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Antimicrobial Resistance in Oral biofilm: Challenges and Alternatives

Satish Kumar Sharma*, Shmmon Ahmad

Department of Pharmacology, Glocal School of Pharmacy, Glocal University, Mirzapur Pole, Saharanpur-247121, Uttar Pradesh, India

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ABSTRACT

Bacterial biofilm has been a major contributor to severe bacterial infections in humans. Oral infections have also been associated with biofilm-forming microbes. Several antimicrobial strategies have been developed to combat bacterial biofilms. However, the complexity of the oral cavity has made it difficult to use common drug treatments. Most effective ways to control normal bacterial infections are rendered ineffective for bacterial biofilms. Due to limited drug concentration availability, drug neutralization or altered phenotype of bacterial cells, different drug have been ineffective to identify the target cells. This leads to the development of the multifaceted phenomenon of antimicrobial resistance (AMR). Biofilm research done so far has been focused on using antimicrobial drugs to target molecular mechanisms of cells. The severity and resistance mechanisms of extracellular matrix (ECM) have been underestimated. The present study describes different antimicrobial strategies with respect to their applications in dental or oral infections. A prospective strategy has been proposed targeting ECM which is expected to provide an insight on biofilm obstinacy and antimicrobial resistance.

*Corresponding Author

Name: Satish Kumar Sharma
Phone: 9958166931
Email: satishdipsar55@gmail.com

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INTRODUCTION

Biofilms are one of the most obstinate forms of natural existence displayed by aggregated microbial communities. Microorganisms rarely persist as pure cultures, mostly they form microbial aggregates on biotic and abiotic surfaces. Most of the bacterial biofilms are pathogenic capable of causing more than 60% of chronic health infections (Jamal *et al.*, 2018). In 1985, J. W. Costerton had introduced the term 'biofilm'. The significance of biofilm infections got highlighted when aggregated form of *Pseudomonas aeruginosa* cells was observed in sputum samples and lungs of cystic fibrosis patients (Høiby, 2017). Some of the most common infectious diseases such as vaginosis, ear infections, gingivitis, endocarditis, catheter infections, chronic wound infections and UTIs (Urinary Tract Infections) have been found to be associated with biofilms formation.

Oral biofilms are aggregated community of microbes encased inside extracellular matrix stably organized on surface of teeth. These have been recognized as characteristic features of many oral diseases like periodontitis, peri-implantitis, dental caries and gingivitis. Bacterial attachment on surfaces involves a course of steps involving cell attachment, biofilms maturation and biofilm dispersal. The attachment of the primary layer of bacteria cells is mediated by different adhesive proteins released by bacteria. The attached cell layer self-organize into aggregates via secretion of different biomolecules such as proteins, polysaccharides, extracellular DNA (eDNA) to ensure structural stability. These biomolecules

collectively form an extracellular matrix (ECM) of biofilm which serves as niche for other bacterial cells. The process of biofilm formation occurs in response to several environmental stress. Therapeutic practices that can obstruct any of these steps are considered as a potential anti-biofilm approach.

As compared to non-living surfaces, the oral cavity is complex and due to salivary clearance, superficially applied antibacterial drugs lose their effect and desired concentration in a short span of time. Usually, bacterial biofilm is composed of two types of cells — planktonic cells and biofilm cells. The planktonic cells are usually far from ECM defence and hence can be easily targeted by antimicrobial drugs (Van Acker *et al.*, 2014). However, biofilms pose resistance to antimicrobials through different mechanisms (Figure 1). Due to this, most common and effective ways to control bacterial infections are rendered ineffective for bacterial biofilms. A group of studies have shown that inexplicable resistant mechanisms exist in bacterial biofilms when antimicrobial resistance (AMR) is concerned. The biofilm development process involving multicellular mechanisms is responsible for antimicrobial resistance. However, the molecular mechanism of biofilm ECM to obstruct antimicrobial effect on bacterial cells is relatively less explored.

