Biofilms in endodontic infections

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Biofilm biology has become an expanding field of research in human, industrial and environmental ecosystems. The knowledge accumulated suggests that organisms growing in biofilms develop properties different to those dwelling in the planktonic state. On surveying the endodontic literature it is obvious that this realization and the fact that biofilms afford the resident microorganisms protection/resistance against harmful exogeneous influences including anti-microbial agents, is rather new to endodontology. Hence, the conditions under which biofilms occur in endodontic infections, and the measures that ought to be taken for their eradication, are not well understood. In this review the biofilm concept is presented and how it may apply to endodontic infections is discussed.

Introduction

The term biofilm was introduced to designate the thin-layered condensations of microbes (e.g. bacteria, fungi, protozoa) that may occur on various surface structures in nature. 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 substrate where planktonic microorganisms prevail in a water-based solution. In dental contexts, a well-known and extensively studied biofilm structure is established during the attachment of bacteria to teeth to form dental plaque. Here, bacteria free in saliva (planktonic organisms) serve as the primary source for the organization of this specific biofilm (1).

The excretion of adhesive substances viz. polysaccharides and proteins is crucial for the initial attachment of organisms as well as for holding the biofilm bacteria together. The structure per se will then provide protection and may allow a better resistance to adverse external influences for the organisms incorporated as compared with the planktonic state (2). Phenotypically the organisms may also take on a different character. In addition, 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 (3).

As far as endodontic infections are concerned, the biofilm concept has thus far gained limited attention. It has been discussed mainly within the framework of bacterial appearances on root tips of teeth with non-vital pulps (4–8). Such bacterial aggregations have been thought to be the cause of therapy-resistant apical periodontitis (4, 9). Although not described in great detail, bacterial condensations on the walls of infected root canals have been observed (10, 11) suggesting that mechanisms for biofilm formation may also exist inside the root canal space. In fact, biofilms have been experimentally produced in root canals of extracted teeth with mixed cultures of anaerobic bacteria (12) or pure cultures of Enterococcus faecalis (13, 14). Regardless of whether they are present on the external root surface or inside root canals, aggregations of microorganisms in biofilms are likely to have distinct clinical implications especially from a treatment point of view. 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.

Biofilm formation occurs at surfaces in aquatic systems in nature

In any natural environment, macromolecules and microorganisms are rarely just free floating but have a strong tendency to become associated with surfaces.
and form adherent microbial communities. Thus, biofilm formation will occur at surfaces in any system that comes in contact with natural liquid (15). Although the structural organization of biofilms, the composition and activities of the colonizing microorganisms in various environments may be different, the establishment of a micro-community on a surface seems to follow essentially the same series of developmental stages, including deposition of a conditioning film, adhesion and colonization of planktonic microorganisms in a polymeric matrix, co-adhesion of other organisms, and detachment of biofilm microorganisms into the surroundings (Fig. 1).

Over the last decades, numerous in vitro and in vivo studies of dental plaque have significantly contributed to our present knowledge of biofilm formation and microbial community interactions taking place in biofilms (1, 16). The earliest stage of biofilm formation involves the adsorption of macromolecules in the planktonic phase to the surface, leading to the formation of a conditioning film. On tooth surfaces, the conditioning film is comprised of proteins and glycoproteins from saliva and gingival crevicular fluid, and some secreted microbial products (17). This conditioning film is always formed prior to the arrival of microorganisms and selectively promotes adhesion of certain microorganisms. Numerous microorganisms in the planktonic phase will be transported to the surface but it is the properties of the conditioning film that determine those microorganisms, which attach, and thereby influence the microbial composition of the biofilm. The second stage involves adhesion and co-adhesion of microorganisms and attachment may be strengthened through polymer production and unfolding of cell surface structures. Often, in biological contexts, many organisms are involved in building up a biofilm structure and specific early colonizers seem to be crucial for the subsequent co-adhesion of other organisms. For example, streptococcal strains are among the early colonizers on tooth surfaces and appear to provide important features for the subsequent attachment of both Gram-positive and Gram-negative organisms (18). The third stage involves multiplication and metabolism of attached microorganisms that ultimately will result in a structurally organized mixed microbial community. During this stage the inherent characteristics of the microorganisms and the nature of the microenvironment influence growth and succession of microorganisms in the biofilm.

The rate of detachment of microorganisms from dental biofilms is not clear. Keeping in mind that the number of microorganisms in the planktonic phase (saliva) averages ten to hundred million per milliliter and that these microorganisms originate from dental and soft tissue biofilms, the detachment of microorganisms should be seen as a continuous process during development. It is known from in vitro studies that monolayers of oral bacteria release enzymes that mediate their detachment (19). Thus, it is likely that localized detachment of microorganisms starts after initial adhesion and increases with time as it is related to the number of microorganisms present in the biofilm. The fact that microorganisms detach regularly has implications for their spreading and colonization to other sites.

