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Effect of a solution containing citrate/Methylene Blue/parabens on *Staphylococcus aureus* bacteria and biofilm, and comparison with various heparin solutions

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***Objectives*: Some antibiotic solutions increase bacterial resistance and may cause toxic side effects. Heparin, frequently used as an anticoagulant in catheter lock solutions, may cause bleeding and stimulate biofilm formation. The aim of this study was to investigate the effect of a new antibacterial/antithrombotic solution, citrate/Methylene Blue/parabens (C/MB/P), versus various heparin solutions on the viability and the structure of preformed mature biofilms of *Staphylococcus aureus* bacteria. The degree of eradication of both planktonic and sessile microorganisms was evaluated.**

***Methods*: The changes in the structure of biofilms after exposure to C/MB/P and several concentrations of heparin were analysed by means of confocal laser scanning microscopy. COMSTAT image analysis was utilized to compare biofilm biomass, average and maximum height, surface coverage and roughness coefficient. Viability studies were performed on both biofilms and supernatant solutions.**

***Results*: C/MB/P, in contrast to heparin solutions, significantly reduced biofilm biomass and thickness and reduced viability by 5 log when compared with saline treatment. No viable planktonic bacteria were detected and the few remaining biofilm cells appeared to be lysed. In contrast, most heparin solutions only reduced viability up to 1.0 log and failed to eradicate planktonic bacteria.**

***Conclusions*: C/MB/P has a rapid bactericidal effect on the preformed, mature biofilm of *S. aureus*. The structural changes of biofilms treated with C/MB/P, together with the observed log reduction of viable biofilm cells, confirmed the high potential of this solution to eliminate sessile bacteria. Furthermore, the tested solution entirely eliminated planktonic bacteria detached from the biofilm.**

Keywords: catheter infection, CRBSI, haemodialysis, lock solution, antimicrobial effect

Introduction

Bacteria have the ability to attach onto solid surfaces creating communities known as biofilms. Encased in a matrix of hydrated extracellular polymeric substances (EPSs), biofilm maintains a complex heterogeneous structure[1] that constitutes an essential and protective lifestyle.[2] The resistance of biofilms to antibiotics and other antimicrobial agents is a cause of concern in the medical community,[1,3] since sessile bacteria can survive antibiotic concentrations 1000–1500 times higher than those tolerated by planktonic microorganisms.[4]

One promising approach to eradicating biofilms is an attempt to disrupt biofilm structure by inhibiting cell-to-cell communication known as 'quorum sensing', which is essential for the formation and progression of biofilm and its survival.[5] A large group of detachment-promoting agents have been used with mixed results for controlling bacterial biofilms. These substances, being enzymes, chelating agents (e.g. citrate, EDTA) or other chemical compounds, may reduce EPS cohesiveness through a variety of actions. Recently, bacteriophage treatment has been suggested as a method for controlling bacterial biofilms.[6]

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The use of indwelling devices, especially central venous catheters (CVC), in hospitalized patients drastically increases the risk for nosocomial bloodstream infection through catheter-related bloodstream infection (CRBSI). Bacterial contamination takes place through colonization of either the external surface of the CVC by skin flora or contamination of the inner lumen during insertion or subsequent catheter handling. In CVC used for dialysis access, the majority of CRBSI is the result of intraluminal contamination. Bacterial colonization then contributes to catheter clotting and poor flow, complications that limit the utility and safety of catheters. It is estimated that 200000–400000 episodes of CRBSI occur annually in the USA with an attributable mortality ranging from 12% to 25% and average prolongation of hospital stays by 7 days.[7] Thus, there is a need for a novel and effective treatment preventing microbial colonization and/or eradicating existing biofilms in catheters. This is particularly important when considering the dramatic increase in antibiotic resistance and the finding that antibiotic treatment can induce the formation of biofilms.[8,9]

To diminish the incidence of CRBSI, recent efforts have focused on using antibacterial catheter lock solutions (CLSs) based upon antiseptics rather than antibiotics. Antiseptics kill bacteria through physical effects rather than specific biochemical pathways and may not induce microbial resistance. In this study, we compared the efficacy of a novel solution containing citrate/Methylene Blue/parabens (C/MB/P) in eliminating preformed *Staphylococcus aureus* biofilms developed in a flow cell bioreactor with those of various heparin CLSs. The biofilm architecture before and after treatment with tested lock solutions was studied by means of confocal laser scanning microscopy (CLSM), one of the best tools for exploration of biofilm growing in a flow cell reactor.

