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(54) Title: SOLUBLE β -N-ACETYLGLUCOSAMINIDASE BASED ANTIBIOFILM COMPOSITIONS AND USES THEREOF

(57) Abstract: The present invention provides compositions comprising an antibiofilm enzyme, a soluble β -N- acetylglucosaminidase similar to the dspB gene (DispersinBTM), and an antimicrobial for preventing growth and proliferation of biofilm-embedded microorganisms in acute and chronic wounds, and methods of treatment. The invention further provides methods for preparing medical devices, and in particular, wound care devices using DispersinBTM-based antimicrobial compositions.



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DISPERSINB™ ENZYME-BASED ANTIBIOFILM COMPOSITIONS AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. provisional application no. 60/829,420, filed on October 13, 2006, U.S. provisional application no. 60/870,762, filed December 19, 2006, U.S. provisional application no. 60/890,320, filed February 16, 2007, U.S. provisional application no. 60/945,474, filed June 21, 2007, U.S. provisional application no. 60/950,416, filed July 18, 2007 and U.S. provisional application no. 60/969,355, filed August 31, 2007.

Field of the Invention

The present invention relates to antibiofilm enzyme DispersinB™-based antimicrobial compositions that inhibit growth and proliferation of biofilm-embedded microorganisms, and methods of administering the compositions.

Background

From a microbiological perspective, the primary function of normal, intact skin is to control microbial populations that live on the skin surface and to prevent underlying tissue from becoming colonized and invaded by potential pathogens. Exposure of subcutaneous tissue (i.e. a wound) provides a moist, warm and nutritious environment that is conducive to microbial colonization and proliferation.

Since wound colonization is mostly polymicrobial, involving numerous microorganisms that are potentially pathogenic, any wound is at some risk of becoming infected. In the event of an infection a wound fails to heal, the patient suffers increased trauma as well as increased treatment costs. General wound management practices become more resource demanding. Wounds are an enormous problem worldwide. Approximately 1% of the world's population suffers a venous leg ulcer (Ruckley, 1997. *Angiology*, 48: 67-69). Friedberg et al. estimated the annual cost for dealing with venous leg ulcers in 192 patients to be \$1.26 million (Friedberg et al., 2002. *J. Wound. Ostomy. Continence. Nurs.* 29: 186-192). This equals 6.5 billion of direct wound care cost for every 1 million venous leg ulcer patients. Pressure ulcers are a common and expensive wound care problem in acute care, nursing

homes and home care populations. For decubitus ulcer, Stausberg et al. (2005) demonstrated 1% incidence rate along with a 5% prevalence rate for hospital patients (Stausberg et al., 2005. *Adv. Skin Wound. Care*, 18: 140-145). Bennett et al. found that the management of decubitus ulcers costs approximately 3-4 billion dollars annually in the United Kingdom, which is over 4% of the total National Health Service expenditure in the United Kingdom (Bennett et al., 2004. *Ageing*, 33: 230-235). In the United States, diabetic foot ulcers in 2004 consumed approximately 10 billion dollars in direct cost (approximately 4% of the total personal health spending of the United States) and another \$5 billion in indirect cost (disability, nursing homes, etc.). Diabetic foot ulcers caused over 100,000 major diabetic limb amputations. The cost for each amputation when factoring in associated costs was \$100,000 in 2005, resulting in \$10 billion in direct cost (Heyneman and Lawless-Liday, 2002. *Critical Care Nurse*, 22: 52-60). Wounds are becoming an increased portion of the cost of the healthcare system.

Thus, concern among health care practitioners regarding the risk of wound infection is justifiable not only in terms of increased trauma to the patient but also in view of its burden on financial resources and the increasing requirement for cost-effective management within the health care system. Most wound infections are caused by *Staphylococcus aureus* (20%), *Staphylococcus epidermidis* (14%), *Enterococci* spp. (12%), *Escherichia coli* (8%), *Pseudomonas aeruginosa* (8%), *Enterobacter* spp. (7%), *Proteus* spp. (3%), *Klebsiella pneumoniae* (3%), *Streptococci* (3%) and *Candida albicans* (3%) (CDC Report on common bacterial species associated with wound infections, 1996).

In recent years, there have been numerous efforts to use antibiotics and antimicrobials for the treatment of non-healing, clinically infected wounds. These antimicrobial agents are of varying chemical composition and can include peptides (Zaleski et al., 2006, *Antimicrob. Agents Chemother.*, 50: 3856-3860), antiseptics (US patent No. 6,700,032), antibiotics (Rothstein, et al., 2006, *Antimicrob. Agents Chemother.* 50: 3658-3664; Rittenhouse, et al., 2006, *Antimicrob. Agents Chemother.* 50: 3886-3888), silver ions/compounds (US patent appl. pub. no. 2005/0035327), chitosan (US patent appl. pub. no. 2006/0210613; US patent no. 6,998,509), nitrofurazone (Munster, 1984, *J. Trauma* 24: 524-525), bismuth thiols (Domenico, et al., 2000, *Infect. Med.* 17: 123-127), and xylitol (WO 2005/058381).

There have been various attempts by others to create wound care devices such as dressings or bandages, gels and ointments comprising antimicrobial agents. For example,

U.S. Patent No. 3,930,000 discloses the use of a silver zinc allantoinate cream for killing bacteria and fungi associated with burn wounds. Another example is silver sulfadiazine (SILVA-DINE®), which has been shown to be effective when tested *in vitro* against 50 strains of methicillin resistant *S. aureus* (MRSA). Numerous products are commercially available with different trade names that employ silver as antimicrobial agents such as STERIPURE®, A.M.Y., ACTICOAT™, ACTISORB®, and SILVERLON®.

U.S. Patent No. 7,091,336 teaches the process of making a gel containing gellan gum that increases in viscosity once applied to the wound to form an immobile gel. One example of a commercially available wound gel is INTRASITE®, contains carboxymethyl cellulose as a main ingredient. U.S. patent No. 6,700,032 discloses the application of triclosan in wound dressing fabricated from a natural or synthetic film-forming material, such as hydrophobic polymeric membrane. DeBusk and Alleman disclose a wound dressing that has been infused with a suspension of starch hydrolysate containing collagen and α -tocopherol acetate (U.S. patent appl. Pub. No. 2004/0001878). Wounds, in particular those occurring in the skin as second and third degree burns, stasis ulcers, tropic lesions, such as decubitus ulcers, severe cuts and abrasions that are commonly resistant to the natural healing process, may be treated with the infused dressing. Progress has been made on developing wound care devices, but each of the wound etiologies are increasing at double digit rates annually, causing the number of wounds to double every 4-5 years (Drosou et al., 2003, *Wounds*, 15:149-166).

Wounds often have multiple barriers to healing. Wound healing and infection is influenced by the relationship between the ability of bacteria to create a stable, prosperous community within a wound environment and the ability of the host to control the bacterial community. Since bacteria are rapidly able to form their own protective microenvironment (biofilm) following their attachment to a surface, the ability of the host to control these organisms is likely to decrease as the biofilm community matures. Within a stable biofilm community, interactions between aerobic and anaerobic bacteria are likely to increase their net pathogenic effect, enhancing their potential to cause infection and delay healing. Over the last few years, some have linked biofilm to chronic wounds (Mertz, 2003, *Wounds*, 15: 1-9). Microscopic evaluation of chronic wounds showed well organized biofilm with extracellular polymeric substance adhered around colony bacteria in at least 60% of the chronic wounds (Mertz, 2003, *Wounds*, 15: 1-9).

In addition to a direct effect on wound healing by the production of destructive enzymes and toxins, mixed communities of microorganisms may also indirectly affect healing by promoting a chronic inflammatory state. Prolonged exposure to bacteria within a chronic wound leads to a prolonged inflammatory response, resulting in the release of free radicals and numerous lytic enzymes that could have a detrimental effect on cellular processes involved in wound healing. Proteinases released from a number of bacteria, particularly *Pseudomonas aeruginosa*, are known to affect growth factors and many other tissue proteins that are necessary for the wound healing process (Steed et al., 1996, *J. Am. Coll. Surg.*, 183: 61-64; Travis et al., 1995, *Trends Microbiol.* 3: 405-407). The increased production of exudates that often accompanies increased microbial load has been associated with the degradation of growth factors and matrix metalloproteinases (MMPs), which subsequently affect cell proliferation and wound healing (Falanga et al., 1994, *J. Invest. Dermatol.* 1: 125-127).

Dental plaque is a host-associated biofilm that adheres to the tooth surface both above and below the gingival margin. Dental plaque consists mainly of microorganisms with a small number of epithelial cells, leukocytes, and macrophages in an intracellular matrix. It has been postulated that there are approximately 300 to 400 different bacterial species in dental plaque (Moore, 1987, *J. Periodont. Res.* 22: 335-341). Periodontal disease comprises a collection of inflammatory conditions of the periodontium (gingiva, periodontal ligament, cementum, and alveolar bone) due to a chronic bacterial infection, i.e., dental plaque. Over 90% of the population of the United States is affected by periodontal disease (Brown et al., 1996, *J. Dent. Res.* 75: 672-683).

In addition to periodontal diseases, other conditions/diseases caused by biofilms include cystic fibrosis, pneumonia, native valve endocarditis and otitis media (Costerton et al. Science 1999 284:1318-1322). Biofilm is also implicated in the infection of various medical devices such as urinary catheters, mechanical heart valves, cardiac pacemakers, prosthetic joints, and contact lenses (Donlan, R.M. 2001 *Emerging Infect. Dis.* 7:277-281). For example, urinary tract infection (UTI) is the most common hospital-acquired infection, accounting for up to 40% of all nosocomial infections. The majority of cases of UTIs are associated with the use of urinary catheters, including trans-urethral Foley, suprapubic, and nephrostomy catheters. These urinary catheters are inserted in a variety of populations, including the elderly, stroke victims, spinal cord-injured patients, post-operative patients and

those with obstructive uropathy. Despite adherence to sterile guidelines for the insertion and maintenance of urinary catheters, catheter-associated UTIs continue to pose a major problem. For instance, it is estimated that almost one-quarter of hospitalized spinal cord-injured patients develop symptomatic UTIs during their hospital course. Gram-negative bacilli
5 account for almost 60-70%, *Enterococci* for about 25%, and *Candida* species for about 10% of cases of catheter-associated UTI.

Furthermore, indwelling medical devices including vascular catheters are becoming essential in the management of hospitalized patients by providing venous access. The benefit
10 derived from these catheters as well as other types of medical devices such as peritoneal catheters, cardiovascular devices, orthopedic implants, and other prosthetic devices is often offset by infectious complications. The most common organisms causing these infectious complications are *Staphylococcus epidermidis* and *Staphylococcus aureus*. In the case of vascular catheters, these two organisms account for almost 70-80% of all infectious
15 organisms, with *Staphylococcus epidermidis* being the most common organism. Fungi also form biofilms of clinical significance. *Candida albicans*, a fungal agent, accounts for 10-15% of catheter infections.

Bacteria and fungi growing in biofilms exhibit increased resistance to antimicrobial
20 agents and are nearly impossible to eradicate using known techniques. The present invention teaches applications of an antibiofilm enzyme DispersinB™-based antimicrobial composition in devices, methods for preparing such devices, and methods of treating wounds and oral infections.

Summary of the Invention

25 In one embodiment, the present invention provides a composition for preventing and/or inhibiting growth or proliferation of biofilm-embedded microorganisms comprising: (a) a first compound comprising DispersinB™, an active fragment or variant thereof that disperses a biofilm; and (b) a second compound comprising an antimicrobial agent active
against bacteria or fungi.

30 In an embodiment, DspB is in a concentration of about 5 to about 500 µg/ml. In another embodiment, DspB is in a concentration of about 10 to about 250 µg/ml. In another embodiment, DispersinB™ is in a concentration of about 25 ng/ml to about 100 µg/ml.

In another embodiment, an antimicrobial agent can include triclosan, antibiotics (such as rifampicin, cefamandole nafate and ciprofloxacin), nitrofurazone, bismuth-thiols [such as bismuth ethanedithiol (BisEDT)], chitosan, epigallocatechin gallate (EGCG), sodium usnate, antineoplastic agents (such as 5-fluorouracil), detergents (such as SDS, benzalkonium chloride), chlorhexidine, chelating agents (such as EDTA), silver compounds, bacteriophage, antimicrobial enzymes (such as glucose oxidase and lactoperoxidase), sugar alcohols (such as xylitol), maleimides [such as *N,N*-(1,2 phenylene) dimaleimide (oPDM) and *N*-(1-pyrenyl) maleimide (PyrM)], cadexomer iodine, methylene blue, gentian violet, or medium chain dextrans (such as honey).

In a further embodiment, an antimicrobial agent can include triclosan, and can be in a concentration of about 0.1 $\mu\text{g/ml}$ to about 50 mg/ml. In another embodiment, the concentration is about 0.2 $\mu\text{g/ml}$ to about 25 mg/ml and in a still further embodiment, the concentration is about 0.325 $\mu\text{g/ml}$ to about 10 mg/ml.

In yet another embodiment, an antimicrobial agent can include, but is not limited to,

- (i) rifampicin in a concentration of about 0.1 to about 1000 $\mu\text{g/ml}$, preferably about 1 to about 100, and more preferably about 10 to about 50 $\mu\text{g/ml}$;
- (ii) cefamandole nafate in a concentration of about 0.01 to about 10 $\mu\text{g/ml}$, preferably about 0.05 to about 5 $\mu\text{g/ml}$, and more 0.1 to about 2 $\mu\text{g/ml}$;
- (iii) nitrofurazone in a concentration of about 0.01 to about 1 mg/ml, preferably about 0.1 to about 1 mg/ml, and more preferably about 0.5 to about 1 mg/ml;
- (iv) bismuth ethanedithiol (BisEDT) in a concentration of about 0.01 to about 5 mM, preferably about 0.1 to about 2 mM, and more preferably about 1 mM;
- (v) ciprofloxacin in a concentration of about 0.01 to about 1.0 mg/ml; preferably about 0.05 to about 0.5 mg/ml and more preferably about 0.1 mg/ml;
- (vi) epigallocatechin gallate in a concentration of about 10 to about 100 $\mu\text{g/ml}$, preferably about 25 to about 50 $\mu\text{g/ml}$, or more preferably about 50 $\mu\text{g/ml}$;
- (vii) sodium usnate in a concentration of about 10 to about 750 $\mu\text{g/ml}$, preferably about 100 to about 500 $\mu\text{g/ml}$ or more preferably 200 to about 400 $\mu\text{g/ml}$;
- (viii) ovotransferrin in a concentration of about 10 to about 1000 mg/ml, preferably about 25 to about 500 mg/ml or more preferably about 50 to about 200 mg/ml;
- (ix) sodium doccyl sulfate in a concentration of about 0.01 to about 100 mg/ml, more preferably about 0.05 mg/ml to about 50 mg/ml and more preferably about 0.1 mg/ml to about 10 mg/ml;
- (x) 5-fluorouracil in a concentration of about 1 to about 1000 $\mu\text{g/ml}$, preferably about 10 to about 500 $\mu\text{g/ml}$ and more preferably about 25 to 100 $\mu\text{g/ml}$;
- (xi) chlorhexidine in a concentration of about 0.01

$\mu\text{g/ml}$ to about 100 $\mu\text{g/ml}$, preferably about 0.1 $\mu\text{g/ml}$ to about 10 $\mu\text{g/ml}$, and more preferably about 1 $\mu\text{g/ml}$ to about 5 $\mu\text{g/ml}$; (xii) benzalkonium chloride 0.01 $\mu\text{g/ml}$ to about 100 $\mu\text{g/ml}$, preferably about 0.1 $\mu\text{g/ml}$ to about 10 $\mu\text{g/ml}$, and more preferably about 1 $\mu\text{g/ml}$ to about 5 $\mu\text{g/ml}$; (xiii) EDTA in a concentration of about 1 to about 1000 $\mu\text{g/ml}$, preferably about 10 to about 100 $\mu\text{g/ml}$ and more preferably about 25 to about 50 $\mu\text{g/ml}$; or (xv) silver nanopowder in a concentration of 0.01 $\mu\text{g/ml}$ to about 100 $\mu\text{g/ml}$, preferably about 0.1 $\mu\text{g/ml}$ to about 10 $\mu\text{g/ml}$, and more preferably about 1 $\mu\text{g/ml}$ to about 5 $\mu\text{g/ml}$.

An embodiment of the invention includes a method of inhibiting biofilm-embedded microorganisms comprising administering an effective amount of DispersinBTM, an active fragment, or variant thereof that disperses a biofilm; and an effective amount of an antimicrobial agent or a mixture of an antimicrobial agent.

In another embodiment, the DispersinBTM, an active fragment, or variant thereof is administered prior to administration of the antimicrobial agent and the antimicrobial agent is sodium doecyl sulfate, chlorhexidine, or benzalkonium chloride.

An embodiment of the invention includes a method of treating an infection by administering a composition comprising (a) DispersinBTM, a DispersinBTM fragment, or variant thereof; and (b) an antimicrobial agent or a mixture of an antimicrobial agent.

In yet another embodiment, a DispersinBTM-based antimicrobial composition can treat various kinds of wounds, including, but not limited to, cutaneous abscess, surgical wounds, sutured lacerations, contaminated lacerations, burn wounds such as partial and full thickness burns, decubitus ulcers, stasis ulcers, leg ulcers, foot ulcers, venous ulcers, diabetic ulcers, ischemic ulcers, and pressure ulcers.

In another embodiment, a DispersinBTM-based antimicrobial composition can treat an oral infection. Oral infections include microorganisms in the subgingival and supragingival plaque. Subgingival plaque comprising microorganisms can cause periodontal disease. The compositions of the present invention can be used in the treatment of periodontal disease. The compositions of the present invention can be used in the treatment of localized juvenile periodontitis.

Biofilm microorganisms can be bacteria, such as gram-negative *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Bacteroides* spp., *Porphyromonas* spp., *Prevotella*

spp., *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*), *Treponema denticola*, or *Pseudomonas aeruginosa*, and gram-positive *Enterococcus faecalis*, *Enterococcus cloacae*, Vancomycin Resistant Enterococci (VRE), *Streptococcus* spp., *Peptostreptococcus* spp., *Staphylococcus epidermidis*,
5 or *Staphylococcus aureus*. Furthermore, a wound-associated microorganism can be fungi, such as *Candida albicans*.

One embodiment of the present invention includes providing methods of using a DispersinBTM-based composition or compositions in wound care devices such as non-resorbable gauze/sponge dressing, hydrophilic wound dressing, occlusive wound dressing,
10 hydrogel wound, and burn dressing. The present invention also includes use of a spray-applicator containing a DispersinBTM-based antimicrobial composition as a wound care device. Another embodiment of the invention includes a wound care device comprising a DispersinBTM based composition or compositions.

An additional aspect of the present invention includes wound care ointments, gels,
15 and lotions comprising DispersinBTM and an antimicrobial agent. An embodiment of the present invention also includes wound care sutures coated with DispersinBTM and an antimicrobial agent.

Furthermore, a composition can comprise binders, wetting agents, odor absorbing agents, levelling agents, adherents, thickeners, and the like. Other additives may be present
20 on and/or within a fabric of bandage including antistatic agents, optical brightening compounds, opacifiers (e.g., titanium dioxide), nucleating agents, antioxidants, UV stabilizers, fillers, permanent press finishes, softeners, lubricants, curing accelerators, adhesives, and the like.

In another embodiment, the present invention includes wound gel compositions for:
25 (a) DispersinBTM antimicrobial wound gel with a viscosity improving agent; and (b) Triclosan-DispersinBTM antimicrobial wound gel with a viscosity improving agent. A DispersinBTM or Triclosan-DispersinBTM wound gel can include DispersinBTM, an active fragment or variant thereof.

30 In another embodiment, an antimicrobial agent can include, but is not limited to, triclosan, antibiotics (such as rifampicin, cefamandole nafate and ciprofloxacin)

nitrofurazone, bismuth-thiols [such as bismuth ethanedithiol (BisEDT)], chitosan, Epigallocatechin gallate (EGCG), sodium usnate, antineoplastic agents (such as 5-fluorouracil), detergents (such as SDS, benzalkonium chloride), chlorhexidine, chelating agents (such as EDTA), silver compounds, bacteriophage, antimicrobial enzymes (such as glucose oxidase and lactoperoxidase), sugar alcohols (such as xylitol), maleimides [such as *N,N*-(1,2 phenylene) dimaleimide (oPDM) and *N*-(1-pyrenyl) maleimide (PyrM)], cadexomer iodine, methylene blue, gentian violet, medium chain dextrans (such as honey), and mixtures thereof can be used in combination with DispersinBTM.

In a further embodiment, a Triclosan-DispersinBTM wound gel comprises of about 1 to about 10% triclosan, preferably of about 5 to about 10% triclosan and more preferably, about 1% triclosan. In a further embodiment, a DispersinBTM wound gel and a Triclosan-DispersinBTM wound gel can optionally further comprise a gelling agent and/or a viscosity increasing agent.

Triclosan-DispersinBTM wound gel can be prepared in polyethylene glycol (PEG)/ethanol. PEG of different molecular weights ranging from about 200 to about 511,000 can be used in a gel formulation. In an embodiment, a Triclosan-DispersinBTM wound gel is prepared in 10% PEG-400/10% ethanol.

In a further embodiment, gelling agents in a wound gel include, but are not limited to, gums, polysaccharides, alginates, synthetic polymeric compounds, natural polymeric compounds, and mixtures thereof.

DispersinBTM-based antimicrobial wound gels of the present invention can be used to inhibit the proliferation of biofilm-embedded gram-negative and gram-positive bacteria, which include, but are not limited to, *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, *Providentia sturtii*, *Serratia marcescens*, *Enterococcus faecalis*, Vancomycin Resistant *Enterococci* (VRE), *Peptostreptococcus* spp., *Corynebacterium* spp., *Clostridium* spp., *Bacteriodes* spp., *Prevotella* spp., *Streptococcus pyogenes*, *Streptococcus viridans*, *Micrococcus* spp., Beta-hemolytic streptococcus (group C), Beta-hemolytic streptococcus (group B), *Bacillus* spp., *Porphyromonas* spp., *Enterobacter cloacae*, *S. epidermidis*, *S. aureus*, *Staphylococcus agalactiae*, and *Staphylococcus saprophyticus*.

Additionally, DispersinBTM based antimicrobial compositions of the invention can also be used to inhibit proliferation of biofilm-embedded fungi, such as *Candida albicans*, *Candida parapsilosis*, and *Candida utilis*.

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In one embodiment, a DispersinBTM-based antimicrobial wound gel can be used for treating wounds including, but is not limited to, a cutaneous abscess, surgical wound, sutured laceration, contaminated laceration, blister wound, soft tissue wound, partial thickness burn, full thickness burn, decubitus ulcer, stasis ulcer, foot ulcer, venous ulcer, diabetic ulcer, ischemic ulcer, pressure ulcer, or combinations thereof.

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Another embodiment, the present invention provides a method of preparing a device comprising treating at least one surface of the device with a composition as herein described. For example, the composition can be incorporated into polymers, wherein said polymers are used to form the device. Another aspect of the present invention is a method of preparing a device comprising coating the composition as herein described onto the inner and/or outer surface of a device.

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In another embodiment, the DispersinBTM is about 0.1 to about 500 $\mu\text{g/ml}$ of the composition, preferably about 1 to about 350 $\mu\text{g/ml}$ of the composition or more preferably about 10 to about 100 $\mu\text{g/ml}$ of the composition. In yet another embodiment, the antimicrobial agent is triclosan, rifampicin, cefamandole nafate, nitrofurazone, ciprofloxacin, minocycline, gentamycin, silver compounds, chlorhexidine, 5-fluorouracil or a bisphosphonate, preferably rifampicin, cefamandole nafate, nitrofurazone, or triclosan, more preferably triclosan. In one embodiment, the triclosan is in a concentration of about 0.01 to about 100 mg/ml of the composition, preferably about 0.1 to about 100 mg/ml of the composition or more preferably about 1 to about 100 mg/ml of the composition. In another embodiment, the antibacterial agent is rifampicin in a concentration of about 10 to about 1000 $\mu\text{g/ml}$ of the composition, preferably about 100 to about 1000 $\mu\text{g/ml}$ of the composition or more preferably about 10 to about 100 $\mu\text{g/ml}$ of the composition. In another embodiment, the antibacterial agent is cefamandole nafate in a concentration of about 0.05 to 5 $\mu\text{g/ml}$ of the composition, preferably about 0.5 to about 5 $\mu\text{g/ml}$ of the composition, or more preferably, about 1 to about 5 $\mu\text{g/ml}$ of the composition. In another embodiment, the antibacterial agent is nitrofurazone in a concentration of about 0.01 to about 1 mg/ml of the

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composition, preferably about 0.1 to about 1 mg/ml of the composition, and more preferably about 0.5 to about 1 mg/ml of the composition. In one embodiment of the present invention, the composition comprises effective amounts of DispersinBTM and triclosan. In another embodiment of the present invention, the composition comprises effective amounts of DispersinBTM and rifampicin. In yet another embodiment of the present invention, the composition comprises effective amounts of DispersinBTM and cefamandole nafate. In yet another embodiment of the present invention, the composition comprises effective amounts of DispersinBTM and nitrofurazone.

