MINIREVIEW

Anti-biofilm agents: recent breakthrough against multi-drug resistant Staphylococcus aureus

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Staphylococcus aureus biofilms are a major health concern. In this review, Chung & Toh discuss recent progress in preventing and eradicating these biofilms and discuss future potential anti-S. aureus biofilm therapies

Keywords
Staphylococcus aureus; anti-biofilm agents; quorum sensing.

Abstract
Staphylococcus aureus is a Gram-positive pathogen that causes potentially life-threatening nosocomial- and community-acquired infections, such as osteomyelitis and endocarditis. Staphylococcus aureus has the ability to form multicellular, surface-adherent communities called biofilms, which enables it to survive in various sources of stress, including antibiotics, nutrient limitations, heat shock, and immune responses. Biofilm-forming capacity is now recognized as an important virulence determinant in the development of staphylococcal device-related infections. In light of the projected increase in the numbers of elderly patients who will require semi-permanent indwelling medical devices such as artificial knees and hips, we can anticipate an expanded need for new agents and treatment options to manage biofilm-associated infections in an expanding at-risk population. With better understanding of staphylococcal biofilm formation and growth, novel strategies that target biofilm-associated infections caused by S. aureus have recently been described and seem promising as future anti-biofilm therapies.

Introduction
Staphylococcus aureus is one of the most important biofilm-forming pathogens that cause complications ranging from minor to life-threatening infections. Multidrug-resistant S. aureus which are isolated from clinical environments have a high probability of forming biofilms in indwelling medical devices (Kwon et al., 2008) and increase the probability of development into persistent, chronic, and recurrent infections (Francois et al., 2000). The ability of S. aureus to form biofilm has drawn considerable interest from researchers over the past decade, particularly biofilms formed on catheters or implanted devices, bone, and prosthetic heart valves (Kiedrowski & Horswill, 2011; Fig. 1).

Currently, biofilm infections are usually treated with combinations of antibiotics. In device-related biofilm infections, the device often has to be removed and replaced surgically, which involves risk and complications (Holby et al., 2011). However, novel strategies in preventing and eradicating biofilm formation have recently been reported. In this review, we will summarize the features of staphylococcal biofilm, the most recent advances in the elimination of biofilms and discuss the potential of these promising developments.

Biofilm formation
Biofilms can be defined as structured aggregation of surface-attached microorganisms encased in an extracellular matrix. The staphylococcal biofilm life cycle is believed to occur in four stages, that is the initial attachment of cells to a surface, formation of microcolonies on the surface of interest, maturation of the microcolonies into an established biofilm, and dispersal of the bacteria from the biofilm. In the initial stage, the planktonic phenotype of the bacteria attach reversibly to a solid living or non-living substratum (O’Neill et al., 2008) by van der Waal forces, steric interactions, and electrostatic (double layer) interaction, collectively known as the DLVO (Derjaguin, Verwey, Landau, and Overbeek)
forces (Garrett et al., 2008). The surface of the substratum is conditioned by the host matrix proteins (fibrinogen, fibronectin, and collagen), forming a conditioning film that facilitates adhesion by the bacteria (Francois et al., 2000). A number of the reversibly adsorbed cells remain immobilize and become irreversibly adsorbed as a result of the hydrophobic and hydrophilic interaction between the bacteria and the surface (Liu et al., 2004). These bacteria then grow, multiply, and form microcolonies (Stoodley et al., 2008). Once microcolonies are formed and in optimal growth conditions, the biofilm undergoes the maturation stage where a more complex architecture of biofilm is established with water channels equipped to aid the flow of nutrients into the interior of the biofilm. Due to the availability of different physicochemical conditions in terms of oxygen availability, diffusible substrates and metabolic side products, pH, and cell density, cells from different regions of a biofilm can show different gene expression patterns. In the final stage of development, some of the bacteria cells can be dispersed from the biofilm, via physical detachment or signaling events followed by the hydrolysis of exopolysaccharide (EPS), and return to the planktonic state to enable the occupancy of new niches (Boles & Horswill, 2011).

