Biofouling and Anti-Fouling of Medical Devices

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Abstract

Biofouling of medical devices involves the formation of a biofilm by bacteria. The infecting bacteria produce extensive exopolysaccharides to form a confluent biofilm. Bacteria within a biofilm can be 1,000 times more resistant to antimicrobials than their planktonic counterparts and less susceptible to host immune systems. According to an estimate by the NIH (USA), about 80% of all human bacterial infections involve biofilms. Over 45% of all hospital-acquired infections in the U.S. (>2 million/year) are device-related. Therefore, development of anti-infective products possessing both antibiofilm and antimicrobial activity is of utmost importance for the effective control of biofouling in clinical settings. We have developed the following unique synergistic antimicrobial compositions with antibiofilm activity for coating indwelling medical devices and disinfecting dental unit waterlines:

(i) A combination of protamine sulfate (PS) and chlorhexidine (CHX) has shown a significant synergistic antimicrobial and antibiofilm activity against nosocomial infection associated Escherichia coli, Pseudomonas aeruginosa, Staphylococcus epidermidis, Staphylococcus aureus, Enterococcus faecalis, Klebsiella pneumoniae, Enterococcus faecium, Proteus mirabilis and Candida albicans. Protamine sulfate enhances the activity of CHX by increasing the permeability of cell membrane and inhibits as well as moderately disperses biofilms;

(ii) N-acetyl-D-glucosamine-1-phosphate acetyltransferase (GlmU) is involved in the biosynthesis of lipopolysaccharides in both gram-positive and gram-negative bacteria. An antimicrobial composition comprising GlmU inhibitor (GUI) and PS is effective against catheter-associated uropathogens, including E. coli and P.
P. aeruginosa. The GUI interferes with cell wall formation and exhibits antimicrobial activity. PS enhances GUI activity;

(iii) The composition comprising ovotransferrin (OT), PS and EDTA is effective against the growth and biofilm formation of both gram-positive and gram-negative bacteria associated with medical devices. While chelating agents OT and EDTA sequester iron and magnesium, calcium plus iron, respectively and thus making them unavailable for bacterial growth, the PS increases membrane permeability;

(iv) The synergistic combination of a broad-spectrum antimicrobial triclosan (TCSN) and DispersinB® (DspB), an antibiofilm enzyme, has antimicrobial and antibiofilm activity against vascular catheter associated bacteria. DspB inhibits as well as disperses biofilms and thus makes the CVC-associated bacteria more susceptible to TCSN killing;

(v) The combination of sodium bicarbonate (SB), sodium metaperiodate (SMP) and SDS is effective in reducing the growth as well as biofilm formation in dental unit waterline (DUWL)-associated microorganisms such as Legionella pneumophila, P. aeruginosa, S. aureus, K. pneumoniae, C. albicans, Mycobacterium avium and Mycobacterium chelonae. The biofilm dispersal activity of SMP makes biofilm-embedded bacteria and yeasts more susceptible to the antimicrobial action of SDS and SB.

1. INTRODUCTION

Biofouling involves the formation of biofilm by microorganisms. Biofilms can develop on solid surfaces under conditions which facilitate microbial growth thus biofilms are ubiquitous in nature [1]. Biofilms may form on a wide variety of surfaces such as living tissues, indwelling medical devices industrial or potable water system piping or natural aquatic systems [2]. The first observed biofilm was unknowingly seen by Van Leeuwenhoek as bacteria growing on tooth surfaces [2]. Heukelekian and Heller [3] observed this same phenomenon in the marine environment and Zobell [4] observed this in seawater.

The development of a microbial biofilm involves three main steps; irreversible attachment of bacteria to a surface, development of biofilm by attached bacteria and detachment of single cells or large pieces from the biofilm [1, 5, 6, 7, 8]. Production of extracellular polymer substances (EPS) by bacteria in the biofilm is an important factor for biofilm maturation. The EPS includes polysaccharides, proteins and nucleic acids [8]. The EPS of biofilm can house noncellular materials such as mineral crystals, corrosion particles, clay, silt, blood, protein, nucleic acid as well as host factors such as fibrin, fibronectin depending on the environment in which the biofilm has developed [6, 8, 9, 10]. The physical properties of the biofilm are largely determined by the EPS while the physiological properties are determined by the bacterial cells [1]. Biofilm microorganisms can be distinguished from its planktonic counterparts by the fact that they are associated with a surface, high population density, presence of extracellular polymer slime matrix, a wide range of physical, metabolic and chemical heterogeneities and also by their gene transcription profile [1, 2].

Biofilms cause a wide range of problems in both the health and industrial sectors. Attachment and colonization of microorganisms on biomaterial surfaces poses a risk of local and systemic infections in patients [11, 12]. Microbial cells living in biofilms are much more difficult to eradicate than their non-biofilm counterparts [1]. Biofilm embedded micro-
organisms are highly resistant to antimicrobial treatments and less susceptible to the hosts’ immune system [6]. Biofilm associated microorganisms may elicit disease processes by detachment of individual or aggregates of cells from the device surface, production of endotoxin and providing a niche for the development of antibiotic-resistant organisms [6, 7]. It has also been suggested that the negatively charged exopolysaccharide is very effective in protecting bacterial cells from cationic antibiotics by restricting their permeation [13]. The suboptimal efficacy of antimicrobial agents against biofilm-embedded organisms may be attributed, at least in part, to the relatively slow bacterial growth within the biofilm, inhibition of antimicrobial activity by exopolysaccharide material, and poor penetration of some antimicrobial agents into the biofilm matrix. [14, 15]. The insensitivity of biofilm bacteria to antibiotics is a function of cell wall composition, surface structure, and phenotypic variation in enzymatic activity [16, 17]. This insensitivity of biofilm bacteria to antibiotics also seems to be attributed to four major mechanisms of action: slow antimicrobial penetration, the stress response, substrate limitation and the presence of persister cells [18].

