Therapeutic Strategies Targeting Cariogenic Biofilm Microenvironment

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Abstract

Cariogenic biofilms are highly structured microbial communities embedded in an extracellular matrix, a multifunctional scaffold that is essential for the existence of the biofilm lifestyle and full expression of virulence. The extracellular matrix provides the physical and biological properties that enhance biofilm adhesion and cohesion, as well as create a diffusion-modulating milieu, protecting the resident microbes and facilitating the formation of localized acidic pH niches. These biochemical properties pose significant challenges for the development of effective antibiofilm therapeutics to control dental caries. Conventional approaches focusing solely on antimicrobial activity or enhancing remineralization may not achieve maximal efficacy within the complex biofilm microenvironment. Recent approaches disrupting the biofilm microbial community and the microenvironment have emerged, including specific targeting of cariogenic pathogens, modulation of biofilm pH, and synergistic combination of bacterial killing and matrix degradation. Furthermore, new “smart” nanotechnologies that trigger drug release or activation in response to acidic pH are being developed that could enhance the efficacy of current and prospective chemical modalities. Therapeutic strategies that can locally disrupt the pathogenic niche by targeting the biofilm structure and its microenvironment to eliminate the embedded microorganism and facilitate the action of remineralizing agents may lead to enhanced and precise anticaries approaches.

Keywords: EPS, matrix, acidic pH, dental caries, nanotechnology, antibiofilm therapeutics

Introduction

Biofilms: More Than Just a Cluster of Bacteria

Although early studies mainly focused on microbial composition of biofilms, it is now clear that the residing organisms are embedded and surrounded by an extracellular matrix of polymeric substances, such as exopolysaccharides, proteins, and nucleic acids (Flemming et al. 2016). The critical roles of the matrix for microbial interactions and virulence, as well as for antimicrobial tolerance, are being increasingly recognized. The matrix production enhances bacterial cell adhesion and cohesion (resulting in densely packed cell aggregates), providing mechanical stability. Furthermore, its diffusion-modulating properties help create chemical and nutrient gradients within the biofilm, while providing a protected environment against antimicrobials (Fig. 1). Thus, the matrix allows cells to organize into multicellular ecosystems, where resident microorganisms interact within heterogeneous yet compartmentalized microenvironments, including pH and redox, modulating microbial composition and virulence (Flemming et al. 2016; Koo and Yamada 2016). The matrix mediates the assembly of cariogenic oral biofilms that “cling” on teeth despite exposure to shear forces and form acidic microenvironments despite being “bathed” by buffering saliva.

The Cariogenic Biofilm and Its Complex Biochemical Microenvironment

Dental caries is a biofilm-induced disease that causes the destruction of the mineralized tooth. The presence of microorganisms is

necessary, but not sufficient, to cause dental caries because the formation of cariogenic biofilms is dependent on the host diet. In the mouth, the microbial interactions start with early colonizers that can rapidly adhere to the pellicle-coated tooth surface and then coadhere with other microorganisms. During this process, the various species interact physically and metabolically to shape the initial biofilm community. Certain interactions are beneficial as commensals (e.g., *Streptococcus gordonii* and *Streptococcus salivarius*) can compete against cariogenic bacteria (e.g., *Streptococcus mutans*) by secreting hydrogen peroxide and bacteriocins as “chemical weapons” or counter the deleterious effects of acidification by producing alkali (Liu et al. 2012; Merrit and Qi 2012; Qi and Kreth 2017). However, the balance between commensals and pathogens can be disrupted by frequent sugar consumption and poor oral care (Fig. 1).