In all these phases of biofilm formation, quorum-sensing (QS) system is involved in the regulation of population density and metabolic activity. Generally, QS system is a central component of bacterial cell-to-cell communication (Asad & Opal, 2008) which acts as a language for the interaction among the neighboring bacteria that collectively and genetically respond to the extracellular, diffusible small molecule signals released in a cell-density dependent manner (Kalai & Purohit, 2011). As such, the production of molecule signals can be controlled and helps the bacteria in overwhelming the host defenses by secreting exotoxins after sufficient colonization in the host has taken place. Molecule signals or autoinducers which are used in staphylococci are autoinducing peptides (AIP) such as AgrD peptide which are regulated by the agr locus (Pan & Ren, 2009).

Principal strategies in the management of biofilms

The challenge in treating staphylococcal biofilm infection is the increased resistance of the bacteria within the biofilm structures to antimicrobial agents and host defense mechanisms (del Pozo & Patel, 2007). Resistance to antimicrobial agents is mediated through a dormant phenotype caused by adaptation to an anoxic environment and nutrient deprivation. As a result, the metabolic levels of the bacterial cells are low and cell division occurs at radically down-regulated rates (Lewis, 2010), producing many slow-growing cells and a subpopulation of persisters cells that are tolerant to high levels of antimicrobial agents. Therefore, antibiotics such as β-lactams which are only active against dividing staphylococcal cells are not very efficient at eradicating biofilm infections (Hoiby et al., 2010).

In addition, the EPS matrix may act as a diffusion barrier to delay the infiltration of some antimicrobial agents (Xu et al., 2000). The reactive chlorine species in a number of these agents may be deactivated at the surface layers of the biofilm before they are able to disseminate into the interior of the biofilm (de Beer et al., 1994). A recent study showed that oxacillin, cefotaxime, and vancomycin had reduced penetration throughout S. aureus and S. epidermidis biofilms (Singh et al., 2010).

With the emergence of multidrug-resistant S. aureus, the need for more effective treatments of biofilm-associated infections becomes imperative. Three principal strategies have been developed to thwart biofilm formation or target different biofilm developmental stages (Fig. 2). The first principal strategy is inhibiting the adhesion of bacteria to living or non-living surfaces at the initial stage, thus reducing the chances of further development and establishment of biofilm. The second strategy is aimed at the disruption of biofilm architecture during the maturation process (Kalai & Purohit, 2011). The third strategy is an antipathogenic or signal interference approach, which involves the inhibition of QS. Staphylococcus aureus coordinates biofilm formation and expression of virulence factors via QS to enhance their ability to survive in a specific environment (Wright et al., 2004). A disruption of QS system or quorum quenching (QQ) will eventually affect the expression and dissemination of virulence factors.

Inhibition of attachment

Attachment of bacteria to surfaces is mediated by a number of factors such as adhesion surface proteins, pil or fimbriae, and specific exopolysaccharides (Maira-Litran et al., 2002; Conrady et al., 2008). In general, adhesion occurs most readily on surfaces that are rougher, more hydrophobic, and coated with surface conditioning films (Donlan, 2002). Catheters coated with minocycline and rifampin have been shown to significantly decrease the incidence of central line-associated bloodstream infection by S. aureus in a medical intensive care unit in a manner that was independent and complementary to the infection control precautions (Ramos et al., 2011). Thus, altering the surface properties of...
the indwelling medical devices, such as coating the surface with bactericidal or bacteriostatic substances, could prevent biofilm-associated infections. One of the most commonly used alternative agents is silver in the form of nanoparticles. Small molecules such as aryl rhodanines and chelating agents are also shown to inhibit staphylococcal biofilm formation.