Biofilms on indwelling medical devices such as catheters may be composed of gram-positive or gram-negative bacteria or yeasts. The organisms that commonly contaminate and develop biofilms on central venous and urinary catheters are Enterococcus faecalis, Staphylococcus epidermidis, S. aureus, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa and Candida albicans [6]. It has been shown that more than 25 different bacterial species, fungi and protozoa can colonize on medical devices such as dental unit water lines [19]. The majority of these microbes are the same gram-negative bacterial species that survive in small numbers in municipal water systems [19, 20]. Pathogens such as Pseudomonas aeruginosa, Legionella pneumophila, Mycobacterium spp., Candida spp., Actinomyces spp., and Fusobacterium spp. have been recovered from dental unit water, so it is evident that these medical devices provide a supportive environment for opportunistic pathogens [21, 22, 23, 24, 25, 26].

Despite the fact that device related infections are the most common and serious complication, they constitute an increasingly essential component of modern healthcare. The increasing use of long-established and new medical devices has further magnified the problem of device-associated infections in patients, particularly in those who are inherently at high risk of infection [27]. Device-associated infections that are generally cumbersome to manage, expensive to treat and necessitate extended stays in hospitals have great impact on the quality of life [28, 29, 30, 31]. There are over two million hospital-acquired infections in the United States each year, of which 80% are associated with indwelling medical devices. The urinary catheter is the most commonly utilized medical device as well as the second most common cause of nosocomial infections which are associated with a relatively high rate of colonization and infection [15, 32, 33]. More than 900,000 episodes of catheter-associated urinary tract infections occur annually in acute-care hospitals in the United States, accounting for 40% of all nosocomial infections and involving between 10 and 30% of patients with indwelling urinary catheters [34]. Catheter-associated urinary tract infection prolongs the hospital stay between an estimated 2.4 and 4.5 days, with resultant increased healthcare costs [35, 36]. Most cases of bloodstream infection arise from vascular catheters of which over 250,000 episodes of nosocomial bloodstream infection occur each year in the United States and are associated with a mortality rate between 12 and 25% with a treatment cost of approximately $25,000 per survivor [30, 37]. Vascular catheter-bloodstream infection can
also result in disseminated infection; for instance, one-third to one-half of episodes of nosocomial endocarditis has been linked to infected intravascular catheters [38].

Dental unit water system commonly used to irrigate the oral cavity during dental treatment is another medical device which is prone to biofouling. Despite the lack of epidemiological evidence of infectious outcomes associated with DUWL biofilms, concerns about the microbial colonization of DUWL have led to the development of some guidelines for reducing the bacterial numbers in DUWL. In the United States, the American Dental Association (ADA) and the Centres for Disease Control and Prevention have proposed a guideline for dental unit water of < 200 colony forming units (CFU)/ml [19]. Martin [39] showed that abscesses caused by two different strains of *P. aeruginosa* in two immunocompromised patients had the same pyocin types as isolates recovered from the dental unit water, suggesting that the dental unit water was the source of the infections. Although *P. aeruginosa* is not a normal resident of the oral cavity, Martin [39] isolated this bacterium from the oral cavities of 78 healthy patients for 3–5 weeks following exposure to the dental unit water contaminated with *P. aeruginosa*. Atlas *et al.* [21] reported the death of a dentist from California resulting from Legionnaire’s disease, possibly due to exposure to dental unit water contamination with *Legionella* sp.

According to an estimate by the National Institute of Health (NIH, USA), about 80% of all human bacterial infections are caused by biofilms [40]. Biofilms can put patients at risk for local and systemic infectious complications including local site infection, catheter-related bloodstream infection and endocarditis [8]. These facts explain why there is a pressing need to effectively prevent catheter-related infections. The serious medical complications, problematic management and economic sequelae of device-related infections have prompted a keen interest in exploring innovative preventive approaches [41]. In the last decade, several strategies, including using topical antimicrobial ointments, minimizing the length of time of catheterization, using catheters provided with a surgically implanted cuff [42], and coating the catheter lumen with antimicrobial agents [43, 44, 45, 46, 47, 48] have been suggested to control biofilm growth on medical devices.

Coating medical devices with different antimicrobial agents with variable success is one of the many strategies that have been proposed to prevent bacterial adherence to the catheter surface. It has been reported that antibiofilm compounds such as *N*-acetylcysteine, ethylenediaminetetraacetic acid (EDTA) [49], ovotransferrin, lactoferrin [50], proteolytic and polysaccharide degrading enzymes, RNAIII inhibitory peptide (RIP) [51], sodium metaperiodate [52] and gallium [53] improve the action of antimicrobial agents when used in combination [54]. Therefore, there is a need to develop antibiofilm-antimicrobial activity compositions that are broad-spectrum, environmentally friendly, medically acceptable, and relatively economical to manufacture on a commercial scale for preventing biofilm formation and fouling of biomedical devices.

### 2. Current Methods of Anti-Fouling

The current methods that are being used for anti-fouling on catheters and DUWLs are discussed in this section.
2.1. Urinary Catheter

There are three types of urinary catheter: uncoated, polymer coated, and antimicrobial coated. The uncoated urinary catheter is made of latex, silicone or a combination of latex/silicone. Latex catheters are not suitable for individuals who are sensitive or allergic to latex. Silicone coated or 100% silicone catheters prove to be safer than latex, but are more expensive. The polymer coated catheter has a layer of polymer that is bound to the catheter surface that can absorb water to create a thick, smooth, and a slippery surface. These types of catheters (uncoated and polymer coated) rely on the catheter surface to slow down biofouling or biofilm formation. The antimicrobial catheters currently in use are either coated with Nitrofurazone (ReleaseNF catheter; Rochester Medical Stewartville, MN, USA) or silver (BARDEX® I.C. Foley Catheter; C. R. Bard, Murray Hill, NJ, USA). The nitrofurazone coated catheter releases the drug to the surrounding aqueous environment of the urethral tract. The nitrofurazone coated catheter has shown a 10-fold reduction in bacteria related to catheter-associated urinary tract infection (CAUTI) at 3 days and a 6-fold reduction at 5 days [55]. This catheter has been effective against a broad spectrum of bacterial pathogens known to cause urinary tract infections. The silver alloy coated catheter is anti-infective and proven to reduce the average CAUTI by 25% to 47% in over 30 clinical trials [33, 56, 57]. The catheters coated with silver based hydrogels gradually release the silver ions from the matrix to the surrounding and interfere with the integrity, metabolism, and reproduction of bacteria. The antimicrobial catheters rely on the antimicrobial nitrofurazone or silver to inhibit bacterial growth both on the catheter surface and surrounding area.

