

Impact of slime dispersants and anti-adhesives on *in vitro* biofilm formation of *Staphylococcus epidermidis* on intraocular lenses and on antibiotic activities

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Objectives: Infectious endophthalmitis has occurred despite the use of antibiotics in irrigating solutions during implantation of intraocular lenses (IOLs). This infection is generally resistant to antibiotic therapy and, therefore, removal of the implant is necessary before eradication of the infection. This study was designed to assess the role of chosen dispersants and anti-adhesives in inhibiting *Staphylococcus epidermidis* hydrophobicity, adhesion, slime production and subsequently biofilm formation on IOLs.

Methods: The relative activity of several potential slime dispersants and anti-adhesives on slime production, hydrophobicity and the adherence of *S. epidermidis* to IOLs and the degrees to which their effects enhance antibiotic activities were investigated.

Results and conclusions: The MBCs of antibiotics against *S. epidermidis* strains in a biofilm increased 10–16 times compared with those against bacterial strains in suspension. Addition of slime dispersants or anti-adhesives reversed the susceptibility of the strains in a biofilm to that of bacteria in suspension. Slime production by *S. epidermidis* strains was significantly diminished by dispersants. Anti-adhesives, hyaluronan, heparin and carboxymethyl cellulose 934 exerted less effects on slime production than dispersants. Addition of slime dispersants or anti-adhesives to cell cultures resulted in a significant reduction in bacterial surface hydrophobicity compared with control untreated cultures (at $P < 0.001$). Reduction of slime production and bacterial surface hydrophobicity led to a marked decrease in the adherence of *S. epidermidis* to IOLs. Slime dispersants were more effective at reducing bacterial adherence than anti-adhesives. Simultaneous use of antibiotics with slime dispersants or anti-adhesives will exert a more beneficial effect during IOL implantation.

Keywords: hydrophobicity, adhesion, slime production

Introduction

Infectious endophthalmitis is the most feared and uncommon complication after cataract surgery and intraocular lens (IOL) implantation.¹ Although this condition typically occurs in the early post-operative period, a less common manifestation of post-operative endophthalmitis has a chronic course.¹

Staphylococcus epidermidis is commonly found in normal eyes and conjunctival sacs and is usually associated with post-operative endophthalmitis.² There are two main characteristics

of *S. epidermidis* that allow persistence of infection: (i) the ability of the bacteria to adhere onto surfaces of prosthetic devices in multilayered cell clusters; and (ii) the production of a mucoid substance commonly known as slime. The adherent bacteria and slime are collectively known as biofilm.³ Bacteria can adhere to IOLs by either a non-specific adhesion mechanism (governed by the surface properties of both bacterial cells and implant) or a specific adhesion binding reaction. Once embedded in this biofilm layer, the microorganisms are protected from the host's immune cells and from the action of antimicrobial agents.

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Inhibition of *S. epidermidis* biofilm production by dispersants and anti-adhesives

In many cases, the only effective therapy for these infections is the removal and replacement of the lens.³ An alternative approach to overcome this problem is based on the prevention of biofilm formation.³ Anti-adhesives are among the substances that can greatly reduce bacterial cell adhesion *in vitro* and in several *in vivo* applications.⁴ Also, slime dispersants are agents with the potential to reduce slime viscosity and thus promote the diffusion of most antibiotics through slime.⁵ The aims of this study were to: (i) assess the *in vitro* reducing effect of anti-adhesives and dispersants on the adherence of bacteria to polymethylmethacrylate (PMMA) IOLs; and (ii) assess the changes in the susceptibility of residual adherent bacteria to antimicrobial agents.

Materials and methods

Bacterial strains

A clinical strain of *S. epidermidis* was isolated from a patient with endophthalmitis in the Department of Ophthalmology, Faculty of Medicine, King Saud University, Riyadh, Saudi Arabia. The isolate was identified by Gram staining, catalase reaction and the tube coagulation test.⁶ A standard slime producer strain (ATCC 35984) was also used throughout this investigation.

Chemicals

All chemicals were purchased from Sigma Chemical Co, St Louis, MO, USA, except for carbopol 934, which was purchased from BF Goodrich, Cleveland, OH, USA. EDTA and EGTA were used at a concentration of 0.001 M, *N*-acetyl cysteine (NAC) was used at a concentration of 0.02 M and sodium citrate and sodium chloride were used at a concentration of 0.1 M. Hyaluronan and carbopol were used at concentrations of 0.75% and 0.25%, respectively. Heparin was added at a concentration of 2000 IU/mL.