When viewed by confocal laser scanning microscopy, significant structural features of mature biofilms

Fig. 1. Stages of biofilm formation.
include micro-colonies of microorganisms embedded in a matrix that contains open water channels within which a flow of bulk water occurs. This open architecture has also been demonstrated for dental biofilms with channels transversing from the enamel surface to the oral cavity (20, 21) contrasting earlier documentation by electron microscopy. According to live/dead stains, the vitality of microorganisms varies throughout the biofilm with the most viable bacteria lining the channels (22). The suggested open structure has implications for the penetration and distribution of nutrients and metabolic end products within the biofilm.

There are several benefits of a community lifestyle for biofilm bacteria. For example, bacteria functioning together in biofilms are able to degrade large complex nutrient molecules that could not be efficiently degraded by an individual bacterium. Glycoproteins and proteins supplied by saliva or gingival crevicular fluid act as the primary source of nutrients for the microbial communities of dental biofilms (23). The breakdown of these nutrients requires sequential action of a range of proteases, peptidases and glycosidases. Distinct oral bacterial species possess restricted repertoires of these extracellular enzymes and consequently grow poorly in the presence of complex nutrients in monospecies cultures. In contrast, higher biomasses can be reached when groups of oral bacteria with complementary extracellular enzyme profiles co-operate to degrade macromolecules (24). Similar nutrient conditions are likely to prevail in infected root canals of non-vital pulps and be tempered by variations in nutritional streams. Whether microorganisms in the root canal cooperate as true consortia remain to be investigated. However, this would explain why biofilm populations in root canals survive periods of starvation and recover rapidly following access to increased nutritional supply.

The phenotype of biofilm bacteria is distinct from that of planktonic bacteria

Today, considerable evidence exists to show that the physiological properties of bacteria in biofilms are different from those of the same bacterium in liquid culture. Certain bacterial species have been found to display new, and more virulent types when growing in biofilms, and most importantly, bacteria within biofilms have an inherently increased resistance to antimicrobial agents compared with the same bacteria grown under planktonic conditions (25–27).

Much current research is focused on modification of bacterial functions related to the transition from the planktonic to the biofilm state. It is becoming increasingly clear that the adhesion of microorganisms to a surface triggers an altered expression of a large number of genes and their phenotypes are thus changed. The character of such surface-induced changes is dependent both on the microorganisms and the nature of the surface involved. In this context, one of the most studied microorganisms is Pseudomonas spp. Early research, using reporter gene technology, showed that attachment to a glass surface up-regulated the gene for alginate production within 15 min resulting in more firmly attached cells (28). Furthermore, adhesion was found to be accompanied by dramatic quantitative alterations of protein synthesis, as well as, synthesis of novel proteins expressed only in adherent cells (29). Similar surface-associated shifts in gene and protein expressions have been reported for oral bacteria. For example, the exposure of Streptococcus gordonii to saliva results in induction of genes encoding proteins that binds to salivary glycoproteins (30). Thus, it is possible that attachment per se gives rise to new phenotypes in dental biofilms. However, altered gene expressions and protein synthesis, i.e. new phenotypes, might also arise because of local environmental conditions within the biofilm. For example, in Streptococcus mutans, the genes associated with synthesis of extracellular polymers were found to be differently expressed in mature biofilm compared with initial (31). The authors concluded that this was an effect of environmental pH and amount of carbohydrate in the biofilm (31).

It is evident that oral microorganisms have the capacity to respond and adapt to changing environmental conditions (1). Individual microorganisms are able to sense and process the chemical information from the environment and thereby adjust their phenotypic properties. As biofilms form on a surface, the nature of the biofilm gives rise to diverse physical-chemical gradients with examples including the concentration of nutrients, metabolic end products, oxygen, growth factors and biocides (24). With respect to the environmental heterogeneity within biofilms, it is likely that a particular biofilm harbours a number of different phenotypes of a single species (Fig. 2).
Quorum sensing, a bacterial cell-to-cell communication mechanism for controlling cellular functions, is of particular interest because of the presence of dense aggregates of bacteria in biofilms. The signaling is mediated by diffusible molecules which, when present in sufficient concentrations, serve to modify gene expression in neighboring microorganisms. Quorum-sensing signaling is known to be involved in the regulation of several microbial properties, including virulence and the ability to form biofilms, incorporate extracellular DNA and cope with environmental stress (32). The known peptide-signal molecules produced by oral streptococci are primarily used for intra-species communication. However, other signals of unknown nature and actual function released by oral microorganisms serve as inter-species communication (33). Because many of the oral bacteria found in root canals, e.g. *S. gordonii*, *Streptococcus mitis*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Prevotella intermedia* possess the ability to communicate through quorum sensing, it is likely that signal molecules operate as additional environmental factors that alter gene expression to optimize phenotypic properties of biofilm bacteria in root canals.

