Catheter lock solutions influence staphylococcal biofilm formation on abiotic surfaces

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Abstract

Background. Microbial biofilms form on central venous catheters and may be associated with systemic infections as well as decreased dialysis efficiency due to catheter thrombosis. The most widely used anticoagulant catheter lock solution in the US is sodium heparin. We have previously shown that sodium heparin in clinically relevant concentrations enhances Staphylococcus aureus biofilm formation. In the present study, we examine the effect of several alternative catheter lock solutions on in vitro biofilm formation by laboratory and clinical isolates of S. aureus and coagulase-negative staphylococci (CNS).

Methods. Lepirudin, low molecular weight heparin, tissue plasminogen activator, sodium citrate, sodium citrate with gentamicin and sodium ethylene diamine tetra-acetic acid (EDTA) were assessed for their effect on biofilm formation on polystyrene, polyurethane and silicon elastomer.

Results. Sodium citrate at concentrations above 0.5% efficiently inhibits biofilm formation and cell growth of S. aureus and Staphylococcus epidermidis. Subinhibitory concentrations of sodium citrate significantly stimulate biofilm formation in most tested S. aureus strains, but not in CNS strains. Sodium EDTA was effective in prevention of biofilm formation as was a combination of sodium citrate and gentamicin. Low molecular weight heparin stimulated biofilm formation of S. aureus, while lepirudin and tissue plasminogen activator had little effect on S. aureus biofilm formation.

Conclusions. This in vitro study demonstrates that heparin alternatives, sodium citrate and sodium EDTA, can prevent the formation of S. aureus biofilms, suggesting that they may reduce the risk of biofilm-associated complications in indwelling catheters. This finding suggests a biological mechanism for the observed improvement in catheter-related outcomes in recent clinical comparisons of heparin and trisodium citrate as catheter locking solutions. A novel and potential clinically relevant finding of the present study is the observation that citrate at low levels strongly stimulates biofilm formation by S. aureus.

Keywords: adherence; anticoagulant; bacteria; biofilm; catheter lock

Introduction

Bacterial biofilms are complex microbial communities which have a significant impact upon human health [1–3]. Bacteria in biofilms may have enhanced pathogenic capability relative to bacteria in solution by virtue of sessile behaviour, increased resistance to antimicrobial agents and the potential for detachment and distal embolization of biofilm fragments [4]. There are few clinical situations where this is more relevant than infections associated with indwelling intravenous catheters for dialysis and other purposes. It is estimated that there are over 400 000 vascular catheter-related bloodstream infections each year in the US [5]. Each such episode is expensive to treat and potentially complicated by systemic sepsis and other foci of infection.

Bacterial biofilms are defined as communities of bacteria attached to surfaces [6]. Biofilms defy most antimicrobial treatments and represent a nidus for chronic infections. In a previous study, we found that sodium heparin, which is the most widely used catheter lock solution, stimulates Staphylococcus aureus biofilm formation in vitro [7]. Catheter lock solutions are placed in the catheter lumen between dialysis sessions to maintain catheter patency. Heparin is widely used in the US for this purpose. Heparin is effective, but has...
several disadvantages. It is relatively expensive and of increasing concern in the dialysis setting in the development of heparin-associated and initiated thrombocytopenia and thrombosis (HIT/HAT) mediated by antiheparin antibodies. Our previous work has shown that heparin, at clinically relevant concentrations, enhances S. aureus biofilm formation. Several previous studies have suggested that solutions of sodium citrate, ethylene diamine tetra-acetic acid (EDTA) and sodium citrate with gentamicin as catheter lock solutions are as efficacious as heparin in preventing thrombosis and may be superior for maintaining catheter patency [8,9]. A recent randomised control trial demonstrates that 30% trichloroacetic acid reduced the risk of catheter-related sepsis in dialysis patients by 75% when compared with heparin as a lock solution [9]. We have explored the effect of alternative catheter lock solutions upon S. aureus biofilm formation.