### Small molecules

Aryl rhodanines specifically inhibit biofilm formation of *S. aureus* and other Gram-positive bacteria, but not Gram-negative bacteria. Preliminary studies revealed that aryl rhodanines specifically inhibit the early stages of biofilm development by preventing attachment of the bacteria to the surfaces (Opperman *et al.*, 2009). Interestingly, these molecules do not exhibit antibacterial activity against both the Gram-positive and Gram-negative bacteria. The absence of antibacterial activity reduces the selective pressure against biofilm formation, thus decreases the likelihood of the development of resistance. There were variable responses to calcium chelators ethylene glycol tetraacetic acid (EGTA) and trisodium citrate (TSC) on biofilm formation in different *S. aureus* strains (Abraham *et al.*, 2012). In some strains, the chelators prevented biofilm formation, while in others, they had no effect or actually enhanced biofilm formation. Thus, it is important to use these agents appropriately so that inhibitory doses are achieved consistently.

### Silver ions and nanoparticles

Metallic silver, silver ions, and silver nanoparticles have been used as antimicrobial agents in the treatment of burns and chronic wounds. Silver ions are effective against bacteria such as *E. coli*, *S. aureus*, Klebsiella species, *P. aeruginosa*, *Salmonella typhimurium*, and *Candida albicans* (Chernousova & Epple, 2013). The exact mechanism of action of silver on microorganisms is still not known but can be observed by the structural and morphological changes. Silver nanoparticles showed efficient antimicrobial property due to their extremely large surface area, which provides better contact with the microorganisms. The nanoparticles attach to the cell membrane and penetrate the bacteria. The particles then interact with the sulfur-containing proteins in the cell membrane and the phosphorus-containing molecules such as DNA (Rai *et al.*, 2009). Silver also interacts with thiol group compounds found in the respiratory enzymes of bacterial cells. As a result, silver treatment inhibits DNA replication, expression of ribosomal and other cellular proteins, and interferes with the respiration process, finally leading to cell death (Feng *et al.*, 2000; Klasen, 2000; Yamanaka *et al.*, 2005). Studies in rabbits showed that nanoparticle silver ion-coated implants inhibited *S. aureus* biofilm formation without causing silver accumulation in host tissues, even 28 days after impregnation (Secinti *et al.*, 2011). An implant coated with silver oxide-containing hydroxyapatite (Ag-HA) in the medullary activity of rat tibiae showed better results for

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![Fig. 2 Strategies in the management of biofilms (adapted from wikipedia.org/wiki/File:Biofilm.jpg).](https://academic.oup.com/femspd/article-abstract/70/3/231/567248/fig2)
abscesses, bone resorption, and destruction of cortical bone, indicating that Ag-HA coatings may help prevent surgical-site infections associated with joint replacement (Akiyama et al., 2013). In the presence of silver nanoparticles, antibiotics such as penicillin G, amoxicillin, erythromycin, clindamycin, and vancomycin showed increased antibacterial activity against *S. aureus* (Shahverdi et al., 2007).

However, studies performed *in vitro* with fresh platelet-rich blood plasma demonstrated that the presence of silver nanoparticles on medical devices correlates with accelerated thrombin formation and stronger platelet activation, which could increase the thrombosis risk in patients (Stevens et al., 2009). In a recent clinical trial on critically ill patients, the use of silver nanoparticles-impregnated triple-lumen central venous catheter (CVC) has been reported to have no significant effect on CVC colonization and related bloodstream infections (CRBSI), CRBSI incidence, or intensive care unit (ICU) mortality (Antonelli et al., 2012). Therefore, much effort is still needed to address the exact mechanism of interaction of silver nanoparticles with the bacterial cells and the effect of surface area of the nanoparticles on the killing activity.

**Disruption of biofilm architecture**

Mature biofilms are tolerant to antimicrobial agents due to the altered growth rate of the organisms in the biofilm (Donlan & Costerton, 2002) and emergence of resistant subpopulations (Ito et al., 2009). In addition, biofilms also promote horizontal transfer of antibiotic resistance genes in *S. aureus* (Savage et al., 2013). Thus, agents that interfere with the biofilm structure and have great potential in the management of biofilm-mediated infections are being developed by many research groups.

