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Abstract

Electron microscopy is useful in studying the interactions between S. aureus and polyurethane nanoparticles as good models for bacteria-polymer relations. A valuable ensemble of investigation tools allows not only to understand the cellular dynamics, but provides information about nanoparticles delivery (to host cells and consequently to tissues and organs) as well.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.

Index terms— S. aureus, nanoparticles, electron microscopy, polyurethane, biodestruction, endocytosis, bacteria-host cell interaction, toxicology.

1 Introduction

lectron 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 E 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 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.

2 II.

3 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 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. **??**). 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 ?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 **??**wing, 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.

4 III.

5 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. **??** 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 nmthick, respectively.

Polyurethane nanoparticles have higher electron density than the cell biological components, so they appear darker than the surrounding medium in BF images (Figs. **??**, In Fig. **??** 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. ?? 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. Global Journal of A portion of the same sample as in Fig. ??, with better magnification, is shown in Fig. ?? (TEM BF) and Fig. ?? (STEM HAADF). Fig. ?? gives a focus on the vesicles that go from the ruffle and across the cell, lying in a row; Fig. ?? 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 ?? : TEM BF image of a portion of the same sample as in Fig. ??, 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 ?? : Same image as in Fig. ?? rielaborated with GIMP in order to better display internal vesicles Figure ?? : STEM HAADF image of a portion of the same sample as in Fig. ??, with higher magnification. Details of a ruffle and nanoparticle loaded vesicles are present

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7 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. ?? is 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; Askar'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 nanoparticlesbacteria 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 [Skruzny 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- 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 [Teterycz et al., 2010]. Moreover, nanoparticles released in the body by the bacteria-host cells chain (Fig. **??**1) 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. ne 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; **??**alani, 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., 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 [Teterycz 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. Electron Microscopy Furthers the Investigation of Bacteria-Nanoparticles Interactions Sub-Cellular [1] [2]

Figure 1: [

[1]Electron Microscopy Furthers the Investigation of Bacteria-Nanoparticles Interactions Sub-Cellular

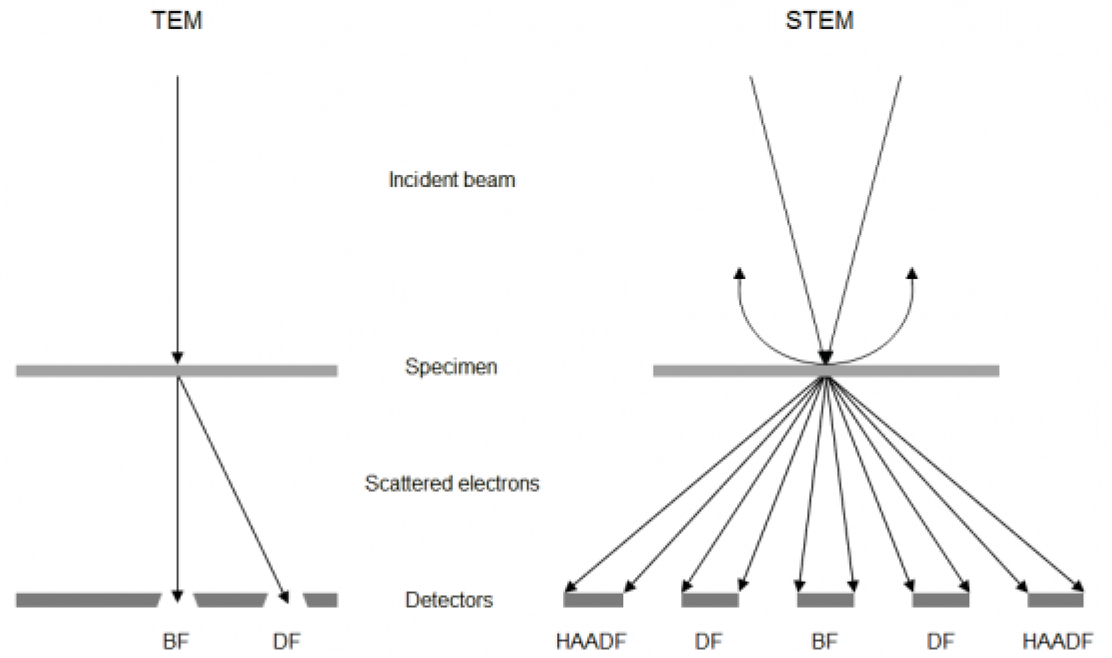

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Figure 2: Figure 2 :