Materials and methods

Composition of a new CLS and heparin solutions

C/MB/P comprises 7% (w/v) sodium citrate buffer (0.24 M of pH 6.2), 0.05% Methylene Blue (MB), 0.15% methyl paraben (MP) and 0.015% propyl paraben (PP). This solution has a density of 1.040, which is very close to the density of the blood of patients with end-stage renal disease (ESRD) and mild anaemia. The following heparin solutions (Abraxis Pharmaceutical Products, Schaumburg, IL, USA) were used: heparin (5000 U/mL, 5 K) containing 1.5% benzyl alcohol as a preservative, heparin (10000 U/mL, 10 K) containing 0.15% MP and 0.015% PP, heparin (5000 U/mL, 5 K) containing 0.075% MP and 0.0075% PP, and heparin (1000 U/mL, 1 K) without preservatives. Heparin 5 K with parabens was obtained by dilution of 10 K heparin solution with saline.

Biofilm growth

Both structural changes of biofilm and viable cell enumeration were studied at intervals of 0 until 48 h. To do so, a previously described[10] continuous culture single-pass flow cell was configured to observe the growth and development of *S. aureus* ALC2085 (strain RN6390 containing pALC2084)[11] biofilms attached to a glass substratum. The strain chosen expressed green fluorescence protein (GFP) when exposed to 50 ng/mL tetracycline under flowing conditions. Diluted Luria–Bertani medium (0.2×) was used as a growth medium. Four millilitres of suspension with bacteria cells ($\sim 3\times10^7$ cfu/mL, obtained by dilution with saline of a stationary phase culture) served as the inoculum and was injected into a septum 4 cm upstream from the flow cell. Bacteria were allowed to attach to the glass substratum for 1 h prior to initiating flow. The flow rate of the system was adjusted to 0.1 mL/min. Flow through the chamber was laminar with a Reynolds number of <0.5, having a fluid residence time of 60 min. Biofilms were grown under flowing conditions at 37°C in 5% CO_2.

Treatment of biofilms

To determine the effect of heparin and C/MB/P on mature *S. aureus* biofilms, biofilms were allowed to mature before initiation of treatment. Under flowing conditions, *S. aureus* established mature biofilms within 3 days of growth. Then the medium was replaced with 5 mL of C/MB/P or heparin solutions or saline (control). To be certain that the cell was filled with the full strength of the tested solution, the lock solution was routinely replaced 2 h after initial treatment and left in the flow cell. The mature biofilms were exposed to all tested solutions under static conditions for a period of 48 h simulating the time period between uses and during 'lock' of a CVC for dialysis. Upon initiation of treatment, images were acquired over a period of 60 min at the same location to better visualize the effect of antimicrobial/antithrombotic CLSs. Then image stacks were acquired at random over the entire length of the flow cell after 60 min, 6 h, 1 and 2 days of treatment. Experiments were carried out in triplicate.

Visualization of biofilm architecture

Flow cell-grown biofilms were visualized by CLSM using an LSM 510 Meta inverted microscope (Zeiss, Heidelberg, Germany). Images were obtained with an LD-Apochrome ×40/0.6 lens and the LSM 510 Meta image acquisition software (Zeiss). No staining of the biofilm cells was necessary for visualization due to fluorescence of GFP produced by the *S. aureus* strain. To follow *S. aureus* biofilm development and to ensure the timing of *S. aureus* biofilms reaching maturity, an average of six image stacks was acquired at 2 and 6 h and 1, 2, 3 and 4 days after initiation of flow. Images were taken at random along the length of the flow cell. To visualize the effect of treatment with heparin, C/MB/P and saline on biofilm architecture/structure, an average of six image stacks was acquired prior to treatment, after 3 days of biofilm growth and at 1, 6, 24 and 48 h post-initiation of treatment under static conditions. All experiments were carried out in triplicate.

COMSTAT analysis

To confirm the visual observations of the effect of saline, heparin and C/MB/P over time on biofilm structure, we utilized the COMSTAT image analysis program. A total of five variables were used to evaluate biofilm architecture: biofilm biomass, average and maximum height, surface coverage and roughness coefficient. The roughness coefficient provides a measure of biofilm heterogeneity and, therefore, how structured the biofilm architecture is. For each timepoint (0–48 h), a total of six image stacks were acquired and analysed using the COMSTAT image analysis program as described by Heydorn *et al.*[12]

Viability

To visualize the effect of heparin, C/MB/P and saline 48 h post-treatment under static conditions, the remaining biofilms were

stained using the Live/Dead® BacLight™ stain from Invitrogen (Carlsbad, CA, USA) to visualize cells that are alive (stained green) and cells that are dead (stained red). In addition, biofilms were sacrificed after 2 days of treatment under static conditions to determine viability. To do so, the bulk liquid was drained from the flow cell and centrifuged. The captured cells were then washed with saline and resuspended in 1 mL of saline. Biofilms were subsequently harvested by scraping the biofilms off the glass coverslip, resuspended in 1 mL of saline, and next homogenized for 15 s to disaggregate biofilm clusters. The bacterial suspensions were subsequently serially diluted 10-fold and the cfu determined by (i) spread plate and (ii) drop plate methods. To determine log reduction, *S. aureus* biofilms were harvested after 3 days of growth (before and after treatment with saline for 2 days) and cfu/biofilm quantified as described earlier.