**Small molecules**

Cis-2-Decenoic acid (C2DA) is a medium-chain fatty acid chemical messenger produced by *Pseudomonas aeruginosa* that can induce the dispersion in biofilms in *S. aureus*, in addition to other Gram-positive and Gram-negative bacteria (Davies & Marques, 2009). In a pilot study carried out by Jennings et al. (2012), C2DA could potentially control initiation of biofilm formation in addition to dispersion of existing biofilm. The same study also showed that combination of C2DA may have additive or synergistic effects on biofilm formation. Davies & Marques (2009) demonstrated that C2DA inhibited biofilm in methicillin-resistant *S. aureus* (MRSA) but did not completely eliminate it. Recently, a mixture of D-amino acids reportedly triggered biofilm disassembly in *S. aureus*, as well as *B. subtilis* and *P. aeruginosa*. The incorporation of these acids into the peptidoglycan results in the release of amyloid fibers which is the proteinaceous component of the extracellular matrix (ECM) that linked cells together in the biofilm matrix (Kolodkin-Gal et al., 2010; Jermy, 2012). Although the *in vitro* data are encouraging, the mechanism of biofilm inhibition of these small molecules is still unknown.

**Matrix-targeting enzymes**

Dispersion and degradation of the matrix components, such as polysaccharide, DNA, and proteins, can weaken and disperse biofilms. Dispersin B, a biofilm-releasing enzyme produced by the Gram-negative periodontal pathogen *Actinobacillus actinomycetemcomitans* (Kaplan et al., 2004) could eliminate the biofilm in half of the catheter tested in a sheep model for port-related bloodstream infection when combined with teicoplanin (Serrera et al., 2007). Recently, dispersin B was reported to inhibit and disperse biofilm by depolymerizing a polysaccharide, β-1,6-N-acetyl-d-glucosamine (PGA) which is essential for the formation of biofilm in some species of staphylococci (Itoh et al., 2005). In experiments with *S. aureus* and *S. epidermidis* grown as biofilms, it has been demonstrated that dispersin B was able to significantly enhance the antimicrobial and anti-biofilm activity of antibiotic cefamandole nafate (CEF) by improving the diffusion of CEF into bacterial clusters and promoting the reaching of antibiotic cell targets (Donelli et al., 2007). In another study with vascular catheters, the combination triclosan and dispersin B showed synergistic and broad-spectrum antimicrobial and anti-biofilm activity against *S. aureus*, *S. epidermidis*, and *E. coli* significantly reduced bacterial colonization and generally demonstrated a prolonged superior antimicrobial activity compared to chlorhexidine-silver sulfadiazine (SH-SS; Darouiche et al., 2009).

DNase I cleaves DNA in the biofilm matrix and prevents biofilm formation on abiotic surfaces, such as glass, plastic, and titanium surfaces (Mann et al., 2009; Kedrowski & Horswill, 2011). In combination with dispersin B, DNase I inhibited biofilm formation 3.4-fold relative to untreated biofilms, while treatment with either enzyme alone decreased biofilm formation 1.6–2.8-fold (Lynch & Abbanat, 2010). Lysostaphin is a glycyglycine endopeptidase which specifically cleaves the pentaglycine cross-bridge in the staphylococcal peptidoglycan and disrupts the extracellular matrix of *S. aureus* biofilms. When applied to biofilms of *S. aureus* clinical isolates grown *in vitro*, lysostaphin markedly reduced biomass thickness (Wu et al., 2003). Kokai-Kun et al. (2009) demonstrated that lysostaphin is an effective treatment for established biofilm infections on implanted jugular veins catheters in mice, particularly in combination with nafcillin. Proteinase K cleaves the surface or matrix proteins and inhibits biofilm formation or dispersal of established biofilms. Chaignon et al. (2007) suggested that treatment with dispersin B followed by Proteinase K or trypsin could be capable of eradicating biofilms of a variety of staphylococcal strains on inert surfaces. As with the small molecules, the *in vivo* efficacy of these enzymatic treatments in the elimination of established biofilms is not well established, as treatment of host with proteins could cause inflammatory and allergic reactions (Chen et al., 2013), thus limiting the therapeutic potentials of these enzymes.