Sodium citrate is a potent antimicrobial agent and is effective as an anticoagulant catheter lock solution [10]. Anticoagulant concentrations of sodium citrate ranging from 0.3 to 47% have been described [10]. We found that sodium citrate in this range effectively prevents the formation of staphylococcal biofilms. The effect of sodium EDTA, lepirudin (Refludan), tissue plasminogen activator (Alteplase) and low molecular weight heparin (LMWH; Lovenox) on S. aureus biofilm formation was also assessed.

Subjects and methods

Strains and medium

Staphylococcal strains used in this study are listed in Table 1. Bacterial cells were grown in tryptic soy broth (TSB) or on tryptic soy agar (TSA). For all phenotypic assays, we used 66% TSB + 0.2% glucose, as this medium promotes robust biofilm formation (data not shown, 20g/l of Difco Bacto Tryptic Soy Broth, Becton Dickinson, Sparks, MD). Henceforth in this article ‘TSB’ will signify 66% TSB.

Biofilm studies

Biofilm formation on polystyrene was performed similar to the previously described methods [7]. Overnight cultures of staphylococci were grown in TSB. Cultures were diluted to OD600 of 0.01 in TSB + glucose (0.2%) with experimental solutions added at 10% vol/vol to final concentrations indicated for each experiment in the respective figures. One hundred microlitres of cells plus medium, plus or minus tested substance, were added to individual wells of a tissue culture-treated, 96-well polystyrene microtitre dishes (Costar 3595, Corning Inc., Corning, NY). These were incubated in a closed, humidified plastic container for 8 h at 37°C, then assayed for biofilm formation. Non-adherent cells were removed and adherent cells were stained with 0.1% crystal violet as previously described [7]. Photographs of the inverted plate were taken with a digital camera (Nikon 990, Nikon, Melville, NY), then crystal violet was solubilized using 30% glacial acetic acid for 15 min. Relative biofilm formation was assayed by reading optical density at 550 nm using a Molecular Devices Vmax kinetic microplate reader (Sunnyvale, CA). Six or seven wells were used for each dilution, and each experiment was done at least three times, except when testing the group of strains listed in Table 1, when experiments were done at least two times.

Reagents specifically tested in these experiments were sodium citrate (S279–500, Fisher Scientific, Fair Lawn, NJ), lepirudin (Refludan NDC 50419-150-01 Berlex Laboratories, Wayne, NJ), tissue plasminogen activator (Alteplase or Cathflo Activase, NDC 5024-041-65, Genentech, Inc., South San Francisco, CA), LMWH (Lovenox NDC 0075-0624-30, Aventis Pharmaceuticals, Bridgewater, NJ), EDTA (10378-23-1, Fisher Scientific, Pittsburgh, PA) and gentamicin sulfate (G-3632, Sigma-Aldrich Co., St Louis, MO).

Adherence to silicone elastomer and polyurethane

A 1 mm thick sheet of silicone elastomer was acquired from Goodfellow Cambridge Limited (LS269875, Huntingdon, UK). Coupons (1/8” diameter) were made using a hole punch (FSK-2351, Fiskars Inc., Madison, WI) and three of these coupons were glued to the bottom of a well in a 24-well costar dish with silicone sealant (super silicone sealant 08661, 3M Inc., St Paul, MN). TSB + 0.2% glucose + bacterial cells were added as with the microtitre dish assay, except that 1.0 ml was added per well. Bacterial cells were incubated with the coupons for 24 h, then were washed five times with sterile PBS, stained with crystal violet, washed three times with sterile PBS and allowed to dry overnight. Individual crystal violet stained coupons were then moved to individual wells of a 96-well plate containing 0.125 ml of 30% glacial acetic acid for 15 min. An aliquot of 0.1 ml of solubilized crystal violet were then moved to 96-well dishes and A550 readings were determined as noted previously.

Adherence to polyurethane was determined just as earlier except using coupons punched from a 1.6 mm thick polyurethane sheet (Precision Urethane and Machine, Hempstead, TX).