Statistical analysis

All experiments described were performed at least in triplicate. *P* values were calculated using ANOVA and Student's *t*-test (two-tailed, paired). Error bars indicate 1 SD.

Results

Biofilm development

The biofilm biomass increased over a period of 3 days after which the biomass appeared to reach a steady-state. The same trend was observed for the average and maximum thickness and surface area of *S. aureus* biofilms (Figure 1). All of these changes were statistically significant ($P<0.01$) for each period up to 3 days of biofilm development. No significant increase in biofilm biomass and biofilm thickness was observed between 3 and 4 days of growth under flowing conditions. This steady-state suggested that after 3 days, *S. aureus* had formed mature biofilms.

The roughness coefficient, a measure of biofilm heterogeneity, was found to remain constant over the course of biofilm formation. On average, a roughness coefficient of ~0.6 was detected, indicating that *S. aureus* biofilms were mostly uniform and even in height. The findings indicated that under the conditions tested, *S. aureus* biofilms reach maturity within 3 days of growth and are fairly uniform in thickness. We therefore carried out all other experiments with *S. aureus* biofilms after growth for 3 days.

Viability studies

To determine the efficacy of tested solutions (C/MB/P, heparin) on biofilm viability and disaggregation of biofilms following 48 h of treatment under static conditions (this time simulates the period between dialysis sessions when CLS fills the catheter), the total cfu from both biofilms and bacteria present in the bulk liquids was determined. Treatment with saline was used as the control.

Our studies demonstrated that with increasing concentration of heparin and the preservative parabens, fewer viable *S. aureus* cells were recovered from both biofilms and the bulk liquid ($P<0.05$ for biofilm versus saline, $P<0.01$ for biofilm versus bulk liquid and bulk liquid versus saline) (data not shown). In the heparin group, the highest log reduction of biofilm cells was observed for 5 K heparin containing 1.5% benzyl alcohol, but only during treatment with this heparin solution more viable bacterial cells were detected in the bulk liquid than in the biofilm itself. In contrast, after treatment with other heparin solutions containing parabens or, as in the case of 1 K heparin

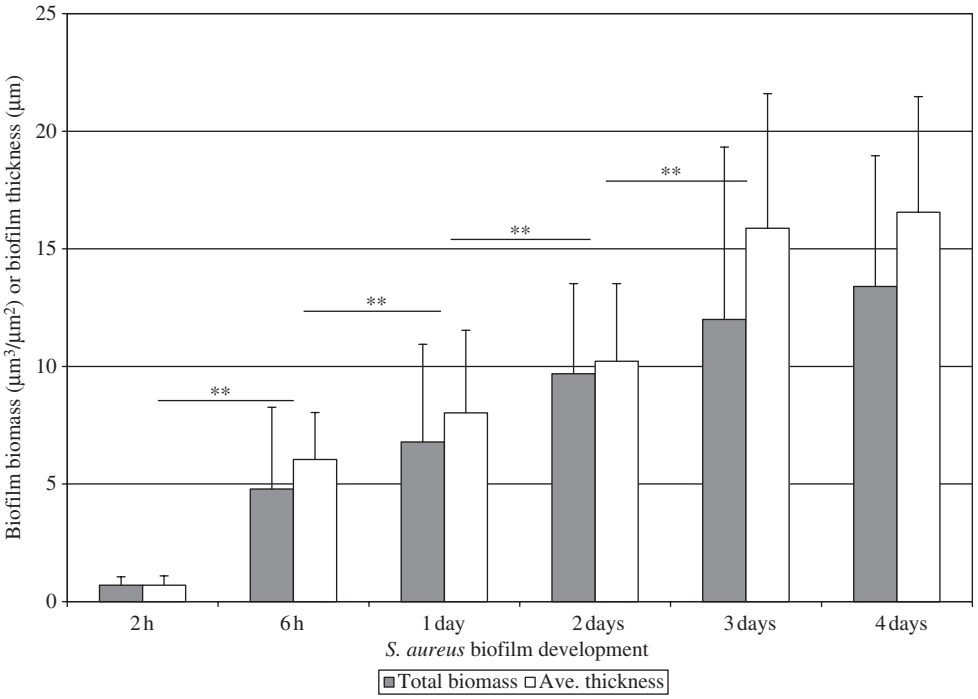

Figure 1. Biofilm biomass and biofilm average thickness of *S. aureus* biofilms grown in flow cells over a period of 4 days under flowing conditions. Comparison is between timepoints (i.e. 2 and 6 h, 6 h and 1 day, etc.) $^{**}P<0.01$. Error bars indicate 1 SD.

containing no preservatives, 5–6 times more cells were detected in biofilms than in bulk liquids of the flow cell. This suggests that the heparin concentration has a small effect on biofilm viability (at most a 1 log reduction in biofilm cells), and does not result in more biofilm cells being dislodged into the bulk liquid. The data also suggest that the benzyl alcohol at 1.5% concentration may have an effect in lowering the amount of viable cells in biofilm but also increases the quantity of cells in the bulk liquid.

