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Scanning Electron Microscopy (SEM) and Environmental SEM: Suitable Tools for Study of Adhesion Stage and Biofilm Formation

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1. Introduction

For most of the history of microbiology, microorganisms have primarily been characterized as planktonic, freely suspended cells and described on the basis of their growth characteristics in nutritionally rich culture media. The discovery of microorganisms, 1684, is usually ascribed to Antoni van Leeuwenhoek, who was the first person to publish microscopic observations of bacteria. The direct quantitative recovery techniques showed unequivocally that more than 99.9% of the bacteria grow in biofilms on a wide variety of surfaces. Although the most common mode of growth for microorganisms on earth is in surface associated communities (Stoodley et al., 2002; Sutherland, 2001), the first reported findings of microorganisms "attached in layers" were not made until the 1940s. During the 1960s and 70s the research on "microbial slimes" accelerated but the term "biofilm" was not unanimous formulated until 1984 (Bryers, 2000). Biofilm has three-dimensional (3D) structured, heterogeneous community of microbial cells enclosed in an exopolysaccharide matrix (also called glycocalyx) that are irreversibly attached to an inert or living surface. As establish, biofilm formation has a serious implications in public health and medicine. In the case of human health, a number of microbial infections are associated with surface colonization not only on live surfaces (sinusitis, pulmonary infection in cystic fibrosis patients, periodontitis, etc. (Hall-Stoodley et al., 2004) but also on medical implants (contact lenses, dental implants, intravascular catheters, urinary stents) etc. (Donlan, 2001; Hall-Stoodley et al., 2004). Biofilms affect heat exchangers, filters, etc. because they induce biocorrosion and biofouling, producing damages on metallic surfaces and the efficiency loss in industrial set-up (Dunne, 2002; Garret et al., 2008). However,biofilms have also useful applications in bioremediation (Vidali, 2001) of different environments (microorganisms degrade and convert pollutants into less toxic forms) and biolixiviation (bacteria can efficiently dissolve minerals used in industry, to obtain copper and gold).

In order that we may gain a greater insight into the ecology of the microorganisms that exist in biofilm, it is necessary not only to be able to isolate them by traditional culture methods but also to have some understanding of the way in which these individual microorganisms interact in situ in their environment. Different microscopic techniques for biofilm monitoring including Scanning Electron microscopy (SEM) have been proved to be suitable tools in order to follow the study of adhesion stage and biofilm formation. Scanning electron microscopy as a specialized field of science that employs the electron microscope as a tool and uses a beam of electrons to form an image of a specimen allowing imaging and quantification of surface topographic features.

The scope of this chapter is to illustrate the importance of scanning electron microscopy and environnemental scanning electron microscopy in biofilm examination and control. Furthermore, although we are conscious about the vast variety of biofilms in natural, clinical and industrial environments, this chapter will mainly concentrate on imaging application of SEM and ESEM biofilms.

2. Step of biofilm formation

Planktonic cells are able to attach on the surfaces and form biofilm through a process that include several steps:

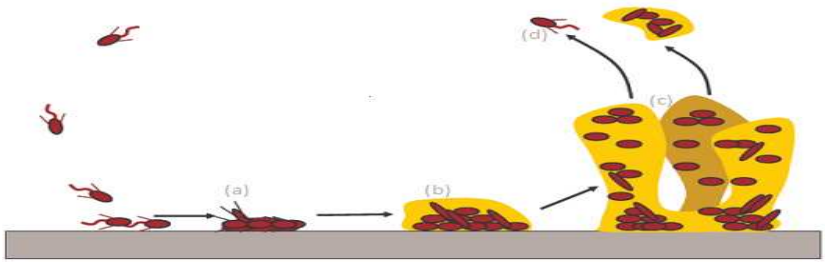

Fig. 1. Schematic illustrations of biofilm formation and development. (Filloux & Vallet, 2003).

2.1 Attachment/colonization

The primary adhesion stage constitutes the beneficial contact between a conditioned surface and planktonic microorganisms. During the process of attachment, the organism must be brought into close proximity of the surface, propelled either randomly or in a directed fashion via chemotaxis and mobility (Prakash et al., 2003). This step is reversible and it is characterized by a number of physicochemical variables that defines the interaction between the microbial cell surface and the conditioned surface of interest (An et al., 2000; Liu et al., 2004; Singh et al., 2002).