Among other coating technologies that has potential for medical use; a silicone catheter coated with hydrogel containing bacteriophages specific to S. epidermidis [58] has shown significant reduction in biofilm formation in an in vitro model system. Biofilm formation on both hydrogel-coated and serum/hydrogel coated silicone catheters were significantly reduced over 24 hrs in the presence of phage 456. A liposomal/ciprofloxacin hydrogel coating [59] has also shown reduction in biofilm formation. An acoustic-wave-activated Foley Catheter [60] which works by emitting low-energy surface acoustic waves at the catheter surface is repulsive to bacteria and prevents initial phases of microbial biofilm development.

2.2. Central Venous Catheter

There are two types of central venous catheters; the short term and long term. While the short term central venous catheters (STCVC) are used for nutrition therapy, delivering multiple medications simultaneously and additional patient monitoring, the long term central venous catheter (LTCVC) are used for administration of nutrition and medication, and for chemotherapy. Central venous catheters are also used for haemodialysis. The catheters coated with antimicrobial composition that are currently on the market contains silver sulfadiazine combined with chlorhexidine (ARROWg+ard Blue and ARROWg+ard Blue PLUS, Arrow International Inc., Reading, PA), oligon agent which is a combination of natural silver, platinum metals, and black carbon (Vantex; Edwards Lifesciences, Irvine, CA), benzalkonium chloride and heparin (AMC Thromboshield; Edwards Lifesciences, Irvine, CA) and minocycline combined with rifampin (COOK SPECTRUM®; Cook Medical Inc., Bloomington, IN). While Arrowg+ard Blue catheter has coating only on the external surface,
ARROWgard Blue Plus has coating on both the internal and external surfaces. A randomized clinical trial has shown that ARROWgard Blue catheters are 50% less likely to be colonized and 80% less likely to produce a bloodstream infection than the uncoated catheters. Neither any adverse effects of using antimicrobial catheter nor any in vitro resistance to chlorhexidine and silver sulfadiazine by isolates from infected catheters were reported [55]. ARROWgard Blue Plus catheters showed in vitro durability for more than 7 days against CVC associated pathogens such as Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Enterobacter cloacae, and Candida albicans.

Edwards Life Sciences produces the Vantex and the AMC Thromboshield. Vantex catheters have both their internal and external surfaces coated with the oligon agent containing silver, a natural antimicrobial. Patients showed a decreased allergic response to Vantex catheters then to those coated with silver sulfadiazine and chlorhexidine combination. Prospective randomized trials performed at ten institutions showed that Vantex catheters significantly reduced catheter colonization rate and catheter related infections by 48% [61]. Both the internal and external surfaces of AMC Thromboshield catheters are coated with benzalkonium chloride combined with heparin. Benzalkonium chloride provides antimicrobial properties while heparin reduces thrombus formation. In vitro studies performed on AMC Thromboshield catheters showed durability for 8 days against catheter associated pathogens such as S. aureus, S. epidermidis, and Streptococcus faecalis. Cook Spectrum catheter (COOK Company) which has its internal and external surfaces coated with minocycline and rifampin antibiotic combination works synergistically to provide broad-spectrum activity against bacteria and yeasts. Clinical trials indicate that Cook Spectrum catheters were twelve fold less likely to produce catheter-related bloodstream infection and three fold less likely to be colonized when compared to ARROWgard Blue catheter [44].

2.3. Dental Unit Water Line (DUWL)

There are a plethora of automated flushing systems, filters, water disinfectants, independent bottle water systems and even fully detachable autoclavable DUWL on the market [19, 20]. Inactivation of microorganisms and/or detachment of biofilms are the objectives of the majority of studies that have been conducted to treat waterlines. Chemicals may be introduced either intermittently or continuously. The major advantage of intermittent chemical use is that the active agent can be purged from the system before patient treatment. The disadvantages include the potential rebinding of microorganisms surviving in biofilms back on the waterline, staff exposure to chemicals, and adverse effects on metal, rubber and synthetic dental unit components. Continuous treatment reduces the likelihood of recolonization of waterlines but may cause damage to the equipment. The chronic health effects due to constant exposure of the health care workers to the cleaning agent must be seriously considered [20]. It is also possible that enamel and dentin bond strength of dental adhesive material may also be affected [62].

The extensive use of chlorine or sodium hypochlorite for cleaning can produce carcinogens like tri-halomethanes by reacting with biofilm organic polymers, corrode the metal components, and damage rubber and synthetic materials. Other agents being used for cleaning DUWLs include chlorhexidine gluconate, hydrogen peroxide, iodophors and commercial mouthrinses [20]. Controlling microbial contaminations by flushing the DUWLs
with chemical agents alone only reduces the number of organisms suspended but does not guarantee predictable and effective removal of adherent biofilm [20]. The use of micropore membrane filters for removal of microorganisms from water and solutions in DUWLs is another option that has not received much attention. The controlled conditions and sterility needed for these systems is impractical in dental theatre settings. Filtration methods can reduce or eliminate chemical usage, damage to dental unit’s components and staff exposure to chemical residues. However, use of filters cannot eliminate biofilm growth in prefiltration segments of waterlines [20].

