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Review Article

Battling Against Biofilm

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ABSTRACT

Biofilm biology has become an expanding field of research in human, industrial and environmental ecosystems. Various researches suggest that organisms growing in biofilms are better protected from adverse environmental changes and other antimicrobial agents. The structure per se will provide protection and allow better resistance to external influences for the organisms compared with the planktonic state. This article highlights the concept endodontic biofilm in root canal infections, biofilm-associated organisms and its control strategies.

Keywords: biofilm, e. faecalis, irrigant, periapical, planktonic

INTRODUCTION

The term biofilm denote a thin-layered condensation of microbes, which include bacteria, fungi, and protozoa. Costerton et al stated that biofilm consist of single cells and microcolonies, cell embedded in a highly hydrated, predominantly anionic exopolymer matrix. It is characterized by surface attachment, structural heterogeneity, genetic diversity, complex community interactions, and an extracellular matrix of polymeric substance. [1] It acts as a protective environment for microorganisms in which bacteria lives as a community. It also acts a reservoir for accumulating and concentrating nutrients required for the survival of microorganisms. [2,3] The concept of biofilms was not recognized until 1978 and it almost took 2 decades to accept this concept. It was highlighted by Nobel laureate, Joushua Lederburg, in 1996, in a conference called "microbial ecology and infectious disease" hosted by national institute of dental research, Bethesda. With the advent of scientific research such as atomic fluorescence or confocal microscopy, which is assisted with digital imaging technology, a detailed description about biofilm and its composition was elucidated.

BIOFILMS IN DENTISTRY

Pellicle formation, bacterial colonization and biofilm maturation leads to the formation of oral biofilm. The organic substances surround microorganisms that contain carbohydrates, proteins and lipids. [3] The inorganic elements found in a biofilm are calcium, phosphorus, magnesium and fluoride. These inorganic substances are found to be in higher concentration in biofilms than in saliva. Salivary micelle-like globules (SMGs) from saliva acts to remove enamel pellicle on the tooth surface and this salivary micelle-like globules act as a "foundation" for the future biofilm formation. [3] Calcium helps in the formation of larger globules by connecting the negative charges on the subunits. Oral diseases such as Dental caries, gingivitis,

periodontitis, peri-implantitis, and apical periodontitis are few examples caused due to the formation of the biofilm rather than single organisms.

HISTORY OF BIOFILM

- Van Leeuwenhoek described that surface associated microorganisms (biofilms) exhibit an unique phenotype with respect to gene transcription and growth rate. They exhibit specific mechanisms for initial attachment to a surface, development of a community structure and ecosystem and detachment. [4]
- Miller (1894) published his findings on the pulp bacteriology. He observed different microorganisms in pulp space exhibiting different morphology in coronal, middle, and apical parts of root canal system and realized some were uncultivable. [5]
- Costerton in 1978, put forth a theory of biofilm describing the mechanisms of microorganisms adhering to living and non-living materials. [6]
- Kakehashi et al exposed the dental pulps of conventional and germ free rats to oral cavity and found that conventional rats showed high amounts of pulp necrosis and peri-radicular lesions. [7] Thus, bacteria may be unaffected by endodontic disinfection procedure in some area that includes isthmuses, ramifications, deltas, irregularities and dentinal tubules.

DEFINITION OF BIOFILM

According to Percival et al, it s defined as "microbial cells immobilized in a matrix of extracellular polymers acting as an independent functioning ecosystem, homeostatically regulated". Biofilm is defined as polysaccharide matrix enclosed sessile or pedunlated microbial population composed of tower or mushroom shaped microcolonies, containing cells irreversibly adherent to each other or to substratum or interface.

CRITERIA FOR A BIOFILM

Caldwell et al described four characteristics of biofilm as follows: [8]

- Autopoiesis – Must possess the ability to self-organize.
- Homeostasis - Resist environment perturbations.
- Synergy –Must be more effective in association than in isolation.
- Communality – Ability to react to the environmental changes as a unit rather than as single individual. The ideal example of a biofilm is a dental plaque.