2.2 Irreversible adhesion

The second step is the irreversible adhesion during which bacteria start to express adhesion protein such as curli or fimbriae to adhere to the surface. Microorganisms starts to produce intercellular connections (intercellular curli for example) and a polymeric matrix, usually

called extracellular polymeric substances (EPS). This matrix is a complex hydrogel embedding the bacteria community and building up in three dimensions. The backbone of this gel is mainly composed of polysaccharides produced by bacteria (such as colanic acid, chitosan, alginate), other components such as enzymes, DNA, RNA, nutrients, proteins, surfactants (Flemming et al., 2007). The exact role of the matrix is not yet completely elucidated but it has been demonstrated that the matrix acts as a protective layer (Fux et al., 2005) and is microenvironment-conservative (Beech, 2004).

After the adherence of microorganism to the inert surface, the association becomes stable for micro-colonies formation (Bechmann & Eduvean, 2006; O'Toole et al., 2000). The microorganism begin to multiply while sending out chemical signals that intercommunicate among the bacterial cells. In this way, the bacteria multiply within the embedded exopolysaccharide matrix, thus giving rise to formation of a micro-colonies (Prakash et al., 2003).

2.3 Maturation of biofilm

Once bacteria have irreversibly attached to a surface, the process of biofilm maturation begins. The overall density and complexity of the biofilm increase as surface-bound organisms begin to actively replicate and extracellular components generated by attached bacteria interact with organic and inorganic molecules in the immediate environment to create the glycocalyx (Carpentier & Cerf, 1993). The maturation of biofilm generate many process already having taken place, such as quorum sensing (Nadell et al., 2008), gene transfer (Molin, 2003), persister development (Lewis, 2005) etc. All of these processes contribute to the community life of the biofilm and play an important role in biofilm survival and biofilm spreading, since they allow also detachment of biofilm parts and release of free bacteria, which is the most common way for biofilm to spread (Kaplan et al., 2003).

2.4 Detachment and dispersal of biofilm cells

As the biofilm gets older, cells detach, disperse and colonize a new niche. This detachment can be due to various factors including, fluid dynamics and shear effects of the bulk fluid (Brugnoni et al., 2007). At some point of biofilms may partially dissolve releasing cells that more away to other where a new cycle begins (Prakash et al., 2003; Singh et al., 2002).

3. Imaging application

SEM is a well-established basic method to observe the morphology of bacteria adhered on a material surfaces, the morphology of the material surface, and the relationships between them (Peters et al., 1982). SEM has been used for enumeration of adhered bacteria or tissue large number of samples. It is as a key technique that provides also information about the morphology of biofilm, presence of EPS and the nature of corrosion products (crystalline or amorphous).

3.1 SEM applied of adhesion stage

Microbial adhesion is the first step of the formation of biofilm and an extremely complicated process that is affected by many factors. In this regard, detailed investigation of microbial

adhesion involved in the developmental process from single sessile bacteria to multicellular biofilm is crucial to elaborate strategies to control biofilm development. Moreover, submicrometer-scale cell surface polymers and appendages, such as curli, flagella, and exocellular polymers, have been shown to play essential roles during cell adhesion and biofilm formation (Busscher et al., 2008; Dufrêne, 2008; Rodrigues & Elimelech, 2009). A SEM image of such a curli is depicted in Figure 2.

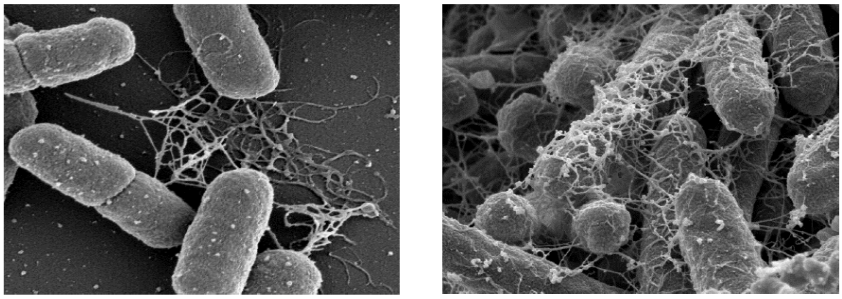

Fig. 2. SEM images of *E.coli* K-12 MG 1655 ompR234 producing curli (Olsen et al., 1989)