Some of the current products on the market for cleaning DUWLs contain compounds such as chlorine dioxide, silver salt, sodium citrate, proprietary iodine formulation, sodium percarbonate, silver nitrate and cationic surfactants, ethanol and chlorhexidine, glycerin and chlorhexidine gluconate, alkaline proxygen with phase transfer catalyst, botanical extract from citrus and sodium chlorite. The filter/pump systems that use filters to purify the water supply have active compounds such as elemental iodine ionized silver resin and electrolyte solution, and generator to produce hypochlorous acid. Sterisil, Inc. has 7 products on the market: CitriSil™, Sterilex®Liquid Ultra, Sterilex®Ultra Powder, PureLine50, PureTube™ BR360 Cartridge, PureTube PLUS™, Sterilox™ (www.sterisil.com). Pelton and Crane have 5 products on the market: Teamvista™ Waterline Cleaner Combo, VistaClean™ Irrigant Solution concentrate, Vista DayTab™ Waterline Irrigant Tablets, VistaClear™ Integrated System #2970, VistaClear™ Waterline Treatment System (www.pelton.net)

3. BIOFILMS IN BIOFOULING

The process of biofilm formation of urinary catheters, central venous catheters and dental unit water lines are discussed in this section. The development of biofilms on devices is a multi-step process which involves the device, bacteria and the host [5, 27]. The medical device prior to being used is a clean, sterile device often made of various polymers also called biomaterial. Immediately following the device being implanted it becomes surrounded by the bodily fluids such as blood, urine, saliva and mucus. The types of components that adhere to the device depend on the surface characteristics such as the surface chemistry, charge and hydrophobicity. Components from the bodily fluids interact with the surface of the device to form a conditioning film. The conditioning film is vital to the bacteria as they are not able to adhere directly to the implant. In the case of urinary catheters the presence of sodium or magnesium ions in urine could act as a bridge between the negatively charged conditioning film and the negatively charged microbial surface thereby facilitate the approach and attachment of microorganisms [5]. For solutions such as urine that is high in ionic strength, the electrostatic repulsion may be completely balanced, allowing bacteria to irreversibly adhere to the conditioning surface [5].

Biofilms on medical devices may be composed of bacteria or yeast which originates from the skin of patients, healthcare workers, tap water or other environmental sources where the device is being used. Bacteria commonly associated with medical devices include the gram-positive Enterococcus faecalis, Staphylococcus aureus, S. epidermidis and Streptococcus viridans and the gram-negative E. coli, Klebsiella pneumoniae, Proteus mirabilis and P. aeruginosa. Biofilms on urinary catheters may be composed of a single or multiple species of
microorganisms. Biofilm development generally starts with a single species and becomes multispecies with time. The bulk of biofilm structure is made up of polysaccharides which are produced by bacteria. The polysaccharides act as a filter to retain minerals and host produced serum components and also make the biofilm highly resistant to antimicrobials. The longer the catheter remains in place the greater the tendency for biofilm development. The adhesion of bacteria is dependent on the surface properties of the catheter and the hydrophobicity of the bacteria. Catheters displaying both hydrophobic and hydrophilic regions are prone to be colonized by a wide variety of organisms. Urease producing organisms such as *Proteus mirabilis* hydrolyze urea in urine to ammonium hydroxide which causes an increase in pH and precipitation of minerals at the biofilm urine interface. These mineral containing biofilms form encrustations that can completely block the inner lumen of the catheter [6].

Patient skin, health care workers and contaminated infusates are found to be the sources of biofilm-forming organisms on central venous catheters. Microbes gain access to the catheter by migrating externally from the skin along the exterior catheter surface or internally from the catheter hub or post and colonize rapidly. It has been reported that short term (<10 days) catheterization developed greater biofilm on the external surface whereas long term (30 days) catheterization developed more biofilm on the catheter inner lumen. Dariouche [27] reported that the fluid going through the catheter also has an effect on the biofilm formation. In addition to general factors, some specific factors such as adhesions and host factors specific to certain bacterial species help to form a biofilm. In the case of *S. epidermidis*, the cells rapidly attach to a device surface by non-specific factors such as surface tension, hydrophobicity and electrostatic forces or by specific adhesions. After this initial attachment, bacteria adhere to each other to form the biofilm which is mediated by polysaccharide production. In the case of *S. aureus* adhesion appears to be more dependent on the presence of host tissue ligands such as fibronectin, fibrinogen and collagen. Bacteria adhere to these factors via surface components. Also, the device itself may have particular characteristics which help biofilm development. The same bacterial species adhere differently to different device materials and also different bacteria species adhere differently to the same device material. The same device material with properties (e.g. hydrophobic, hydrophilic) and placed under different conditions (flow type and temperature) causes the same bacteria to adhere differently. Not only are the parameters of the device and the bacteria a challenge but the fact that the *in vitro* testing does not insure anti-infective efficacy *in vivo*. This fact compounded with the clinical benefit of a particular surface modifying approach may vary between applications all factor into device design [27]. Thus there are a multitude of factors to be considered when designing, testing and implementing a new coating technology. However, the factors affecting the biofouling and biofilm development of DUWLs are slightly different than that of the catheter applications. The materials commonly used to deliver water to dental hand pieces and air/water syringes are excellent substrates for the attachment of bacteria and proliferation of biofilm. While most organisms recovered from dental units are water inhabitant bacterial species that have demonstrated little potential to cause disease in immunocompetent hosts, potential pathogenic species recovered are believed to have reached dental water systems from outside [19, 20]. *Pseudomonas* and *Legionella* are the most significant pathogenic organisms of concern. *Pseudomonas aeruginosa*, the causal agent of pneumonia in hospitalized patients, is associated with a wide range of opportunistic infections. It is highly resistant to disinfectants such as chlorhexidine and iodophors, and also thrives in low nutrient environments such as distilled water. *Burkholderia*, a species related to
Pseudomonas, is also associated with pneumonia in cystic fibrosis patients. Legionella pneumophila, an intracellular parasite of protozoa, that causes Legioniernes’ disease and a related condition known as Pontiac fever are ubiquitous in the environment and found in all types of water. Aquatic non-tuberculous Mycobacterium species associated with pulmonary disease and opportunistic wound infections also have been recovered in DUWLs [19, 20]. Endotoxin has been detected in dental water with reported mean counts of 80 endotoxin units/ml which is far exceeding the 0.25 units/ml limit set by USA Pharmacopoeia for irrigation sterile water. The bacterial cell wall of gram-negative bacteria is a potent source of endotoxin. Endotoxin can cause localized inflammation, fever and shock [19, 20].