A diet rich in sugars fuels the assembly of the exopolysaccharides (EPS) matrix and enhances the accumulation of an acidogenic-aciduric microbiota (Takahashi and Nyvad 2011;

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Figure 1. The matrix and targets of current and prospective antibiofilm approaches. The matrix is a “multifunctional scaffold” essential for biofilm lifestyle, which benefits its entire life cycle from initial microbial colonization and accumulation to biofilm maturation. Therapeutic strategies can be designed to (I) promote healthy oral communities on teeth by favoring the colonization and/or fitness of commensal organisms against cariogenic pathogens. If conditions are conducive to development of dental caries (e.g., sugar-rich diet), exopolysaccharides (EPS)–mediated bacterial adhesion-cohesion and further accumulation ensues, (II) promoting the initiation of cariogenic biofilms (III), which would require EPS-targeting strategies in addition to antimicrobials against increasing proportions of cariogenic pathogens. Once mature biofilm is established (IV), more aggressive measures, including physical disruption and EPS matrix degradation combined with antimicrobials, may be needed to enhance access and killing efficacy against the embedded bacteria.

Koo et al. 2013). Cariogenic bacteria such as *S. mutans* has an exceptional capacity to use a wide variety of carbohydrates to produce EPS and acids and to reside within biofilms (Takahashi and Nyvad 2011; Koo et al. 2013; Moya et al. 2014). When sucrose is available, EPS-producing exoenzymes such as *S. mutans*–derived glucosyltransferases (Gtfs) present in the pellicle and also bound to different microorganisms (including commensal streptococci, *Actinomyces* spp., *Candida albicans*) produce large amounts of glucans in situ (Paes Leme et al. 2006; Bowen and Koo 2011). The surface-formed EPS provide avid binding sites for adhesion and coadhesion, which mediates bacterial clustering on the tooth surface, while assembling a polymeric matrix that embeds the cells as observed in microscopic images of cariogenic human plaque (Hajishengallis et al. 2017). Although EPS are major matrix components, extracellular DNA (eDNA), amyloid-like proteins, and host-derived proteins/glycoproteins have been also identified (Paes Leme et al. 2006; Klein et al. 2015).

The matrix significantly changes the physicochemical characteristics of the biofilm, modifying the diffusion properties and enhancing adhesive strength, making biofilm recalcitrant

to antimicrobials and difficult to remove from surfaces (Peterson et al. 2015). If the biofilm remains on teeth and the consumption of carbohydrate-rich diets persists, the amount of EPS and extent of acidification increase. The diffusion-modifying properties of the matrix combined with metabolic activities of embedded organisms help create highly acidic pH microenvironments (Guo, McLean, Lux, et al. 2015; Hwang et al. 2016). Such conditions elicit ecological and structural changes that favor acid producers and EPS-binding microbes that can synergize with each other, while also benefiting those that use lactate as a carbon source (e.g., *Veillonella*) (Hajishengallis et al. 2017). However, if the acidified microenvironment persists, the microbial diversity is further reduced where acid-sensitive species perish and even more potent acidogenic-aciduric microbiota flourish (Takahashi and Nyvad 2011; Marsh and Zaura 2017). The low-pH condition at the tooth-biofilm interface promotes demineralization of the enamel, leading to the onset of cavitation, a late manifestation of the disease that requires costly intervention and restoration. Thus, dental caries is a result of profound changes in the biofilm structure and microbial ecology, where diet plays a key

role by fueling EPS-producing pathogens (*S. mutans*) to build up “a cariogenic habitat.” In turn, ecological changes ensue within a protected milieu where polymicrobial synergies promote localized acidification and demineralization of the tooth surface. Further understanding of these processes would help develop new and effective antibiofilm/anticaries therapeutics.

Challenges and Limitations for Current Antimicrobial Approaches

Despite significant advances in the prevention of dental caries, particularly with the use of fluoride, controlling cariogenic biofilms remains challenging. Major hurdles include the following: 1) microorganisms within biofilms are enmeshed and protected in an EPS-rich matrix, making them difficult to kill or remove; 2) EPS-embedded microbes create highly acidic microenvironments that promote cariogenic biofilm buildup and reduce drug efficacy; and 3) topically applied agents are poorly retained on teeth or within biofilms due to rapid clearance in the mouth. For example, the EPS can bind cationic agents such as chlorhexidine and antimicrobial peptides, preventing penetration into the deeper layers of the biofilm and thereby reducing bacterial killing efficacy (Hope and Wilson 2004; Xiao et al. 2012; Liu et al. 2016). Hence, therapeutic approaches against cariogenic biofilms that could both disrupt the matrix and simultaneously kill the bacteria within biofilms with enhanced efficacy at acidic pH values would be desirable.