Adhesion phenomena has been evaluated as function of substratum, liquid medium, carbone source, pH and hydrodynamics parameters including flow rate. Many of the conclusions about biofilm development, composition, distribution, and relationship to substratum have been derived from scanning electron microscopy (Bragadeewaran et al., 2010; Herald & Zottola, 1988; Pinna et al., 2000). We report here several investigations made in our laboratory used scanning electron microscopy to study adhesion phenomena. Hamadi et al., (2005) have investigated the adhesion of *Staphylococcus aureus* ATCC 25923 to glass at different pH values using scanning electron microscopy and image analysis with the Mathlab® program is shown in Figure 3.

The surface topography has been widely discussed as a parameter influencing microbial adhesion. In this regard, experiments made by Kouider et al., (2010) using SEM to determine the effect of stainless steel surface roughness on *Staphylococcus aureus* adhesion shown that adhesion level was found to largely depend on the substrate roughness with maximum at Ra = 0.025µm and minimum at Ra= 0.8µm. Mallouki et al., (2007) have studied the anti-adhesive effect of fucans by SEM and a MATLAB program to determine the number and characteristics of adhered cells.

3.2 SEM applied of biofilm formation

Scanning electron microscopy (SEM) is a useful technique for the investigation of surface structure of biological samples (Duckett & Ligrone, 1995; Minoura et al., 1995; Motta et al., 1994). For instance, much of the current knowledge about biofilms is due to the advances in imaging studies, especially the SEM. Early microscopic techniques used in biofilm monitoring, mainly applied during the 1980s, include scanning electron microscopy. SEM has been previously used to show a clear visualization of bacteria within a biofilm and is capable of demonstrating even a single bacterium and the relation of the biofilm to the underlying surface.

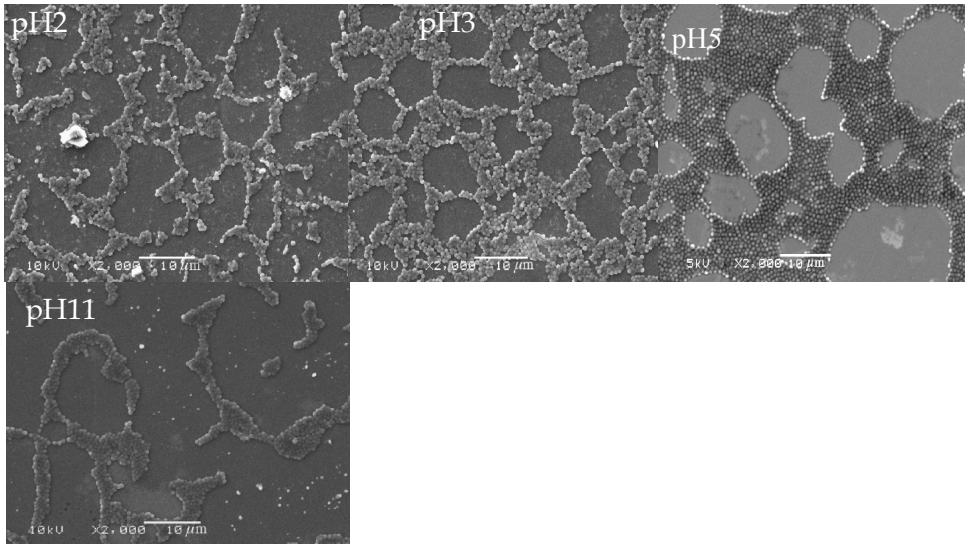

Fig. 3. SEM images of *S. aureus* adhered to glass as a function of pH (Hamadi et al., 2005)

