REVIEW ARTICLE

Endodontic biofilm – an unsolved mystery
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Abstract

Microbial biofilm is a thin layered condensation of microbes (e.g. bacteria, fungi, protozoa) that may form on various surface structures in nature. Free-floating bacteria existing in an aqueous environment, the planktonic microorganisms, are a prerequisite for biofilm formation. Bacteria in a biofilm state show distinct capacity to survive tough growth and environmental conditions. This film is a mechanism of survival of bacteria in nature and is considered as an important etiology in establishing pulp and periradicular pathology. The aim of this article is to review mechanism of formation and resistance of biofilm, its role in pulp and periradicular disease, its mechanism of antimicrobial resistance, effects of various root canal irrigants and medicaments as well as lasers on biofilm.

Keywords
biofilm, quorum sensing, intracanal medicaments, endodontic irrigants, ozone, sonic ultrasonic agitation, lasers.

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Introduction

Biofilm is defined as a sessile multicellular microbial community characterized by cells that are firmly attached to a surface and enmeshed in a self-produced matrix of extracellular polymeric substrate (EPS usually a polysaccharide) (1).

Free-floating bacteria existing in an aqueous environment, the planktonic microorganisms, are a prerequisite for biofilm formation (2) (Fig 1). Such films may become established on any organic or inorganic surface substrate where planktonic microorganisms prevail in a water-based solution. This film is a mechanism of survival of bacteria in nature (3). The extracellular polymeric matrix accounts for 85% of the volume of a biofilm. In dental context, a well-known and extensively studied biofilm structure is established during the attachment of bacteria to teeth to form dental plaque.

As far as endodontic infections are concerned, the biofilm concept has far gained limited attention. It has been discussed mainly within the framework of bacterial appearances on root tips of teeth with nonvital pulps (4). Such bacterial aggregations have been thought to be the cause of therapy-resistant apical periodontitis (5). Although not described in great detail, bacterial condensations on the walls of infected root canals have been observed (6) suggesting that mechanisms for biofilm formation may also exist inside the root canal space.

Regardless of whether they are present on the external root surface or inside
root canals, aggregation of microorganisms in biofilms are likely to have distinct clinical implications especially from a treatment point of view.

Mechanism of resistance
The structure of the biofilm per se provides protection and resistance against adverse external influences for the residing organisms as compared with the planktonic state (7). Phenotypically the biofilm organisms differ from their planktonic counterparts (Fig.2).

Bacteria in the biofilm state show distinct capacity to survive tough growth and environmental conditions. This unique capacity of biofilm bacteria is due to its structure which : 1) Protects the residing bacteria from environmental threats. 2) Allows trapping of nutrients and metabolites cooperatively between resident cells of same species and/or different species 3) Display organized internal compartmentalization, which allows bacterial species with different growth requirements to survive in each compartment 4) Allows the bacterial cells in a biofilm community to communicate and exchange genetic material to acquire new traits.

With the changes in the environmental conditions microorganisms adapt to the biofilm lifestyle by regulating set of genes thus are able to optimize phenotypic properties for the particular environment.

Quorum Sensing—Bacterial Intercommunication
Quorum sensing describes a system based on cell-cell communication that regulate gene expression in a cell density–dependent manner (9). Quorum-sensing systems capacitate bacteria to behave collectively as a group. This phenomenon has been described in both gram-positive and gram negative bacterial species. Quorum sensing involves the production, release, and subsequent detection of diffusible signaling molecules called auto inducers. As members of a bacterial population producing and releasing auto inducers multiply, the extracellular concentration of these signaling molecules also increases. When auto inducers reach a crucial threshold level, the group responds with a population-wide alteration in gene expression, activating or repressing target genes (10) since alterations in gene expression are linked to the presence of auto inducers, bacteria can perform specific functions only when living in groups (Fig 3). Such a behavior provides some advantages to a bacterial population, affording adaptability to and protection against threatening environment. Quorum-sensing systems are known to regulate virulence, competence for DNA uptake, entry into stationary phase and biofilm formation (11). Using quorum sensing, bacteria can amass a high cell density before virulence factors are expressed, and in doing so, the bacteria are able to make a consolidated attack to overcome the host defenses and establish infection (12).