In comparison, treatment with C/MB/P resulted in a ~5 log reduction in biofilm viability compared with untreated biofilms (Figure 2) ($P<0.01$). Moreover, no viable cells were detected in the bulk liquid following treatment with C/MB/P. As can be seen, 5 K heparin with benzyl alcohol can reduce *S. aureus* biofilm by an average of 2.5 log. However, if considering the total cfu/flow cell by taking into account the viable *S. aureus* cells present in both the biofilm and the bulk liquid, treatment with this solution produces only a ~1.5 log reduction when compared with almost a 5 log reduction in the case of C/MB/P treatment. As shown in Figure 2, higher concentrations of heparin and parabens resulted in higher log reductions in total biofilm cells compared with saline-treated cells (fold reductions in the range of 0.25–1.0, $P<0.01$ within group and versus saline). The log reductions observed with any of the tested heparin solutions were appreciably smaller than the nearly 5 log reduction with C/MB/P. Biofilm cells were dislodged into the bulk liquid without being eliminated/eradicated, contrary to treatment of biofilm with C/MB/P.

Live/dead staining of biofilms

In order to visualize the effect of heparin and C/MB/P treatment on viability of bacteria within biofilms, biofilms were stained following treatment for 48 h using a live/dead stain. As shown in Figure 3(a), saline-treated biofilm cells were mostly stained green indicating that the majority of biofilm cells were still alive. Only a few cells stained red (indicative that cells are dead) were detected (<10%).

In contrast, the proportion of viable bacteria detectable in biofilms upon treatment with heparin varied with the heparin concentration and the presence of preservative. Approximately 50% of 5 K heparin benzyl alcohol-treated biofilm cells were red suggesting that treatment resulted in killing of half of the biofilm population (Figure 3b). Treatment with 10 and 5 K heparin containing parabens only resulted in ~25% and 10% of the biofilm cells, respectively, being stained red (Figure 3c and d), while only very few dead cells (~1%) were detectable upon treatment with 1 K heparin (Figure 3e).

The C/MB/P-treated biofilms were predominantly stained red, indicating the presence of dead cells (Figure 3f). In addition, while coccoid-shaped cells were easily detectable in heparin- or saline-treated biofilms, no defined coccoid-shaped cells stained red were identified, though a few coccoid-shaped cells were stained green. We therefore assume that the biofilm material on the substratum represents lysed cells and released DNA to which the propidium iodide dye adhered. This is supported by the finding during removal that the remaining biofilm biomass was very viscous. The microscopic assessment of live/dead-stained bacterial cells is usually simplified to either 'green'- (live) or 'red'-labelled (dead) cells. It seems correct to assume that membrane compromised bacterial cells can be considered dead; however, the reverse (intact cells are active cells) is not necessarily true.

Biofilm variables and biofilm architecture after exposure to tested solutions

Treatment with heparin solutions caused an initial modest reduction in biofilm biomass. This reduction was more

