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Biofilms-The Unforgiving Film in Dentistry (Clinical Endodontic Biofilms)

Aruna Kanaparthy[1]* and Rosaiah Kanaparthy[2]

[1]Conservative Dentistry and Endodontics, People's Dental Academy, Bhopal, Madhya Pradesh, India
[2]Department of periodontics, People's Dental Academy, Bhopal, Madhya Pradesh, India

Abstract

It is evident that oral microorganisms have the capacity to respond and adapt to changing environmental conditions. Individual microorganisms are able to sense and process the chemical information from the environment and thereby adjust their phenotypic properties. The term biofilm is used to indicate the presence of a film of condensed microorganisms on any surface. Bacterial condensations on the walls of infected root canals have been observed suggesting that mechanisms for biofilm formation also exist inside the root canal space. A mature biofilm is a metabolically active community of microorganisms where individuals share duties and benefits.

A growing body of knowledge suggests that organisms in biofilms assume a stronger pathogenic potential than those in a planktonic state. From these aspects, the formation of biofilms carries particular clinical significance because not only host defense mechanisms, but also therapeutic efforts including chemical and mechanical anti-microbial treatment measures, have a most difficult task to deal with organisms that are gathered in a biofilm. Such bacterial aggregations have been thought to be the cause of therapy-resistant apical periodontitis. The aim of this communication is therefore to give an overview of the biofilm concept and to discuss how it may apply to endodontic infections.

Keywords: Biofilms; Planktonic; Microorganism; Apical periodontitis

Introduction

Costerton et al. [1] stated that biofilm consists of single cells and microcolonies, all embedded in a highly hydrated, predominantly anionic exopolymer matrix. The new definition of a biofilm is a microbially derived community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extra-cellular polysaccharides that they have produced and exhibit an altered phenotype with respect to growth rate and gene transcription. Free-floating bacteria existing in an aqueous environment, so-called planktonic microorganisms are a prerequisite for biofilm formation. Such films may thus become established on any organic or inorganic surface substrates. Bacteria can form biofilms on any surface that is bathed in a nutrient-containing fluid. The three major components involved in biofilm formation are bacterial cells, a solid surface, and a fluid medium.

Ultrastructure of biofilm

The basic structural units of a biofilm are the colonies or cell clusters formed by the surface adherent bacterial cells. Colonies are discrete units of densely packed bacterial cell aggregates. There is a spatial distribution of bacterial cells of different physiological and metabolic states with in a biofilm. A glycocalyx matrix made up of extra-cellular polymeric substances surrounds the microcolonies and anchors the bacterial cell to the substrate. Probably up to eighty-five percent by volume of the biofilm structure is made up of matrix material, while 15% is made up of cells. The structure and composition of a matured biofilm is known to modify according to the environmental conditions (growth conditions, nutritional availability, nature of fluid movements, physicochemical properties of the substrate, etc. Generally, a viable, fully hydrated biofilm appears as "tower-" or "mushroom" shaped structures adherent to a substrate. The water channels, which are regarded as a primitive circulatory system in a biofilm, intersect the structure of biofilm to establish connections between the microcolonies. Presence of water channels facilitates efficient exchange of materials between bacterial cells and bulk fluid, which in turn helps to coordinate functions in a biofilm community [2,3]. The structural feature of a biofilm that has the highest impact in chronic bacterial infection is the tendency of microcolonies to detach from the biofilm community. During the detachment, the biofilm transfer particulate constituents (cells, polymers, and precipitates) from the biofilm to the fluid bathing the biofilm. There are two main types of detachment process: erosion (the continual detachment of single cells and small portions of the biofilm) and sloughing (the rapid, massive loss of biofilm). Detachment has been understood to play an important role in shaping the morphological characteristics and structure of mature biofilm. It is also considered as an active dispersive mechanism (seeding dispersal); these detached cells, which have acquired the resistance traits from the parent biofilm community, can be source for persistent infection figure 1 [4].

Composition of biofilm

The organic substances surround the microorganisms of biofilm and contain primarily carbohydrates, proteins, and lipids. Among inorganic elements in biofilms are calcium, phosphorous, magnesium and fluoride [1,5].

Factors affecting formation of biofilm

Formation of the detailed structure of a bacterial colony is a combination of intrinsic and extrinsic factors. Intrinsic factors are products of the genetics of the cell itself. They determine the morphology of the individual cell, the mode of cell reproduction, the possession of extracellular appendages (flagella, fimbriae, pili, etc.) production of extracellular products (exopolysaccharides, proteins, etc.), motility, energy metabolism, pigment formation and so on.