Microscopy

Microscopy was performed as described previously [7]. Cell viability of adherent bacterial cells was determined with the Live/Dead BacLight Bacterial viability kit (L7012, Molecular Probes, Eugene, OR) following the manufacturers’ directions.

Ion concentration determination

Free calcium and sodium levels were determined using fluorescent ion detection kits from Molecular Probes (Eugene, OR) as prescribed by the manufacturer. Calcium was detected with Fluo-5F and sodium levels with CoroNa (Eugene, OR) as prescribed by the manufacturer. Free calcium and sodium levels were determined using Student’s t-test with Excel software.
CNS strains

Strain description | Biofilm formation with citrate %
--- | ---
| | 0 | 0.2% | 2.0% | 4.0% |
*S. aureus* strains
MZ100 laboratory strain | 1.85 ± 0.20 | 3.48 ± 0.09 | 0.01 ± 0.02 | 0.13 ± 0.03 |
Col MRSA | 0.20 ± 0.03 | 0.25 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 |
SH1000 Rub1+ laboratory strain | 0.41 ± 0.03 | 2.08 ± 0.58 | 0.07 ± 0.03 | 0.27 ± 0.05 |
8325-4 laboratory strain | 0.79 ± 0.10 | 1.12 ± 0.09 | 0.42 ± 0.07 | 0.14 ± 0.03 |
RN6390 | 0.92 ± 0.10 | 3.60 ± 0.02 | 0.07 ± 0.14 | 0.14 ± 0.01 |
Newman clinical isolate | 0.03 ± 0.03 | 0.21 ± 0.05 | 0.10 ± 0.04 | >0.01 |
NARSA240* osteitis | 0.25 ± 0.07 | 2.37 ± 0.22 | 0.02 ± 0.02 | 0.06 ± 0.01 |
NARSA228 osteomyelitis | 0.08 ± 0.04 | 0.40 ± 0.11 | 0.02 ± 0.01 | 0.09 ± 0.02 |
NARSA227 hip arthritis | 0.18 ± 0.04 | 0.78 ± 0.13 | 0.12 ± 0.05 | 0.05 ± 0.02 |
NARSA249 endocarditis MRSA | 0.15 ± 0.07 | 0.46 ± 0.07 | 0.07 ± 0.01 | 0.11 ± 0.01 |
NARSA235 endocarditis | 0.19 ± 0.03 | 0.54 ± 0.12 | 0.02 ± 0.01 | 0.06 ± 0.01 |
NARSA234 endocarditis | 0.41 ± 0.05 | 1.72 ± 0.45 | 0.05 ± 0.01 | 0.15 ± 0.02 |
Db blood isolate | 0.92 ± 0.20 | 3.40 ± 0.08 | 0.09 ± 0.03 | 0.13 ± 0.02 |
SMC92 clinical isolate | 1.82 ± 0.33 | 1.06 ± 0.22 | 0.03 ± 0.03 | <0.01 |
SMC760 clinical isolate | 0.62 ± 0.03 | 3.18 ± 0.05 | 0.34 ± 0.03 | 0.34 ± 0.06 |
SMC761 clinical isolate | 0.04 ± 0.01 | 0.18 ± 0.07 | 0.016 ± 0.02 | 0.13 ± 0.06 |
SMC762 clinical isolate | 1.17 ± 0.20 | 3.06 ± 0.17 | <0.01 | <0.01 |
SMC763 clinical isolate | 1.49 ± 0.10 | 3.49 ± 0.01 | 0.10 ± 0.03 | 0.24 ± 0.03 |
SMC764 clinical isolate | 0.85 ± 0.17 | 1.99 ± 0.45 | 0.03 ± 0.01 | 0.06 ± 0.01 |
SMC765 clinical isolate | 0.37 ± 0.06 | 0.50 ± 0.15 | 0.02 ± 0.01 | 0.12 ± 0.02 |
SMC766 clinical isolate | 0.22 ± 0.03 | 1.02 ± 0.39 | 0.04 ± 0.01 | 0.02 ± 0.01 |
SMC767 clinical isolate | 0.48 ± 0.07 | 1.18 ± 0.38 | 0.05 ± 0.01 | 0.05 ± 0.01 |
SMC768 clinical isolate | 0.34 ± 0.06 | 0.54 ± 0.10 | 0.04 ± 0.01 | 0.05 ± 0.01 |
SMC769 clinical isolate | 0.26 ± 0.04 | 1.37 ± 0.25 | 0.03 ± 0.01 | 0.05 ± 0.02 |
SMC770 clinical isolate | 0.37 ± 0.06 | 2.01 ± 0.25 | 0.07 ± 0.02 | 0.16 ± 0.02 |
SMC771 clinical isolate | 0.57 ± 0.11 | 2.47 ± 0.30 | 0.13 ± 0.02 | 0.13 ± 0.01 |
SMC772 clinical isolate | 0.35 ± 0.06 | 1.81 ± 0.34 | 0.11 ± 0.04 | 0.13 ± 0.03 |
SMC773 clinical isolate | 0.46 ± 0.03 | 0.96 ± 0.17 | 0.12 ± 0.02 | 0.19 ± 0.05 |
SMC774 clinical isolate | 0.58 ± 0.10 | 2.20 ± 0.40 | 0.19 ± 0.05 | 0.19 ± 0.02 |
SMC775 clinical isolate | 0.26 ± 0.04 | 1.57 ± 0.14 | 0.16 ± 0.03 | 0.11 ± 0.02 |
*CNS* strains
*S. epidermidis* O-47 | 1.00 ± 0.06 | 1.22 ± 0.17 | 0.04 ± 0.03 | <0.01 |
*S. epidermidis* RP62A | 1.58 ± 0.06 | 0.45 ± 0.17 | <0.01 | 0.01 ± 0.01 |
*S. epidermidis* ATCC R97-03* | 2.38 ± 0.11 | 1.77 ± 0.09 | 0.13 ± 0.04 | 0.03 ± 0.03 |
*S. epidermidis* ATCC R94-10 | 1.08 ± 0.09 | 0.92 ± 0.06 | 0.08 ± 0.03 | 0.05 ± 0.03 |
RMQS161 CNS catheter isolate* | 0.30 ± 0.04 | 0.23 ± 0.05 | 0.09 ± 0.05 | <0.01 |
*S. saprophyticus* PIC. #4654* | 0.19 ± 0.06 | 0.07 ± 0.04 | <0.01 | <0.01 |