In one aspect of the present invention, the device is a medical device, such as a catheter, for example, an indwelling catheter such as a central venous catheter, a peripheral intravenous catheter, an arterial catheter, a peritoneal catheter, a haemodialysis catheter, an umbilical catheter, precutaneous nontunneled silicone catheter, a cuffed tunneled central venous catheter, an endotracheal tube, a subcutaneous central venous port, urinary catheter, a peritoneal catheter, a peripheral intravenous catheter or a central venous catheter.

In another embodiment of the present invention, the medical devices are catheters, pacemakers, prosthetic heart valves, prosthetic joints, voice prostheses, contact lenses, a shunt, heart valve, penile implant, small or temporary joint replacement, urinary dilator, cannula, elastomer, or intrauterine devices.

In another embodiment of the present invention, the device is a catheter lock, a needle, a Leur-Lok[®] connector, a needleless connector, a clamp, a forcep, a scissor, a skin hook, a tubing, a needle, a retractor, a scaler, a drill, a chisel, a rasp, a surgical instrument, a dental instrument, a tube, an intravenous tube, a breathing tube, a dental water line, a dental drain tube, a feeding tube, a bandage, a wound dressing, an orthopedic implant, or a saw.

Another embodiment of the present invention is a method of preparing a device comprising coating a composition herein described onto at least one surface of the device.

Another embodiment of the present invention is a device coated, impregnated, or treated with a composition as herein described, for example, a medical device such as a catheter, for example an indwelling catheter such as a central venous catheter, a peripheral intravenous catheter, an arterial catheter, a peritoneal catheter, a haemodialysis catheter, an

umbilical catheter, precutaneous nontunneled silicone catheter, a cuffed tunneled central venous catheter, an endotracheal tube, a urinary catheter, a peritoneal catheter, a peripheral intravenous catheter and central venous catheter or a subcutaneous central venous port.

5 A device may also be catheters, pacemakers, prosthetic heart valves, prosthetic joints, voice prostheses, contact lenses, a stent, heart valve, penile implant, small or temporary joint replacement, urinary dilator, cannula, elastomer, intrauterine devices, catheter lock, a needle, a Leur-Lok[®] connector, a needleless connector, a clamp, a forcep, a scissor, a skin hook, a tubing, a needle, a retractor, a scaler, a drill, a chisel, a rasp, a surgical instrument, a dental instrument, a tube, an intravenous tube, a breathing tube, a dental water line, a dental drain
10 tube, a feeding tube, a bandage, a wound dressing, an orthopedic implant, or a saw.

Another embodiment of the present invention is a method of preventing device or catheter-related infection in a mammal, said method comprising coating, incorporating, or treating a device or catheter to be implanted with a composition as herein described. Another
15 embodiment of the present invention is a method of preventing an infection caused by a device or catheter in a mammal, said method comprising coating, incorporating or treating the device or catheter with a composition as herein described.

Another embodiment of the present invention is the use of a composition as herein described in the preparation of a medical device for implantation in a mammal. In one embodiment, a medical device may be coated, incorporated, or treated with a composition. In
20 another embodiment, the composition may prevent urinary tract infection. Another aspect of the present invention is the use wherein the composition prevents urinary or vascular infection.

In another embodiment, the present invention provides a composition for inhibiting biofilm-embedded microorganisms comprising: (a) DispersinB[™], an active fragment or
25 variant thereof that disperses a biofilm; and (b) a bacteriophage. The composition can comprise about 10^8 bacteriophage. The bacteriophage can comprise more than one species of bacteriophage.

In another embodiment, the present invention provides a composition for inhibiting biofilm-embedded microorganisms comprising a recombinant bacteriophage, wherein the

recombinant bacteriophage displays DispersinBTM. The displayed DispersinBTM can be fused to a phage coat protein. The DispersinBTM can be fused to the major coat protein or the minor coat protein. In another embodiment, the present invention provides a fusion protein comprising at least a portion of a phage coat protein bonded to DispersinBTM.

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Brief Description of the Figures

Figure 1 is a bar graph showing the effect of DispersinBTM on *Escherichia coli*, *Staphylococcus epidermidis* and *Staphylococcus aureus* biofilm formation. All three bacterial strains were grown separately in a media without DispersinBTM as a negative control.

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Figure 2 shows the effect of DispersinBTM in polystyrene tubes on *S. epidermidis* biofilm dispersal.

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Figure 3 is bar graph showing an enhanced inhibitory effect of a DispersinBTM and Triclosan (TCSN) combination on *S. epidermidis* biofilm formation. Planktonic (□) and biofilm (■) *S. epidermidis* growth was measured in media with no antimicrobials (control), DispersinBTM (25, 50, and 100 ng/ml), TCSN (25, 50, and 100 µg/ml), and the combination of DispersinBTM (25, 50, or 100 ng/ml) and TCSN (25, 50, and 100 µg/ml).

20

Figure 4 is bar graph showing an enhanced effect of DispersinBTM on the sensitivity of biofilm-embedded *S. epidermidis* to rifampicin. *S. epidermis* growth was measured in media with no antimicrobials (control), DispersinBTM (20 µg/ml), rifampicin (100 µg/ml), and a combination of DispersinBTM (20 µg/ml) and rifampicin (100 µg/ml).

25

Figure 5 is bar graph showing an enhanced effect of DispersinBTM on the sensitivity of biofilm-embedded *S. epidermidis* to cefamandole nafate. *S. epidermidis* growth was measured in media with no antimicrobials (control), DispersinBTM (20 µg/ml), cefamandole nafate (0.1 µg/ml), and a combination of DispersinBTM (20 µg/ml) and cefamandole nafate (0.1 µg/ml).

Figure 6 is bar graph showings an enhanced effect of DispersinBTM on the scnsitivity of biofilm-embedded *S. epidermidis* to nitrofurazone. *S. epidermis* growth was measured in media with no antimicrobials (control), DispersinBTM (20 µg/ml), nitrofurazone (25 µg/ml), and a combination of DispersinBTM (20 µg/ml) and nitrofurazone (25 µg/ml).

Figure 7 is bar graph showing an enhanced effect of DispersinBTM on the sensitivity of biofilm-embedded *S. epidermidis* to bismuth ethanedithiol (BisEDT). *S. epidermidis* growth was measured in media with no antimicrobials (control), DispersinBTM (20 µg/ml), BisEDT (0.5 mM), and a combination of DispersinBTM (20 µg/ml) and BisEDT (0.5 mM).

5 Figure 8 is bar graph showing an enhanced effect of DispersinBTM on the sensitivity of biofilm-embedded *S. epidermidis* to ciprofloxacin (CI^r). *S. epidermidis* growth was measured in media with no antimicrobials (control), DispersinBTM (20 µg/ml), CF (200 µg/ml), and a combination of DispersinBTM (20 µg/ml) and CF (200 µg/ml).

10 Figure 9 is a bar graph showing an enhanced effect of DispersinBTM on the sensitivity of biofilm-embedded *S. epidermidis* to lactoferrin (Lf). *S. epidermis* growth was measured in media with no antimicrobials (control), DispersinBTM (20 µg/ml), Lf (5 mg/ml), and a combination of DispersinBTM (20 µg/ml) and Lf (5 mg/ml).

15 Figure 10 is bar graph showing an enhanced effect of DispersinBTM on the sensitivity of biofilm-embedded *S. epidermidis* to conalbumin/ovotransferrin (OT). *S. epidermis* growth was measured in media with no antimicrobials (control), DispersinBTM (20 µg/ml), OT (10 mg/ml), and a combination of DispersinBTM (20 µg/ml) and OT (10 mg/ml).

20 Figure 11 is bar graph showing an enhanced effect of DispersinBTM on the sensitivity of biofilm-embedded *S. epidermidis* to gallium (III) nitrate. *S. epidermis* growth was measured in media with no antimicrobials (control), DispersinBTM (20 µg/ml), gallium (III) nitrate (5 mg/ml), and a combination of DispersinBTM (20 µg/ml) and gallium (III) nitrate (5 mg/ml).

25 Figure 12 is bar graph showing an enhanced effect of DispersinBTM on the sensitivity of biofilm-embedded *S. epidermidis* to chitosan. *S. epidermidis* growth was measured in media with no antimicrobials (control), DispersinBTM (20 µg/ml), chitosan (2 mg/ml), and a combination of DispersinBTM (20 µg/ml) and chitosan (2 mg/ml).

Figure 13 is bar graph showing an effect of DispersinBTM and Epigallocatechin gallate (EGCG) alone and in combination on *S. epidermidis* biofilm formation. *S. epidermidis* growth was measured in media with no antimicrobials (control), DispersinBTM (50 µg/ml), EGCG (100 ng/ml), and a combination of DispersinBTM (50 µg/ml) and EGCG (100 ng/ml).

Figure 14 is bar graph showing an effect of DispersinBTM and Epigallocatechin gallate (EGCG) alone and in combination on *S. aureus* biofilm formation. *S. aureus* growth was measured in conditions of no antimicrobials (control), DispersinBTM (50 µg/ml), EGCG (100 ng/ml), and a combination of DispersinBTM (50 µg/ml) and EGCG (100 ng/ml).

5 Figure 15 is bar graph showing an effect of DispersinBTM and triclosan alone and in combination on biofilm-embedded *S. epidermidis*. *S. epidermidis* growth was measured in media with no antimicrobials (control), DispersinBTM (20 µg/ml), triclosan (1 mg/ml), and a combination of DispersinBTM (20 µg/ml) and EGCG (1 mg /ml).

10 Figure 16 is bar graph showing an effect of DispersinBTM and sodium (Na) usnate alone and in combination on biofilm-embedded *S. epidermidis*. *S. epidermidis* growth was measured in media with no antimicrobials (control), DispersinBTM (50 µg/ml), Na usnate (500 µg/ml), and a combination of DispersinBTM (50 µg/ml) and Na usnate (500 µg/ml).

15 Figure 17 is bar graph showing an enhanced inhibitory effect of DispersinBTM and Triclosan (TCSN) combination on coagulase-negative Staphylococci (CNS) biofilm formation. Planktonic (□) and biofilm growth (■) were measured in media with no antimicrobials (control), DispersinBTM (25, 50, and 100 ng/ml), TCSN (0.325, 0.625, and 1.25 µg/ml), and a combination of DispersinBTM (25, 50, and 100 ng/ml) and TCSN 0.325, 0.625, and 1.25 µg/ml).

20 Figure 18 is bar graph showing enhanced effect of DispersinBTM on the sensitivity of biofilm-embedded *S. epidermidis* to 5-fluorouracil (5-FU). *S. epidermidis* growth was measured in media with no antimicrobials (control), DispersinBTM (20 µg/ml), 5-FU (100 µg/ml), and a combination of DispersinBTM (20 µg/ml) and 5-FU (100 µg/ml).

25 Figure 19 is bar graph showing the increased susceptibility of biofilm-embedded *S. epidermidis* pretreated with DispersinBTM to killing by sodium dodecyl sulfate (SDS). *S. epidermidis* growth was measured in media with no antimicrobials (control), DispersinBTM (20 µg/ml), SDS (0.2 mg/ml), and a combination of DispersinBTM (20 µg/ml) and SDS (0.2 mg/ml).

Figure 20 is bar graph showing the increased susceptibility of biofilm-embedded *S. epidermidis* pretreated with DispersinBTM to killing by chlorhexidine (CHX). *S. epidermidis*

growth was measured in media with no antimicrobials (control), DispersinBTM (20 µg/ml), CHX (0.2 µg/ml), and a combination of DispersinBTM (20 µg/ml) and CHX (0.2 µg/ml).

Figure 21 is bar graph showing the increased susceptibility of biofilm-embedded *S. epidermidis* pretreated with DispersinBTM to killing by benzalkonium chloride (BKC). *S.*

5 *epidermidis* growth was measured in media with no antimicrobials (control), DispersinBTM (20 µg/ml), BKC (0.4 µg/ml), and a combination of DispersinBTM (20 µg/ml) and BKC (0.4 µg/ml).

Figure 22 is bar graph showing an enhanced inhibitory effect of DispersinBTM and EDTA combination on *S. epidermidis* biofilm formation. Planktonic (□) and biofilm (■) *S.*

10 *epidermidis* growth was measured in media with no antimicrobials (control), DispersinBTM (100 ng/ml), EDTA (25 and 50 µg/ml), and combinations of DispersinBTM (100 ng/ml) and EDTA (25 or 50 µg/ml).

Figure 23 is bar graph showing the increased susceptibility of biofilm-embedded *S. epidermidis* pretreated with DispersinBTM to killing by silver nanopowder (SNP). *S.*

15 *epidermidis* growth was measured in media with no antimicrobials (control), DispersinBTM (20 µg/ml), SNP (0.03125 µg/ml), and a combination of DispersinBTM (20 µg/ml) and SNP (0.03125 µg/ml).

Figure 24 is line graph showing the increased susceptibility of biofilm-embedded *E. coli csrA luxCDABE kan^r* over time to a combination of DispersinBTM and a phage cocktail

20 of FF3, K20, T7, and U3 (▲). Biofilm-embedded *E. coli csrA luxCDABE kan^r* grown in media without antimicrobials or DispersinBTM was used as a control (◇). Biofilm-embedded *E. coli csrA luxCDABE kan^r* treated with DispersinBTM only (■) had growth similar to the control. Biofilm-embedded *E. coli csrA luxCDABE kan^r* were individually treated with phage FF3 (*), K20 (●), T7 (+), and U3 (-). Treatment with the phage produced a short term
25 decrease in *E. coli* metabolic activity, but the *E. coli* metabolic activity returned to near control levels after 4 days. Biofilm-embedded *E. coli csrA luxCDABE kan^r* treated with a phage cocktail of FF3, K20, T7, and U3 (X) alone produced similar results as the treatments with the individual phages.

Figure 25 is a schematic diagram of the construction of recombinant λ phage for
30 DispersinBTM display.

Figure 26 is a schematic diagram of the construction of recombinant λ phage for DispersinBTM display.

Figure 27 is a schematic diagram of the construction of recombinant M13 phage for DispersinBTM display.

5 Figure 28 shows the biofilm growth and detachment of *A. actinomycetemcomitans* strains CU1000 (wild-type) and HW1018 (PGA mutant) in polystyrene tubes and 96-well microtiter plates. All tubes and microplate wells were stained with crystal violet. Biofilm formation at 0 h and 24 h in tubes (panel A) and microplates (panel B). The biofilms on the right were rinsed with water and treated with SDS (0.1% in PBS) or DispersinBTM (20 μ g/mL in PBS) for 5 min prior to crystal violet staining. (C) Detachment of CU1000 biofilms from
10 microplates by SDS. Wells on the bottom were pre-treated with DispersinBTM for 30 min prior to the SDS treatment.

Figure 29 is line graph showing the detachment of *A. actinomycetemcomitans* strain CU1000 (wild-type) biofilms from 96-well microtiter plates by SDS. Biofilms were pre-
15 treated with PBS (mock pretreatment) or DispersinBTM (20 μ g/mL in PBS) for 30 min, and then treated with increasing concentrations of SDS for 5 min. Biofilms were then rinsed and stained with crystal violet. We quantitated the amount of bound crystal violet dye, which is proportional to biofilm biomass, by measuring its absorbance at 590 nm. Values are the mean absorbance for duplicate wells. Error bars indicate range of standard deviation.

20 Figure 30 is bar graph showing that pre-treatment of *A. actinomycetemcomitans* CU1000 (wild-type) biofilms with DispersinBTM increased sensitivity to killing by SDS. Biofilms grown in polystyrene tubes were rinsed with PBS and treated with 1 mL of PBS (mock pre-treatment) or DispersinBTM (20 μ g/mL in PBS) for 5 min (black bars) or 30 min (gray bars), and then treated with PBS (-) or SDS (0.01% in PBS; +) for 5 min. Colony
25 forming units (CFU) were enumerated by dilution plating. Values indicate the log₁₀ of the mean number of CFU per tube for duplicate tubes. Error bars indicate range of standard deviation.

Figure 31 is bar graph showing that pretreatment of *A. actinomycetemcomitans* CU1000 biofilms with DispersinBTM increases their sensitivity to killing by cetylpyridinium
30 chloride (CPC). Biofilms grown in polystyrene tubes were rinsed with PBS and treated for 30 min with PBS (mock pretreatment) or PBS containing 20 μ g/mL of DispersinBTM B, and then

treated for 5 min with 0.02% CPC. CFU were enumerated by dilution plating. Values indicate the log₁₀ of the mean number of CFU/tube for duplicate tubes. Error bars indicate range of standard deviation.

Figure 32 is bar graph showing the effect of DispersinBTM antimicrobial wound gel on
5 *Staphylococcus epidermidis* growth and biofilm formation.

Figure 33 is a bar graph showing the effect of DispersinBTM antimicrobial wound gel on *Staphylococcus epidermidis* biofilm dispersal.

Figure 34 is a bar graph showing the synergistic inhibitory effect of DispersinBTM and Triclosan (TCSN) combination on *Staphylococcus epidermidis* biofilm formation.

10 Figure 35 is a bar graph showing the synergistic inhibitory effect of DispersinBTM and Triclosan (TCSN) combination coated silicone catheters on *Escherichia coli* colonization.

Figure 36 is a bar graph showing the synergistic inhibitory effect of DispersinBTM and Triclosan (TCSN) combination coated silicone catheters on *Staphylococcus epidermidis* colonization

15 Figure 37 is a bar graph showing the antibiofilm activity of DispersinBTM and Triclosan (TCSN) combination-coated catheters against catheter-associated microorganisms

Figure 38 is a line graph showing the durability of inhibitory activity of DispersinBTM and Triclosan (TCSN) combination-coated polyurethane catheters.

20 Figure 39 is a line graph showing the durability of inhibitory activity of DispersinBTM and Triclosan (TCSN) combination-coated polyurethane catheters in plasma (tested against *Staphylococcus epidermidis*).

Figure 40 a bar graph showing the durability of inhibitory activity of DispersinBTM and Triclosan (TCSN) combination-coated polyurethane catheters in TSB containing 20% Bovine Serum (tested against *Staphylococcus aureus*).

25 Figure 41 is a line graph showing the durability of inhibitory activity of DispersinBTM and Triclosan (TCSN) combination-coated polyurethane catheters in TSB containing 20% Bovine Serum (tested against *Staphylococcus aureus*).

Figure 42 is a line graph showing the inhibitory activity of DispersinBTM and Triclosan (TCSN) combination coated silicone catheters in synthetic urine (tested against *Staphylococcus aureus*).

5 Figure 43 is a line graph showing the durability of DispersinBTM and Triclosan (TCSN) combination coated coated silicone catheters in synthetic urine.

Figure 44 is a bar graph showing the *in vivo* efficacy of DispersinBTM and Triclosan (TCSN) combination coated central venous catheters.

Figure 45 is a bar graph showing the effect of DispersinBTM and xylitol alone and in combination on *Staphylococcus epidermidis* biofilm formation.

10 Figure 46 is a bar graph showing the effect of DispersinBTM and glucose oxidase alone and in combination on *Staphylococcus epidermidis* biofilm formation.

Figure 47 is a bar graph showing the effect of DispersinBTM and N-(1-pyrenyl)malcimide (PyrM) alone and in combination on *Staphylococcus epidermidis* biofilm formation.

15 Figure 48 is a bar graph showing the effect of DispersinBTM and N,N-(1,2-phenylene)dimalcimide (oPDM), alone and in combination on *Staphylococcus epidermidis* biofilm formation.

Figure 49 is a bar graph showing the antibiofilm activity of DispersinBTM and cefamandole nafate (CFN) combination-coated catheters.

20 Figure 50 is a bar graph showing the antibiofilm activity of DispersinBTM and 5-fluorouracil (FU) combination-coated catheters.

Figure 51 is a bar graph showing the antibiofilm activity of DispersinBTM and sodium usnate (SU) combination-coated catheters.

25 Figure 52 is a bar graph showing the antibiofilm activity of DispersinBTM and benzalkonium chloride (BKC) combination-coated catheters.

Figure 53 is a bar graph showing the antibiofilm activity of DispersinBTM and chitosan combination-coated catheters.

Detailed Description

Definitions

The term "active fragment" refers to smaller portions of the DispersinB™ polypeptide that retains the ability to disperse bacteria or fungi.

- 5 The term "antimicrobial" means a compound or a composition that kills or slows/stops the growth of microorganisms, including, but not limited to bacteria and yeasts, and but not including agents which specifically disperse bacteria or fungi. Some examples of antimicrobials are triclosan, rifampicin, or cefamendole nafate.

- 10 The term "biofilm embedded microorganisms" refers to any microorganism that forms a biofilm during colonization and proliferation on a surface, including, but not limited to, gram-positive bacteria (e.g., *Staphylococcus epidermidis*), gram-negative bacteria (e.g., *Pseudomonas aeruginosa*), and/or fungi (e.g., *Candida albicans*).

The term "biofilm formation" means the attachment of microorganisms to surfaces and the subsequent development multiple layers of cells.

- 15 A "composition" refers to of this invention can comprise (a) DispersinB™, an active fragment or variant thereof that disperses a biofilm; and (b) an antimicrobial agent active against bacteria or fungi, optionally in combination with a physiologically acceptable carrier. The composition can further comprise an additional antimicrobial agent.

- 20 The term "detergent" is used to mean any substance that reduces the surface tension of water. A detergent may be a surface active agent that concentrates at oil-water interfaces, exerts emulsifying action and thereby aids in removing soils e.g., common sodium soaps of fatty acids. A detergent may be anionic, cationic, or nonionic depending on their mode of chemical action. Detergents include linear alkyl sulfonates (LAS) often aided by "builders." A LAS is preferably an alkyl benzene sulfonate ABS that is readily decomposed by
25 microorganisms (biodegradable). A LAS is generally a straight chain alkyl comprising 10 to 30 carbon atoms. A detergent may be in a liquid or a solid form.

A "viscosity increasing agent", "viscosity improving agent" or "gelling agent" refers to agents that increase viscosity thereby making compositions, such as wound gels, thick and stable. Examples of a viscosity improving agents include, but are not limited to, natural

products such as alginic acid, sodium alginate, potassium alginate, ammonium alginate, calcium alginate, agar, carrageenan, locust bean gum, pectin, gelatin, carboxymethyl cellulose (CMC), and chemically synthesized polymers, such as carbopol.

The term "disperse" or "disperse a biofilm" refers to individual bacterial or fungal cells detaching from a surface or detaching from a biofilm. The term "disperse" also refers to disaggregation of autoaggregating bacterial or fungal biofilm cells. "Disperses a biofilm" does not require all biofilm embedded microorganisms to detach, but rather a portion to detach from a surface or a biofilm.

The term "inhibition" or "inhibiting" refers to a decrease of biofilm associated microorganism formation and/or growth. The microorganisms can include bacteria (e.g., streptococci) or fungi (e.g., *Candida* spp.)

"Modulating detachment" as used herein, is meant to be inclusive of increases as well as decreases in bacterial or fungal biofilm detachment or release of bacterial or fungal cells from a biofilm. Further, "modulating detachment", is also meant to be inclusive of changes in the ability of the bacteria or fungal to attach as a biofilm. For example, as demonstrated herein, DispersinBTM modulates detachment of *S. epidermidis*, *Staphylococcus aureus* and *Escherichia coli* not only by promoting detachment but also by inhibiting the ability of the bacteria to attach to surfaces and form a biofilm.

The term "mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic, farm, sport and zoo animals, or pet animals, such as dogs, horses, cats, cattle, pigs, sheep, etc. Preferably, the mammal is human.