Minerals particularly calcium carbonate in drinking water deposit on water bearing surfaces and subsequently organic molecules (carbon) concentrate on these hydrophobic surfaces and promote adhesion of bacteria suspended in municipal water system. Biofilms are also present in the water mains and lines that link the faucet to the municipal water system. Surface/volume ratios explain why dental unit water is more contaminated than faucet water. The volume of water line tubing in most dental units is somewhat less than 100 ml, if there is 100 ml volume of water in a 10 inch diameter tube, there is only 4 inches of tube surface available for biofilm formation. This same 100 ml volume in a 1/16” diameter tubing now has more that 400 square inches of surface available for colonization [20]. The pattern of flow of fluid through narrow bore tubing known as laminar flow causes the tubing surface to exert frictional force, and slow down the movement of fluids until flow at the surface is stabilized. This creates an environment conducive for biofilm formation. At the busiest times of the day, the flow rate in a dental hand piece is between 2 and 10 milliliters per minute. During the stagnant periods like weekends and evenings, chlorine in standing water rapidly dissipates and allows rapid colonization. (www.micrylium.com).

4. ANTIBIOFILM TECHNOLOGIES

Kane Biotech has developed and tested a range of broad-spectrum anti-infective compositions which can be used to coat medical devices to prevent the formation of biofilm and remove preformed biofilm. These synergistic combinations have both antibiofilm and antimicrobial activity. One of the combinations has possible applications as a dental unit water line disinfectant, catheter lock solution or for cleaning food industry and paper mill equipment. This section details the in vitro and some in vivo results of testing the synergistic combinations against common medical device associated bacteria.

4.1. Protamine Sulfate and Chlorhexidine (PS/CHX) Combination

The PS/CHX combination is a formulation that uses PS which presumably impairs bacterial biofilms by binding to polysaccharides and disrupting the microbes’ hydrophilic surface coat [63] together with CHX which has a broad-spectrum antimicrobial activity [64].
4.1.1. Protamine Sulfate (PS)

PS (CAS# 53597-25-4, Synonyms: Salmine sulfate) with the amino acid sequence “mprrrsr prrrrr srssrrgr rrr” (NCBI accession # P69014) is an FDA-approved compound for human use. The use of PS in the neutralization of heparin is well known and well established. Heparin is used to prevent blood clots from forming. PS is given when there is excessive bleeding following the administration or accidental overdose of heparin [65, 66]. The safety of PS has been demonstrated over time as it has been successfully used as an intravenous injection for almost 50 years. It is currently marketed by Eli Lily. PS has antimicrobial activity against a range of gram-positive and gram-negative bacteria, yeasts and moulds. PS works by altering the permeability of the microbial cell membrane and the dilation of ion channels, which in turn facilitates the transport of antimicrobial compounds to the cytoplasm [67, 68]. In addition, antimicrobial activity of PS is presumably due to electrostatic attraction between the positively charged molecule and the negatively charged cell envelop, and appropriate concentrations cause growth inhibition or cell death due to the leakage of K+, ATP and intracellular enzymes [69]. Furthermore, PS has been reported to prevent *Staphylococcus* sp. adhesion to stainless steel [70]. PS has been shown to enhance both *in vitro* and *in vivo* antimicrobial as well as antibiofilm activities of antibiotics and non-antibiotic compounds [47, 63, 71, 72, 73, 74].

4.1.2. Chlorhexidine (CHX)

CHX is an FDA-approved compound for human use with the Molecular Formula C\textsubscript{22}H\textsubscript{30}Cl\textsubscript{2}N\textsubscript{10}, Melting point 134°C, Mol. Wt: 505.46 and CAS# 55-56-1 for chlorhexidine base. It has broad-spectrum antimicrobial and antiviral activity [64] that is partly attributed to the disruption of the cellular plasma membrane [75]. CHX is a membrane-active agent, causing protoplast and spheroplast lysis. At higher concentrations, it causes precipitation of proteins and nucleic acids [76]. The safety of CHX has also been demonstrated over time since it has been used as a topical disinfectant since mid-1970. CHX is an effective broad-spectrum antimicrobial agent and can be found in oral care products such as mouth rinses and toothpastes (Peridex, Periochip, PerioGard, Chlorohex, Savaco, etc.), antiseptic skin creams and disinfectants used to prepare the skin for surgical procedures (Oronine, Avagard, Hibiclens, Hibiscrub, ChloraPrep, and Exidine). It is also a component of the famous household antiseptic Savlon. CHX has been demonstrated to successfully prevent clinical infections associated with catheters [55]. Currently there are numerous medical devices on the market that have CHX as their active ingredient. For example, CHX in combination with silver sulfadiazine is successfully used for coating central venous catheters (Arrow International, PA, USA) [77]. ARROWg+ard Blue central venous catheters are externally surface coated with chlorhexidine acetate and silver sulfadiazine. Compared to the original ARROWg+ard Blue catheters the ARROWg+ard Blue Plus external surface treatment represents a three-fold increase in the amount of chlorhexidine acetate and an unchanged amount of silver sulfadiazine. In the early 1990’s, the FDA cleared the following three medical devices containing CHX: (i) intravenous catheters (ARROWg+ard Blue and ARROWg+ard Blue Plus), (ii) topical antimicrobial skin dressings (BIOPATCH and BACTIGRAS), and (iii) an implanted surgical mesh (GORE DUALMESH® PLUS) [FDA Public Health Notice http://www.fda.gov/cdrh/chlorhex.html].
4.1.3. PS/CHX Combination Test Results

The PS/CHX combination was initially tested using the minimal inhibitory concentration (MIC) assay for its antimicrobial effect on nosocomial infection associated uropathogens. The PS/CHX combination was more effective against *E. coli*, *K. pneumoniae*, *S. aureus*, and Vancomycin resistant *E. faecium* than the individual compounds [78]. The PS/CHX combination was then tested using an *in vitro* biofilm assay. The combination had a synergistic inhibitory effect on biofilm formation of *E. coli*, *P. aeruginosa* and *S. epidermidis*. Further, the PS/CHX combination was used to coat urinary catheters. The *in vitro* antimicrobial activity of PS/CHX coated urinary catheters was studied using the zone of inhibition assay. The catheters coated with PS/CHX combination were durable for 7 days against *E. coli*, *K. pneumoniae*, *S. epidermidis*, *S. aureus*, *E. faecalis* and *C. albicans*. Furthermore, the *in vitro* durability was also tested in artificial urine for 7 days. The PS/CHX-coated catheters were able to prevent ≥ 80% of *E. coli* and *S. epidermidis* colonization after 7 days.

