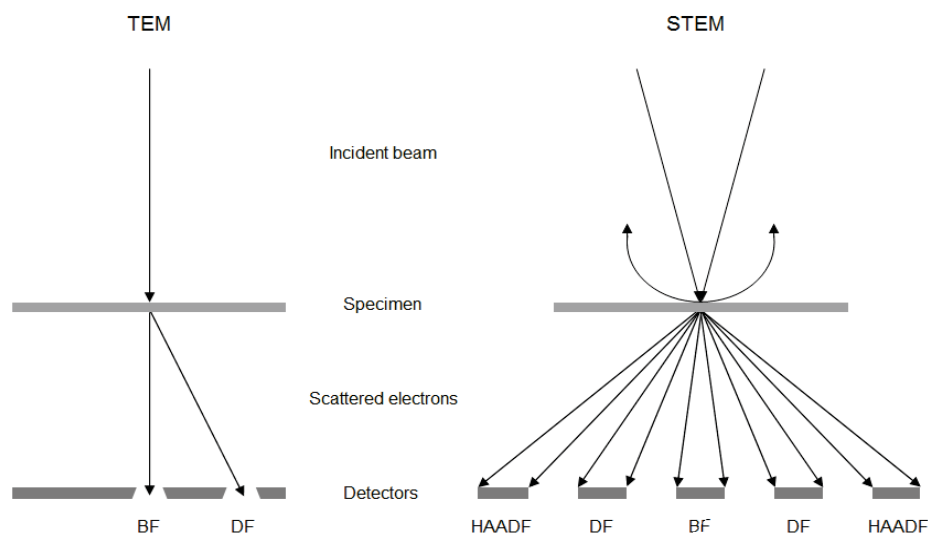
In this work we would like to show how electron microscopy is a valid tool to investigate the *in vitro* interactions between bacteria and polymeric materials (polyurethane).

*S. aureus* is a Gram positive bacterium, normally present in the oral cavity, able to operate biodestruction over polyurethane prostheses [Didenko *et al.*, 2012]. *In vitro* experiments show that the incubation of polyurethane with *S. aureus* results in the formation of a biofilm [Arciola *et al.*, 2012] and, in the last stage, in the biodestruction of the polymeric material [Didenko *et al.*, 2012; Howard, 2011; Zachinyaev *et al.*, 2009]. The starting point of biofilm formation is the bacterial attachment to the polyurethane surface [Ghannoum and O'Toole, 2004; Smirnova *et al.*, 2010; Park *et al.*, 1998]. It has been demonstrated that the rougher is the surface, the faster is the adhesion [Satriano *et al.*, 2006; Yoda *et al.*, 2014]. After a long incubation time (> 25 days) the whole surface of polyurethane (both rough and smooth) is covered with biofilm [Didenko *et al.*, 2012]. After the irreversible adhesion of microorganisms to the plastic material, bacteria start the production of a matrix of extracellular polymeric substance (EPS) [Flemming and Wingender, 2010]. The EPS is generally composed of extracellular DNA, proteins and polysaccharides, and its functions are to protect and hold together the bacterial cells and to

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**Figure 1 :** The scheme shows how differently the incident beam works and how signals are collected in Transmission Electron Microscope (TEM) and Scanning Transmission Electron Microscope (STEM). When an electron beam hits a sample, electrons are scattered to different angles. Bright Field (BF), Dark Field (DF) and High Angle Annular Dark Field (HAADF) images are acquired collecting different signals

promote the communication among them [Bhattacharyya and Choudhury, 2008]. The following step is the maturation of the biofilm and the formation of microcolonies [Ghannoum and O'Toole, 2004; Smirnova *et al.*, 2010]. The number of bacterial cells increases, therefore there is a shortcoming of nutrients; moreover there is an increment of the environment acidity, due to the bacterial metabolic activity. Hence the polyurethane is weakened and bacteria attack the plastic material, already deteriorated by the low pH of the environment, in order to get some source of nourishment. The polyurethane degradation operated by *S. aureus* implies the detachment of little scraps of material that range from micrometers to nanometers. This has been demonstrated removing the biofilm from the polyurethane in a FIB/SEM (Focused Ion Beam/Scanning Electron Microscope) through ultrasounds and analyzing the polymeric surface [Didenko *et al.*, 2012]. It appears deeply modified, micro- or nano-patterned, and looks lacy.