The term "therapeutically effective amount" refers to an amount of a composition of this invention effective to "alleviate" or "treat" a disease or disorder in a subject or mammal. A "therapeutically effective amount" as used herein includes a prophylactic amount, for example, an amount effective for preventing or protecting against infectious diseases, and symptoms thereof, and amounts effective for alleviating or treating infectious diseases, related diseases, and symptoms thereof. A "therapeutically effective amount" as used herein also includes an amount that is bacteriostatic or bacteriocidal, for example, an amount effective for inhibiting growth of biofilm associated bacteria or killing biofilm associated bacteria, respectively. A "therapeutically effective amount" as used herein also includes an amount that is fungistatic or fungicidal, for example, an amount effective for inhibiting

further growth of biofilm associated fungi or killing biofilm associated fungi, respectively. By administering a DispersinBTM compound suitable for use in methods of the invention concurrently with an antimicrobial compound, the therapeutic antimicrobial compound may be administered in a dosage amount that is less than the dosage amount required when the therapeutic antimicrobial compound is administered as a sole active ingredient. By administering lower dosage amounts of the active ingredient, the side effects associated therewith should accordingly be reduced.

The term "treatment", "treating", or "alleviating" refers to an intervention performed with the intention of preventing the development or altering the pathology of a disorder. Accordingly, "treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

A chronic wound defined herein is a wound that fails to progress through an orderly and timely sequence of repair or a wound that does not respond to treatment and/or the demands of treatment are beyond the patient's physical health, tolerance or stamina. Many wounds that are first considered to be acute wounds ultimately become chronic wounds due to factors still not well understood. One significant factor is the transition of planktonic bacteria within the wound to form a biofilm.

In the context of wound treatment, "biofilm disruption" or "inhibition of biofilm reconstitution" refers to biofilm clearance from a chronic or acute wound, or to inhibit reconstitution of a biofilm mass from remnants remaining after debridement and thereby promote healing of a wound.

A "wild type" or "reference" sequence or the sequence of a "wild type" or "reference" protein/polypeptide, such as a coat protein, or a CDR or variable domain of a source antibody, maybe the reference sequence from which variant polypeptides are derived through the introduction of mutations. In general, the "wild type" sequence for a given protein is the sequence that is most common in nature. Similarly, a "wild type" gene sequence is the sequence for that gene which is most commonly found in nature. Mutations may be introduced into a "wild type" gene (and thus the protein it encodes) either through natural processes or through man induced means. The products of such processes are "variant" or "mutant" forms of the original "wild type" protein or gene.

A "variant" of a polypeptide refers to a polypeptide that contains an amino acid sequence that differs from a wild type or reference sequence. A variant polypeptide can differ from the wild type or reference sequence due to a deletion, insertion, or substitution of a nucleotide(s) relative to said reference or wild type nucleotide sequence. The reference or wild type sequence can be a full-length native polypeptide sequence or any other fragment of a full-length polypeptide sequence. A polypeptide variant generally has at least about 80% amino acid sequence identity with the reference sequence, but may include 85% amino acid sequence identity with the reference sequence, 86% amino acid sequence identity with the reference sequence, 87% amino acid sequence identity with the reference sequence, 88% amino acid sequence identity with the reference sequence, 89% amino acid sequence identity with the reference sequence, 90% amino acid sequence identity with the reference sequence, 91% amino acid sequence identity with the reference sequence, 92% amino acid sequence identity with the reference sequence, 93% amino acid sequence identity with the reference sequence, 94% amino acid sequence identity with the reference sequence, 95% amino acid sequence identity with the reference sequence, 96% amino acid sequence identity with the reference sequence, 97% amino acid sequence identity with the reference sequence, 98% amino acid sequence identity with the reference sequence, 98.5% amino acid sequence identity with the reference sequence, 99% amino acid sequence identity with the reference sequence, or 99.5% amino acid sequence identity with the reference sequence.

"Percent (%) nucleic acid sequence identity" is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in a reference polypeptide-encoding nucleic acid sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software. Appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared can be determined by known methods.

For purposes herein, the % nucleic acid sequence identity of a given nucleic acid sequence C to, with, or against a given nucleic acid sequence D (which can alternatively be phrased as a given nucleic acid sequence C that has or comprises a certain % nucleic acid

sequence identity to, with, or against a given nucleic acid sequence D) is calculated as follows:

$$100 \text{ times the fraction } W/Z,$$

where W is the number of nucleotides scored as identical matches by the sequence alignment program in that program's alignment of C and D, and where Z is the total number of nucleotides in D. Where the length of nucleic acid sequence C is not equal to the length of nucleic acid sequence D, the % nucleic acid sequence identity of C to D will not equal the % nucleic acid sequence identity of D to C.

The term "protein" has an amino acid sequence that is longer than a peptide. A "peptide" contains 2 to about 50 amino acid residues. The term "polypeptide" includes proteins and peptides. Examples of proteins include, but are not limited to, antibodies, enzymes, lectins and receptors; lipoproteins and lipopolypeptides; and glycoproteins and glycopolypeptides.

A "phage coat protein" comprises at least a portion of the surface of the phage virus particle. Functionally, a coat protein is any protein that associates with a virus particle during the viral assembly process in a host cell and remains associated with the assembled virus until infection. A major coat protein is that which principally comprises the coat and is present in 10 copies or more copies/particle; a minor coat protein is less abundant. A phage coat protein may be a variant coat protein. Some variant coat proteins have improved display of the fused polypeptide.

A "fusion protein" and a "fusion polypeptide" refer to a polypeptide having two portions covalently linked together, where each of the portions is a polypeptide having a different property. The property may be a biological property, such as activity *in vitro* or *in vivo*. The property may also be a simple chemical or physical property, such as binding to a target antigen, catalysis of a reaction, etc. The two portions may be linked directly by a single peptide bond or through a peptide linker containing one or more amino acid residues. Generally, the two portions and the linker will be in reading frame with each other. Preferably, the two portions of the polypeptide are obtained from heterologous or different polypeptides.

The term "phage display" is a technique by which polypeptides are displayed as fusion proteins to at least a portion of coat protein on the surface of phage, e.g., filamentous phage, particles. A utility of phage display lies in the fact that large libraries of randomized protein variants can be rapidly and efficiently sorted for those sequences that bind to a target antigen with high affinity. Display of peptide and protein libraries on phage has been used for screening millions of polypeptides for ones with specific binding properties. Polyvalent phage display methods have been used for displaying small random peptides and small proteins through fusions to either gene III or gene VIII of filamentous phage (Wells & Lowman, *Curr. Opin. Struct. Biol.*, 3:355-362 (1992)).

"PCR" refers to the technique in which minute amounts of a specific piece of nucleic acid, RNA and/or DNA, are amplified as described in US Patent No. 4,683,195. PCR can be used to amplify specific RNA sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences, etc.

DNA is "purified" when the DNA is separated from non-nucleic acid impurities. The impurities may be polar, non-polar, ionic, etc.

The term "nucleic acid" as used herein includes (but is not limited to) unmodified RNA or DNA or modified RNA or DNA. Thus, by nucleic acid it is meant to be inclusive of single- and double- stranded DNA, DNA that is a mixture of single- and double- stranded regions, single- and double- stranded RNA, and RNA that is a mixture of single- and double- stranded regions, hybrid molecules containing DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. Further, the DNA or RNA sequences of the present invention may comprise a modified backbone and/or modified bases. A variety of modifications to DNA and RNA are known in the art for multiple useful purposes. The term "nucleic acid" as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of nucleic acids, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including simple and complex cells.

By the term "allelic variant" as used herein it is meant one of two or more alternative naturally occurring forms of a gene, each of which comprises a unique nucleic acid sequence. Allelic variants encompassed by the present invention encode proteins with similar or identical enzymatic activities.