Scientists have been trying to understand the battleground of antimicrobials and bacterial biofilms. Among different approaches to treat biofilm infection, the concept of dispersing biofilm components and attacking biofilm cells followed by prevention of recurrent biofilm seems to be most common. Apart from conventional antibiotics, the novel treatment strategies are based on combating the antimicrobial resistance. However, there are very few strategies which target ECM and bacterial cells in combination. This review compares different anti-biofilm strategies employed in the field of dental biofilms and provides a novel prospective strategy to tackle obstinate biofilms through the dual-target approach.

Oral biofilm development and antimicrobial resistance

The oral cavity is a niche for microbes and existential competition between beneficial and pathogenic organism determines the state as healthy or diseased. During biofilm formation, nutritional support is required for bacterial cells to attach and grow their community. In the oral cavity, salivary proteins and polysaccharides serve as a nutritional source for pathogens, responsible for pellicle formation. Different interactions such as hydrophobic interactions, van der Waals forces, electrostatic, ionic interactions and covalent forces initiate pellicle formation and undergo conformational changes to attract bacterial cells. Consequently, the mechanism of biofilm development involves steps such as — Cell Adhesion, Niche maturation and Cell dispersion. The series of steps lead to obstinate biofilm formation and its movement to spread infection from one location to another.

Cells adhesion

Pellicle formed on tooth surface expose cell-binding proteins such as alpha-amylase and proline-rich glycol-proteins. Bacterial cell surface receptors recognize these binding proteins and attach to the pellicle. The early attachment phase is usually based on weak interactions and hence the binding is reversible (Characklis and Mcfeeters, 1990). Later, chemical forces dominate the binding process leading to the formation of strong bacterial niche where other cells are adhered with stronger forces.

Niche Maturation

With the growing number of bacterial cells, chemical interactions become predominant and cells attached to pellet secrete extracellular polysaccharides, proteins, DNA is collectively known as the extracellular matrix. ECM helps in bacterial aggregation by the cell to cell recognition and binding happens by polysaccharide recognition among bacterial cells. Co-aggregation among different bacteria is dependent on the receptor to adhesion protein binding between two cells. Additionally, protein aggregation has recently emerged as a potential reason for the mechanical stability of biofilm. The overall ECM and aggregated cells provide structural as well as functional support to make the biofilm stable against physical and chemical stress.

Cells dispersion

After a certain period of biofilm maturation, bacterial cells independently or in combination, leave the biofilm niche usually in nutrient deficiency situations. Cells dispersion in case of nutrient deficiency at one location and finding new sources of nutrients is referred to as active dispersion. Whereas, sometimes there is a competition between different cell types over limited nutrition termed as passive dispersion. The passive dispersion is mostly a characteristic of the host's defence mechanism such as salivary shear strength to prevent biofilm formation.

Several therapeutic drugs and clinical practices have been practically explored. The mechanism of biofilm formation has provided different targets for therapeutic interventions that have been researched in various domains. Usually, two types of the bacterial population exist in biofilm, i.e. planktonic cells and biofilm attached cells. Planktonic cells are loosely