Through electron microscopy we were able to investigate that *S. aureus* can internalize the nanosized debris of polyurethane (less than 10 nm). The internalization process of the polymeric material into bacterial cells occurs through endocytosis following a general scheme. In literature several types of internalization processes are discussed with different names, but globally they present the same general features [Doherty and McMahon, 2009; Iversen *et al.*, 2011]. Thanks to the electron images we collected shots of the several steps: approach of nanoparticles to the bacterial cell, formation of a vesicle for the absorption of

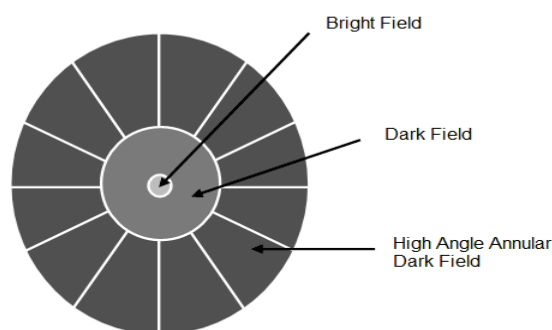
nanoparticles and englobing of nanoparticles in vesicles inside the bacterial cell. Hence the issue of the toxicity of nanosized polyurethane raises [Revell, 2006; Gatti, 2004; Hoet *et al.*, 2004], in fact the same material in the bulk form is not toxic [Howard, 2011]. Electron images point out that, as a consequence of the endocytic process, polyurethane nanoparticles accumulate into bacterial cells.

With this work we would like to draw attention to the problem of the material toxicity, since it has been demonstrated the actual uptake and storage of polyurethane nanoparticles by *S. aureus*. Moreover, considering the possible dynamics of bacteria-host cells interactions, we can suggest mechanisms of nanoparticles spreading from bacteria to host cells and therefore to an entire organism.

## II. MATERIALS AND METHODS

Bacterial cells (*S. aureus*) were isolated from a patient suffering from a periodontal disease. A part of them was incubated in a nutrient broth as a control sample; the remaining part was incubated in a broth with polyurethane. The polymeric material, provided by Dentalur Russia, had different types of surfaces. The role of the polyurethane roughness is discussed in Satriano *et al.*, 2006 and in Yoda *et al.*, 2014. The control sample of the polymer was a smooth polyurethane slice in broth. Polyurethane and bacteria were incubated from 1 to 45 days at 37°C. Samples were prepared for the observation in TEM. Details of the preparation method can be found in Didenko *et al.*, 2012 and in Curia *et al.*, 2014.

TEM and STEM images were acquired on a FEI TECNAI F20 X-TWIN (FEI Company, USA) equipped with a 200 kV FEG column and a CCD detector. Using only an



**Figure 2 :** Description of an annular CCD detector and its zones, as seen in the plane perpendicular to the incident beam

instrument for both the TEM and STEM modes, we were able to obtain two images of the very sample spot, therefore we could analyze the TEM image and the STEM one, capturing the several details that the two modes bring out. Moreover the images were collected with two different techniques: Bright Field (BF) and High Angle Annular Dark Field (HAADF). These techniques differ from the way in which electrons hit the sample and build up the images (Fig. 1). In BF modality an aperture lets only the direct beam hit the sample, blocking the scattered electrons; as a result in BF images thin areas appear brighter than the thicker ones. When an electron beam hits an ultrathin sample, most of the electrons are scattered into high angles or backwards. In HAADF modality annular detectors (Fig. 2) collect the scattered electrons up to angles higher than 50 mrad. In HAADF images denser zones (objects) which have a higher atomic number  $Z$  and thus scatter stronger, appear bright, whereas thinner areas result darker than the thicker ones [Krumeich]. With these different techniques it is possible to obtain images with better contrast and resolution.