Figure 3:

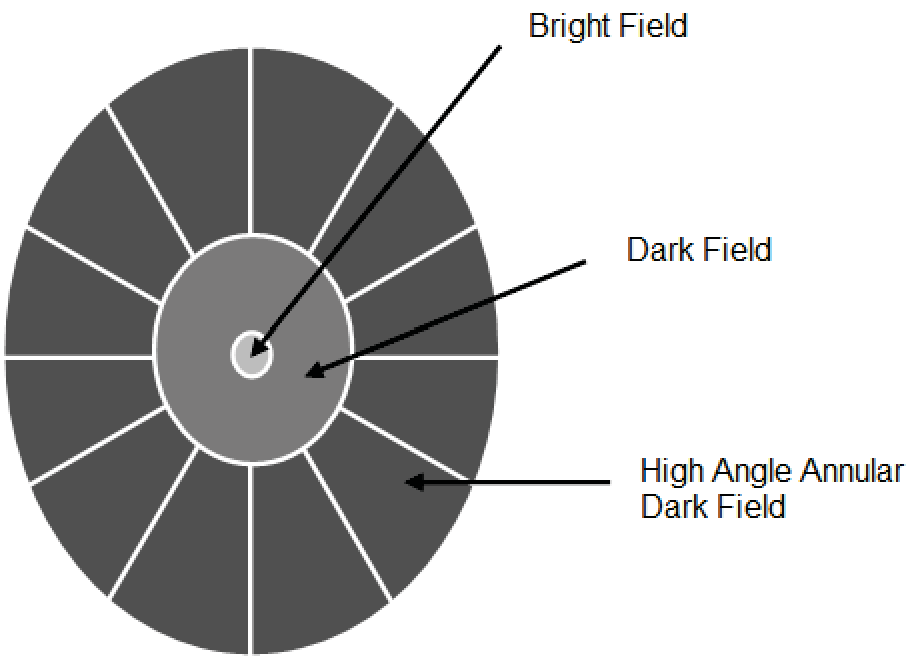

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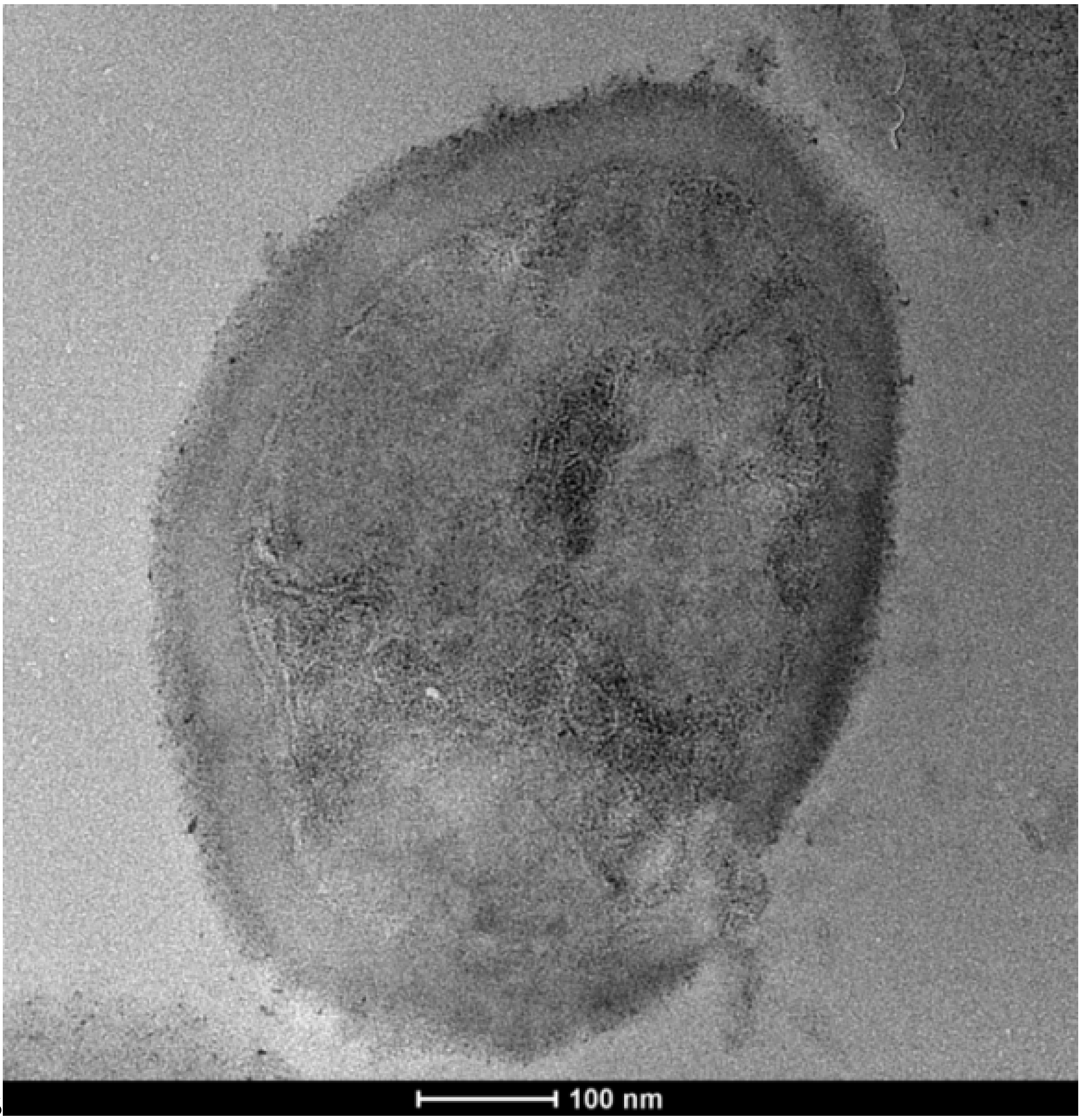
Figure 4: Figure 3 :Figure 4 :

