Inhibitory Effects of 2,2′-Dipyridyl and 1,2,3,4,6-Penta-O-Galloyl-b-D-Glucopyranose on Biofilm Formation in Contact Lens Cases

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Purpose. This study observed biofilm formation by *Pseudomonas aeruginosa* and *Staphylococcus aureus* in contact lens cases and investigated the inhibitory effects of 2,2′-dipyridyl (2DP) and 1,2,3,4,6-penta-O-galloyl-b-D-glucopyranose (PGG).

Methods. Biofilm formation of *P. aeruginosa* ATCC 9027 and *S. aureus* ATCC 25923 and ATCC 6538 in contact lens cases was determined for a range of initial inocula and incubation times using crystal violet staining. The effects of 2DP and PGG on biofilm were evaluated alone and in combination by their incorporation into the media at commencement of incubation.

Results. At 24 hours, biofilm production was related to initial concentration. However, with extended incubation, higher initial concentrations affected formation in *S. aureus*. Presence of 312 μM 2DP significantly inhibited *P. aeruginosa* biofilm formation, but had little effect on that of *S. aureus*. In contrast, PGG (50 μM) inhibited *S. aureus* biofilm formation, but had much less effect on that of *P. aeruginosa*. Combination of the agents effectively inhibited biofilm formation of all three organisms throughout a week-long incubation period with OD levels barely exceeding cell-free controls.

Conclusions. Biofilm formation of *P. aeruginosa* could be prevented by 2DP, while biofilm formation of *S. aureus* was inhibited by PGG. However, combining these agents showed better inhibition of biofilm production than use of either agent alone on both species. This combination may be useful in prevention of biofilm in contact lens cases, thereby reducing infection risk due to poor compliance with lens case cleaning and replacement. Further work is needed to confirm compatibility with multipurpose solutions and investigate cytotoxicity to ocular tissues.

Keywords: biofilm, inhibition, 2,2′-dipyridyl (2DP), 1,2,3,4,6-Penta-O-galloyl-b-D-glucopyranose (PGG), contact lens case, *Staphylococcus aureus*, *Pseudomonas aeruginosa*

Microbial keratitis is a rare, but a sight-threatening complication associated with contact lens wear.[1] *Pseudomonas aeruginosa* and *Staphylococcus aureus* are common pathogens associated with microbial keratitis,[2,3] and may be isolated from used contact lens cases.[2,4,5] Noncompliance in contact lens wear leads to contamination of the lenses and accessories, in particular the lens cases, and is a major obstacle to safe contact lens wear.[1,5–7] High rates of contamination of contact lens cases have been reported in users of both soft and rigid lenses.[7–9]

Commercially available contact lens multipurpose solutions to be approved by the US Food and Drug Administration (FDA) have to achieve at least a 3-log reduction of cell viability of three strains of bacteria designated in the FDA guidelines: *S. aureus* ATCC 6538, *Serratia marcescens* ATCC 13880, and *P. aeruginosa* ATCC 9027.[10] Some multipurpose solutions have been shown to have activity against other organisms, including clinical isolates.[11] However, the FDA testing method only requires solutions to be active against planktonic bacteria,[10] but both *P. aeruginosa* and *S. aureus* can form biofilms on surfaces of medical devices.[12,13]

The formation of a biofilm involves the attachment and accumulation of bacterial cells on a solid surface.[12] Organisms in the biofilm have been shown to be more resistant to killing by disinfectants and other antimicrobial agents through several mechanisms, such as reducing access of toxic agents to the cells within the biofilm, production of neutralizing chemicals, gene mutation or genetic exchange between cells in the biofilm, and cells with lower metabolic rate.[14,15] Szczotka-Flynn et al.[16] demonstrated that some multipurpose solutions (MPS) used currently had little to no effect (log reduction less than 1) on the biofilms of *P. aeruginosa*, *S. marcescens*, and *S. aureus*.

Iron is an essential nutrient for bacterial growth and is crucial for bacterial energy production, nucleotide synthesis, and regulation of gene expression.[17] Iron regulation of biofilm formation has been demonstrated in many bacterial species.[17,18] As in many other bacteria, the ferric uptake regulator protein in *P. aeruginosa* plays a major role in biofilm development as a global regulator of iron-responsive genes.[19] Singh et al.[20] demonstrated that iron acted as an environmental signal for the development of *P. aeruginosa* biofilm that can be