It is important to note that, under the same conditions of signal and detector, in STEM the resolution is a  $\sqrt{2}$  factor better than in TEM. Moreover, as no lenses are used to form STEM DF images, they are less noisy than TEM DF ones [Utsunomiya and Ewing, 2003].

The images acquired have been processed with GIMP [available from <http://www.gimp.org>], an open source image editing software. Our aim was to locate nanoparticles and bacterial internal structures. We worked mostly balancing the contrasts and enhancing the edges, unfortunately, in our case GIMP did not resulted as efficient as a visual analysis, since the software was effective only in the identification of structures provided with large contours. Nanoparticles'

edges detection cannot be faced with GIMP and we were able to identify only the membranous vesicles.

### III. RESULTS

Thanks to the electron images we were able to capture some of the events that occur in the bacterial cell and its surroundings in presence of polymers.

Fig. 3 is a TEM BF image of a cell of *S. aureus* after a long term *in vitro* incubation with polyurethane. It sums up the power of electron microscopy that, with only one image, can bring out a lot of information. In this image it is possible to observe polymeric nanoparticles (derived from biodestruction) out of the cell, on the cell wall and inside the cell, enclosed in vesicles. This shows which could be the possible course of nanoparticles from out the cell to inside the bacterium. In this shot it is also visible a ruffle, a possible step in the uptake process [Doherty and McMahon, 2009], in which are present vesicles loaded with nanoparticles. It is evident that vesicles can contain one or more nanoparticles. From the image we can deduce that not all the nanoparticles around the cell (whose size can be as large as 100 nm, as seen in Didenko *et al.*, 2013) enter *S. aureus*, but only those which measure less than 10 nm. They are enveloped in vesicles whose diameters and membrane are approximately 30 nm and 5 nm-thick, respectively.

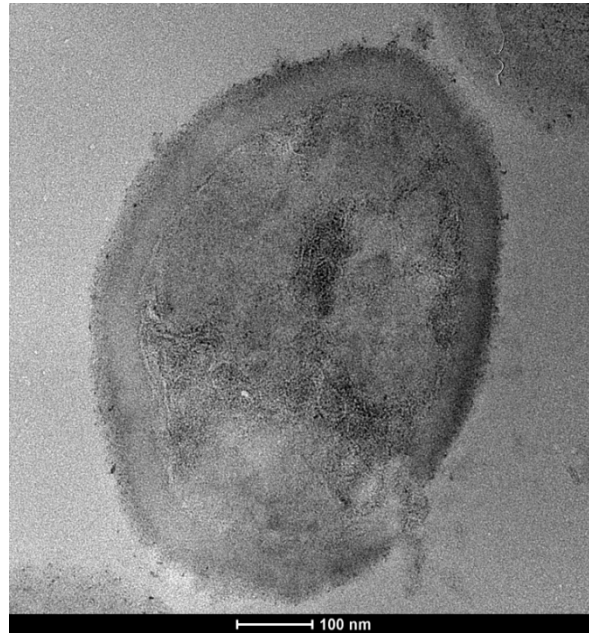
Polyurethane nanoparticles have higher electron density than the cell biological components, so they appear darker than the surrounding medium in BF images (Figs. 3, 4, 6 and 7) and brighter in HAADF ones (Figs. 8 and 9) [Didenko *et al.*, 2013].

In Fig. 4 it is possible to single the vesicles out, spotting them both in the ruffle and in the cell. We obtained this images working with GIMP on the original one, balancing contrasts and enhancing the contours of the objects, within the bacterial cell, we were interested into (vesicles).

