Bismuth-Ethanedithiol:

A Potential Drug to Treat Biofilm Infections of Medical Devices Produced by *Staphylococcus Epidermidis* and *Proteus Mirabilis*

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Bismuth-Ethanedithiol:A Potential Drug to Treat Biofilm Infections of Medical Devices Produced by Staphylococcus Epidermidis and Proteus Mirabilis

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ABSTRACT

Staphylococcus epidermidis is the leading cause of hospital-acquired infections associated with implanted medical devices. Likewise, *Proteus mirabilis* is one of the leading causes of nosocomial Urinary Tract Infections (UTIs) and is associated with urinary catheter blockage. Both organisms produce biofilms, which make them less susceptible to antibiotics. Therefore, these infections are often notoriously difficult to treat and in many cases lead to complications. Preventing biofilm formation or killing a pre-existing biofilm would be useful solutions since biofilm formation is a phenomenon commonly observed in these organsims.

This study investigated the *in vitro* efficacy of Bismuth-ethanedithiol (BisEDT) against biofilms produced by *P. mirabilis* and *S. epidermidis*. Anti-biofilm activity of BisEDT was evaluated in terms of inhibiting/preventing biofilm formation and eradicating/killing pre-formed biofilm produced by single species *S. epidermidis* and *P. mirabilis* as well as dual species biofilms produced by these two organisms. The following assays were used to assess: 1) Polystyrene bead assay. 2) Agar diffusion susceptibility assay. 3) Spectrophotometric bead assay. The assays showed that 0.1 μg/ml of BisEDT inhibited *S. epidermidis* biofilm formation by 99.9% in single species biofilms. One μg/ml of BisEDT inhibited single species *P. mirabilis* biofilm formation by 99.9%. Dual species biofilms of both organisms were inhibited by 99.9% by five days of exposure to 0.5 μg/ml. 99.9% biofilm eradication in single and dual species biofilms of both organisms was achieved by 5 μg/ml of BisEDT.

These results suggest that BisEDT was highly successful in inhibiting and eradicating biofilms. Therefore, this drug may be of use to treat device related infections caused by *S. epidermidis* and *P. mirabilis*.

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PREFACE

The following thesis is written in the manuscript format and conforms to guidelines established by the Graduate School of the University of Rhode Island and the *ASM Style Manual for Journals and Books* (American Society for Microbiology, 2000.)

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INTRODUCTION

A recent announcement from the National Institutes of Health reported that “more than 60% of all microbial infections are caused by biofilms” (19). This percentage represents biofilm-associated medical conditions such as dental plaque, gingivitis, urinary tract infections, middle ear infections as well as bacterial colonization of indwelling medical devices. The last is a major medical concern due to significant rates of patient morbidity/mortality and high economic cost (25). This is partly due to modern medicine’s increased dependency on indwelling devices such as intravascular catheters, cerebrospinal fluid shunts, continuous ambulatory peritoneal dialysis catheters, orthopedic devices, artificial joints, prosthetic heart valves and cardiac pacemakers as tools of diagnosis and/or therapy for various medical conditions (17). Colonization of these devices contribute to nosocomial (hospital-acquired) infections, particularly endocarditis and blood-stream infections. A recent survey revealed that up to 2 million patients a year are affected by nosocomial infections, causing 88,000 deaths and costing over $4.5 billion to the health care system (26).

Coagulase-negative staphylococci (CNS), especially *Staphylococcus epidermidis* is the most common cause of infections from both temporarily and permanently inserted medical devices (17, 21). For instance, this organism is the culprit of up to 67% of central nervous system shunt complications, 70% of catheter-related infections, 50% of prosthetic cardiac valve infections and 50% of joint replacement infections (22). Treatment of these infections is further complicated by the emergence of antibiotic resistance. With the onset of

methicillin-resistant CNS, vancomycin became the drug of hope, which also in recent years has exhibited decreased sensitivity to CNS (29, 33).