Current approaches against cariogenic biofilms are mostly limited to conventional, broad-spectrum antimicrobials that are incapable of degrading the protective matrix or affecting the physicochemical aspects of dental caries. Although capable of killing planktonic bacteria and reducing microbial accumulation on teeth, chlorhexidine is far less effective against established biofilms, does not prevent caries, and is not suitable for daily use due to adverse effects, including calculus formation and tooth staining (Autio-Gold 2008). Likewise, hydrogen peroxide (H_2O_2), a readily available antiseptic agent, also has antimicrobial effects yet limited activity on biofilms even at high concentrations (>3%). Thus, antimicrobials have limited efficacy for caries prevention or treatment if not combined with other modalities. Furthermore, frequent use of broad-spectrum antimicrobials may potentially cause ecological imbalance, increasing susceptibility to reinfection by opportunistic pathogens, including *S. mutans* (Guo, McLean, Yang, et al. 2015). Nevertheless, when conditions are not conducive for dental caries, currently available antimicrobials (including essential oils) can help maintain biofilm accumulation to levels compatible with a healthy mouth (Marsh 2012).

Conversely, fluoride is currently the mainstay of caries prevention (O'Mullane et al. 2016), but it does not offer complete protection (Featherstone and Doméjean 2012). Fluoride exerts its major effect by enhancing remineralization and reducing tooth enamel demineralization but has limited effects against biofilms despite inhibiting bacterial metabolism (Marquis et al. 2003). Alternatively, EPS-degrading enzymes like dextranase and mutanase can digest biofilm EPS matrix (Pleszczyńska et al. 2015; Liu et al. 2016) but have no antibacterial activity

and limited clinical efficacy when used alone in addition to practical issues, including high cost and low enzyme stability. Furthermore, natural products have shown multiple biological effects, including antibacterial, antiadhesion, and glucan synthesis inhibition, many of which demonstrated promising therapeutic effects using in vivo caries models (Jeon et al. 2011). However, it is a challenging approach owing to complex chemistry and composition variability of natural products as well as multistep isolation procedures to derive active compounds, although advances in analytical/chemical separation methods may mitigate these limitations.

Nanomaterials have received increased attention for biofilm control and caries prevention through intrinsic antibacterial properties, as a carrier to deliver bioactive molecules or even as remineralizing agents such as calcium phosphate nanostructures (Allaker and Memarzadeh 2014; Besinis et al. 2015). However, most antibacterial nanomaterials are designed to inhibit bacterial adhesion rather than disrupting existing biofilm. Moreover, these materials are not designed to break down the biofilm matrix or to specifically target the pathological environments found in cariogenic biofilms (with few exceptions). Therefore, new approaches for enhanced anticaries effects should eradicate biofilms locally and, at the same time, prevent acid dissolution of the adjacent tooth enamel.

Effective New Therapeutic Approaches Are Needed

Recent approaches have emerged to disrupt the biofilm microbial composition and the microenvironment (Fig 1). For example, an *S. mutans*-specific targeting approach was developed using synthetic antimicrobial peptide (AMP) consisting of dual-functionally independent moieties (termed C16G2): a broad-spectrum, novispirin-derived AMP “killing” region (G2) attached to a *S. mutans*-specific peptide pheromone that provides “targeting specificity” (C16) (Guo, McLean, Yang, et al. 2015). An increased rate of G2 accumulation on the bacterial surface, mediated by the targeting peptide, led to rapid and more selective killing of *S. mutans* versus other bacteria. This approach reduced the population of *S. mutans* from multispecies biofilm communities, while increasing the abundance of commensal streptococci, including *Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus sanguinis* (Guo, McLean, Yang, et al. 2015). Similarly, AMP containing a *S. mutans*-targeting domain to the ComC signaling peptide fused with a killing domain composed of broad-spectrum AMP pleurocidin also demonstrated enhanced specificity against *S. mutans* while sparing *S. sanguinis* or *Streptococcus gordonii* cells in vitro (Mai et al. 2011). Such targeted killing approach may remove specific pathogens from cariogenic biofilms to promote a “healthy-like microbiome” although in vivo studies are needed to further understand the mechanisms and its efficacy for caries prevention.