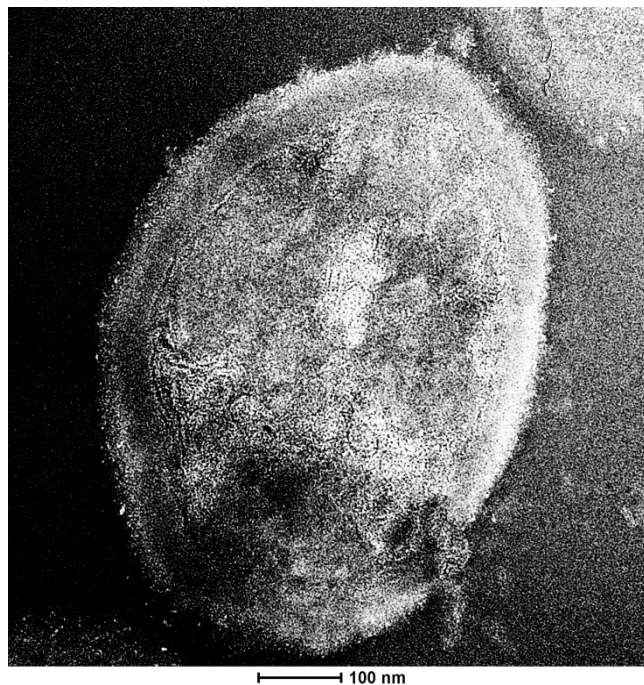
Looking at Figs. 3 and 4 in detail, one can clearly see that vesicles follow a line, as they were in a row driven by a supporting structure. In these images it is not evident what is carrying the vesicles, but the way in which they are disposed make us think that it is a cytoskeletal-like structure, probably a microtubule, whose existence and role are suggested in Amos *et al.*, 2004 and in Cabeen and Jacobs-Wagner, 2007. Moreover this points out the presence of a *continuum* of vesicles from the ruffle to the whole cell, strengthening the theses that the ruffle derives from an uptake process stimulated by the nanoparticles, and that the bacterial cytoskeleton is actually involved in the capture and internalization of the nanomaterial. The scheme of the vesicles' position is clarified in Fig. 5.

In the scheme displayed in Fig. 5 we have underlined the presence of the EPS matrix that has an important role in the approach of nanoparticles to the

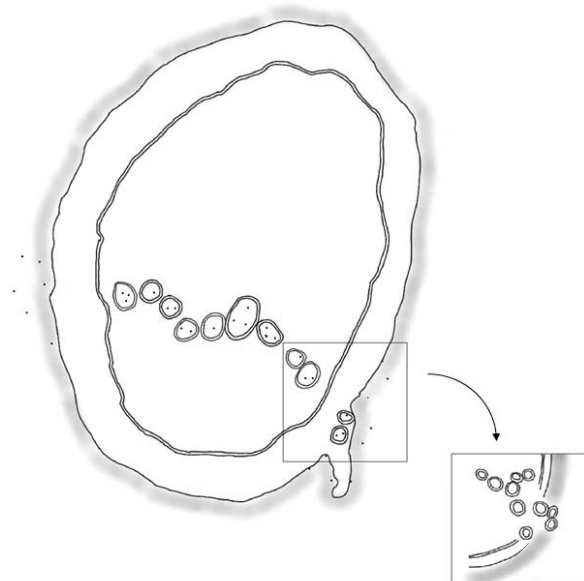
cell, in the nanoparticles coating (protein corona), and in the internalization process, where the ruffle enters in connection with the matrix during endocytosis.



*Figure 3* : TEM BF image of *S. aureus* after incubation with polyurethane. Polymeric nanoparticles can be found outside the cell, on the cell wall and inside the cell, surrounded by vesicles. Details of a ruffle and internal structures are present



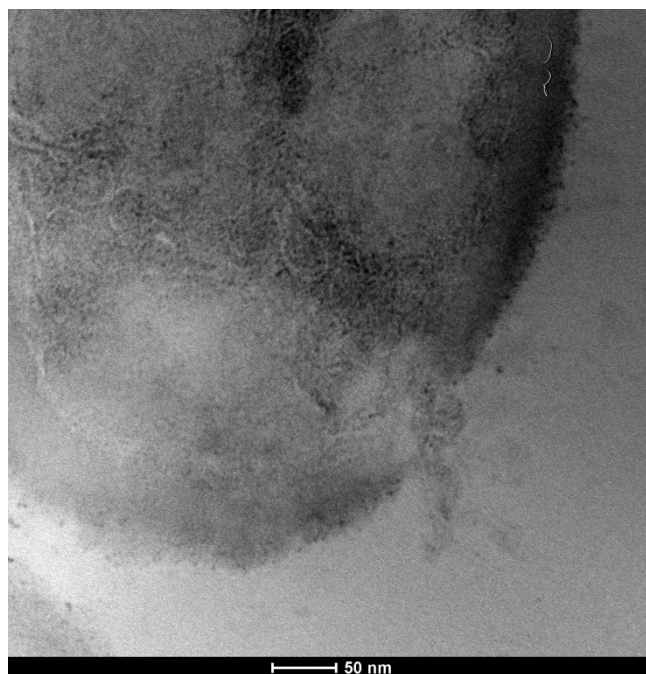
*Figure 4* : Same image as in Fig. 3 ri elaborated with GIMP in order to better display internal structures (vesicles)