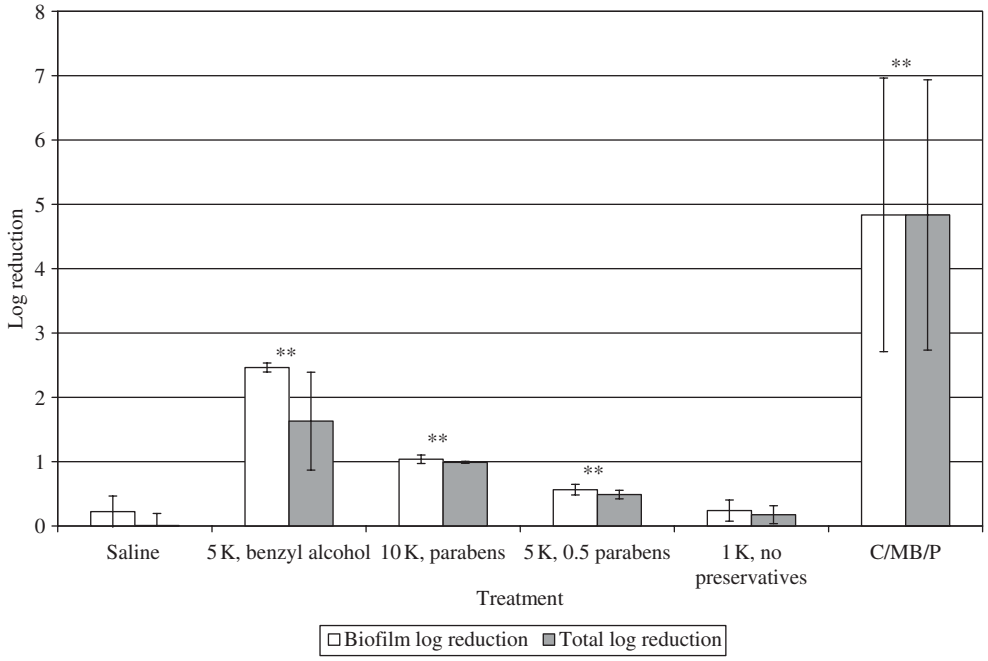

Figure 2. Susceptibility of *S. aureus* biofilms to various tested solutions (heparins, C/MB/P) following 48 h of treatment under static conditions. Log reduction for flow cell-grown biofilms (white bars) and for flow cell-grown biofilms plus *S. aureus* cells present in the bulk liquid (grey bars) was determined by viability counts. $^{**}P<0.01$. Error bars indicate 1 SD.

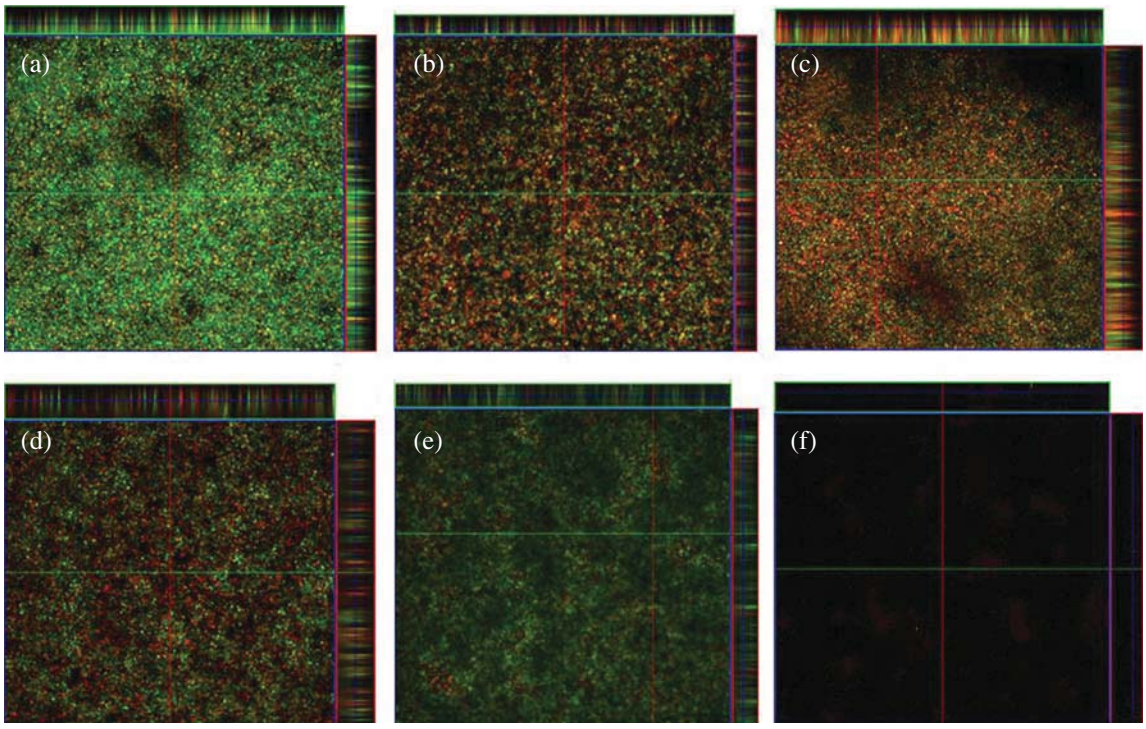

Figure 3. Representative confocal images of *S. aureus* biofilms obtained after 48 h of treatment under static conditions with various tested solutions. Images were acquired after treatment with (a) saline, (b) 5 K heparin/benzyl alcohol, (c) 10 K heparin/parabens, (d) 5 K heparin/0.5× parabens, (e) 1 K heparin without preservatives and (f) C/MB/P. The CLSM images show the $x-y$ and $x-z$ planes.

