Microbial Biofilm Imaging ESEM vs. HVSEM

Complementary Information and Minimization of Artifacts

Microbial biofilm was imaged by SEM in high vacuum mode after standard preparation, and in environmental mode without any sample pre-treatment. ESEM represents an effective technique for detecting high-magnification hydrated biofilm without the introduction of dehydration artifacts, while HVSEM allows easier cell discrimination. The combination of both HVSEM and ESEM information can provide a complete description of the cellular and extracellular matrix components of the biofilm.

Introduction

Microbial biofilms (MB) are live sessile communities formed by bacteria cells irreversibly attached to a substratum, embedded in a matrix of extracellular substances that they have produced [1]. Biofilms are quite widespread in aquatic ecosystems as well as on implanted medical devices and living tissues, and are cause of concern as they are much more resistant to antimicrobial agents than their planktonic counterparts. The basic MB structural unit is called microcolony and is composed of bacteria (10 ÷ 25 % by volume) and extracellular polymeric substances (EPS) (75 ÷ 90 % by volume) [2]. The EPS polymeric matrix enclosing the cells presents a high water content (about 95 % by wt) and is mainly made of polysaccharides.

Different techniques have been employed for the morphological and structural characterization of biofilms, including electron microscopy (SEM and TEM), and confocal laser scanning microscopy (CLSM) [1]. High Vacuum SEM (HVSEM) allows high-resolution imaging of MB, but requires sample fixation, dehydration, freeze- or critical point-drying, and sputtering. These treatments can deeply affect the original biofilm morphology. Conversely, CLSM allows in-situ and in-vivo examination of biofilms but lower magnification and resolution are available.

Environmental SEM (ESEM) is a recently introduced technique that represents a powerful alternative to conventional HVSEM, as it allows the imaging of biological samples in their original hydrated conditions at relatively high resolution [3]. ESEM is based on the application of differential-pumping systems and pressure-limiting apertures, which allow the introduction in the specimen chamber of gases (e.g.
water vapor) at quasi-ambient pressure (5-20 Torr) [4]. For ESEM observation, wet samples are maintained at controlled temperature (typically 3-4 °C) by a Peltier-effect cooling stage. In such conditions, the dew point of water is lowered (5.7-6.1 Torr) and the pressure in the imaging chamber can thus be adjusted to maintain the sample hydrated. The ionization of vapor molecules induces the amplification of the low-energy secondary electrons originating from the sample and permits the neutralization of the electric charge accumulated on the surface of insulating samples, thus avoiding the need for conducting coatings.

Taking into account the peculiar characteristics of MB, this study aims to image biofilm morphology and structure in environmental conditions and to examine advantages and disadvantages of ESEM in comparison to HVSEM.

Biofilm Growth And Imaging

Three environmental strains (*Aquaspirillum peregrinum*, *Acidovorax delafieldii* and *Mycobacterium chelonae*) were isolated from a contaminated circuit for the production of microfiltered sterile water used in health facilities. An experimental flow bioreactor was specifically developed to grow microbial biofilms in dynamic low-nutrient conditions. Strain capability of producing biofilm in mono- and poly-microbial culture was assessed and the steps of biofilm growth were monitored. Glass coverslips and medical grade silicon tubes were used as reference and real substrates for bacteria adhesion, respectively. At fixed time intervals (from 5 to 17 days), biofilm-coated substrates were aseptically collected from the flow-chambers of the bioreactor. Some samples were checked for morphological identification by Gram-staining and conventional optical microscopy. The samples for HVSEM were fixed with 2.5 % glutaraldehyde in 0.1 M PBS (pH 7.2), repeatedly washed with PBS, dehydrated by ethanol-graded series, freeze-dried and gold-sputtered. Differently, no sample preparation was required for ESEM analysis: fresh biofilm samples grown on silicon tubes or glass coverslips were directly placed on the ESEM sample holder. During ESEM observations, the
vapor pressure in the specimen chamber was regulated to maintain a relative humidity ranging from 100 to 85 %. A FEG-ESEM XL30, equipped with a field emission gun, secondary, backscattered and gaseous secondary electron detectors were used for samples imaging, operating both in HV and environmental modes. All images were collected at an accelerating voltage of 9-10 kV.