**Figure 5 :** Scheme of the spotting of some of the bacterial internal structures of interest according to different dimensional scales (nanoparticles, vesicles, vesicles' membrane, bacterial plasmatic membrane). This schematization, carried out through visual analysis and mixing of Figs. 3, 6 and 8 (the last two at a higher magnification), proves the existence of a *continuum* of vesicles from the ruffle to the whole cell

A portion of the same sample as in Fig. 3, with better magnification, is shown in Fig. 6 (TEM BF) and Fig. 8 (STEM HAADF). Fig. 6 gives a focus on the vesicles that go from the ruffle and across the cell, lying

in a row; Fig. 8 offers a better possibility to observe the vesicles in the ruffle, showing in the meantime the clear contours of the vesicles inside the cell.



**Figure 6 :** TEM BF image of a portion of the same sample as in Fig. 3, with higher magnification. Details of a ruffle and nanoparticle loaded vesicles are present

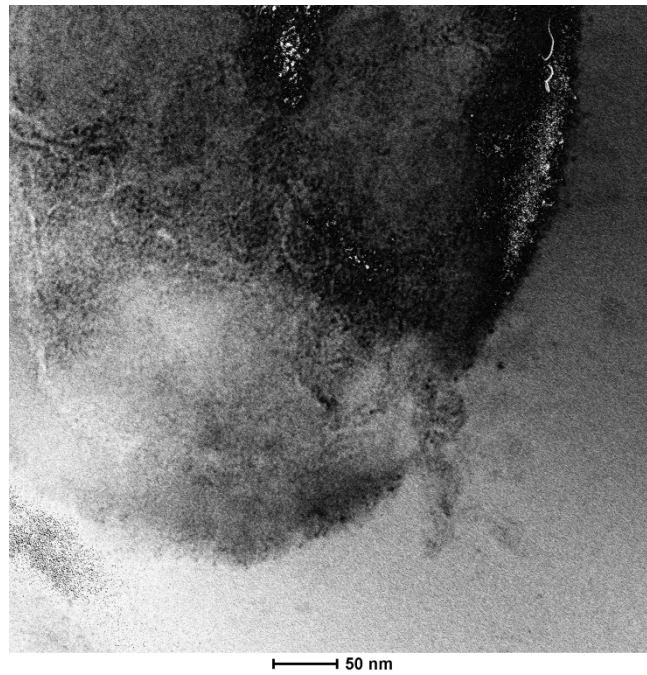
Figs. 7 and 9 show our attempts to improve the visibility of internal structures with the use of GIMP. While in these images it is possible to identify the improved edges of some of the vesicles present, we can state that

the result as a whole is not satisfying. In fact in this case the tools of GIMP do not allow to improve the identification of the internal structures, whereas a visual inspection is more efficient.