Proteus mirabilis is the third most common pathogen involved in Urinary Tract Infections (UTIs; after *Escherichia coli* and *Klebsiella* spp.) (4). UTIs make up almost 40% of nosocomial infections of which, as much as 80% are associated with indwelling urinary catheters. The risk of death, once catheter-related bateriuria occurs increases by 2.8 fold and additional cost of hospitalization is increased by $2800 (2, 27). Urinary catheters colonized by *P. mirabilis* produce encrustations (due to urease activity), which not only blocks the catheter but may also cause bladder stones (2, 31). Treatment of *P. mirabilis* infections is also affected by antibiotic resistance. Stickler et al. (30) showed that this organism survived well in the presence in chlorhexidine. Likewise *P. mirabilis* has been shown to develop resistance after 14 days of exposure to mandelic acid, a drug widely used to treat UTIs (2).

Both nosocomial *S. epidermidis* and *P. mirabilis* involved in device-related infections produce biofilms. Two leading scientists in the field have provided classic definitions of biofilms; Costerton et al. defines biofilms as “a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface” (7). Elder et al. defines biofilms as “a functional consortium of microorganisms organized within an extensive exopolymer matrix” (16). Apart from bacterial cells and the exopolymer matrix (also known as Extracellular Polymeric Substance (EPS), glycocalyx or more commonly ‘slime’, which is primarily composed of polysaccharides and proteins), a biofilm may contain other materials

depending on the environment in which it is found. For example, inflammatory response proteins, complement, fibrinogen, fibronectin and glycosaminoglycans may be found in biofilms associated with artificial prosthetic devices, while mineral crystals, clay, silt and corrosion particles may be found in industrial or portable water systems and natural aquatic systems (15, 16).

The process of biofilm formation and its metabolism is highly complex. The initial steps involved in bacterial adherence and biofilm production on a surface depends on several factors such as the texture of the surface, chemical alteration of the surface due to materials found in the medium (called 'conditioning'), hydrodynamics, pH, temperature, presence of ions and nutrients in the medium (15). More important are the properties of the bacterial cell such as the presence of flagella and/or pili and the ability to produce EPS. Biofilm production is a cell-density dependent process where quorum-sensing systems sense the number of planktonic cells in close proximity to a conditioned surface and begin to adhere to it as a result of hydrophobic and electrostatic interactions (16, 23). Investigators have shown that a gene cluster (called the *ica* operon in staphylococci species) encodes the production of polysaccharide intercellular adhesion (PIA), which mediates cell aggregation once attachment has occurred (8, 16). Next, the superficially attached cells secrete EPS, which acts as a glue that adheres them to the foreign surface more tightly and provides protection to the cells within the biofilm. The final step in biofilm formation involves the maturation of the multi-layers characterized by the replication of attached organisms (16).

Bacterial cells enclosed within biofilms are resistant to antimicrobial agents and the host immune system than planktonic cells. Researchers have suggested several mechanisms as to how biofilms successfully evade antimicrobial agents. One mechanism is the restricted penetration of antimicrobial agents due to the multi-layer structure of biofilms. Investigators have shown that these layers act as a diffusion barrier or a molecular filter, physically retarding and/or preventing the penetration of antibiotics and antimicrobial proteins (i.e. lysozyme) and complement (19). Further evidence shows that, in some cases (depending on the species and the agent used) the antimicrobial agent is rendered inactive before it can diffuse through the layers of the biofilm, possibly due to binding by EPS (7, 19). Another important observation was made during experiments involving resistance of *Pseudomonas aeruginosa* biofilms to tobramycin and *S. epidermidis* slime to vancomycin and teichoplanin. In both organisms, researchers found the minimum inhibitory concentration (MIC) required to sterilize biofilms were 5 and 15 fold more than their planktonic counterparts, respectively (16, 19).
A second mechanism relates to the growth rate of cells within biofilms. Cells that are buried deep in the layers are metabolically inert (possibly due to nutrient limitations and waste build-up). Therefore, these cells are not actively growing. The function of these cells is primarily to provide a foothold on the surface of the foreign material (to act as a 'suction cup'), so that multi-layers may be formed on them. Most antibiotics target actively dividing cells. For example, cell growth is an absolute requirement for penicillin and ampicillin to be effective. Also, the rate of kill of the target cell is proportional to the rate

of growth of the cell (19). Other speculative reasons that would explain the resistance of biofilms to treatment are lower pH and higher CO_2 within these slime layers, expression of possible resistance genes, changes in cell wall composition and phenotypic variation in enzymatic activity (16, 19, 25).

