

REVIEW

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## Strategies for combating bacterial biofilms: A focus on anti-biofilm agents and their mechanisms of action

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### ABSTRACT

Biofilm refers to the complex, sessile communities of microbes found either attached to a surface or buried firmly in an extracellular matrix as aggregates. The biofilm matrix surrounding bacteria makes them tolerant to harsh conditions and resistant to antibacterial treatments. Moreover, the biofilms are responsible for causing a broad range of chronic diseases and due to the emergence of antibiotic resistance in bacteria it has really become difficult to treat them with efficacy. Furthermore, the antibiotics available till date are ineffective for treating these biofilm related infections due to their higher values of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), which may result in *in-vivo* toxicity. Hence, it is critically important to design or screen anti-biofilm molecules that can effectively minimize and eradicate biofilm related infections. In the present article, we have highlighted the mechanism of biofilm formation with reference to different models and various methods used for biofilm detection. A major focus has been put on various anti-biofilm molecules discovered or tested till date which may include herbal active compounds, chelating agents, peptide antibiotics, lantibiotics and synthetic chemical compounds along with their structures, mechanism of action and their respective MICs, MBCs, minimum biofilm inhibitory concentrations (MBICs) as well as the half maximal inhibitory concentration (IC<sub>50</sub>) values available in the literature so far. Different mode of action of anti biofilm molecules addressed here are inhibition via interference in the quorum sensing pathways, adhesion mechanism, disruption of extracellular DNA, protein, lipopolysaccharides, exopolysaccharides and secondary messengers involved in various signaling pathways. From this study, we conclude that the molecules considered here might be used to treat biofilm-associated infections after significant structural modifications, thereby investigating its effective delivery in the host. It should also be ensured that minimum effective concentration of these molecules must be capable of eradicating biofilm infections with maximum potency without posing any adverse side effects on the host.

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### Introduction

Biofilm refers to the complex communities of microbes that may be found attached to a surface or may form aggregates without adhering to a surface, as seen in *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and some other bacteria<sup>1–3</sup> and buried firmly in an extracellular matrix (ECM). The biofilm lifestyle allows the bacteria to withstand hostile environmental conditions like starvation, desiccation and makes them capable to cause a broad range of chronic diseases. Hence, it is considered as a major cause of persistent nosocomial infections in immune-compromised patients.<sup>4,5</sup> Around 50% of the nosocomial infections are confined to the patients by indwelling devices used for the purpose of medical treatments such as catheters, cardiac pacemakers, joint

prosthesis, dentures, prosthetic heart valves and contact lenses.<sup>6,7</sup> These foreign bodies provide an ideal surface for the attachment of bacterial cells. Thus a significant increase in biofilm formation has been observed in the presence of implants.<sup>8</sup> In many cases, the use of antibiotics like imipenem, colistin and many more can only reduce the biofilms but cannot eliminate the entire biofilm. Due to their toxic and side effects it is not possible to reach the minimal concentration of antibiotic *in-vivo*. The higher values of MIC and MBC for the biofilm bacterial cells have therefore made the antibiotic treatment less adequate.<sup>6,9–11</sup>

Moreover, biofilms protect the invading bacteria against the immune system of host via impaired activation of phagocytes and complement system<sup>12–14</sup> and also

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increase their resistance against the conventional antibiotics by around 1000-fold.<sup>2,6,15-20</sup> Some other factors may also account for this antimicrobial tolerance. Previous experimental works revealed several reasons behind the resistance, which includes nature and structure of biofilm, nutrient and oxygen availability to the bacterial cells and intrinsic and acquired bacterial resistance. The involvement of biofilm in providing resistance was made evident from a study on *P. aeruginosa* where the mucoid nature of biofilm was found responsible for high resistance toward tobramycin.<sup>21</sup> The metabolic state of biofilm-associated bacteria is another potential reason of antimicrobial resistance. Cells of the nutrient depleted zones (slow growing state) in the biofilm may lead to dormancy like the stationary phase which makes the bacteria insensitive to antibiotics since they divide very infrequently.<sup>22,23</sup> Dividing cells are sensitive to some antibiotics including  $\beta$  lactams, thereafter making them unfit for use. Walters et al. reported that antibiotic resistance was also influenced by limited oxygen supply as observed in case of *P. aeruginosa*, where antibiotic was effective at the air-biofilm interface, the part of the biofilm exposed to oxygen (50–90  $\mu\text{m}$  in the biofilm).<sup>24</sup> Moreover, studies also demonstrate that biofilm cells undergo a higher rate of mutation than their planktonic counterparts resulting in a 10-fold increase in the efficiency of transfer of plasmid having antibiotic resistance gene, when biofilm is exposed to a sub-lethal concentration of that antibiotic.<sup>25</sup>

Bjarnsholt et al. demonstrated that mucoid biofilms observed in the samples of cystic fibrosis lungs were not found adhered to the lung epithelia, instead were adhered to the neighboring bacteria embedded in a biopolymeric matrix known as aggregated form.<sup>2</sup> These findings were subsequently confirmed in the studies performed by other researchers<sup>1,3</sup> suggesting that non-surface attached aggregates exhibit similar levels of tolerance to various antibiotics and polymorphonuclear leukocytes (PMN) as surface attached biofilms. The properties of non-surface attached aggregates despite being similar to that of biofilms, exhibit some differences like they have higher metabolic activity than cells in biofilm as well as planktonic cells. These aggregates have also been reported to be involved in chronic infections and wounds<sup>26,27</sup> and middle ear infections.<sup>28</sup> So, the necessity to find new effective drugs that could disperse and eliminate the biofilm is of major concern to prevent and treat various infections caused by generation of biofilms.

Therefore, the study of biofilm and the strategies to eliminate them is one of the most important fields of research in the present days. Many reviews on anti-biofilm compounds have already been done, but this review focuses especially on different strategies or targets of biofilm inhibition. A recent review by Wu et al. has

discussed few strategies to combat biofilm. These strategies include the removal of infected foreign bodies like stents and implants and replacing them with new uninfected ones, inhibition of the quorum sensing pathway and modification of c-di-GMP to reduce biofilm infections.<sup>6</sup> Here, we have made an effort to compile all the known strategies or targets for combating biofilm, which may help the researchers to design new molecules having anti-biofilm activity. Here, we have also discussed the mechanism of biofilm formation with respect to different models followed by various biofilm detection methods along with detailed discussion of the mechanism of action of anti-biofilm molecules found till date. Many of the compounds that exhibit anti-biofilm activity need to undergo further modifications and *in-vivo* tests followed by clinical trials before using them commercially.

## Biofilm formation

Biofilm formation on any surface involves mainly 3 stages. The first stage involves attachment of cells to a surface followed by assembly of the cells to form microcolonies and finally differentiation of biofilm into a mature structure. After the complete development of biofilm, its disassembly or dispersion takes place through both mechanical and active processes.<sup>29</sup> Deposition of bacteria is especially mediated by sedimentation, Brownian motion and hydrodynamic forces, whereas adhesion to the substratum is governed by Lifshitz–Van der Waals, acid–base, hydrophobic, electrostatic interaction forces.<sup>30</sup> Certain surface associated proteins like OmpA, fibronectin binding proteins,<sup>31</sup> protein A,<sup>32</sup> SasG,<sup>33,34</sup> biofilm associated protein (BAP)<sup>35,36</sup> and many other factors are involved in the formation of biofilms, particularly, during initial attachment stages. Some species cannot attach to a surface but can anchor themselves to the matrix or directly to the earlier colonies. Small signaling molecules with the help of cell-cell communication systems mediate this colonization. This phenomenon is generally referred to as quorum sensing.<sup>37</sup> Biofilm formation is a major quorum-sensing controlled phenotype.<sup>38</sup> In biofilms, the bacterial cells are enclosed in an extracellular matrix, which is a complex and highly polar mixture of biomolecules including proteins, polysaccharides, nucleic acids and lipids.<sup>39</sup> The matrix provides protection from various stress conditions such as antimicrobial exposure or immune cells attack. However, the matrix of the biofilm does not act as a mechanical barrier for the antimicrobial agent.<sup>40</sup> This was confirmed by a study which shows that biofilm formed by  $\beta$ -lactamase-deficient strain of *K. pneumoniae*, allowed the penetration

of ampicillin whereas in wild type *K. pneumoniae* strain possessing  $\beta$ -lactamase, ampicillin was unable to infiltrate biofilm,<sup>40</sup> suggesting that in the latter case, ampicillin was rapidly degraded by  $\beta$ -lactamase before infiltrating the wild type biofilm. Once the bacteria start secreting extracellular polysaccharide substance (EPS), second stage of development of biofilm comes in process, which is an irreversible process. The secretion of EPS is continuous till the third stage of formation ensuring the safe attachment of bacteria to the surface inside a thickly complex bio-molecular layer.<sup>41</sup> The fully matured biofilm now takes on a tower-like structure having 3 dimensions. These towers comprise of small channels, which transport nutrients, water and waste, and the small cavities present in the towers provide shelter for the planktonic bacteria. Studies also demonstrate that the organization and architecture of biofilms vary greatly for different bacteria. Exact reason for this variation remains unclear. However, the adhesive protein LapA governs the biofilm formation of *P. putida*<sup>42-44</sup> while exopolysaccharides Pel and Psl govern biofilm formation in other pseudomonads including *P. aeruginosa*.<sup>45-47</sup> Hence, difference in extracellular matrix (ECM) component may give rise to the variations in the structure of biofilm. Finally, these towers either erode (small parts) or are sloughed off (large parts) and get detached, emptying the cavities containing non-surface attached bacteria. This is followed by the release of fresh bacteria into the environment.<sup>48,49</sup>

Some recent studies on various bacterial species such as *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Yersinia pestis*, *Escherichia coli*, *Vibrio cholerae*, *Burkholderia cenocepacia*, *Salmonella enterica*, *Clostridium difficile*, *Klebsiella pneumoniae*, *Vibrio cholerae* and *Bacillus subtilis* demonstrate that increase in c-di-GMP level, an intracellular secondary messenger designates the initiation of biofilm formation and virulence.<sup>42,43,50-58,59-63</sup> c-di-GMP was first described as a novel secondary messenger in the allosteric activation of cellulose synthase of *Gluconacetobacter xylinus*.<sup>55</sup> Several types of c-di-GMP diguanylate cyclase and phosphodiesterases that are synthesized by bacteria participate in different c-di-GMP circuits.<sup>64</sup> c-di-GMP functions by binding to a wide range of receptors which include enzymes, adaptor proteins, transcription factors and riboswitches.<sup>61</sup> It has also been reported that various environment causes and transducer mechanisms lead to an increase in the c-di-GMP level in the cell. This not only leads to the production of adhesins but also helps in the secretion of extracellular matrix.<sup>65,66</sup> In *P. aeruginosa*, the level of c-di-GMP positively regulates the production of extracellular matrix components such as CdrA

adhesin, alginate exo-polysaccharide, Pel and Psl.<sup>53,67</sup> Along with c-di-GMP, small regulatory RNAs (sRNA) also regulate the formation of biofilm in several bacterial species.<sup>68</sup>

Certain bacterial strains have the ability to form planktonic aggregates, which depend on growth conditions. Previous studies suggest that some strains of *S. aureus* form large aggregates and the formation process starts in the early exponential growth phase. A cluster of about 20 cells form a structured population when cell density is low. However, at higher density these structures are larger forming aggregates up to diameter of 1000 $\mu$ m. Extracellular polysaccharide intracellular adhesin (referred as polymers of  $\beta$  1-6 N-acetylglucosamine or PNAG after determination of the chemical structure),<sup>69</sup> and *spa* encoding Protein A are reported to be responsible for the extensive aggregation.<sup>3</sup> Studies by Alhede et al. 2011 suggested that the matrix of aggregates of *P. aeruginosa* comprises of DNA and mannose-rich extracellular polysaccharide like Psl.<sup>1</sup>