Detection of slime production

Production of slime from *S. epidermidis* was detected by culturing the strains onto Congo Red agar (CRA) plates.⁷ CRA plates were incubated at 37°C for 24 h and then at room temperature overnight. On CRA, slime-producing strains form black colonies, while non-producing strains develop red colonies.⁷

Biofilm assay on microtitre plates

The ability of *S. epidermidis* strains to form biofilm on abiotic surfaces was quantified by the method of O'Toole and Kolter.⁸ Briefly, *S. epidermidis* strains were grown overnight in tryptic soy broth (TSB) with 0.25% glucose at 37°C. The culture was diluted 1:40 in TSB/0.25% glucose in the absence or presence of dispersants or anti-adhesives, and 200 µL of this cell suspension was used to inoculate sterile 96-well polystyrene U-bottom microtitre plates (Sterilin, UK). After 48 h at 37°C, wells were gently washed three times with 200 µL of PBS, dried in an inverted position and stained with 200 µL of 1% Crystal Violet for 15 min. Wells were rinsed again, and Crystal Violet was solubilized in ethanol/acetone (80:20, v/v). Optical density (OD) or absorbance (Abs) of the well content was determined⁶ at 595 nm (OD₅₉₅) using a microplate reader (Bio-Tek, VT, USA).

Effect of slime dispersants and anti-adhesives on bacterial adhesion to IOLs

PMMA IOLs (Pharmacia Production B.V., The Netherlands) were used as adherence supports. They were placed in glass tubes containing PBS with bacteria at 10⁷ cfu/mL and different slime dispersants or anti-adhesives. Tubes containing no dispersant or anti-adhesive were used as controls. The tubes were statically incubated at 37°C for 4 h. To remove non-adherent bacteria after incubation, each lens was washed three times with 5 mL of sterile distilled water. Then, each lens was placed in a glass tube containing 1 mL of PBS and sonicated briefly for 3 min at 40 kHz in an ultrasonic cleaner (Branson cleaning instrument; Shelton, CT, USA). Quantitative cultures were performed by plating 100 µL of the solution on Mueller–Hinton agar. The results were expressed as numbers of cfu/100 µL of adhered bacterial suspension. The rate of residual bacterial adhesion, expressed as a percentage, is given as the number of adherent bacteria in treated samples divided by the number in control specimens.⁹

Effect of anti-adhesives and dispersants on hydrophobicity

Cells in logarithmic growth (6 h) in Mueller–Hinton broth (MHB) were collected by centrifugation and adjusted by a spectrophotometer (Ultraspec II spectrophotometer; Pharmacia, Rockville, MD, USA) to 10⁶ cfu/mL in PBS. Different anti-adhesives or dispersants were added to the culture tubes and incubated at 37°C for 1 h. Then, the cells were harvested by centrifugation for the determination of hydrophobicity using the method of Rosenberg *et al.*¹⁰ The pellet was suspended in 1 mL of PBS, and the absorbance at 530 nm (*A* value) was adjusted to 0.4 by the addition of PBS.¹¹ One hundred microlitres of hexadecane was added in 10 µL increments to the 1 mL suspension. After each addition, the suspension was vortex-mixed at a high speed for 15 s. The resultant *A*₅₃₀ was immediately read using a spectrophotometer. The decrease in '*A*' readings caused by the addition of hexadecane reflected the surface hydrophobicity of the organism. These assays were performed six times, and the mean values were calculated. Controls were treated similarly without the addition of anti-adhesives or dispersants.

MBCs of antibiotics for suspended bacteria

The MBC tests were performed in duplicate on suspended bacteria by the microdilution method. Bacterial inocula were standardized against the 0.5 McFarland turbidity standard and diluted with MHB supplemented with calcium (MHBSC) at a concentration of 15 mg/100 mL to produce a final concentration of ~10⁴ cfu per well in each of a series of log₂ antibiotic dilutions. MBCs were determined after 24 h of incubation at 37°C. MBC was defined as the lowest drug concentration that resulted in at least a 99.9% reduction in the number of cfu per well relative to control levels.¹²