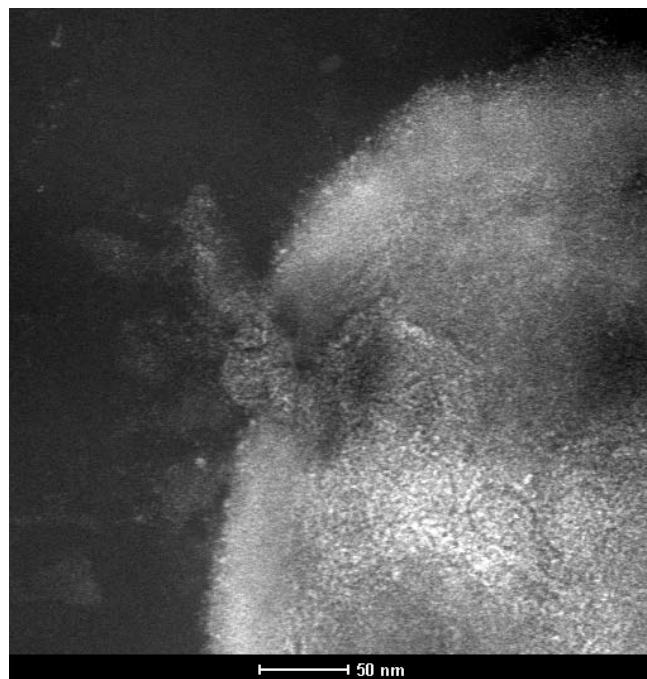
The limits of the use of editing image software are proved not only in Figs. 7 and 9 where it is impossible to clearly identify all the bacterial structures of interest, but in all of the other images as well, since GIMP allowed to focus only on objects provided with

large contours (vesicles) and not on nanoparticles where edge detection is not feasible.

The above mentioned limits prove that in our research an automatic process for structures' recognition is far from reach.



*Figure 7 :* Same image as in Fig. 6 rielaborated with GIMP in order to better display internal vesicles



*Figure 8 :* STEM HAADF image of a portion of the same sample as in Fig. 3, with higher magnification. Details of a ruffle and nanoparticle loaded vesicles are present

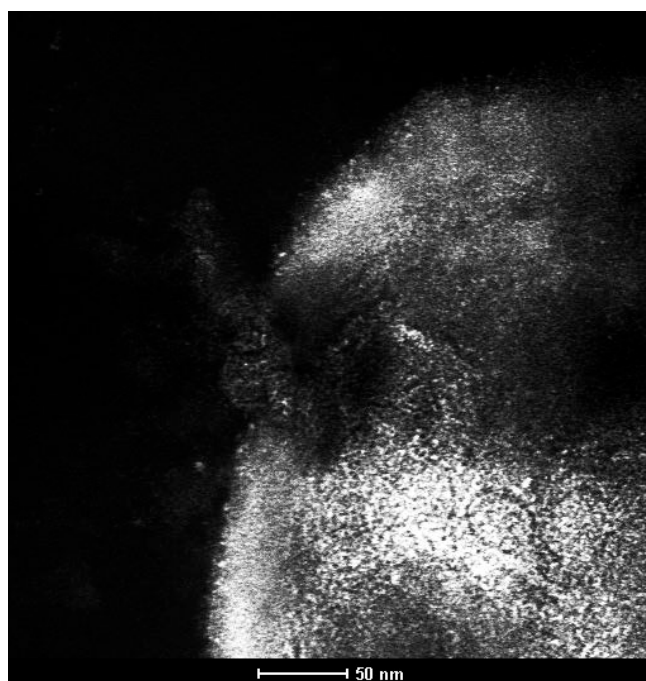


Figure 9 : Same image as in Fig. 8 rielaborated with GIMP in order to better display internal vesicles

#### IV. DISCUSSION

The images in the previous section prove the importance of electron microscopy. Electron images suggest the possible endocytic pathway that nanoparticles take from outside the bacterium to the interior of the cell. Fig. 3 is shot in a fixed time and helps us to understand the space-time cellular dynamics. It is notable that from a single image we get information about the whole course of the polymeric material. The first step is the approaching of nanosized particles to the bacterial cell. Polyurethane nanoparticles can be positively or negatively charged, as well as neutral [Urquhart *et al.*, 1995; Watanabe *et al.*, 2003]. In all cases, the proximity of nanoparticles to the bacterial membrane brings into play electromagnetic forces. In fact, when approached by charged or neutral nanoparticles, the membrane high electric field (up to 10GV/m) interacts with permanent nanoparticle dipole moments (if present) and/or induces electrical dipoles [Korobeynikov *et al.*, 2002; Pekker and Shneider, 2014; Fröhlich, 1975; Davydov, 1982; Del Giudice *et al.*, 1985; Del Giudice *et al.*, 1986; Askari'yan, 1962; Ho *et al.*, 1994]. Moreover nanoparticles can be free or enveloped by a protein corona (not visible in our images). The corona is a protein cover, probably derived from the EPS matrix, which encloses nanoparticles [Lynch *et al.*, 2009; Lundqvist *et al.*, 2008; Walczyk *et al.*, 2010; Wang *et al.*, 2013]. Thus the protein corona, while covering its inner load to the bacterial cell, modifies the electromagnetic parameters of the nanoparticles-bacteria system. The role of the EPS matrix, consequently, is not limited to holding together and protecting bacterial cells, but it plays a fundamental role