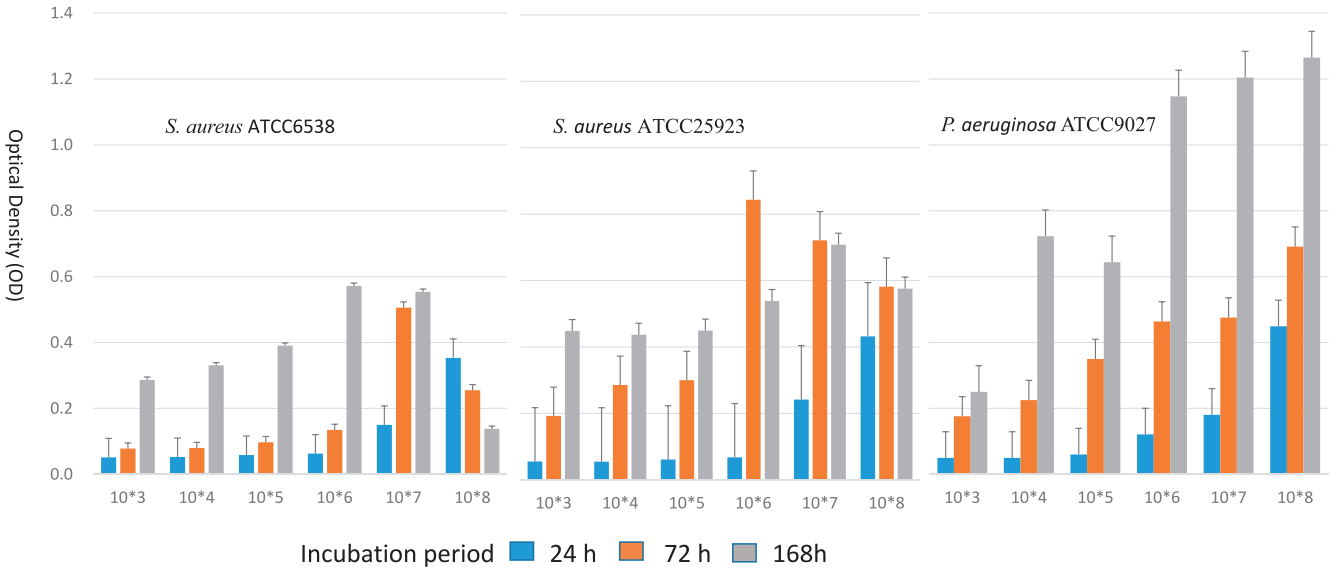

Figure 1. Mean optical densities of *S. aureus* ATCC 6538, *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 9027 biofilms produced in contact lens cases after 24-, 72-, and 168-hour incubation. Biofilms were stained before being resuspended and the optical density measured. Results shown are the mean of three repeats performed on different days.

restricted by lactoferrin, an iron chelator. In *S. aureus*, iron is also required for biofilm formation.[21]

Iron-chelating agents 2,2′-dipyridyl (2DP) and 1,2,3,4,6-penta-O-galloyl-b-D-glucopyranose (PGG) have been shown to inhibit biofilm formation on smooth surfaces by *S. aureus* and *P. aeruginosa*.[17,22] To facilitate contact lens removal from lens case wells, most lens case designs include ridges on the surface of the wells. These ridges make the cases more difficult to clean, facilitating biofilm formation.

The purpose of this study was to investigate the formation of biofilm by incubation of *S. aureus* and *P. aeruginosa* in the contact lens cases and the effects of 2DP and PGG against such biofilm formation. Two strains of *S. aureus* were utilized as they differ in their ability to produce biofilms, which is related to their production of exopolysaccharides.[23]

Methods

Bacterial Strains and Culture Conditions

Three bacterial strains were used in this study: *S. aureus* ATCC 6538, *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 9027. The first two strains are those recommended for testing MPS by FDA and are widely used in studies involving effectiveness of disinfection in optometry. The second *S. aureus* strain is recognized as producing more biofilm than the FDA recommended strain.[23] It is used as the standard strain for testing antimicrobial susceptibility. Tryptone Soya Agar (TSA; Oxoid, Basingstoke, UK) was used for cultivation of the bacterial strains. The plates were incubated at 37°C for 16 to 18 hours.

Chemicals

The iron chelators used were 2DP and PGG, which were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA), dissolved in phosphate buffered saline, and used over a range of concentrations (0–2500 and 0–50 μM, respectively).