MBCs of antibiotics for adherent bacteria

Bacterial strains were grown in MHBSC for 6 h at 37°C. The bacteria were adjusted with MHBSC against the 0.5 McFarland turbidity standard, diluted with MHBSC to ~10⁶ cfu/mL and then transferred to sterile microplates. IOLs were placed into bacterial suspensions with or without anti-adhesives and incubated at 37°C. After 24 h, the lenses were removed with sterile forceps and washed twice with 5 mL of PBS, pH 7.2, to remove non-adherent bacteria. Each lens was transferred to a well in a 96-well plate in a series of duplicate log₂ dilution steps of antibiotic in MHBSC and maintained

at 37°C. Additional colonized lenses were placed in either drug-free MHBSC wells (controls) or different dispersants in antibiotic-containing MHBSC wells. After 24 h of incubation, the lenses were removed and washed twice with 5 mL of PBS to remove non-adherent bacteria. The lenses were placed in 10 mL of PBS, sonicated for 3 min in a low output cleaning sonicator then vortexed for 30 s. Supernatants were serially diluted with PBS, and 100 µL from each tube was spread onto a blood agar plate. Bacterial colonies were counted after 24 h of incubation at 37°C. MBC was defined as the lowest drug concentration that resulted in at least a 99.9% reduction in the number of cfu per lens relative to control levels.¹²

Results

The clinical isolate of *S. epidermidis* was identified on a CRA plate as a slime producer. The adherence of clinical and standard strains of *S. epidermidis* to IOLs was more strongly inhibited by the dispersants than the anti-adhesives (Table 1). Adherence of strains to IOLs was decreased to <10% when treated with the slime dispersants EDTA, EGTA and NAC compared with control untreated cells. However, the anti-adhesives hyaluronan and heparin reduced bacterial adhesion to IOLs to ≥30% compared with control untreated cells (Table 1).

As Table 2 reveals, exposure of *S. epidermidis* strains to dispersants or anti-adhesives for 1 h resulted in a significant decrease in bacterial surface hydrophobicity compared with control untreated cultures (*t*-test, *P* < 0.001 for all comparisons). Bacterial surface hydrophobicity was reduced by ≥70% when treated with EDTA, EGTA and NAC.

Figures 1 and 2 reveal the effect of anti-adhesives and dispersants on slime production by clinical and standard strains. Slime production of clinical and standard strains was significantly decreased by EDTA, EGTA and NAC. Hyaluronan and heparin exerted the most reducing effect on slime production among the anti-adhesives.

Growth of standard and clinical strains of *S. epidermidis* adherent to IOLs increased the MBCs of gentamicin 12 times (details not shown). Meanwhile, the MBCs of vancomycin against the adherent standard strains increased 16 times, while

Table 1. Effect of slime dispersants and anti-adhesives on bacterial adhesion to IOLs

Treatment	Residual adherent bacteria (%)	
	clinical strain	standard strain (ATCC 35984)
Control (PBS)	100	100
Dispersants		
EDTA	2.4	3.5
EGTA	2.7	4.1
<i>N</i> -acetyl cysteine	4.2	6.1
sodium citrate	26.8	27.4
sodium chloride	28.3	29.3
Anti-adhesives		
hyaluronan	30.6	33.2
heparin	31.9	36.6
carbopol 934	39.1	42.3

Table 2. Effect of dispersants and anti-adhesives on hydrophobicity of *S. epidermidis* strains

Treatment	%Hydrophobicity ^a (mean ± SD)	
	ATCC 35984	clinical strain
Control	63.1 ± 1.3	57.32 ± 2.2
Dispersants		
EDTA	14.19 ± 1.6 (<0.001) ^b	13.12 ± 1.4 (<0.001)
EGTA	14.34 ± 0.9 (<0.001)	13.16 ± 1.1 (<0.001)
<i>N</i> -acetyl cysteine	18.8 ± 0.7 (<0.001)	17.29 ± 1.45 (<0.001)
sodium citrate	27.9 ± 1.27 (<0.001)	26.2 ± 1.13 (<0.001)
sodium chloride	28.1 ± 2.12 (<0.001)	27.19 ± 0.8 (<0.001)
Anti-adhesives		
hyaluronan	30.2 ± 0.6 (<0.001)	30.1 ± 2.1 (<0.001)
heparin	31.1 ± 0.3 (<0.001)	30.9 ± 1.9 (<0.001)
carbopol 934	34.3 ± 1.4 (<0.001)	33.9 ± 2.3 (<0.001)

^aEach experiment was repeated six times.