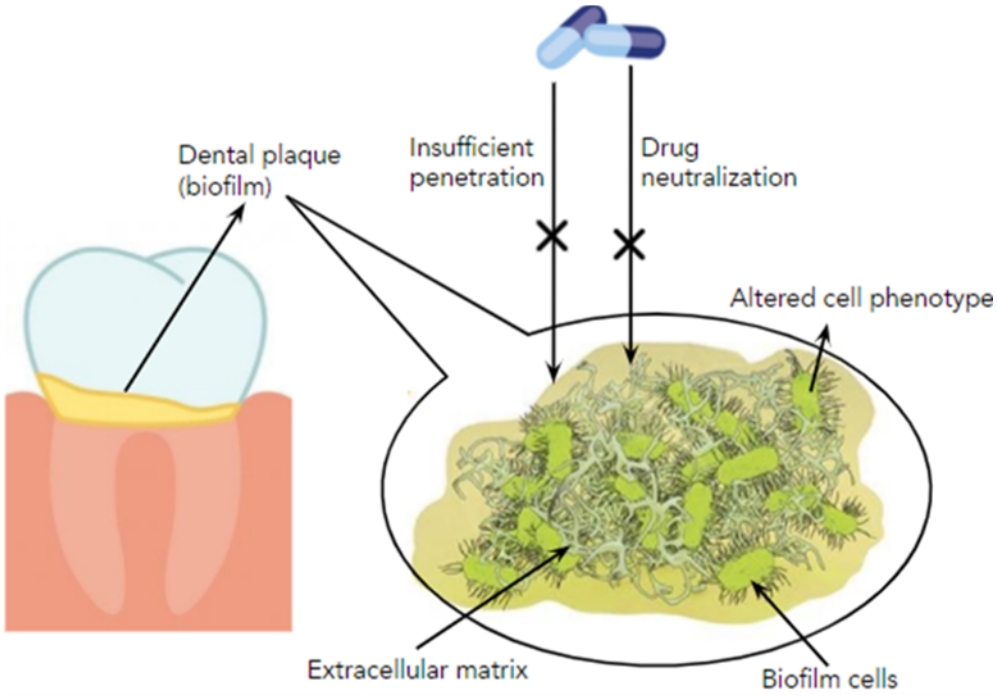

Figure 1: Biofilm defence mechanisms contributing to antimicrobial resistance (AMR) in dental infections

attached cells which exist in relatively uniform environmental conditions whereas biofilm cells are shielded by extracellular matrix and exhibit irreversible attachment. Unlike planktonic counterparts, biofilm cells are exposed to nutrient gradient due to ECM intervention. With frequent exposure to antimicrobials and antibiotics, biofilm cells experience non-uniform exposure which eventually develops metabolic changes as defence mechanisms leading to resistance development. Biofilm resistance has been associated with polymorphic mutations and resistance can be transferred from one cell to another by an exchange of specific genetic element. Moreover, the limited penetration of antimicrobials to biofilm cells develops resistance mechanisms in biofilm cells (Vrany *et al.*, 1997).

The complex composition of ECM and chemical interactions pose obstructions in diffusing antimicrobials inside biofilm (Figure 1). It has been reported that *P. aeruginosa* secrete Ps1 as exopolysaccharide and Ps1 is responsible for sequestration of antibiotics through electrostatic interactions as defence (Billings *et al.*, 2013). Sometimes, different ECM enzymes such as amylase, DNAase, lactamase, secreted by biofilm cells are responsible for the degradation of antibiotics and prevent their target interaction. eDNA (extracellular DNA) in biofilm ECM has been related to quorum sensing mechanisms for cells aggregation. According to reports, eDNA has been involved in imparting antimicrobial resistance to biofilm cells encased within ECM (Chiang *et al.*, 2013). Interesting, bacteriophage genes have also been reported to be expressed by biofilm cells. Expression of phage genes leads to cell death which results in eDNA release into ECM and develops small colonies with tolerance against antibiotics. Overall, antibiotics target the metabolic mechanisms of bacterial cells. However, inability to penetrate within the biofilm ECM leads to exposure of decreased antibiotic concentration, developing antimicrobial resistance in biofilm cells.

Antimicrobial Resistance Prevention

With the logarithmically growing scale of ineffective antimicrobials due to AMR, it is required to regulate the use of antibiotics. It is necessary that antibiotics exposure to genetically altered cells should be reduced to control the spread of resistance. Alternative therapeutic targets should be considered instead of increasing the antibiotics dose in an unregulated manner. For antibiotics resistance associated with biofilm, ECM could be a potential target to inhibit biofilm formation. In order to make antibiotics effective on biofilm cells, ECM mechanical stability should be targeted in priority. Several therapies in the form of enzymes, surfactants and small molecules that can disrupt ECM stability can be coupled with antimicrobial compounds.