Biofilm and Adhesion Assay

After 24 hours incubation on TSA plate, bacterial colonies were harvested and suspended in PBS (Sigma-Aldrich Corp.) that contained 20% nutrient broth (PBS-NB; Oxoid) and adjusted with the use of a spectrophotometer to achieve an optical density (OD) reading of 0.1 ($\sim 10^8$ CFU/mL) at 600 nm wavelength. Subsequently, the concentration of each inoculum was adjusted to approximately 10^3 to 10^7 per mL using serial dilution in PBS-NB, of which 4 mL was added to the wells of unused contact lens cases and incubated for 24, 72, and 168 hours at room temperature. We used PBS-NB without bacteria as control (blank). Biofilm formation in each well was analyzed by using a crystal violet staining method followed by measurement of OD.[23,24] Each experiment was repeated three times.

To determine the effects of the chelators 2DP and PGG on the biofilm formation, the chelators were incorporated into the medium at the start of the incubation period at a range of concentrations with approximate initial inoculums of 10^7 CFU/mL *S. aureus* ATCC 6538 and *S. aureus* ATCC 25923 and 10^6 CFU/mL *P. aeruginosa* ATCC 9027. The biofilms were subsequently analyzed at 24, 72, and 168 hours. Following assessment of the effects of the chelators alone, further testing was performed using a combination of 2DP (312 μM) and PGG (50 μM). All assessments were performed in triplicate.

Statistical analysis

Statistical analyses were performed using statistical software (SPSS system for Windows version 16.0; SPSS, Inc., Chicago, IL, USA). An unpaired *t*-test was used to compare the amount of biofilm formed between control and inhibitors, alone and in combination, as well as between inhibitors alone and in combination. A value of $P < 0.05$ was considered significant.

Results

Figure 1 shows that after 24 hours incubation, the OD of biofilm of *S. aureus* ATCC 6538, *S. aureus* ATCC 25923, and *P. aeruginosa* ATCC 9027 formed was related to the initial