## Biofilm models

Study of various biofilm model systems enhances the knowledge regarding the biofilm biology. The biofilms are studied using both *in-vivo* and *in-vitro* model systems. *In-vitro* biofilm model systems are broadly classified into 3 major types including closed or static model, open or dynamic models and microcosms. The most frequently used closed model systems are microtitre plate-based model systems which uses static and batch growth conditions.<sup>70</sup> In this model, there is no flow of media, product or waste materials into or out of the reactor, so the experimental conditions changes gradually in the wells like accumulation of signaling components, increase of bacterial population and depletion of nutrients in media. Since, it is cost effective and require small volume of reagents therefore, numerous tests can be performed at a single time.<sup>71</sup> Additionally, microtitre plate-based models can be used to differentiate between biofilm-deficient mutants and biofilm forming wild type strains,<sup>72,73</sup> determine the antimicrobial and anti-biofilm effects of different antimicrobial compounds, identify the factors involved in the biofilm initiation such as adhesins, pili, flagella, enzymes involved in cyclic-di-GMP metabolism and genes responsible for extracellular polysaccharide production.<sup>74,75</sup> Among the open and dynamic models, flow displacement biofilm model is most commonly used to study biofilms. Unlike the microtitre plate method, in this model system, addition of nutrients and release of waste products can occur.<sup>70,76</sup> The dynamic model of biofilm formation using perfused silicone tubes is one of the most important models for

studying biofilms as it initiates *in-vivo* conditions very closely. Biofilms are formed in a silicone tube system under dynamic condition followed by cutting of the tube in small pieces for further treatment and investigation.<sup>77</sup> Microcosms constitute another *in-vitro* model system for studying biofilms that mimic with the *in situ* conditions in controlled environment, such as for studying wound biofilm, oral biofilm, stream biofilm and dental biofilm.<sup>78-80</sup> Both *in-vitro* and *in-vivo* systems can be turned into a microcosm by using the same medium and creating an artificial environment to assess the cell metabolism and behavior. Apart from this there exists an *ex-vivo* model system, which deals with the tissues and organs extracted from organisms for the further analysis and experimentation in artificial environment. This model can be useful to monitor the bacterial colonization and progression in the given tissue or organ. To validate the simplified results provided by the *in-vitro* model studies, certain *in-vivo* model system studies should be performed. To address various therapeutic and diagnostic challenges the studies of mammalian models closer to the humans is necessary. These tissue-associated model systems are being used for studying mainly lung infections, urinary tract infections as well as the wound infections.<sup>74,81</sup> Different other models such as central venous catheter models; subcutaneous foreign body infection models; intra-peritoneal foreign body infection models; urinary tract infection models; ear, nose and throat infection models; respiratory tract infection models and osteomyelitis infection models have been used for the study of these infections.<sup>74</sup> The use of mammalian model possess some difficulties that has made researchers to switch over to the non-mammalian model system such as *Drosophila melanogaster*, *Caenorhabditis elegans* or *Danio rerio*.<sup>82</sup> The advantages of these models reside in the very short generation time and their lower cost. Moreover their small sizes provide the ease to maintain them in microtitre plates thus making it easier for high throughput screening of biofilm formation.

### Methods for quantification and structural assessment of biofilm

Biofilm production can be assessed by several methods. The standard assay for screening the presence of biofilms is crystal violet (CV) assay by quantifying the dye bound to cells on polystyrene and other hydrophobic substratum. However, the limitation of crystal violet assay includes its indirect nature, and requires repeated washings, which may cause loss of cells and above all the biofilm has to be disrupted.<sup>83</sup> Tissue Culture Plate (TCP) method<sup>84</sup> is also one of the most commonly practiced standard method and a

more reliable process as compared with Congo Red Agar method (CRA)<sup>85</sup> and Tube method (TM).<sup>84</sup> Other methods for detection of biofilm include bioluminescent assay,<sup>86</sup> piezoelectric sensors,<sup>87</sup> and Percentage Transmission (%T) method.<sup>88</sup> The advances in biofilm imaging technology have been proved to be very crucial to understand the complexity and dynamics of biofilms. These optical techniques include fluorescent microscopic examination,<sup>89</sup> scanning electron microscopy (SEM),<sup>80</sup> confocal scanning laser microscopy (CSLM), light microscopy, infrared spectroscopy, reflectance spectroscopy and optical fluorometry, which can be used to check the existence and visualize the 3D structure of biofilm.<sup>90-92</sup> However, SEM is expensive and quantitation of the biofilm is difficult. Information on biofilm heterogeneity and cell localization can be obtained via fluorescence staining coupled with CSLM followed by high-speed computing. Reflectance assay is a semi-quantitative, inexpensive, and nondestructive optical assay for biofilms on abiotic surfaces and to some extent on biotic surfaces. This assay is capable of examining the status and morphology of biofilm formation and also reveals the biofilm forming ability of bacteria.<sup>93-95</sup> To characterize the chemistry of biofilm NMR and FTIR can be taken into use. Nuclear Magnetic Resonance (NMR) imaging results demonstrate the water dynamics, molecular dynamics and biomolecule diffusion within biofilms<sup>96,97</sup> and analysis of Fourier Transform Infrared Spectroscopy (FTIR) along with Raman imaging of biofilm permits the characterization of extracellular and cellular components. Raman imaging generates detailed chemical image based on sample's Raman spectra. Raman spectrometry along with CSLM provides information on the spatial distribution of biomass, water and chemical composition of *P. aeruginosa* biofilms.<sup>98,99</sup> One of the most important quantitative assay is XTT ((2,3-bis (2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) reduction assay, where the tertazolium dye, XTT is converted to water soluble colored formazan due to normal metabolic activities of cell.<sup>88,100</sup> It not only facilitates the study of intact biofilm but also investigates biofilm drug susceptibility keeping the biofilm structure undisturbed.<sup>101</sup>

For the routine diagnosis of biofilm infections several microbiological techniques are commonly used. Proper sonication of indwelling devices is found to be an efficient technique for the detection of biofilm followed by 16S rRNA sequence detection for identifying the strain of the organism involved.<sup>102,103</sup> In some cases, the identification of biofilms can be done by examining blood leukocyte count, C-reactive protein,

interleukin-6 and pro-calcitonin level.<sup>104,105</sup> In addition, fluorescent *in situ* hybridization (FISH) is used for the diagnosis of biofilm infections in cystic fibrosis and chronic wounds using either traditionally labeled DNA probes or the Peptide Nucleic Acid (PNA) probes, the latter having better attributes thereafter providing information on community structure of biofilms.<sup>106-112</sup> To measure anti-biofilm activity, viability and matrix biomass is assessed. For this, resazurin and crystal violet staining are performed sequentially in the same plate. Wheatgerm agglutinin-Alexa Fluor 488 fluorescent conjugate are generally used to stain the matrix. It is essential to measure the biofilm matrix, biomass and viability to investigate the efficiency of antibiotic treatment.<sup>113</sup> The different methods of biofilm detection with their principles are enlisted in the Table 1.

### Strategies to combat biofilm formation

Since biofilm formation contributes to the bacterial pathogenicity and resistance toward antibiotics, there must be certain strategies to deal with this problem. Recently, Wu et al has reported the use of foreign bodies as a major cause of increase in biofilm infections.<sup>6</sup> So, to treat such biofilm associated infections it is imperative to remove the indwelling medical equipment followed by replacement with new uninfected ones along with sensitive and aggressive administration of antibiotics. Moreover, the implant removal should be timed properly so that the new or

replaced implant does not get infected when inserted in the patient's body. In cases where removal is not possible a long-term administration of antibiotics is recommended so as to avert the biofilm from growing. According to the previous reports, the premature biofilms can be treated more effectively with antibiotics than that of the mature biofilms. However, the inefficiency to diagnose premature biofilms in the body is the most crucial reason for the occurrence of clinical conditions, which are mostly related to mature form of biofilms.<sup>9-11,114</sup> The antibiotic used for the treatment of biofilms should be legitimately selected on the basis of sensitivity as well as the capacity to penetrate properly through the biofilm matrix.<sup>6</sup> It is evident from the previous studies that bacteria underneath biofilms are more resistant to antibiotics than their planktonic counterparts. Therefore, the use of combinatorial therapy is more preferable instead of antibiotic monotherapy.<sup>115</sup> The combination of agents is advantageous due to different functioning of the individual agents; for example, one may be effective against dormant cells and other against the growing cells. Moreover, the therapy also requires proper dispensation of antibiotic in terms of dosages and duration. The discovery of antifouling or antimicrobial surfaces can be another possible approach to prevent biofilm formation.<sup>116</sup> Polymeric hydrophilic coatings such as PEG are used for building antifouling surfaces as they minimize or hamper the microbial adhesion. Building of antimicrobial surfaces involves impregnation with antibiotics or

**Table 1.** Different methods used for biofilm quantification.

S. No.	Methods of biofilm detection	Principle	References
1	Tissue culture plate method	TCP method is a standard method for biofilm detection. It simply involves the staining of cells with crystal violet dye.	84
2	Tube method	Crystal violet staining → A visible lining appears on the bottom and wall of tube → confirms biofilm formation	84
3	Congo red agar method	Congo red staining → black colonies in crystalline form appears → confirms biofilm production	85
4	Bioluminescent assay	This assay is based on the signaling based detection of metabolically active cells. It involves the catalysis of ATP and luciferin by luciferase.	86
5	Crystal violet assay (CV assay)	The CV assay quantifies the dye bound to biofilm. It actually quantifies all biomass (live, dead and also matrix of biofilm)	83
6	XTT reduction assay	It is mainly used for the quantification of Candida biofilms. The reagent XTT: (2,3-bis (2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide is involved here. XTT is an age dependent assay hence mature biofilms gives low intensity of color with XTT due to less viability of cells.	88,100
7	Scanning Electron Microscopy	This is used to study the morphology of bacteria attached on the surface and for enumeration of adhered bacteria.	253
8	Fluorescent <i>In-situ</i> Hybridization (FISH)	This is used to visualize the patterns of microbial colonization and the composition of microbial communities.	108
9	Confocal scanning laser microscopy	This gives the 3-dimensional view of the microbial community. It can show the focused part as well as the part out of focus.	90-92
10	Infrared spectroscopy	This technique is used to study molecules such as proteins, polysaccharides, metabolites essential for biofilms. It also gives the information about different hydrogen bonding states of water. Using Attenuated Total Reflectance Infrared (ATR-IR) spectroscopy, the early biofilm development stages including bacterial attachment and growth can be studied.	94,95
11	Piezoelectric sensors	These monitors the shift in the frequency due to accumulation of mass on the surface of sensor	87

disinfectants, mainly polyurethane polymers, which are loaded with different antibiotics.<sup>117,118</sup> Coating with nanoparticles such as silver nanoparticles, anti-oxidant nanoparticles can also be used for the prevention of biofilm formation.<sup>69,119</sup> However, the coating strategy has encountered difficulties as the surface quickly gets eroded and hence becomes available for the formation of biofilms. Photodynamic therapy (PDT) has potential applications in prevention of wound biofilm infections. Here, a photoactive dye is used followed by irradiation in the presence of oxygen, thus killing the bacteria.<sup>120</sup> During therapy, proper care should be taken that patient's eyes are not exposed to laser light. With respect to photosensitizer and photochemical reactions, it is very crucial that the therapy should be used carefully to stain and kill the bacterial cells only without affecting the surrounding tissues of the patient's body. Another uprising strategy is the use of effective anti-biofilm molecules or the biofilm dissolving substances.<sup>121</sup> The anti-biofilm molecules interfere with bacterial signaling pathways in both Gram-positive and Gram-negative bacteria. The anti-biofilm molecules may be any enzyme, a peptide, an antibiotic, polyphenols etc. In the present review, we have discussed about the different anti-biofilm molecules discovered against different bacterial infections. We have also highlighted the mechanism of action of different available anti-biofilm molecules. This review will help to understand the targets for anti-biofilm molecules and help researchers working in the discovery of new antibiotics for Gram-negative bacteria.

### Anti-biofilm molecules and their mechanism of action

Anti-biofilm molecules belong to diverse compounds thereby inhibiting the biofilm formation. The identified anti-biofilm compounds are mainly isolated from the natural sources,<sup>122</sup> some synthetic compounds, chelating agents, and lantibiotics also have been found to possess anti-biofilm activity. The different anti-biofilm molecules along with their target microorganisms are listed in the Table 2. These anti-biofilm molecules follow different mechanisms to inhibit biofilm formation in different bacteria as listed in Table 3.