aBiofilm formation of 6–8 h biofilms assayed by the microtiter biofilm assay. Each strain was tested at least twice and six or more replicates were assayed per measurement, and one SD is shown.
bStrains that do not show a significant increase in biofilm formation in the presence of 0.2% citrate (*P* > 0.5) are shown in bold.
cNARSA, network on antimicrobial resistance in *S. aureus*; MRSA, methicillin-resistant *S. aureus*; ATCC, American type culture collection; CNS, Coagulase negative *Staphylococcus*; PIC, Presque Island Cultures.

### Results

**Sodium citrate concentrations ≥0.5% prevent *S. aureus* biofilm formation, but do not destroy existing biofilms**

*Staphylococcus aureus* biofilm formation on polystyrene was assayed with the addition of sodium citrate over a concentration range of 0–4% (Figure 1A). We found that biofilms were stimulated at low concentrations of sodium citrate particularly at 0.125 and 0.25% (Figures 1A–C and 2C–D). At higher concentrations, biofilm formation was largely prevented (Figures 1A–C and 2E–F).

To determine whether existing biofilms could be eliminated by the addition of sodium citrate, we allowed biofilms to form for 8 h in the absence of sodium citrate. Then we added sodium citrate (0–4%) and incubated for 16–18 h at 37°C. Remaining biofilms were then quantified. We determined that even 4% citrate did not disrupt pre-existing biofilms (data not shown).