Teleologically, it appears bacteria were able to strategically wait for a critical number of cells to be reached and then start inducing damage, not giving the host sufficient time to mount an effective defense response. In reality, with regard to energy yield, it would be difficult for bacteria to produce some virulence factors before a critical community density is reached. For instance, production of proteinases by a few cells are not expected to have significant impact on the host environment, representing “wasted energy” for the growing population.
Biofilm in Endodontics

While the oral cavity is the common source for microorganisms that may colonize root canals after loss of pulpal vitality, there is rather scant knowledge as to the formation of biofilms in endodontic infections. Possibly the first identification of biofilm structures in infected root canals was carried out by Nair (6). Various studies have demonstrated dense aggregates of rods, cocci and filaments sticking to the canal walls and forming thin to thick layers of bacterial condensations.

Scanning electron microscopic (SEM) observations of the apical 2 mm of infected root canals show that cocci and rods and/or filaments often formed microcolonies in this area into which spirochetes were interspersed. However, rarely did spirochetes gather in clusters. Cocci were also seen attached to filaments assembled into the so-called ‘corn-cob’-like structures, which are also described for dental plaque (6).

The presence of biofilm on root tips of extracted teeth with ‘refractory periapical pathosis’ and gutta-percha points was observed by Noiri et al. (2002) using Scanning Electron Microscope (13). The root tips of extracted teeth with various pulpal conditions such as vital pulps, necrotic pulps without apical lesion and necrotic pulps with apical lesions using SEM. The biofilm was found only on teeth with apical periodontitis. These biofilms were composed of different bacterial morphotypes viz. cocci, bacilli and filaments(14).

One re-treatment case of a maxillary premolar with fistula and a periapical lesion was studied using SEM. Cocci and fungal forms on the resected root tip and in resorption lacunae were identified. A true biofilm structure of the bacterial mass was, however, not confirmed (4).

Endodontic biofilms can be classified as follows:

1) Intracanal biofilms that are generally present on the root canal dentin of an endodontically infected tooth.
2) Extraradicular microbial biofilms that are formed on the root (cementum) surface adjacent to the apex of endodontically infected teeth.
3) Periapical microbial biofilms are isolated biofilms found in the periapical region of an endodontically treated tooth.
4) Biomaterial centered infection that is formed when bacteria adheres to an artificial biomaterial surface such as root canal obturating material, thereby forming a biofilm. These biofilms could be intraradicular or extraradicular, depending on the apical extent of the obturation (15).

As far as endodontic infections are concerned, acute exacerbations of endodontic lesions may be explained by a shift in the flow of nutrients to the root canal space, giving rise to ecological changes. This environmental change may favour growth of various proteolytic bacteria, which outcompete other members of the micro-community to become pathogenic by virtue of a numerical dominance.

Following the initial instrumentation of a primary infected root canal, injury of the periapical tissue by over instrumentation may release inflammatory exudates into the root canal and cause growth of proteolytic bacteria that may have survived the endodontic treatment procedure. Similarly, an over-instrumentation in a re-treatment case may break the starvation conditions that often exist in treated root canals for bacteria that may have become entrapped by the root canal filling. If the canal system is opened again in conjunction with a re-treatment attempt, inadvertent enlargement of the foraminal structures may increase the nutritional supply considerably and negatively impact upon the outcome of the procedure.

Another case in point, causing a potential shift in the local root canal environment, occurs when coronal restorations are broken down and a direct pathway to the oral cavity is established. Such a pathway leading to so-called coronal leakage (16) may not only bring nutritional elements that can revive dormant microorganisms in unfilled spaces of the root canal system but also new organisms.

From an ecological perspective, the root canal can be considered as a highly controlled environment with a limited number of niches (17). Therefore, for bacteria to endure endodontic treatment and be detected in post-treatment samples, they must resist intracanal disinfection procedures and adapt to the
drastically changed environment. Several strategies may help bacteria to resist treatment. Bacteria can adhere to the root canal walls, accumulate, and form biofilms. Several factors can affect bacterial attachment to a solid substrate. These factors include surface energy of the substrate, temperature, pH, flow rate of the fluid passing over the surface, length of time the bacteria are in contact with the surface, surface hydrophobicity, and nutrient availability.

**Antimicrobial resistance of biofilm**

There are several mechanisms for biofilms to resist antimicrobial agents. The polysaccharide matrix of biofilms can retard diffusion of the antibiotics. In addition, extracellular enzymes such as β-lactamase may become trapped and concentrated in the matrix, thereby inactivating β-lactam antibiotics (18). Furthermore, quorum sensing (communication with one another) can influence the structure of the biofilm by encouraging the growth of species beneficial to the biofilm. It has been shown that subpopulations of bacteria in a biofilm form a phenotypic state (altered gene expression) where they are highly protected (19).