### Inhibition of AHL-Mediated quorum sensing

N-acyl homo-serine lactones (AHLs) are used as the signaling molecules by numerous bacteria especially, Gram-negative bacteria during quorum sensing to control their

population density as well as facilitate swarming motility. These signaling molecules vary in their length, substitutions on acyl side chains<sup>123</sup> and are synthesized by a LuxI-type synthase. Binding of these molecules at certain critical concentrations, to a cognate LuxR-type transcriptional activator protein regulates the target gene expression.<sup>124,125</sup> A secondary metabolite derivative, synthetic halogenated furanone (Fig. 2) compound is derived from natural furanone produced by the Australian macroalga, *Dilsea pulchra*. This compound has the capability to interfere with bacterial signaling processes and motility of swarm cells. It was also hypothesized that similarity in the structure of *D. pulchra* furanones and AHL molecules is responsible for affecting the interaction of putative regulatory protein with AHL molecules via binding competitively to the receptor. Furanones inhibit surface aggregation traits in ecologically relevant bacteria in ecologically relevant concentrations.<sup>126</sup> Transcription of *lasB-gfp* (ASV) reporter fusion, regulated by quorum sensing is interfered by furanone 56 decreasing the extracellular chitinase and elastase activity and having almost no effect on the growth of the bacteria or in the protein synthesis. Studies suggested that furanone targets the *rhl* system which is involved in the quorum sensing and also penetrates the biofilm matrix of *P. aeruginosa* thereby affecting the expression of genes related to quorum sensing biofilm maturity. This molecule alters the structure of the biofilm which facilitates bacterial detachment at an increased rate and results in the loss of biomass of the bacteria from substratum.<sup>123</sup> It was also discovered that furanone mediates displacement of AHL molecules from Lux R,<sup>127</sup> which suggested the competence of furanone with the cognate AHL signal for the LuxR receptor site. At present, there are several experimental evidences which support the observations about furanones such as, repression of AHL-dependent expression of bioluminescence,<sup>127</sup> inhibition of production and pathogenesis of AHL-controlled virulence factor,<sup>123,128</sup> and inhibition of quorum sensing-controlled luminescence.<sup>129</sup> Some polyphenols (like EGCG, tannic acid, ellagic acid) (Fig. 2) are believed to follow the similar mechanism to inhibit biofilm formation but due to their less efficiency than furanones, they are required in higher concentration.<sup>38</sup> Quercetin (Fig. 2), a flavonoid, also influences quorum sensing, hence acts as an anti-biofilm compound against *S. aureus*. It inhibits alginate production in a concentration dependent manner; resulting into declination in the adherence during biofilm formation. It also reduces exopolysaccharide (EPS) production required for the initial attachment of bacteria and leads to induction of swarming motility.<sup>130</sup> Apart from quercetin, 2 more synthetic flavanoids are also identified, which act as potential antimicrobial agent against the dispersed

Table 2. Different anti-biofilm molecules and their target bacteria.

S. N.	Anti-biofilm molecules	Source	Target bacteria	MIC/MBIC/MBIC <sub>50</sub> values	Reference
1.	Epigallocatechin gallate (EGCG)	Camellia sinensis (Green tea)	<i>Acinetobacter baumannii</i> , <i>Pseudomonas aeruginosa</i> , <i>Staphylococcus aureus</i> , <i>Escherichia coli</i> , <i>Stenotrophomonas maltophilia</i>	MIC = 64–512 µg/ml MBC = 64–1024 µg/ml	254
2.	Ellagic acid	<i>Camellia sinensis</i>	<i>Streptococcus dysgalactiae</i>	MIC = 4 µg/ml	245
3.	Esculetin	<i>Santolina oblongifolia</i> , <i>Alchemilla speciosa</i> , <i>Tagetes lucida</i>	<i>S. aureus</i>	MIC > 512 µg/ml	245
4.	Fisetin	<i>Fragaria ananassa</i> , <i>Malus domestica</i> , <i>Vitis vinifera</i> , <i>Allium cepa</i> , <i>Solanum lycopersicum</i> , <i>Cucumis sativus</i>	<i>S. aureus</i>	MIC = 64 µg/ml	245
5.	Reserpine	<i>Rauwolfia vomitoria</i> , <i>Rauwolfia serpentina</i>	<i>Streptococcus dysgalactiae</i> <i>Klebsiella pneumoniae</i>	MIC = 1000 µg/ml MBIC = 15.6 µg/ml	255
6.	Quercetin	<i>Usnea longissima</i>	<i>P. aeruginosa</i> , <i>K. pneumoniae</i>	MIC = 80 µg/ml	256
7.	Linoleic acid	<i>Hydrastis canadensis</i> , <i>Coptis chinensis</i> , <i>Berberis aquifolium</i> , <i>B. vulgaris</i> , <i>B. aristata</i>	<i>K. pneumoniae</i>	MIC = 250 µg/ml MBIC = 31.2 µg/ml	255
8.	Berberine	<i>Berberis aquifolium</i> , <i>B. vulgaris</i> , <i>B. aristata</i>	<i>K. pneumoniae</i>	MIC = 2000 µg/ml MBIC = 62.5 µg/ml	255
9.	Chitosan	Chitin	<i>K. pneumoniae</i>	MIC = 500 µg/ml MBIC = 62.5 µg/ml	255
10.	Eugenol	<i>Ocimum plants</i> , <i>Syzigium aromaticum</i>	<i>K. pneumoniae</i>	MIC = 250 µg/ml MBIC = 62.5 µg/ml	255,257
11.	Curcumin	<i>Curcuma longa</i>	<i>S. mutans</i> <i>K. pneumoniae</i>	MIC = 0.3125 µg/ml MIC = 12500 µg/ml MBC = 250 µg/ml	255
12.	Synthetic halogenated furanone (F-56)	Derived from natural furanone	<i>Helicobacter pylori</i> <i>P. aeruginosa</i> , <i>Serratia liquifaciens</i> <i>P. aeruginosa</i>	MBC = 8 µg/ml —	123
13.	Peptide 1018	-	<i>E. coli</i>  <i>A. baumannii</i>	MIC = 64 µg/ml MBIC <sub>50</sub> = 5 µg/ml MBIC <sub>100</sub> = 10 µg/ml MIC = 32 µg/ml MBIC <sub>50</sub> = 8 µg/ml MBIC <sub>100</sub> = 10 µg/ml MIC = 128 µg/ml MBIC <sub>50</sub> = 2 µg/ml MBIC <sub>100</sub> = 10 µg/ml	134
14.	CFT073 group-I capsular polysaccharide (Serotype K2)	Produced by extra intestinal <i>E. coli</i> of phylogenetic group B2 or D	<i>K. pneumoniae</i> , <i>S. aureus</i> , <i>S. typhimurium</i> , <i>Burkholderia cenocepacia</i>	MIC > 256 µg/ml MBIC <sub>50</sub> = 2 µg/ml MBIC <sub>100</sub> = 10 µg/ml	225
15.	PeI polysaccharide	<i>P. aeruginosa</i>	<i>E. coli</i> , <i>P. aeruginosa</i> , <i>K. pneumoniae</i> ,	—	225,229,258
16.	PsI polysaccharide	<i>P. aeruginosa</i>	<i>S. aureus</i> , <i>S. aureus</i> <i>S. aureus</i>	—	225,229,258

17. Sphorolipid (Biosurfactant)	Produced on microbial cells or excreted extracellular hydrophobic and hydrophilic moieties	<i>Cupriavidus necator</i> , <i>Bacillus subtilis</i> , <i>S. aureus</i> <i>S. maltophilia</i>	5% (v/v)	191
18. Colistin (Polymyxine)	<i>Paenibacillus polymyxa</i>			254
Polymyxin B	—	<i>P. aeruginosa</i> , <i>S. aureus</i> , <i>E. coli</i> <i>S. aureus</i> , <i>Staphylococcus epidermis</i>	MIC = 158 µg/ml MBC = 256 µg/ml MIC = 158 µg/ml MBC = 256 µg/ml	259 189,190
19. Lantibiotics: Nisin	<i>Lactococcus lactis</i>	<i>Staphylococcus epidermis</i> <i>Lactococcus lactis</i> <i>Lactococcus lactis</i>	MIC=1 µg/ml MIC = 4–8 µg/ml MIC = 0.5 µg/ml MIC = 2.0 µg/ml MIC = 0.5 µg/ml	259,260,171,179,201-203
20. Antimicrobial peptide (AMP): LL-37	Human cationic host defense peptide	<i>S. aureus</i> , <i>E. coli</i> <i>P. aeruginosa</i> , <i>S. aureus</i> , <i>E. coli</i>	MIC = 2–16 µM	
Lytic peptide (PTP-7)	Synthetic analog from Gaegurin 5	<i>P. aeruginosa</i> , <i>S. aureus</i> , <i>E. coli</i>		
Sushi peptides	Derived from sushi-3 domain of Factor C, which is a LPS-sensitive serine protease of horseshoe crab coagulation cascade	<i>P. aeruginosa</i> , <i>S. aureus</i> ,	—	
PMAP-23	Cathelicidin-derived peptide identified from porcine leukocytes	<i>E. coli</i> <i>P. aeruginosa</i> , <i>S. aureus</i> ,	—	
PR-39	Isolated from the pig's small intestine	<i>E. coli</i> <i>P. aeruginosa</i> , <i>S. aureus</i> , <i>E. coli</i>	MIC = 0.94 µM	
Buforin-II	Derived from Buforin-I (stomach tissue of <i>Bufo bufo gargarizans</i> )	<i>P. aeruginosa</i> , <i>S. aureus</i> , <i>E. coli</i>	MIC = 0.25–4.0 µg/ml	
Indolicidin	From cytoplasmic granules of bovine neutrophils	<i>P. aeruginosa</i> , <i>S. aureus</i> , <i>E. coli</i>	MIC = 50 µg/ml	
Pyrrhocoricin		<i>P. aeruginosa</i> , <i>S. aureus</i> , <i>E. coli</i>	IC <sub>50</sub> < 0.3 µM	261
Microcin B17	Post-translationally modified peptide that is produced by <i>E. coli</i> containing the plasmid-borne MccB17 operon	<i>E. coli</i>	IC <sub>50</sub> = 0.9 µM	260
21. Chelating agents: (a) Sodium citrate (b) Tetrasodium EDTA (c) Disodium-EDTA		<i>Staphylococcus</i> species, <i>P. aeruginosa</i> <i>S. aureus</i>	MIC ≥ 0.5%	146 121,260
22. Tannic acid	<i>Caesalpinia spinosa</i> , <i>Rhus semialata</i> , <i>Quercus infectoria</i> , <i>Rhus coriaria</i>	<i>Staphylococcus and Enterococcus</i> <i>Streptococcus pyogenes</i>	MIC = 0.04–0.08 µg/ml MBC = 0.02–0.08 µg/ml MBC = 1.25–5 µg/ml MBC = 100000–150000 µg/ml	150 193
23. Enzymes: Deoxyribonuclease I, glycoside hydrolase (dispersin B)		<i>P. aeruginosa</i> , <i>S. proteamaculans</i> <i>P. aeruginosa</i> , <i>S. aureus</i>		193
24. Bacteriophage-encoded endolysin (PlyC)				
25. Silver				
26. Octenidine hydrochloride				

(Continued on next page)



Table 2. (Continued).