***Corresponding author:** Aruna Kanaparthy, Reader, Conservative Dentistry and Endodontics, People's Dental Academy, HIG-3, PDA Staff quarters, Peoples campus, Bhanpur, Bhopal, Madhya Pradesh, India, Tel: 9165232031; E-mail: draruna15@gmail.com, draruna2008@rediffmail.com

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Extrinsic factors include the prevailing chemical environment which influences the physiology of the cell plus the transport of solutes into and out of the growing colony and the inevitable formation of solute diffusion gradients within the colony and the surrounding medium.

Development of biofilm

Stage 1: The first step involved in the development of biofllm is the adsorption of inorganic and organic molecules to the solid surface creating a conditioning layer.

Stage 2: The next step in biofilm formation is the adhesion of microbial cells to this layer. There are many factors that affect bacterial attachment to a solid substrate. These factors include pH, temperature, surface energy of the substrate, flow rate of the fluid passing over the surface, nutrient availability, length of time the bacteria is in contact with the surface, bacterial growth stage, as well as bacterial cell charge, and surface hydrophobicity. Physicochemical properties such as surface energy and charge density determine the nature of initial bacteria. The microbial adherence to a substrate is also mediated by bacterial surface structures such as fimbriae, pili, flagella, and glycocalyx (Figure 2).

Stage 3: During this stage, the monolayer of microbes attracts secondary colonizers forming microcolonies and the collection of colonies gives rise to the final structure of biofilm. Microcolony formation and co-adhesion take place before a monolayer is established along lateral and vertical growth of indwellers gives rise to micro colonies similar to towers [6]. Two types of microbial interactions occur at the cellular level during the formation of biofilm. One is the process of recognition between a suspended cell and a cell which is already attached to substratum. This type of interaction is termed co-adhesion. In the second type of interaction, genetically distinct cells in suspension recognize each other and clump together. This type of interaction is called coaggregation. These associations are highly specific and occur between coaggregating partners only. Although genetic makeup of bacteria is the main determinant of coaggregation, the physicochemical characteristics of the environment also play a crucial role.

Resistance of microbes in biofilm to antimicrobials

The nature of biofilm structure and physiological characteristics of the resident microorganisms offer an inherent resistance to antimicrobial agents, such as antibiotics, disinfectants, or germicides. The resistance to antimicrobial agents has been found to amplify more than thousand times for microbes in biofilm, when compared to planktonic cells. The mechanisms responsible for the resistance to antimicrobial agents may include the following:

- Resistance associated with the extracellular polymeric matrix.
- Resistance associated with growth rate and nutrient availability.

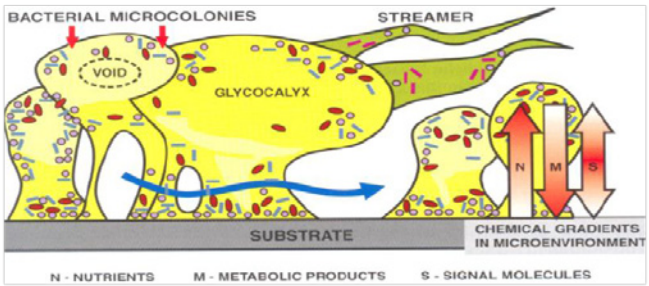

Figure 1: Schematic representation of the structure of a mature biofilm.

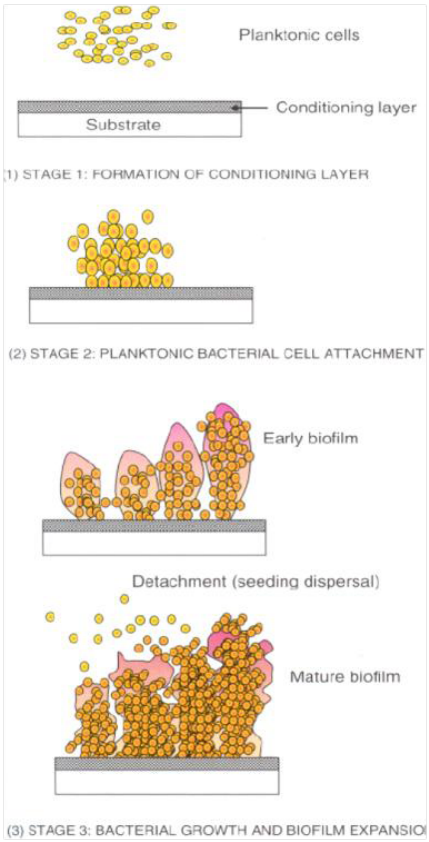

Figure 2: Environmental heterogeneity within biofilms, it is likely that a particular biofilm a number of different phenotypes of a single species.

- Resistance associated with the adoption of resistance phenotype.

It is apparent that these mechanisms act in concert within the biofilm and amplify the effect of small variations in susceptible phenotypes.

