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Unravelling Mystery of Biofilm

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Abstract
Biofilms are microbially derived sessile communities characterized by the cells that are irreversibly attached to a substratum or to each other. The oral microbiota attach and grows as interactive microbial communities in the form of structurally and functionally organized biofilms. The bacteria interact, both synergistically and antagonistically. Bacteria combine metabolic forces in order to breakdown complex host macromolecules (eg, mucins) in order to obtain nutrients. Cell-cell signaling occurs using a range of diffusible molecules which facilitates the coordination of gene expression among members of the microbial community. Taking in account these considerations, here, the most commonly used, as well as some innovative methods for analytical studies on biofilm are reviewed. Understanding the complex interactions between bacteria that occur within an oral biofilm will provide insight necessary for improving diagnosis, treatment and prevention of periodontal disease. Dental practitioners should be aware of emerging diagnostic techniques and should strive to work in concert with researchers to harness new technologies for improving biofilm management.
Keywords: *Biofilm, Bacteria, Aassays, Mucins, Community, Cell siganilling.*

Introduction

Biofilms are microbially derived sessile communities characterized by the cells that are irreversibly attached to a substratum or to each other. They are embedded in a matrix of extracellular polymeric substances (EPS) produced by them , and exhibit an altered phenotype with respect to growth rate and gene transcription.[8] Within a biofilm, bacteria communicate with each other by production of chemotactic particles known as quorum sensing. [23]

Today biofilms are defined as a diverse community of microorganisms living as a structural unit, with complex communication pathways between species.[1]

Development of Dental Biofilms

The oral microbiota attach and grows as interactive microbial communities in the form of structurally and functionally organized biofilms.[16] All surfaces of the oral cavity are covered by a layer of adsorbed molecules of bacterial and

salivary origin (termed the acquired pellicle), and the initial colonizing bacteria attach to this layer. Initially, these "pioneer" species are held reversibly through weak, physicochemical interactions between charged molecules on the cell and oral surfaces. This interaction can become permanent via strong, short-range stereochemical interactions between adhesins on the bacterium and complementary receptors in the acquired pellicle.[3] The early colonizers are generally streptococci, and as they grow, they modify the local environment and make conditions suitable for colonization by more fastidious organisms. Secondary colonizers attach to receptors on these already attached bacteria (coadhesion), and gradually the diversity of the biofilm increases over time to form a multispecies community. The attached bacteria synthesize a range of extracellular polymers to form a biofilm matrix; this matrix is more than a structural scaffold because it can retain and bind many molecules, including enzymes, and so is biologically active.[15] The bacteria interact, both synergistically and antagonistically. Bacteria combine metabolic forces in order to breakdown complex host macromolecules (eg, mucins) in order to obtain nutrients. Cell-cell signaling occurs using a range of diffusible molecules which facilitates the coordination of gene expression among members of the microbial community. Thus, these oral biofilms become structurally and functionally organized, and their biological properties are greater than the sum of the individual species.[2] These biofilms are also more tolerant of antimicrobial agents and host defenses.

Methods Used For Study of Biofilms

Considering the complexity and heterogeneity of biofilm structure, the exact objective of investigation must be taken into account. The amount of extracellular polymeric substances (EPS), the total number of bacterial cells embedded in biofilm or the effective number of "living bacteria" in biofilm must be considered as different "targets" requiring different experimental approaches.[4] Taking in account these considerations, here, the most commonly used, as well as some innovative methods for analytical studies on biofilm are reviewed.

Staining Assays

Crystal violet assay - It is the first method used for biofilm biomass quantification. In nutshell, this method consists in staining negatively charged molecules by the basic dye crystal violet.[5] CV binds indifferently to negatively charged bacteria and polysaccharides of the EPS . After staining, the adsorbed CV is diluted using a solvent (e.g. ethanol or acetic acid). The amount of dye solubilised by the solvent (measured by optical absorbance at 590 nm) is directly proportional to biofilm size. The limitations of this method are related to the low reproducibility of the method, i.e.: the experimental condition of biofilm growth, the specific nature and concentration of the solvent and the dilution time are crucial steps.