Since bacterial biofilms show poor response to antibiotics, they are notoriously difficult to treat without the removal of the infected device. As a result, several remedies are currently being developed in order to eradicate/inhibit colonization of medical devices. The use of electromagnetic fields, ultrasound, genetic manipulation of 'biofilm genes' and coating or impregnating implants with antimicrobial agents are several options that are being investigated (19, 22, 25). This study proposes a relatively new antimicrobial agent that can be effectively used against catheter-related infections.

Bismuth-ethanedithiol (BisEDT) is a new class of anti-biofilm agents that has demonstrated remarkable activity against a broad spectrum of biofilm-producing organisms. Several investigators using Bismuth-dimercaprol (BisBAL), Bismuth-ethanedithiol and Bismuth-3,4-dimercaptotoluoene (BisTOL) have shown inhibition of *Klebsiella* spp. capsule production, reduction of alginate expression by *P. aeruginosa* and inhibition of slime-producing *S. epidermidis* (10, 14, 18, 35). Bismuththiols, while having up to 1000-fold greater antimicrobial activity than other bismuth salts, inhibit slime expression in both gram positive and negative organisms at non-toxic concentrations (10).

To date, the effects of BisEDT on *S. epidermidis* slime production has not been investigated by a polystyrene bead adherence assay. The effects of BisEDT on *P. mirabilis* biofilms have not been reported. Furthermore, treatment of a mixed biofilm of both organisms with BisEDT has not been attempted. Therefore, this study investigates the *in vitro* efficacy of BisEDT in:

Study #1: Inhibiting/preventing biofilm production by *S. epidermidis*, *P. mirabilis* and a mixture of both organisms.

Study #2: Eradicating/killing pre-formed biofilm produced by the above organisms.

MATERIALS AND METHODS

Bacterial strains: Both *S. epidermidis* RP62A (ATCC 35984) and *P. mirabilis* (ATCC 12453) were obtained from the American Type Culture Collection (ATCC.)

Preparation of BisEDT (1:1): Both liquid and powder forms were prepared. For the liquid form, 4.85 mg of bismuth nitrate (BN) was dissolved in 1 ml of 1,2-propanediol, while 100 µl of 1,2-ethanedithiol (EDT) was dissolved in 900 µl of 1,2-propanediol. 1000 µg/ml working stock BisEDT was prepared by adding 50 µl of BN, 5.8 µl of concentrated HCl (12.1N) and 4.2 µl of EDT to 940 µl of 1,2- propanediol. For the non-liquid form, 1 mg of BisEDT powder was placed in 990 µl of 1,2 propanediol and 10 µl of concentrated HCl. For both types, a water bath sonicator (Fischer FS 3 ultrasonic cleaner, Pittsburgh, PA) was employed to break up particles. (Five micromoles of BT is approximately equal to 1 µg of bismuth/ml.) The working stock was diluted

1:10 and 1:100 in sterile saline. BN was made fresh bimonthly while EDT was made biweekly.

Preparation of initial inoculum: Both bacterial strains were grown overnight, shaking at 150 rpm (Model G-10, New Brunswick Scientific Co., Inc., Edison, NJ) at 37˚C in trypticase soy broth (TSB; Difco, Detroit, MI.) The OD_{600} of the overnight cultures was adjusted to 0.01 (~10^7 CFU/ml) and 15 µl aliquots were used as the inocula for each culture tube. RP62A or *P. mirabilis* inoculum was used for single-species biofilm, while both were used to establish a dual-species biofilm.