Biofilm morphology and mass are important characteristics that control the kinetics of substrate removal by biofilms. SEM is a powerful technique for revealing the fine structure of living systems and has been applied to biofilms (Eighmy et al., 1983; Richards and Turner, 1984; Weber et al., 1978). It has also been of special importance in elucidating biofilm structure for understanding the physiology and ecology of these microbial systems (Blenkinsopp & Costerton 1991). For example, electron-microscopic studies proved that the biofilm is composed of bacterial cells "wrapped" in a dense "glycocalyx", i.e. exopolysaccharide matrix (Blenkinsopp & Costerton, 1991; Eighmy et al. 1983). In medical applications, for example, Storti et al., (2005) used scanning electron microscopy and reported that the extracellular biofilm matrix appears as an amorphous material on the catheter surface. In the same context, scanning electron microscopy (SEM) images of matrix-enclosed microbial assemblages on leaf surfaces (Surico, 1993) have led some authors to suggest that biofilms occur in the phyllosphere (Beattie and Lindow, 1995). Morris et al., (1997) have been to observe microbial biofilms directly on leaf surfaces. Bacterial aggregates in the phyllosphere have been observed previously with SEM (Surico,1993), but most have been very small (less than 20 mm long) or have lacked an obvious exopolymeric matrix (Surico,1993). Previous studies have claimed to demonstrate the presence of biofilms in situ on plant aerial surfaces using SEM (Gras et al., 1994).

Biofilm thickness is also especially important for calculation of heat exchange or diffusion rates of antimicrobials or nutrients through a biofilm and for evaluation of the mechanical properties of a biofilm (Korstgens et al., 2001). As reported elsewhere, SEM sample (freeze-dried cross-section of Foley bladder catheter) revealed the thickness of biofilm and also the layers of embedded of slime by different strains and species of bacterial cells (Ganderton et al., 1992).

In general, other application of SEM techniques may be mentioned. Akernan et al., (1993) used scanning electron microscopy of nanobacteria - novel biofilm producing organisms in

blood. Indeed, nanoscale characterization of *Escherichia coli* Biofilm formed in the glass surface using scanning electron microscopy has been reported by Lim et al., (2008). He showed reticular structures on the surface of biofilms. The reticular structures consist of nanopores having diameter ranging from 14 nm to 100 nm.

Scanning electron microscopy (SEM) is one of the many methods available for the visual the effect of antibacterial or antifungal on biofilm development (Camargo et al., 2005; McDowell et al., 2004; Sasidharan et al., 2010; Sevinç & Hanley, 2010; Zameer & Gopal, 2010; Zeraik & Nitschke, 2010). Sasidharan et al., (2010) used SEM for studied The effects of potential antifungal extracts from natural sources in *Candida albicans* biofilm (Figure.4).

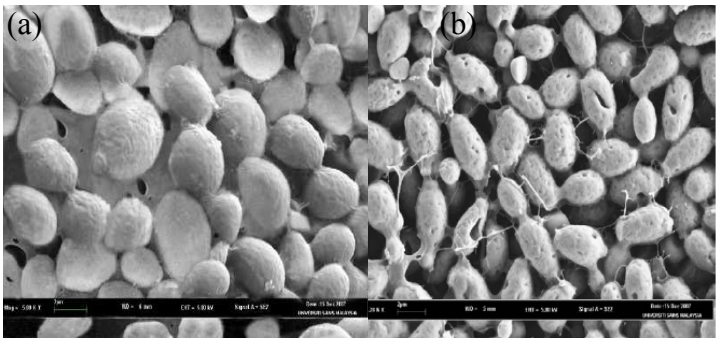

Fig. 4. Scanning electron micrograph reduction in *Candida albicans* biofilm after 36 h treatment. (a) Control and (b) Cassia spectabilis extract treated *C. albicans* cells.

3.3 Advantages and disadvantages of SEM

In part, it is true that Scanning electron microscopy (SEM) present a many advantages, the more important are: (i) higher resolution of visualization microbial biofilms (Walker et al., 2001) than other imaging techniques, typically 3.5 nm, (ii) able to measure and quantify data in three dimensions. However, this technique utilizes graded solvents (alcohol, acetone, and xylene) to gradually dehydrate the specimen prior to examination, since water of hydration is not compatible with the vacuum used with the electron beam. While any pretreatment can alter specimen morphology, drying appears to significantly alter biofilms due to EPS polymers collapsing (Fassel & Edmiston, 1999; Little et al., 1991). The dehydration process results in significant sample distortion and artifacts; the extracellular polymeric substances, which are approximately 95% water and the liquid loss led them to appear more like fibers surrounding the cells than like a gelatinous matrix (Characklis & Marshall, 1990). Several ultrastructural studies have used conventional scanning electron microscopy (SEM) to investigate the glycocalyx, but these studies (Costerton et al., 1981; Fassel et al., 1991; Marshall et al., 1971) were hampered by low resolution and also by the inability to use low voltages (<5 keV), which yield increased information from small topographical features (Pawley & Erlandsen, 1989).