^b*P* values in parentheses are for treated cultures compared with untreated controls (*t*-test).

those against adherent clinical strains increased 10 times. Dispersants and anti-adhesives were able to reduce the MBCs of antibiotics for bacterial strains to the regular MBCs for the suspended bacteria.

Discussion

S. epidermidis commonly colonizes eyelid margins and conjunctiva and is the most common organism causing post-operative endophthalmitis.¹

Several approaches should be developed to prevent endophthalmitis, which is the uncommon complication of IOL implantation.

Bacterial adherence to IOLs is a prerequisite in infectious endophthalmitis. Thus, the best prophylactic approach would be to prevent bacterial adhesion. PMMA is used widely in the manufacture of IOLs. It has the major disadvantage of promoting bacterial adherence.⁹

Various factors affect the interaction between bacteria and biomaterials according to environmental conditions and the surface characteristics of both material and bacterium.⁶

Adherence is mediated by a variety of non-specific factors, such as hydrophobicity, electrostatic charge and slime production, and specific factors, such as bacterial adhesion.¹³

Our previous studies^{11,14} show that antibiotics at subinhibitory or high concentrations during intraocular implantation reduce the adherence of *S. epidermidis* to IOLs. We also elucidated how rare endophthalmitis could have happened despite antibiotics being used in irrigating solutions.¹¹ The persistence of microorganisms with the use of antibiotics in irrigating solutions was explained by heavy contamination, and none of the tested antibiotics (at high doses) was able to eradicate the microorganisms and the subsequent biofilm formation. We suggest the use of adjuvant therapy such as dispersants or anti-adhesives, in addition to the antibiotics in irrigating solutions.

Inhibition of *S. epidermidis* biofilm production by dispersants and anti-adhesives

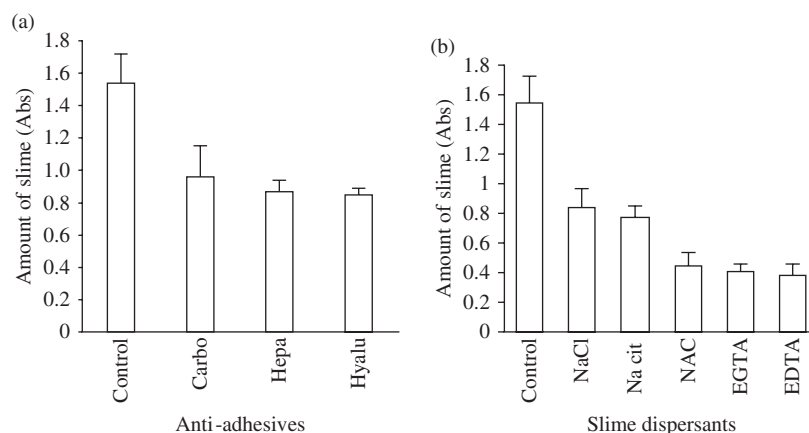


Figure 1. Effect of (a) anti-adhesives and (b) slime dispersants on slime production of *S. epidermidis* standard strain. Carbo, carbopol; Hepa, heparin, Hyalu, hyaluronan; NaCl, sodium chloride; Na cit, sodium citrate; NAC, *N*-acetyl cysteine.

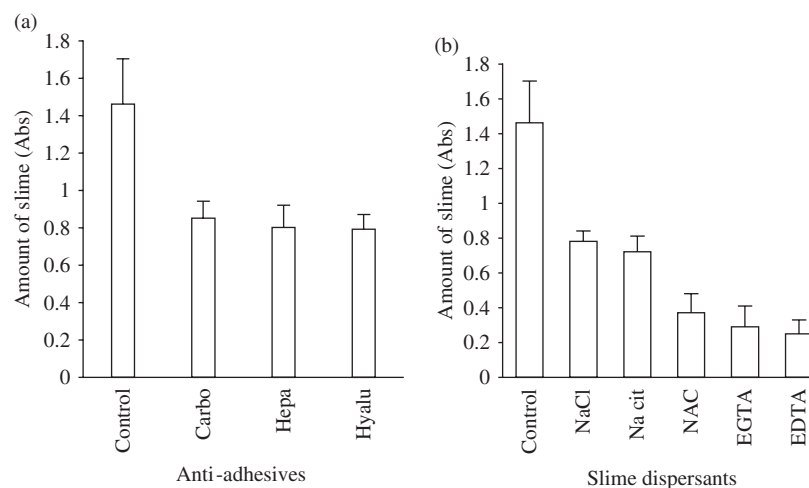


Figure 2. Effect of (a) anti-adhesives and (b) slime dispersants on slime production of *S. epidermidis* clinical strain. Carbo, carbopol; Hepa, heparin; Hyalu, hyaluronan; NaCl, sodium chloride; Na cit, sodium citrate; NAC, *N*-acetyl cysteine.