Statistical analysis: All tests were done in duplicate in three different trials. The statistical significance of the data was determined by a paired t-test using Slide Write Plus software (Version 4.0 by Advanced Graphics Software, Inc., Carlsbad, CA.) A *P* value of < 0.05 was considered to be statistically significant. The ± standard error of the mean (SEM) was also recorded.

MIC determinations of BisEDT: The minimum inhibitory concentration of BisEDT against planktonic cells of both organisms was determined by a broth microdilution method as recommended by the National Committee for Clinical Laboratory Standards (NCCLS; 34). The broth medium used was cation-adjusted Mueller Hinton broth (CAMHB; Difco.)

Study #1: <u>Inhibition of biofilm formation</u>

Method #1: Polystyrene bead assay

Adherence assay: Each culture tube contained 15 μl inoculum, one sterile polystyrene bead (5.5mm diam., Precision Plastic Ball Co., Franklin Park, Illinois) and 150 μl of CAMHB. The following concentrations (final) of BT were added to the culture tubes:

Tube	BisEDT (final conc. μg/ml)
Reference	0
#1	0.1 (only for single-species RP62A)
#2	0.5
#3	1
#4	5

The final volume of all tubes was brought up to 300 μl with sterile de-ionized water, mixed well and incubated at 37˚C with shaking at 150 rpm for 1, 3 and 5 days.

Following incubation, the liquid from all tubes was aspirated, the beads were washed 3 times with sterile phosphate-buffered saline (PBS, pH 7) and 500 μl of a solution containing 0.5% Tween 80 (polyxyethylene-sorbitan monooleate; Sigma, St. Louis, MO) and 10mM EDTA (ethylenediamine tetraacetic acid; Sigma) was added to each tube. After 10 minutes of Tween 80-EDTA treatment, bacteria adhered to the beads were released by vigorous vortexing (Fischer Vortex-Genie 2™ Model G-560, Scientific Industries, Inc., Bohemia, NY) for 3 minutes in the Tween 80-EDTA solution. An ultrasonic bath was not used to release adhered cells since previous studies indicate the

vortex only method to have a recovery efficiency of 97% (24). The liquid was serially diluted and the number of biofilm producing cells that adhered to the beads were spot plated and enumerated by the viable plate count method on selective media. Mannitol salts agar (MSA; Difco) plates were used to select for the growth of *S. epidermidis* while inhibiting the growth of Gram negative, *P. mirabilis.* Xylose lysine deoxycholate (XLD; Difco) plates were used to select for *P. mirabilis* and to inhibit Gram positive, *S. epidermidis* growth. Preliminary data (not shown) showed insignificant differences between the number of colonies grown on selective and non-selective, i.e. blood agar media (Difco.) All plates were incubated overnight at 37˚C and the colonies counted to determine CFU/ml.

Determination of treatment effects: Treatment effects were determined by the relative inhibition of biofilm production (expressed as a mean percentage):

$$100 - [(\text{CFU of treated bead} \div \text{CFU of reference bead}) \times 100]$$

The CFUs generated by the untreated/reference bead served as the reference inoculum for that set of experiments.

Method #2: Agar diffusion susceptibility test

Disk preparation: Mueller-Hinton agar (Difco) plates were streaked for confluent growth with RP62A or *P. mirabilis* for single-species biofilm. Sterile filter paper disks (6 mm diameter) soaked with10 μl of 0, 0.1, 0.5, 1, 5 and 10 μg BisEDT were placed on the surface of agar plates and incubated overnight at 37˚C.

Determination of treatment effects: After incubation, the diameter of the zone of inhibition created by the diffusion of the drug was measured in millimeters (mm) using a scientific ruler (Sorvall, Norwalk, CT.)