**Immunotherapy**

Currently, there are no approved immunotherapies for treating staphylococcal infections. The discovery and
development of effective vaccines are particularly difficult as *S. aureus* possess nearly 70 virulence factors which are transiently expressed (Harro et al., 2010) and armed with multiple factors to evade host immune response (Proctor, 2012). The vast majority of research in this area focuses on the protection from acute, planktonic-associated *S. aureus* infections. Studies have shown that gene expression and protein production between these two modes of biofilm and planktonic growth differ greatly (Beenken et al., 2004; Brady et al., 2006; Resch et al., 2006); thus, development of a universal *S. aureus* vaccine that protects against multiple modes of growth is particularly challenging. One vaccine which showed such potential in treating chronic infections is a quadrivalent vaccine comprising cell wall and membrane-associated proteins that has significantly reduced MRSA osteomyelitis infection in rabbits when co-administered with vancomycin (Brady et al., 2011). Several novel antigens are being tested as potential anti-*Staphylococcus aureus* vaccine, including cell-anchored adhesion proteins and exotoxins (Schaffer & Lee, 2009).

**Bacteriophage therapy**

In the treatment of infections associated with biofilms, phages offer advantages, that is, they are inexpensive, highly specific, do not affect the normal microflora in the environment in which they are introduced to, and improve the treatment of biofilm-related infections with conventional antibiotics (Yang et al., 2011). Phage may carry on their surface very specific enzymes that degrade bacterial polysaccharides and rapidly destroyed the integrity of biofilms (Sutherland et al., 2004). A cell wall-degrading enzyme SAL-2 from a *S. aureus* bacteriophage SAP-2 exhibits specific lytic activity with minimum inhibitory concentration (MIC) of 1 mg mL\(^{-1}\) and can efficiently remove *S. aureus* biofilms (Son et al., 2010). An induced phage SAP-26 which was isolated from a clinical strain of *S. aureus* showed a wide spectrum of lytic activity against both MRSA and meticillin-susceptible *Staphylococcus aureus* (MSSA). In the combined therapy of the phage with antimicrobial agents, particularly rifampicin, the phages are able to penetrate the biofilm layers through the pores and channels, causing induced structural changes and subsequent destruction in the biofilm matrix (Hughes et al., 1998). The bacterial cells are released as planktonic cells and then attacked by both the phages and antibiotics (Rahman et al., 2011). In another study, staphylococcal phage K has demonstrated the potential to prevent biofilm formation by *S. aureus* and reduce established biofilm density in a time-dependent manner, with complete inhibition of biofilm formation over a 48-h period (Kelly et al., 2012).

**Signal transduction interference**

Quorum sensing (QS) relies on a sequence of events including signal production, detection, and gene activation/ inactivation. Interruption of any of these steps could render the QS to fail and potentially cause detrimental consequences on the survival and pathogenesis of the bacteria (Pan & Ren, 2009). *S. aureus* regulate biofilm formation and dispersal using the agr QS system. Recent studies have shown that inhibition of *agr* causes *S. aureus* to become more adherent due to increased biofilm formation, while addition of autoinducing peptides (AIP) or glucose depletion reactivates *agr* in established biofilm, leading to complete disassembly and conversion of biofilm-associated cells back to a planktonic phenotype (Boles & Horswill, 2008). Activation of the *agr* system can result in increased levels of staphylococcal proteases that cut cell surface proteins and disrupt cell–cell interactions within the biofilm to cause biofilm dispersal (Kiedrowski & Horswill, 2011) and is also known to induce expression of phenol-soluble modulins (PSMs), which have recently emerged as a novel toxin family that contributes to biofilm development and dissemination of biofilm-associated infections (Peschel & Otto, 2013). Most importantly, dispersion of the cells from the biofilm restores the cells sensitivity to antibiotics such as rifampicin. Despite the success at clearing *in vitro* biofilms via the activation of the *agr* system, the mechanisms are not well defined.