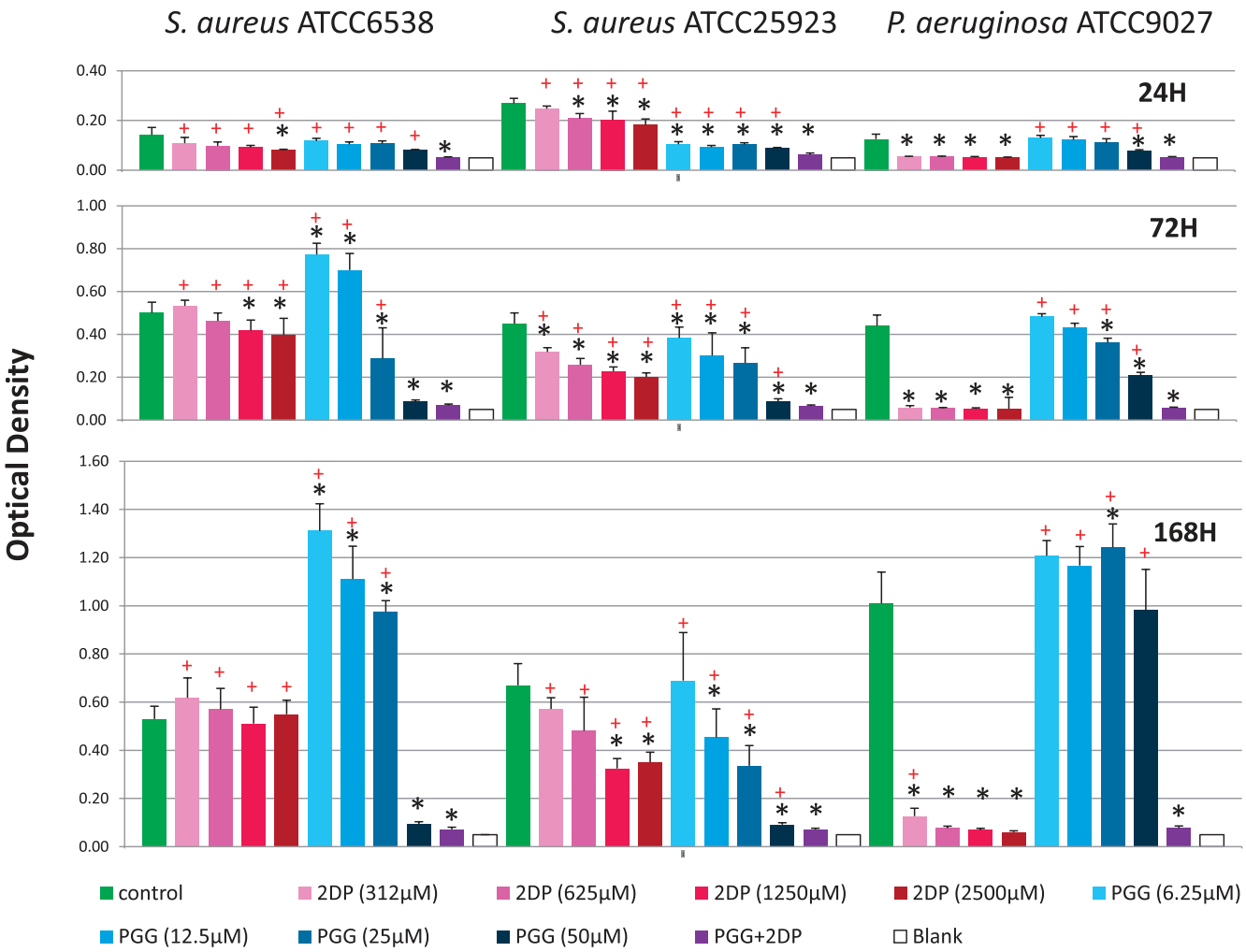

Figure 2. Effect of 2DP and/or PGG on biofilm formation of *S. aureus* ATCC 6538, *S. aureus* ATCC 25923, and *P. aeruginosa* ATCC 9027 in comparison to control growth of biofilm at 24, 72, and 168 hours. Each *error bar* indicates one standard deviation. *Significantly different from control. +Significantly different from combination (PGG + 2DP); $P < 0.05$ indicates significance.

concentration of bacteria, and a sharp increase of growth occurred in the presence of initial densities $\geq 10^7$ CFU/mL of *S. aureus* and $\geq 10^6$ CFU/mL *P. aeruginosa*. The optical density of *P. aeruginosa* biofilm was related to both the initial inoculum density of the bacteria and incubation period, with longer incubation and increased initial inoculums resulting in increased biofilm production. However, for both *S. aureus* ATCC 6538 and *S. aureus* ATCC 25923, optimal conditions for biofilm formation appeared to be an initial density of 10^6 CFU/mL, as biofilm production was reduced at higher initial inoculum densities if incubation was prolonged. At 10^6 CFU/mL, there was rapid formation of biofilm after 72-hour incubation period for *S. aureus* ATCC 6538; but there was greater and more rapid biofilm formation for *S. aureus* ATCC 25923, which did not increase much after 72 hours incubation (Fig. 1).

Exposure of *P. aeruginosa* to 2DP led to significant in biofilm formation at all concentrations tested and at all three incubation periods decrease (24 hours: $P = 0.004$–0.006; 72 hours: <0.001–0.002; 168 hours: <0.001; Fig. 2). At a concentration of 312 μM 2DP for 24- and 72-hour incubation periods, and 625 μM 2DP for a 168-hour incubation period, the OD readings were similar to the blank (Fig. 2). In contrast, 2DP had far less effects on the amount of biofilm produced by both strains of *S. aureus*. Even with the addition of 2500 μM 2DP after 72- and 168-hour incubation periods, a considerable amount of the biofilm accrued on the lens case.

The results for PGG differed from those of 2DP. At all concentrations tested, there was no complete inhibition of biofilm formation by *P. aeruginosa* with the amount formed, even at the highest concentration of PGG, OD was not significantly different from the control ($P = 0.727$). In contrast, for both strains of *S. aureus,* exposure to PGG at a concentration of 50 μM resulted in reduced biofilm formation. This inhibition was maintained for the entire 168-hour incubation period, with significantly less growth present than the control ($P = 0.18$; see Fig. 2). However, at low PGG concentrations (6.25 and 12.5 μM), extension of incubation to 72 and 168 hours, there was no inhibition of biofilm formation and the presence of PGG appeared to significantly promote growth. This phenomenon was more pronounced with *S. aureus* ATCC 6538 than *S. aureus* ATCC 25923.

The combination of 2DP (312 μM) and PGG (50 μM) was able to inhibit biofilm formation of both *P. aeruginosa* and the two *S. aureus* strains. At all three incubation periods, the ODs of the test wells (24 hours: 0.052–0.063; 72 hours: 0.057–0.069; 168 hours: 0.072–0.077) were similar to those of the blank (0.050), indicating there was minimal biofilm formation and this combination could successfully inhibit biofilm production.