DispersinBTM

Biofilm-embedded *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans* can release individual cells into liquid medium. These detached cells can attach to the surface of a culture apparatus and start a new colony. The *dspB* gene encodes a 381 amino acid soluble β -N-acetylglucosaminidase that is responsible for the detachment/dispersion of *A. actinomycetemcomitans*. This polypeptide is referred to as DispersinBTM. The first 20 amino acids are a signal peptide, and amino acids 21-381 are the mature polypeptide. The mature DispersinBTM polypeptide has the following sequence (SEQ ID NO:11; Accession No. AY228551.1):

```

10      1 nccvkgnsiy pqktstkqtg lmltdiarhfy speviksfid tislsggnfl hlfhsdheny
      61 aieshllnqr aenavqgkdg iyinpytgkp flsyrliddi kayakakgie lipeldspnh
      121 mtaifklvqk drgvkylqgl ksrqvdeid itnadsitfm qslmsevidi fgdtsgbfhi
      181 ggdefgysve snhefityan klstyflkkk lktrmwndgl ikntfeqinp nieitywayd
      241 gdtgdkneaa errdmrvslp ellakgftvl nynsylyiv pkasptfsqd aafaakdvik
15      301 nwdlgwdgr ntknrvqnth eiagaalsiw gedakaikde tiqkntksll eavinhktngd
      361 e

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The closely related *Actinobacillus pleuropneumoniae* also encodes a DispersinBTM, which is a 377 amino acid polypeptide that includes a signal peptide from amino acids 1 to 34. The *A. pleuropneumoniae* DispersinBTM has the following full polypeptide sequence (SEQ ID NO:12; Accession No. AY618481.1; AAT46094.1 GI:48727581):

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      1 mkkaitlftl lcavllsfst atyanamdlp kkesgltdi arrfytvdti kqfidtthqa
      61 ggtfihlhfs dhenyalese yleqreenat eknqtyfnpk tnkpfltykq lneiyyake
      121 rnleivpevd spnhmtaifd lltlkhgkey vkgkspya eeidinnpea veviktligc
25      181 viyifghser hfhiggdefe yavennhefi ryvntlnfdi nskglitrwv ndgliknls
      241 elnknieity wsydgdaqak ediqyrreir adlpellang fkvlnysy lyfvpksgen
      301 ihndgkyaae dvlennwtlgk wdgknssnhv qntqniigss lsiwgerssa lneqtiqqas
      361 knllkaviqk tndpksh

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Embodiments of the invention also include active fragments and variants of SEQ ID No:11 and SEQ ID No:12. DispersinBTM active fragments and variants only include those fragments and variants that retain an ability to disperse a bacterial or fungal cell from a biofilm.

A substrate for both DispersinBTM is a high-molecular weight hexosamine-containing extracellular polysaccharide adhesin encoded in the *pgaABCD* locus and *pgaCD* in *A. actinomycetemcomitans* and *A. pleuropneumoniae*, respectively (Kaplan et al., 2004, *J.*

Bacteriol. 186:8213-8220). These polysaccharide adhesins are a component of the *Aggregatibacter* biofilm. A PGA component of the biofilm functions as a protective barrier for cells of a biofilm. *Aggregatibacter* PGA is structurally and functionally similar to *E. coli* PGA and *S. epidermidis* PIA, both polysaccharides comprising N-acetyl-D-glucosamine residues in a $\beta(1,6)$ linkage (Kaplan et al., 2004). Thus, embodiments of this invention can be used to detach bacterial cells other than *A. actinomycetemcomitans* or *A. pleuropneumoniae*.

Nucleic acid sequences encoding orthologs of DispersinBTM protein have been identified in *A. ligniersii* strain 19393, *A. actinomycetemcomitans* strain IDH 781, *Haemophilus aphrophilus* strain NJ8700 and *A. pleuropneumoniae* strain IA5 and are depicted in SEQ ID No: 3, 5, 7, and 9, respectively. Accordingly, preferred isolated nucleic acid sequences of the present invention comprise SEQ ID No. 1, 3, 5, 7 or 9.

Also included within the present invention are allelic variants of the exemplified DispersinBTM nucleic acid sequence for SEQ ID No: 1, 3, 5, 7, or 9 encoding proteins with similar enzymatic activities to DispersinBTM and nucleic acid sequences with substantial percent sequence identity to the exemplified DispersinBTM nucleic acid sequences of SEQ ID NO: 1, 3, 5, 7 or 9 encoding proteins with similar enzymatic activities

There are similarities between the amino acid sequence of DispersinBTM and these orthologs and the consensus sequence of the family 20 glycosyl hydrolase. More specifically, amino acid residues 40 to 297 of the predicted DispersinBTM protein sequence are homologous to the catalytic domain of the family 20 glycosyl hydrolases (NCBI Conserved Domain Database accession Number pfam00728). This family of enzymes includes bacterial chitinases, chitobias and lacto-N-biosidases (Sano et al. *J. Biol. Chem.* 1993 268:18560-18566; Tews et al. *Gene* 1996, 170:63-67; Tsujibo et al. *Biochim. Biophys. Acta* 1998, 1425:437-440.), and eukaryotic hexosaminidases (Graham et al. *J. Biol. Chem.* 1988 263:16823-16829). A protein related to *A. actinomycetemcomitans* DispersinBTM is lacto-N-biosidase of *Lactococcus lactis* (GenBank accession no. AAK05592), which displays 28% identity over 281 amino acid residues not counting gaps and terminal extensions.

Similarity between DispersinBTM and lacto-N-biosidases is high in the regions surrounding Arg47 and the acidic amino acid pair Asp202 and Glu203. These residues have

been shown to participate in substrate binding and catalysis in other family 20 glycosyl hydrolases (Mark et al. J. Biol. Chem. 2001, 276:10330-10337; Mark et al. J. Biol. Chem. 1998, 273:19618-19624; Prag et al. J. Mol. Biol. 2000, 300:611-617). The C-terminal half of DispersinBTM contained three Trp residues that were conserved in *L. lactis* lacto-N-biosidase (positions 236, 279, and 353). Multiple Trp residues are present in the C-terminal regions of the catalytic domains of all family 20 glycosyl hydrolases (Graham et al. J. Biol. Chem. 1988, 263:16823-16829; Tews et al. Gene 1996, 170:63-67). These Trp residues line the part of the substrate binding pocket that is complementary to the hydrophobic surfaces of the hexosamine sugar ring (Tews et al. Nature Struct. Biol. 1996, 3:638-648). It is expected that mutation of amino acids in these regions of DispersinBTM and its orthologs will alter enzymatic activity.

In a preferred embodiment an isolated amino acid sequence of the present invention comprises SEQ ID NO: 2, 4, 6, 8, 10, 11 or 12 or an active fragment or variants thereof. Preferred active fragments are those comprising a portion of the amino acid sequence of SEQ ID NO: 2, 4, 6, 8, 10, 11 or 12 with similarities to the consensus sequence of the family 20 glycosyl hydrolase.

"Active variants or "functionally equivalent variants" as used herein are polypeptide sequences structurally different from the DispersinBTM protein, but having no significant functional difference from the protein. For example, when orthologous polypeptide sequences from various strains of *A. actinomycetemcomitans* are aligned, divergence in amino acid sequence is observed, usually 0 to 10 percent (Kaplan et al. Oral Microbiol. Immunol. December 2002, 17:354-359; Kaplan et al. Infect. Immun. 2001, 69:5375-5384). Proteins displaying this amount of divergence are considered functionally equivalent variants because of the fact that mixing of genetic alleles that encode these variants is often observed in populations (Kaplan et al. Oral Microbiol. Immunol. December 2002 17:354-359). The DispersinBTM sequence from *A. actinomycetemcomitans* strain IDH781 (SEQ ID NO:6), therefore, is expected to be a functionally equivalent or active variant of SEQ ID NO:2, and is included in the scope of the present invention. Similarly, DispersinBTM sequences from other strains of *A. actinomycetemcomitans*, such as those that exhibit different serotypes, restriction fragment length polymorphism genotypes, 16S ribosomal RNA genotypes, or arbitrarily-primed PCR genotypes that are commonly observed among phylogenetically diverse strains isolated from different subjects (Kaplan et al. J. Clin. Microbiol. 2002 40:1181-1187;

Kaplan et al., Oral Microbial. Immunol. December 2002 17:354-359), are also expected to be functionally equivalent or active variants of SEQ ID NO:2, and are included in the scope of the present invention.

Similarly, orthologous proteins from phylogenetically diverse species of bacteria are usually functionally equivalent or active variants, as evidenced by the fact that a common method for cloning genes of interest into plasmids is to screen a plasmid library for plasmids that complement a genetic mutation in a different species of bacteria (Kaplan et al. J. Mol. Biol. 1985 183:327-340). This is especially true of bacterial enzymes. Orthologous enzymes of different bacterial species can exhibit up to 50% divergence or greater, yet still utilize the identical substrate, catalyze the same chemical reaction, and produce the same product. This sequence divergence results from genetic drift coupled with fixation of selected genetic changes in the population. The genetic changes that are selected and fixed are those that alter characteristics of the enzyme other than substrate, reaction, and product, as for example, reaction rate, pH optimum, temperature optimum, level of expression, and interactions with other enzymes, such that these genetic changes confer upon a bacterial cell a selective advantage in its environment. Since *A. actinomycetemcomitans* is genetically closely related to *A. pleuropneumoniae* (Dewhirst et al. J. Bacteriol. 1992 174:2002-2013) and produces a biofilm similar to that produced by *A. actinomycetemcomitans*, which detaches upon contact with *A. actinomycetemcomitans* DispersinBTM, it is expected that the *A. pleuropneumoniae* DispersinBTM homologue identified in SEQ ID NO:10 is a functionally equivalent or active variant of SEQ ID NO:2, and is included in the scope of the present invention. Similarly, since *Actinobacillus lignieresii* is genetically closely related to *Actinobacillus pleuropneumoniae* (Dewhirst et al. J. Bacteriol. 1992 174:2002-2013) and *Haemophilus aphrophilus* is genetically closely related to *A. actinomycetemcomitans* (Dewhirst et al. J. Bacteriol. 1992 174:2002-2013; Kaplan et al. J. Clin. Microbiol. 2002 40:1181-1187), and since both *A. lignieresii* and *Haemophilus aphrophilus* produce biofilms similar to that produced by *A. actinomycetemcomitans*, it is expected that the *Actinobacillus lignieresii* and *Haemophilus aphrophilus* DispersinBTM homologues identified in SEQ ID NO:4 and SEQ ID NO:5, respectively, are functionally equivalent or active variants of SEQ ID NO:2, and are included in the scope of the present invention.

The above mentioned examples demonstrate functionally equivalent or active variants of *A. actinomycetemcomitans* DispersinBTM that occur in nature. As will be understood by

those of skill in the art upon reading this disclosure, however, artificially produced genes that encode functionally equivalent or active variants of *A. actinomycetemcomitans* DispersinBTM can also be produced routinely in accordance with the teachings herein using various well known genetic engineering techniques. For example, a genetically engineered dispersin enzyme that lacks 20 N-terminal amino acid residues, and also contained a 32 amino acid residue C-terminal tail, which is functionally equivalent to the natural DispersinBTM enzyme has been produced. It has also been shown that the methionine residue at the N-terminus of this genetically engineered DispersinBTM enzyme, when expressed in *E. coli*, was removed by the action of methionine aminopeptidase, yet the absence of the methionine did not affect enzyme activity. It has also been shown that cleavage of the C-terminal 28 amino acid residues from this genetically engineered DispersinBTM enzyme has no effect on enzyme activity. These examples demonstrate that artificial genes can be produced that encode functionally equivalent variants of *A. actinomycetemcomitans* DispersinBTM. These artificially produced functionally equivalent variants of *A. actinomycetemcomitans* DispersinBTM are included in the scope of the present invention.

The above mentioned examples demonstrate genetically-engineered, functionally equivalent variants of *A. actinomycetemcomitans* DispersinBTM that contain either a deletion of amino acid residues at the N-terminus of the protein, or the fusion of an additional polypeptide at the C-terminus of the protein. It is expected that other genetically-engineered alterations, such as the fusion of an additional polypeptide at the N-terminus of the protein, a deletion of amino acid residues at the C-terminus of the protein, internal deletions and insertions of amino acid residues, and amino acid substitutions, would also result in functionally equivalent variants of *A. actinomycetemcomitans* DispersinBTM. Information about which deletions, insertions, and amino acid substitutions would produce functionally equivalent variants of *A. actinomycetemcomitans* DispersinBTM can be obtained from amino acid sequence alignments, and from commonly available computer software that predicts polypeptide secondary structures based on both primary amino acid sequences and on amino acid sequence alignments with homologous proteins having known three-dimensional structures. *A. actinomycetemcomitans* DispersinBTM, for example, is a member of the family 20 glycosyl hydrolases, a family that includes several well-studied enzymes, and a family represented by numerous homologous primary amino acid sequences in the public databases. In some cases, three-dimensional structures of family 20 glycosyl hydrolases are known (Tews et al. Nature Struct. Biol. 1996 3:638-648).

All family 20 glycosyl hydrolases exhibit a $(\alpha\beta)_8$ -barrel motif (also known as a TIM-barrel motif; Tews et al., Nature Struct. Biol. 1996 3:638-648; Prag et al. J. Mol. Biol. 2000 300:611-617), which is by far the most common enzyme fold in the Protein Data Bank (PDB) database of known protein structures. It is estimated that 10% of all known enzymes have this domain (Wiercenge, R. K., FEBS Lett. 2001 492:193-198). The $(\alpha\beta)_8$ -barrel motif is seen in many different enzyme families, catalyzing completely unrelated reactions. The availability of numerous homologous primary amino acid sequences, combined with the availability of the three-dimensional structures of several *A. actinomycetemcomitans* DispersinBTM homologues, forms the basis of these sequence alignments and secondary structure predictions. For example, the $(\alpha\beta)_8$ -barrel motif consists of eight α -helices and eight β -strands such that eight parallel β -strands form a barrel on the inside of the protein, which are covered by eight α -helices on the outside of the protein. Based on the above mentioned protein sequence alignments and structural predictions, it is expected that the eight β -strands in *A. actinomycetemcomitans* DispersinBTM comprise the amino acid residues surrounding positions 41-44, 69-81, 130-134, 169-171, 189-200, 253-256, 288-300, and 348-360 of SEQ ID NO:2. Any alteration in the amino acid sequence that disrupts the β -strand architecture of these eight regions would be expected to result in a decrease in enzyme activity because of a concomitant disruption in the three-dimensional structure of the $(\alpha\beta)_8$ -barrel of the enzyme. Similarly, based on the above mentioned protein sequence alignments and structural predictions, it is expected that the eight α -helices in *A. actinomycetemcomitans* DispersinBTM comprise the amino acid residues surrounding positions 52-63, 89-93, 143-149, 176-183, 214-228, 269-284, 309-321, and 361-374 of SEQ ID NO:2. Any alteration in the amino acid sequence that disrupts the β -helical architecture of these eight regions would be expected to result in a decrease in enzyme activity because of a concomitant disruption in the three-dimensional structure of $(\alpha\beta)_8$ -barrel of the enzyme.

Similarly, because the α -strands consist of four inward pointing side chains (pointing into the $(\alpha\beta)_8$ -barrel) and four outward pointing side chains (pointing towards the α -helices), it is expected that alterations in the inward-pointing amino acid residues will reduce enzyme activity because of concomitant alterations to the substrate binding pocket inside the $(\alpha\beta)_8$ -barrel, and that alterations in the outward-pointing amino acid residues will reduce enzyme activity when they interfere with the interactions, between the β -strands and the α -helices. Similarly, the active site of family 20 glycosyl hydrolases is always located at the C-terminal end of the eight parallel β -strands of the barrel. It is expected that alterations in the

homologous region of *A. actinomycetemcomitans* DispersinBTM will affect enzyme activity. Similarly, it is predicted that the introduction of insertions and deletions into the regions between the α -helices and the β -strands, namely in positions 45-51, 64-68, 82-88, 94-129, 135-142, 150-168, 172-175, 182-188, 201-213, 229-252, 257-268, 285-287, 301-308, 322-347, and 351-360, in SEQ ID NO:2, will not effect enzyme activity. Similarly, it is expected that almost any alteration of residues 47 (Arginine), 203 (Aspartate) and 204 (Glutamate) will result in complete loss of enzyme activity, because these three residues have been shown to participate directly in substrate binding and catalysis in all family 20 glycosyl hydrolases (Mark et al. J. Biol. Chem. 1998 273: 19618-19624; Prag et al. J. Mol. Biol. 2000, 300:611-617; Mark et al., Biol. Chem. 2001 276:10330-10337). Similarly, it is expected that alteration of the three tryptophan residues at positions 236, 257 and 350, to any non-aromatic amino acid residue will result in a decrease in enzyme activity because these three tryptophan residues have been shown to line part of the substrate- binding pocket that is complementary to the hydrophobic surfaces of the substrate hexosamine sugar ring (Tews et al. Nature Struct. Biol. 1996 3:638-648). As a result of the locations of these essential amino acid residues in *A. actinomycetemcomitans* DispersinBTM, it is predicted that no more than 46 amino acid residues can be deleted from the N- terminus, and no more that 31 amino acids can be deleted from the C-terminus, without loss of enzyme activity. All of those genetic alterations that result in functionally equivalent variants are included in the scope of the present invention.

Genes encoding functionally different variants of *A. actinomycetemcomitans* DispersinBTM can also be produced in accordance with the teachings of the instant application using well known genetic engineering techniques. For example, as mentioned above, it is expected that almost any alteration of residues 47 (Arginine), 203 (Aspartate) and 204 (Glutamate) in SEQ ID NO:2 will result in complete loss of enzyme activity. Alternatively, variants of *A. actinomycetemcomitans* DispersinBTM that exhibit characteristics that may be useful in a clinical setting could also be artificially produced. For example, the temperature optimum of *A. actinomycetemcomitans* DispersinBTM is 30°C. It may be desirable to produce a genetically-engineered variant of DispersinBTM that exhibits a temperature optimum of 37°C, thereby resulting in an increased effectiveness of the enzyme or decreased cost of treatment, such variants can be artificially produced by first creating random mutations in the *A. actinomycetemcomitans* DispersinBTM gene sequence, for example by using UV light or a chemical mutagen like nitrosoguanidine and then screening large numbers of these random variants, for example in a quantitative 96-well microtiter plate

assay (Kaplan et al. J. Bacteriol. 2003 185:4693-4698), for ones that exhibit higher temperature optima. An alternative method is to utilize directed evolution of sequences by DNA shuffling (Christians et al. Nature Biotechnol. 1999 17:259-264; Dichek et al. J. Lipid Res. 1993 34:1393-1340), combined with a high-throughput robotic screen based upon a
5 quantitative 96-well microtiter plate assay (Kaplan et al. J. Bacteriol. 2003 185:4693-4698) to identify variants with increased temperature optima. The aforementioned methods can also be used to produce variants of *A. actinomycetemcomitans* DispersinBTM that exhibit increased substantivity to biomaterials, increased pH optima, increased stability in aqueous solutions, increased reaction rate, increased stability upon desiccation, and other characteristics that
10 could result in increased effectiveness of the enzyme or decreased cost of treatment. An alternative method that can be used to produce useful variants is site-directed mutagenesis. For example, it is expected that the eight α -helices of the ($\alpha\beta$)₈-barrel in *A. actinomycetemcomitans* DispersinBTM contain many amino acid residues that are exposed on the outer surface of the enzyme, and that altering the outward-pointing amino acid residues of
15 the eight α -helices will alter the outer surface properties of the enzyme, thereby potentially increasing the substantivity of the enzyme for biomaterials without affecting enzyme activity. Accordingly, these outward pointing amino acid residues can be systematically mutated, for example from polar residues to charged residues, and the resulting mutants screened to identify variants with increased substantivity to biomaterials. Functionally different variants
20 of *A. actinomycetemcomitans* DispersinBTM that are intended to improve the clinical efficiency or cost effectiveness of the enzyme, when applied to detaching bacterial or fungal cells from biofilms, are included in the scope of the present invention.

Compositions

Antibiofilm enzyme-based antimicrobial compositions comprising DispersinBTM or
25 an active fragment or variant thereof, and an antimicrobial agent, can inhibit biofilm formation as well as biofilm growth. In particular, a composition comprising DispersinBTM or an active fragment or variant thereof, and triclosan, a broad-spectrum antimicrobial has enhanced antibiofilm and antimicrobial activity. Such compounds are effective for inhibiting growth and proliferation of biofilm-embedded microorganisms, including both bacterial and
30 fungal species. An enhanced antimicrobial activity of antimicrobials used in combination with DispersinBTM enzyme is evidenced by the low concentration of each compound required to inhibit bacterial growth effectively. In particular, it is possible to use small amounts of

DispersinBTM, or an active fragment or variant thereof, which are biologically acceptable, and a small amount of triclosan, which is biologically acceptable at lower concentrations.

It will be appreciated that DispersinBTM or active fragments or variants thereof and antimicrobial agents can be used together in the form of a single composition in one
5 embodiment or together in the form of separate compositions for inhibiting growth and proliferation of biofilm-embedded microorganisms in another embodiment. In embodiments wherein separate compositions comprising DispersinBTM or an active fragment or variant thereof and antimicrobial agents are employed, the separate compositions can be used at the same time or sequentially. In a preferred embodiment, a composition comprising
10 DispersinBTM or an active fragment or variant thereof is administered separately to a biofilm to be treated followed by separate administration of a composition comprising an antimicrobial agent for inhibiting growth and proliferation of biofilm-embedded microorganisms. In a further preferred embodiment, the composition comprising an antimicrobial agent, comprises sodium doceyl sulfate, benzalkonium chloride or
15 chlorhexidine as the antimicrobial agent.

Accordingly, an embodiment of the present invention provides compositions for preventing growth and proliferation of biofilm embedded-microorganisms comprising: (a) DispersinBTM, an active fragment, or variant thereof; and (b) triclosan.

Also, other antimicrobials including, but not limited to, triclosan, antibiotics (such as
20 rifampicin, cefamandole nafate and ciprofloxacin) nitrofurazone, bismuth-thiols [such as bismuth ethanedithiol (BisEDT)], chitosan, epigallocatechin gallate (EGCG), sodium usnate, antineoplastic agents (such as 5-fluorouracil), detergents (such as sodium doceyl sulfate (SDS), benzalkonium chloride), chlorhexidine, chelating agents (such as EDTA), silver compounds, bacteriophage, antimicrobial enzymes (such as glucose oxidase and
25 lactoperoxidase), sugar alcohols (such as xylitol), maleimides [such as *N,N*-(1,2 phenylene) dimaleimide (oPDM) and *N*-(1-pyrenyl) maleimide (PyrM)], cadexomer iodine, methylene blue, gentian violet, medium chain dextrans (such as honey), and mixtures thereof can be used in combination with DispersinBTM.

An enhanced antimicrobial composition of the invention requires remarkably small
30 amounts of active ingredients (compared to that used in the past) to be effective against the microbial growth and biofilm formation. A composition according to the invention may have

properties that include those of separate compounds but go beyond them in efficacy and scope of application. Extremely low levels, and hence increased efficacy, of active compounds or ingredients, make embodiments of this invention very desirable and relatively economical to manufacture, although higher concentrations of these compounds can be used if it is desired for certain applications. A further advantage of using these compositions is the effectiveness for preventing growth of biofilm embedded bacteria and fungus, and in particular, bacterial and fungal species that colonize wounds.

DispersinBTM-based antimicrobial compositions of the invention can be used to inhibit the proliferation of biofilm-embedded gram-negative and gram-positive bacteria, which include, but are not limited to: *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, *Providentia stuartii*, *Serratia marcescens*, *Enterococcus faecalis*, Vancomycin Resistant *Enterococci* (VRE), *Peptostreptococcus* spp., *Corynebacterium* spp., *Clostridium* spp., *Bacteroides* spp., *Prevotella* spp., *Streptococcus pyogenes*, *Streptococcus viridans*, *Micrococcus* spp., β -hemolytic streptococcus (group C), Beta-hemolytic streptococcus (group B), *Bacillus* spp., *Porphyromonas* spp., *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Treponema denticola*, *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Staphylococcus saprophyticus*.

Additionally, DispersinBTM-based antimicrobial compositions of the invention can also be used to inhibit the proliferation of biofilm-embedded fungi, such as *Candida albicans*, *Candida parapsilosis*, and *Candida utilis*.

In one aspect, a DispersinBTM-based antimicrobial composition can treat various kinds of wounds, including, but not limited to, cutaneous abscesses, surgical wounds, sutured lacerations, contaminated lacerations, blister wounds, soft tissue wounds, partial thickness and full thickness burns, decubitus ulcers, stasis ulcers, leg ulcers, foot ulcers, venous ulcers, diabetic ulcers, ischemic ulcers, and pressure ulcers.

Another aspect includes methods of using DispersinBTM-based antimicrobial compositions in wound care devices including, but not limited to, non-resorbable gauze/sponge dressing, hydrophilic wound dressing, occlusive wound dressing, hydrogel wound and burn dressing, spray-applicator, and also in ointments, lotions, and suture.

Suitable substrates for receiving a topically applied DispersinBTM-based antimicrobial composition finish include, without limitation, fibres, fabrics, and alginates. A fabric may be formed from fibres such as synthetic fibres, natural fibres, or a combination thereof. Synthetic fibres include, for example, polyester, acrylic, polyamide, polyolefin, polyaramid, polyurethane, regenerated cellulose (i.e., rayon), and blends thereof. Suitable polymeric materials include but are not limited to silastic or other silicone-based material, polyethyleneterephthalate (PET), Dacron[®], knitted Dacron[®], velour Dacron[®], polyglacin, chromic gut, nylon, silk, bovine arterial graft, polyethylene (PE), polyurethane, polyvinyl chlorides silastic elastomer, silicone rubber, PMMA [poly-(methylmethacrylate)], latex, polypropylene (PP), polyolefin, cellulose, poly vinyl alcohol (PVA), poly(hydroxyethyl methacrylate (PHEMA), poly(glycolic acid), poly (acrylonitrile) (PAN), fluoroethylene-cohexa-fluoropropylene (FEP), Teflon[®] (PTFE), Cobalt-Chromium alloys, copolymers thereof and mixtures thereof.

A method of incorporating a therapeutically active DispersinBTM-based composition of the present invention into the polymeric material includes direct compounding of a therapeutically active substance into a plastic resin before casting or the like.

In addition, a DispersinBTM-based antimicrobial composition can further comprise binders, wetting agents, odour absorbing agents, levelling agents, adherents, thickeners, and the like. Other additives may also be present on and/or within a fabric of bandage including antistatic agents, optical brightening compounds, opacifiers (such as titanium dioxide), nucleating agents, antioxidants, UV stabilizers, fillers, permanent press finishes, softeners, lubricants, curing accelerators, adhesives, and the like.