The effect of sodium citrate on *S. aureus* biofilm formation was also assessed on silicone elastomer and polyurethane surfaces, polymers used in the manufacture of many catheters. When incubated with 0.2% sodium citrate, a greater than 2-fold increase in biofilm formation was observed on silicone elastomer coupons at 20 h (Figure 1B and C). However, 2 and 4% sodium citrate inhibited biofilm formation on silicone elastomer (Figure 1B and C). A similar trend was observed on polyurethane though the cells were better able to adhere to polyurethane. The adherence to polyurethane at 0% citrate determined by spectrophotometric absorbance of crystal violet staining was 0.6 ± 0.17,
at 0.2% citrate adherence increased to 1.12 ± 0.09, and decreased to 0.33 ± 0.05 and 0.23 ± 0.08 at 2 and 4% citrate, respectively. In all cases, the difference between the citrate treated conditions and the absence of citrate was significantly different with *P* < 0.001.

We repeated the above experiments, but instead observed biofilms directly with phase-contrast and fluorescent microscopy rather than with the crystal violet microtitre dish assay (Figure 2A–F). A clear decrease in biofilm formation was seen at citrate levels above 0.25%. While at 0.125 and 0.25% sodium citrate levels, biofilm formation was greatly enhanced (Figure 2C and D). These hyper-biofilms were very thick and devoid of much of the architecture seen in untreated biofilms (see microcolony indicated by the arrow in Figure 2A). Adherent *S. aureus* cells were stained with Syto-9, a fluorescent dye that preferentially stains living bacterial cells, and propidium iodide (PI), which stains bacterial cells with disrupted membranes. Syto-9 and PI staining indicates that the few bacterial cells able to adhere to abiotic surfaces in the presence of 2 and 4% sodium are viable (Figure 2E, F and data not shown).

**Sodium citrate inhibits S. aureus growth**

The reason for decreased biofilm formation at high concentration of sodium citrate could be a consequence of growth inhibition of *S. aureus* by citrate. The kinetics of bacterial growth were determined by measuring culture turbidity over time in TSB + glucose + citrate at various concentrations. These are the same media conditions in which biofilm formation was assessed. We found that *S. aureus* growth was inhibited at 1, 2 and 4% but not at 0.2% (1% is not shown, Figure 3A). This result is consistent with the greatly reduced rates of biofilm formation at 1–4% sodium citrate being a consequence of the inhibition of population growth by citrate.

The lack of enhanced cell growth in the presence of 0.2% citrate indicates that the increase in biofilm formation is not a consequence of more bacterial cells in the culture. This suggests that citrate, rather than increasing biomass, acts either directly or indirectly to modify the way bacterial cells interact with surfaces.

Sodium citrate can be a chelator of cations such as calcium, magnesium and iron. We found that the addition of 10 or 100 mM CaCl₂ or MgCl₂, but not FeCl₂, could rescue both culture growth and biofilm formation of *S. aureus* in the presence of 2% sodium citrate (data not shown). These data are consistent with sodium citrate inhibiting growth due to either depletion of cations from the growth medium or by removal of essential cations from bacterial cells. Helander and Mattila-Sandholm [11] have reported that the membranes of several Gram-negative bacteria were permeabilized by sodium citrate, and could be rescued by MgCl₂, and concluded that citrate toxicity stemmed from cation chelation. Free calcium levels were assessed with fluorescent indicators at a range of citrate levels and only small variations were observed. Calcium levels were 8.6 μM at 0% citrate, 8.3 μM at 0.2%, 7.1 μM at 2.0% and 6.5 μM at 4.0% citrate. This suggests that calcium levels are not
Calcium was added to the medium supplemented with 0 and 0.2% citrate to detect whether the reduction in calcium was responsible for increased biofilm formation. Likewise, other divalent cations, manganese and magnesium, were separately added to 0.2% citrate supplemented medium to test if they could prevent stimulation of biofilm formation. Biofilm formation was still strongly stimulated by 0.2% citrate in the presence of these cations (Figure 3B). These data are consistent with the hypothesis that the citrate-induced change in pH is responsible for growth inhibition is unlikely.