Another strategy to modulate biofilm microbiota ecology includes alkali production by specific oral bacteria using L-arginine (Liu et al. 2012). Presumably, the alkali-producing bacteria protect against plaque acidification and further growth/

dominance of cariogenic bacteria that thrive in acidic conditions which cause tooth demineralization (Kleinberg 2002). The use of exogenous amino acids to affect both biofilm metabolism and alkaligenic potential has been investigated (Nascimento et al. 2014; Kolderman et al. 2015; Koopman et al. 2017). L-arginine (L-Arg) serves as a substrate for alkali production by arginolytic bacteria (e.g., *S. gordonii*), which can neutralize acids and modulate pH homeostasis within clinical oral biofilms (Nascimento et al. 2014). Treatment of multispecies biofilms comprising *S. mutans*, *S. gordonii*, and *Actinomyces naeslundii* with L-Arg suppressed *S. mutans* growth and resulted in substantial reduction in insoluble EPS amounts and altered biofilm architecture (He et al. 2016). In addition to pH modulatory effects, L-Arg was also capable of repression of genes involved in the production of insoluble EPS and bacteriocin in *S. mutans* while increasing hydrogen peroxide production by *S. gordonii* (He et al. 2016). L-Arg also destabilized multispecies oral biofilms, reducing viability and increasing susceptibility to cetylpyridinium chloride (Kolderman et al. 2015). Thus, arginine-containing formulations can promote favorable modification in the microbiota, affecting cariogenic biofilm development.

Once cariogenic biofilm is allowed to form, the presence of extracellular matrix, with its local barriers and acidified microenvironment, reduces drug access and triggers bacterial tolerance to antimicrobials while enhancing its mechanical stability. High-velocity water sprays can both mechanically dislodge tightly adherent biofilms (including those in the interproximal space) and enhance delivery of antimicrobials by combining forced advection into the EPS matrix and physical restructuring (Fabbri et al. 2016). Recently, a low-cost approach using plant-made antimicrobial peptide (PMAMP)–Protegrin (PG1) was developed for topical use to control biofilms (Liu et al. 2016). PMAMP-PG1, with complex secondary structure (hairpin), rapidly killed *S. mutans* and impaired biofilm formation following a single topical application of the tooth-mimetic apatite surface. Importantly, a synergistic strategy using PG1 combined with EPS-degrading enzymes (dextranase + mutanase) was capable of digesting the protective matrix barrier of preformed biofilms, facilitating peptide access into the structure, and killing the exposed bacterial clusters (Liu et al. 2016). Biopharmaceuticals made via plant-chloroplast technology are affordable because they eliminate prohibitively expensive fermentation, purification, and cold transportation/storage (Kwon and Daniell 2015). Recent Food and Drug Administration (FDA) approval of plant-produced protein drugs augurs well for clinical advancement of this novel biofilm-disrupting approach.

Targeting the Complexity of the Cariogenic Biofilm Microenvironment

Given the complexity of the oral cavity, targeting the specific pathogenic microenvironments (or niches) using agents that are activated in response to low pH or bacterial products could be an attractive approach against cariogenic biofilms. Recent technological breakthroughs in nanoengineering provide new insights to develop novel nanomaterials to control biofilms.