5 

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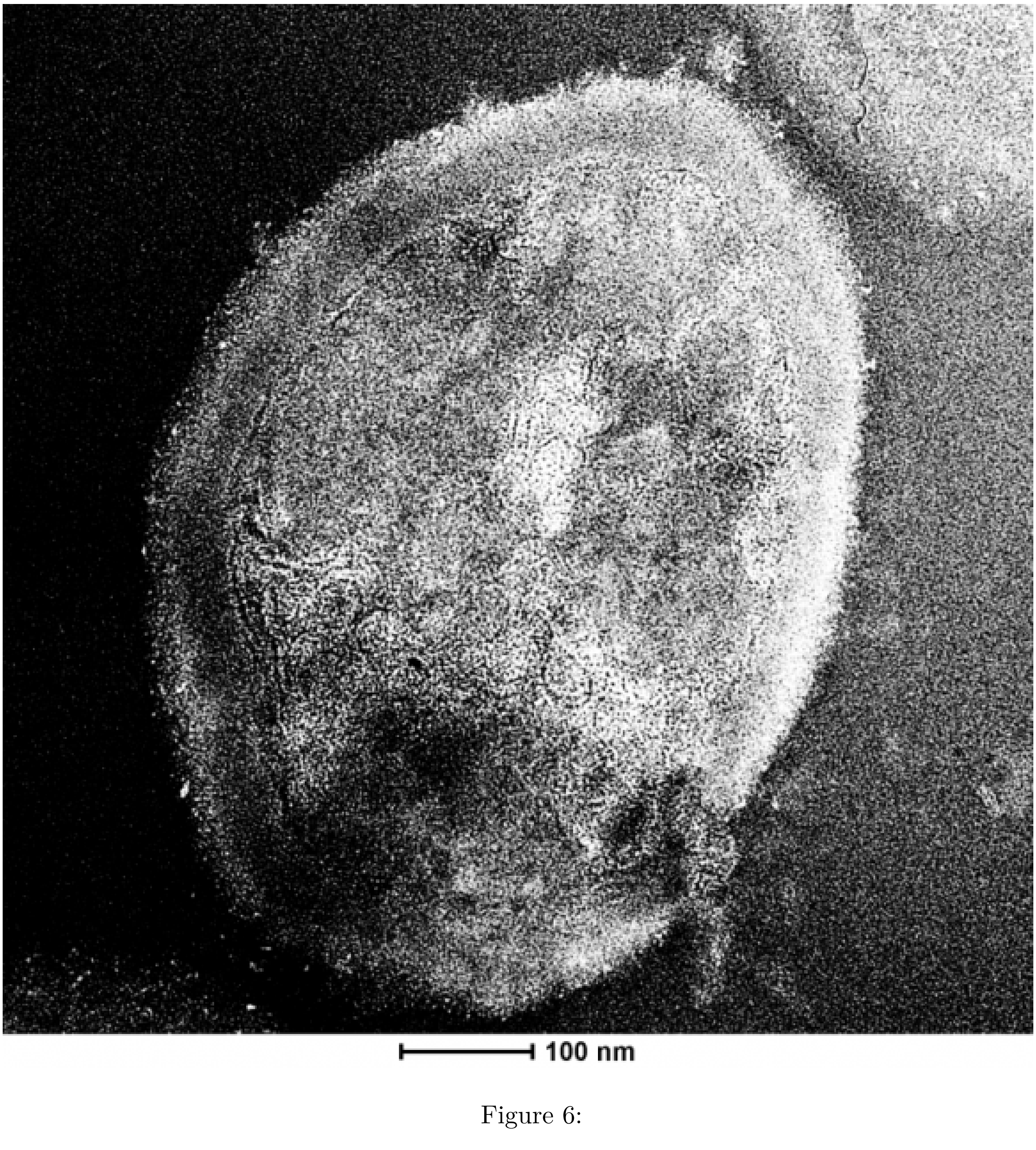