Dimethyl methylene blue assay - This is a colorimetric assay used to quantify specifically *Staphylococcus aureus* biofilm using the 1,9-dimethyl methylen blue (DMMB) . In nutshell, this method is based on the consideration that the main constituent of *S. aureus* biofilm EPS is the intercellular polysaccharide adhesion (PIA), composed of poly-b-1,6-linked- N-acetylglucosamine.[21] Therefore PIA has been considered as a logical target for *S. aureus* biofilm detection. After complexation of DMMB with polysaccharides of *S. aureus* biofilm, then a decomplexation solution, was added to quantify spectrophotometrically the amount of DMMB-dye released and, in an indirect way, the amount of matrix biofilm. The main limitation of this method seems to be that it is limited to only those few bacterial species (i.e. some *S. aureus*) possessing the PIA related biofilm matrix.

Fluorescein-di-acetate assay - This technique uses the colourless fluorescein-di-acetate (FDA), a cell membrane soluble dye. After bacterial uptake, FDA is hydrolyzed by cellular esterases to

fluorescein which is fluorescent yellow. The signal can be measured spectrophotometrically. Dead cells are not able to metabolise FDA so that there is no fluorescent signal. This method has been used to quantify *Candida albicans* biofilm growth at the surface of silicone disks. Considering the limited field of examination and the thickness of biofilm, this method is not particularly suitable for quantitative studies on mature biofilm, yielding only semiquantitative results[22] .

Live/Dead BacLight assay. This method is based on the use of two different nucleic acid binding stains. The first dye is the green fluorescent , able to cross all bacterial membranes and bind to DNA of both Gram-positive and Gram-negative bacteria. The second dye is red-fluorescent propidium-iodide that crosses damaged bacterial membranes only. The stained samples are observed using a fluorescent optical microscopy to evaluate live and dead bacterial population. As matter of fact, live bacteria fluoresce in green and dead bacteria fluoresce in orange/red.[23] The main drawback of this method is the need of observing statistically relevant portion of the sample, representative of the total population

Metabolic Assays

Resazurin assay. Resazurin (7-hydroxy-3H-phenoxazin-3-one-10- oxide) is a biological dye that does not damage living cells.[7] The blue-non fluorescent resazurin also known as Alamar Blue, can be reduced by cellular metabolic activity and converted in pink-fluorescent resorufin (irreversible process) to reach to a completely reduced colourless state (reversible process). Pink-fluorescent resorufin can be measured spectrophotometrically. For these properties, resazurin has been used to detect viable microorganism in many studies on antimicrobials as well as to quantify the actual number of viable cells in biofilm. The limitation is that test is highly susceptible to bacterial respiratory efficiency which in turn is related to the growth phase, and to age and thickness of the microbial biofilm.

XTT assay. This method uses a redox indicator to enumerate spectrophotometrically viable cells in biofilm. This method is based on the observation that microbial respiratory metabolism of viable cells is able to reduce the 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide salt (XTT) to a water-soluble formazan. The number of viable bacteria in biofilm can be deduced measuring the absorbance of supernatant after the metabolic reduction of XTT . The main limitations of this method are related to the complexity and heterogeneity of biofilm structure and composition showing different metabolism gradients and to the predisposition of mature biofilm to slow down or partially retain the reduction and release of XTT and formazan, respectively.

Bio Timer assay - BTA is a colorimetric assay allowing counting viable bacteria in biofilm life-style. BTA employs a specific reagent containing phenol red. The colour of the specific reagent switches from red-to-yellow. The time required for colour switch of BTA specific reagent is correlated to initial bacterial concentration. Therefore, the time required for colour switch determines the number of bacteria present in the sample at time zero through a genus-specific correlation line. Noteworthy, BTA does not require sample manipulation. The main limitation relies on the difficulty in applying BTA for the evaluation of multispecies biofilm.

Genetic Assays

Polymerase chain reaction assay - is widely used as diagnostic method. In biofilm, this method allows to identify efficiently the presence of specific genetic sequences related to individual bacterial species.[6] Moreover, due to its high sensitivity, false positive results can be expected from natural contamination. To overcome these problems, "Real Time Quantitative-Reverse Transcription- PCR" (**qRT-PCR**) has been

adopted. The qRT-PCR is one of the most powerful and sensitive gene analysis techniques available at now. While in traditional PCR analysis, results are collected at the end of the reaction, during qRT-PCR, the fluorescent signal is measured in real time at each amplification cycle and is directly proportional to the number of amplicons generated. The limitations of this method are due to the high costs and the difficulty of execution, requiring expensive scientific equipment and skilled technical staff.