**HVSEM**

The three tested microbial strains showed different tendencies to form biofilm structures, and presented different growth rates, and morphologies (fig. 1). Macroscopic biofilm features were revealed by optical microscopy in Gram-stained samples and similar patterns were imaged by low magnification SEM (fig. 2). The characteristic web-like distribution of *A. delafieldii* biofilms, clearly oriented in the direction of flow, showed the influence of the medium shear stress on MB morphology and underlines the importance of testing MB in dynamic conditions. At high magnification, HVSEM allowed the detailed visualization of sessile MB revealing the three-dimensional -micro-architecture and the distribution of single microbial cells within the microcolonies. Mature biofilm presented mushroom-like structures with external MB portions ready to detach and to colonize new clean surfaces [1-2], as well as water channels useful for supplying nutrients to the internal cells [1-2] (fig. 3).

**ESEM**

Environmental SEM provided the description of fresh biofilm morphology and allowed the imaging of both the cellular and extra-cellular biofilm components preserving the EPS in a wet state. In ESEM mode, MB appeared to be closely attached to the substrate thanks to a continuous layer of EPS. Moreover, the extension of the area covered by EPS could be precisely appreciated due to the capability of revealing thin layers of extra-cellular matrix on the substrate (fig. 4). Indeed, ESEM is particularly sensitive to the EPS surface, which is imaged as a semi-transparent coating encapsulating the bacteria. This fairly impaired the visualization of the bacterial cells, but favored the detection of the EPS amount and volume, which appeared as more compatible with the typical volume percent reported in literature [2].

**HVSEM vs. ESEM: Findings and Perspectives**

HVSEM micrographs of thick biofilm layers frequently presented artifacts as EPS collapsing, cells shrinking and cracks (fig. 5). Structural modifications in MB architecture, and, particularly, an overall loss of matrix volume were appreciable
when comparing HVSEM to ESEM images. Alcoholic dehydration and freeze-drying procedures hardly affected the MB morphology by causing the EPS matrix to collapse and by considerably decreasing the MB volume. According to Sutton et al., the comparison of different dehydration techniques showed that freeze-dried samples presented a drastic detachment of the MB from the substrate material, but also more complex dehydration procedures as critical point drying caused the almost complete disappearance of the bio-film EPS matrix [5]. Although proper fixation processes were applied, the detrimental collapse of the biofilm structure upon dehydration procedures is mainly imputable to the absence of a self-sustaining scaffold in the EPS matrix. Differently, bacteria cells preserved their shape and dimensions in vacuum after fixation and could be clearly identified by HVSEM. In ESEM mode, the semi-transparent appearance of the EPS could represent a limitation for cell discrimination. Moreover, the low signal-to-noise ratio caused by ESEM imaging of MB at high pressures results in a limitation of the actual resolution. Therefore, in case of samples requiring high relative humidity in the microscope chamber, a high-brilliance source, like that obtained by a field emission gun, is preferable.

As recently reported in literature, a procedure to improve ESEM topographic resolution in biofilms and EPS imaging or to enhance the contrast of microbial structures preserving samples hydration consists in the use of specific staining (i.e. ruthenium red, osmium tetroxide), increasing the electron density of the targeted sample portion [6]. These staining procedures allow easily discriminating polymeric matrix or having a better contrast on bacterial encapsulated cells.

In conclusion, ESEM represents an effective technique for detecting highly hydrated bacterial biofilms by preserving the massive EPS component without introducing common HVSEM artifacts. Conversely, HVSEM allowed a detailed and intuitive localization of cellular components and favored the detection of three-dimensional hollow structures, but failed in showing the actual biofilm architecture comprehensive of the large volume of EPS matrix surrounding the cells. The combined use of HVSEM and ESEM techniques can therefore provide complementary information on different biofilm components, enhancing bacterial cells and extra-cellular matrix, respectively.

References:
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