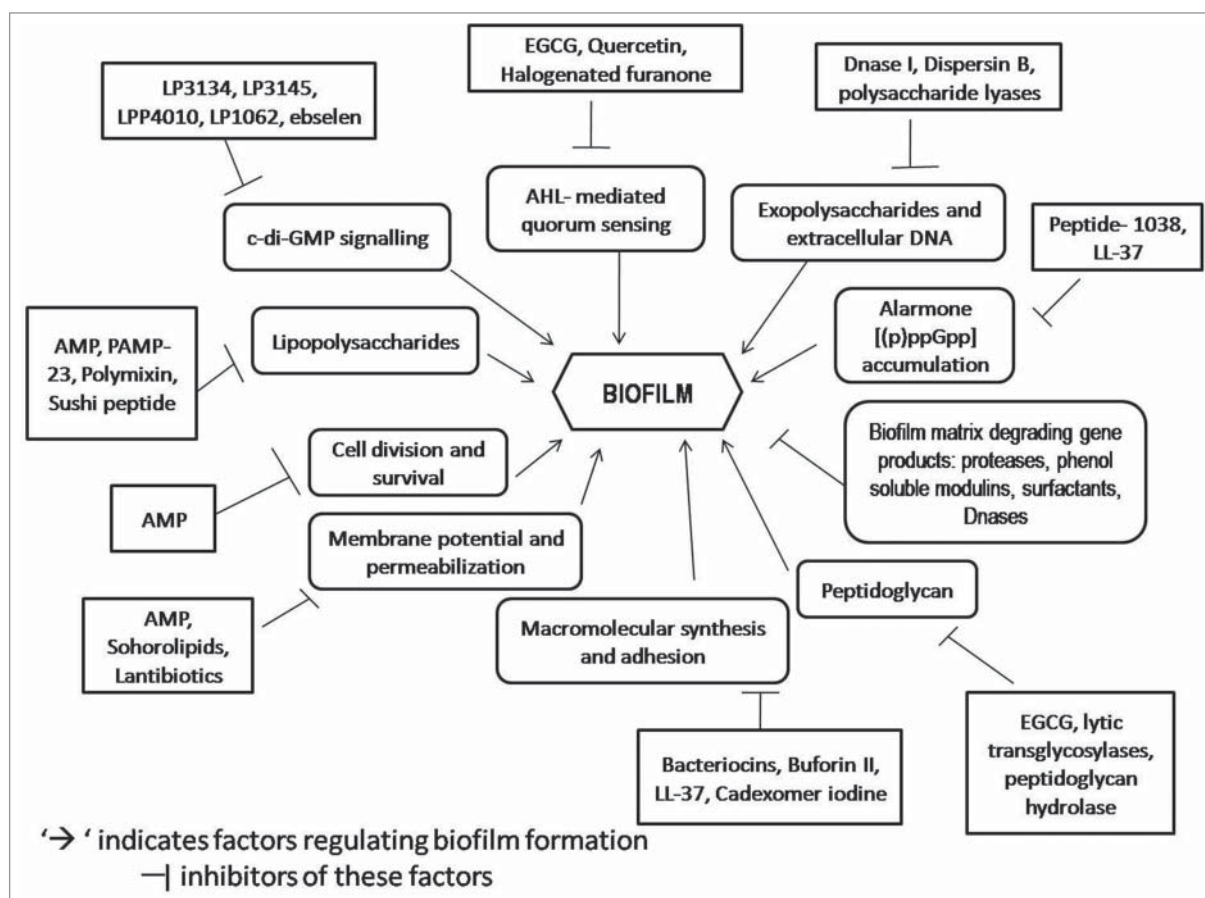
S. N.	Anti-biofilm molecules	Source	Target bacteria	MIC/MBC /MBIC/IC <sub>50</sub> values	Reference
27	Chlorhexidine		<i>P. aeruginosa</i> , <i>S. aureus</i>	—	193
28	Cadexomer iodine		<i>S. aureus</i>	—	193
29	Polyhexamethylene biguanide		<i>P. aeruginosa</i>	—	193
30	Usnic acid	A secondary lichen metabolite	<i>S. aureus</i> , <i>C. albicans</i>	—	132,175

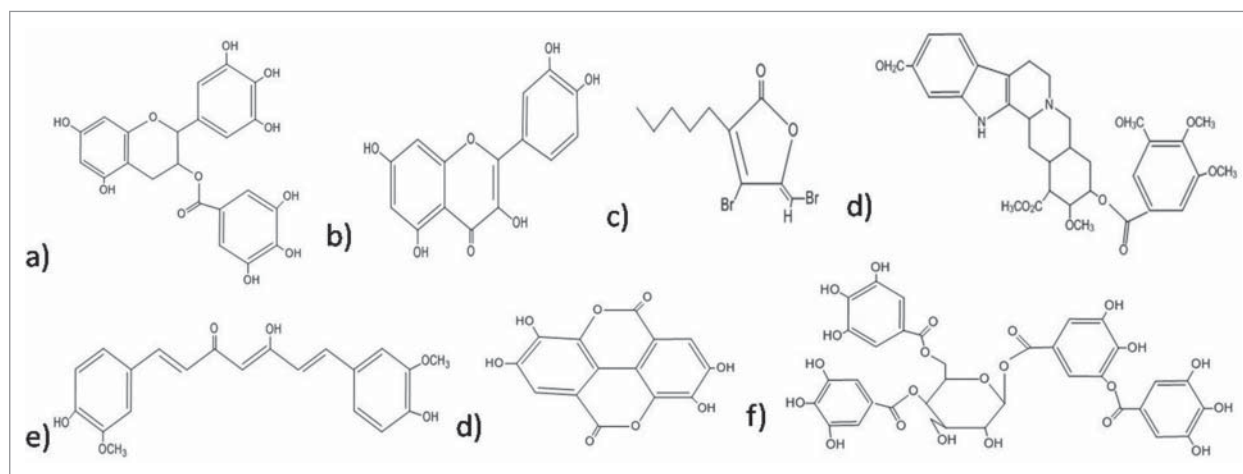
**Table 3.** Mechanisms followed by different Anti-biofilm molecules.

S. N.	Mechanism of action	Molecules associated	Reference
1.	Inhibition of AHL-mediated quorum sensing pathway	Halogenated furanone compounds, Quercetin	123,256
2.	Inhibition of (p)ppGpp regulated stringent response	Peptide-1018, Peptide-1038	134,140
3.	Dispersion of Extracellular Polymeric Substance (EPS) of biofilm	Deoxyribonuclease I and glycoside hydrolase dispersin B	259
4.	Cleavage of peptidoglycan	Tannic acid, Endolysins (PlyC), Epigallocatechin gallate (EGCG)	146,150,159
5.	Biofilm disassembly	A cyclic autoinducing peptide (AIP), Nuclease, extracellular proteases (eg. sarA, sigB, Esp), antiamyloid molecules (AA-861, parthenolides), D-Tyrosine, Ethyl-pyruvate	77,160,168,172
6.	Neutralization/disaggregation of LPS	Polymyxin (B and E), Gramicidin S, Sushi peptides, PMAP-23	177,180,262
7.	Alteration of membrane permeabilization	Lantibiotics (nisin, gallidermin), Lytic peptides (PTP-7), Sophorolipids, Polyhexamethylene biguanide, Chlorhexidine, Pentasilver hexaaxoiodate	179,186,191,192
8.	Inhibition of cell division or cell survival	Pyrrhocoricin, Microcin B17	194,198
9.	Inhibition of macromolecule synthesis and adhesion of cells	Buforin II, PR-39, Indolicidin, LL-37, Bacteriocins, Cadexomer iodine, Mannosides, Plicicides	39,193,201,202,204,209,221,222
10.	Inhibition of biofilm by polysaccharides	EPS273, Psl and Pel, K2, PAM galactan, A101, PslG, Polysaccharides of algae, plants and animals	224,225,227,228,230,235
11.	Inhibition of c-di-GMP signaling system	LP 3134, LP 3145, LP 4010, LP 1062, ebselen, ebselen oxide Desformylflustra bromine	6,238,239
12.	Inhibition of curli biosynthesis	Analogues of FN075 and BibC6 of ring-fused 2-pyridones	169

cells as well as biofilm of *S. aureus*.<sup>131</sup> Some other reports also suggested that usinic acid show inhibitory effect on the *S. aureus* biofilm and affected the morphology of biofilm produced by *P. aeruginosa*.

Researchers have hypothesized that this may be due to any interference in quorum sensing, but the exact mechanism of action is still unclear.<sup>132</sup> Curcumin (Fig. 2), a phytochemical from the rhizome of Curcuma

**Figure 1.** Schematic representation of overview of the targets of anti-biofilm molecules.



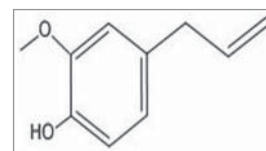
**Figure 2.** Structures of the anti-biofilm molecules that inhibit AHL-mediated quorum sensing. (a) EGCG<sup>263</sup>, (b) Quercetin<sup>264</sup>, (c) Synthetic halogenated furanone<sup>265</sup>, (d) Reserpine<sup>266</sup>, (e) Curcumin<sup>267</sup>, (f) Ellagic acid<sup>268</sup>, (g) Tannic acid<sup>269</sup>.

longa exhibit potent antibiofilm effect by the modulation of expression of genes involved in quorum sensing and related virulence factors like alginate production, and swarming motility.<sup>133</sup>

### Inhibition of stringent response by bacteria

A peptide named 1018-peptide works by inhibiting the alarmone accumulation, which is a part of stringent response by bacteria in response to nutritional stress. During their stress conditions bacteria synthesize alarmones, namely; guanosine tetraphosphate and guanosine pentaphosphate, collectively termed as (p)ppGpp.<sup>134,135</sup> Generally, 2 enzymes regulate the (p)ppGpp metabolism: the (p)ppGpp-synthetase/hydrolyase, RelA (orRsh) with dual function and the small alarmone synthetase, RelQ. Previous works by researchers suggest that during stress conditions, rapid accumulation of (p)ppGpp is regulated by RelA whereas RelQ is found responsible for low level of expression of (p)ppGpp under no stress conditions. The absence of (p)ppGpp, in the case of a  $\Delta$ relA- $\Delta$ relQ double mutant strain, leads to reduced antibiotic tolerance and attenuated virulence.<sup>136</sup> Studies also revealed that any change in (p)ppGpp pool can severely affect the bacterial biofilm formation, development and maintenance of its stable form *in-vitro*. The RelA-mediated stringent response and helps in the cell survival as during starvation conditions it can optimize gene expression for growth and survival. Some results prove the importance of the (p)ppGpp synthetase, RelQ in biofilm homeostasis. In case of  $\Delta$ relA  $\Delta$ relQ strain, there occurs some severe defects during the formation of biofilm, its maturation and viability due to uncontrolled consumption of energy resource, NAD/NADH ratio imbalance, or amassing of the metabolic

end products.<sup>137</sup> Hence, the amphipathic cationic peptide 1018 must establish direct contact with (p)ppGpp by crossing through the cell membrane and reaching protoplasm. The peptide disrupts the biofilm in 3 possible manners. First, it prevents formation of biofilm when added before initiation step. Second, at much specified low concentration it disrupts and kills the bacteria present in biofilm without affecting the planktonic cells. Third, it can disperse mature biofilm which is 2 d old.<sup>134</sup> It was also reported to exhibit significant synergistic effect with antibiotics against biofilm formed by different bacteria.<sup>138</sup> The peptide 1018 and its derivatives HE4 and HE10 are found to be active against *P. aeruginosa* and *B. cenocepacia* at concentrations much below the MIC of their planktonic growth. In contrast to the parent molecule 1018, these derivatives exhibit equal or decreased level of anti-biofilm activity against biofilm formed by *P. aeruginosa*, but increased activity against methicillin resistant *S. aureus* biofilms.<sup>139</sup> Peptide 1037 was also evident to reduce biofilms formed by many Gram-positive and Gram-negative bacteria.<sup>140</sup> Another peptide named, Peptide 1038<sup>140</sup> induce twitching motility (which destroy biofilm), and inhibit adhesion and quorum sensing of *Pseudomonas* involved in biofilm formation. A secondary metabolite from *Syzygium aromaticum*, eugenol (Fig. 3) treatment to *S. mutans* causes the downregulation of gene, *relA*, involved in the control of stringent



**Figure 3.** Structures of the anti-biofilm molecules that inhibit the stringent response. (a) Eugenol<sup>270</sup>.

response in biofilm formation as well as acid tolerance.<sup>141</sup>

### Dispersion of extracellular polysaccharide substance of biofilm by enzymes

The Extracellular Polysaccharide Substance (EPS) of the biofilm protects the microorganisms from various antimicrobial agents. The disorganization of the EPS would expose the released as well as residual biofilm cells to these agents. There are certain enzymes such as polysaccharide lyases and DNases that are capable of disrupting the exo-polysaccharides.<sup>142</sup> Likewise, DNase I and Dispersin B are the major enzymes that functions as potential anti-biofilm agents.<sup>143,144</sup> DNase I is capable of digesting the extracellular DNA (eDNA) which is present within the biofilm structure whereas Dispersin B, a glycoside hydrolase works by cleaving polymers of  $\beta$  1–6 N-acetylglucosamine (PNAG), an extracellular polysaccharide substance which facilitates aggregation of bacteria. Also, it can disperse EPS layers present on medical devices.<sup>143,144</sup> These biofilm-dispersing enzymes are more efficient when administered in combination with antimicrobial agents in killing the bacteria embedded in the EPS.<sup>145</sup>

### Cleavage of peptidoglycan

The cleavage of peptidoglycan, which is present in the cell wall of most of the bacteria inhibit biofilm generation. Tannic acid, a polyphenolic compound, inhibits biofilm formation without affecting bacterial growth in *Staphylococcus aureus*.<sup>146</sup> The mechanism of action was found to depend on the putative lytic transglycosylase, an immune-dominant *Staphylococcal* Antigen A (IsaA), which acts by cleaving peptidoglycan.<sup>147</sup> These transglycosylases are lysozyme-like enzyme which catalyzes the cleavage of the  $\beta$ -1,4 glycosidic bond between N-acetyl muramic acid (MurNAc) and N-acetyl glucosamine (GlcNAc).<sup>148</sup> Tannic acid inhibits the formation of biofilms by increasing the extracellular level of IsaA.<sup>146</sup> Cleavage of peptidoglycan reduces biofilm formation by several ways, such as; it alters the composition of proteins and teichoic acids present on the cell wall. Peptidoglycan cleavage may also result in the release of signaling molecules<sup>149</sup> that can modulate the biofilm-related gene expression. Bacteriophages encode a unique class of peptidoglycan hydrolases referred as endolysins,<sup>150</sup> which digest the cell wall of bacteria so as to release progeny bacteriophage. Endolysins usually work in a species-specific manner. They bind with the cell wall and cleave it, which ultimately leads to hypotonic lysis and bacterial death.<sup>151</sup> Endolysins work on the multiple antibiotic

resistant strains. *PlyC*, a specific streptococcal bacteriophage endolysin,<sup>152-156</sup> functions by disrupting the *in-vitro* biofilms. This bacteriophage therapy requires the knowledge of the bacteria causing infections, for which specific bacteriophages are needed to be design properly. Another molecule, epigallocatechin gallate, a polyphenol, inhibits bacteria by causing the cell wall damage via binding with the peptidoglycan,<sup>157,158</sup> thus interferes with initial docking phase (mainly due to hydrophobic interactions) of biofilm formation.<sup>159</sup>

### Inhibition through biofilm disassembly

Biofilm disassembly is a multistep process that involves deterioration of the extracellular matrix and change in the physiology of cell which prepares them to sustain in conditions persisting beyond the boundary of the biofilm.<sup>160</sup> Several bacterial species takes into account a primary mechanism of disassembly of biofilms that include the production of extracellular enzymes or surfactants that leads to degradation as well as solubilization of adhesive components in the matrix of biofilm. The matrix keep the cells enclosed within the biofilm colony, thus, upon its deterioration cells get detached from this colony and are released in the environment. The active biofilm dispersal is mediated by certain matrix-degrading gene products such as proteases, deoxyribonucleases (DNases) and surfactants.