Characteristics of biofilm

Bacteria in a biofilm state show distinct capacity to survive tough growth and environmental conditions. This unique capacity of bacteria in a biofilm state is due to the following features:

- Biofilm structure protects the residing bacteria from environmental threats.
- Structure of biofilm permits trapping of nutrients and metabolic cooperativity between resident cells of same species and/or different species.
- Biofilm structures display organized internal compartmentalization, which allows bacterial species with different growth requirements to survive in each compartment.
- Bacterial cells in a biofilm community may communicate and exchange genetic materials to acquire new traits [7,8].

Bacterial biofilm provides a setting for the residing bacterial cells to communicate with each other. Some of these signals, produced by cells, may be interpreted not just by members of the same species, but by other microbial species too. Communications between bacterial

cells residing in a biofilm is attained through signaling molecules, by a process called quorum sensing. Quorum sensing is mediated by low molecular weight molecules, which in appropriate concentration can alter the metabolic activity of neighboring cells, and coordinate the functions of resident bacterial cells within a biofilm. Exchange of genetic material between bacterial species residing in a biofilm will result in the evolution of microbial communities with different traits.

Biofilms in dentistry

Oral bacteria have the capacity to form biofilms on distinct surfaces ranging from hard to soft tissues. The characteristics of the biofilm formed depend upon the residing bacterial species, the surface or substratum composition, and the conditioning layer coating the surfaces on which they are formed. Oral biofilms are formed in three basic steps, namely, pellicle formation, bacterial colonization, and biofilm maturation. The chemical composition of biofilm differs among individuals, between tooth surfaces, in an individual and with age. The organic substance surrounds the microorganisms of the biofilm and contains primarily carbohydrates, proteins, and lipids. Carbohydrates are produced by many bacteria, and they include glucans, fructans, or levans. They contribute to the adherence of microorganisms to each other and are the stored form of energy in biofilm bacteria.

The proteins found in the supragingival biofilm are derived from saliva, while the proteins in the subgingival biofilm are derived from gingival sulcular fluid. The lipid content may include endotoxins (LPS) from Gram-negative bacteria. The inorganic elements found in a biofilm are calcium, phosphorus, magnesium, and fluoride. The concentrations of these inorganic elements are higher in biofilm than in saliva. Human saliva contains proline-rich proteins that aggregate together to form micelle like globules called salivary micelle-like globules (SMGs). SMGs from saliva get adsorbed to the clean tooth surface to form acquired enamel pellicle, which acts as a "foundation" for the future multi layered biofilm. The globular micelles of acquired enamel pellicles are characterized by a negatively charged (calcium binding) surface and hydrophobic interior. Presence of calcium facilitates the formation of larger globules by bridging the negative charges on the subunits. The initial attachment of bacteria to the pellicle is by selective adherence of specific bacteria from oral environment. Innate characteristics of the bacteria and the pellicle determine the adhesive interactions that cause a specific organism to adhere to the pellicle. Dental biofilm consists of a complex mixture of microorganisms that occur primarily as microcolonies. The population density is very high and increases as biofilm ages. The prospect of developing dental caries or gingivitis increases as the number of microorganisms increases. The acquired pellicle attracts Gram-positive cocci such as *S. mutans* and *S. sanguis*, which are the pioneer organisms in the plaque formation. Subsequently, filamentous bacterium such as *F. nucleatum* and slender rods adheres to primary colonizers. Gradually, the filamentous form grows into the cocci layer and replaces many of the cocci. Vibrios and spirochetes appear as the biofilm thickens. More and more gram-negative and anaerobic organisms emerge as the biofilm matures. Interestingly, it is not only the surface of tooth that can be attached by bacterial cells. The surface of some bacteria (bacilli and spirochetes) also can serve as attachment sites for certain smaller coccoids. This coaggregation of *F. nucleatum* with coccoid bacteria gives rise to "corn cob" structure, which is unique in plaque biofilms. The presence of these bacteria makes it possible for other non-aggregating bacteria to coexist in the biofilm, by acting as coaggregating bridges. Calcified dental biofilm is termed as calculus. It is formed by the precipitation of calcium phosphates within the organic plaque matrix. Factors that regulate the deposition of minerals on dental biofilms are physicochemical factors such as plaque pH, local saturation of Calcium, Phosphate, and availability of fluoride ions and biological factors such as presence of crystallization nucleators/inhibitors from either bacteria or oral fluids. Localized super saturation of calcium and phosphate ions provides the driving force for mineralization. The inorganic or mineral fraction consists of calcium phosphates, magnesium, fluoride, and carbonate, and they make up 70% to 80% weight of dental calculus [8]. Various mineral phases namely, HAP, whitlockite, octacalcium phosphate, and brushite have been reported in calculus.