According to Rendueles et al., certain polysaccharides have been reported to exhibit antimicrobial properties in the form of bio-surfactants for

both gram-positive as well as gram-negative bacteria (Valle *et al.*, 2006). Interestingly, polymers that initiate biofilm formation have shown an anti-biofilm effect for different species. Naturally produced enzymes such as dispersin B have been reported to disrupt biofilm ECM. Other class of ECM inhibiting compounds could be quorum sensing inhibitors. Different traditional Chinese medicines have also been reported as potential quorum sensing inhibitors in biofilm. The strategy can be essential in preventing biofilm formation. Consequently, anti-ECM agents can be used in combination with antimicrobials to combat the problem of antimicrobial resistance in biofilms. Alternatively, certain adhesion proteins and cell mobility elements can be targeted to prevent biofilm initiation and reduce infection spread. Thus, changing the therapeutic target towards ECM and other players of biofilm mechanism can help in reviving ineffective antimicrobials as well as control AMR.

Antimicrobial agents, coupled with dental material

In order to ensure biofilm prevention in the oral cavity, cell adhesion and biofilm stabilization are the most important targets. The process of developing dental materials equipped with antimicrobial properties has been optimized through strategies of releasing antimicrobial agent and/or killing the cell by contact with an antimicrobial agent. Colton et al. developed first-ever dental resin-filled antibiotic drugs. The strategy has been implemented in preventing biofilm infections. Antibiotics or silver compounds are mostly used as antimicrobial agents released over time to prevent biofilm formation. Silver compounds are known for their broad-spectrum effect involving bacterial membranes and enzymes as targets. Due to limited repository of antimicrobials and regulatory drug release, the results have been short term.

Nanomaterials have been extensively researched for anti-biofilm activities. Nanoparticles made up of silver, titanium, zinc-oxide, chitosan ad quaternary ammonium compounds have been incorporated into polymers and used as dental fillers to control biofilm formation. Nanoparticle-based applications in dentistry involve filling, enamel polishing and dental implants with the sole purpose of preventing biofilm formation. Based on a study conducted on *S. mutans* biofilm, nanoparticles loaded with farsenol were found to display drug release in response to pH changes during biofilm formation (Jiao *et al.*, 2017). Although nanoparticles display flexibility in terms of size and charge, human toxicity can be a limiting factor to their applications. Dental materials have also been combined with quorum sensing inhibitors to obstruct signaling pathways of biofilm-forming bacteria. These inhibitors prevent bacterial adhesion and subsequent biofilm formation. Overall, a balance between antimicrobial effectiveness and cellular toxicity need to be maintained along with long term regulated drug release.

Another range of antimicrobials used against dental biofilms includes natural antimicrobial peptides (AMPs) and synthetic chemicals like polycations and quaternary ammonium compounds (QACs). QACs have a history of being used as antiseptics and disinfecting compounds. Structurally, QACs are cationic surfactant based antimicrobials effective against both grams positive and gram-negative bacteria. Due to road spectrum antimicrobial characteristics and low toxicity, QACs have been used as anti-biofilm components in mouthwash liquids. The concept was then repurposed to dental composites.

The compounds have also been used to prevent dental caries and plaque. QACs display antimicrobial property through electrostatic interaction between positively charged QAC and negatively charged bacterial membrane and promoting membrane disruption (Beyth *et al.*, 2006). The presence of reactive groups in the compounds enables them to be easily coupled with dental resins and polymers. According to Imazato *et al*., 12-methacryloyloxydodecylpyridinium bromide (MDPB) has a strong anti-biofilm effect on S*. mutans, E.faecalis, F. nucleatum,* and *P. nigrescens.* Also, MDPB has been coupled with methacryloxylethyl cetyl dimethyl ammonium chloride to inhibit adherence of oral microbes while the mixture was incorporated into dental composite.