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Discussion

Biofilms are commonly observed in contact lens cases and contribute to persistent contamination of contact lens cases.[4,25] Microorganisms within the biofilm are more likely to survive after disinfection and may act as potential pathogens of microbial keratitis.[14,16] Typically, the development of a biofilm is a three-step process involving an initial attachment, a subsequent maturation phase, and final dispersion.[26,27] Our results showed that biofilm production of *P. aeruginosa* was greater than that of *S. aureus* in the strains used. This may be due to differences in structure between *P. aeruginosa* and *S. aureus*, as *P. aeruginosa* cells have flagella allowing motility and aid adhesion to a substrate or other *P. aeruginosa* cells, which may help initial biofilm formation.[28] Chan et al.[29] showed that *P. aeruginosa* adhered to soft contact lenses in higher numbers compared with *S. aureus*.

Our results demonstrated that the biofilm formation of *P. aeruginosa* increased gradually with the extension of incubation periods and higher initial inoculum concentrations. However, the maximum OD achieved appeared to plateau with higher initial inocula which may be related to insufficient nutrient supply at higher cell concentrations. This outcome was more obvious for both strains of *S. aureus* in which the higher initial inocula displayed no increase in biofilm OD at 168 hours compared with 72 hours or even a reduction in OD (*S. aureus* ATCC 6358 at 10^8 CFU/mL). Quorum-sensing systems play a significant role in the development of biofilm formation in bacteria, including *S. aureus* and *P. aeruginosa,*[30,31] by allowing cells to detect bacterial density through their quorum sensing systems. This allows coordination of gene expression. *P. aeruginosa* has three different quorum sensing systems: *Las*, *Rhl*, and *PQS*.[30]

In *S. aureus*, the accessory gene regulator (*agr*) system, has been recognized to play a role in quorum sensing. Regulation of *agr* is unrelated to the systems controlling biofilm production in *P. aeruginosa* and mainly involves the upregulation of adhesion factors such as microbial surface components recognizing adhesive matrix molecules when the cell density is low.[31] Production of *lyt*M is controlled by *agr* and its upregulation increases biofilm production.[23] Biofilm formation of *S. aureus* has also been shown to require the presence of the intracellular adhesion (*ica*) locus, which encodes for the production of polysaccharide intercellular adhesin. Strains lacking this gene are unable to produce biofilm.[32] Expression of the gene may be affected by presence of other agents such as ethanol and expression varies between strains. Strain ATCC 6538 has been shown to have lower expression of *ica*D than ATCC 25923, resulting in poorer adhesion.[23]

The biofilm formation of *P. aeruginosa* decreased significantly in the presence of at least 312 μM 2DP; but PGG at the highest concentration (50 μM) failed to prevent biofilm formation in the lens case. Previously, 2DP has been shown as an effective biofilm inhibitory compound.[17] It has been suggested that this is due to the iron chelating properties of 2DP that reduce available iron for *P. aeruginosa*, which is closely related to its QS systems.[19,30] Although the biofilm formation of *S. aureus* involves the uptake of iron, the synthesis of an extracellular polysaccharide substance and polysaccharide intercellular adhesin may be more essential steps in this process for this organism.[21,26] Therefore, exposure to high levels of 2DP did not lead to effective inhibition of biofilm formation in the contact lens cases, though there was some reduction in production at higher concentration in *S. aureus* ATCC 25923 (Fig. 2).

As has been previously reported for other materials,[22] our results showed that *S. aureus* biofilm was greatly reduced by the presence of 50 μM PGG. It has been proposed that PGG may inhibit the formation of *S. aureus* biofilm during the initial attachment stage.[22] However, at low concentrations, PGG did not inhibit biofilm formation of *S. aureus*, but even enhanced growth for ATCC 6538. Although PGG is an iron chelator and therefore should inhibit growth, it appears that the role of iron in biofilm formation in *S. aureus* is complex and is affected by several variables.[33]

Agents 2DP and PGG appeared to inhibit biofilm formation through different mechanisms and showed varying effects depending on the strains tested. It is clear than one of these agents would be inadequate to prevent biofilm formation in the multiorganism environment of the contact lens case. Our combination of 2DP and PGG resulted in excellent inhibitory effect of biofilm formation of both *P. aeruginosa* and *S. aureus*. The effects were greater than the individual agents alone on the organisms, suggesting that there is an additive effect of their properties leading to an improved inhibition of biofilm formation.

In conclusion, the combination of inhibitory agents on biofilm production of two important ocular pathogens showed promising results, but further work is needed to determine if these agents are compatible with multipurpose solutions for contact lens disinfection and if their presence in the solution causes any change to cytotoxicity. In addition, their actions need to be assessed on biofilm produced in cases of contact lens wearers in which a wide variety of organisms may be present. This is particularly important for rigid contact lens wearers, such as orthokeratology, as these wearers need to use lens cases for disinfection and it has been shown that compliance with cleaning and replacement of lens cases is poor.[5]

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