In another embodiment, a DispersinBTM-based antimicrobial composition can include a detergent. A detergent may be anionic, cationic, or non-ionic. Detergents can include: sodium dodecyl sulfate (SDS) (also known as lauryl sulfate, sodium salt (other salts are also useful including lithium and potassium salts); sodium cocomonoglyceride sulfonate; sodium lauryl sarcosinate; sodium cholate; sodium deoxycholate; octylglucoside; dodecyldimethylamine oxide; 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS); dodecyltriethylammonium bromide (DTAB); cetyltrimethylammonium bromide (CTAB); polyoxyethylene-p-isooctylphenyl ether (e.g., Triton[®] X-20, Triton[®] X-100, Triton[®] X-114); alkyl sulfate; alkyl sulfonate; quaternary amines; octyldodecyldimethylammonium chloride; dioctyldimethylammonium chloride; didecyldimethylammonium chloride;

cetylpyridinium chloride; benzalkonium chloride; benzyldodecyldimethylammonium bromide; thonzonium bromide; cholic acid; chenodeoxycholic acid; glycodeoxycholic acid sodium salt; cremophor EL; N-Nonanoyl-N-methylglucamine; saponin; surfactin; protamine, and colistin.

5 Therapeutic Use for Treating Oral Infections

In an embodiment, a DispersinB[™]-based antimicrobial composition can treat an oral infection. Oral infections include microorganisms in the subgingival and supragingival plaque. Subgingival plaque comprises microorganisms can cause periodontal disease. Periodontal disease includes gingivitis, periodontitis, acute necrotizing ulcerative gingivitis
10 (ANUG), and localized juvenile periodontitis (LJP). Symptoms of periodontal disease include inflammation of the gingiva, deepening periodontal pockets, and alveolar bone loss.

A. actinomycetemcomitans is the principal etiologic agent of LJP and is considered a putative etiologic agent for generalized periodontitis, also referred to as adult periodontitis. *Prevotella intermedia* is considered the chief etiologic agent for ANUG and is also
15 considered a putative etiologic agent of adult periodontitis. *Porphyromonas gingivalis* is considered the main etiologic agent of chronic and severe adult periodontitis, but other microorganisms are thought to contribute to adult periodontitis as well. Other etiologic agents of periodontal diseases include *Fusobacterium nucleatum*, *Treponema denticola*, *Eikenella corrodens*, *P. nigrescens*, *Campylobacter rectus*, *Prevotella nigrescens*, and
20 *Bacteroides forsythus*.

In an embodiment, a DispersinB[™]-based composition can be used to treat oral infections. Preferably, an oral infection would include dental plaque that causes periodontal disease. In another embodiment, an oral infection includes *Streptococcus mutans*, the etiologic agent of caries.

25 In an embodiment, a method includes administering a composition comprising (a) DispersinB[™], an active fragment or variant thereof that disperses a biofilm; and (b) an antimicrobial agent. The antimicrobial agent can be an amount to kill or inhibit microorganisms that cause periodontal disease. In another embodiment, the antimicrobial agent can be an amount to kill or inhibit *S. mutans*.

A structural matrix established during biofilm formation can make colonizing cells able to withstand normal treatment doses of an antimicrobial. In a biofilm, a glycocalyx matrix serves as a barrier that protects and isolates microorganisms from antimicrobials and host defenses (e.g., antibodies, macrophages, etc.) (Costerton et al., 1981, *Ann. Rev. Microbiol.* 35:299-324). In one study, biofilm-associated bacteria were able to survive a concentration of antibiotic 20 times the concentration effective to eliminate the same species of bacteria grown in planktonic culture (Nickel et al., 1985, *Antimicrob. Agents Chemother.* 27:619-624). Higher doses of antimicrobials necessary to eliminate biofilm growth may not be well tolerated in a mammal, particularly a human. A DispersinBTM-based composition can overcome this structural protection of biofilm-embedded microorganisms. DispersinBTM can break up a biofilm matrix, whereby the antimicrobial then has access to the microorganisms.

Gel Formulations

In another embodiment, the present invention provides antibiofilm enzyme-based wound gel compositions comprising DispersinBTM, or an active fragment or variants thereof, and an antimicrobial agent, can inhibit biofilm formation as well as biofilm growth. In particular, a composition can include (a) DispersinBTM, an active fragment or a variant thereof, and (b) triclosan or a broad-spectrum antimicrobial. Such compositions are effective in inhibiting growth and proliferation of biofilm-embedded microorganisms, including both bacterial and fungal species. A composition can further comprise a viscosity improving agent.

Accordingly, an embodiment of the present invention provides wound gel compositions for: (a) DispersinBTM antimicrobial wound gel with a viscosity improving agent (gelling agent); and (b) Triclosan- DispersinBTM antimicrobial wound gel with a viscosity improving agent. In both the wound gels DispersinBTM or an active fragment or variants thereof could be used.

Also, other antimicrobials including, but not limited to, triclosan, antibiotics (such as rifampicin, cefamandole nafate and ciprofloxacin) nitrofurazone, bismuth-thiols [such as bismuth ethanedithiol (BisEDT)], chitosan, epigallocatechin gallate (EGCG), sodium usnate, antineoplastic agents (such as 5-fluorouracil), detergents (such as SDS, benzalkonium chloride), chlorhexidine, chelating agents (such as EDTA), silver compounds, bacteriophage, antimicrobial enzymes (such as glucose oxidase and lactoperoxidase), sugar alcohols (such as

xylitol), maleimides [such as *N,N*-(1,2 phenylene) dimaleimide (oPDM) and *N*-(1-pyrenyl) maleimide (PyrM)], cadexomer iodine, methylene blue, gentian violet, medium chain dextrans (such as honey), and mixtures thereof can be used in combination with DispersinBTM.

5 According to another embodiment, a Triclosan- DispersinBTM wound gel comprises about 1% triclosan. In a further embodiment, a DispersinBTM wound gel and a Triclosan- DispersinBTM wound gel can optionally further comprises a gelling agent and/or a viscosity improving agent.

10 Triclosan-DispersinBTM wound gel can be prepared in polyethylene glycol (PEG)/ethanol. PEG of molecular weights ranging between 200 and 511000 can be used in the gel formulation. According to another embodiment, a Triclosan- DispersinBTM wound gel is prepared in 10% polyethylene glycol (PEG) 400 plus 10% ethanol.

15 According to another embodiment, a viscosity increasing agent is an alginate based material. There are a number of suitable viscosity increasing agents available and, as previously indicated, preferred embodiments of the present invention will rely on gelling agents. A number of gelling agents are available including various gums and polysaccharides, alginates, and both synthetic and natural polymeric compounds. Such gelling agents are well known in the art, in particular in the food and medical arenas and will not be discussed in any specific detail herein apart from some representative examples given later herein. Some
20 useful prior art referencing the use of gelling agents in medical type applications include U.S. Pat. No. 4,948,575, U.S. Pat. No. 5,674,524, U.S. Pat. No. 5,197,954, U.S. Pat. No. 5,735,812, U.S. Pat. No. 5,238,685, U.S. Pat. No. 5,470,576, U.S. Pat. No. 5,738,860, U.S. Pat. No. 5,336,501, U.S. Pat. No. 5,482,932. Reference is made to these documents as a background to various viscosity increasing agents, which may find with the present invention.

25 A DispersinBTM based antimicrobial wound gel can be used to inhibit the proliferation of biofilm-embedded gram-negative and gram-positive bacteria, which include, but are limited to: *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, *Providentia sturtii*, *Serratia marcescens*, *Enterobacter cloacae*, *Enterococcus faecalis*, Vancomycin Resistant *Enterococci* (VRE),
30 *Peptostreptococcus* spp., *Corynebacterium* spp., *Clostridium* spp., *Bacteriodes* spp., *Prevotella* spp., *Streptococcus pyogenes*, *Streptococcus viridans*, *Micrococcus* spp., Beta-

hemolytic streptococcus (group C), Beta-hemolytic streptococcus (group B), *Bacillus* spp., *Porphyromonas* spp., *Staphylococcus epidermidis*, *S. aureus*, *S. agalactiae* and *S. saprophyticus*.

Additionally, a DispersinBTM based antimicrobial composition can also be used to
5 inhibit the proliferation of biofilm-embedded fungi, such as *Candida albicans*, *Candida parapsilosis*, and *Candida utilis*.

Use of Gel Formulations

DispersinBTM based antibiofilm gel formulations can be administered to subjects to inhibit biofilms. Such biofilms can include bacteria, fungi, or a mixture of bacteria and fungi.
10 Biofilms can be associated with wounds. Administration of a DispersinBTM based antibiofilm wound gel can also be achieved wherein a wound dressing or device comprises said DispersinBTM based antibiofilm gel formulations.

A DispersinBTM based antibiofilm gel formulation that is administered to treat a biofilm can also include an antimicrobial, such as triclosan. As further described in the
15 examples, a triclosan-DispersinBTM antibiofilm formulation significantly, if not totally, ablates biofilm growth and/or survival.

In one aspect, a DispersinBTM based antibiofilm wound gel can be used for treating a wound that includes but is not limited to, a cutaneous abscess, surgical wound, sutured laceration, contaminated laceration, blister wound, soft tissue wound, partial thickness burn,
20 full thickness burn, decubitus ulcer, stasis ulcer, foot ulcer, venous ulcer, diabetic ulcer, ischemic ulcer, pressure ulcer, or combinations thereof.

A wound gel is preferably applied following wound debridement. Although biofilm bacteria cannot be completely eradicated from a wound area by debridement, decreasing biofilm mass and providing increased exposure of the debrided tissue and remaining biofilm
25 bacteria to a wound gel increases wound healing. The slough that fills a chronic wound, previously thought to be comprised of dead cells, cellular debris, bacteria, and tissue fluid, has recently been demonstrated to be comprised primarily of a mixed-species bacterial biofilm. It is therefore of benefit to debride the slough from the wound as completely as possible. Debridement can be performed by surgical, mechanical, autolytic, enzymatic, or a
30 combination of means known to those of skill in the art of wound care.

A wound gel could be applied on chronic wounds along with systemic administration of antibiotics. At present antibiotics are not effective against some chronic wounds as biofilm embedded cells are more resistant to antibiotics. Application of a wound gel with antibiofilm activity will disrupt biofilm embedded cells and systemically administered antibiotics will kill dispersed cells. Therefore, a wound gel of present invention will improve the activity of antibiotics.

A DispersinBTM wound gel could be used sequentially along with antimicrobial agents, which are not compatible with enzymes such as detergents. A DispersinBTM wound gel can be applied on wounds first to disperse biofilm embedded cells and then antimicrobial agents.

A wound gel of the present invention utilizes alginate salts to form a product of the desired viscosity (e.g. gel, putty or pliable sheet, etc.). Alginates appear to be especially suitable for use with a wound gel since physical properties of a gel product appear to be relatively easily controlled. Introduction of polyvalent cations helps to form a gel product of desired consistency. Any moulding, extruding, or forming processes should also be performed at this time so that a final product could be formed into desired configuration. Machining (e.g. slicing) into a final form, such as sheets cut from a block, can also be incorporated into any manufacturing process.

Alginates can also have other potentially realisable advantages by introducing cations or cations that are already a part of the selected alginate. For instance, calcium containing alginates may be selected where there is bleeding, as calcium can promote blood clotting. Another example of advantageous cation exchange by an alginate includes alginate fibre dressings that are high in mannuronic acid, wherein the fibre dressings can readily exchange calcium ions for sodium ions. This increases fluid uptake by the dressing, which consequently forms a soft gel that can be easily flushed away with saline. Fibre dressings high in guluronic acid form stronger gels that keep their shape, making removal in one piece possible.

Alginates can exhibit gelling and cross linking properties promoted by the presence of polyvalent cations. These often tend to form tougher and less soluble alginate materials and thus may find use in a number of products for altering physical characteristics. Such a

modification can be used for a sheet-like embodiment, particularly as a way of increasing the strength or solubility properties of a resulting sheet.

Polyvalent cations may be introduced in a number of ways, including introduction of a soluble solution of polyvalent cations during the blending procedure. Preferably, this should be after gelling of a blend has been initiated to avoid thickening reactions, which interfere with the dispersion and hydrating of all of the sodium (or other) alginate being blended with DispersinB™ and triclosan. However, adding polyvalent cations at different points can theoretically substantially alter the characteristics of the resulting product and thus a number of options open to the user to allow them to tailor the physical characteristics of products according to the intended end use and user requirements. It is anticipated that soluble calcium salts, such as calcium chloride, may be introduced at relatively low concentrations to promote the various gelling and cross reactions.

Sheets from wound gels can be formed by placing wound gel in between sheets of a non-wettable material and rolling it to uniform thickness. As a variation, a gauze fabric or other suitable material may be placed on top of a lower non-wettable sheet prior to pouring a wound gel. The rolling procedure is completed with a sheet-like gel bonded to gauze. Various materials could be used to apply DispersinB™ based wound gel including, without limitations, fibres, and fabrics. A fabric may be formed from fibres such as synthetic fibres, natural fibres, or combinations thereof. Synthetic fibres include, for example, polyester, acrylic, polyamide, polyolefin, polyaramid, polyurethane, regenerated cellulose (i.e. rayon), and blends thereof. Suitable polymeric materials include but are not limited to silastic or other silicone-based material, polyethyleneterephthalate (PET), Dacron®, kitted Dacron®, velour Dacron®, polyglacin, chromic gut, nylon, silk, bovine arterial graft, polyethylene (PE), polyurethane, polyvinyl chlorides silastic elastomer, silicone rubber, PMMA[poly-(methylmethacrylate), latex, polypropylene (PP), polyolefin, cellulose, poly vinyl] alcohol (PVA), poly(hydroxymethyl) methacrylate (PHEMA), Poly(glycolic acid), poly(acrylonitrile) (PAN), fluorooctylenc-cohexa-fluoropropylene (FEP), Teflon® (PTFE), Cobalt-Chromium alloys, copolymers thereof and mixtures thereof.

Other potentially useful gelling agents include hydrocolloids and hydrogels. These components tend to absorb moisture to form a moist healing environment and tend to absorb less fluid than the alginates. Consequently it is envisaged that they would not be used for embodiments for heavily exuding wounds in which alginates would tend to offer better

performance. However, it is envisaged that combinations of various viscosity increasing agents may be used in particular embodiments, particularly each imparts a slightly difference property which helps fulfil a particular specification required by the user. For instance the hydrocolloids or hydrogels may be incorporated into gelling blends to vary properties such as the amount of fluid absorbed from a wound, etc.

In addition, DispersinBTM based wound gels can further comprise binders, wetting agents, odour absorbing agents, levelling agents, adherents, thickeners, coupling agents, pH adjusters, and the like.

A formulation of the present invention may be used for human wound therapy or for veterinary use. A formulation may be applied topically to one or more wounds of, for example, a dog, cat, or other mammal. A formulation may be applied to a bite wound to protect a human from developing an ulcerated wound as the result of infection (often with biofilm fragments from the mouth of the animal).

Compositions of the invention can also include quorum sensing inhibitors (QSIs). Quorum sensing is a means of communication between bacteria, most notably in a biofilm. Quorum sensing is mediated by *N*-acyl-homoserine lactones (AHLs) in gram-negative bacteria and mostly through small peptides in gram positive bacteria (March & Bentley, *Curr. Opin. Biotechnol.* 15: 495-502 (2004)). Quorum sensing inhibitors can inhibit AHL expression, dissemination, and signal reception. For instance, the *Bacillus* enzyme AiiA hydrolyzes AHLs (Dong et al., *Proc. Natl. Acad. Sci. USA* 97: 3526-3531 (2000)). Other QSIs can include AHL analogs that compete and/or interfere with AHL binding to a receptor (e.g., LuxR). These antagonists AHLs can include AHLs with a longer acyl side chains (e.g., extended with at least one methylene), AHLs with decreased acyl side chain rotation (e.g., introduction of an unsaturated bond close to the amide linkage), or a substitution to the phenyl ring (e.g., para-bromo). Other QSIs include furanone compounds (Wu et al., *J. Antimicrob. Chemother.* 53: 1054-1061 (2004)) such as (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone (Jones et al., *J. Infect. Dis.* 191: 1881-1888 (2005)), 4-nitro-pyridine-*N*-oxide, garlic extract, *p*-benzoquinone, 2,4,5-tri-bromo-imidazole, 3-amino-benzen-sulfonamide, and 3-nitro-benzen-sulfonamide (Rasmussen et al., *J. Bacteriol.* 187: 1799-1814 (2005)).

Compositions of the invention can also include RNAIII inhibitory peptide (RIP) (U. S. Pat. No. 6,291,431). RIP is a heptapeptide (YSPWTNF-NH₂; SEQ ID NO: 5) that inhibits *S. aureus* and *S. epidermidis* adhesion to surfaces (e.g., epithelial cells, polymers).

Compositions can also include bacterial transcription inhibitors which are known to be active
5 against biofilms (Guillot et al., 2007, *Antimicrob. Agents Chemother.* 51:3117-3121).

Methods to modulate biofilm detachment can include DispersinBTM and other molecules mentioned above. DispersinBTM can be administered to a biofilm concurrently or prior to administering QSIs and/or an antimicrobial. Further, a combination of DispersinBTM and QSIs can be administered concurrently or prior to administering an antimicrobial.

10 Therapeutic Delivery of Bacteriophages

"Bacteriophage" or "phage" are viruses that infect bacteria. Many phage have the ability to lyse bacteria, usually occurring after viral assembly is completely so fully assembled virus can exit the host cell.

Phage display is a system in which a protein and small peptides are displayed on the
15 surface of a phage as a fusion with one of the coat proteins of the virus. Phage display is a powerful tool that allows the discovery and characterization of proteins that interact with a desired target. Phage display peptide libraries are produced with billions of unique displayed proteins (see, e.g., U.S. Patent No. 5,702,892). Phage display libraries are well known and extensively used to investigate ligand-receptor binding.

20 Due to accessibility to solvents, displayed proteins and peptides frequently adopt their native conformation, and behave essentially as it is not attached to the surface of a phage. Therefore, most proteins and peptides attached to phage surfaces are biologically active, and can be used directly without time consuming purification and refolding steps that is otherwise needed for proteins expressed using bacterial and eukaryotic expression systems.

25 Using phages as antimicrobial agents for infection control has been demonstrated in animal models for *Escherichia coli* (Merrill et al., *Proc. Natl. Acad. Sci. USA* (1996) 93: 3188-3192; Smith & Huggins, *J. Gen. Microbiol.* (1982) 128: 307-318; Smith & Huggins, *J. Gen. Microbiol.* (1983) 129: 2659-2675; Smith et al., *J. Gen. Microbiol.* (1987) 133: 1111-1126; Smith et al., *J. Gen. Microbiol.* (1987) 133: 1127-1135); *Pseudomonas aeruginosa*
30 (Soothill, *J. Med. Microbiol.* (1992) 37: 258-261; Soothill, *Burns* (1994) 20: 209-211);

Salmonella enterica serovar *typhimurium* (Berchieri et al., *Res. Microbiol.* (1991) 142: 541-549); *Staphylococcus aureus* (Matsuzaki et al., *J. Infect. Dis.* (2003) 187: 613-624); and *Vibrio vulnificus* (Cervený et al., *Infect. Immun.* (2002) 70: 6251-6262). Using bacteriophage to treat biofilm-associated infection, however, is problematic because a biofilm acts as a diffusion barrier to the phage (Doolittle et al., *Can. J. Microbiol.* (1995) 41: 12-18; Doolittle et al., *Ind. J. Microbiol.* (1996) 16: 331-341).

Routes of administration of phage therapy include but are not limited to: oral, aerosol or other device for delivery to the lungs, nasal spray, intravenous, intramuscular, intraperitoneal, intrathecal, vaginal, rectal, topical, lumbar puncture, intrathecal, and direct application to the brain and/or meninges. Excipients which can be used as a vehicle for the delivery of phage are well known. For example, free phage could be in lyophilized form and be dissolved just prior to administration by IV injection. Dosage of administration is contemplated to be about 10^6 pfu/kg/day, about 10^7 pfu/kg/day, about 10^8 pfu/kg/day, about 10^9 pfu/kg/day, about 10^{10} pfu/kg/day, about 10^{11} pfu/kg/day, about 10^{12} pfu/kg/day, or about 10^{13} pfu/kg/day. Phage can be administered until successful elimination of pathogenic bacteria is achieved.

With respect to aerosol administration, antimicrobial phage can be incorporated into an aerosol formulation specifically designed for administration to the lungs by inhalation. Many such aerosols are well known, and the present invention is not limited to any particular formulation. An example of such an aerosol is the Proventil™ inhaler manufactured by Schering-Plough, the propellant of which contains trichloromonofluoromethane, dichlorodifluoromethane, and oleic acid. Concentrations of propellant ingredients and emulsifiers are adjusted if necessary based on the phage being used in the treatment. The number of phage to be administered per aerosol treatment can be about 10^6 pfu, about 10^7 pfu, about 10^8 pfu, about 10^9 pfu, about 10^{10} pfu, about 10^{11} pfu, about 10^{12} pfu, or about 10^{13} pfu.

Treatment of Devices

In a further embodiment, a composition(s) of the present invention can be used to inhibit the growth and proliferation of biofilm embedded microorganisms on devices, and in particular, medical devices. The compositions of the present invention can be used in the preparation of medical devices for implantation in a mammal. A medical device to be

implanted can be coated, incorporated or treated with a composition(s) of the present invention. A composition(s) of the present invention can also be used to prevent infections caused by an implanted medical device, including but not limited to urinary tract infections and vascular infections.

5 In one embodiment, a composition comprises DispersinBTM or an active fragment thereof in combination with triclosan. An amount of DispersinBTM included in a composition is preferably between about 0.1 and 500 µg/ml and more preferably about 40 µg/ml. The higher end of this range can be used to prepare a concentrated product which may be diluted prior to use. The amount of triclosan included in a composition is preferably between about
10 0.1 and 100 mg/ml and more preferably about 10 mg/ml. The higher end of this range can be used to prepare a concentrated product which may be diluted prior to use.

 In another embodiment of the present invention, the composition comprises effective amounts of DispersinBTM and rifampicin. In yet another embodiment of the present invention, the composition comprises effective amounts of DispersinBTM and cefamandole
15 nafate. In yet another embodiment of the present invention, the composition comprises effective amounts of DispersinBTM and nitrofurazone.

 Higher concentrations of a compound can be used for certain applications depending on targeted bacteria and a device to be treated. Suitable working concentrations can easily be determined using known methods.

20 In an embodiment of the present invention, wound dressings including but not limited to sponges or gauzes can be impregnated with the isolated DispersinBTM protein or active fragment or variant thereof to prevent or inhibit bacterial or fungal attachment and reduce the risk of wound infections. Similarly, catheter shields as well as other materials used to cover a catheter insertion sites can be coated or impregnated with a DispersinBTM protein or active
25 fragment or variant thereof to inhibit bacterial or fungal biofilm attachment thereto. Adhesive drapes used to prevent wound infection during high risk surgeries can be impregnated with the isolated protein or active fragment or variant thereof as well. Additional medical devices which can be coated with a DispersinBTM protein or active fragment or variant thereof include, but are not limited, central venous catheters, intravascular catheters,
30 urinary catheters, Hickman catheters, peritoneal dialysis catheters, endotracheal catheters, mechanical heart valves, cardiac pacemakers, arteriovenous shunts, scleral buckles,

prosthetic joints, tympanostomy tubes, tracheostomy tubes, voice prosthetics penile prosthetics, artificial urinary sphincters, synthetic pubovaginal slings, surgical sutures, bone anchors, bone screws, intraocular lenses, contact lenses, intrauterine devices, aortofemoral grafts and vascular grafts. Exemplary solutions for impregnating gauzes or sponges, catheter shields and adhesive drapes or coating catheter shields and other medical devices include, but are not limited to, phosphate buffered saline (pH approximately 7.5) and bicarbonate buffer (pH approximately 9.0). In yet another embodiment, an isolated DispersinBTM protein or active fragment or variant thereof can be incorporated in a liquid disinfecting solution. Such solutions may further comprise antimicrobials or antifungals such as alcohol, providone-iodine solution and antibiotics as well as preservatives. These solutions can be used, for example, as disinfectants of the skin or surrounding area prior to insertion or implantation of a device such as a catheter, as catheter lock and/or flush solutions, and as antiseptic rinses for any medical device including, but not limited to catheter components such as needles, Leur-Lok[®] connectors, needleless connectors and hubs as well as other implantable devices. These solutions can also be used to coat or disinfect surgical instruments including, but not limited to, clamps, forceps, scissors, skin hooks, tubing, needles, retractors, scalers, drills, chisels, rasps and saws. In a preferably embodiment, the composition comprising DispersinBTM, an active fragment, or a variant thereof, and triclosan, is used to coat a medical device, such as a catheter. Alternatively, the composition comprising DispersinBTM, an active fragment or a variant thereof, and triclosan, can be incorporated into the medical device as it is being made, for example, through an extrusion process. Compositions of the invention can be prepared using known methods. Generally, components are dissolved in a suitable solvent, such as water, glycerol, organic acids, and other suitable solvents

Compositions of the invention useful for the treatment of devices may include any number of well known active components and base materials. Such compositions may further comprise ingredients such as, but not limited to: suitable solvents such as water; antibiotics such antibacterials and antifungals; binding, bonding, or coupling agent, cross-linking agent; or a pH adjuster.

Compositions of the invention useful for the treatment of devices may further comprise additional antimicrobial ingredients such as bis-phenols, biguanides, anilides, diamidines, halogen-releasing agents, metallic ions, chelating agents, cationic peptides/polypeptides, N-substituted maleimides, and quaternary ammonium compounds.

Examples of bis-phenols useful for preparing compositions of the present invention include, but are not limited to, triclosan and hexachlorophene. Examples of biguanides useful for preparing compositions of the present invention include, but are not limited to, chlorhexidine, chlorhexidine salts, alexidine and polymeric biguanides. Examples of anilides useful for preparing compositions of the present invention include, but are not limited to, triclocarban. Examples of diamidines useful for preparing compositions of the present invention include, but are not limited to, propamidine and dibromopropamidine. Examples of halogen-releasing agents useful for preparing compositions of the present invention include, but are not limited to, iodine compounds, silver compounds, silver nanoparticles and halophenols. Examples of metallic ions useful for preparing compositions of the present invention include, but are not limited to, gallium and other related metal derivatives. Examples of chelating agents useful for preparing compositions of the present invention include, but are not limited to, lactoferrin, ovotransferrin, scrotransferrin, EDTA and EGTA. Examples of cationic peptides/polypeptides useful for preparing compositions of the present invention include, but are not limited to, protamine sulfate, lysozyme and polylysine. Examples of N-maleimides useful for preparing compositions of the present invention include, but are not limited to: N-ethylmaleimide (NEM), 5,5-dithiobis-(2-nitrobenzoic acid)(DTNB), N-phenylmaleimide (PhcM), N-(1-pyrenyl) maleimide (PyrM), naphthalene-1,5-dimalcicide (NDM), N,N'-(1,2-phenylene) dimaleimide (oPDM), N,N'-1,4-phenylene dimaleimide (pPDM), N,N'-1,3-phenylene dimaleimide (mPDM), and 1,1-(methylenedi-4,1-phenylene) bismaleimide(BM). Examples of quaternary ammonium compounds useful for preparing compositions of the present invention include, but are not limited to benzalkonium chloride, tridodecyl methyl ammonium chloride, cetrimide and didecyl dimethyl ammonium chloride.