Method #3: Spectrophotometric assay

Adherence assay: Culture tubes with polystyrene beads were inoculated, the same concentrations of BisEDT were added and incubated under conditions described in method #1. Following incubation, non-adherent cells were removed by rinsing with PBS. Biofilm on the beads was stained with 350 μl of 1% crystal violet (Fisher) for 25 min. followed by further rinsing with distilled water. The cell-associated stain was solubilized in 400 μl of dimethyl sulfoxide (DMSO; Fisher) for 5 min. as described in (3).

Determination of treatment effects: The resulting solution was quantified by measuring the OD at 570 nm using an UV spectrophotometer (Pharmacia-Biotech.) An uninoculated/untreated bead stained with crystal violet and subsequently solubilized in DMSO was used as the blank.

Study #2: <u>Eradication of pre-formed biofilm</u>

Method #1: Polystyrene bead assay

Adherence assay: 15 μl aliquots of the inocula were added to culture tubes containing a sterile polystyrene bead and 150 μl of CAMHB followed by overnight incubation at 37˚C with shaking at 150 rpm. The next day the following concentrations (final) of BisEDT were added to the tubes containing the pre-formed biofilm:

Tube	BisEDT (final conc. μg/ml)
Reference	0
#1	5
#2	10

The final volume of all tubes was brought up to 300 μl with sterile de-ionized water, mixed well and incubated at 37˚C with shaking at 150 rpm for 1, 3 and 5 days. The removal of adherent bacterial cells and enumeration was done as previously described.

Determination of treatment effects: Treatment effects were determined by the relative eradication/killing of pre-formed biofilm (expressed as a mean percentage):

$$100 - [(\text{CFU of treated bead} \div \text{CFU of reference bead}) \times 100]$$

The CFUs generated by the untreated/reference bead served as the reference inoculum for that set of experiments.

Method #2: Spectrophotometric assay

Adherence assay: Culture tubes with polystyrene beads were inoculated and incubated under conditions described in method #1. 1, 5 and 10 μg/ml final concentrations of BisEDT were added to the existing biofilm-coated beads. Following incubation, removal of non-adherent cells, biofilm staining and solubilizing was done as described in study #1.

Determination of treatment effects: The resulting solution was quantified by measuring the OD at 570 nm using an UV spectrophotometer. An uninoculated/untreated bead was used as the blank.

Examination of bacterial antagonism: 15 µl aliquots of RP62A and *P. mirabilis* were used to inoculate polystyrene beads and incubated overnight as previously described, but without the addition of BisEDT. Following incubation, the adherent cells were released, serially diluted and plated on corresponding media as described previously. Bacterial antagonism exhibited by one organism on the other was calculated as a mean percentage:

$$100 - \left[\left(\underset{\text{with both organisms}}{\text{CFU of bead inoculated}} \div \underset{\text{with one organisms}}{\text{CFU of bead inoculated}}\right) \times 100\right]$$

RESULTS

MIC determinations. The MIC of BisEDT against planktonic *S. epidermidis* RP62A was 0.1 µg/ml and 0.5 µg/ml against planktonic *P. mirabilis* (Table 1 and Table 2).

<u>Inhibition/Prevention of biofilm formation</u>

Polystyrene bead assay. Both 0.1 and 0.5 µg/ml of BisEDT inhibited *S. epidermidis* biofilm. Biofilm inhibition by 0.1 µg/ml was 90.6%(±5.6), 69%(±13.9) and 99.6%(±0.3) at 1, 3 and 5 days respectively. At 0.5 µg/ml, 97.1%(±2.3), 97.5%(±2.2) and 99.9%(±0.01) inhibition was seen at 1, 3 and 5 days, respectively (Table 3, Figure 1). However, 0.5 µg/ml was not statistically