Bacterial cells protect themselves by being located within the interior part of a biofilm; hence, medicaments will only act on the microorganisms in the peripheral portion of the biofilm. Additionally, bacterial cells residing within a biofilm grow more slowly than planktonic cells, and as a result, antimicrobial agents act more slowly.

Depletion of nutrients or accumulation of waste products can result in bacteria entering a non-growing state which protects the bacteria from the antibiotics. Pajkos et al. (2004) revealed that biofilm bacteria exist in a low metabolic state, with a slower growth rate and production of exopolysaccharides (20). Chemical changes to the environment in the biofilm such as the lack of oxygen inhibits some antibiotics and accumulated acidic waste leads to a difference in pH which has an antagonizing effect on the antibiotics (21).

**Effects of endodontic irrigants and medicaments on biofilms**

Chavez et al. 2007 recognized that microbial communities are noticeably resistant to and difficult to eradicate with antimicrobials (17). Deep-seated microorganisms, in particular, may escape killing and remain viable for remultiplication when conditions are favourable and, thus, cause an endodontic treatment failure. An in vitro study showed that wild-strain bacteria of endodontic origin grown over 8 days on dentin slices of extracted teeth to generate biofilms were not possible to eradicate using ampicillin, doxycycline, clindamycin, azithromycin, or metronidazole (22).

In situ effect of antimicrobials and alkali on biofilms of Enterococcus faecalis, Lactobacillus paracasei, Streptococcus anginosus and Streptococcus gordonii isolated from root canals with persistent infections was studied by Luis E et al. (2010). The effect of chlorhexidine, pH12, EDTA, and NaOCl was tested on the removal of biofilm cells in the aforementioned organisms. The most efficient removal action was achieved by sodium hypochlorite. The effects were substratum dependent, and most organisms displayed increased resistance to the antimicrobials on collagen-coated surfaces (23).

Effectiveness of three concentrations of NaOCl (6%, 3%, and 1%), 2% CHX, and BioPure MTAD on apical dentin biofilms was evaluated Clegg et al. (2006). Their findings indicated that 6% NaOCl was the only irrigant capable of both rendering the bacteria nonviable and physically removing the biofilm (24).

Ozdemir et al. (2010) demonstrated that combination of EDTA and NaOCl significantly reduced the amount of intracanal biofilm in both young and old age groups. However, the bacterial counts of E. faecalis in the old group were still higher (25). Soares et al. revealed that the irrigation regimen based on the alternating use of NaOCl and EDTA seems to be promising for elimination of root canal E. faecalis biofilms (26).

Naturally occurring substances with antibiofilm effects have been suggested for treatment of biofilm related diseases, including caries and chronic wound infections (27). Examples of these substances include those that target bacterial attachment (lactoferrin and salicylic acid) and those that block formation or cause degradation of the biofilm matrix (xylitol and farnesol). By
partially disrupting the biofilm structure, the remaining bacteria can become more vulnerable to antimicrobial agents. Therefore, substances that affect biofilm biomass may be of great utility for the treatment of biofilm infections.

The antibacterial effect of triple antibiotic paste (TAP), double antibiotic paste (DAP), and calcium hydroxide \[\text{Ca(OH)}_2\] against Enterococcus faecalis and Porphyromonas gingivalis biofilm was compared by Alaa H.A. Sabrah et al. (2013). Biofilm formation by these bacteria was significantly decreased with TAP and DAP at all tested dilutions \((P<.0001)\) compared with control groups; however, TAP and DAP biofilm formation were not significantly different from each other. \[\text{Ca(OH)}_2\] significantly decreased bacterial biofilm formation compared with the control, but it was significantly less than TAP and DAP \((P<.05)\). Thus, both TAP and DAP were more effective than \[\text{Ca(OH)}_2\] against E. faecalis and P. gingivalis. DAP can be considered an effective and comparable antibacterial substitute for TAP (28).

**Effects of Ozone, Sonic-Ultrasound agitation and Non-equilibrium Plasma on Endodontic Biofilms**

A variety of different measures have been used to eradicate root canal bacteria, including chemomechanical instrumentation techniques, irrigation, and intracanal medicaments. However, it has been shown that complete eradication of microorganisms is difficult to achieve. Therefore, new methods are continuously being developed to eradicate all bacteria and secure healing.