Other possible components of the composition include, but are not limited to, buffer solutions, phosphate buffered saline, saline, polyvinyl, polyethylene, polyurethane, polypropylene, silicone (e.g., silicone lassosers and silicone adhesives), polycarboxylic acids, (e.g., polyacrylic acid, polymethacrylic acid, polymaleic acid, poly-(maleic acid monoester), polyaspartic acid, polyglutamic acid, aginic acid or pectimic acid), polycarboxylic acid anhydrides (e.g., polymaleic anhydride, polymethacrylic anhydride or polyacrylic acid anhydride), polyamines, polyamine ions (e.g., polyethylene imine, polyvinylamine, polylysine, poly-(dialkylamineoethyl methacrylate), poly-(dialkylaminomethyl styrene) or poly-(vinylpyridine), polyammonium ions (e.g., poly-(2-methacryloxyethyl trialkyl ammonium ion), poly-(vinylbenzyl trialkyl ammonium ions), poly-(N,N-alkylpyridinium

ion) or poly-(dialkyl octamethylene ammonium ion) and polysulfonates (e.g. poly-(vinyl sulfonate) or poly-(styrene sulfonate), collodion, nylon, rubber, plastic, polyesters, DacronTM (polyethylene terephthalate), TeflonTM (polytetrafluoroethylene), latex, and derivatives thereof, elastomers and Dacron (sealed with gelatin, collagen or albumin, cyanoacrylates, methacrylates, papers with porous barrier films, adhesives, e.g., hot melt adhesives, solvent based adhesives, and adhesive hydrogels, fabrics, and crosslinked and non-crosslinked hydrogels, and any other polymeric materials which facilitate dispersion of the active components and adhesion of the biofilm penetrating coating to at least one surface of the medical device. Linear copolymers, cross-linked copolymers, graft polymers, and block polymers, containing monomers as constituents of the above-exemplified polymers may also be used.

Examples of biofilm embedded bacteria that may be inhibited using compositions according to the invention include gram-negative bacteria such as, but not limited to: *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, *Providentia stuartii*, or *Serratia marcescens* and gram-positive bacteria such as, but not limited to: *Enterococcus faecalis*, Vancomycin Resistant *Enterococci* (VRE), *Streptococcus viridans*, *Staphylococcus epidermidis*, and *Staphylococcus aureus* or *Staphylococcus saprophyticus*. These bacteria are commonly found associated with medical devices including catheters.

Compositions according to the invention can also be used to inhibit the growth and proliferation of biofilm embedded fungus such as *Candida albicans*, *Candida parapsilosis*, and *Candida utilis*. In another aspect, the present invention provides a method of preparing a device comprising treating at least one surface of the device with an effective amount of DispersinBTM, an active fragment or variant thereof, and an effective amount of triclosan, according to the invention.

The term "effective" refers to a sufficient amount of active components to substantially prevent growth or proliferation of biofilm embedded microorganisms on at least one surface of a medical device coated with an embodied composition; and as a sufficient amount of the active components to substantially penetrate, or break-up, a biofilm on at least one surface of a medical device, thereby facilitating access of active components, antimicrobial agents, and/or antifungal agents to microorganisms embedded in a biofilm, and thus, removal of substantially all microorganisms from at least one surface of a medical

device treated with a solution of an embodied composition. An amount will vary for each active component and upon known factors such as pharmaceutical characteristics; type of medical device; degree of biofilm embedded microorganism contamination; and use and length of use.

- 5 Examples of devices that can be treated using the compositions of the invention include medical devices such as tubing and other medical devices, such as catheters, pacemakers, prosthetic heart valves, prosthetic joints, voice prostheses, contact lenses, and intrauterine devices.

- 10 Medical devices include disposable or permanent or indwelling catheters, (e.g., central venous catheters, dialysis catheters, long-term tunneled central venous catheters, short-term central venous catheters, peripherally inserted central catheters, peripheral venous catheters, pulmonary artery Swan-Ganz catheters, urinary catheters, and peritoneal catheters), long-term urinary devices, tissue bonding urinary devices, vascular grafts, vascular catheter ports, wound drain tubes, ventricular catheters, hydrocephalus shunts heart valves, heart
15 assist devices (e.g., left ventricular assist devices), pacemaker capsules, incontinence devices, penile implants, endotracheal tubes, small or temporary joint replacements, urinary dilator, cannulas, elastomers, hydrogels, surgical instruments, dental instruments, tubings, such as intravenous tubes, breathing tubes, dental water lines, dental drain tubes, and feeding tubes, fabrics, paper, indicator strips (e.g., paper indicator strips or plastic indicator strips),
20 adhesives (e.g., hydrogel adhesives, hot-melt adhesives, or solvent-based adhesives), bandages, wound dressings, orthopedic implants, and any other device used in the medical field.

- Medical devices also include any device which may be inserted or implanted into a human being or other animal, or placed at the insertion or implantation site such as the skin
25 near the insertion or implantation site, and which include at least one surface which is susceptible to colonization by biofilm embedded microorganisms.

 Medical devices for the present invention include surfaces of equipment in operating rooms, emergency rooms, hospital rooms, clinics, and bathrooms.

- Implantable medical devices include orthopedic implants, which may be inspected for
30 contamination or infection by biofilm embedded microorganisms using endoscopy.

Insertable medical devices include catheters and shunts, which can be inspected without invasive techniques such as endoscopy.

Medical devices may be formed of any suitable metallic materials or non-metallic materials. Examples of metallic materials include, but are not limited to, titanium, and stainless steel, and derivatives or combinations thereof. Examples of non-metallic materials include, but are not limited to, thermoplastic or polymeric materials such as rubber, plastic, polyesters, polyethylene, polyurethane, silicone, GortexTM (polytetrafluoroethylene), DacronTM (polyethylene tetrathalate), TeflonTM (polytetrafluoroethylene), latex, elastomers, and DacronTM sealed with gelatin, collagen, or albumin, and derivatives or combinations thereof.

In a preferred embodiment, the method of treating at least one surface of a medical device comprises contacting a medical device with a composition according to the invention. As used herein, the term "contacting" includes, but is not limited to: coating, spraying, soaking, rinsing, flushing, submerging, and washing. A medical device is contacted with a composition for a period of time sufficient to remove substantially all biofilm embedded microorganisms from a treated surface of a medical device.

In a more preferred embodiment, a medical device is submerged in a composition for at least 5 minutes. Alternatively, a medical device may be flushed with a composition. In the case of a medical device being tubing, such as dental drain tubing, a composition may be poured into a dental drain tubing and both ends of the tubing clamped such that the composition is retained within the lumen of the tubing. The tubing is then allowed to remain filled with the composition for a period of time sufficient to remove substantially all of the microorganisms from at least one surface of the medical device, generally, for at least about 1 minute to about 48 hours. Alternatively, tubing may be flushed by pouring a composition into the lumen of the tubing for an amount of time sufficient to prevent substantial growth of all biofilm embedded microorganisms. Concentrations of active components in a composition may vary as desired or necessary to decrease the amount of time the composition is in contact with a medical device.

In another embodiment of a method for treating a surface of a device, a composition of the invention may also include an organic solvent, a medical device material penetrating agent, or adding an alkalizing agent to the composition, to enhance reactivity of a surface of

the medical device with the composition. An organic solvent, medical device material penetrating agent, and/or alkalizing agent are those which preferably facilitate adhesion of a composition to at least one surface of a medical device.

Another aspect provides a method of coating a composition of the invention onto at least one surface of a device. Preferably, the device is a medical device. Broadly, a method for coating a medical device includes the steps of providing a medical device; providing or forming a composition coating; and applying the composition coating to at least one surface of the medical device in an amount sufficient to substantially prevent growth or proliferation of biofilm embedded microorganisms on at least one surface of the medical device. In one specific embodiment, a method for coating a medical device includes the steps of forming a composition of the invention of an effective concentration for activating an active component, thereby substantially preventing growth or proliferation of microorganisms on at least one surface of the medical device, wherein the composition of the invention is formed by combining an active component and a base material. At least one surface of a medical device is then contacted with a composition of the invention under conditions wherein the composition of the invention covers at least one surface of the medical device. The term "contacting" further includes, but is not limited to: impregnating, compounding, mixing, integrating, coating, spraying and dipping.

In another embodiment of a method for coating a medical device, a composition coating is preferably formed by combining an active component and a base material at room temperature and mixing the composition for a time sufficient to evenly disperse active agents in the composition prior to applying the composition to a surface of the device. A medical device may be contacted with a composition for a period of time sufficient for a composition to adhere to at least one surface of the device. After a composition is applied to a surface of a device, it is allowed to dry.

A device is preferably placed in contact with a composition by dipping the medical device in the composition for a period of time ranging from about 30 seconds to about 180 minutes at a temperature ranging from about 25°C to about 60°C. Preferably, a device is placed in contact with a composition by dipping the medical device in the composition for about 60 minutes at a temperature of about 37°C. A device is removed from a composition and then allowed to dry. A medical device may be placed in an oven or other heated environment for a period of time sufficient for a composition to dry.

Although one layer, or coating, of a composition is believed to provide a desired composition coating, multiple layers are preferred. Multiple layers of a composition are preferably applied to at least one surface of a medical device by repeating steps discussed above. Preferably, a medical device is contacted with a composition three times, allowing the composition to dry on at least one surface of the medical device prior to contacting the medical device with the composition for each subsequent layer. Thus, a medical device preferably includes three coats, or layers, of a composition on at least one surface of the medical device.

In another embodiment, a method for coating medical devices with a composition coating includes the steps of forming a composition coating of an effective concentration to substantially prevent the growth or proliferation of biofilm embedded microorganisms on at least one surface of a medical device by dissolving an active component in an organic solvent, combining a medical device material penetrating agent to the active component(s) and organic solvent, and combining an alkalinizing agent to improve reactivity of the material of the medical device. A composition is then heated to a temperature ranging from about 30°C to about 60°C to enhance adherence of a composition coating to at least one surface of the device. A composition coating is applied to at least one surface of a medical device, preferably by contacting the composition coating to the at least one surface of the medical device for a sufficient period of time for the composition coating to adhere to at least one surface of the medical device. A medical device is removed from a composition coating and allowed to dry, preferably, for at least 18 hours at room temperature. A medical device may then be rinsed with a liquid, such as water and allowed to dry for at least 2 hours, and preferably 4 hours, before being sterilized. To facilitate drying of a composition of the invention onto a surface of a medical device, a medical device may be placed into a heated environment such as an oven.

In another aspect, the invention provides a method of incorporating a composition according to the invention into a device. Preferably, a device is a medical device and a composition is incorporated into a material forming the medical device during formation of the medical device. For example, a composition may be combined with a material forming the medical device, e.g., silicone, polyurethane, polyethylene, Gortex™ (polytetrafluoroethylene), Dacron™ (polyethylene terephthalate), and Teflon™ (polytetrafluoroethylene), and/or polypropylene, and extruded with the material forming the

medical device, thereby incorporating the composition into material forming the medical device. In this embodiment, the composition may be incorporated in a septum or adhesive, which is placed at the medical device insertion or implantation site. One example of a medical device having a composition incorporated into the material forming the medical device in accordance with this embodiment is a catheter insertion seal having an adhesive layer described below in greater detail. Another example of a medical device having a composition incorporated into the material is an adhesive. A composition of the invention can be integrated into an adhesive, such as tape, thereby providing an adhesive, which may prevent growth or proliferation of biofilm embedded microorganisms on at least one surface of the adhesive.

EXAMPLES

Example 1: Effect of DispersinBTM on biofilm formation

An *in vitro* microplate assay was performed to determine the effect of DispersinBTM on the growth and biofilm formation of *E. coli*, *S. epidermidis*, and *S. aureus*. *E. coli* biofilm was grown in colony forming antigen (CFA) medium. Purified DispersinBTM was obtained from Jeffrey Kaplan (University of Medicine and Dentistry of New Jersey) and was produced as described in Kaplan et al., 2003, *J. Bacteriol.* 185: 4693-4698. *S. epidermidis* and *S. aureus* biofilm was grown in tryptic soy broth (TSB). Bacteria were separately grown in 96-well microtiterplate in the absence and presence of DispersinBTM at different concentrations. The *E. coli* plate was incubated at 26°C for 24 hours. The *S. epidermidis* and *S. aureus* biofilm plates were incubated at 37°C for 24 hours. Growth of planktonic cells based on the absorbance at 600 nm was determined using Labsystems Multiskan Ascent microplate reader. Biofilm was measured by discarding the medium; rinsing the wells with water (three times), and staining bound cells with crystal violet. The dye was solubilized with 33% acetic acid, and absorbance at 630 nm was determined using a microtiter plate reader. For each experiment, background staining was corrected by subtracting the crystal violet bound to uninoculated control (Figure 1). The tests showed an appreciable effect by DispersinBTM on biofilm formation among all the cultures tested.

Example 2: Dispersal of *S. epidermidis* biofilm by DispersinBTM

Dispersal of *S. epidermidis* biofilm by DispersinBTM was demonstrated by growing *S. epidermidis* biofilm in a tube. The biofilm growth from the surface was scraped from the

bottom of the tube and transferred to another tube (Figure 2). Under these condition cells formed a sticky aggregate that rapidly settle to the bottom of the tube. Treatment of the cell aggregates with DispersinBTM resulted in uniformly turbid cell suspensions indicating that the treatment with DispersinBTM detaches the biofilm.

5 **Example 3: Enhanced inhibitory effect of DispersinBTM and Triclosan (TCSN) combination on *Staphylococcus epidermidis* biofilm**

An *in vitro* microplate assay was performed to determine the effect of DispersinBTM and triclosan (an antimicrobial agent) on the growth and biofilm formation of *S. epidermidis*. An overnight culture of *S. epidermidis* in Tryptic Soy Broth (TSB) was used as inoculum.

- 10 Bacteria were grown in TSB on a 96-well microtiterplate in the absence and presence of each compound (DispersinBTM or TCSN) at different concentrations separately and together (DispersinBTM + TCSN). Concentrations of DispersinBTM included 25 ng/ml, 50 ng/ml, and 100 ng/ml. Concentrations of TCSN included 25 µg/ml, 50 µg/ml, and 100 µg/ml. The plate was incubated at 37°C for 24 hours. The growth and biofilm was measured as explained in
- 15 Example 1. The combination of DispersinBTM and TCSN (50 ng/ml + 50 µg/ml, respectively) showed enhanced inhibitory effect on *S. epidermidis* biofilm formation (Figure 3).

Example 4: Antimicrobial activity of DispersinBTM and Triclosan (TCSN) combination against wound infection- associated pathogens

- 20 The combined antimicrobial activity of DispersinBTM and triclosan was studied by determining minimal inhibitory concentrations (MIC) in a 96 well microtiter plate. Briefly, serial two-fold dilutions of triclosan and in presence of DispersinBTM (40 µg/ml) were performed in TSB. A suspension of each microorganism from Table 1 was added to wells at a concentration of 5×10^5 CFU/mL, and the microtiter plates were incubated at 37°C. The MIC
- 25 was defined, as the lowest concentration of an antimicrobial required for total inhibition of a test microorganism at 37°C. Triclosan in combination with DispersinBTM was active against all the pathogens tested.

Table 1: MIC of triclosan in the presence of DispersinB™ (40 µg/ml) enzyme against wound infection associated pathogens

Cultures	MIC (µg/ml)
<i>S. aureus</i>	< 0.097
<i>S. epidermidis</i>	3.125
<i>E. faecalis</i>	100
<i>E. faecium</i> Vancomycin resistant	25
<i>E. faecium</i>	12.5
<i>E. coli</i>	6.25
<i>Enterobacter cloacae</i>	25
<i>P. mirabilis</i>	12.5
<i>K. pneumoniae</i>	25
<i>C. albicans</i>	100

Example 5: Enhancing effect of DispersinB™ on the sensitivity of biofilm-embedded *Staphylococcus epidermidis* to antimicrobials

An *in vitro* biofilm-dispersal assay was performed to determine whether DispersinB™ increased sensitivity of biofilm-embedded *S. epidermidis* to rifampicin and/or cefamandole nafate. *S. epidermidis* biofilm was grown in 1.5 ml polypropylene microcentrifuge tubes (200 µl culture volume), was rinsed with 200 µl of fresh medium and then treated with 200 µl medium containing 100 µg/ml of rifampicin or 0.1 µg/ml cefamandole nafate, each alone or in combination with 20 µg/ml of DispersinB™. After 3 hours at 37°C, 10 µl of 200 µg/ml DispersinB™ was added to each tube, and tubes were incubated for additional 5 min to detach biofilm. Serial dilutions of cells were plated on TSA. DispersinB™ enhanced the inhibitory effect of rifampicin and cefamandole nafate on biofilm-embedded *S. epidermidis* (Figures 4 and 5). DispersinB™ dispersed *S. epidermidis* biofilm and made it susceptible to rifampicin and cefamandole nafate.

Example 6: Enhancing effect of DispersinB™ on the sensitivity of *Staphylococcus epidermidis* biofilm to nitrofurazone

An *in vitro* biofilm dispersal assay was performed to determine the effect of DispersinB™ on enhancing the sensitivity of *S. epidermidis* biofilm to nitrofurazone (NF). *S. epidermidis* biofilm grown in 1.5 ml polypropylene microcentrifuge tubes was rinsed with

200 µl of fresh medium and then treated with 200 µl medium containing 25 µg/ml of NF and/or 20 µg/ml of DispersinBTM. Biofilm detachment and plating biofilm embedded cells were performed as described in Example 5. When DispersinBTM was used in combination with NF, there was increased sensitivity of *S. epidermidis* biofilm to NF (Figure 6). Thus, the DispersinBTM and NF combination had an enhanced inhibitory effect on biofilm-embedded *S. epidermidis*.

Example 7: Enhancing effect of DispersinBTM on the sensitivity of *Staphylococcus epidermidis* biofilm to Bismuth ethanedithiol (BisEDT)

An *in vitro* biofilm dispersal assay was performed to determine the effect of DispersinBTM on enhancing the sensitivity of *S. epidermidis* biofilm to bismuth ethanedithiol (BisEDT). *S. epidermidis* biofilm grown in 1.5 ml polypropylene microcentrifuge tubes was rinsed with 200 µl of fresh medium and then treated with 200 µl medium containing 0.5 mM of BisEDT and/or 20 µg/ml of DispersinBTM. Biofilm detachment and plating biofilm embedded cells were performed as described in Example 5. DispersinBTM in combination with BisEDT increased the sensitivity of *S. epidermidis* biofilm to BisEDT (Figure 7). Thus, the DispersinBTM and BisEDT combination had an enhanced inhibitory effect on biofilm-embedded *S. epidermidis*.

Example 8: Enhancing effect of DispersinBTM on the sensitivity of *Staphylococcus epidermidis* biofilm to ciprofloxacin (CF)

An *in vitro* biofilm dispersal assay was performed to determine the effect of DispersinBTM on enhancing the sensitivity of *S. epidermidis* biofilm to Ciprofloxacin (CF). *S. epidermidis* biofilm grown in 1.5 ml polypropylene microcentrifuge tubes was rinsed with 200 µl of fresh medium and then treated with 200 µl medium containing 200 µg/ml of CF and/or 20 µg/ml of DispersinBTM. Biofilm detachment and plating biofilm embedded cells were performed as described in Example 5. DispersinBTM in combination with CF increased the sensitivity of *S. epidermidis* biofilm to CF (Figure 8). Thus, the DispersinBTM and CF combination had an enhanced inhibitory effect on biofilm-embedded *S. epidermidis*.

Example 9: Effect of DispersinBTM on the sensitivity of *Staphylococcus epidermidis* biofilm to lactoferrin (Lf)

An *in vitro* biofilm dispersal assay was performed to determine the effect of DispersinBTM on the sensitivity of *S. epidermidis* biofilm to lactoferrin (Lf). *S. epidermidis* biofilm grown in 1.5 ml polypropylene microcentrifuge tubes was rinsed with 200 µl of fresh

medium and then treated with 200 μ l medium containing 5 mg/ml of Lf and/or 20 μ g/ml of DispersinBTM. Biofilm detachment and plating biofilm embedded cells were performed as described in Example 5. DispersinBTM in combination with Lf, did not increase the sensitivity of *S. epidermidis* biofilm to Lf (Figure 9). Thus, the DispersinBTM and Lf combination did not have an enhanced inhibitory effect on biofilm-embedded *S. epidermidis*.

Example 10: Enhancing effect of DispersinBTM on the sensitivity of *Staphylococcus epidermidis* biofilm to conalbumin/ovotransferrin (OT)

An *in vitro* biofilm dispersal assay was performed to determine the effect of DispersinBTM on enhancing the sensitivity of *S. epidermidis* biofilm to ovotransferrin (OT). *S. epidermidis* biofilm grown in 1.5 ml polypropylene microcentrifuge tubes was rinsed with 200 μ l of fresh medium and then treated with 200 μ l medium containing 10 mg/ml of OT and/or 20 μ g/ml of DispersinBTM. Biofilm detachment and plating biofilm embedded cells were performed as described in Example 5. DispersinBTM in combination with OT slightly increased the sensitivity of *S. epidermidis* biofilm to OT (Figure 10). Thus, the DispersinBTM and OT combination had a slightly enhanced effect on biofilm-embedded *S. epidermidis*.

Example 11: Effect of DispersinBTM on the sensitivity of *Staphylococcus epidermidis* biofilm to gallium (III) nitrate

An *in vitro* biofilm dispersal assay was performed to determine the effect of DispersinBTM on enhancing the sensitivity of *S. epidermidis* biofilm to gallium (III) nitrate. *S. epidermidis* biofilm grown in 1.5 ml polypropylene microcentrifuge tubes was rinsed with 200 μ l of fresh medium and then treated with 200 μ l medium containing 5 mg/ml of gallium (III) nitrate and/or 50 μ g/ml of DispersinBTM. Biofilm detachment and plating biofilm embedded cells were performed as described in Example 5. DispersinBTM in combination with gallium (III) nitrate did not increase the sensitivity of *S. epidermidis* biofilm to gallium (III) nitrate (Figure 11). Thus, DispersinBTM and gallium (III) nitrate combination did not have an enhanced inhibitory effect on biofilm-embedded *S. epidermidis*.

Example 12: Enhancing effect of DispersinBTM on the sensitivity of *Staphylococcus epidermidis* biofilm to chitosan

An *in vitro* biofilm dispersal assay was performed to determine the effect of DispersinBTM on enhancing the sensitivity of *S. epidermidis* biofilm to chitosan. *S. epidermidis* biofilm grown in 1.5 ml polypropylene microcentrifuge tubes was rinsed with 200 μ l of fresh medium and then treated with 200 μ l medium containing 2 mg/ml of chitosan

and/or 20 µg/ml of DispersinBTM. Biofilm detachment and plating biofilm embedded cells were performed as described in Example 5. DispersinBTM in combination with chitosan slightly increased the sensitivity of *S. epidermidis* biofilm to chitosan (Figure 12). Thus, the DispersinBTM and chitosan combination had a slightly enhanced inhibitory effect on biofilm-embedded *S. epidermidis*.

Example 13: Effect of DispersinBTM and Epigallocatechin gallate (EGCG) on biofilm formation of *Staphylococcus epidermidis* and *Staphylococcus aureus*

An *in vitro* microplate assays were performed to determine the effects of a DispersinBTM and EGCG combination on the growth of biofilm embedded *S. epidermidis* and *S. aureus*. Overnight cultures of each bacterial strain grown in Tryptic Soy Broth (TSB) were used as inoculum. Biofilm was developed in TSB on a 12-well microplate in the absence and presence of each test compound (50ng DispersinBTM or 100 ng/ml EGCG) separately and together (DispersinBTM+EGCG). The plates were incubated at 37°C for 24 hours. Medium containing planktonic cells in each well was removed gently and rinsed with sterile water. A known volume of water was added to each well and sonicated for 30 seconds. The contents of each well was transferred into a sterile tube, vortexed for a minute, followed by 10-fold serial dilution, and plated on agar plates using a spreader. After incubating the plates at 37°C for 24 hours, colony forming units (CFU) were counted. Although EGCG was more effective than DispersinBTM in inhibiting the growth of biofilm embedded test microorganisms, together the combination of DispersinBTM and EGCG did not have an enhanced inhibitory effect on biofilm-embedded *S. epidermidis* and *S. aureus* (Figures 13 and 14).

Example 14: DispersinBTM increased the sensitivity of biofilm-embedded *Staphylococcus epidermidis* to triclosan

An *in vitro* biofilm dispersal assay was performed to determine the effect of DispersinBTM on the sensitivity of a *S. epidermidis* biofilm to triclosan. A *S. epidermidis* biofilm grown in 1.5 ml polypropylene microcentrifuge tubes was rinsed with 200 µl of fresh medium and then treated with 200 µl medium containing 1 mg/ml of triclosan and/or 20 µg/ml of DispersinBTM. A biofilm dispersal assay was performed as described in the Example 5. When DispersinBTM was used in combination with triclosan, sensitivity of biofilm-embedded *S. epidermidis* to triclosan increased (Figure 15). The DispersinBTM and triclosan combination had an enhanced inhibitory effect on biofilm-embedded *S. epidermidis*.

Example 15: Enhancing effect of DispersinBTM on the sensitivity of biofilm-embedded *Staphylococcus epidermidis* to sodium usnate

An *in vitro* biofilm dispersal assay was performed to determine the effect of DispersinBTM on the sensitivity of *S. epidermidis* biofilm to sodium usnate. A *S. epidermidis* biofilm grown in 1.5 ml polypropylene microcentrifuge tubes was rinsed with 200 µl of fresh medium and then treated with 200 µl medium containing 500 µg/ml of sodium usnate and/or 50 µg/ml of DispersinBTM. A biofilm dispersal assay was performed as described in Example 5. When DispersinBTM was used in combination with sodium usnate, the sensitivity of biofilm-embedded *S. epidermidis* to sodium usnate increased (Figure 16). The DispersinBTM and sodium usnate combination had an enhanced effect on biofilm-embedded *S. epidermidis*.