Typically, SEM imaging requires a high vacuum, $\leq 10^{-8}$ Torr (reviewed in Stewart, 1985), having first been chemically fixed, dehydrated, and coated with a conductive material (e.g. gold) to prevent charge buildup from the electron beam. Few biological specimens tolerate

these conditions without rapid collapse (Heslop-Harrison, 1970) and fewer still survive (Read & Lord, 1991). Uncoated non-conductors build up local concentration of electron, referred to as-charging- that prevent the formation of usable images. Energy X-ray Spectroscopy (EDS) can be used to determine the elemental composition of surface films in the SEM, but EDS analyses must be completed prior to deposition of the thin metal coating EDS data are typically collected from an area, the specimen must be removed from the specimen chamber and coating with a conductive layer, and returned to the SEM.

To allow observations under the high vacuum conditions of SEM, many preparations of biological samples have been developed, e.g., glutaraldehyde fixation, negative staining, the Sputter–Cryo technique, and coating with gold or osmium (Allan-Wojtas et al., 2008; Hassan et al., 2003; Lamed et al., 1987). Moreover, these preparations have some positive effects on the biological sample; for instance, they enhance contrast, reduce damage, and are uncharged up by the electron beam.

4. Biofilm formation: Environmental Scanning Electron Microscopy (ESEM)

A new SEM technique is now available which allows overcoming these obstacles. a modified, low-vacuum scanning electron microscopy technique for biofilm monitoring that enables imaging of hydrated specimens, termed environmental scanning electron microscopy (ESEM) also called variable pressure SEM (VP-SEM),was introduced in the mid-1990s (Little et al., 1991). The environmental SEM (reviewed in Stokes & Donald, 2000) uses a series of pressure limiting apertures (Muscariello et al., 2005) while preventing gas leakage from the specimen chamber, which can be maintained at 1–20 Torr. The ESEM is based upon the gaseous detection device (GDD). The main feature distinguishing ESEM from conventional SEM is the presence of a gas in the specimen chamber. Gases may include nitrous oxide, helium, argon and other, but water vapour is the most efficient amplifying gas found and the most common gas used in ESEM. The ionization GDD uses the ionization of the gas for the detection of secondary electrons from the specimen surface. It is a conical electrode about 1 cm in diameter that is positioned with the apex downward and concentric with the beam at the bottom of the pole piece. Secondary electrons emitted from the sample collide with water molecules in the chamber producing additional electrons and positive ions. The positive ions are attracted to the sample surface and eliminate the charging artifacts. A proportional cascade amplification of the original secondary electron signal results. With the GDD both secondary and backscattered electron images can be produced. Detailed technical explanations about this device can be found elsewhere (Danilatos, 1990).

The balance of gas flows into and out of the ESEM sample chamber determines its pressure.

The multiple apertures are situated below the objective lens and separate the sample chamber from the column. This feature allows the column to remain at high vacuum while the specimen chamber may sustain pressures as high as 50 Torr. The temperature and humidity of the sample can also be manually controlled to provide a suitable environment for maintaining the biological samples in their natural state.

The relative humidity in an ESEM specimen chamber can be controlled (Stokes & Donald, 2000), so ESEM is particularly useful for hydrated materials (Muscariello et al., 2005; Stokes & Donald, 2000; Stokes, 2001). A gaseous secondary electron detector (GSED) exploits the gas in the specimen chamber for signal amplification. BSED operation produces positive

ions that have the added benefit of limiting charging of non-conductive specimens (Stokes & Donald, 2000). It does not require prior fixing and staining of the biofilm, minimizes biofilm dehydration and thus preserves native morphologies including surface structures (Walker et al., 2001) and native morphologies of bacteria and biofilms (e.g. Priester et al., 2007) and is able to achieve high magnifications, comparable with SEM. Shrinkage is prevented and artefact formation is reduced.

Additional advantages of ESEM include minimal processing of samples. It results in shorter time scales and lower costs while reducing the possibility of introducing artefacts. Samples can be preserved in saline in a common refrigerator (in fresh) if examination is to be deferred a few hours (Ramírez-Camacho et al., 2008). ESEM provides spatial resolutions of 10 nm or less. Compared to SEM, ESEM produces different, perhaps complementary, information for biological specimens (Doucet et al., 2005; Surman et al., 1996). Cell structures are visible with SEM, but external polymers around cells are more apparent in ESEM (Callow et al., 2003; Doucet et al., 2005; S. Douglas & D.D. Douglas, 2001).