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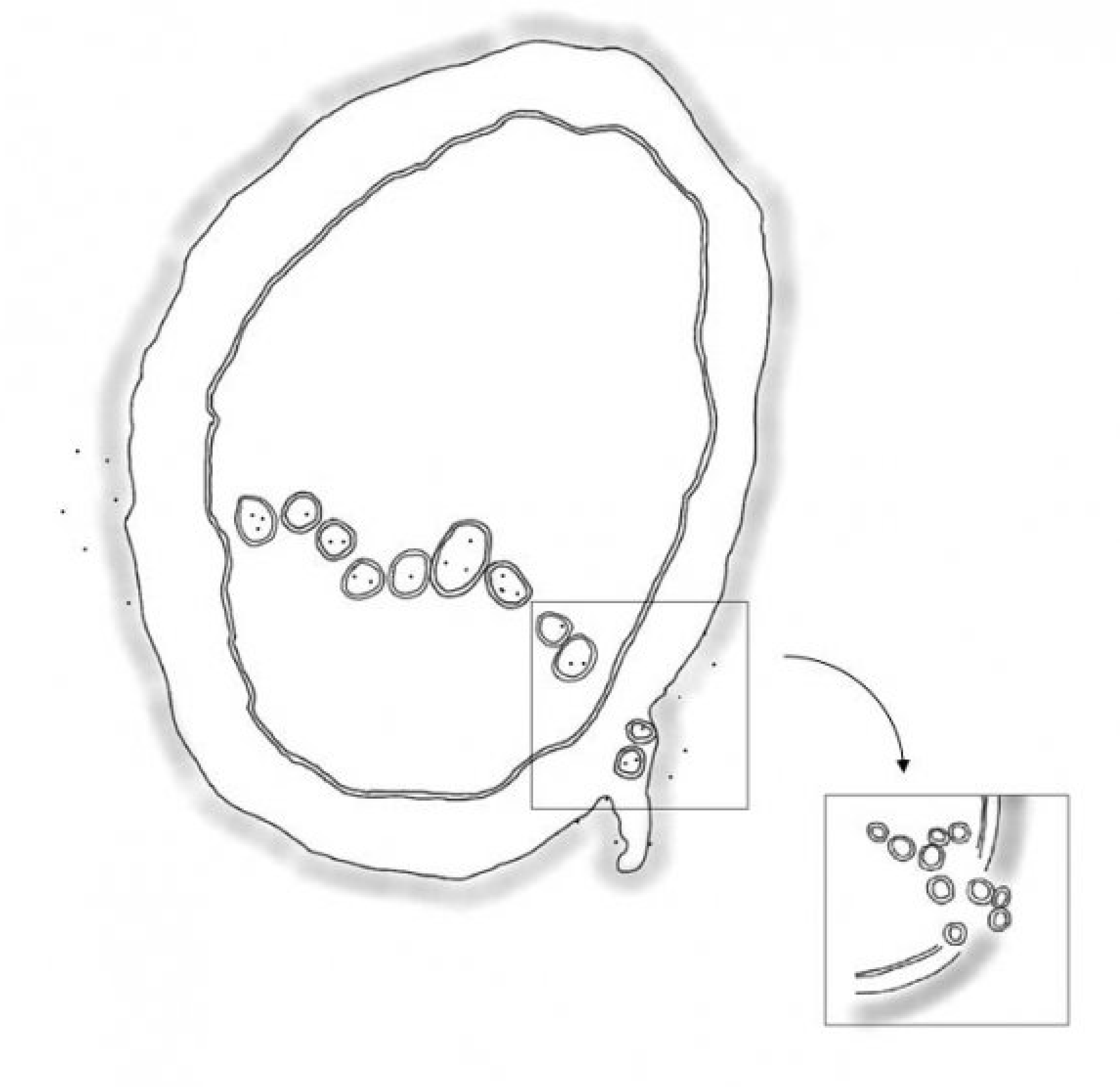