Ozone is thermodynamically unstable short-lived oxygen decomposes to pure oxygen and generates oxygen-free radicals when in an aqueous solution. It is a strong oxidizing agent that causes lipid peroxidation and alters membrane permeability and function. The bactericidal and virucidal properties of ozone are well recognized (29) and have shown to reduce the level of viable E. faecalis in dentine tubules. Several endodontic pathogens including E. faecalis are rendered nonviable upon exposure to 2 and 4 ppm (mg/L) ozone in water (30). The main limiting factor in conventional irrigation is the complexity of the root canal anatomy, the ultrastructure of the dentin, and the characteristics of the bacterial biofilms (31,32). Attempts to surmount these limitations have recently led to renewed interest in the application of sonic and ultrasonic agitation of the irrigants within the root canal system.

In a study by Andrew Halford et al. (2012), effects of microbubble emulsion (ME) combined with sonic or ultrasonic agitation on irrigation dynamics and reduction of biofilm bacteria within root canal models were examined. Enhanced bubble dynamics and reduced E. faecalis biofilm bacteria beyond the level achieved by sonic or ultrasonic agitation of \[\text{NaOCl}\] suggested a synergistic effect of ME combined with ultrasonic agitation (33).

Plasma is the fourth state of matter after solid, liquid, and gas and is an ionized gas containing free charge particles (electrons and ions), active radicals, and excited molecules (34). Nonequilibrium plasma exists at atmospheric pressure and room temperature and does not inflict thermal damage to nearby objects. Recently, nonequilibrium plasma has attracted particular interest because it can be used for disinfection when in direct contact with living tissue.

The preliminary results of nonequilibrium plasma against endodontic bacteria in planktonic culture and biofilms have been promising (35). Du et al (2012) reported that the killing activity of non equilibrium plasma against Enterococcus faecalis biofilm was similar to 2% chlorhexidine digluconate (CHX) (36).

The efficacy of hand, rotary nickel-titanium, and self-adjusting file (SAF) instrumentation in biofilm bacteria removal was quantified by James Lin et al. (2013). It was found that a smaller area remained occupied by bacteria after the use of the SAF compared with the ProFile and the K-file (3.25%, 19.25%, and 26.98%, respectively). SAF reduced significantly more bacteria within the apical groove. However, no technique was able to remove all bacteria. This biofilm model represents a potentially useful tool for the future study of root canal disinfection (37).

**Effect of lasers on biofilms**

The specific characteristics of laser light, together
with the possibility that light can better reach the intricacies of the root canal system, have raised interest in its use for root canal disinfection. Both the use of high-power lasers and the combination of low-power laser light with a photosensitizer have been proposed for this purpose.

The effect of photodynamic therapy (PDT) on endodontic pathogens in planktonic phase as well as on E. faecalis biofilms in experimentally infected root canals of extracted teeth was investigated by Soukos et al. (2006). Strains of microorganisms were sensitized with methylene blue (25 μg/ml) for 5 min, followed by exposure to red light of 665 nm with an energy influence of 30 J/cm². Methylene blue fully eliminated all bacterial species with the exception of E. faecalis (53% killing). The same concentration of methylene blue in combination with red light (222 J/cm²) was able to eliminate 97% of E. faecalis biofilm bacteria in root canals using an optical fiber with multiple cylindrical diffusers that uniformly distributed light at 3600 (38).

The antimicrobial efficacy of two high power lasers (Nd:YAG and Er:YAG) and two commercial antimicrobial photodynamic therapy (aPDT) systems with that of sodium hypochlorite (NaOCl) action on Enterococcus faecalis biofilms grown on dentine discs was compared by Meire MA et al. (2012). Within the limitations of this particular laboratory set-up, NaOCl was found to be the most effective in E. faecalis biofilm elimination, while Er:YAG laser treatment (100 mJ pulses) also resulted in high reductions in viable counts. The use of both commercial aPDT systems resulted in a weak reduction in the number of E. faecalis cells. Nd:YAG irradiation was the least effective (39).

**Conclusion**

The fact that surface-associated growth of microorganisms is the cause of most infections has put an emphasis on virulence properties and survival strategies of biofilm bacteria. Given that most of our current knowledge about the microbial behavior of root canal bacteria originates from research using pure cultures, grown in nutrient-rich media under optimal conditions, extrapolation of results from such conventional studies to the real-life situation can be highly misleading. Thus, one future challenge for research in endodontology is to assess virulence expression in in vivo and in situ models with microenvironments resembling the real-life condition in the root canal.

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