4.1 ESEM applied of biofilm formation

Sutton et al., (1994) used this technique to study the structure of a *Streptococcus crista* CR3 biofilm. Gilpin & Sigee (1995) showed that biological samples can be imaged in the ESEM in wet or partially hydrated states with a minimum of sample damage and changes in specimen morphology. This gave the possibility to the visualization of biofilm surfaces in their natural wet anaerobic state (Darkin et al., 2001). Recently, Schwartz et al., (2009) used ESEM imaging to obtain information about the bacterial composition, matrix composition, and spatial biofilm structures of natural biofilms grown on filter materials at waterworks.

Scanning electron microscopes are frequently equipped with an energy dispersive x-ray analyser. This equipment permits elemental analysis with a high horizontal resolution of the inspected specimens. In this same context, mineral structures formed by bacterial and microalgal biofilms growing on the archaeological surface in Maltese hypogea were studied using Energy Dispersive X-Ray Spectroscopy (EDS) coupled to Environmental Scanning Electron Microscopy (ESEM), are reported by Zammit et al., (2011). These techniques have shown that mineral structures having different morphologies and chemical composition were associated with the microorganisms in the subaerophytic biofilm (Figure.5).

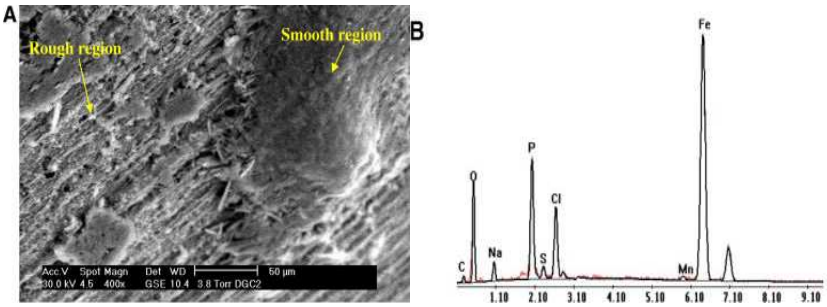

Fig. 5. ESEM and EDS analysis for the system under SRB-biofilm influence. (A) SEM Image of carbon steel exposed to sterile artificial seawater (supplemented with nutrients) and with SRB, (B) EDS analysis corresponding to the ESEM smooth region.

Interestingly, Shen et al., (2011) have been proposed a novel method for measuring an adhesion force of single yeast cell based on a nanorobotic manipulation system inside an environmental scanning electron microscope (ESEM) and Dubey & Ben-Yehuda (2011) report the identification of analogous nanotubular channels formed among bacterial cells grown on solid surface. They demonstrate that nanotubes connect bacteria of the same and different species, thereby providing an effective conduit for exchange of intracellular content.

5. Conclusion

Scanning electron microscopy is a key tool to study the effect of physicochemical properties on adhesion phenomena (pH, roughness, topography, temperature, etc). SEM plays also a paramount role for assessing the microbial populations, three-dimensional structure, physiology, thickness, etc.

SEM proved to be an invaluable method for ultra-structural investigation, allowing imaging of the overall appearance and/or specific features of biofilms formed in different environments , e.g. microbial colonies and individual cells, the glycocalyx, and the presence of inorganic products within the biofilm.

Surely, Scanning Electron Microscope (SEM) is a powerful research tool, but since it requires high vacuum conditions, the wet materials and biological samples must undergo a complex preparation that limits the application of SEM on this kind of specimen and often causes the introduction of artifacts. The introduction of Environmental Scanning Electron Microscope (ESEM), working in gaseous atmosphere, represented a new perspective in biofilm monitoring with high resolution without prior fixing and staining.

ESEM could be useful as a complementary technique to help in the characterization of the structure and architecture of biofilms. In fact, ESEM could reveal the exact topography of intact, live and fully hydrated biofilms, with a higher magnification than the other microscopy techniques. In general, a combination of several techniques is to be recommended when investigating biofilms as the different techniques offer distinctly valuable information about different aspects of biofilm development.

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