In addition, the *in vivo* efficacy of PS/CHX combination on coated catheters was compared with control uncoated catheters and commercially available silver-hydrogel catheters using a rabbit model of *E. coli* infection. Two out of 28 (7%) PS/CHX coated catheters, 25/28 (89%) silver-hydrogel coated catheters, and 18/28 (64%) uncoated catheters became colonized with *E. coli*. The PS/CHX coated catheters were significantly less likely to be colonized than either silver hydrogel-coated catheters or the uncoated catheters. Furthermore, 1/28 (4%) PS/CHX coated catheters, 12/28 (43%) silver hydrogel-coated catheters, and 14/28 (50%) uncoated catheters resulted in infection due to *E. coli*. The PS/CHX coated catheters were significantly less likely to cause infection than either silver hydrogel-coated catheters or uncoated catheters.

4.2. GlmU Inhibitor and Protamine Sulfate (oPDM/PS) Combination

*N-acetyl-D-glucosamine-1-phosphate acetyltransferase* (GlmU) enzyme is involved in the synthesis of activated nucleotide sugar UDP-GlcNAc, a precursor for synthesis of β-1,6- *N-acetyl-D-glucosamine polysaccharide adhesin* that is required for biofilm formation in *E. coli* and *S. epidermidis* and bacterial cell wall synthesis. This enzyme could provide a novel target for the development of antibiofilm agents. GlmU is a bifunctional enzyme with acetyltransferase and uridyltransferase activities. Its acetyltransferase activity is inactivated in the presence of thiol-specific reagents, such as iodoacetamide and *N*-substituted maleimides [79, 80]. GlmU inhibitors belong to a maleimide class of compounds that are quite reactive against sulfhydryl-containing enzymes [80] and have poor specificity, making them broad-spectrum antimicrobial compounds [81, 82]. In the recent past, GlmU enzyme inhibitors, which belong to a thiol-specific reagent group, were reported to inactivate bacterial pathogens [81, 82]. The GlmU inhibitors are iodoacetamide (IDA), *N*-ethylmaleimide (NEM), and the following maleimide analogs: *N,N*(1,2-phenylene) dimaleimide (oPDM), *N*(1-pyrenyl) maleimide (PyrM) and *N*-phenyl maleimide (NPM). The GlmU inhibitor and PS combination is a formulation that uses the maleimide analog oPDM which interferes in bacterial cell wall synthesis and biofilm formation and PS which disrupts the microbes’ hydrophilic surface coat [63].
4.2.1. GlmU Enzyme Inhibitors

IDA is an irreversible inhibitor of many cysteine proteases with the Molecular Formula \( \text{C}_2\text{H}_4\text{INO} \), Melting point 93-96°C, Mol. Wt: 185.0 and CAS #144-48-9. IDA is not highly specific for the active site cysteine residue of proteases and can inhibit many enzymes [81]. NEM is an enzyme that has quantitative reactions with thiol groups with the Molecular Formula \( \text{C}_6\text{H}_7\text{NO}_2 \), Melting point 45°C, Mol. Wt: 125.1 and CAS#128-53-0. NEM binds human haemoglobin and it has an effect on oxygen equilibrium [83], inactivation of isocitrate dehydrogenase by modification of cysteinyi residues [84], inhibition of galactosyl transferase [85] and yeast alcohol dehydrogenase [86]. NEM has been reported to be an inhibitor of numerous other enzymes [87]. oPDM is a maleimide analog with the Molecular Formula \( \text{C}_{14}\text{H}_8\text{N}_2\text{O}_4 \), Melting point 245-247°C, Mol. Wt. 268.2 and CAS#13118-04-2. PyrM is a maleimide analog with the Molecular Formula \( \text{C}_{20}\text{H}_11\text{NO}_2 \), Melting point N/A, Mol. Wt. 297.3 and CAS #42189-56-0. NPM is a maleimide analog with the Molecular Formula \( \text{C}_{10}\text{H}_7\text{NO}_2 \), Melting point 85-87°C., Mol. Wt. 173.2 and CAS #941-69-5.

4.2.2. oPDM/PS Test Results

Initially the GlmU inhibitors NEM and IDA were tested against \( \text{E. coli} \) P18 biofilm. It was observed that NEM was effective in reducing biofilm formation by 80%. After this initial result the NEM analogs (NPM, PyrM and oPDM) were tested and showed reduction in biofilm formation against catheter associated \( \text{E. coli} \), \( \text{K. pneumoniae} \) and \( \text{S. epidermidis} \) [71]. The addition of protamine sulfate to oPDM enhanced anti-biofilm activity and catheters coated with both compounds were virtually free from bacterial colonization. The visualization of oPDM/PS coating on \( \text{S. epidermidis} \) biofilm formation on silicone catheters was determined using confocal scanning light microscopy (CSLM). The confocal image showed uniform colonization on uncoated catheters, but the catheters coated with oPDM/PS showed little or no colonization [71].

4.3. Ovotransferrin, Protamine Sulfate and Ethylenediaminetetraacetic Acid (OT/PS/EDTA) Combination

The OT/PS/EDTA composition comprising a natural antimicrobial OT, PS with both antimicrobial and antibiofilm activity and EDTA, a chelating agent.

4.3.1. Ovotransferrin (OT)

OT also called conalbumin is a natural antimicrobial which is generally regarded as safe (GRAS) with the CAS #1391-06-6. The iron-sequestering glycoproteins, such as lactoferrin from milk and ovotransferrin (conalbumin) from egg white are iron-binding glycoproteins, which inhibit the growth of certain bacteria by making iron unavailable for bacterial metabolism (88).