It has been reported that antimicrobial dental materials do not show long term effect. According to Chen et al., polymer dental materials lose the antimicrobial property after 14 days after storage in saline buffer (Chen *et al.*, 2018). This was contrasting as compared to long term applicability expected from antimicrobial dental materials. Along with the discovery of new antimicrobial dental materials, the dental microbial colonies can begin displaying antimicrobial resistance due to unregulated use and discard of used/unused antimicrobial materials. According to different reports, antimicrobial resistance has been proved in eight oral bacteria including *S. mutans, S. sanguinis, S. gordonii, E. faecalis, A. actinomycetemcomitans, F. nucleatum, P. gingivalis,* and *P. intermedia* when frequently treated with two types of QAC compounds. Overall, antimicrobial dental materials have some limitations in terms of less options of drugs with broad-spectrum, sustained action for a longer time period and antimicro-

bial resistance.

Anti-biofilm peptides against oral pathogens

Antimicrobial peptides have always been considered an important therapeutic strategy against bacterial infections. The human body has a natural ability of synthesizing defence peptides or short chain of amino acids in response to bacterial pathogens. These peptides have been researched and recognized as antimicrobial peptides (AMPs). There have been reports on *in-vitro* isolation of AMPs from plants, fungi and bacteria as well. Polylysine is one such natural AMP isolated from *S. albuus* and being used for applications in food industries. Polylysine, along with nisin, has been found to be synergistically active against *S. mutans*, a common oral pathogen (Najjar *et al.*, 2009). AMPs are expressed on the surface of epithelial cells and cause repulsion from bacteria, fungi and viruses, thus inhibiting pathogen invasion. Different AMPs work through multiple mechanisms of action and their structural orientations have been studied as a template for designing synthetic antimicrobial peptides *in-vitro*.

With the goal of enhancing antimicrobial properties of AMPs to make them effective against biofilms, the idea of developing synthetic peptides evolved. Several physicochemical amendments have been studied to eliminate obstructions related to natural peptides. A study had shown that the bactericidal effect of B1CTcu5, a frog secreted antibacterial peptide was observed when four N-terminal amino acids were removed (Abraham *et al.*, 2015). In another study, substituting fatty acids with amino acids in dermaseptin S4 can be effective for oral disease-causing microbes. Among synthetic peptides, it has been reported that alpha-helical peptide KSL (KKVVFKVKFK) has shown a broad range of anti-biofilm activity against microbial strains related to dental caries. The dose-dependent action of KSL targets bacterial membrane through electrostatic interactions and cause membrane destabilization (Leung *et al.*, 2009). KSL has been reported to inhibit the logarithmic growth of cells for 45 hours of culturing period. Additionally, there have been reports where AMPs have shown anti-biofilm results for over three days of the study period with a concentration-dependent effect. Hence, these AMPs hold potential for being utilized in the development of anti-biofilm therapies against dental biofilms.

Periodontal disease, an inflammatory disease caused by multispecies biofilms formation on teeth, dental implants and oral tissues. The stabilization of biofilms niche by bacterial aggregates releases toxins and induce inflammatory immune processes that cause gingival tissue destruction, peri-implantitis and osseointegration loss. In order to prevent peri-implantitis, antibiofilm peptides have been used in titanium-based implant surfaces. According to Yoshinari et al., quartz crystal microbalance technique can be used to link AMPs and Ti-binding peptides onto implant surfaces. Consequently, down-regulation of metabolic pathways was observed in *Porphyromonas gingivalis.* Subsequent studies confirmed the anti-biofilm activity of the Ti-implant on several biofilms forming oral pathogens (Yoshinari *et al.*, 2010). In spite of several advancements in anti-biofilm implants, limited time peptide exposure was a major limitation. The improvement in peptide exposure time requires complex implant design procedures, thus imposing limitations on therapeutic utility.

The anti-biofilm effect has been reduced in exposure to biological fluids like plasma, serum, saliva (Silva *et al.*, 2012). Along with being expensive and complex, parenteral use of AMPs is difficult due to quick clearance through the kidney. The difficulties in regulation of toxicity towards host cells and severe inflammatory response are some of the added limitations. Alternatively, AMPs have been part of bacteria defence mechanism against many antimicrobials. Due to this, AMPs can exhibit protein degradation and cell surface protein alteration which can lead to further problems of antimicrobial resistance in target bacteria. Hence, careful screening of AMPs needs to be done to ensure efficiency without non-target reactions.