Endodontic biofilms

Although the microbes in the normal oral flora participate in mutually beneficial relationships, they are opportunistic pathogens if they gain access to the normally sterile dental pulp and produce a polymicrobial disease. The bacterium in the infected root canal system is a restricted group compared to oral flora and largely comprised of facultative bacteria and strict anaerobes. Progression of infection alters the nutritional and environmental status within the root canal. The initially polymicrobial environment of the infected root canal apparently becomes more anaerobic and the nutritional level will be depleted. These changes will offer a tough ecological niche for the surviving microorganisms. Studies of endodontically treated teeth requiring retreatment show a prevalence of facultative bacteria especially *E. faecalis* and fungi which are more resistant to treatment.

Clinical investigations have shown that the complete disinfection of root canal is very difficult to achieve. Microbes are found to persist in the anatomical complexities such as isthmuses and deltas and in the apical portion of root canal system. Often, bacterial activities may not be confined to intracanal spaces, but also access regions beyond the apical foramen. These anatomical and geometrical complexities in the root canal systems shelter the adhering bacteria from cleaning and shaping procedures. Because biofilm is the manner of bacterial growth to survive unfavorable environmental and nutritional conditions, the root canal environment in both primary and post-treatment infections will favour biofilm formation. Additionally, biofilm mode of bacterial growth offers other advantages such as,

- Resistance to antimicrobial agents, increase in the local concentration of nutrients, opportunity for genetic material exchange.
- Ability to communicate between bacterial populations of same and/or different species, and produce growth factors across species boundaries.

Endodontic bacterial biofilms can be categorized as:

- Intracanal biofilms
- Extra radicular biofilms
- Periapical biofilms
- Biomaterial centered infections

The characteristic features in cell-cell and microbe-substrate interactions were explained based on the phenomena of microbial adherence [9-11]. Studies have established the ability of *E. faecalis* to resist starvation and develop biofilms under different environmental and nutrient conditions (aerobic, anaerobic, nutrient-rich, and nutrient-deprived conditions). However, the physicochemical properties of *E. faecalis* biofilms were noted to modify according to the prevailing environmental and nutrient conditions. *E. faecalis* under

nutrient-rich environment (aerobic and anaerobic) produced typical biofilm structures with characteristic surface aggregates of bacterial cells and water channels. Viable bacterial cells were present on the surface of the biofilm. Under nutrient-deprived environment (aerobic and anaerobic), irregular growth of adherent cell clumps were observed. Laser scanning confocal microscopy displayed many dead bacterial cells and pockets of viable bacterial cells in this biofilm structure. In vitro experiments have revealed distinct stages in the development of *E. faecalis* biofilm on root canal dentine.

Stage 1: *E. faecalis* cells adhered and formed microcolonies on the root canal dentine surface.

Stage 2: They induced bacterial-mediated dissolution of the mineral fraction from the dentine substrate. This localized increase in the calcium and phosphate ions promotes mineralization (or calcification) of the *E.faecalis* biofilm.

Stage 3: The mature biofilm structure formed after 6 weeks of incubation showed signs of mineralization and subtle but distinct compositional difference. The mineralized *E.faecalis* biofilm showed carbonated-apatite structure as compared to natural dentine which had carbonated fluorapatite structure. A consequence of the interaction of bacteria and their metabolic products on dentine [12-14] in a recent investigation has highlighted the ability of *E.faecalis* clinical isolate to coaggregate with *F. nucleatum*. The coaggregation interactions between *E. faecalis* and *F. nucleatum* suggested the ability of these microorganisms to coexist in a microbial community and contribute to endodontic infection. Apical biofilm is clinically important because microbial biofilms are inherently resistant to antimicrobial agents and cannot be removed by biomechanical preparation alone. Numerous studies have shown the presence of rods, cocci, bacilli and spirochetes on root surfaces in cases of refractory periodontitis [15,16].

Anti-microbial agents and biofilms

Anti-microbial agents have often been developed and optimized for their activity against fast growing, dispersed populations containing a single microorganism. The slow growth rate of microorganisms in established biofilms can result in cells being more resistant to the agent than faster dividing cells. The structure and dense organization of the biofilm population within the polymeric matrix might restrict the penetration of the agent into the biofilm leaving microorganisms in the depths of the biofilm unaffected. The agent might also be inactivated in the biofilm. Biofilm bacteria may also display a distinct phenotype that accounts for the enhanced resistance [17-19]. For example, biofilm bacteria might not express the drug target or use different metabolic pathways than planktonic bacteria.

Conclusion

The formation of biofilms carries particular clinical significance because not only host defense mechanisms, but also the therapeutic efforts including chemical and mechanical anti-microbial treatment measures, have a most difficult task to deal with organisms that are gathered in a biofilm. As far as endodontic infections are concerned, the biofilm concept has thus far gained limited attention. Model development and studies are needed to explore the conditions that may affect the efficacy of endodontic anti-microbials *in vivo* so that their clinical effects can be better predicted.

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