**Oral diseases as consequences of ecological changes in biofilms**

While seemingly a contradictory quality, dental biofilms are essential for maintenance of both oral health and oral disease conditions. Indeed, caries, gingivitis and chronic periodontitis are caused by commensal microorganisms and not by classical microbial pathogens. Currently their development is considered to be a consequence of ecologically driven imbalances in dental microbial biofilms (34). In the case of caries for example, a low pH environment caused by microbial fermentation of carbohydrates selects populations of acid-tolerant and acid-producing strains that in turn increase acid formation and may result in demineralization of the tooth structure. In the case of marginal periodontitis, accumulation of dental plaque enhances inflammation and increases the flow of gingival crevicular fluid. This environmental change may favor growth of various proteolytic bacteria, which outcompete other members of the micro-community to become pathogenic by virtue of a numerical dominance. As far as endodontic infections are concerned the flare-up lesion could have a similar mechanism. Hence, 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, which promote growth of proteolytic bacteria. Several such instances are likely to occur. Following the initial instrumentation of a primary infected root canal, injury of the periapical tissue by over-instrumentation may release entry of inflammatory exudates into the root canal and cause growth of proteolytic bacteria that may not 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 (35). 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 (36) may not only bring nutritional elements that can revive dormant microorganisms in unfilled spaces of the root canal system but also new organisms.

**Evidence for biofilm structures in endodontic infections**

While the oral cavity is the common source for microorganisms that may colonize root canals after loss
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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 (10). On the basis of transmission electron microscopy (TEM) he examined the root canal content of 31 teeth, which had gross coronal caries and to which the periapical inflammatory process was attached upon extraction. He noted, in addition to his observations of the microstructure of the inflammatory tissue, that the major bulk of the organisms existed as ‘loose collections’ of cocci, rods, filaments and spirochetes. While most of these organisms appeared suspended, in what he felt was a moist canal space, dense aggregates were also observed sticking to the canal walls and forming thin to thick layers of bacterial condensations. Amorphous material filled the inter-bacterial spaces and was interpreted as an extracellular matrix of bacterial origin. When they occurred, the bacterial condensations showed a palisade structure similar to the one for dental plaque on external tooth surfaces, suggesting similar mechanisms for bacterial attachment as those for dental plaque (37).

Emploing a similar set of extracted teeth as Nair (10), Molven et al. (11) noted by scanning electron microscopic (SEM) observations of the apical 2 mm of infected root canals that cocci and rods and/or filaments often formed micro-colonies 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 (37).

Sen et al. (38) examined untreated extracted teeth with apical periodontitis by SEM. The root canals were heavily infected and microorganisms were observed ‘in all areas of the canals’. Cocci and rods predominated and formed colonies on the root canal walls and also, to a varying degree, penetrated the dentinal tubules. However, the pictures displayed did not reveal any typical biofilm structure. Fungal hyphae structures were seen in four of the 10 specimens examined.

To these reports should be added some recent efforts at elucidating mechanisms that may allow binding of *E. faecalis* to the inner dentin walls of root canals. Utilizing autoclaved root specimens of extracted teeth, Hubble et al. (14) provided evidence for a significant role of collagen-binding protein and serine protease produced by this organism. In another report from the group it was noted that pure cultures of *E. faecalis* added to calcium hydroxide medicated and non-medicated root canals were able to form a biofilm structure on the canal walls (13). The authors suggested that biofilm formation may be a mechanism, which allows this organism to resist treatment.

Better described are biofilm-like structures on the external root surface of the apical portion of extracted teeth with infected root canals. In a study of cases resisting treatment, the so-called refractory endodontic cases, Tronstad et al. (4) examined the surfaces of the root tips removed during surgical intervention by SEM. They noted that the apex of the roots adjacent to the apical foramen was coated with a continuous, smooth, structure-less layer containing a variety of bacterial forms. In irregularities of the surfaces and in crypts and holes, bacteria were seen held together by an extracellular material. The organisms were identified as cocci and rods with some presence of fibrillar forms.