Sodium citrate has different effects on S. aureus and coagulase-negative staphylococci (CNS) biofilm formation

A range of S. aureus clinical isolates was tested for biofilm formation with various concentrations of sodium citrate (0, 0.2, 2 and 4%) in TSB + glucose. We assessed biofilm formation of 24 S. aureus clinical isolates from various sources, and observed that 21 were stimulated in biofilm formation at 0.2% citrate, and all were inhibited in biofilm formation at 2 and 4% citrate (Table 1). Five commonly used laboratory strains including the methicillin-resistant S. aureus (MRSA) strain COL. were also tested; all were inhibited for biofilm formation at 2 and 4% citrate (Table 1). The average increase in biofilm formation (0.2% citrate biofilm levels above 0% biofilm levels) was 253% for all S. aureus strains tested. The maximum increase induced by 0.2% citrate was 848% (9.5-fold) above biofilms formed in the absence of citrate. The average decreases in S. aureus biofilm formation observed in the presence of 2 and 4% citrate were 79 and 63%, respectively.

Six CNS strains were also tested. None were stimulated in biofilm formation by 0.2% citrate, though all were inhibited at higher concentrations (Table 1). On average the presence of citrate inhibited CNS biofilm formation at 0.2, 2 and 4%. At these concentrations CNS biofilm formation was reduced by 40, 90 and 97%, respectively.

Sodium citrate and gentamicin together are effective at preventing high levels of biofilm formation

The combination of sodium citrate and gentamicin or other antibiotics in catheter locks has been suggested responsible for the cell death phenotype observed at 2–4% citrate.

Calcium was added to the medium supplemented with 0 and 0.2% citrate to detect whether the reduction in calcium was responsible for increased biofilm formation. Likewise, other divalent cations, manganese and magnesium, were separately added to 0.2% citrate supplemented medium to test if they could prevent stimulation of biofilm formation. Biofilm formation was still strongly stimulated by 0.2% citrate in the presence of these cations (Figure 3B). These data are consistent with the hypothesis that the citrate-dependent biofilm stimulation phenotype exhibited by S. aureus is mediated by a response to citrate levels rather than cation-depletion.

The addition of trisodium citrate clearly would increase the amount of sodium in solution, and sodium is known to effect biofilm formation. High salt levels have previously been shown to stimulate biofilm formation in S. aureus and Staphylococcus epidermidis [12,13]. Free sodium increased from 98 mM at 0% to 107 mM at 0.2% citrate, and to 156 mM at 2% citrate. To test whether the increase in biofilm formation at 0.2% citrate was due to the increase in sodium levels, we added 10 mM of sodium and found that there was no increase in biofilm formation until >100 mM was added (0 mM Na supplement yielded A550 biofilm formation of 0.85±0.15, 0.78±0.14 at 10 mM added sodium, 0.96±0.11 at 100 mM and 1.73±0.1 at 1000 mM, Figure 3B). These data show sodium at >150 mM was insufficient to prevent bacterial growth and found that cells grew well at levels of sodium added with inhibitory citrate levels (Figure 3B and data not shown). This is not surprising, as it is well known that S. aureus can withstand very high levels of NaCl.

Trisodium citrate has little effect upon the pH under our experimental conditions. The pH of medium supplemented with 0 and 0.2% citrate was 7.2, indicating that a change in pH is not the cause of biofilm stimulation at 0.2% citrate. The pH of the medium increases to 7.4–7.5 in medium supplemented with 2–4% trisodium citrate. As this pH is well tolerated by S. aureus, the hypothesis that a citrate-induced change in pH is responsible for growth inhibition is unlikely.
for use [14,15]. Because of this, we tested the efficacy of this combination to prevent biofilm formation. To test this, we used sodium citrate concentration of 0, 0.2, 2.0 and 4.0%, and varied gentamicin concentration from 0 to 40 mg/ml in the biofilm formation medium (TSB + 0.2% glucose). These high concentrations of gentamicin have been suggested in the literature. The ability of MZ100 to form biofilms with the addition of even the lowest concentration of gentamicin (2.5 mg/µl) was strongly inhibited (Figure 4A).