Dentifrices and mouth-rinse solutions against periodontal biofilms

Essential oils have been known for broad antimicrobial domain comprising Gram-positive bacteria, Gram-negative bacteria and yeast strains. Usually, the bactericidal property is essential, but mouth-rinse solutions work by forming an antibacterial film on the tooth surface and prevent bacterial adhesion. Essential oils have shown to affect bacterial multiplication, causing a reduction in bacterial load and plaque mass. Their bacterial cell killing mechanism involves cell wall rupturing and inhibition of enzymatic action (Fine *et al.*, 1996). According to a study performed to analyze essential oil mouth-rinse solution's penetration in dental plaque, 78.7% of plaque bacteria were found to be dead in comparison with 27.9% in control (without essential oils). Using essential oil mouth-wash rinse twice in a day has been found to reduce microbial levels of aerobic and anaerobic species in gingivitis (Fine *et al.*, 2005). This shows that essential oils are capable of penetrating obstinate plaque mass and deliver a bactericidal effect on bacterial cells.

Table 1: Different anti-bio strategies employed against oral biofilms

Anti-Biofilm Strategy	Dental Application	Limitations
Antimicrobial dental material	Metals/ nanomaterials/ quaternary ammonium compounds coated dental implants	Procedural complexity, Cytotoxicity, Short term impact, Antimicrobial resistance
Antimicrobial peptides	Dental implants, Gingivitis	Cytotoxicity
Dentifrice and mouth-rinse	Dental caries and plaques	Significance with respect to mechanical cleaning
Small compounds	Oral cancer, Dental caries	Antimicrobial resistance
Amino acids	Endodontic diseases	Ambiguous action mechanism

Dentifrices are formulations that are used for teeth cleaning and polishing. The formulations containing triclosan and maleic acid copolymer have exhibited antimicrobial activity against plaques and gingivitis. At bacteriostatic concentrations, triclosan inhibits amino acid uptake by the bacterial cells and destabilization of the bacterial membrane. The conjugation of triclosan and maleic acid copolymer helps in triclosan retention on teeth and surface of oral tissues. According to a study, significant effects of triclosan and copolymer dentrifice were observed in one-week when dentrifice was used twice a day. 88% to 96% decrease in anaerobes population of *Veillonella* spp. and *Fusobacteria* sp. was observed at three sample sites in comparison with the control group (Fine *et al.*, 2006). In a three-year-long clinical study, the authors combined fluoride dentrifice with mechanical cleaning in the form of a powered toothbrush and failed to observe the significant clinical benefit of the dentrifice.

After careful examination of results by using DNA-DNA hybridization checkboard, both fluoride dentifrice and mechanical cleaning resulted in similar DNA probe count reduction (Bogren *et al.*, 2007). Hence, it can be interpreted that combined use of mechanical cleaning and dentrifice renders the effect of dentrifice's anti-biofilml ingredient to minimal. Therefore, anti-biofilm dentrifices can be used in the case of cavities severely damaged by biofilms inflammatory response where mechanical cleaning can be painful. Briefly, anti-biofilm strategies discussed so far either have complex development procedures, an insignificant antimicrobial effect for a longer duration or fail to regulate cell toxicity (Table 1), which limits their applications in oral procedures.

Anti-biofilm effect of small molecules and amino acids

In order to control biofilms formation, small molecules have recently emerged as a novel strategy against oral biofilms. *S. mutans* has been famous for being associated with oral cancer-causing biofilm formation. The microbe can rapidly metabolize sucrose from human diet and synthesize extracellular matrix (ECM) with polysaccharides as a major constituent. Different glucosyltransferases (Gtfs) have been found to be involved in ECM formation and providing a specific binding site to bacterial colonies. These Gtfs have become therapeutic targets for biofilms inhibition. A study suggests screening of different small molecules virtually to mark compounds that can target the catalytic domain of Gtfs. Consequently, two small molecules quinoxaline derivative and unnamed compound #G43 was found to selectively inhibit *S. mutans* biofilm formation (Zhang *et al.*, 2017). Overall, the compounds were effective in reducing severe dental caries. Natural antimicrobial products such as ginger, turmeric, garlic etc. have inspired scientists to develop small molecules with anti-bofilm properties and negligible drug resistance.