An accessory gene regulatory (*agr*) system present in several bacteria, control the synthesis of biofilm matrix degrading enzymes. The *agr* system is mediated by a cyclic auto-inducing peptide (AIP). At critical threshold concentration (in the low nanomolar range), a 2-component signal transduction cascade is activated by AIP which results into the generation of virulence factors.<sup>161</sup> The extracellular proteome of *agr* includes several proteases and small pore-forming toxins known as phenol-soluble modulins (PSMs).<sup>29,162,163</sup> Thus, activation of the *agr* system prevents biofilm maturation, performing an inhibitory role.<sup>29</sup>

The production of extracellular proteases (eg. *sarA*, *sigB*, *Esp*) has been found to be associated with the biofilm disassembly mechanism.<sup>162,164,165</sup> During biofilm disassembly, nuclease acts as an endogenous mediator. It is an effective DNase (a thermonuclease or micrococcal nuclease) that helps in the separation of cells from biofilms.<sup>166</sup> In some species, when the DNases and restriction enzymes are added exogenously, the biofilms get readily dispersed from microplate wells, which indicates that extracellular DNA (eDNA) is a major biofilm matrix moieties there.<sup>144,166</sup> The amyloid-like fibers and the secretion of *TasA* protein have also been reported to play an important role in the formation of biofilms.<sup>167,168</sup> The

detachment of these amyloid-like fibers from the cell surfaces lead to the disassembly of biofilms.<sup>168</sup> To screen the anti-amyloid activities of molecules, *B. subtilis* biofilms, being the simplest biologic system are mainly preferred. AA-861 and parthenolide exhibit inhibitory properties against biofilms by *B. subtilis*, *E. coli* and *Bacillus cereus* by interfering with the polymerization of TasA into amyloid-like fibers.<sup>168</sup> *E. coli* and other species of *Enterobacteriaceae* produce functional amyloid fibers named curli. Type 1 pili and curli plays significant roles in promoting biofilm in *E. coli*. Some earlier studies suggested that 2 analogs of FN075 and BibC6 of ring-fused 2-pyridones are peptidomimetic that target the protein–protein interactions in macromolecular assembly, blocking the synthesis of curli in *E. coli*. Bacterial virulence is significantly attenuated in a mouse urinary tract infection model when *E. coli* was pretreated with FN075.<sup>169</sup> Additionally, Connolly et al.<sup>170</sup> and Park et al.<sup>171</sup> reported the use of cysteine protease SpeB and proteases from Group A *Streptococcus* and *P. aeruginosa*, respectively, for the biofilm dispersal.

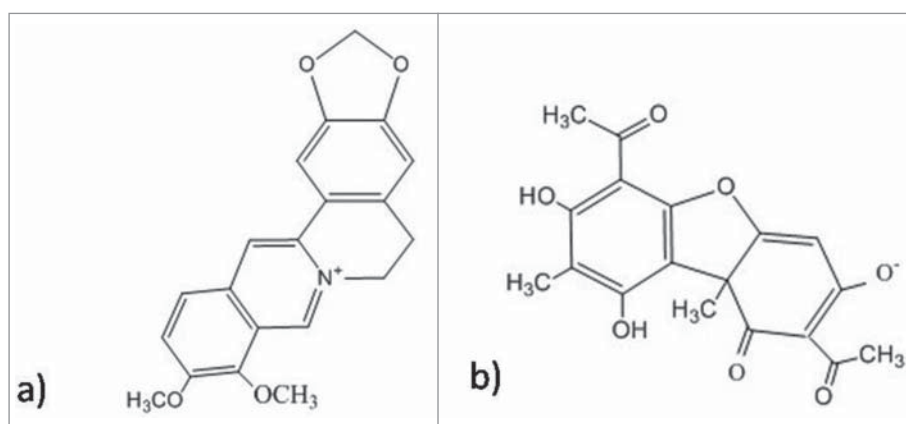
D-tyrosine leads to a significant decrease in cell attachment thus, preventing the formation of biofilm. It also causes biofilm disassembly at a very low concentration in both *B. subtilis* and *P. aeruginosa*. Impact of D-tyrosine on EPS production and extracellular protein is concentration specific and varies greatly in Gram-positive and Gram-negative bacteria. The concentration of extracellular proteins increased in *B. subtilis* biofilms and reduced in the biofilms of *P. aeruginosa*. Moreover, EPS production increased when *P. aeruginosa* was treated with low concentration of D-tyrosine and decreased at higher concentrations but no change was observed in *B. subtilis*.<sup>172</sup> Hence, it is very crucial to decide the dosage carefully before recommending D-tyrosine for treatment of biofilms. *In-vitro* studies also suggested that D-histidine, D-cysteine and D-tryptophan inhibit 35–86% biofilm formation in *A. baumannii* at a very low concentration of 2 mM and D-cystine, D-tryptophan and D-tyrosine inhibit 10–30% biofilm formation in *P. aeruginosa* at 4 mM. However, it is also demonstrated in this study that significant effects of D-amino acids were not observed *in-vivo* as the effective *in-vitro* concentration produced toxic effects and even fatal when tested on mouse models. The use of D-amino acids with antibiotics should be further investigated.<sup>173</sup> Some recent *in-vitro* and *ex-vivo* studies suggested nagZ, a protein involved in peptidoglycan recycling, also reduces preformed biofilm in *Neisseria gonorrhoeae* but the exact mechanism of biofilm dispersal is still unclear.<sup>174</sup>

A lichen secondary metabolite, usnic acid (Fig. 4), has the potential to inhibit 65% biofilm formation and yeast to hyphal transition. This compound not only prevents adhesion but also reduces various sugars in EPS. Light microscopic studies revealed that usnic acid stops the transition from yeast to hyphal state thereby reducing the thickness of matured biofilm.<sup>175</sup> Some other reports also suggested that usnic acid show inhibitory effect on the *S. aureus* biofilm and affected the morphology of biofilm produced by *P. aeruginosa*. Researchers have hypothesized that this may be due to any interference in quorum sensing, but the exact mechanism of action is still unclear.<sup>132</sup>

In some recent studies, ethyl pyruvate (EP), an anti-tumor and anti-trypanosome drug, has been reported to possess significant antimicrobial and anti-biofilm activity. The architecture of the biofilm matrix is especially stabilized by DNA and negatively charged polymeric sugars, bridged by Ca<sup>2+</sup> ions. EP acts as a potential Ca<sup>2+</sup> ions chelating agent due to its dicarbonyl structure, which destabilizes the biofilm matrix. Moreover, this specific structural element plays a crucial role in inhibiting enzymes pyruvate kinase of glycolytic and glyoxalase-1 of para-glycolytic pathway. This explains the antimicrobial activity of EP. The study reveals EP to be better anti-biofilm agent for being tissue protective, showing no side-effects in clinical studies, harmless to symbionts, inhibiting a broad spectrum target such as bacteria, fungi, parasites and mold, lower chance of developing resistance, inhibiting adhesion and maturation of biofilm and the dissolution of pre-formed biofilm matrix.<sup>77</sup>

### Neutralization or disassembly of lipopolysaccharides

The antimicrobial peptide (AMP) is an alternative of conventional antibiotics and is considered as an effective anti-biofilm agent. AMPs are evolutionary conserved proteins with low molecular weight and exhibits antimicrobial activity against fungi, bacteria and viruses. They are generally positively charged and contain both hydrophilic and hydrophobic sides which make them capable of penetrating the lipid bilayer as well as solubilizing in aquatic environment.<sup>176</sup> Antimicrobial peptides usually bind electrostatically with lipopolysaccharides (LPS) involving interaction between 2 cationic amino acids (lysine and arginine) and their respective head groups. The complex is stabilized through hydrophobic interactions between the hydrophobic amino acids of the peptide and fatty acyl chains of LPS<sup>177,178</sup> and



**Figure 4.** Structure of anti biofilm molecules that disassemble the biofilm. (a) Berberine<sup>271</sup>, (b) Usnic acid<sup>272</sup>.

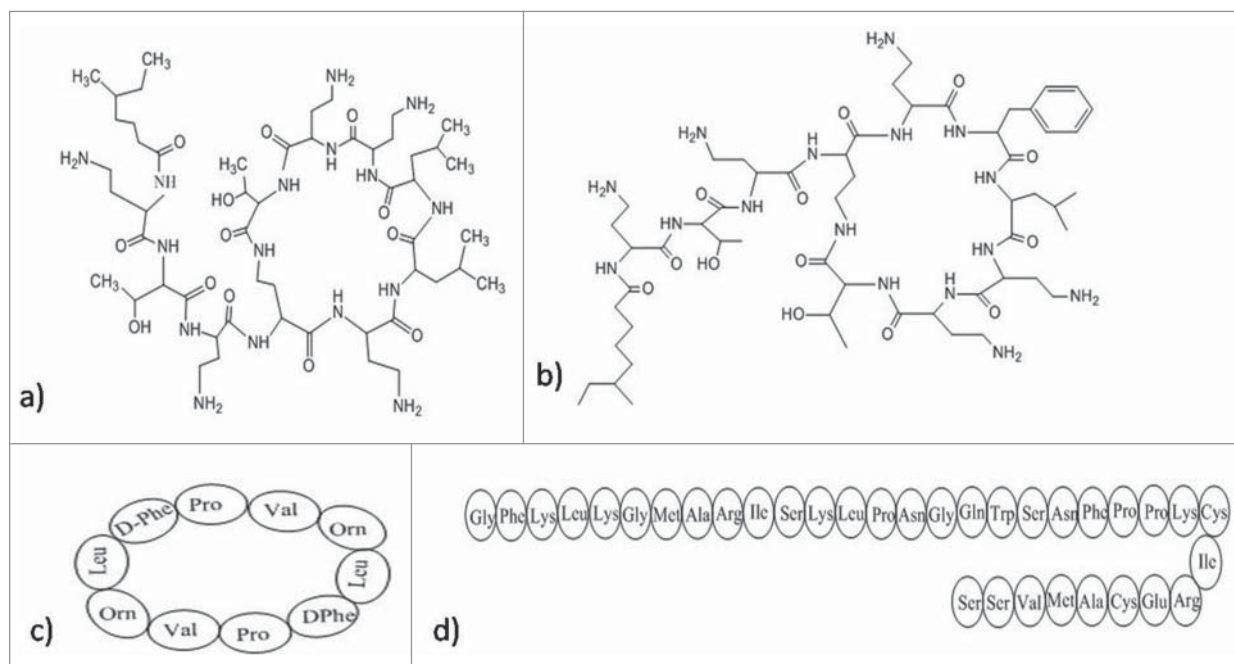
resulting into destabilization of lipid head groups by multiple pore formation, thereby disrupting the integrity of cellular membrane. PTP-7, an example of lytic peptide is a synthetic analog from Gaegurin 5. Despite being a cationic peptide, its activity is not affected by acidic pH, negatively charged extracellular polysaccharides in biofilm matrix or high metal ion concentrations. Rather it is capable of entering deep in the biofilm and kill bacteria very efficiently.<sup>179</sup> Polymyxins, especially polymyxin E or colistin and polymyxin B (Fig. 5) (pentabasic decapeptide antibiotic) bind to lipid A of LPS in Gram-negative bacteria making the outer membrane permeabilized. Along with this, Gramicidin S (Fig. 5) also disturbs the membrane integrity of the Gram-positive and Gram-negative bacteria. Both these cationic cyclic peptides possess specific targets in cell membrane leading to interference in the hydrophobic interactions at ligand binding sites of the enzymes. Further improvement of toxicity, structural analysis and clinical tests should be performed for using it clinically.<sup>180</sup> Likewise, sushi peptides, a derivative of Factor C (Fig. 5) (LPS-sensitive serine protease of the horseshoe crab coagulation cascade) follow detergent-like mechanism for the disruption of LPS aggregates. They have LPS-neutralizing activity too. They act very specifically with palmitoyl-oleoyl-phosphatidylglycerol (POPG). Unsaturated POPG renders fluidity and ultimately increases the entry of peptides in lipid bilayer, completely disrupting membrane stability.<sup>177,181</sup>