in the approach of nanoparticles to the cell and in the nanoparticles coating (protein corona).

The following step is the uptake of nanoparticles from *S. aureus*. This process occurs through endocytosis [Doherty and McMahon, 2009; Iversen *et al.*, 2011; Jermy, 2010] and starts with the folding of the plasma membrane: the bacterial membrane ruffles and the cell alters its connections with the EPS matrix. Subsequent events consist in the formation of membranous vesicles that surround the polymeric material and if present the protein corona as well, and in the actual internalization of the polyurethane into the bacterial cell. The action of the cytoskeleton components backs the whole endocytic pathway [Skrzyny *et al.*, 2012] from the ruffling of the membrane to the formation and absorption of vesicles. *S. aureus* is able to englobe the foreign nano-material as a whole, incorporating it into vesicles. The existence of a bacterial cytoskeleton [Amos *et al.*, 2004; Cabeen and Jacobs-Wagner, 2007] and its important role are supported and underlined by electron images that show vesicles laying in a row, on a linear structure that could be a microtubule. Therefore electron microscopy illustrates not only the dynamics of the internalization processes, but provides information useful in the understanding of the highly controversial issues of bacterial cytoskeleton-like structures and functions [Amos *et al.*, 2004; Cabeen and Jacobs-Wagner, 2007], trying to clear up how they are involved in endocytosis.

The absorption of nanoparticles by bacterial cells (Fig. 10) studied *in vitro* suggests that similar events could occur *in vivo* as well.

*S. aureus* is a pathogen agent capable of reaching almost all the body districts, having various

targets such as tissues and organs, and able to provoke infections. Its pathogenicity is even worse when it is associated with resistance to antibiotics [Lowy, 2000; Malani, 2014; Sansonetti, 1993].

The uptake of nanoparticles by *S. aureus* has implications in the toxicological field [Curia *et al.*, 2013]. First of all because the nanoparticles we are taking into consideration are not engineered [Kettiger *et al.*, 2013; Simkó and Mattsson, 2010], in fact we are dealing with nanoparticles derived from the bacterial action against polymers (in our case polyurethane dental prostheses). It has already been assessed that bulk polyurethane is not toxic [Howard, 2011], but it is known that the properties of the same material change when its size approaches the nanoscale. The higher surface/volume

ratio of nanoparticles compared to that of bigger particles, makes nanoparticles more readily absorbable by a cell [Revell, 2006; Gatti, 2004; Hoet *et al.*, 2004]. Moreover the uptake of nanoparticles does not affect the bacterial viability, so that *S. aureus* continues its course undisturbed [Didenko *et al.*, 2012; Didenko *et al.*, 2013; Curia *et al.*, 2013; Curia *et al.*, 2014].