COMPOSITION OF BIOFILM:

A matured biofilm posses a heterogeneous arrangement of microbial cells and surface adherent bacterial cells forms the basic structural unit, micro colonies or cell clusters. Water constitutes 80% of the oral biofilm, while the organic and inorganic fractions form approximately 20% of the biofilm structure. They are composed of matrix material consisting of proteins, polysaccharides, nucleic acid, and salt which makes up 85% by volume, while 15% is made up of cells. [8,9] The structure and composition of biofilm get matured and modifies according to various environmental conditions. These environmental conditions include growth conditions, nutrition, temperature, and fluid nature. Due to these impacts they get easily detached and cause chronic infections. During the process of detachment, biofilms transfer cells, polymers and precipitates from the biofilm to fluid, which contributes for the change in morphological characters and structure of mature biofilm (seeding dispersal or dispersive mechanism).

The Extracellular Polymeric Substances (EPS) secreted by biofilms which provides unique characteristics to the biofilm community and is essential for physiologic activity of biofilm. [10] The important functions includes:

1. It acts as "biologic glue" by enhancing the adhesive property of biofilms
2. It enables extracellular enzymes to exert nutrient acquisition and co-operative degradation of complex macromolecules

3. It allows interactions like quorum sensing (signaling molecules), genetic exchanges and pathogenic synergism
4. It maintains highly hydrated environment by retaining water
5. It provides mechanical stability to the biofilm
6. It acts as nutrient source during the periods of nutrient deprivation.
7. It plays protective role against host defense cells and molecules as well as antimicrobial agents

Ca2+, Mg2+,and Fe3+ readily bind and precipitate within an ionic biofilm under a favorable environment and helps in biofilm-mediated mineralization.

Factors affecting attachment of biofilm:

Attachment of microorganisms to a surface is a complex process regulated by diverse characteristics of the growth medium, substratum, and cell surface. [11] There are many factors that affect the bacterial attachment to a solid surface. These factors include, pH, temperature, nutrient availability, length of time the bacteria is in contact with the surface, surface energy of the substrate, bacterial growth stage, flow rate of the fluid passing over the surface, bacterial cell surface charge and surface hydrophobicity. [12]

STAGES IN DEVELOPMENT OF BIOFILM

Bacterial cells, fluid medium and solid surface are the three main components involved in biofilm formation. [1] **Schematic representation of stages of biofilm [13] is illustrated in Figure 1.**

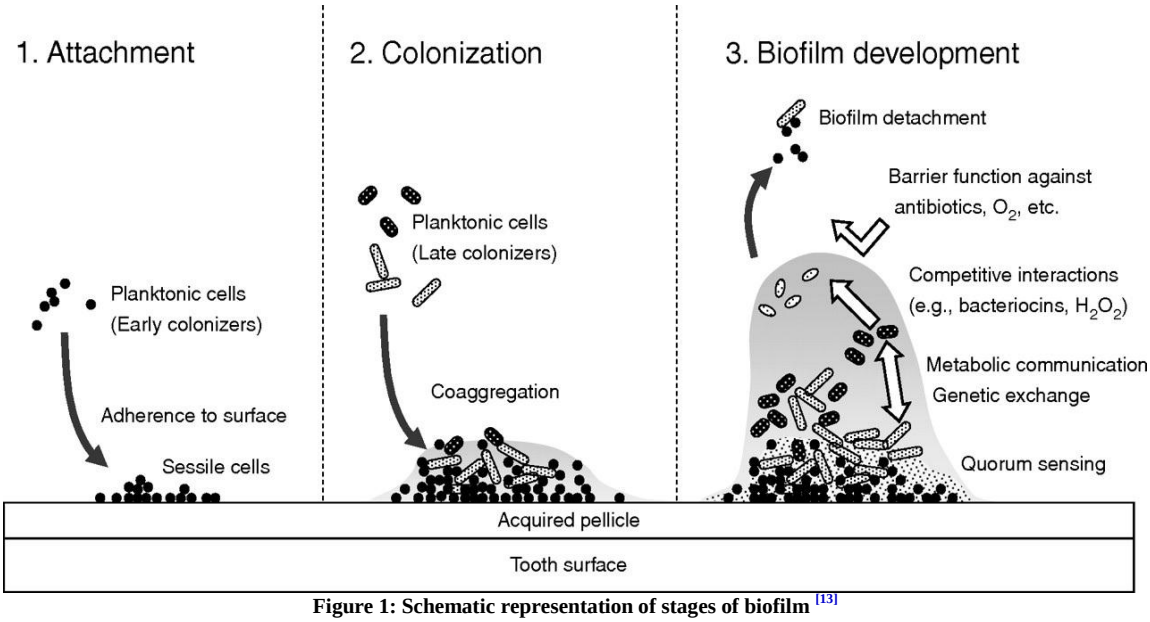

Figure 1: Schematic representation of stages of biofilm [13]