### Alteration of membrane potential or membrane permeabilization

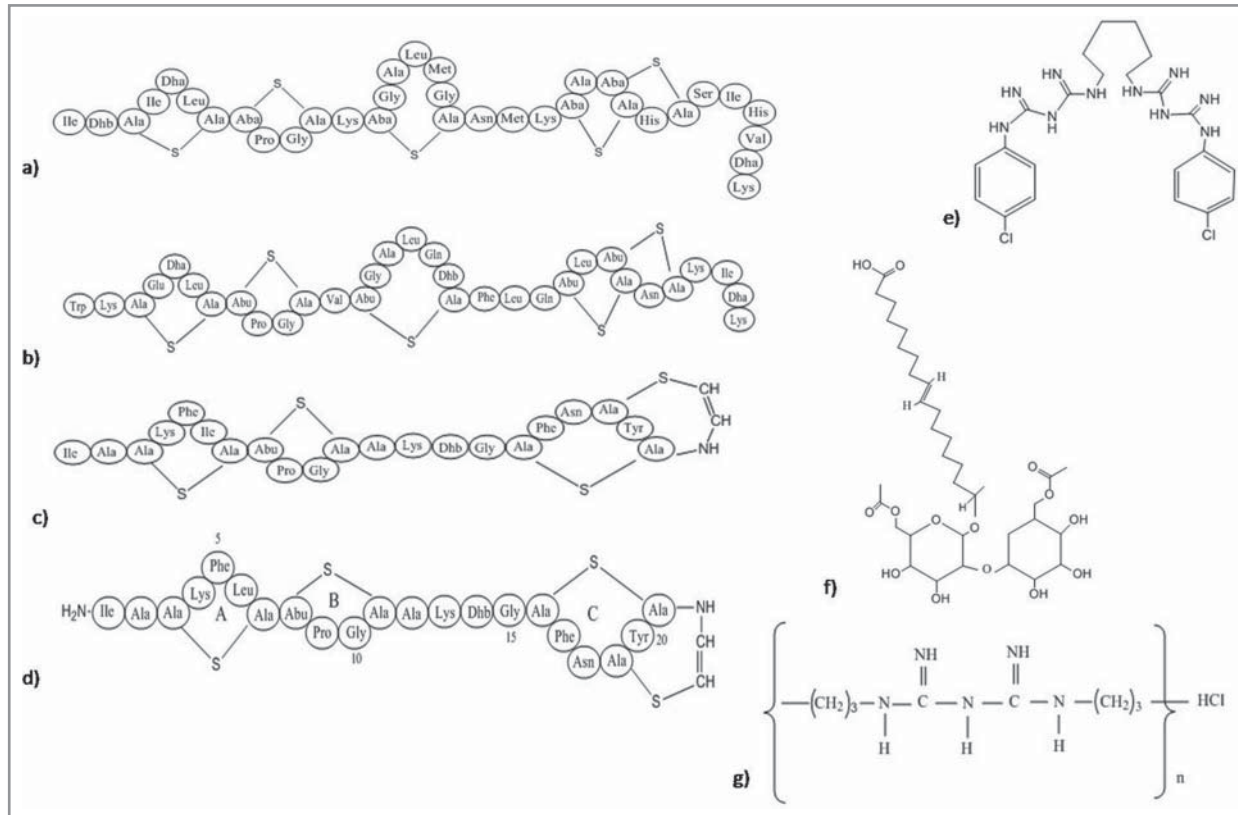
The alteration of membrane potential or membrane permeabilization is yet another mode of action of antimicrobial peptides. This results into disruption of the

cytoplasmic membrane via pore formation either through a barrel-stave,<sup>182</sup> a toroidal pore,<sup>183,184</sup> or through a non-pore carpet-like mechanism<sup>185</sup> that result into the efflux of intracellular materials.

The lantibiotics are the class of the peptide antibiotics with ring structure, linked via thioester containing lanthionine and methylanthionine, or unsaturated amino acids dehydro alanine or 2-amino isobutyric acids. They are synthesized by ribosomes and modified post-translationally in Gram-negative bacteria and serve as anti-biofilm agents. These peptides comprises of an intra-molecular ring structure and can inhibit a wide-range of bacteria.<sup>186</sup> These compounds pose an inhibitory effect on bacteria by damaging its bacterial membrane thus, inhibiting the production of enzymes. The most well known lantibiotic, nisin (Fig. 6), forms complex with lipid I and II thus, resulting in the inhibition of the cell wall biosynthesis.<sup>187,188</sup> Nisin can also induce permeability to the cytoplasmic membrane by producing pores with short life-span.<sup>186</sup> Subtilin (Fig. 6), another pore-forming lantibiotic structurally similar to nisin, acts by dissipating the transmembrane proton motive force resulting in the release of cytoplasmic solutes from *Staphylococcus simulans* and *B. subtilis* cells and from membrane vesicles. Subtilin binds to bactoprenyl pyrophosphate and causes membrane permeabilization in a lipid II-dependent fashion. *In-vitro* modifications were successfully used to insert thioester rings in various biologically active peptides. Clinical use of these modified lantibiotics can be ensured after proper *in-vivo* tests.<sup>189</sup> Epidermin and gallidermin have the same putative lipid II binding motif as nisin; however, differing only in size with 22 amino acids, as compared with 34 in nisin. These 2 lantibiotics interfere with lipid II biosynthesis and interact with lipid-I, lipid-II and their intermediates that ultimately



**Figure 5.** Structures of the anti-biofilm molecules that inhibit lipopolysacharides. (a) Colistin (Polymixin E)<sup>273</sup>, (b) Polymixin B<sup>274,275</sup>, (c) Gramicidin S<sup>276</sup>, (d) Sushi peptide (S1 domain)<sup>181</sup>.



**Figure 6.** Structures of the anti-biofilm molecules that alter the membrane potential or membrane permeabilization. (a) Nisin<sup>277,278</sup>, (b) Subtilin<sup>279</sup>, (c) Epidermin<sup>280</sup>, (d) Gallidermin<sup>281</sup>, (e) Chlorhexidine<sup>282</sup>, (f) Sophorolipid<sup>283</sup>, (g) Polyhexamethylene biguanide<sup>284</sup>.

prove to be fatal for the bacteria. Studies show that gallidermin efficiently inhibits biofilm formation by *Staphylococci*, which might be due to repression of genes involved in biofilm formation, such as *atl* (major autolysin) and *ica* (intercellular adhesin). However, the effect of gallidermin on mature (24-h and 5-day-old) biofilm was significantly reduced.<sup>190</sup>

Biosurfactants are amphipathic molecules that possess antibacterial activity, inhibit bacterial cell-surface adhesion and hence disrupt biofilm. Sophorolipids are a class of biosurfactants that act against biofilms by increasing the permeability of membrane. In *B. subtilis*, sophorolipids (Fig. 6) disrupt the bacterial cells followed by release of an intracellular enzyme, malate dehydrogenase causing efflux of their cytoplasmic contents. They also inhibit biofilm produced by single as well as mixed culture of *B. subtilis* and *S. aureus* at very low concentration. This result indicated the use of sophorolipids as an adjuvant with other antibacterial agents that inhibit bacterial growth or disassemble the biofilm of some pathogens.<sup>191</sup> Biofilm can also be destroyed by the use of polyhexamethylene biguanide (Fig. 6); a cationic antimicrobial agent disrupts the membrane and hampers the cell permeability without cell wall lysis. Chlorhexidine (Fig. 6) changes the osmolarity of the cell by binding with the negatively charged component. Compared to these 2, a silver compound penta-silver hexaoxiodate (Ag(5)IO (6)) is much more efficient in killing of a broad spectrum planktonic organisms, inhibition of microbial adhesion to surface for longer time period and disassemble and eradicate mature biofilms of *C. albicans*, *P. aeruginosa*, and *S. aureus*. The reason behind the high efficiency may be due to structure of the nanomaterial, which possess silver in both cation and anion along with the protection of anion by iodate. This compound may consider to be used as a potent antimicrobial agent for disinfecting medical devices like catheters, implants, ventilators and wound dressing.<sup>192</sup>

### Inhibition of cell division and survival

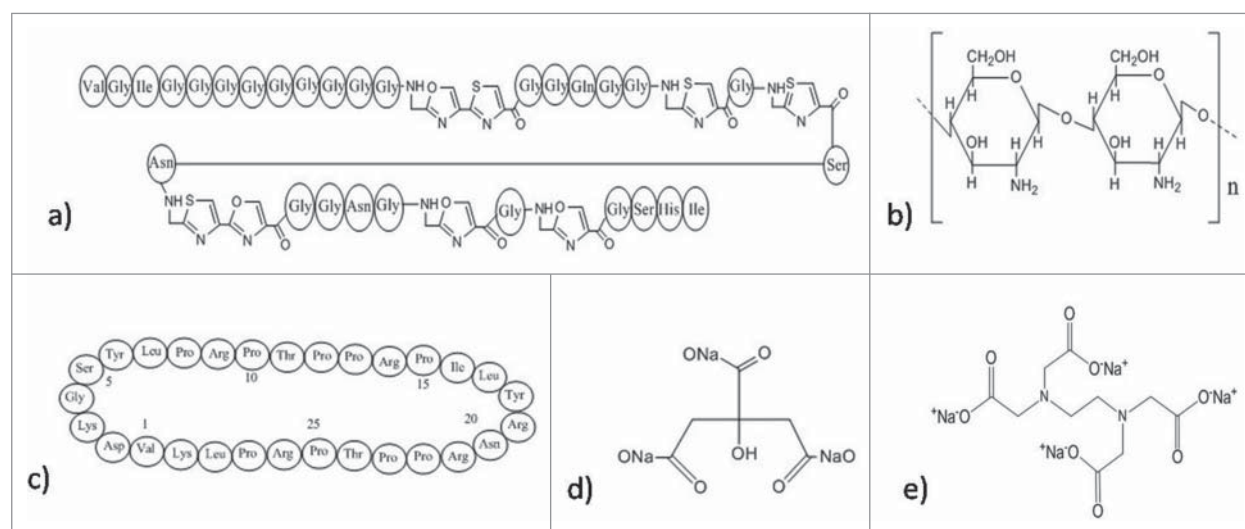
Cell division is very crucial for the survival of bacteria in biofilm and their further spread to a new area. Silver accumulates within the intracellular vacuoles resulting in the damage of plasma membrane followed by alteration in the electric potential, thereby preventing cell division.<sup>122,193</sup> Some antimicrobial peptides function by inhibiting cytoplasmic proteins, which have role in cell division and survival. These peptides penetrate into the cytosol of bacteria either by flip-flop method or channel formation in the outer membrane protein. Some antibacterial peptides are rich in proline such as pyrrolicorin (Fig. 7),<sup>194</sup> apidaecin<sup>195</sup> and drosocin.<sup>196</sup> All these

peptides are capable to bind with multi-helical lid region of DnaK (a heat shock protein of bacteria) and interfere in the initiation step of chromosomal DNA replication. Moreover, they also interfere in the interaction of DnaK with DnaJ that causes bacterial death. Pyrrolicorin enters into bacterial cytosol via C-terminus and the N-terminus is responsible for inhibition of ATPase activity of DnaK protein.<sup>194</sup> In addition to this, proline-rich AMPs actively enter the bacterial cell and interfering in translation initiation via binding to the tunnel of ribosome.<sup>197</sup> Microcin B17 (Fig. 7), a ribosomally synthesized antimicrobial peptide from *Enterobacteriaceae* inhibits DNA gyrase followed by inhibition of DNA replication. Also, it is the first peptide that has the capability to inhibit a type II DNA topoisomerase.<sup>198</sup> Apart from this, chelating agents like EDTA (Fig. 7) are able to potentiate the cell wall, thereafter destabilizing the biofilms via sequestering iron, zinc, magnesium, and calcium. This makes them suitable for the management of biofilms.<sup>199</sup> Chitosan, a natural polymer, due to its cationic nature has the capability to disrupt negatively charged cell membranes as soon as microbes settle on the surface.<sup>200</sup>