4.3.2. Ethylenediaminetetraacetic Acid (EDTA)

EDTA is a chelating agent with a Molecular Formula (HOCOCH\(_2\))\(_2\)N(CH\(_2\))\(_2\)N(HOCOCH\(_2\))\(_2\), Melting point 220-240°C, Mol. Wt. 292.3 and CAS# 60-00-4. EDTA exerts its antimicrobial activity by depriving the micro-organisms from magnesium, calcium and iron
needed for growth and membrane stability. Furthermore, EDTA chelation of magnesium, calcium and iron enhances the detachment of cells from the biofilm, and also facilitates the killing of biofilm cells by chelating magnesium associated with lipopolysaccharides [89]. EDTA is a chelating agent used in a wide variety of food products to prevent oxidation and other deterioration reactions catalyzed by metal ions [72].

4.3.3. OT/PS/EDTA Test Results

Initially the OT, PS and EDTA alone and in combinations were tested against growth and biofilm formation on catheter associated pathogens. The OT/PS/EDTA combination showed a synergistic effect on *K. pneumoniae*, *P. aeruginosa* and *S. epidermidis* biofilm formation [74].

The OT/PS/EDTA combination was further tested by coating it onto clear vinyl catheters. The OT/PS/EDTA coated vinyl catheters had an inhibitory effect on biofilm formation showing a 79-95% reduction of *P. aeruginosa* and *S. epidermidis*.

4.4. Triclosan and DispersinB® (TCSN/DspB) Combination

The TCSN/DspB is a combination of a broad spectrum antiseptic TCSN, and DispersinB® antibiofilm enzyme.

4.4.1. Triclosan (TCSN)

TCSN also called Irgasan is a broad spectrum antiseptic with a Molecular Formula C_{12}H_{7}Cl_{3}O_{2}, Melting point 56-58°C, Mol Wt. 289.5 and CAS# 3380-34-5. TCSN is an antiseptic used in many household products; it possesses a broad-spectrum antimicrobial activity by inhibiting the activity of enoyl-acyl-carrier-protein (ACP) reductase enzyme involved in bacterial fatty acid biosynthesis [90, 91]. Triclosan has been reported to be safe for topical and surface-coating applications [92, 93, 94].

4.4.2. DispersinB® (DspB)

DspB is an antibiofilm enzyme, which inhibits and disperses biofilms of major bacterial pathogens. It was discovered by Dr. Jeffrey B. Kaplan, Department of Oral Microbiology, New Jersey Dental School, NJ, USA. The unique ability of this enzyme to specifically inhibit biofilm formation without affecting bacterial growth and to disperse preformed biofilms makes it a first of its kind antibiofilm enzyme. DspB is a naturally occurring N-acetylglucosaminidase enzyme produced by a periodontal disease-associated oral bacterium, *Aggregatibacter actinomycetemcomitans*. This 41 kDa enzyme consists of a single chain containing 361 amino acid residues, is a highly active and stable glycoside hydrolase that functions in a narrow pH range. It is readily soluble in water. Structurally, the bowl-shaped DspB molecule has a large central cavity, which exhibits a negative electrostatic potential. It consists of a single domain and can further be subdivided into substrates. The major structure (residues 1-60 and residues 91-358) folds into a β/α structure with a typical TIM-barrel, which contains minor substrates as an insertion. DspB is an antibiofilm enzyme, which has been shown to inhibit and disperse biofilms [95, 96].

The DspB amino acid sequence is as follows:
DspB specifically hydrolyses the glycosidic linkages of poly-β-1, 6-N-acetylglucosamine in polysaccharide adhesins of bacteria needed for biofilm formation, and also in preformed biofilm (a polysaccharide matrix) without affecting the growth [95, 97]. DspB inhibits biofilm formation as well as disperses preformed biofilm of human and animal pathogens such as gram-negative *Escherichia coli*, *Aggregatibacter actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*), *Actinobacillus pleuropneumoniae*, *Pseudomonas fluorescens*, *Bordetella pertussis*, *Bordetella parapertussis*, *Bordetella bronchiseptica*, *Xanthomonas axonopodis*, *Yersinia pestis*, *Yersinia pseudotuberculosis*, *Yersinia enterocolitica*, and gram-positive *Staphylococcus epidermidis* and *Staphylococcus aureus* [95, 96, 97, 98, 99]. The safety of this naturally occurring enzyme has been shown by Donelli [100] where by it was demonstrated to be non-cytotoxic by an *in vitro* cytotoxicity study on eukaryotic cells (Hep-2 cell culture). DspB did not cause any alteration in the cell cytoskeleton. Testing of DspB in a haemolytic activity test showed no haemolytic activity observed at a concentration as high as 2 mg/ml in rabbit blood, and as high as 1 mg/ml in human blood.

### 4.4.3. TCSN/DspB Test Results

The TCSN/DspB combination significantly inhibited biofilm formation in *S. aureus*, *S. epidermidis*, Coagulase-negative *Staphylococci*-42 (CoNS-42) and *E. coli*, as compared to control, triclosan alone or DspB alone, thereby indicating a synergistic antibiofilm activity by the TCSN/DspB combination [101]. The TCSN/DspB coated CVC segments demonstrated an anti-adherence ability against *S. aureus*, *S. epidermidis*, and *C. albicans*. The growth of tested organisms on TCSN/DspB coated catheter segments was significantly lower than control uncoated catheter segments. The TCSN/DspB coating on catheter segments reduced catheter adhesion by 2 logs as compared to uncoated control catheter segments.