Stage1: Creating a conditioning layer – Initially, there is adsorption of various macro-molecules in the planktonic phase to the solid surface leading to the formation of a conditioning layer

Stage 2: Planktonic bacterial cell attachment - Adhesion and cohesion of microbial cells to this layer. It involves various phases in stage 2 biofilm formation are:

Phase1: (transport of microbe to the substrate surface): Bacteria-substrate interaction is determined by physicochemical properties such as surface energy and charge density. Bacterial adherence to a substrate is achieved with the help of fimbriae, pili, flagella and EPS (glycocalyx). They form the bridges between bacteria and conditioning film.

Phase 2: (initial non-specific microbial-substrate adherence phase): Molecular specific interactions between bacterial surface structures and substrate become active. These bridges are a combination of

covalent and hydrogen bonding, dipole interaction, electrostatic attraction, and hydrophobic interaction. Porphyromonas gingivalis, Streptococcus mitis, Streptococcus mutans, Streptococcus salivarius, Prevotella intermedia, Prevotella nigrescens, and Actinomyces naeslundii are some of the oral bacteria possessing surface structures.

Phase3: (specific microbial – substrate adherence phase): Polysaccharide adhesion or ligand formation enables the binding property of the receptors on the substrate, specific bacterial adhesion with a substrate is produced.

Stage 3: There is bacterial growth and biofilm expansion occurs. The microcolony is formed by the monolayer of microbes, which attracts secondary colonizers leading to the formation of final structure of the biofilm. [14] The two types of microbial interactions that occur at the cellular level are co-adhesion and co-aggregation. Thus, multiplication and metabolism of attached microorganisms ultimately result in a structurally organized mixed microbial community.

Stage 4: During the process of detachment, the biofilm transfer participate constituents (cells, polymers, and precipitates) to the fluid bathing biofilm. It can be either dispersed or detached and it is of 3 types:

1. Clumping dispersal: It is known as physical detachment. A micro-colony gets detached and is carried to a new location to initiate a new sessile population. This detachment can be of either continuous detachment from a single cell (erosion), rapid detachment of the biofilm (sloughing) or detachment due to collision of particles from the bulk fluid with the biofilm (abrasion).
2. Seeding dispersal: It is known as programmed detachment and occurs due to hydrolysis of EPS matrix and these detached cells are responsible for persistent infection.
3. Quorum sensing: It is the process of communication between bacterial cells residing in a biofilm attained through signaling molecules. [15] It is a intraspecies communications which is mediated by low molecular weight molecules, which can alter the metabolic activity of neighboring cells and coordinate the function of resident bacterial cells within a biofilm. Quorum sensing can also regulate the microbial property such as virulence factor and extracellular DNA incorporation.

TYPES OF ENDODONTIC BIOFILMS:

1. INTRACANAL BIOFILMS: This microbial biofilm is formed on the root canal dentin of infected tooth. It was first identified and reported by Nair in 1987, under Transmission Electron Microscopy. [16,15,3] It exists as loose collections of cocci, rods, filaments, and spirochetes. Moreover, the bacterial condensation occurs as a palisading structure similar to dental plaque on tooth surface and show distinct morphologies.
2. EXTRARADICULAR BIOFILM: Extraradicular biofilms (root surface biofilms) are formed on root surface adjacent to the root apex of endodontically affected tooth. The most favorable sites are teeth with asymptomatic periapical periodontitis and chronic periapical abscess associated with sinus tract. Filamentous and fibrillar forms were most commonly observed and are dominated by cocci and short rods. The extracted teeth under SEM revealed the structureless smooth biofilm with multiple species of bacteria and had varying degree of extracellular matrix.
3. PERIAPICAL MICROBIAL BIOFILMS: Periapical microbial biofilms are found in the periapical region of an endodontically infected teeth. They have a capacity to overcome host defense mechanisms, which in turn result in periapical lesions. It is even associated with asymptomatic root canal infections. Commonly involved microorganisms are Actinomyces and