Fluorescence *in situ* Hybridization - It is a genetic approach using oligonucleotide probes labelled with fluorescent dyes. These probes can be specifically designed to bind rRNA, particularly abundant in viable cells or to bind a specific molecule representative of a specific target of interest. It can detect viable but not cultivable bacteria or bacteria with low metabolism (dormient) in biofilm. The main limits are related to the complex preparation procedure and to the fact that the technique is time consuming and expensive.

Physical Assays

Mass spectrometry assay – It is a powerful analytical technique used to quantify known materials and to reveal chemical properties of different molecules. In this method, the substance to be studied is crossed by a beam of electrons so that all molecules are ionized with the production of gaseous ions. Ions are then separated in the mass spectrometer and are characterized by their mass/charge ratios and relative abundances. This technique has both qualitative and quantitative chance of being able to identify and quantify unknown compounds. However, many steps in MS are highly invasive for the sample: high vacuum environment, aggressive chemical solvent etc. To overcome this problem, the Desorption-Electro-Spray-Ionization **(DESI)** assay has been proposed. DESI spectrometry analysis is carried out at atmospheric pressure and the sample is maintained under ambient conditions. This characteristic allows direct and non-destructive analysis of complex samples allowing chemical characterization of microbial biofilm in different growth state and conditions. However, MS and DESI methods are expensive and time consuming methods and require advanced equipment and skilled personnel.

Confocal Laser Scanning Microscopy - It is an optical microscope equipped with a laser beam, particularly useful in biology and life sciences to study thick samples. Confocal laser scanning microscope technology make it possible to scan a thick biological sample, e.g. a microbial biofilm, by processing images, line by line, in X, Y and Z axes. Biological samples are often stained with specific fluorescent dye so that the fluorescent light from the illuminated spot is collected and transformed by a photodiode in electrical signal processed by a computer. The optical reconstruction of all the pixel information was assembled yielding a high contrast and high resolution three-dimensional image. This technique has been widely used in the study of biofilm, especially to study EPS components. The main limitations of this method are that CLSM allows only a semiquantitative investigation and that only few fluorescent stains can be employed simultaneously showing just a couple of component in the same image.

Confocal-Raman Microscopy- In Raman spectroscopy an electromagnetic laser beam with known wavelength hits the sample to be analyzed. Measuring its scattered radiation and the shift in energy, information on the chemical characteristics of the sample can be derived. Using Raman spectroscopy, we can acquire information on chemical fingerprint of different biofilms.[14] However, a deep investigation inside thickness of the biofilm is a difficult challenge. The main limitation is related to the set up of experimental condition to achieve high quality signals.

Electron microscopy – It has advantage of the higher resolution allowed by the use of an electron beam, i.e., of short-wavelength and high-energy radiation. In practice, magnification can be

achieved from the one typical of optical microscopy to that needed to resolve nanometer details. Transmission electron microscopy (**TEM**) shows unique capability for the imaging of the inner of biofilms and intracellular features, but requires the sample to be prepared as ultra-thin slices . Conversely, scanning electron microscopy (**SEM**) has been widely used to visualize the surface of microcolonies as well as old biofilm .[24] Moreover, SEM can be used in synergy with focused ion beam (**FIB**), the latter enabling the milling of selected areas of the sample, to investigate the inner of biofilms by removing the exposed surface layers and/or cutting cross-sections .

In addition, SEM-FIB and TEM can be supported by energy-dispersive X-ray spectroscopy (**EDX**) to acquire local compositional spectra and maps of bacterial cells and biofilms . The main limitation of SEM is the need for tedious samples preparation to dehydrate and make them suitable for vacuum operations. These limitations have been – at least partially – overcome by **cryo-SEM** and environmental SEM (**ESEM**).

X-ray microscopy - The sample is illuminated with a soft X-ray focused radiation, either mono- or poly-chromatic. Different XMs have been proved to enable high-resolution imaging and compositional mapping of biological samples without any specific preparation and with reduced radiation damage with respect to EM. In particular, scanning transmission X-ray microscopy (**STXM**) has been widely used to investigate the composition of bacterial cells and biofilms with nominal resolution of 25 nm . STXM may also operate in synergy with other X-ray based methods, such as X-ray fluorescence. In particular, these two techniques have been recently used to detect arsenic in Fe(II)-oxidizing freshwater bacteria . Clearly, the need for complex and expensive instrumentations represents the major limit to the diffusion of XM techniques.