Example 16: Antimicrobial activity of DispersinBTM and Triclosan (TCSN) combination against clinical isolates of wound-associated pathogens

The antimicrobial activity of DispersinBTM and triclosan combination was studied by determining the minimal inhibitory concentrations (MIC) in 96 well microtiter plates as described in Example 4. Triclosan in combination with DispersinBTM was active against all the pathogens tested (Table 2).

Table 2: MIC of triclosan in the presence of DispersinBTM, 40 µg/ml) enzyme for clinical isolates of wound-associated pathogens

Cultures	MIC (µg/ml)
<i>S. aureus</i> Gav 16A	< 0.39
<i>S. aureus</i> 120	< 0.39
<i>S. aureus</i> 68	< 0.39
Coagulase-negative staphylococci (CNS 11)	25
CNS 82	< 0.39
CNS 42	0.625

Example 17: Enhanced inhibitory effect of DispersinBTM and Triclosan (TCSN) combination on Coagulase-Negative Staphylococci (CNS) Biofilm

An *in vitro* microplate assay was performed to determine the effect of DispersinBTM and triclosan combination on the growth and biofilm formation of coagulase-negative Staphylococci-42 (CNS-42). The experiment was performed as explained in Example 1. The

combination of DispersinBTM and TCSN (100 ng/ml + 1.25 µg/ml, respectively) showed enhanced inhibitory effect on CNS-42 biofilm formation (Figure 17).

**Example 18: Enhancing effect of DispersinBTM on the sensitivity of
biofilm-embedded *Staphylococcus epidermidis* to 5-fluorouracil**

5 An *in vitro* biofilm dispersal assay was performed to determine the effect of DispersinBTM on the sensitivity of biofilm-embedded *S. epidermidis* to 5-fluorouracil (5-FU). *S. epidermidis* biofilm grown in 1.5 ml polypropylene microcentrifuge tubes was rinsed with 200 µl of fresh medium and then treated with 200 µl medium containing 100 µg/ml of 5-FU and/or 20 µg/ml of DispersinBTM. Biofilm detachment and plating biofilm embedded cells
10 were performed as described in Example 5. When DispersinBTM was used in combination with 5-FU, there was increased sensitivity of biofilm-embedded *S. epidermidis* to 5-FU (Figure 18). Thus, the DispersinBTM and 5-FU combination had an enhanced inhibitory effect on biofilm-embedded *S. epidermidis*.

**Example 19: Increased susceptibility of biofilm-embedded *Staphylococcus epidermidis*
pretreated with DispersinBTM to killing by SDS**

15 An *in vitro* biofilm assay was performed to determine the effect of DispersinBTM pre-treatment on the susceptibility of *S. epidermidis* to SDS. *S. epidermidis* biofilm grown in tubes were pretreated with PBS or DispersinBTM (20 µg/ml) for 30 min, and then treated with SDS (0.2 mg/ml) for 5 min at 37°C. The untreated, DispersinBTM alone, or SDS alone did not
20 significantly kill biofilm-embedded *S. epidermidis* (Figure 19). However, SDS caused a 1.5 log unit decrease in the number of CFUs in tubes pretreated with DispersinBTM pre-treatment made biofilm embedded cells more susceptible to SDS. This shows that sequential application of DispersinBTM enzyme and an antimicrobial agent is possible, when an antimicrobial agent is not compatible with the enzyme.

**Example 20: Increased susceptibility of biofilm-embedded *Staphylococcus epidermidis*
pretreated with DispersinBTM to killing by Chlorhexidine (CHX)**

25 An *in vitro* biofilm assay was performed to determine the effect of DispersinBTM pre-treatment on the susceptibility of biofilm-embedded *S. epidermidis* to CHX. *S. epidermidis* biofilm grown in tubes were pretreated with PBS or DispersinBTM (20 µg/ml) for 30 min, and
30 then treated with CHX (0.2 µg/ml) for 5 min at 37°C. The untreated, DispersinBTM alone, or CHX alone did not significantly kill *S. epidermidis* biofilm (Figure 20). However, CHX

caused a 1.6 log decrease of CFU in tubes pretreated with DispersinBTM. DispersinBTM pretreatment made biofilm-embedded cells more susceptible to CHX. This shows that sequential application of DispersinBTM enzyme and an antimicrobial agent is possible, when an antimicrobial agent is not compatible with the enzyme.

5 **Example 21: Increased susceptibility of biofilm-embedded *Staphylococcus epidermidis* pretreated with DispersinBTM to killing by benzalkonium chloride (BKC)**

An *in vitro* biofilm assay was performed to determine the effect of DispersinBTM pre-treatment on the susceptibility of *S. epidermidis* to BKC. *S. epidermidis* biofilm grown in tubes were pretreated with PBS or DispersinBTM (20 µg/ml) for 30 min, and then treated with
10 BKC (0.4 µg/ml) for 60 min at 37°C. The untreated, DispersinBTM alone, or BKC alone did not significantly kill biofilm-embedded *S. epidermidis* (Figure 21). However, BKC caused a 2.2 log decrease in CFU in tubes pretreated with DispersinBTM. DispersinBTM pre-treatment made biofilm-embedded cells more susceptible to BKC. This shows that sequential application of DispersinBTM enzyme and an antimicrobial agent is possible, when an
15 antimicrobial agent is not compatible with the enzyme.

Example 22: Enhanced inhibitory effect of DispersinBTM and EDTA combination on biofilm-embedded *Staphylococcus epidermidis*

An *in vitro* biofilm assay was performed to determine the effect of DispersinBTM and EDTA on the growth and biofilm formation of *S. epidermidis*. The experiment was
20 performed as explained in Example 1. The 100 ng/ml DispersinBTM in combination with EDTA at two concentrations such as 25 µg/ml and 50 µg/ml showed enhanced inhibitory effect on *S. epidermidis* biofilm formation (Figure 22).

Example 23: Increased susceptibility of biofilm-embedded *Staphylococcus epidermidis* pretreated with DispersinBTM to killing by silver nanopowder (SNP)

25 An *in vitro* biofilm assay was performed to determine the effect of DispersinBTM pre-treatment on susceptibility of *S. epidermidis* to silver nanopowder (SNP). *S. epidermidis* biofilm grown in tubes were pretreated with PBS or DispersinBTM (20 µg/ml) for 30 min, and then treated with SNP (0.03125 µg/ml) for 60 min at 37°C. The untreated, DispersinBTM alone, or SNP alone did not significantly kill biofilm-embedded *S. epidermidis* (Figure 23).
30 However, SNP caused a 1.5 log decrease in CFU in tubes pretreated with DispersinBTM. DispersinBTM pre-treatment made biofilm embedded cells more susceptible to SNP treatment.

This shows sequential application of DispersinBTM enzyme and an antimicrobial agent is possible, when an antimicrobial agent is not compatible with the enzyme.

Example 24: Enhanced susceptibility of biofilm-embedded *E. coli* to a combination therapy of DispersinBTM and bacteriophage

5 An *E. coli* biofilm was tested for survival after treatment with bacteriophage, DispersinBTM, a bacteriophage cocktail, and a combination of DispersinBTM and a bacteriophage cocktail.

E. coli TRMG1655 [*csrA::kan^r*] strain was transformed with transposon, mini-TN5 *luxCDABE::kan^r* for luciferase expression (Kadurugamuwa et al., 2005, *Infect. Immun.* 73: 10 3878-3887), and the operon integrated into the chromosome. Integration was confirmed by amplification of the genomic DNA upstream of the transposon by inverse PCR using *SspI*-digested genomic DNA. Primers UTCF1 (5'-GTGCAATCCATTAATTTTGGTG-3'; SEQ ID NO:13) and UTCR (5'-CATACGTATCCTCCAAGCC-3'; SEQ ID NO:14) were used to 15 amplify the upstream region using *Pfu* DNA polymerase (Sigma-Oligosynthesis, St. Louis, MO). The *lux* operon is derived from *Photobacterium luminescence* and was obtained from Xenogen Inc. (Alameda, CA). These bioluminescent bacteria allow real-time monitoring by noninvasive imaging of biofilms, either *in vitro* or *in vivo*.

A cell suspension of 10⁶ *E. coli csrA luxCDABE kan^r* was used to inoculate a filter disc (Millipore Corporation, Billerica, MA). The *E. coli* biofilm was maintained in minimal 20 media (M9) supplemented with 50 µg/ml kanamycin and 100 µg/ml ampicillin. The media were changed every day by transferring the disc to new plate. After 3 days, chemoluminescence activity of the established biofilm was measured using a TyphoonTM imaging scanner (General Electric Healthcare Life Sciences) and ImageQuant TL software (Amersham Biosciences, Sunnyvale, CA). The measure luminescence directly correlates 25 with the metabolic activity of the biofilm.

Luminescence was measured at day 0 and was used as the control. Following the measurement of luminescence at day 0, each disc was treated with one of the following-- media alone (control) and media containing:

- 1) 50 µg/ml DispersinBTM;
- 30 2) 10⁸ FF3;
- 3) 10⁸ K20;

- 4) 10^8 T7;
- 5) 10^8 U3;
- 6) a phage cocktail of 10^8 of K20, FF3, T7, and U3; and
- 7) 50 $\mu\text{g/ml}$ DispersinBTM plus a phage cocktail of total 10^8 of K20, FF3, T7, and U3.

5

Luminescence was measured every 24 hours followed by replacing the media. Thus, every 24 h following the measurement of the luminescence, 10 μl each of the different solutions was applied to the plate and the biofilm disc was placed on top of the drop everyday. The quantitative analysis of the biofilm luminescence from each day was used to compare the biofilm activity after treatment with the different treatments. Table 3 (corresponds to Figure 24) provides the raw data as measured in relative light units (RLU). These data show the relative increase or decrease in luminescence after the particular treatment.

Table 3

	Treatment	Day 0	Day 1	Day 2	Day 3	Day 4
15	Control	948.72	1131.8	1135.14	1447.05	1520.07
	DispersinB TM	948.72	1167.19	1297.42	1487.76	1550.01
	DispersinB TM					
	+Phage Cocktail	948.72	24.42	29.48	22.72	18.99
20	Phage Cocktail	948.72	287.82	974.62	1308.72	1381.05
	FF3	948.72	252.72	684.26	1072.27	1186.36
	K20	948.72	449.21	522.73	742.37	1196.76
	T7	948.72	212.8	987.28	1217.28	1457.48
	U3	948.72	321.62	803.7	1057.51	1283.72

25

The experiments were repeated using the same *E. coli* strain *csrA luxCDABE kan^r* and protocols. Biofilm discs were pulsed with media alone (control) and media containing:

- 1) BisEDT (50 $\mu\text{g/ml}$);
- 2) BisEDT (50 $\mu\text{g/ml}$) plus a phage cocktail of 10^8 of K20, FF3, T7, and U3;
- 30 3) BisEDT (50 $\mu\text{g/ml}$) plus 50 $\mu\text{g/ml}$ DispersinBTM;
- 4) BisEDT (50 $\mu\text{g/ml}$) plus 50 $\mu\text{g/ml}$ DispersinBTM plus a phage cocktail of 10^8 of K20, FF3, T7, and U3;
- 5) a phage cocktail of 10^8 of K20, FF3, T7, and U3;
- 6) 50 $\mu\text{g/ml}$ DispersinBTM; and
- 35 7) 50 $\mu\text{g/ml}$ DispersinBTM plus a phage cocktail of 10^8 of K20, FF3, T7, and U3.

Table 4

	Day 0	Day 1	Day 2	Day 3	Day 4
Control	1815.26	2151.4	1864.41	2318.77	2096.8
BisEDT	1815.26	245.27	83.51	8.02	47.62
BisEDT+Cocktail	1815.26	256.32	25.11	2.53	13.1
BisEDT+ DispersinB TM	1815.26	4.11	32.08	13.35	5.52
BisEDT+ DispersinB TM +Cocktail	1815.26	0.22	3.16	6.7	22.77
Cocktail	1815.26	188.3	899.35	1485.75	2127.73
DispersinB TM	1815.26	2137.73	1911.9	2982.02	2032.06
DispersinB TM +Cocktail	1815.26	287.93	462.37	521.27	429.25

The data indicate that the combination of DispersinBTM and an antimicrobial agent, including phage), provides a longer term anti-biofilm effect. Phage therapy alone produces an initially large decrease in RLU followed by a steady increase over time back to control levels. However, the combination of DispersinBTM and phage therapy produces a sharp decrease in RLU, which is maintained over 4 days.

Example 25: Phage Display of DispersinBTM

In the present work, we introduce biofilm dispersal capabilities to phage by expressing DispersinBTM on the phage surface (phage display of DispersinBTM). This allows the production of DispersinBTM at the site of bacterial infections using phage replication machinery that infect bacteria, and controls specifically targeted pathogenic bacteria. Choosing a lytic phage for displaying DispersinBTM helps to dissolve bacterial biofilms and facilitate the movement of phage to a host organism. Conversely, using a temperate phage (lysogenic) for displaying DispersinBTM would not eliminate host bacteria but allow constant multiplication of phage-displayed DispersinBTM at the infection site and thereby helping to

dissolve bacterial biofilm more rapidly. Once the biofilm is dissolved, the bacteria at the infection site are easily accessible to antimicrobial compounds and phages.

Application of phage-displayed DispersinBTM has certain advantages over purified DispersinBTM for infection control. In general, phages replicate at the site of infection and are available in abundance where they are most required (Smith & Huggins, 1982). Use of phage displayed DispersinBTM eliminates time consuming, expensive and elaborate purification process that is required for production of pure DispersinBTM. Purified DispersinBTM has a shelf life of approximately 12 months. Once phage-displayed DispersinBTM is applied to an infection site, the phage should multiply exponentially using existing host bacteria, and progressively and effectively dissolve the biofilm by reaching its deeper layers. As an additional advantage, displaying DispersinBTM on lytic phages facilitates lytic phage to dissolve biofilm and kill biofilm-embedded bacteria. Furthermore, a specifically targeted bacterial species can be eliminated and biofilm can be dissolved by choosing specific lytic phages to display DispersinBTM.

The lytic phage λ and the lysogenic phage M13 are modified to display DispersinBTM on their surfaces to test this hypothesis.

Materials and methods

Creation of recombinant phage λ , M13, and plasmid vectors for DispersinBTM display

Head decorating protein gene D (gpD) and left arm fragments (nucleotide position 1–100086, and 20040–33498) of bacteriophage λ , *lamB* gene of *E. coli*, *rrnB* terminator sequence of plasmid vector pQE60, gene VIII and III sequences of M13 phage, DispersinBTM gene of *A. actinomycetemcomitans* are amplified by PCR. Specific restriction sites, linker sequence (GGGSGGGS), and P_{lac} sequences are incorporated to PCR fragments with oligonucleotide primers.

The *pfu* DNA polymerase, Klenow fragment of DNA polymerase, and restriction endonucleases are purchased from MBI Fermentas (Burlington, ON, Canada). T4 DNA ligase and Shrimp Alkaline Phosphatase (SAP) are from New England Biolabs (Mississauga, ON, Canada) and Roche Diagnostics (Laval, QC, Canada), respectively. Synthetic oligonucleotides are obtained from Sigma Genosys (Oakville, ON, Canada). All enzymatic reactions and *in vitro* packaging are performed according to manufacturers' instructions. *E.*

coli cells are transformed by heat shock using frozen competent cells prepared using calcium chloride method described in *Molecular Cloning* (Sambrook et al., 2001). Plasmid DNA is extracted from *E. coli* following the alkaline lysis method of Sambrook et al. (1989). Bacteriophage DNA is extracted following the proteinase K and SDS method described in *Molecular Cloning* (2001). A Initially the recombinant bacteriophage M13-VIII DispersinBTM and M13-III DispersinBTM are introduced to host bacteria by electroporation.

Plasmid p^{QDispersinBTMMgpD-1} and p^{QgpDDispersinBTM-2} are high copy number plasmids with cloned DispersinBTM fused to the N-terminus and C-terminus, respectively, of *gpD* gene and expression is controlled in both plasmids by the P_{lac} promoter (Figure 25). To aid purification of the fusion protein, the DispersinBTM gene in vectors p^{QDispersinBTMMgpD-1} and p^{QgpDDispersinBTM-2} carry a histidine tag (6xHis) at their N- and C-terminus, respectively. λDispersinBTMMgpD⁻¹ and λgpD DispersinBTM-2 develop by incorporating promoterless DispersinBTM-gpD and gpD- DispersinBTM fusion cassettes of p^{QDispersinBTMMgpD-1} and p^{QgpDDispersinBTM-2}, respectively. In both λ DispersinBTM gpD⁻¹ and λgpD DispersinBTM-2, the expression of the DispersinBTM fusion peptide is under the expression signal of the original *gpD* promoter. Since the *gpD* gene of phage λ DispersinBTM gpD⁻¹ and λgpD DispersinBTM-2 are replaced with DispersinBTM-gpD, 100% of head decorating protein molecules carry DispersinBTM as a displayed protein. Phage λgpD- is defective in infection since the *gpD* gene is deleted. λgpD- can become infective by trans supply of the D protein. Therefore, λgpD- is used for screening expression cassettes (i.e. p^{QDispersinBTMMgpD-1}, p^{QgpDDispersinBTM-2}, p^{QDispersinBTMMgpD-3}, p^{QgpDDispersinBTM-4}) that produce the DispersinBTM-gpD fusion (Figures 25 and 26). Plasmids p^{B8LDSVIII} and p^{B31LDSIII} are high copy number plasmids that carry DispersinBTM inserted between the leader sequence and the sequence that codes for the mature protein of coat proteins VIII and III, respectively, of phage M13 (Figure 27). Both expression cassettes are under the control of the P_{lac} promoter, and the *rrnB* terminator sequence is placed before the P_{lac} promoter to terminate any transcription from upstream promoters. To aid purification of the fusion protein, the DispersinBTM gene in vectors p^{B8LDSVIII} and p^{B31LDSIII} carry a histidine tag (6xHis) at their N terminus. Phage M13-VIII DispersinBTM and M13-III DispersinBTM carry the DispersinBTM expression cassettes of p^{B8LDSVIII} and p^{B31LDSIII} respectively (Figure 27). Since both phages M13-VIII DispersinBTM and M13-III DispersinBTM also carry their wild type copy of genes VIII and III, only a fraction of major coat protein VIII and minor coat protein III of M13-VIII DispersinBTM and M13-III DispersinBTM, respectively, are fused to DispersinBTM.

Growth of bacteria and Phage

All bacterial strains are cultured at 37°C with agitation at 200 rpm in LB medium that contains 10 g/L each of bactotryptone, 10 g/L sodium chloride, and 5 g/L yeast extract in prepared in distilled deionized water.

- 5 Stocks of M13 phages are prepared by first inoculating 20 ml LB culture with 1 ml from a fresh overnight culture of host bacteria. The culture is shaken for 1 hr, after which 10¹⁰ plaque forming units (pfu) of the phage are added and the culture is shaken for an additional 3 hrs. The culture is cleared by centrifugation, and phage are precipitated by addition of 4% (w/v) polyethylene glycol (PEG) 8000 and 3% NaCl (w/v), incubating on ice
10 for 1 hr, and centrifugation at 10000 rpm for 30 min. The phage pellet is resuspended in 1 ml PBS (50 mM phosphate, pH 7.2, 150 mM NaCl) and is microcentrifuged to pellet the debris. The supernatant containing phage is transferred to new tube.

- Stocks of λ phages are prepared by infecting 0.1 ml fresh overnight culture of host bacteria cultured in LB with 10⁶ pfu of the phage in 50-100 μ l volume. The infected culture is
15 incubated for 20 min at 37°C in 4 ml of LB with vigorous shaking until the cells are completely lysed, usually for 8-12 hrs. The lysate is supplemented with 100 μ l of chloroform, incubating for 15 minutes at 37°C, and is centrifuged at 4000 g for 10 minutes at 4°C. The supernatant containing phage is separated and further purified by centrifugation through a glycerol step gradient (40% & 5%) at 35000 rpm for 60 minutes at 4°C.

- 20 The oncentration of phage in final stocks is determined by extracting DNA and subjecting the samples to electrophoresis on 1% agarose gels, where known quantities of similar DNA is used as standards. Plaque-forming units per ml are calculated with soft agar overlay method.

Purification of DispersinBTM and DispersinBTM-phage fusion proteins

- 25 *E. coli* Tuner (DE3)pLacI are transformed with plasmids expressing DispersinBTM. A single colony carrying transformed plasmid is cultured in 500 ml LB media containing 50 mg/L ampicillin and used for extraction of DispersinBTM. Bacterial cells are harvested by centrifugation at 5000 rpm for 15 minutes, and the cell pellet is taken up in 20 ml of lysis buffer (20 mM Tris-HCl (pH 8.0, 500 mM NaCl, 1 mM PMSF, 2 mg/ml lysozyme 0.1%
30 Igepal[®]). Cells are disrupted by sonication three times, each 10 seconds at 30% capacity. The

cell lysate is supplemented with RNaseA and DNaseI to a final concentration of 10 µg/ml and 5 µg/ml, respectively, and is incubated for 30 minutes at room temperature with gentle agitation. The cell debris is pelleted by centrifugation at 13000 rpm for 30 minutes, and the cleared lysate is used for isolation of DispersinBTM by Ni-affinity chromatography. The clear
5 cell lysate is passed through a column packed with Ni-CAMTM HC Resin (10 cm pack volume) and is equilibrated with wash buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl). The column is washed with 3 column volumes of wash buffer containing 5 mM imidazole followed by another 3 column volumes of wash buffer containing 20 mM imidazole. DispersinBTM is eluted in one ml fractions with 20 ml elution buffer (wash buffer containing
10 100 mM imidazole). DispersinBTM containing fractions are pooled and dialyzed against 4 L of 100 mM phosphate buffer (pH 5.9) containing 200 mM NaCl. Purified DispersinBTM is stored in storage buffer (50 mM Phosphate buffer (pH 5.9), 50 mM NaCl, 50% Glycerol) at -20°C.

DispersinBTM enzyme assay

15 The activity of DispersinBTM displayed on phage and purified DispersinBTM fusion peptides is measured by following the β-1,6-N-acetyl D-glucosaminidase (DispersinBTM) assay as described by Kaplan et al. (2003, *J. Bacteriol.* 185: 4693-4698). The enzyme reaction is carried out in total 1 mL reaction volume that contains 500 µl of 10 mM substrate stock (5 mM 4-nitrophenyl N-acetyl-D-glucosaminide), 3.7 µg DispersinBTM solution or
20 known volume of purified DispersinBTM display phage, 50 mM sodium phosphate buffer pH 5.9 containing 100 mM NaCl and ddH₂O to bring the total reaction volume to 1 ml. The reaction mix is incubated at 30°C for 30 min and supplemented with 5 µl of 10 N NaOH to stop enzyme reaction. The amount of p-nitrophenol produced in the reaction is determined spectrophotometrically at 405 nm using a standard curve constructed.

25 Cell Count Assay

A fresh overnight culture of bacteria grown in LB is diluted to 5% in LB, and 1.8 ml is added per well of 12-well tissue culture polystyrene plates (Corning Inc., New York, NY). 200 µl of different dilutions of an aqueous test solution containing purified DispersinBTM or its fusion peptides or phages with or without displayed DispersinBTM are added per well individually.
30 200 µl water is added to negative control wells. After incubating for 24 hrs, the medium containing planktonic cells in each well is removed, and the biofilm is rinsed with PBS. After

adding 2 ml of PBS to each well, the plate is sonicated for 1 minute, and the dislodged biofilm is mixed well with the pipette tip. The 1 ml suspension from each well is then serially diluted and 100 μ l aliquots from each dilution are inoculated to LB plates. The plates are incubated at 37°C for 24 hrs, and colonies are enumerated. **Biofilm assay** Fresh overnight grown cultures are diluted to 5% in LB medium, and 180 μ l are added to each well of a 96-well microtitre plate (Corning Inc.). 20 μ l of different dilutions of an aqueous test solution containing purified DispersinBTM or DispersinBTM-phage fusion peptides or phages with or without displayed DispersinBTM are added to microtiter plate wells individually. dd H₂O serves as control. The plates are incubated at 37°C for 16–18 h under stationary condition and the growth is measured at 600 nm. Biofilm formation is determined by measuring the absorbance at 630 nm. Biofilms are assayed by crystal violet staining, as described by Jackson et al. (2002, *J. Bacteriol.* 184: 290–301.).

Example 26: DispersinBTM in combination with detergents

A major component of the *A. actinomycetemcomitans* biofilm matrix is a hexosamine-rich polysaccharide that is functionally and genetically related to extracellular polysaccharide adhesins produced by *S. aureus*, *S. epidermidis*, *E. coli* and *A. pleuropneumoniae* (Kaplan et al., 2004). These polysaccharides, usually referred to as PNAG, PIA (polysaccharide intercellular adhesin), or PGA, consist of linear chains of N-acetyl-D- glucosamine (GlcNAc) residues in β (1,6) linkage (hereafter referred to as PGA). PGA has been shown to play a role in abiotic surface attachment and intercellular adhesion (Wang et al., *J. Bacteriol.* 186: 2724–2734 (2004); Izano et al., *Microb. Pathogen.* 43: 1–9 (2007); Agladze et al., *J. Bacteriol.* 187: 8237–8246 (2005); Heilmann et al., *Mol. Microbiol.* 20: 1083–1091 (1996); McKenney et al., *Infect. Immun.* 66: 4711–4720 (1998)), protection from killing by antibiotics, antimicrobial peptides and phagocytes (Izano et al., 2007; Vuong et al., *Cell. Microbiol.* 6: 269–275 (2004)) and virulence (Kropec et al., *Infect. Immun.* 73: 6868–6876 (2005)). In *A. actinomycetemcomitans*, PGA has been shown to mediate intercellular adhesion and resistance to killing by the anionic detergent sodium dodecyl sulfate (SDS) (Kaplan et al., 2004).

The effect of a detergent on the sensitivity of biofilm-embedded *A. actinomycetemcomitans* was investigated.

Methods

Reagents. Recombinant dispersinB protein was purified from an overexpressing strain of *E. coli* as previously described (Kaplan *et al.*, *J. Bacteriol.* 2003, 185: 4693-4698). The enzyme had a specific activity of $\sim 10^3$ units *per* mg of protein. Sodium dodecyl sulfate (SDS) was purchased from Fluka (St. Gallen, Switzerland). Phosphate-buffered saline (PBS; 138 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) was purchased from Sigma Chemical Company (St. Louis, MO, USA).

Bacterial strains, media, and growth conditions. *A. actinomycetemcomitans* strain CU1000 (serotype f) was isolated from a 13-year-old African-American female with localized aggressive periodontitis (Fine *et al.*, *Microbiol.* 1999, 145: 1335-1347). Strain CU1000 exhibits a rough-textured colony morphology on agar and a strong biofilm formation phenotype in broth, both of which are characteristic of fresh clinical isolates (Fine *et al.*, 1999). An isogenic PGA mutant strain HW1018 (CU1000 *pgaC::IS903* ϕ Kan) was isolated by randomly mutagenizing CU1000 with transposon *IS903* ϕ Kan and selecting mutants that produced white colonies on Congo red agar, as previously described (Kaplan *et al.*, 2003; Kaplan *et al.*, *J. Bacteriol.* 2004, 186: 8213-8220). Like other *pgaC* mutant strains, HW1018 was completely deficient in PGA production, but still formed tenacious biofilms on plastic surfaces (Kaplan *et al.*, 2004). Bacteria were grown in trypticase soy broth supplemented with 6 g yeast extract and 8 g/L glucose. Solid medium was supplemented with 15 g/L agar. All cultures were incubated statically at 37°C in 10% CO₂.

Preparation of Inocula. Approximately 10 colonies from a 48-hour-old agar plate were transferred to a 1.5-mL polypropylene microcentrifuge tube containing 200 μ L of fresh broth. The cells were homogenized with 10 strokes of a disposable pellet pestle (Kontes), transferred to a 15-mL conical centrifuge tube containing 2 mL of fresh broth, subjected to high-speed vortex agitation for 15 sec, and then passed through a 5- μ m-pore-size PVDF syringe filter (Millipore). The resulting filtrate (~ 1 mL) contained $> 99\%$ single cells at a concentration of 10^7 to 10^8 colony-forming units (CFU)/mL (Kaplan & Fine, *Appl. Environ. Microbiol.* 2002, 68: 4943-4950).

Biofilm Cultures. Biofilms were grown in 17 mm x 100 mm culture tubes (untreated polystyrene; Falcon #352051) or 96-well microtiter plates (tissueculture-treated polystyrene,

flat bottoms; Falcon #353072). Culture vessels were inoculated with a 1:10 dilution of inoculum in fresh broth (1 mL for tubes or 200 μ L for microplates) and incubated for 24 h.

Crystal Violet Assay. Biofilm biomass was visualized and quantitated by means of a crystal violet binding assay as previously described (Kaplan *et al.*, *Antimicrob. Agents Chemother.* 2004, 48: 2633-2636). Briefly, biofilms were rinsed with water to remove loosely attached cells, stained for 1 min with Gram's crystal violet (200 μ L for microplates and 1 mL for tubes), rinsed, dried, and photographed. For quantitation of biofilms grown in microplates, biofilms were de-stained with 200 μ L of 33% acetic acid for 5 min, and the absorbance of the crystal violet solution was measured directly in the plate by means of a BioRad Benchmark microtiter plate-reader set at 590 nm. Crystal violet binds to bacterial biofilms, but not to polystyrene (O'Toole & Kolter, *Mol. Microbiol.* 1998, 28: 449-461).

Biofilm Detachment Assay. Biofilms were rinsed with water and treated with 200 μ L (for microplates) or 1 mL (for tubes) of DispersinBTM (20 μ g/mL in PBS) or SDS (0.001-1% in PBS). After a five- or 30-minute incubation at 37°C, biofilms were rinsed with water and stained with crystal violet as described above. In some assays, biofilms were first treated with DispersinBTM for 5 or 30 min, rinsed, and then treated with SDS. All detachment assays were performed in duplicate wells or tubes. All assays were performed on at least 3 separate occasions, with similar results.