For example, nanoparticles can be used to coat existing surfaces (e.g., teeth) and exogenously introduced surfaces (e.g., restorative or implant materials) for prevention of bacterial adhesion and cariogenic biofilm formation (Paula and Koo 2017). Specifically, functionalized nanoparticles can be conceived to carry and selectively release or activate antimicrobial agents at the surface of attachment or within oral biofilms. The latter mechanism includes “smart release or activation” of agents when triggered by pathogenic microenvironments (e.g., acidic pH) that could simultaneously kill bacteria and dismantle the biofilm matrix.

Recently, functional polymeric nanostructures (PNs) were developed for enhanced drug delivery when triggered by acidic pH values (Horev et al. 2015). These nanostructures are formed from diblock copolymers and 2-propylacrylic acid that self-assemble into cationic nanoparticles. By tuning the PNs’ outer corona surface, they display outstanding adsorption affinities to pellicle and EPS-coated apatitic surfaces due to strong electrostatic interaction (Horev et al. 2015) (Fig. 2). Owing to hydrophobic cores, these “nanocarriers” can encapsulate non-polar antibacterial drugs such as farnesol with high efficiency while making them soluble in aqueous solution due to hydrophilic outer corona. With this specific polymer conjugation, the nanoparticles undergo core destabilization and drug release in a pH-responsive manner, triggered by the acidification of the biofilm microenvironment in cariogenic conditions. These pH-activated polymeric nanocarriers enhanced the antibiofilm activity of farnesol by 4-fold (vs. free farnesol), significantly improving its efficacy against caries severity in vivo (>10-fold) under twice-daily topical treatment regimen.

Another promising approach employs pH-responsive catalytic inorganic nanoparticles (CAT-NPs) with antibiofilm and anticaries properties. Nanoparticles containing biocompatible iron-oxide (Fe_3O_4) with peroxidase-like activity were developed to catalyze H_2O_2 in a pH-dependent manner (high catalytic activity at low pH vs. no activity at neutral pH) (Gao et al. 2016), as shown in Figure 3. CAT-NPs can bind and retain within biofilms following topical treatment and maintain catalytic activity. Under acidic pH, the catalytic nanoparticles can generate free radicals from H_2O_2 in situ that simultaneously degrade the protective EPS matrix barrier and kill the embedded bacteria with exceptional efficacy within 5 min (>5-log reduction of viable cells vs. control and 5,000-fold more effective than 1% H_2O_2 alone) (Gao et al. 2016). Interestingly, CAT-NPs also reduced apatite demineralization, providing an additional pH-dependent mechanism to help prevent the development of dental caries. Notably, the components in CAT-NPs are biocompatible, while activation of H_2O_2 is triggered only under acidic (pathologic) conditions, which may alleviate safety concerns regarding unmitigated free radical production that could damage the host oral tissues at physiological pH values. Using 1-min topical daily treatments, CAT-NPs in combination with H_2O_2 suppressed caries development and prevented the onset of cavitation while sparing normal tissues in vivo (Gao et al. 2016). Considering that CAT-NPs can be produced at low cost on large scale and H_2O_2 is readily available and extensively used in over-the-counter products, the combination of CAT-NPs

Figure 2. Structure and function of pH-activated nanocarrier for antibiofilm drug delivery. Scheme depicting **(A)** the self-assembly of diblock copolymers that results in cationic polymeric nanoparticle carrier (nanocarrier) of around 21 nm. The polymer conjugation and its subsequent physicochemical features results in a positively charged hydrophilic outer surface and hydrophobic core, in which hydrophobic antibacterial agent (e.g., farnesol) can be incorporated. The pH-responsive core expedites drug release at acidic pH. **(B)** The nanocarrier has high affinity to both the pellicle and exopolysaccharide matrix (multisite affinity). Microchemical environment changes toward an acidic pH trigger farnesol release and bacterial killing within biofilms. Adapted from Horev et al. (2015) with the permission of the American Chemical Society. The nanocarrier design and biofilm diagrams were designed by Michael Osadciw/University of Rochester. EPS, exopolysaccharides.

and H_2O_2 has potential for development of feasible products for clinical use (albeit full toxicity studies should be conducted to examine the long-term effects of topical applications).