Scanning probe microscopy - The topography of the sample is reconstructed analyzing the signal from a sharp probe with nanometrical dimension, which scans the sample in close vicinity of its surface. Among the SPM techniques, atomic force microscopy (**AFM**) is the most widely diffused for the characterization of microbiological samples like bacterial cells and biofilms. AFM enables high resolution down to nanometer scale, nondestructive analysis, operations both in air and in water, and does not require for any specific sample preparation. Due to these characteristics, AFM has been used to visualize the surface of bacteria and biofilm , as well as to monitor (also in real time) the processes connected to the bacterial multiplication , EPS production and predation, e.g., by *Bdellovibrio bacteriovorus* and *Escherichia coli*. In addition, the AFM probe can be used to exert ultra-low loads on the sample surface, thus enabling the quantitative measurement and mapping of biofilm elasticity. The main limitation of SPM techniques is that only the sample surface and the inner portion immediately close to it can be analyzed. So, the ideal samples for SPM seem to be young bacterial colonies and biofilms, while old biofilms with thickness of tens of microns or more can be hardly studied except for their (near) surface layers.

Emerging methods

A number of recently developed techniques have been implemented for microbial identification, and these methods show potential for future applications in the study of oral biofilms. Denaturing high– performance liquid chromatography (DHPLC) is a PCR–based method which is followed by separation based on partial denaturation of the amplified DNA. This technique can be used to detect DNA sequence changes, such as point mutations. DHPLC has been previously utilized in other areas of research, such as intestinal microbiota, and has more recently been applied for analysis of dental biofilms and bacteria.[18] Techniques used in chronic wound biofilm analysis may also become useful for oral biofilm research and diagnosis.[20] Pyrosequencing, a rapid sequencing method that can simultaneously identify microbes and detect antibiotic resistance, has been applied for the

determination of bacterial diversity in chronic wound biofilms such as in diabetic foot ulcers, venous leg ulcers and pressure ulcers.[9] Recently, the pyrosequencing method was applied to the analysis of saliva and supragingival plaque samples, and it was estimated that 19,000 different microbial species are present in the mouth. Studies which utilize these next–generation methods arerevealing that original approximations of oral microbial diversity were highly underestimated.[11]

16S rRna Gene Sequencing

The 16S ribosomal RNA gene is highly conserved and can be used in the formation of phylogenetic trees or genetic relationships. This discovery, along with the advent of PCR techniques, has allowed the analysis of oral biofilms on a genetic level. 16S RNA is present in almost all bacterial species, with unique sequence differences allowing discrimination between species.[13] Amplification methods, such as 16S rRNA sequencing, have eliminated the requirement for culture based techniques, allowing the identification of unculturable species A limitation of this method is low resolution in distinguishing between bacteria at the species level.

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is a PCR and electrophoresis–based approach for analysis of microbial communities. Various marker genes, including 16S rRNA, are amplified using PCR and then analyzed on a denaturing gel. A banding pattern develops based on the denaturation characteristics determined by the sequence composition of each amplified DNA. Each band observed on a DGGE gel theoretically represents a different bacterial population within a community.[10] Thus, DGGE band patterns can illustrate the complexity and diversity of a biofilm sample, and individual bands can be subsequently excised and sequenced to determine species identity. A limitation of DGGE is that sequence differences greater than 1 base pair may fail to separate on a denaturing gel because of similarities in nucleotide proportions that result in identical denaturing characteristics of 2 different sequences.[12] Therefore, excision and sequencing is necessary to confirm the identification of species present within an individual band.

Conclusion

Understanding the complex interactions between bacteria that occur within an oral biofilm will provide insight necessary for improving diagnosis, treatment and prevention of periodontal disease. Dental practitioners should be aware of emerging diagnostic techniques and should strive to work in concert with researchers to harness new technologies for improving biofilm management.[17] Molecular diagnostics of dental biofilms will allow for rapid, focused and personalized treatment, enhancing the traditional methods used by dental hygienists to control and prevent periodontal disease.

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