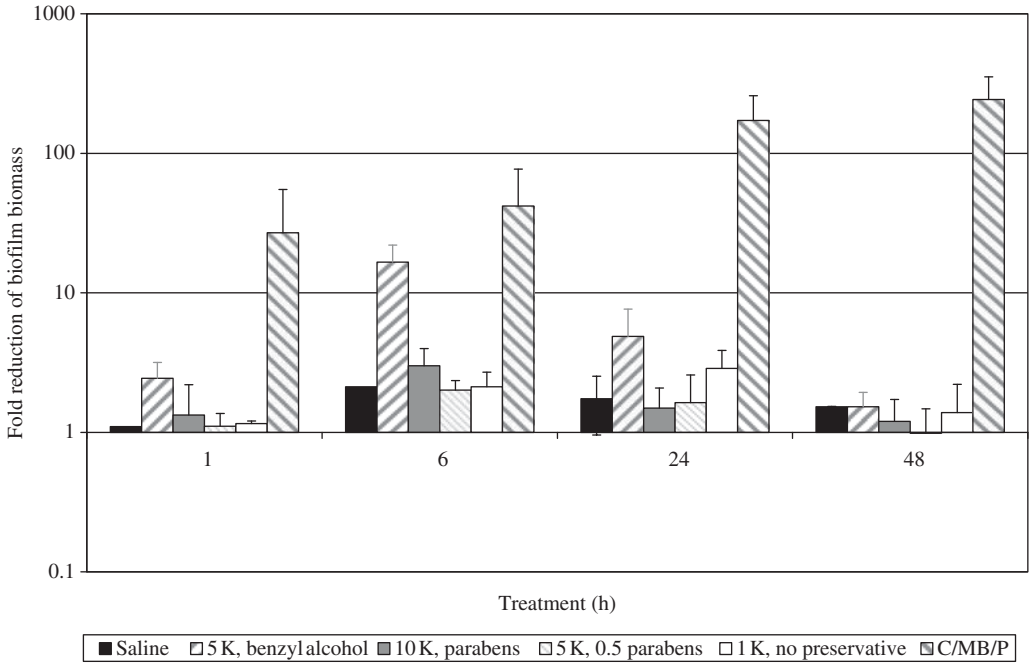

Figure 4. Effect of treatment for 48 h under static conditions on *S. aureus* biofilm biomass. $P<0.001$.

pronounced upon treatment with 5 K heparin/benzyl alcohol. The highest fold reduction (16.6-fold) was observed after 6 h of treatment (Figure 4). However, longer exposure of *S. aureus* biofilms to heparins resulted in an overall increase in biomass. In contrast, treatment of *S. aureus* biofilms with C/MB/P for only 1 h resulted in an overall 27-fold reduction in the biofilm biomass. Continued exposure coincided with a continued decrease in the biofilm biomass (after 6 h, a 42-fold reduction in the biofilm biomass; after 48 h, 243-fold reduction). *P* value of <0.001 for C/MB/P versus saline and heparin solutions (data

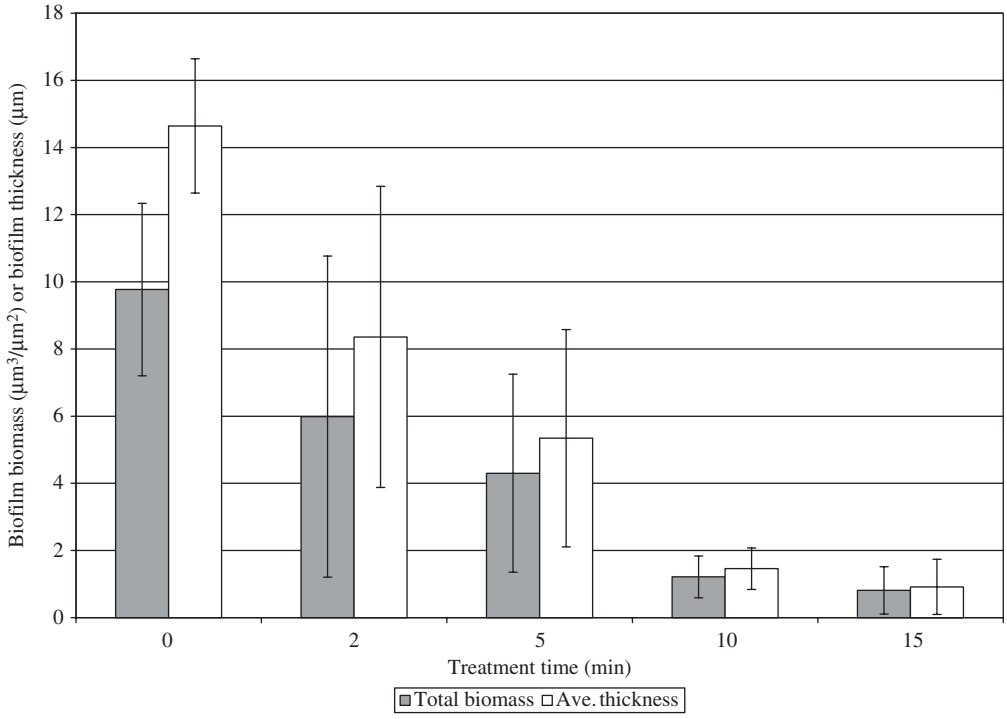

Figure 5. Biofilm biomass and biofilm average thickness of *S. aureus* over the first 15 min of treatment with C/MB/P. $P < 0.001$.