Propionibacterium Propionicum. [17,15] Actinomyces species show presence of yellow granules which appears as ray fungus under scanning electron microscope (SEM). The aggregation of Actinomyces cells are influenced by various factors such as pH, ionic strength and cell concentration. [7]

4. FOREIGN BODY-CENTERED BIOFILM: Foreign body-centered or Biomaterial-centered infection (BCI) occurs when bacteria adheres to artificial biomaterial surface such as root canal obturating materials and forms biofilms in it. [18,15] BCI reveals opportunistic invasion by nosocomial organisms. Coagulase-negative Staphylococcus, S. aureus, Enterococci, Streptococci, P. aeruginosa and fungi are commonly isolated from infected biomaterial surfaces. The bacterial adherence to a biomaterial surface include: initial non-specific adhesion phase and specific adhesion phase. The BCI in endodontics can be either intra-radicular or extra-radicular depending on the position of the obturating material

MICROBES IN ENDODONTIC BIOFILMS: The various methods to isolate microorganisms present in biofilms are culture, microscopy, immunological methods or molecular biology methods, which include PCR, DNA-DNA hybridization, or whole cell protein analysis. [19] Molecular studies investigating the breadth of bacterial diversity in infected root canals have disclosed the occurrence of uncultivated phytotypes belonging to several genera including Synergists, Dialister, Prevotella, Solobacterium, Olsenella, Fusobacterium, Eubacterium, Megasphaera, Veillonella and Selenomonas. [20]

Microorganisms mainly involved in biofilm formation are:

- Enterococcus faecalis
- Coagulase-negative staphylococcus
- Streptococci
- Actinomyces species
- Propionibacterium propionicum
- Others - P. aeruginosa, fungi, Fusobacterium nucleatum, Porphyromonas gingivalis, Tannerella forsynthesis, Actinomyces species.

ROLE OF ENTEROCOCCUS FAECALIS IN BIOFILM

- Enterococcus faecalis, a non-motile organism of enterococci family, has a unique property in biofilm formation. Its physiochemical properties help them to modify according to the prevailing environmental and nutrient conditions. [21] They can grow in extremely alkaline pH, salt concentrated environment and in temperature ranging from 10-45°C and can survive at 60°C for 30minutes.
- Biofilms formed by Enterococcus faecalis have a property to resist destruction by enabling the bacteria to become 1000 times more resistant to phagocytoses, antibodies, antimicrobials than non-biofilm producing bacteria and by maintaining the pH at 11.5 or greater. [9]
- It forms biofilms by adherence and formation of microcolonies by microorganism. Then bacterial mediated dissolution of mineral fraction from dentin will result in release of calcium and phosphate ions leading to initial calcification. In advanced stages they show carbonated-apatite structure, which is more resistant and difficult to eradicate. [7]
- Enterococcus faecalis in conjunction with Fusobacterium nucleatum results in aggravating the endodontic infection by suppressing the action of lymphocytes.

CURRENT THERAPEUTIC OPTIONS FOR BIOFILM:

Root canal irrigation helps to eradicate bacteria and removal of bacterial biofilm from the un-instrumented surfaces. An ideal root canal irrigant should have high efficacy against microorganisms in biofilms but should be non-toxic and non-caustic to the periodontal tissues. Sodium hypochlorite (NaOCl) has excellent antimicrobial activity, caustic and toxic effects to vital

tissues are often noted. [22,23] So plants derived natural and herbal products represent a rich source of antimicrobial compounds has been incorporated in oral hygiene products. However, their application in endodontics is less well documented. [24] Various options that are currently available to eradicate biofilms are: [25]