### Inhibition of adhesion molecule synthesis and function

There are some classes of AMPs that exhibit killing of bacteria by direct interaction with nucleic acids without causing permeabilization of the membrane like, Buforin II.<sup>201</sup> The antimicrobial peptide PR-39 (isolated from the pig's small intestine) can penetrate the outer membrane, and stop the synthesis of DNA and protein, the basic components of biofilms.<sup>202</sup> Another peptide, indolicidin, permeabilizes the membrane without causing the lysis of bacterial cells. It also inhibits DNA synthesis,<sup>203</sup> and exhibits binding specificity with DNA rather than RNA.<sup>204</sup> Studies also reported that a cationic peptide LL-37 (Fig. 8) is present in human as host defense peptide, has the capacity to reduce the bacterial adhesion and promote stimulation of twitching motility mediated by type IV pili. It also stimulates the downregulation of genes related to quorum sensing.<sup>39</sup> It was found active against *S. epidermidis* inhibiting the attachment of bacteria and thereby the formation of biofilms.<sup>205</sup> Studies also revealed that citropin (from green tree frog *Litoria citropa*)<sup>206</sup> and melimine (a non-hemolytic hybrid peptide)<sup>207</sup> have potent activities against *P. aeruginosa* and *S. aureus* and did not pose any toxic effect when tested in animal models. These molecules can be used to prevent bacterial adhesion on medical equipments like catheters and contact lenses. Another modified peptide, cadexomer iodine (Fig. 8),

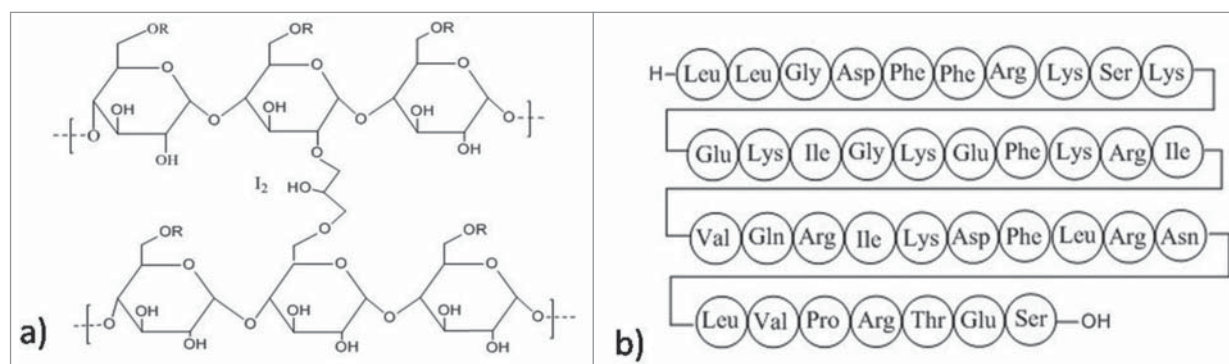




**Figure 7.** Structures of the anti-biofilm molecules that inhibit cell division and survival. (a) Microcin B17<sup>285,286</sup>, (b) Chitosan<sup>200</sup>, (c) Pyrrolic acid<sup>287</sup>, (d) Sodium Citrate<sup>288</sup>, (e) Tetrasodium EDTA<sup>289</sup>.

binds with the cytoplasmic membrane proteins followed by its penetration into the bacterial cell leading to the inhibition of protein synthesis and the disruption of lipid membrane as well as it interferes with the functioning of nucleic acids.<sup>193</sup> Recent studies also demonstrate the ability of AMP to coat the bacteria or the surface of the biomaterials leading to the reduction of adhesion by bacteria as well as reduced biofilm formation.<sup>208</sup> Bacteriocins like bovicin HC5 (produced by *Streptococcus bovis* HC5) and nisin are found to alter the hydrophobicity of the surfaces of *S. aureus* attachment, thus minimizing the adhesion to surfaces of food items, which is thought to be a better option than eradication of the already established biofilms. This facilitates long time storage and preservation of packaged food items.<sup>209</sup> The pili or fimbriae are the long filamentous surface structures that enable bacteria to adhere to the host tissues and are also found to be involved in biofilm formation. PilB and

PilA components of pili are important for biofilm formation but not PilC.<sup>210</sup> Pili are classified into 2 groups. Type I pili comprises of mainly 2 components- FimA (major part) and FimH (minor part). FimH is a mannose-binding adhesion component which facilitates bacterial invasion.<sup>211</sup> Most of the uropathogenic *Escherichia coli* (UPEC) possess type I pili attached with FimH adhesin that facilitates colonization on silicone implants and on surface of urinary bladder leading to CAUTI (Catheter Associated Urinary Tract Infections).<sup>212</sup> This pathogen after entering the host cells bypasses the host immune system and starts aggregating to form large intracellular bacterial communities (IBC) similar to biofilms.<sup>213-215</sup> A peptide from the gingival crevicular fluids and saliva, named lactoferrin, inhibits the attachment of *S. mutans* and *Streptococcus gordonii* and prevents the formation of biofilm in oral cavity.<sup>216</sup> It is also evident from other studies that the presence of



**Figure 8.** Structures of the anti-biofilm molecules that inhibit adhesion molecule synthesis and function (a) Cadexomer iodine<sup>290</sup>, (b) LL-37<sup>291</sup>.

lactoferrin prevents the biofilm formation by *Porphyromonas gingivalis* and *Prevotella intermedia* in the subgingival plaque at a very low concentration of  $\geq 8 \mu\text{g/mL}$ .<sup>217</sup> Mannosides are small molecules that work as an inhibitor of FimH by blocking their functions.<sup>218-220</sup> Murine model was used to investigate the effect of mannosides for treating CAUTI and it was found that the compound effectively inhibited invasion and colonization on the urinary bladder epithelium after infection by UPEC of the implanted bladders. It also enhanced the activity of trimethoprim-sulfamethoxazole when administered orally for the treatment of urinary tract infections.<sup>66</sup> Additionally, mannosides inhibited formation of biofilm on silicone surface in-vitro and if it is used for treatment in humans, it resulted in the reduction of CAUTI rates via inhibiting colonization and invasion of UPEC in bladder and also by not letting to form biofilm on the catheter surface.<sup>221</sup> Another study shows that pili-cides, inhibitor of Chaperone/Usher Pathway pili inhibit type I piliation and dysregulates virulence factors of UPEC thus affecting its growth.<sup>222</sup> Both these molecules are of much clinical relevance and if used to treat UTI or CAUTI current guidelines must be followed. Mannosides may also be used along with other potential antibacterial agents or other preventive compounds before inserting catheter in patient's body as a preventive measure.<sup>223</sup> In addition to these, a plant derived compound, eugenol is suggested to inhibit early biofilm formation and also reduce preformed biofilm of *S. mutans*. It does not affect the bacterial viability, but downregulates the expression of virulence genes involved in the adhesion and formation of biofilm such as comDE, ftf, smu630, vicR, gtfB, relA, gbpB, gtfC, brpA, and spaPat sub-MIC level.<sup>141</sup>

### Inhibition of biofilm by polysaccharides

Extracellular polysaccharides is an essential component of biofilms. Recently, a few exo-polysaccharides have been found to show negative activity against biofilm formation.<sup>224,225</sup> They not only inhibit the biofilm formation but can also lead to the dispersion of the preformed biofilm. A recent experiment have reported an exo-polysaccharide EPS273, obtained from a marine bacterium, *P. stutzeri* 273 that reduces biofilm formation in *P. aeruginosa* by targeting virulence factors which include exoprotease, pyocyanin, and rhamnose. EPS273 interferes with pyocyanin production whose reduction causes the decrease in the production of  $\text{H}_2\text{O}_2$  ultimately inhibiting the release of eDNA which is required for the formation

of stable biofilms.<sup>226</sup> It is also reported that this molecule reduces biofilm related infection in lung cells and embryos of zebrafish and also acts as a potent antioxidant, thereby decreasing the hydroxy radicals and superoxide radicals. Therefore, EPS273 can find its use in healthcare as well as in food industry against *P. aeruginosa*, which is responsible for causing nosocomial infections and spoilage of food respectably. Structural studies revealed that EPS273 has typical characteristics of polysaccharide. 35.4% glucosamine, 28.6% rhamnose, 27.2% glucose, and 8.7% mannose are the predominant monosaccharide units found in EPS273. Molecular weight of this molecule was reported to be 190kDa via HPGPC analysis.<sup>227</sup> Other various anti-biofilm polysaccharides have also been reported. Psl and Pel (Fig. 9) from *P. aeruginosa* PAO1 decrease the ability to form biofilm by *S. epidermidis* in dual-species biofilm in-vitro conditions.<sup>228,229</sup> K2 polysaccharide (Fig. 9) from the capsule of *E. coli* and PAM galactan from strains of *K. kingae* regulate their own biofilm architecture like forming water channels or dispersal of the biofilm thereby inhibiting their own biofilm according to their surrounding environment.<sup>230,231</sup> Another polysaccharide, A101 from *V. cholerae* QY101 causes dispersal of the biofilm formed by *P. aeruginosa*.<sup>224</sup> An exopolysaccharide, PAM galactan from the biofilm of *K. kingae* has also been reported to disperse biofilm of *S. epidermidis*.<sup>230</sup> Many non-bacterial polysaccharides extracted from plant, animal and some algae are also reported to possess anti-biofilm activity.<sup>225</sup> These anti-biofilm polysaccharides, especially the ones that are of bacterial origin portray broad-spectrum anti-biofilm activity while only some are capable of dispersing biofilms in their initial stages before attaining maturity. Different oligosaccharides or polysaccharides exhibiting anti-biofilm properties can be used in industrial and clinical settings which are greatly inhabited by antibiotic resistant biofilms causing variety of nosocomial infections. They can be used as adjuvant with available antibiotics reducing their minimum biofilm eradication concentration,<sup>224</sup> anti-adhesive coating decreasing chances of infections related to medical devices,<sup>230-233</sup> and probiotics to deliver saccharide prebiotics.<sup>234</sup> Another protein PslG produced by *P. aeruginosa*, participates in biosynthesis of Psl, one of the most important polysaccharide of its biofilm matrix. Structural analysis of PslG revealed that it is an endoglycosidase and according to reports endogenous administration of PslG disperses preformed matured biofilm and inhibits biofilm formation by targeting the Psl in the matrix. *Ex-vivo* studies revealed that the treatment with PslG,

increases the susceptibility of biofilms toward antimicrobials and host immune system.<sup>235</sup>