Bacteria loaded with nanoparticles, while attacking the host cells (Fig. 11) of an organism following their natural path [Lowy, 2000; Malani, 2014; Sansonetti, 1993], carry nanosized particles to numerous zones of the body, acting as Trojan Horses. Indeed they hide nanoparticles and, once reached the target, deliver nanosized polyurethane adding a

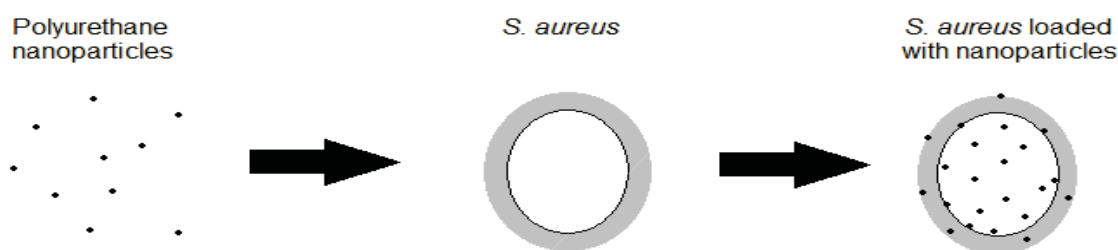


Figure 10 : Scheme of the absorption of polyurethane nanoparticles by *S. aureus*

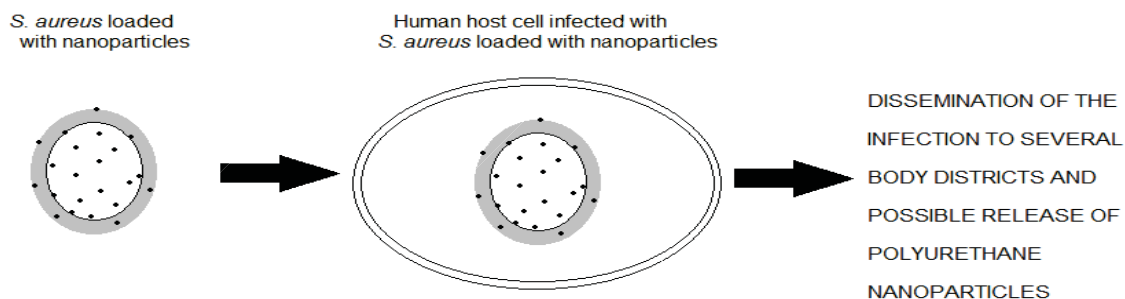


Figure 11 : Representation of the threat that bacteria loaded with nanoparticles stand for an human being

toxicological threat to the infection process [Curia *et al.*, 2013]. *S. aureus*, when in the host cell, can release nanoparticles that adhered to its outer membrane (nanoparticles with or without a protein corona, not enclosed in vesicles), can free nanoparticles without vesicles through exocytosis, or it can die loosening all its internal structures, vesicles filled with nanoparticles included [Curia *et al.*, 2013, Curia *et al.*, 2014]. The question of the existence of exocytic processes is under debate, as some researchers affirm that export processes are absent while others assume that internalization can be a reversible process [Dombu *et al.*, 2010; Salvati *et al.*, 2011].

The dissemination of nanoparticles implies that, due to the infection spreading, numerous organs (even far from one another and not directly exposed to the nanoparticles contamination) are invaded by nanoparticles [Tetrycz *et al.*, 2010]. Moreover, nanoparticles released in the body by the bacteria-host cells chain (Fig. 11) can aggregate [Kasemets *et al.*, 2013] and the possible toxic material could reach high local concentrations, while the average concentration values are below the threshold levels. In the light of this, we suggest that the dosimetry levels of nanoparticles need to be rediscussed.

## V. CONCLUSION

This work illustrates explains how much electron microscopy can contribute in the research of the consequences of biodestruction of medical prostheses operated by bacteria and in the resultant spreading of nanoparticles.

Electron images not only show bacterial internal structures and outline how they are involved in cellular dynamics, but prove the actual existence of nanoparticles uptake processes as well. All these information help to suggest unexplored paths about the nanoparticles delivery resulting from the interplay between *S. aureus* and host cells.

Being polyurethane a material commonly used in medical devices, it is of primary importance to deeply understand the mechanisms of the bacteria-host cells interactions during infectious processes, a threat always associated with implants and common to different bacteria and fungi [Tetrycz *et al.*, 2010]. While electron microscopy manages to answer a few questions clearing up some of the points, it raises important issues about the dissemination of nanoparticles to organs not directly exposed to the menace, and about the potential toxicological concentration that nanoparticles can reach locally, at cellular level, bringing dosimetry up for discussion.

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