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Figure 7: Figure 9 :

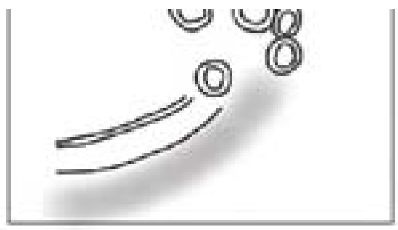

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Figure 8: Figure 10 :Figure 11 :

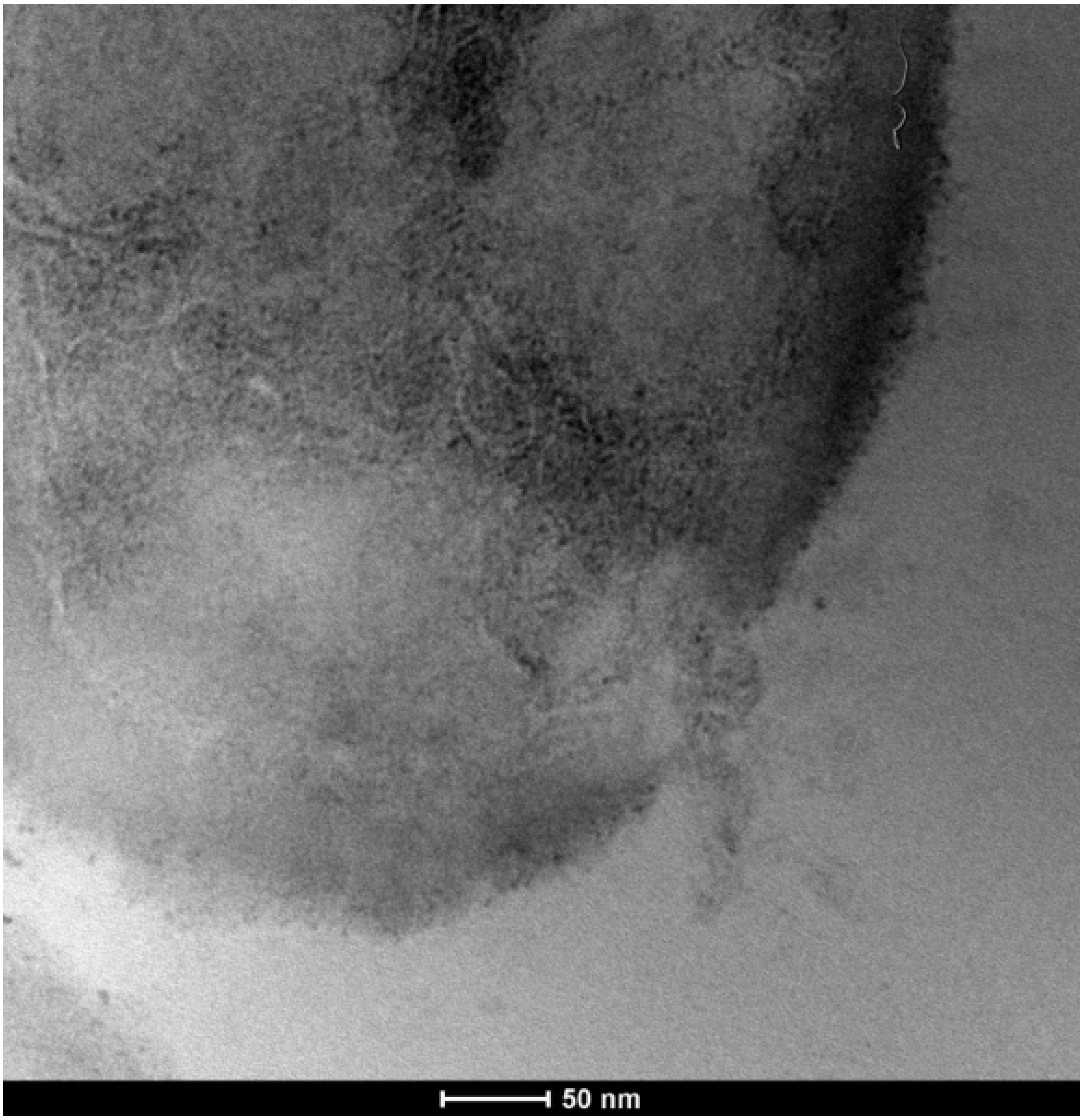

Figure 9:

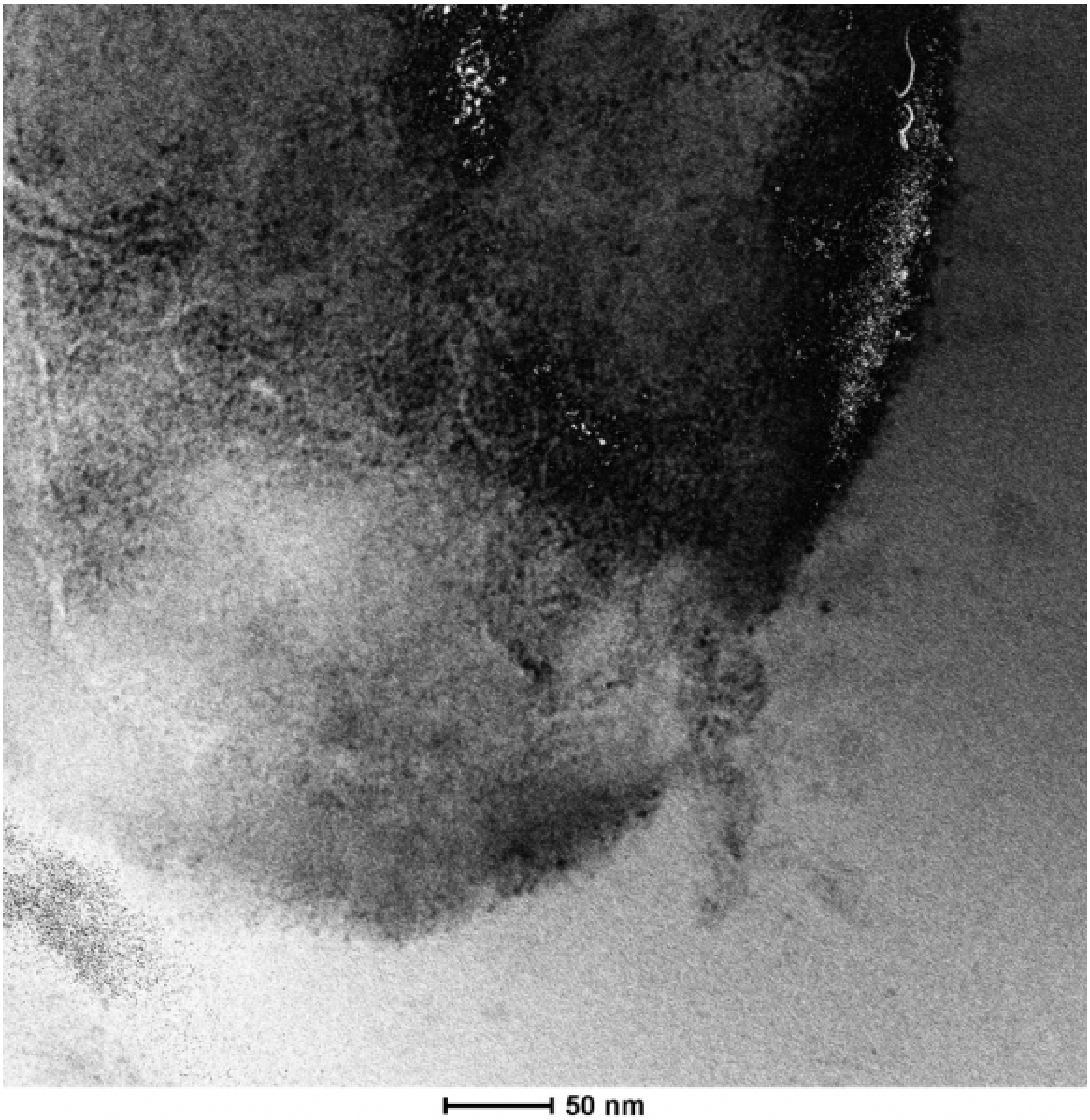

Figure 10:

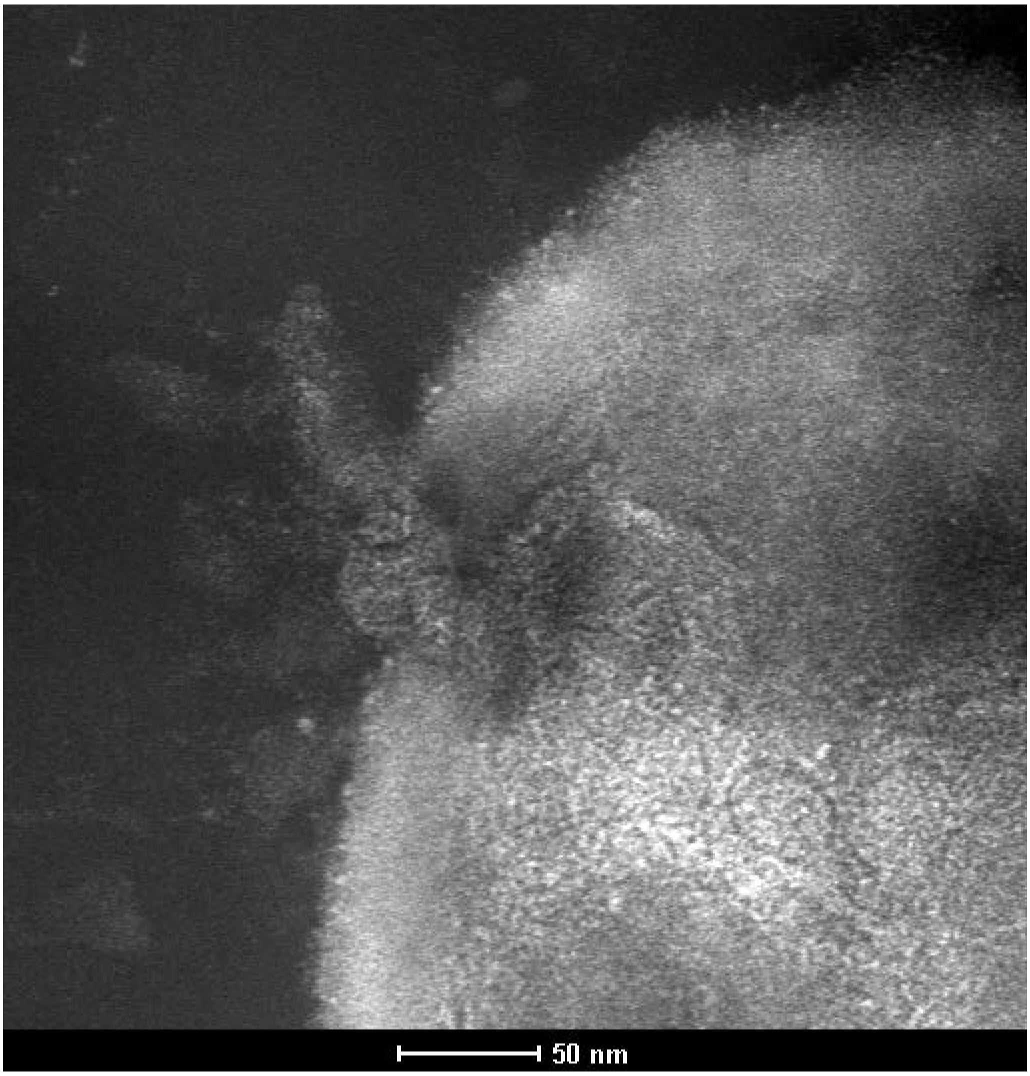

Figure 11:

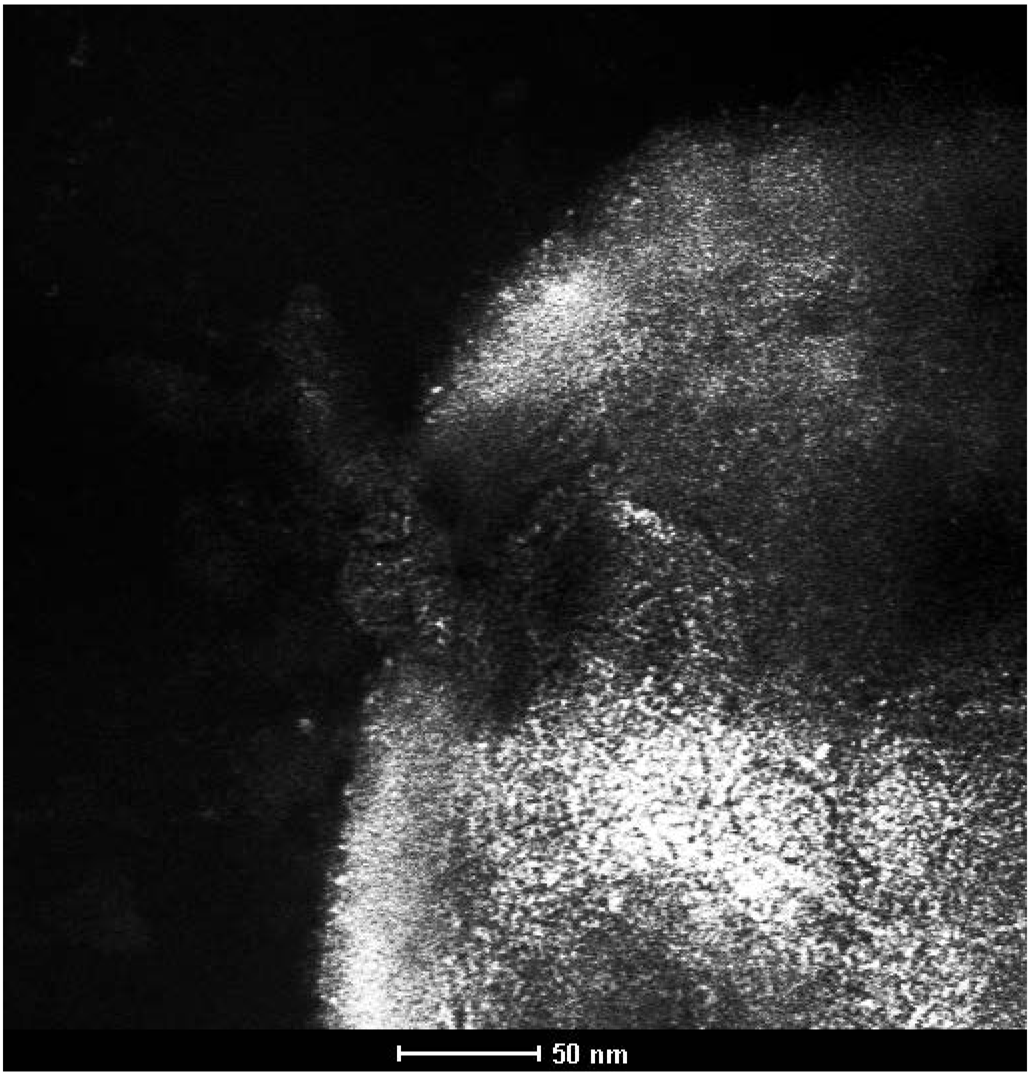

Figure 12:

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