This effect was also assessed microscopically using phase and epifluorescent microscopy. We found a great reduction in the amount of biofilm formation in the presence of both citrate and gentamicin. However, even at the highest concentrations of both compounds (4.0% sodium citrate and 40 mg/ml gentamicin), a small number of adherent bacterial cells remained on the polystyrene surface, and these appeared to be living bacterial cells when assessed by a fluorescent Live/Dead staining procedure (Figure 4B–D and data not shown). The minimum inhibitory concentration of gentamicin for MZ100 in TSB + 0.2% glucose for planktonic bacterial cells was found to be 2.0 µg/ml which is 10 000× less gentamicin than the amount surface-associated bacterial cells appeared to be resistant to based on this observation (Figure 4 and R.M.Q. Shanks and G.A. O’Toole unpublished observations).

**Discussion**

Biofilm formation is strongly influenced by environmental conditions, and biofilms form on several surfaces associated with haemodialysis [21]. Our study demonstrates that the choice of catheter lock solution is likely to have a significant effect on the ability of bacteria to adhere to surfaces. Specifically, we demonstrate that sodium citrate inhibits and hirudin enhances biofilm formation on materials used in the manufacture of indwelling dialysis catheters. This observation provides a biological mechanism for the observed clinical superiority of sodium citrate over hirudin as a catheter lock solution in a prospective randomized study performed in haemodialysis patients [9]. We demonstrate that LMWH stimulates the formation of *S. aureus* biofilms *in vitro*, and sodium citrate and sodium EDTA at high concentrations strongly inhibit biofilm formation. In addition, the combination of sodium citrate and gentamicin at bactericidal concentrations is very effective in reducing the formation of biofilms on abiotic surfaces. We demonstrate that two additional potential catheter lock solutions, lepirudin and tPA, have little effect on *S. aureus* biofilm formation.

**The effect of other anticoagulants on staphylococcal biofilm formation**

A previous study has shown that sodium heparin enhances the accumulation of *S. aureus* on polymer surfaces [7]. LMWH is an effective anticoagulant and has been reported to be less likely to cause heparin-associated complications [16]. We tested whether LMWH incubated with *S. aureus* also increased adherence to polystyrene and found that it increased biofilm formation by more than 2-fold (Figure 5A).

The divalent cation chelator, EDTA, could be used as an anticoagulant lock solution in catheters, and is antibacterial [17]. The effect of EDTA on *S. aureus* biofilm formation was determined over an EDTA concentration gradient from 0.2 to 50 mM. Growth and biofilm formation was inhibited at 0.2 mM and higher levels of EDTA (Figure 5B and data not shown).

The effect of tissue plasminogen activator (tPA) on bacterial adherence was also assessed. This recombinant human serine protease did not stimulate biofilm formation at the tested concentrations and may have slightly inhibited biofilm formation at the highest concentrations (Figure 5C). The preparation we used contained two additives; the amino acid L-arginine and the preservative polysorbate 80. These could formally be responsible for the reduction in biofilm formation seen at high concentrations of tPA.

*Staphylococcus aureus* bacterial cells were exposed to lepirudin, a direct thrombin inhibitor derived from leaches, and biofilm formation was determined. Lepirudin, or hirudin, is expensive, and can be used with patients that are known to have anti-heparin antibodies or those suffering from heparin-induced thrombocytopenia [20]. Lepirudin did not significantly modify *S. aureus* biofilm formation under these experimental conditions (Figure 5D).
The genesis of catheter-related infections is likely to be complex. In addition to bacterial pathogenicity, which includes, but is not limited, to biofilm formation, the development of clinically relevant infection must involve environmental factors including catheter surface characteristics, thrombosis, blood composition and immunological response. Catheter lock solutions are used for two closely related, but separate, purposes: to prevent thrombosis and to prevent infection. Heparin is an effective anticoagulant, and as such, likely contributes to the prevention of dialysis catheter infection by minimizing the local accumulation of host-factors that promote biofilm formation. At the same time, however, heparin is an independent promoter of biofilm formation. This together with the disadvantages of high cost and anti-heparin antibodies emphasizes the importance of considering alternative catheter lock solutions.