In primary endodontic infections, bacterial aggregations may also occur on the external root surface in the foraminal area although their prevalence is not well established. Lomcali et al. (8) used SEM to scan the outer part of root tips of teeth affected by asymptomatic apical periodontitis lesions. Near to and at the exit of the foramen as well as in resorption lacunae, dense chains of bacteria and multilayered bacteria embedded in a heavy extracellular matrix were seen suggesting that, under these conditions, host defense mechanisms are unable to hold back the microorganisms in the root canal space. It was proposed that this ‘structureless smooth material’ may be an important factor sustaining periapical inflammatory lesions and that special treatment precautions ought to be taken for its removal. The type of organisms embedded in this material is not well studied although spherical yeast cells have been identified (8), see further the paper by Waltimo et al. in this volume of Endodontic Topics).

Siqueira and Lopes (6) observed 26 extracted teeth with asymptomatic periradicular lesions. All teeth had extensive caries, radiolucent areas and attached peri-apical tissue processes. SEM showed cocci and rods restricted to the root canal and in only one tooth were bacteria seen beyond the apical foramen. Dense bacterial aggregates were detected close to the apical foramen in only one case, while few bacterial cells were commonly visualized in this area of the other specimens. Most bacteria appeared suspended in the fluid phase of the root canal. It was remarked that the presence of bacteria at or outside the apical foramen...
may not necessarily be a true condition, but rather a function of extrusion of bacterial colonies during tooth extraction. Based on Siqueria’s and Lopes findings, extraradicular infection in terms of root tip aggregations may not be a common occurrence in untreated teeth with infected pulps. The findings by Walton and Arjmand (39) in an experimental study support this view. They induced periapical lesions in monkeys by exposing pulps to the oral environment and observed the position of the bacterial front by histology and Gram stain. In none of the cases were stainable bacteria seen in the lesion per se or on the external root surface after 7 months of exposure to the experimental infection. Two canals of 18 apices demonstrated bacterial masses at the foramen.

Noiri et al. (7) analyzed the presence of biofilm formations on root tips of extracted teeth with ‘refractory periapical pathosis’ and gutta-percha points removed during endodontic treatment by SEM. Gutta-percha points sticking out through the apex were almost completely covered with glycocalyx-like structures. Bacteria, mostly filaments or long rods, were seen on the external root surfaces in the extracted teeth. Leonardo et al. (5) used SEM to compare the root tips of extracted teeth with various pulpal conditions including vital pulps, necrotic pulps without apical lesion and necrotic pulps with apical lesions. They described biofilm formations only on teeth with apical periodontitis. These biofilms were composed of different bacterial morphotypes viz. cocci, bacilli and filaments. Finally, Ferreira et al. (9) recently described one re-treatment case of a maxillary premolar. The tooth had a fistula and a periapical lesion. SEM identified cocci and fungal forms on the resected root tip and in resorption lacunae. A true biofilm structure of the bacterial mass was, however, not confirmed.

These data suggest that the frequency and the conditions under which apical biofilm formations occur are not well understood. Judging from the relatively few case descriptions published, massive infections of the root canal space may be an important precondition, for example, after long-standing oral exposures from caries, poorly treated root canals, or fistulous tracts. Research should address the common denominators for these bacterial aggregations, as they are likely to pose a significant clinical treatment problem.

In conclusion, scanning the literature for descriptions of biofilm structures in endodontic infections indicates that some observations have been made using TEM and SEM. Yet a more systematic analysis of how microorganisms settle in and become organized topographically in and outside the root canal space following pulp necrosis has not been undertaken.

**Hypothesis for root canal biofilm formations**

It is reasonable to assume that the preconditions for biofilm formation in the root canal vary depending on the cause of the pulpal breakdown. An ischemic injury by trauma, leading to pulpal necrosis, is likely to provide totally different prerequisites for the colonization phase than in a caries exposure of the pulp. In the latter case, the inflammatory lesion front may recede successively towards the apex, possibly in bursts, and provide the fluid vehicle by which invading planktonic organisms can multiply and start attaching to the root canal walls.