This study was performed to assess the ability of chosen dispersants and anti-adhesives to inhibit *S. epidermidis* hydrophobicity, adhesion, slime production and biofilm formation on IOLs.

Slime dispersants exerted a profound effect of inhibiting bacterial adherence to IOLs compared with anti-adhesives. *S. epidermidis* adherence was strongly inhibited by EDTA, EGTA and NAC. Active dispersants may be conveniently grouped as chelating agents and sodium salts.⁵

The activity of anti-adhesives was related to strong interaction with water. Where the long hydrophilic chains of these substances bound to the surfaces of biomaterials extend into the aqueous media and form, by trapping water molecules, a highly hydrated layer between the bacteria and the surface of biomaterials, the attachment of bacteria to the surfaces is prevented. Negative charges of some anti-adhesives, e.g. hyaluronan and heparin (hydrophilic polysaccharides), may also play a role in inhibiting adherence.¹ The results of this investigation are consistent with those reported in previous investigations.^{1,4,15} Carpolol 934, a hydrophilic resin, exerts the same mechanism.

A remarkable reduction in bacterial hydrophobicity was obtained when both clinical and standard strains of

S. epidermidis were treated with dispersants and anti-adhesives compared with control cultures (Table 2). The reduction in bacterial hydrophobicity was greater when the cells were treated with dispersants compared with when the cells were treated with anti-adhesives (Table 2). These data are consistent with other studies,^{1,2,9} in that a significant positive correlation between hydrophobicity and adherence to prosthetic biomaterials was found.

Slime production was greatly dissociated or diminished when the strains were treated with dispersants or anti-adhesives, respectively. Noticeably, dispersants reduced slime production more than anti-adhesives (Figures 1 and 2). Dispersant chelating agents, e.g. EDTA, EGTA and NAC, have more potential to reduce slime viscosity than sodium salt dispersants, e.g. sodium citrate and sodium chloride (Figures 1 and 2). The mode of action of EDTA, EGTA and NAC in inhibiting biofilm formation is possibly through their chelating activity on calcium, which is a component essential for the maintenance of the extracellular biofilm matrix, controlling the degree of cross-linking.^{5,16} Reversal of calcium-induced slime formation reduces viscosity and the agents that have this effect might be therapeutically

useful by rendering the bacteria more accessible to antibiotics and phagocytosis. The anti-adhesives hyaluronan and heparin exerted greater reducing effects on slime production by clinical and standard strains compared with carbopol 934 (Figures 1 and 2) by forming a hydrated layer around the bacterial surfaces and/or biomaterials, which enhanced the inhibition of staphylococcal adherence.⁹

Susceptibility of IOL-adherent *S. epidermidis* to antibiotics compared with that of organisms grown in suspension revealed that biomaterial-adherent bacteria exhibited 10–16 times increased resistance to antibiotics. It has been shown that bacteria present in biofilm could exhibit 10–1000 times increased resistance to antibiotics,¹⁷ depending on biofilm age, antibiotic concentration and time of exposure.¹⁸ Slime may constitute a static layer through which an antibiotic must diffuse to reach the surface of the bacterial cell wall. The deeper the static layer, the longer will be the time for equilibration of antibiotic concentration between the external medium and the bacterial cell surface.¹⁹

Addition of dispersants to antibiotics will potentiate the activity of antibiotics against the bacteria adherent to biomaterials (IOLs). This potentiation appears to be a two-step phenomenon: first, a decrease in the number of adherent bacteria; second, for residual adherent bacteria,¹¹ a qualitative modification of binding, resulting in restoration of susceptibility to antibiotic activity.⁹ On the other hand, addition of anti-adhesives to antibiotics prevents the adhesion to and colonization of IOLs by bacteria, leaving them in suspension, vulnerable to antibiotic action.

The simultaneous use of antibiotics with slime dispersants or anti-adhesives in irrigating solutions during implantation of IOLs will enhance the potency of antibiotics and reduce the occurrence of endophthalmitis.

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Transparency declarations

None to declare.

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