- ✓ **Sodium Hypochlorite:** It is effective against biofilms containing P. intermedia, P. micros, S. intermedius, F. nucleatum and E. fecalis as it disrupts oxidative phosphorylation and inhibits DNA synthesis of bacteria. Dunavant et al (2006) concluded that both 1% and 6% were more efficient in killing E. faecalis than other irrigants.
- ✓ **Chlorhexidine Digluconate:** It is effective against both gram-positive and a gram negative bacterium due to its ability to denature the bacterial cell wall by forming pores in the membrane. Various in-vitro studies have demonstrated that the antibacterial activity of CHX is superior than NaOCl in eradicating E. faecalis. It is effective at 0.2 to 2% in 30 seconds or less.
- ✓ **QMiX:** It consists of EDTA, Chlorhexidine, and a detergent. It is equally effective as 6% NaOCl in disrupting the newly formed bacterial biofilm but slightly less effective in older biofilms.
- ✓ **Iodine:** It has a wide range of activity against bacteria, fungi, virus and even spores. It causes cell lysis of the microorganism by denaturing the proteins, fatty acids and nucleotides.
- ✓ **EDTA:** Mechanism behind EDTA is that it extracts surface proteins of the bacteria by combining with metal ions from the envelope of the cell that eventually leads to the bacterial lysis. [26,27]
- ✓ **MTAD:** MTAD has low PH so it acts as a calcium chelator and causes enamel and root surface demineralization. They are absorbed and gradually released from tooth structures such as dentin and cementum.
- ✓ **Tetraclean:** Pappen FG et al (2010) found that tetraclean is more effective than MTAD against E.faecalis and mixed species. The Cetrimide in tetraclean enhances the antimicrobial activity whereas Tween 80% present in MTAD seemed to have a neutral or negative impact on their antimicrobial effectiveness.
- ✓ **Calcium hydroxide:** A commonly used intracanal medicament is shown to be ineffective in killing E.faecalis especially when its high pH is not maintained. But the combination of calcium hydroxide and camphorated paramonochlorophenol completely eliminates E.faecalis. 2% chlorhexidine combined with calcium hydroxide achieves a pH of 12.8 can completely destruct E. faecalis within dentinal tubules. Chlorhexidine and calcium hydroxide when combined together have shown better antimicrobial properties than calcium hydroxide alone.
- ✓ **Ultrasonically Activated Irrigation:** Bhuva B et al (2010) found that use of ultrasonically activated irrigation using 1% sodium hypochlorite, followed by cleaning of root canal and isthmus to remove necrotic materials and biofilm remnants.
- ✓ **Ozone/ Ozonated water:** Viera MR et al (1999) reported that ozone in 0.1 to 0.3 ppm concentration kills bacteria completely after 15 or 30 minutes of contact time with them.
- ✓ **Lasers:** Lasers produce thermal effect resulting in alteration in the bacterial cell was leading to the changes in osmotic gradients of bacteria and cell death. Noiri et al found that Er:YAG laser irradiation produced excellent result due to its ablating capacity and is effective against (apical biofilm) A. naeslundii, E. faecalis, L. casei, P. acnes, F. nucleatum, P. gingivalis, and P. nigrescens.

- ✓ **Plasma Dental Probe:** Plasma Dental Probe is effective for tooth disinfection. Plasma emission spectroscopy identifies atomic oxygen which is more important agent for the bactericidal effect. Complete destruction of endodontic biofilms takes place in 5mm at the depth of 1mm inside root canal.
- ✓ **Photo activated disinfection/Light activated therapy:** It is the latest method to destruct biofilms. It involves killing of microorganisms when a photosensitizer selectively accumulate in the target and is activated by a visible light of appropriate wavelength. [28] The photo sensitizer gets absorbed by microbial cells and gets adheres to it as it is colored and the low-power destructs the target area and inactivate the microbial invaders present inside the canals.
- ✓ **Antibacterial nanoparticles:** Antibacterial nanoparticles bind to negatively charged surfaces and have excellent antimicrobial and antifungal activities. Studies have proved that treatment of root dentin with ZnO nanoparticles, chitosan-layer-ZnO nanoparticles, or Chitosan nanoparticles are effective against E. faecalis adhesion.
- ✓ **Endo activator system:** It penetrates deep into the canal anatomy and effectively removes the smear layer and dislodges simulated biofilm clumps even in curved root canals.

CONCLUSION

Application of the biofilm concept to endodontic microbiology will play a crucial role in helping us to understand the pathogenic potential of root canal microbiota and also new approaches to infection control. [29] Research on microbial biofilms is proceeding on many aspects with particular emphasis on elucidation of the genes expressed by biofilm-associated organisms, [30] evaluation of various control strategies either for preventing or remediating biofilm colonization and development of new methods for assessing the efficacy of new treatment modalities to eradicate biofilms.

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