Figure 3 shows initial bacterial infiltrations of a pulp following a caries exposure, but before any biofilm formation has been established. The events that follow this initial penetration of the pulp can only be speculated upon as it is virtually unknown how the organisms attach and spread further along the root canal. Possibly, it is first after biofilm formation that the infectious process gains sufficient power to cause subsequent destruction of the pulpal tissue. At some point in the breakdown process, however, there has to come to a steady state where the bacterial mass is held up by host defense mechanisms. The demarcation zone may be inside the root canal near the root canal exit (40, 10, 41), at the foramen (10) or as demonstrated by SEM (4, 5, 7, 8) on the external root surface near the exit of the foramen to the periapical tissue environment. It is not unreasonable to assume that organisms may be detached from these positions and occasionally congregate in the lesion per se (42), for a review see (43). It is obvious that most of the lesions observed in Nair’s (10) study were of a rather acute nature as indicated by the observations of heavy infiltrates of PMN’s involved in active phagocytosis of bacterial organisms near the canal exit. Thus the fluid phase for these biofilm-like aggregations may have been exudate derived from the inflammatory lesion front. However, root canals may not always be fluid-filled and the canals of teeth with necrotic pulps often appear dry on entry, at least in their coronal portion. Hence the question remains as to whether bacterial condensations in a
biofilm structure can develop or be retained in sites of the root canal system other than near the bacteria/inflammatory interface zone, where host-derived proteins and bacterially produced adhesive substances may provide the proper prerequisites. Figure 4 demonstrates what appears to be a distinct biofilm structure on the inner walls of root canals in a clinical specimen. Yet, little effort has so far been devoted to studying the pattern by which root canal bacteria may become attached to the root canal walls in progressing pulpal breakdowns and a review of the endodontic literature reveals far from conclusive evidence as to the presence of biofilms in infected root canals.

Anti-microbial agents and biofilms

Central to the theme of biofilm control is the use of surfactants, anti-microbial agents and preservatives. Anti-microbial agents have often been developed and optimized for their activity against fast growing, dispersed populations containing a single microorganism. However, microbial communities grown in biofilms are remarkably difficult to eradicate with anti-microbial agents and microorganisms in mature biofilms can be notoriously resistant for reasons that have yet to be adequately explained. There are reports showing that microorganisms grown in biofilms could be two- to 1000-fold more resistant than the corresponding planktonic form (44, 45). With respect to oral bacteria, the biofilm inhibitory concentrations for chlorhexidine and amine fluoride are 300 and 75 times greater, respectively, when Streptococcus sobrinus is grown as a biofilm compared with the minimum bactericidal concentration for planktonic cells (46). Biofilms of oral bacteria have also been found to be more resistant to amoxycillin, doxycycline and metronidazole (47).
Recalcitrance of biofilm communities to anti-microbials is attributed to a variety of mechanisms (44, 48). 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. In addition, the slow growth rate of microorganisms in established biofilms can result in cells being more resistant to the agent than faster dividing cells. Biofilm bacteria may also display a distinct phenotype that accounts for the enhanced resistance. For example, biofilm bacteria might not express the drug target or use different metabolic pathways than planktonic bacteria.

It is obvious from a survey of the endodontic literature that these relationships have not been taken into proper consideration, when anti-microbials for root canal usage have been evaluated. An exception is a recent study by Spratt et al. (49). They used ‘a simple biofilm model to evaluate the effectiveness of a range of commonly recommended anti-microbial irrigants against monocultures of five root canal isolates’. P. intermedia, Peptostreptococcus micros, Streptococcus intermedius, F. nucleatum, E. faecalis. Cellulose nitrate membrane filters were placed on blood agar and inoculated with a suspension of brain heart infusion broth. After 48 h, membranes with attached organisms were exposed to one of four anti-microbial test agents or controls. NaOCl (2.25%), 0.2% chlorhexidine, 10% povidone iodine and 5 ppm colloidal silver were tested against a PBS control. NaOCl was the most effective anti-microbial followed by the iodine solution. However, as the nature and the influence of root canal surfaces on the expression of novel biofilm phenotypes have not yet been touched upon, conclusions and decisions reached on studies of monocultures on standard laboratory surfaces (membranes, glass, plastic) must be taken with great caution. Such models certainly do not reflect the real world and may give misleading data especially on the effects of anti-microbials on biofilm bacteria. Undoubtedly, 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.

Concluding remarks

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. The published literature clearly suggests that surface adherent communities pose potential health problems that are unrecognized by conventional laboratory culture methods. 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 endodontontology is to assess virulence expression in in vivo and in situ models with microenvironments resembling the real-life condition in the root canal. Knowledge about the microbial composition will not be enough because the same consortium of bacteria is likely to express different phenotypes depending on the heterogenous microenvironment. It may even be questioned if further exploration of the root canal microbiota, for example, by extended genotypic analyses, is meaningful unless the properties and behavior of the organisms in a biofilm context are addressed simultaneously. No doubt, the application of the biofilm concept to endodontic microbiology will play a crucial role in helping us to understand, not only the pathogenic potential of the root canal microbiota, but also the basis for new approaches to infection control. How bacteria adapt their properties under different disease conditions as well as how biofilms are organized in root canals are important issues to be addressed on the road to a clearer understanding of how the root canal bacteria resist endodontic treatment measures.

References