**Quorum-sensing inhibitors (QSI)**

An overview of QSI based on patents submitted between 1999 and 2008 and their applications were reviewed extensively by Pan & Ren (2009). The QSI reported included both natural and synthetic agents and can be mainly categorized into non-peptide small molecules, peptides, and proteins. Hammelitannin (HAM), a non-peptide analog of the quorum-sensing inhibitor RNAIII-inhibiting peptide (RIP), was found to decrease *S. aureus* attachment *in vitro* and *in vivo* (Kong et al., 2006; Kiran et al., 2008). HAM and vancomycin or clindamycin may act synergistically to increase the efficacy of the antibiotics against biofilm-related infections and/or by increasing host survival after infection (Brackman et al., 2011). It has been reported that RIP-coated CVC exhibited significant reduction in the bacterial load in staphylococcal strains, including meticillin- and vancomycin-intermediate-resistant *S. aureus* and *S. epidermidis*. In combination with conventional antibiotics, RIP also enhanced the effect of ciprofloxacin, imipenem, and vancomycin in the treatment of catheter-related *S. aureus* infections (Cirioni et al., 2006). Polymethylmethacrylate (PMMA) beads loaded with RIP implanted in rats were also shown to be able to prevent biofilm formation in orthopedic infections caused by meticillin-resistant *S. aureus* (Anguita-Alonso et al., 2007). In addition, biodegradable gentamycin-releasing poly-trimethylene carbonate (PTMC) beads were demonstrated to be able to inhibit biofilm formation in *S. aureus* by c. 80% over at least 14 days, providing promising alternative in the local treatment of osteomyelitis (Neut et al., 2009).

An antibody against *S. aureus* quorum-sensing peptide AP4 was shown to suppress *S. aureus* pathogenicity in mouse abscess infection model (Park et al., 2007b). Silver nanoparticles synthesized using fresh leaf extract of *Cymbopogon citratus* (lemongrass) have been shown to enhance quorum-quenching activity against *S. aureus* biofilm and...
Anti-biofilm agents against *Staphylococcus aureus*

**Plant-derived natural compounds**

Natural products have played an important role as one of the major sources of new drugs for the past decade due to their incomparable structural diversity (Baker et al., 2007). With state-of-the-art methodologies for separation and isolation procedures, the search of new leads from plants that can be used to develop drugs for human therapy in persistent infections has increased considerably and has led to the discovery of compounds with inhibitory activities on biofilm formation in bacteria. Extracts from *Krameria, Aesculus hippocastanum*, and *Chelidonium majus* yielded four compounds, namely chelerythrine, sanguinarine (Fig. 3), dihydroxybenzofuran, and proanthocyanidin, which are necessary to fully understand the mechanisms of action and suitability for clinical applications of promising QSI.

**Conclusion**

Biofilm formation enables *S. aureus* to endure situations of environmental stress such as immune defenses and conventional antimicrobial therapies. This ability has challenged the treatment of infections caused by this microorganism. Although researches on the formation and dispersal of staphylococcal biofilm are still in its early stages, progress in the development of innovative approaches to eradicate biofilms has been made over the past decade. New approaches such as small molecules, enzyme treatments that weaken the structure of the biofilm, antibodies, and vaccines that targets each important phases of biofilm formation have been developed. However, these promising approaches remain to be validated clinically. As our understanding of the molecular mechanism of biofilm formation and regulation continues to improve, we anticipate that these new approaches will be eventually developed for use in the treatment of problematic biofilm-related infections in the clinical settings.

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**References**