Antibiofilm technologies using environmental stimuli (e.g., pH) activation as exemplified above can be exploited for targeted therapy against dental caries. The presence of a diverse oral microbiota (where commensal organisms coexist with opportunistic pathogens) and surrounding mucosal tissues provide a major challenge to develop antimicrobial strategies against a particular species or a specific group of organisms. In this context, triggering antibiofilm activity in response to pathogenic microenvironments could specifically target where and when the cariogenic biofilms develop, which may lead to antiplaque/anticaries modalities with enhanced precision.

Conclusion and Future Perspectives

Despite great strides since the discovery of fluoride, dental caries continues to be a public health problem. Although effective in reducing tooth enamel demineralization, fluoride has limited

effects against biofilms. Current antimicrobials can help maintain microbial levels compatible with a healthy mouth but lack efficacy when conditions are conducive of dental caries. Indeed, treating cariogenic biofilms is challenging. The bacteria are often embedded in a protective EPS matrix that confers viscoelasticity, making them highly adherent, cohesive and difficult to remove from surfaces. Furthermore, the diffusion-modulating properties of the matrix provide a heterogeneous environment where the resident microbes become recalcitrant to antimicrobials, while polymicrobial interactions occur to cause localized acidification at the biofilm-tooth interface despite the presence of buffering saliva.

The physical and biological complexity of cariogenic biofilms must be taken into consideration when designing new therapeutic approaches. Conventional antimicrobial modalities are limited in addressing these biochemical properties, reducing clinical efficacy to prevent dental caries. In this context, prospective therapeutic strategies can be tuned to specifically target the biofilm microenvironment by targeting both the matrix and the embedded bacteria to eradicate the pathogenic

Figure 3. Schematics of biofilm disruption under acidic condition by catalytic nanoparticle (CAT-NP) activation of hydrogen peroxide (H_2O_2). (1) CAT-NPs bind and retain within biofilm despite brief topical exposure, making them suitable for a topical treatment regimen typically used in oral care products; (2) the enzyme-like activity is pH responsive, rapidly catalyzing low concentrations of H_2O_2 at acidic pH to produce free radicals that simultaneously (3) degrade biofilm matrix and (4) kill embedded bacteria. Furthermore, (5) CAT-NPs can reduce demineralization of tooth hydroxyapatite at acidic pH. EPS, exopolysaccharides.

niche with minimal cytotoxicity to surrounding tissues. Improved drug penetrability into existing biofilms can be achieved by degrading the matrix to enhance antibacterial efficacy and minimize generation of drug resistance.

New strategies with antibiofilm properties have been developed in recent years, including species-specific antimicrobial peptides targeting cariogenic pathogens, modulation of the biofilm pH via arginine metabolism, physical disruption by high-velocity water microspray, and synergistic combination of bacterial killing and EPS digestion. Furthermore, novel (nano) technologies have emerged using “stimuli-triggered release or activation” of agents in response to acidic pH values for enhanced selectivity and controlled drug delivery or potentiation in situ. Other exciting antibiofilm-specific approaches, including nanocoatings, surface modifications, and small molecules from bio/in silico screening, are emerging at a remarkable pace (Allaker and Memarzadeh 2014; Besinis et al. 2015; Paula and Koo 2017). Yet, deeper understanding of the mechanisms of action and toxicity using in vivo models and further efficacy validation in clinical studies are required. The presence of saliva that changes surface chemistries combined with poor retention of topically applied agents due to rapid clearance in the mouth poses yet another challenge. Future directions should focus on achieving maximal efficacy and targeting specificity with minimal toxicity and long-term therapeutic effects. These along with enhanced drug delivery/activation within pathogenic microenvironments may pave the way for an effective therapeutic strategy with high precision against cariogenic biofilms.

Author Contributions

Y. Liu, Z. Ren, contributed to conception and design, drafted the manuscript; G. Hwang, H. Koo, contributed to conception and

design, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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