different than 0.1 μg/ml (*P* > 0.05). *Staphylococcus epidermidis* biofilm inhibition when coexisting as part of a dual species biofilm with *P. mirabilis* was 65.1%(±3.5), 48.6%(±11) and 85.4%(±7.5) at 0.5 μg/ml of BisEDT at 1, 3 and 5 days of exposure. Inhibition at 1 μg/ml was 75.6%(±6.7), 93.4%(±6.5) and 99.9%(±0.06) at 1, 3 and 5 days, respectively. At 5 μg/ml inhibition was 99.5%(±0.3), 99.6%(±0.6) and 99.9%(±0.005) at 1, 3 and 5 days (Table 4, Figure 2). At day 1, 5 μg/ml was statistically more effective than 0.5 or 1 μg/ml (*P* < 0.05). At day 3, 5 μg/ml was significantly better than 0.5 μg/ml (*P* < 0.05), but not 1 μg/ml (*P* > 0.05). By day 5, both 0.5 and 1 μg/ml were equally as effective as 5 μg/ml (*P* > 0.05).

Single species *P. mirabilis* was inhibited 21%(±9.3), 52.4 %(±5) and 54.6%(±11.8) at 1, 3 and 5 days, respectively at 0.5 μg/ml. At 1 μg/ml the inhibition of biofilm formation was 99.7%(±0.1), 60.9%(±12.2) and 75.8%(± 9.4). At 5 μg/ml, inhibition remained at 99.9% through out the 5 days (±0.8, 0.3 and 0.04 for 1, 3 and 5 days, respectively; Table 5, Figure 3). At day 1, 5 μg/ml was significantly better than 0.5 μg/ml (*P* < 0.05), but not 1 μg/ml (*P* > 0.05). At day 3, 5 μg/ml treatment was most effective (*P* < 0.05). At day 5, 5 μg/ml was statistically better than 0.5 μg/ml (*P* < 0.05), but no significantly different than 1 μg/ml (*P* > 0.05). This bacteria in a mixed biofilm with *S. epidermidis* was inhibited by 50.5%(±4.5), 47.9% (±6.7) and 81.9%(±10.1) at 1, 3 and 5 days at 0.5 μg/ml. At 1 μg/ml, 90.8%(±1.8), 77.3%(±7.7) and 99.2%(±0.6) of inhibition was observed. At 5 μg/ml, 95.9%(±1.7), 98.8%(±0.8) and 99.9%(±0) inhibition was seen over the same time period

(Table 6, Figure 4). At days 1 and 3, 5 μg/ml treatment proved to be the most effective ($P < 0.05$). However, at day 5, 5 μg/ml was not statistically better than the other lower concentrations ($P > 0.05$).

Agar disk diffusion assay. An increase in the zone of inhibition was seen with the increase in BisEDT concentration. *S. epidermidis* inhibition was 15 mm(±0.4) at 0.1 μg, 17 mm(±0.8) at 0.5 μg, 20 mm(±0.2) at 1 μg, 21 mm (±0.3) at 5 μg and 24 mm(±0.2) at 10 μg of BisEDT. No inhibition of *P. mirabilis* was seen at 0.1, 0.5 and 1 μg of BisEDT. 8 mm (±0.1) and 10 mm(±0.2) inhibition zones were seen at 5 and 10 μg, respectively (Table 7, Figure 5).

Spectrophotometric bead assay. The absorbance at OD_{570} of the reference (untreated) *S. epidermidis* bead was 1.62(±0.3). When treated with 0.1 μg/ml of BisEDT, the absorbance was 1.64(±0.3). The absorbance was 1.62(±0.2) at 0.5 μg/ml of treatment, 1.35(±0.3) at 1 μg/ml and 1.26(±0.2) at 5 μg/ml (Table 8, Figure 6).

For *P. mirabilis*, absorbance at OD_{570} of the reference/untreated bead was 1.67(±0.2), 1.44(±0.3) when treated with 0.1 μg/ml of BisEDT, 1.73(±0.3) at 0.5 μg/ml, 1.26(±0.2) at 1 μg/ml, 1.58(±0.2) at 5 μg/ml and 1.1(±0.2) at 10 μg/ml of the drug (Table 8, Figure 6).

Eradication/Killing of biofilm

Polystyrene bead assay. There was no statistical difference between BisEDT concentrations 5 μg/ml and 10 μg/ml for both *S. epidermidis* and *P. mirabilis*