Sodium citrate is inexpensive and is very well established as an anticoagulant in transfusion medicine and in continuous veno-venous haemofiltration systems. However, sodium citrate has not gained wide acceptance for use in intermittent haemodialysis in the US primarily because of fear of accidental infusion of highly concentrated solutions of sodium citrate during the lock procedure [22].

When considering the potential for prevention of bloodstream infections, it must be noted that small numbers of viable adherent bacterial cells persist on the material surface even at the highest levels of treatment with sodium citrate and sodium citrate plus gentamicin. These could potentially serve as a source of bloodstream infection. However, it is reasonable to postulate that smaller numbers of bacteria may be more readily cleared by the immune system than larger numbers. In addition, we hypothesize that the prevention of mature biofilm formation in haemodialysis catheters may be central to the prevention of complex infections such as endocarditis, joint and soft tissue infections because these microbial communities can actively detach from surfaces. Detachment behaviour, which is genetically determined, causes rafts of living biofilm or single cells to be released from the surface with the potential to lodge in distant sites [23].

Our data demonstrate that subinhibitory concentrations of sodium citrate actually enhance biofilm formation. This finding is of potential clinical significance because the concentration of citrate, as of all catheter lock solutions, varies at the catheter tip depending on the viscosity of the injected solution [24]. Agharazii and colleagues [25] have shown that up to 75% of the catheter contents may leak in the first 30 min after performing a catheter lock. However, a clinical trial that employed 5% citrate as a catheter lock solution did not show a higher level of infection than catheters locked with 10% citrate, consistent with the increase in biofilm formation caused by low levels of citrate in vitro having little effect upon the rate of infection in clinical practice [26].

It is unclear at this point how low concentrations of sodium citrate cause this stimulation in biofilm formation. This finding is of potential clinical significance because the concentration of citrate, as of all catheter lock solutions, varies at the catheter tip depending on the viscosity of the injected solution [24]. Agharazii and colleagues [25] have shown that up to 75% of the catheter contents may leak in the first 30 min after performing a catheter lock. However, a clinical trial that employed 5% citrate as a catheter lock solution did not show a higher level of infection than catheters locked with 10% citrate, consistent with the increase in biofilm formation caused by low levels of citrate in vitro having little effect upon the rate of infection in clinical practice [26].

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It will be of interest to test the effectiveness of sodium citrate and sodium EDTA in the clinic and on in vitro biofilm formation. There are several reports that indicate sodium citrate is as effective as a catheter lock solution in the clinic as heparin [27]. Likewise, sodium EDTA has been shown by itself or in conjunction with antibiotics to be inhibitory to the formation of biofilms in vitro and in vivo [18,19,28–32].

It has been hypothesized that the bactericidal activity of cationic chelators stems from two activities [11]. First, calcium and magnesium ions are necessary for prokaryotic cell division, and second, they are important for the integrity of the bacterial cell wall. Sodium citrate and EDTA are both capable of chelating calcium and magnesium ions leading to inhibition of bacterial growth.

In conclusion, this study shows that, in contrast to heparin and LMWH, sodium citrate at 2% concentrations or greater powerfully inhibits in vitro biofilm formation by Staphylococcus aureus and CNS. At much lower concentrations, which could theoretically be achieved within or downstream from locked dialysis catheters, sodium citrate actually promotes biofilm formation. These observations, together with recent clinical studies demonstrating the efficacy of sodium citrate as a catheter lock solution in dialysis patients, raise intriguing questions about the pathogenic mechanisms of clinically significant bloodstream infections in haemodialysis patients with indwelling dialysis catheters.

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