The *in vitro* durability of the TCSN/DspB coated CVC was tested in rabbit plasma and bovine serum. In the case of the rabbit plasma the TCSN/DspB coated catheter segments were significantly less colonized by *S. epidermidis* as compared to control catheter segments during the 7 day experiment. In addition the *in vitro* durability of the TCSN/DspB-coated CVC was compared with commercially available antimicrobial catheters coated with a combination of chlorhexidine-silver sulfadiazine against *S. aureus*, *S. epidermidis* and *C. albicans*. The TCSN/DspB-coated CVC generally demonstrated a prolonged superior antimicrobial activity compared with chlorhexidine-silver sulfadiazine-coated catheters. Furthermore, the *in vivo* efficacy of TCSN/DspB-coated catheters compared with control uncoated catheters, ARROWg+ard Blue and ARROWg+ard Blue Plus was assessed by subcutaneous implantation of segments in a rabbit model of *S. aureus*. Testing showed 1/30 (3.3%) TCSN/DspB coated catheters, 4/30 (13.3%) ARROWg+ard Blue catheters, 1/30 (3.3%) ARROWg+ard Blue PLUS catheters, and 29/30 (96.7%) uncoated control catheters.
became colonized with *S. aureus*. Pair-wise comparison of all coated catheter segments against control uncoated catheter segments indicated that coated catheter segments are significantly less likely to become colonized by *S. aureus*. No significant differences were observed when catheter colonization was compared among TCSN/DspB coated, ARROWg+ard Blue and ARROWg+ard Blue PLUS catheter groups. All blood samples collected just prior to euthanasia yielded negative results demonstrating the absence of device-associated bacteremia. The stability and effectiveness of the TCSN/DspB coated polyurethane CVC which were stored for one year at room temperature were tested monthly and showed that the catheters were stable and prevented 90% of adhesion by *S. epidermidis*.

4.5. Sodium Bicarbonate, Sodium Metaperiodate and Sodium Dodecyl Sulphate (SB/SMP/SDS) Combination

The SB/SMP/SDS is a formulation containing SB to maintain an alkaline pH, SMP to degrade polysaccharide adhesins required for biofilm formation and SDS as an anionic surfactant with known bactericidal activity.

4.5.1. Sodium Bicarbonate (SB)

SB with a Molecular Formula NaHCO₃, Melting point 300°C, Mol. Wt. 84.0 and CAS# 144-55-8. SB when added to water maintains an alkaline pH of 8.1 to solubilize fatty acids contained in dirt and grease into a form of soap that can be dissolved in water and rinsed easily. SB also neutralizes odour chemically rather than masking or absorbing them, which helps to provide odour free fresh water [102].

4.5.2. Sodium metaperiodate (SMP)

SMP with a Molecular Formula NaIO₄, Melting point 300°C, Mol. Wt 213.98 and CAS# 7790-28-5. SMP is known to degrade polysaccharide adhesins which is required for biofilm formation. SMP is able to do so, by oxidizing carbons bearing vicinal hydroxyl groups, thereby cleaving the C–C bonds [52, 103].

4.5.3. Sodium Dodecyl Sulphate (SDS)

SDS with a Molecular Formula C₁₂H₂₅O₄SNa, Melting point 204 - 207°C, Mol. Wt. 288.4 and CAS # 151-21-3. SDS is an anionic surfactant and is known to have bactericidal activity by disrupting the cell envelope.

4.5.4. SB/SMP/SDS Test Results

The antibiofilm activity of SB, SMP and SDS alone and in combination was tested against DUWL-associated *P. aeruginosa*, *K. pneumoniae*, *A. naeslundii*, and *C. albicans*. The composition comprising all three compounds showed a significant inhibitory effect only on *P. aeruginosa* biofilm formation as compared with the control and individual compounds [104]. However, the composition dispersed 75% of *P. aeruginosa*, 65–90% of *K. pneumoniae*, 44–60% of *A. naeslundii*, and 56% of *C. albicans* biofilms. The MIC of the SB/SMP/SDS combination was determined against DUWL associated microorganisms such as *Legionella pneumophila*, *P. aeruginosa*, *S. aureus*, *K. pneumoniae*, *C. albicans*, *Fusobacterium*
This combination was effective at inhibiting the planktonic growth of all the cultures at as low as 1 mg/ml of each component.

The biofilm dispersal activity of SB/SMP/SDS was compared with that of commercial DUWL disinfectants: Oxygenal 6, Sterilex Ultra, and PeraSafe against *P. aeruginosa* at 4°C and at room temperature. While the SB/SMP/SDS combination dispersed more than 90% biofilm leaving the microtitre plate wells clean; treatment with the commercial products did not cause biofilm dispersal. Furthermore, the SB/SMP/SDS combination reduced biofilm-embedded *P. aeruginosa* viability by 4–5 log₁₀, whereas Oxygenal 6 was not effective in reducing the viable bacteria embedded in *P. aeruginosa* biofilm. However, the treatment of the biofilms with PeraSafe and Sterilex Ultra caused a complete loss of viability.

### 5. CONCLUSION

Infections associated with medical devices place a burden on healthcare providers and are a significant source of morbidity and mortality among populations. These infections are commonly associated with microorganisms that have colonized the device and formed microbial biofilms. Biofilm prevention and eradication on medical devices have been at the forefront of research for several decades, and countless studies have looked into developing novel materials, surface coatings, antimicrobial combinations, antifouling, antibiofilm agents and physical treatments with clinical benefit against bacterial biofilms. Although, many of these studies have shown powerful *in vitro* and *in vivo* evidence of biofilm inhibition, significant clinical advances in this field remain elusive. This is not entirely surprising as biofilm associated infections show very high tolerance toward antimicrobial agents which has led to the use of treatment strategies that directly target the device surface, with the intent that elevated concentrations of the antimicrobial agent for extended dwell times will kill and eradicate the biofilms and avoid the alternative-removing the device from the patient. However, the potential for the spread of antimicrobial resistance, particularly in very sick patients, argues in favour of newer approaches that are highly effective in dissolution of biofilms while avoiding or greatly minimizing the use of antimicrobial agents.

Currently, GlmU enzyme inhibitors, protamine sulfate, sodium metaperiodate and DispersinB® appear promising molecules that target EPS of bacterial biofilms directly and break down the existing biofilms. Furthermore, their effect is optimal when utilized in combination with antimicrobials, as bacteria can be killed upon release from the biofilm. The promising combination of oPDM plus PS, CHX plus PS and triclosan plus DispersinB® may have broader utility in preventing bacterial colonization of medical devices thereby avoiding medical device biofouling. Further work is needed for more rigorous testing of these coatings from both physical and bacterial challenge standpoints as well as increased preclinical and clinical studies.
6. REFERENCES