Some small molecules derived from marine natural products have been found to inhibit *S. mutans* biofilm *in-vitro.* The molecules were efficient in inhibiting expression of adhesions and Gtfs of S. mutans (Liu *et al.*, 2011). According to a study conducted by Pierce et al., screening of chemical library revealed some small compounds that specifically inhibit oral pathogen *Candida albicans.* The ability of compounds to target biofilms cells more as compared to planktonic cells has made these molecules prospective candidates against oral biofilms.

D-amino acids have evolved as the smallest compounds being able to disperse bacterial biofilms. It has been shown that these D-amino acids destabilize *B. subtilis* biofilms by affecting amyloid fibres formation which is an essential step in ECM stabilization (Oppenheimer-Shaanan *et al.*, 2013). According to Bucher et al., D-Leu competes with D-Ala during essential peptide formation in *B. subtilis* cell membrane and obstructs transpeptidation process. The mechanism of action results in interference in

biofilm formation. Other amino acids like D-Tyr, D-Met, D-Trp, D-Leu can prevent biofilms pellicles formation.

In recent studies, it has been proposed that D-amino acids can be potential biofilms dispersing compounds with applications in endodontic treatments (Kolodkin-Gal *et al.*, 2010). A study conducted by Gahane et al. has introduced Fmoc-amino acids as antimicrobial agents effective against multidrug-resistant Gram-positive bacteria. The study demonstrates the efficacy of other amino acids, as well as the importance of surfactant behaviour to kill bacterial cells (Gahane *et al.*, 2018). The study has been extrapolated to testing these Fmoc-amino acids (Fmoc-F) over biofilm formation and the compound was found to be targeting ECM along with planktonic cells. Overall, synergistic effect of compounds effective in killing bacterial cells and surfactant antimicrobial compounds (Fmoc-F) targeting ECM can be extensively studied on oral biofilms.

CONCLUSION

There have been different strategies against oral biofilms since the past decade. Along with strategic development, antimicrobial resistance has posed several exclamations over long term effectiveness of anti-biofilm compounds. The development of dental elements with anti-microbial compounds has been one of the initial strategies to combat oral pathogens. However, due to complex procedures and lack of toxicity has limited their therapeutic applications. Antimicrobial peptides (AMPs) are another type of anti-biofilm compounds that are relatively simple as well as effective in controlling major disease-causing oral biofilms. However, toxicity regulation is still difficult to achieve in correlation with clinical applications. Dentrifices and mouth-rinse are the most commonly used treatments for dental problems. Incorporation of specific antimicrobial components in denitrifies have been effective in dispersing oral biofilms, but their effectiveness came out to be similar to mechanical cleaning treatments. The significance of anti-biofilm dentifrices could not be explained for long term effects on obstinate biofilms. Small compounds and amino acids emerged out as simple antimicrobial compounds used against various oral pathogens. Host cell toxicity can be reduced in these compounds as the small compounds have flexibility in structural development and amino acids are natural elements. It is possible that these compounds can be a new group of easy to achieve antimicrobials without adverse effects. The target of these compounds has been bacterial cell encased in ECM. There are very few studies targeting ECM as it forms the major structural framework of biofilms. Surfactant based compounds such as Fmoc-amino acids can be utilized to disrupt biofilm ECM and then targeting bacterial cells. Fmoc-amino acids have no reports of cell toxicity and their applications in chronic wound healing makes it less probable to be toxic. The hydrogelation property of Fmoc-amino acids can further broaden the scope of applications. A strategy of using simple antimicrobial compounds targeting cells, in synergy with surfactant-based antimicrobials (Fmoc-amino acids) can be developed in future to treat bacterial biofilms on biotic and abiotic surfaces. This study compares different anti-biofilm strategies and provides novel prospective towards oral biofilms and antimicrobial resistance.

Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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