not shown). Similarly, the fold reduction of average thickness of biofilm after 1 h treatment with C/MB/P was 34 times but only 2.2 times after exposure to heparin/benzyl alcohol and was constantly growing over time, up to 380 at the end of experiment. In contrast, 5 K heparin/benzyl alcohol produced a modest fold reduction of the average thickness within the first 6 h (~11 times) of treatment, but dropped to around 1 after 48 h of treatment.

In addition, C/MB/P treatment resulted in a 56-fold reduction of the surface area of biomass and a ~4-fold reduction of the maximum biofilm thickness. At the same time, the roughness coefficient increased by 0.4, indicating that the biofilm architecture became less heterogeneous. The above-mentioned variables were not considerably changed when *S. aureus* biofilm was in contact with tested heparin solutions or saline. The dynamics of the detachment of biofilm cells from the substratum in the first 15 min of C/MB/P treatment expressed as changes in biofilm biomass and average thickness are shown in Figure 5. All of these results demonstrate that treatment with C/MB/P solution is much more effective than that with heparin solutions in reducing sessile and planktonic populations of *S. aureus* organisms.

Discussion

The antimicrobial study of a new lock solution presented here proves the properties of C/MB/P in the elimination of both sessile and planktonic microorganisms in a short time. The study also demonstrates significant changes in biofilm structure/architecture, ending in removal of the greatest part of the biofilm from the substratum.

Systemic prophylactic treatment has only limited efficacy against catheter-associated biofilms and CRBSI, due to the tolerance of biofilm organisms. Therefore, other strategies such as antibiotic lock solutions have been proposed to diminish the incidence of CRBSI.[13] However, exposure to antibiotics in CLS can result in the development of antibiotic-resistant strains of *Staphylococcus* or *Pseudomonas*,[14] as well as systemic toxicity from persisting antibiotic levels. Disadvantages of heparin, which is frequently part of CLSs, include: the incompatibility of antibiotics such as gentamicin and cephalosporins,[15,16] bleeding risk as a result of systemic anticoagulation and risk of heparin-induced thrombocytopenia.[17,18] In addition, there have been over 150 deaths linked to contamination of heparin during manufacturing processes over the past 6 months that have led to numerous product recalls.[19] To preclude all of these potentially harmful or fatal side effects, some recent catheter locks use different anticoagulation systems and incorporate antiseptic agents or use a mixture of agents to accomplish a synergistic effect.[20]

Citrate and EDTA are effective anticoagulants and can maintain the patency of CVC. Citrate as CLS has been used in a wide range of concentrations from 4% to 46.7%.[17,21,22] In a previous study, we demonstrated antimicrobial activity of sodium citrate, especially in higher concentrations (10% to 47%).[22] Clinical trials have confirmed a decrease in CRBSI using 30% sodium citrate contrasted to heparin,[23] but there are potential risks when citrate is used in high strength,[24] which led to the restriction of the use of a commercially marketed product, Tricitrosol (46.7%), by the FDA. Studies of a dilute sodium citrate formulation as CLS (4% or less) have demonstrated efficacy as an anticoagulant with improved safety and minimal, to no, risk of bleeding.[25,26] However, differences were not detected with respect to the number of infections when comparing 4% citrate with heparin. An *in vitro* study demonstrated that sodium citrate at a concentration >0.5% might inhibit biofilm formation and cell growth of *S. aureus* and *Staphylococcus epidermidis* and thereby reduce the risk of biofilm-associated complications

in indwelling catheters.[27] On the other hand, it was determined that 4% citrate did not disrupt pre-existing biofilms. In recently published *in vitro* studies, the combination of 30% ethanol and 4% trisodium citrate has shown good efficacy in the prevention of bacteria biofilm formation[28] and eradication of many *Candida albicans* isolates obtained from human blood cultures.[29] EDTA, similar to citrate, may have both antibacterial and antithrombotic properties. *In vitro* and *in vivo* studies have shown bactericidal effects when EDTA was used in high concentration[30] or as a mixture with antibiotics.[31]

C/MB/P is a unique combination of several compounds with balanced concentrations to assure safety and efficacy. As we showed previously, the lock solution has excellent anticoagulation properties and capability to prevent growth of many planktonic microorganisms.[32] C/MB/P has also been shown to eradicate bacterial biofilms of many strains grown in 1 day on polymeric or glass coupons and to actively prevent the growth of *S. aureus* biofilm for several days in a flow cell bioreactor.[32] For all tested organisms, the MIC was 25% or less, of the original concentration, and bacterial strains did not develop resistance over more than 40 passages. The C/MB/P is not toxic and has a density very close to the blood of patients with ESRD and mild anaemia, diminishing the leaching of lock solutions into the bloodstream due to gravity effects.[33] During long exposure of catheters to the new CLS, we did not notice any signs of catheter degradation though there was light catheter coloration. Allergic reactions to the components of the lock solution are unknown (citrate) or very rare (MB, parabens).