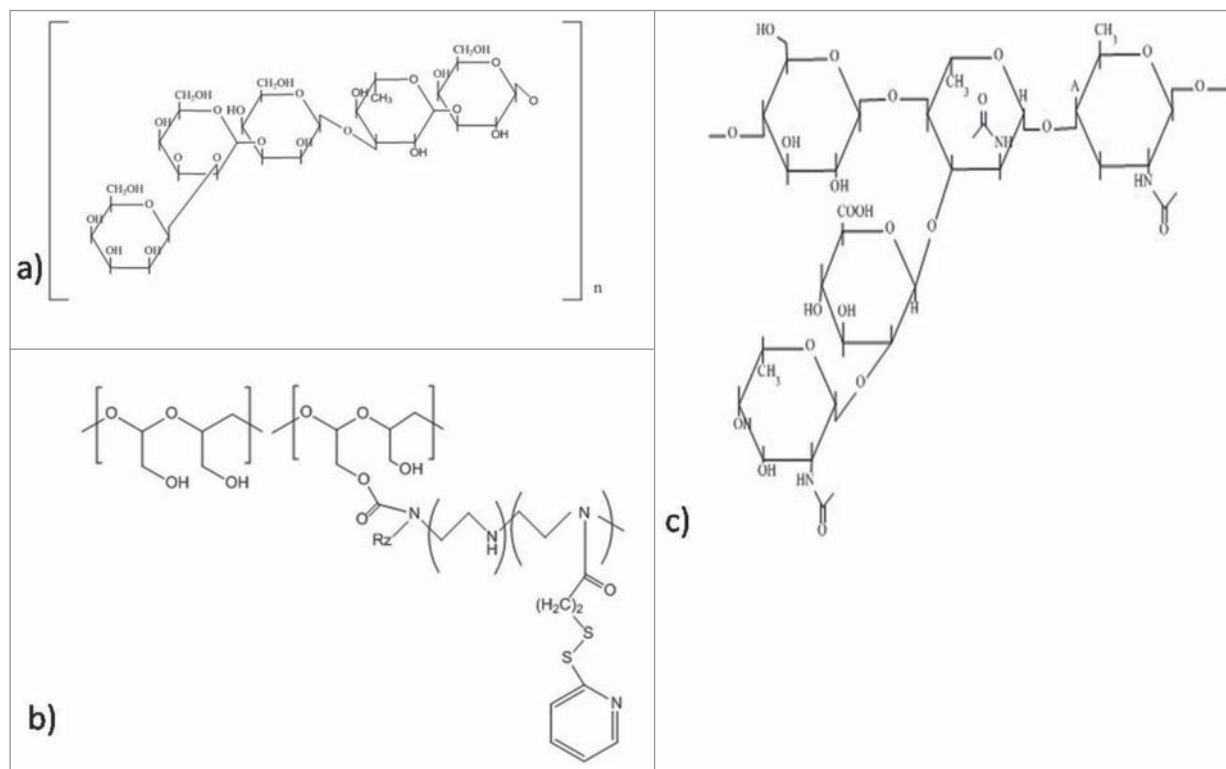
### Inhibition of c-di-GMP signaling system

Bacteria exist in 3 distinct forms namely; planktonic state responsible for acute infections and can be easily eradicated by administration of required dosage of antibiotics, biofilm state responsible for chronic infections and difficult to treat with antibiotics. The third form is the dispersed state, a distinct stage during transition between biofilm and planktonic state. The process of dispersal facilitates biofilm to spread infections within the host and also result in the transmission of bacteria between different hosts. Cyclic di-GMP (c-di-GMP) is a secondary messenger that play role in the biofilm formation. Modification of signaling pathways of c-di-GMP in bacteria can alter the biofilm formation and its dispersal in clinical environment.<sup>236</sup> Under stress conditions such as starvation, nitrosative conditions, etc., the bacterial cells lower the amount of c-di-GMP by the activation of phosphodiesterase leading to the dispersal of biofilm. This study also showed that biofilm-dispersed cells are very distinct from biofilm as well as planktonic cells in physiology as well as in their capacity of pathogenesis. Dispersed cells are found to be more virulent against *C. elegans* and immune cells, due to high level expression of

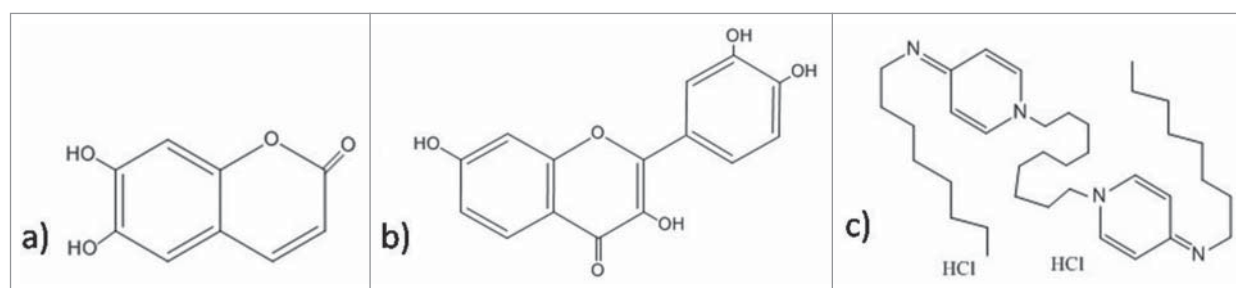
virulence related genes as compared with biofilm and planktonic cells. In addition to reduced c-di-GMP concentration, the biofilm-dispersed cells experience a reduced rsmY and rsmZ expression resulting in low siderophore production by bacterial species.<sup>237</sup> The siderophores function by chelating iron from the environment and are found to be involved in the prevention of biofilm formation by reducing the survival of dispersed cells. On administration of chemicals, the dispersal based anti-biofilm activity gets induced. These dispersed cells can escape the macrophage-mediated phagocytosis, therefore along with the dispersing agents; administration of some antimicrobial agent is preferred, as it would hinder the growth and the spread of dispersed cells. The addition of an iron chelator with the dispersing agent and antimicrobial agent would possibly eradicate the biofilm.<sup>237</sup>

LP 3134, LP 3145, LP 4010 and LP 1062 are the small molecules that inhibit diguanylate cyclase (DGC) that mediates the synthesis of c-di-GMP and hence inhibit biofilm formation in *P. aeruginosa* and *Acinetobacter baumannii*. All of these molecules have been reported to inhibit the biofilm dispersal of *P. aeruginosa*. Among these, only 2 were potential candidates for inhibiting biofilms because they were non-toxic to eukaryotic cells.<sup>6,238</sup>

Some other molecules were also identified as inhibitors of the allosteric binding of c-di-GMP, from studies



**Figure 9.** Structures of the anti-biofilm molecules that inhibit polysaccharides. (a) Psl polysaccharide<sup>292,293</sup>, (b) Pel polysaccharide<sup>294</sup>, (c) CFT073 group-II capsular polysaccharide (Serotype K2)<sup>295</sup>.



**Figure 10.** Structures of the antibiofilm molecules with unknown mechanism of action. (a) Esculetin<sup>296</sup>, b) Fisetin<sup>297</sup>, c) Octenidine hydrochloride<sup>298</sup>.

subjected to differential radial capillary action of ligand assay. DGC activity was reduced by the administration of a synthetic organoselenium drug, ebselen, and binding of c-di-GMP was inhibited by ebselen oxide. Therefore, these 2 molecules can regulate the production of biofilm in *P. aeruginosa*.<sup>239</sup> Indole signaling is considered as one of the most important signaling pathway that is responsible for various pathogenicity related bacterial behaviors such as virulence,<sup>240</sup> acid tolerance,<sup>241</sup> biofilm formation,<sup>242</sup> resistance to antibiotics.<sup>243</sup> Studies performed by Bunders et al. revealed that derivatives of desformylflustra bromine (dFBr) result in the inhibition of production of biofilm via modulating the signaling pathway of indole in *S. aureus* and *E. coli*.<sup>244</sup>

### Molecules with unknown mechanism

Some of the antibiofilm molecules are reported to work very efficiently but their mechanism of action is yet to be discovered. Secondary metabolites fisetin (Fig. 10) and esculetin (Fig. 10) are known to inhibit biofilm. Esculetin treatment affects the structural maturation of biofilm thereby, reducing its thickness. In contrast to this, fisetin not only reduces thickness of mature biofilm but also, interferes with the initiation of biofilm formation, reducing the coverage area. Hence, fisetin is considered better antibiofilm agent than esculetin.<sup>245</sup> A positively charged bispyridinamine, octenidine hydrochloride (Fig. 10) is also suggested to be an effective anti-biofilm agent but its mode of action is still unclear. Studies demonstrate that this compound can be potentially used as sanitizer and antimicrobial lock solution in both treatment and prophylactic activities.<sup>246</sup>

### Cytotoxicity of Anti-Biofilm molecules

Cytotoxicity is the most important factor for assessing any adverse effect of the anti-biofilm molecules before using them commercially for the prevention and removal of biofilm. Various methods are there for testing

cytotoxic effect namely Lactate dehydrogenase (LDH) assay, MTT assay, XTT assay, Trypan blue, crystal violet, colony formation method, DAPI and PI. Naturally derived compounds from plants are usually not toxic. Cytotoxicity studies have been performed in a variety of species, and the results demonstrate that octenidine hydrochloride is not absorbed through the gastrointestinal tract and, mucous membrane with no reported genotoxicity, carcinogenicity, or mutagenicity.<sup>247</sup> Likewise, it has been observed that usnic acid might show little side effects such as allergic contact dermatitis and local irritation. *In-vitro* studies revealed no cytotoxic effects of this compound when tested alone or as a constituent of oral formulation. Moreover no toxic effects were evident in pharmacokinetic studies as well as after oral administration.<sup>132,175</sup> Studies also suggested that AMPs do not show any cytotoxic effects.<sup>248</sup> Similarly, many antibiofilm compounds like S-phenyl-L-cysteine sulfoxide and its derivative diphenyl disulfide (inhibits biofilm via quorum sensing inhibition) was reported to be non-toxic and non-lethal when tested in drosophila-based infection models.<sup>249</sup> Apart from testing cytotoxicity of anti-biofilm molecules, some other studies are also required such as permeability studies, plasma protein binding, efflux studies and solubility (in water and salt) studies.

### Conclusions and future prospective

Till date, a lot has already been studied and understood about the bacterial biofilm formation. Emergence of severe biofilm infections and its resistance to antimicrobial treatment, has posed a great challenge in the medical field. The occurrence of resistant bacteria is mainly seen in human, farm animals, fruits, vegetables, dairy products, sea-foods and poultry products. Hence it is essential to investigate the effective ways to combat this problem and find an alternative among antibiotics. To make through this great challenge of today, biofilm imaging techniques have immensely developed. Fluorescence photo-activated localization microscopy (FPLAM), photo-activated localization microscopy (PLAM)

and stochastic optical reconstruction microscopy (STORM)<sup>250-252</sup> are the techniques which involves super-resolution microscopy. They use fluorescent proteins or probes to produce images with much higher resolution than CSLM. Hence, these techniques can be used more often for studying biofilm. To find a significant and effective alternative against biofilm, focus on the discovery of different anti-biofilm molecules along with modifications in the different signaling pathways associated with quorum sensing is taken under consideration. The higher eukaryotes are devoid of cyclic-di-GMP (c-di-GMP) signaling pathway, which makes it an attractive target for designing naive anti-biofilm agents. Besides these, the role of amyloids in bacterial biofilms has also become prevalent. Targeting these amyloids lessens the adherence property of bacterial cells hence affecting the formation of biofilms.<sup>6</sup> The present study provides the information of the mechanism of action used by different small molecules with anti-biofilm properties. Though, every anti-biofilm molecules have their specific modes of action, but a single molecule may follow more than one mechanism; for example, EGCG works either by inhibiting the AHL-mediated quorum sensing pathway or by degradation of peptidoglycan and membrane disruption. Information regarding mechanism of action provides better understanding about the nature of biofilms, which can be further used to develop new and successful drug molecules with the previously known target of action. This can bring about improvement in the efficacy of the previously known drugs. It can be achieved either by making suitable modifications or by using combinatorial therapy which includes the previously reported less effective drug against bacterial infections along with the potent anti-biofilm agents thereby raising the activity of the antibiotics. According to the present review, EPS273, mannosides and pilicides are the most significant anti-biofilm agents with high clinical relevance but needs further *in-vivo* testing followed by clinical trials. The naturally derived antimicrobials have more biochemical and structural diversity compared with synthetic drugs, thus could be very useful in developing various alternative therapies and *in-silico* pharmaceutical approaches. Additionally, the high complexity of natural products allows enhanced selective binding to the target. The disadvantage of using naturally derived anti-biofilm agents is their high cost, less sustainability; more time consumption and sometimes they show different results once extracted from their sources. In contrary, synthetic drugs are cost effective and consume less time but many of them show adverse side effects. Moreover numerous peptides and polysaccharides due to their large size become inappropriate from a drug perspective. While designing any compound for

combating biofilm, it is very important to observe its proper delivery in any *in-vivo* system at a particular site and the dosage of the anti-biofilm compound is another crucial factor, which needs thorough investigation. However, the discovery of new drugs against new targets is likely to be a prolonged and disconcerting process. Therefore, remodeling the previously used drugs approved against target other than biofilms may prove to be a more appropriate and constructive idea. A wide range of bioinformatic tools are available that might be used for the purpose of screening libraries of the existing drugs and to revamp them as a worthwhile contribution. Further, research has to be performed to find out the small active fragments of these peptides and polysaccharides that can effectively bind to the new identified targets.

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No potential conflicts of interest were disclosed.

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