Based on our confocal microscopy and COMSTAT analysis presented here, contact of mature *S. aureus* biofilm with C/MB/P resulted in a significant reduction of biomass and biofilm thickness. In contrast, treatment with various heparin solutions and saline has a negligible effect on structural factors when compared with those of untreated biofilms. The changes in biofilm structure in the presence of C/MB/P were noticed within minutes after initiation of treatment (Figure 5). These results were supported by live/dead staining experiments. Semi-quantitative analysis revealed that treatment with various tested heparin solutions reduced the amount of living sessile cells in the range of 10% to 50% depending upon the concentration of heparin and preservatives. Treatment with C/MB/P had superior effectiveness in killing sessile bacteria in biofilm, eradicating >99% of the sessile microbes and all planktonic microbes. Microscopic analysis suggested that treatment with C/MB/P resulted in cell lysis. Only a few viable cells stained green were visible within the biofilm. The majority of the viable cells were detected near the substratum close to the glass surface.

In recent publications, heparin has been noted to promote biofilm formation, especially for *Staphylococcus*, by aiding quorum sensing.[34,35] Systemic heparin that flows through the catheter may therefore also be a risk factor.[35] Our studies show no antibacterial effect of heparin on viability of microbes in preformed biofilms. The small effect in lowering cfu observed in 5 K heparin/benzyl alcohol treatment is apparently due to the high concentration of preservative. None of the heparin solutions tested changed the structure of biofilms. High numbers of viable planktonic cells were found in bulk liquids after various heparin treatments, and numbers were comparable to those of saline-treated biofilms. This suggests that heparin may help to detach sessile cells from the substratum but is unable to eliminate planktonic microorganisms.

The present study has some limitations. Because of the established methodology, we were using glass in flow cells as a substratum for biofilm growth, not polymeric materials (e.g. carbothane) commonly utilized in haemodialysis manufacturing. In addition, the *in vitro* environment is different from *in vivo* factors (e.g. blood proteins, platelets etc.) that may contribute to biofilm formation and its resistance, and therefore limits extrapolation of the results to the *in vivo* situation.

In the last few years, eight randomized clinical trials have compared the frequency of catheter-related bacteraemia in patients receiving a prophylactic antimicrobial CLS versus patients receiving standard heparin locks. Six studies used an antibiotic lock (gentamicin, cefazolin with gentamicin, minocycline, minocycline/EDTA or cefotaxime),[15,16,36–39] one study used taurolidine[40] and one used 30% citrate.[17] Each of these trials has shown from 50% to 100% reduction in CRBSI incidence, together with decreases in morbidity and mortality versus use of heparin as CLS.[20,41,42] On the basis of a meta-analysis, it was suggested that these CLSs might be beneficial to prevent the first episode of CRBSI in patients receiving short-term catheter haemodialysis.[43] The gentamicin and minocycline/EDTA locks were as efficacious as broader-spectrum regimens. Also, at the present time, evidence is insufficient to warrant routine use of taurolidine without other preventive measures.[43,44]

Our study revealed massive structural changes of mature biofilms treated with C/MB/P and confirmed the high potential of this solution to eliminate preformed biofilm from a solid surface in a very short time. Further, bulk liquid was free of planktonic bacteria, suggesting that C/MB/P was also able to entirely eliminate planktonic cells or cells detached from the biofilm. These attributes meet the expectations for effective lock solutions discussed by Donlan[45] in a newly published review. Recently, a clinical trial (AZEPTIC) with 415 enrolled patients has been completed, comparing C/MB/P (Zuragen™, from Ash Access Technology) and heparin effects on CRBSI rates and patency of CVC for dialysis. Results of the trial should also provide *in vivo* information regarding safety and efficacy.

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Transparency declarations

J. S. and S. R. A. are both employees and minority shareholders of Ash Access Technology, Inc., a private company. Their role in this study was to assist K. S. with the experimental design and writing of the manuscript. K. S. had independent control of protocol design and execution, data gathering and scientific

interpretation of results based on her experience in the biofilm research field. K. S. does not have any financial conflict of interest.

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