Biofilm Killing Assay. Biofilms grown in polystyrene tubes as described above were washed 3 times with sterile PBS and then treated with 1 mL of SDS (0.01% in PBS) or cetylpyridinium chloride (CPC; 0.02% in PBS). After 5 min, the biofilms were rinsed 3 times with PBS to remove the SDS or CPC, and then treated with 1 mL of DispersinBTM (20 μ g/mL in PBS) for 5 min to detach the cells. Tubes were vortexed for 10 s, and 20 μ L aliquots of the detached biofilms were transferred to the wells of a flat-bottomed 96-well microtiter plate containing 180 μ L of fresh broth. Five serial dilutions (20 μ L into 180 μ L) were performed directly into adjacent wells. Plates were incubated for 48 h and then rinsed and stained with crystal violet as described above. Wells containing 30-300 biofilm colonies were photographed under a dissecting microscope and counted. In some assays, biofilms were pre-treated with 1 mL of DispersinBTM (20 μ g/mL in PBS) for 5 min prior to the SDS treatment. In these assays, a 100 μ L quantity of SDS in PBS (at 10 times the test concentration) was added directly to the DispersinBTM-treated cell suspension and mixed. After 5 min, tubes were vortexed briefly, and 20- μ L aliquots of culture were enumerated as described above.

Killing assays were performed in duplicate tubes on at least 5 separate occasions, with similar results.

Detachment of Biofilms by SDS and DispersinBTM

Crystal violet dye was used to visualize *A. actinomycetemcomitans* biofilm growth and detachment in polystyrene tubes and 96-well microtiter plates (Figure. 28). Both wild-type and PGA mutant strains formed uniform biofilms that covered the bottom surface of the tube or microplate well after 24 hrs (Figures. 28A, 28B). In all cultures, the broth remained optically clear and contained < 1% of the total CFUs after 24 hrs.

A solution of 0.1% SDS had no effect on the attachment of wild-type biofilms, but caused the rapid detachment of PGA mutant biofilms, in both tubes and microplate wells (Figs. 30A, 30B). In contrast, a solution of 20 µg/mL of DispersinBTM caused the rapid detachment of wild-type biofilms from tubes, but not from microplate wells. DispersinBTM had no effect on the attachment of PGA mutant biofilms grown in either culture vessel. Microscopic analyses of biofilms grown in tubes indicated that DispersinBTM caused the biofilms to disaggregate into uniformly turbid suspensions containing > 99% single cells, with very few small clusters of cells (data not shown). Detachment of biofilms from microplate wells could be achieved if higher DispersinBTM concentrations and longer incubation times were used (Kaplan *et al.*, *J. Bacteriol.* 2003, 185: 4693-4698).

Pre-treatment of wild-type biofilms with DispersinBTM rendered them sensitive to detachment by SDS in microplate wells (Figure. 28C). Biofilms were treated with 0.001-1% SDS for 5 min and then biofilm biomass was quantitated by measuring the amount of bound crystal violet dye to measure the concentration of SDS needed to detach *A. actinomycetemcomitans* biofilms from microplate wells (Figure. 29). Wild-type biofilms were resistant to detachment at all concentrations of SDS. A slight, but reproducible, increase in crystal violet staining was exhibited by biofilms treated with 0.04-0.11% SDS. In contrast, wild-type biofilms pre-treated with DispersinBTM were resistant to detachment only at SDS concentrations < 0.04%. When the concentration of SDS was increased from 0.04 to 0.07%, biofilms underwent a transition from SDS-resistant to SDS-sensitive. This concentration of SDS (1.4-2.4 mM) is very close to the critical micelle concentration (CMC) of SDS in physiologic saline at 37°C (Helenius *et al.*, *Methods Enzymol.* 1979, 56: 734-749). PGA

mutant biofilms exhibited a nearly identical transition from SDS resistant to SDS-sensitive between 0.04 and 0.07% SDS (data not shown).

DispersinBTM Increases the Sensitivity of Biofilms to Killing by SDS

The sensitivity of *A. actinomycetemcomitans* biofilms to killing by 0.01% SDS was tested. The 0.01% SDS corresponds to the MIC against *A. actinomycetemcomitans* planktonic cells (Drake *et al.*, *J. Periodontol.* 1992, 63: 696-700; Wade & Addy, *J. Periodontol.* 1992, 63: 280-282), but which is below the concentration required for biofilm detachment (Figure 29). Biofilms grown in tubes were pre-treated with PBS (mock pre-treatment) or DispersinBTM for 5 min or 30 min, and then treated with SDS for 5 min. PBS, DispersinBTM, or SDS alone did not significantly kill *A. actinomycetemcomitans* biofilms (Figure 30). However, SDS caused a 2-log-unit decrease in the number of CFU in tubes pre-treated with DispersinBTM for 5 min, and a 4-log-unit decrease in tubes pre-treated for 30 min.

DispersinBTM Increases the Sensitivity of Biofilms to Killing by CPC

The sensitivity of *A. actinomycetemcomitans* biofilms to killing by 0.02% cetylpyridinium chloride (CPC) was tested. The 0.02% CPC corresponds to 10 times the MIC against *A. actinomycetemcomitans* planktonic cells, but which is below the concentration required for biofilm detachment (data not shown). Biofilms grown in tubes were pretreated for 30 min with phosphate buffer saline (PBS; mock pretreatment) or PBS with 20 µg/mL of DispersinBTM, and then treated with 0.02% CPC for 5 min. Biofilms treated with DispersinBTM or CPC alone exhibited little or no reduction in the number of CFU/tube compared to the mock-treated controls (Figure 31). Biofilms treated with DispersinBTM and then CPC, however, exhibited an approximately 3 log unit decrease in the number of CFUs/tube compared to biofilms treated with DispersinBTM or CPC alone.

These findings suggest that DispersinBTM is a useful agent for sensitizing biofilms to detachment and killing by a detergent such as SDS or CPC, and/or other antimicrobial agents.

Example 27: Formulation of DispersinBTM antimicrobial wound gel

As a general procedure, finely powdered sodium alginate (the use of other alkaline metal alginates may also be considered) was blended with DispersinBTM in distilled water at

room temperature for 6-8 hours. As the alginate slowly dissolved and absorbed water, a gel began to form. Stirring continued during this process so that as any yet unblended alginate did not settle out. The final formulation of wound gel contained 0.01% DispersinB™, 1.5% sodium alginate, and 98.49% water.

5 **Example 28: Formulation of Triclosan- DispersinB™ antimicrobial wound gel**

The solvent system for triclosan comprising polyethylene glycol, ethanol was prepared in distilled water. Triclosan was dissolved in solvent system at 65°C with stirring for 8-10 hours. The solution was cooled to room temperature, and DispersinB™ along with sodium alginate was added. The gel was formed as explained in Example 27. The final gel
10 formulation contained 1% triclosan, 10% polyethylene glycol 400, 10% ethanol, 0.01% DispersinB™, 1.5% sodium alginate and 77.49% water.

Example 29: Effect of DispersinB™ antimicrobial wound gel on *Staphylococcus epidermidis* biofilm formation

An *in vitro* microtiter assay was performed to determine the effect of DispersinB™
15 wound gel on the growth and biofilm formation of *Staphylococcus epidermidis*. *S. epidermidis* biofilm was grown in tryptic soy broth (TSB). *S. epidermidis* was grown in 96-well microtiter plate in the absence and presence of DispersinB™ wound gel at different concentrations. The plate was incubated at 37°C for 24 hours. Growth of planktonic cells based on the absorbance at 600 nm was determined using Labsystems Multiskan Ascent
20 microplate reader. Biofilm was measured by discarding the medium, rinsing the wells with water (three times), and staining bound cells with crystal violet. The dye was solubilized with 33% acetic acid, and absorbance at 630 nm was determined. For each experiment, background staining was corrected by subtracting the crystal violet bound to uninoculated control (Figure 32). The test showed 65%-80% biofilm inhibition at all wound gel dilutions
25 tested without affecting planktonic growth.

Example 30: Effect of DispersinB™ antimicrobial wound gel on *Staphylococcus epidermidis* biofilm dispersal

An *in vitro* microtiter assay was performed to determine the effect of DispersinB™
wound gel on the dispersal of *Staphylococcus epidermidis* biofilm. *S. epidermidis* biofilm
30 was grown in tryptic soy broth (TSB). *S. epidermidis* was grown in 96-well microtiter plate at 37°C for 24 hours. The planktonic growth was discarded and the biofilm was treated with

serial two-fold dilutions of DispersinBTM wound gel at 37°C for 3 hours. After wound gel treatment the microtiter plate was washed and stained as explained in example 2. The test showed 57%-75% *S. epidermidis* biofilm dispersal at all wound gel dilutions tested (Figure 33).

5 **Example 31: Antimicrobial activity of Triclosan-DispersinBTM antimicrobial wound gel against wound associated pathogens**

The antimicrobial activity of Triclosan-DispersinBTM was tested *in vitro*, against wound-associated bacteria such as *Staphylococcus aureus*, *S. epidermidis*, *Enterococcus faecalis*, *Escherichia coli*, *Enterobacter cloacae*, and yeast *Candida albicans* (Vandenbulcke, 10 *et al.* 2006. *Lower Extremity Wounds*, 5: 109-114). The organisms were incubated on Trypticase Soy Agar and the plates were overlayed with 100 µl of wound gel. The plates were incubated at 37°C for 24-48 hours. The number of colony forming units (CFU) per milliliter for each culture was calculated (Table 5). Unexpectedly, there was zero growth (no CFU) on any of the TSA plates treated with Triclosan-DispersinBTM wound gel.

15 **Table 5: Effect of Triclosan-DispersinBTM antimicrobial wound gel on growth of wound-associated pathogens**

Pathogen	CFU/ml	
	Untreated	Treated
Gram-positive bacteria		
<i>Staphylococcus aureus</i>	1.2 x 10 ⁸	0
<i>S. epidermidis</i>	1 x 10 ⁸	0
<i>Enterococcus faecalis</i>	2.3 x 10 ⁴	0
Gram-negative bacteria		
<i>Escherichia coli</i>	2.2 x 10 ⁸	0
<i>Enterobacter cloacae</i>	1.5 x 10 ⁸	0
Yeast		
<i>Candida albicans</i>	4.5 x 10 ⁶	0

Example 32: Inhibitory effect of DispersinBTM and Triclosan (TCSN) combination on *Staphylococcus epidermidis* biofilm

In vitro microplate assay was performed to determine the synergistic effect of DispersinBTM and triclosan (an antimicrobial agent) on the growth and biofilm formation of *S. epidermidis*. Overnight culture of *S. epidermidis* in Tryptic Soy Broth (TSB) was used as inoculum. Bacteria were grown in TSB on a 96-well microtiterplate in the absence and presence of each compound (DispersinBTM or TCSN) at different concentrations separately and together (DispersinBTM+TCSN). The plate was incubated at 37°C for 24 hours. Growth of planktonic cells based on the absorbance at 600 nm was determined using Labsystems Multiskan Ascent microplate reader. Biofilm was measured by discarding the medium; rinsing the wells with water (three times) and staining bound cells with crystal violet. The dye was solubilized with 33% acetic acid, and absorbance at 630 nm was determined using a microtiter plate reader. For each experiment, background staining was corrected by subtracting the crystal violet bound to uninoculated control. The combination of DispersinBTM and TCSN (50 ng/ml + 50 µg/ml, respectively) showed inhibitory effect on *S. epidermidis* biofilm formation (Figure 34).

Example 33: Inhibitory effect of DispersinBTM and Triclosan (TCSN) combination coated silicone catheters on *Staphylococcus epidermidis* and *Escherichia coli* colonization

The adhesion assay was performed to determine the synergistic effect of DispersinBTM and TCSN combination coated silicone catheters on *S. epidermidis* and *E. coli* colonization. The silicone catheter segments (1 cm each) were coated by dipping in DispersinBTM (40 µg/ml) and TCSN (10 mg/ml in 10% Polyethylene glycol) alone and in combination for overnight at 4°C followed by drying at room temperature. The coated and uncoated segments were incubated in *S. epidermidis* and *E. coli* culture in TSB medium at 37°C for 24 hours at 100 rpm. After 24 hours of incubation, the sections were washed three times gently. Each washed section was transferred into a sterile tube containing 1 ml sterile saline and subjected to sonication for 30 seconds and followed by 1 minute vortexing. Further, it was serially diluted using sterile saline and plated using Tryptic Soy Agar (TSA) plates. The plates were incubated at 37°C for 24 hours and the colonies (CFU) were counted. Although triclosan was more effective than DispersinBTM in inhibiting the growth of biofilm-

embedded *S. epidermidis* and *E. coli*, the combination-coated catheters showed an enhanced anti-adherence effect on *S. epidermidis* and *E. coli* (Figures 35 and 36).

Example 34: Antibiofilm activity of DispersinBTM and Triclosan (TCSN) combination-coated catheters against catheter-associated microorganisms

5 The broad-spectrum antibiofilm activity of DispersinBTM and TCSN combination coated catheters against catheter-associated bacteria and yeast was determined. Catheter-associated microorganisms such as *E. coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Enterococcus cloacae*, *Citrobacter diversus*, *S. epidermidis*, *Staphylococcus aureus*, *Staphylococcus saprophyticus*, and *Candida albicans*
10 were grown in TSB for 18 hours. The catheter coating and adherence assay for 24 hours were done as described in Example 33. The TCSN-DispersinBTM combination coated catheters were broad-spectrum in terms of inhibiting Gram +ve, Gram -ve bacteria and yeast colonization on catheters (Figure 37). The combination-coated catheters inhibited > 90% colonization of catheters by test organisms, except *Enterococcus faecalis*.

15 **Example 35: Durability of inhibitory activity of DispersinBTM and Triclosan (TCSN) combination-coated polyurethane catheters**

 The durability of inhibitory activity of DispersinBTM+TCSN coated 1 cm polyurethane catheter segments was assessed using Kirby-Bauer technique as previously described by Sheretz et al. (Antimicrob. Agents Chemother., 33: 1174-1178, 1989). The
20 catheters were coated as described in Example 2. The test organisms such as *Staphylococcus aureus* and *Staphylococcus epidermidis* were grown in TSB for 18 hours at 37°C. An appropriate inoculum of each bacterial strain was used to prepare spread plates. The coated catheter segments were carefully plated. Following incubation for 24 hours at 37°C, the zones of inhibition surrounding each segment were measured at the aspects of perpendicular to the
25 long axes. After measuring the zone of inhibition, the segments were transferred onto fresh spread plates inoculated with respective test organism and incubated for 24 hours at 37°C again. The zones of inhibitions were measured again. This procedure was repeated for determining the durability of inhibitory activity of coated catheter sections against *S. aureus* and *S. epidermidis* (Figure 38). The coated segments showed significant inhibitory activity
30 against *S. aureus* and *S. epidermidis* even after 14 days of passage.

**Example 36: Durability of inhibitory activity of DispersinBTM and Triclosan (TCSN)
combination-coated polyurethane catheters in plasma
(tested against *Staphylococcus epidermidis*).**

5 The ability of DispersinBTM-TCSN coated polyurethane catheters to resist bacterial
colonization for a period of 7 days was tested by exposing uncoated and coated segments to
S. epidermidis. The coated and uncoated catheter segments were incubated in rabbit plasma at
37°C separately for 7 days at 100 rpm prior to challenging with *S. epidermidis*. Both coated
and uncoated catheter segments (in triplicate) were removed at time intervals of 1, 5 and 7
10 days. Further, they were challenged with *S. epidermidis* once at a time. Following the
incubation, the catheter segments were rinsed 3 times gently with sterile water. Each washed
segment was transferred into sterile tube containing 1 ml sterile saline and subjected to
sonication for 30 seconds followed by 1 minute vortexing. Further, it was serially diluted and
plated on TSA. The plates were incubated at 37°C for 24 hours and colony-forming units
15 (CFU) were counted. This procedure was repeated for each time interval. The DispersinBTM-
TCSN coated catheter segments were effective in preventing *S. epidermidis* biofilm
formation over a period of 7 days (Figure 39).

**Example 37: Durability of inhibitory activity of DispersinBTM and Triclosan (TCSN)
combination-coated polyurethane catheters in TSB containing 20% Bovine Serum
(tested against *Staphylococcus aureus*)**

20 The ability of DispersinBTM-TCSN coated polyurethane catheters to resist bacterial
colonization and retain antimicrobial activity for a period of 7 days was tested by exposing
uncoated and coated segments to *S. aureus*. The coated and uncoated segments were
25 incubated in TSB containing 20% bovine serum for 7 days at 100 rpm. The TSB containing
20% bovine serum was replaced every 24 hour. The anti-adherence activity was performed as
explained in Example 36. After 7 days of incubation, the coating prevented > 99% *S. aureus*
biofilm formation (Figure 40). The antimicrobial activity and durability was assessed using
Kirby-Bauer technique as previously described by Sheretz et al. (Antimicrob. Agents
30 Chemother., 33: 1174-1178, 1989). Both coated and uncoated catheter segments (in
triplicate) were removed at time intervals of 1, 5 and 7 days. *S. aureus* was grown in TSB for
18 hours at 37°C. An appropriate inoculum of bacterial strain was used to prepare spread

plates. The coated catheters were carefully plated. Following incubation for 24 hours at 37°C, the zones of inhibition surrounding each segment were measured at the aspects of perpendicular to the long axes. This procedure was repeated for each time interval (Figure 41). The coated catheter segments retained antimicrobial activity even after 7 days of incubation in TSB containing 20% bovine serum.

Example 38: Durability of inhibitory activity of DispersinBTM and Triclosan (TCSN) combination-coated silicone catheters in synthetic urine (tested against *Staphylococcus epidermidis*)

The ability of DispersinBTM-TCSN coating on silicone catheters to resist bacterial colonization and retain antimicrobial activity for 10 days was tested by exposing the uncoated and coated segments to test organisms. The coated and uncoated catheter segments were incubated in sterile artificial urine medium at 37°C for 10-14 days at 100 rpm. The artificial urine in the flask was replaced with fresh artificial urine every 24 hours. Both coated and uncoated catheter segments (in triplicate) were removed at time intervals of 1, 4, 7, 10 and 14 days. Further, they were challenged with *S. epidermidis* one at a time. Following the incubation, the catheter sections were rinsed 3 times gently with sterile water. Each washed segment was transferred into sterile tube containing 1 ml sterile saline and subjected to sonication for 30 seconds followed by 1 minute vortexing. Further, each section was serially diluted and plated on TSA. The plates were incubated at 37°C for 24 hours and colony-forming units (CFU) were counted. This procedure was repeated for each time interval. The DispersinBTM-TCSN coated catheter segments were effective in preventing *S. epidermidis* biofilm formation for more than 10 days (Figure 42). The antimicrobial activity retained by the catheters was studied by Kirby-Bauer technique as previously described by Sheretz et al. (Antimicrob. Agents Chemother., 33: 1174-1178, 1989) against *E. coli*, *P. mirabilis*, *K. pneumoniae* and *S. epidermidis*. The coated catheter segments showed a significant inhibitory activity against *E. coli*, *P. mirabilis*, *K. pneumoniae* and *S. epidermidis* even after 14 days (Figure 43).

Example 39: *In Vivo* Efficacy of DispersinBTM+ Triclosan (DispersinBTM+TCSN) coated central venous catheters (CVC)

In vivo efficacy study was conducted using rabbits to determine the rates of catheter colonization and catheter related infections by *Staphylococcus aureus* with the following four groups of CVCs: (i) catheters coated with DispersinBTM+TCSN, (ii) catheters coated with chlorhexidine+silver sulfadiazine (CH/SS), (iii) chlorhexidine+silver sulfadiazine plus (CH/SS plus), and (iv) uncoated catheters. Thirty 1-cm segments of CVCs from each group were implanted subcutaneously in the back of a total of 20 female New-Zealand white, specific pathogen-free rabbits. Each catheter insertion site was inoculated with 10⁴ colony forming units (CFU) of clinical isolate of *S. aureus*. After 7 days, the rabbits were sacrificed; the catheters were explanted, and cultured by plating on agar plates. Out of 30, 29 (96.7%) uncoated, 1 (3.3%) DispersinBTM+TCSN coated, 4 (13.3%) CH/SS coated and 1 (3.3%) CH/SS plus coated catheters were colonized by *S. aureus* (Figure 44). The DispersinBTM+TCSN, CH/SS and CH/SS plus catheter coatings significantly reduced catheter colonization by *S. aureus* ($p \leq 0.001$) compared to uncoated catheter.

Example 40: Enhancing effect of DispersinB on the sensitivity of biofilm-embedded *Staphylococcus epidermidis* to xylitol

An *in vitro* biofilm-dispersal assay was performed to determine the enhancing effect of DispersinBTM on the sensitivity of biofilm-embedded *S. epidermidis* to xylitol. *S. epidermidis* biofilm was grown in 1.5 ml polypropylene microcentrifuge tubes (200 μ l culture volume) for 24 h and medium containing planktonic cells was discarded. Further, each tube was rinsed with 200 μ l of fresh medium and then treated with 200 μ l medium containing 5% xylitol alone or in combination with DispersinB (20 μ g/ml). After 3 h incubation at 37°C, 10 μ l of 200 μ g/ml DispersinB was added to each tube, and tubes were incubated for additional 5 min to detach biofilm. Serial dilutions of the cells were plated on tryptic soy agar. The DispersinB slightly enhanced the inhibitory effect of xylitol on biofilm-embedded *S. epidermidis* (Figure 45).

Example 41: Enhancing effect of DispersinBTM on the sensitivity of biofilm-embedded *Staphylococcus epidermidis* to antimicrobial enzyme glucose oxidase

An *in vitro* biofilm-dispersal assay was performed to determine the enhancing effect of DispersinBTM on the sensitivity of biofilm-embedded *S. epidermidis* to glucose oxidase. *S. epidermidis* biofilm was grown in 1.5 ml polypropylene microcentrifuge tubes (200 µl culture volume), medium containing planktonic cells was discarded. Further, each tube was rinsed with 200 µl of fresh medium and then treated with 200 µl medium containing 10 U/ml of glucose oxidase alone or in combination with 20 µg/ml of DispersinB. After 3 h incubation at 37°C, 10 µl of 200 µg/ml DispersinBTM was added to each tube, and tubes were incubated for additional 5 min to detach biofilm. Serial dilutions of the cells were plated on tryptic soy agar. DispersinB enhanced the inhibitory effect of glucose oxidase on biofilm-embedded *S. epidermidis* (Figure 46).

Example 42: Effect of DispersinBTM and N-(1-pyrrenyl) maleimide (PyrM) alone and in combination on *Staphylococcus epidermidis* biofilm formation.

In vitro microplate assays were performed to determine the effect of DispersinB and PyrM combination on the growth of biofilm-embedded *S. epidermidis*. Overnight growth of *S. epidermidis* in tryptic soy broth (TSB) was used as inoculum. Biofilm was developed in 12-well microplate in the absence and presence of each test compound (1 µg/ml DispersinB or 8 µg/ml PyrM) separately and together (DispersinB+PyrM). The plates were incubated at 37°C for 24 h. Medium containing planktonic cells in each well was removed gently and rinsed with sterile water. A known volume of water was added to each well and sonicated for 30 seconds. The contents of each well was transferred into a sterile tube, vortexed for a minute, followed by 10-fold serial dilution, and plated on agar plates using a spreader. After incubating the plates at 37°C for 24 h, colony-forming units (CFU) were counted. Although PyrM was more effective than DispersinB in inhibiting the growth of biofilm-embedded *S. epidermidis*, the combination of DispersinB and PyrM had an enhancing inhibitory effect on biofilm-embedded *S. epidermidis* (Figure 47).

Example 43: Effect of DispersinBTM and N, N-(1,2 phenylene) dimaleimide (oPDM) on biofilm formation of *Staphylococcus epidermidis*

In vitro microplate assays were performed to determine the effect of DispersinBTM and oPDM combination on the growth of biofilm embedded *S. epidermidis*. Overnight

growth of *S. epidermidis* in tryptic soy broth (TSB) was used as inoculum. Biofilm was developed in 12-well microplate in the absence and presence of each test compound (1 µg/ml DispersinB or 625 µg/ml oPDM) separately and together (DispersinB+oPDM). The plates were incubated at 37°C for 24 hours. Medium containing planktonic cells in each well was removed gently and rinsed with sterile water. A known volume of water was added to each well and sonicated for 30 seconds. The contents of each well was transferred into a sterile tube, vortexed for a minute, followed by 10-fold serial dilution, and plated on agar plates using a spreader. After incubating the plates at 37°C for 24 h, colony-forming units (CFU) were counted. Although oPDM was more effective than DispersinB in inhibiting the growth of biofilm-embedded *S. epidermidis*, DispersinB and oPDM together had an enhancing inhibitory effect on biofilm-embedded *S. epidermidis* (Figure 48).

Example 44: Antimicrobial activity of catheter coated with DispersinB™ and an antimicrobial

Catheter segments (1 cm) were coated with the solution containing DispersinB™ and antimicrobial by dipping and drying three times. Catheter segments could also be coated sequentially with an antimicrobial agent and DispersinB™. Antimicrobial agents such as benzalkonium chloride, sodium usnate, 5-fluorouracil, cefamandole nafate and chitosan were used separately in combination with DispersinB™ for coating. The solution containing DispersinB and each antimicrobial was prepared in 10 % glycerol as a binding agent. Glycerol could be substituted with polyethylene glycol.

The antimicrobial activity of coated catheter was determined using Kirby-Bauer technique as previously described by Sheretz, *et al.* (Antimicrob. Agents Chemother., 33:1174-1178, 1989). Catheter-associated microorganisms such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *S. aureus* and *Candida albicans* were grown in tryptic soy broth for 18 h at 37°C. An appropriate inoculum of each strain was used to prepare spread plates. The coated and uncoated sections were then carefully pressed onto the center of each spread plate. Following the incubation for 24 h at 37°C, the zone of inhibition surrounding each section was measured at the aspects of perpendicular to the long axes. The catheters coated with DispersinB™-cefamandole nafate and DispersinB™-benzalkonium chloride showed antimicrobial activity against *E. coli*, *S. epidermidis* and *S. aureus* (Table 6). The catheters coated with DispersinB™-5-fluorouracil showed

antimicrobial activity against all the test organisms except *C. albicans*. However, the catheters coated with DispersinB-sodium usnate were selectively active against gram-positive organisms.

5 **Table 6: Antimicrobial activity of catheters coated with DispersinB™ (100 µg/ml) and antimicrobial agents**

Coating	Con. of antimicrobial agent in the coating solution	Zone of inhibition (mm) against test organisms				
		<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. epidermidis</i>	<i>S. aureus</i>	<i>C. albicans</i>
DispersinB™-Cefamandole nafate	50 mg/ml	27.5	0	44	24	0
DispersinB™-5-fluorouracil	10 mg/ml	30.5	15.5	42	27.5	0
DispersinB™-sodium usnate	10 mg/ml	0	0	23	13.5	0
DispersinB™-benzalkonium chloride	100 mg/ml	3.5	0	23.5	19	10.0

Example 45: Antibiofilm activity of DispersinB™ and cefamandole nafate (CFN) combination-coated catheters against catheter-associated microorganisms

10 The antibiofilm activity of DispersinB™ and cefamandole nafate (CFN) combination coated catheters against catheter-associated bacteria and yeast was determined. Catheters were coated with DispersinB™ (100 µg/ml)-cefamandole nafate (50 mg/ml). Catheter-associated microorganisms such as *Escherichia coli*, *Staphylococcus epidermidis* and *S. aureus* were grown in TSB for 18 h. The coated and uncoated catheter segments were placed in 15 ml tubes separately containing 10 ml TSB inoculated with test organism. The tubes
15 were incubated in a water bath at 37°C with gentle shaking. After 24 h incubation, catheter

segments were washed, sonicated, vortexed and the serial dilutions were plated on tryptic soy agar. The DispersinBTM-CFN combination coated catheters inhibited > 99% *E. coli*, *S. epidermidis* and *S. aureus* biofilm formation (Figure 49).

Example 46: Antibiofilm activity of DispersinBTM and 5-fluorouracil (FU) combination-coated catheters against catheter-associated microorganisms

The antibiofilm activity of DispersinBTM and 5-fluorouracil (FU) combination coated catheters against catheter-associated bacteria and yeast was determined. Catheters were coated with DispersinBTM (100 µg/ml)-FU (10 mg/ml). Catheter-associated microorganisms such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *S. aureus* and *Candida albicans* were grown in TSB for 18 h. The coated and uncoated catheter segments were placed in 15 ml tubes separately containing 10 ml TSB inoculated with test organism. The tubes were incubated in a water bath at 37°C with gentle shaking. After 24 h incubation, catheter segments were washed, sonicated, vortexed and the serial dilutions were plated on tryptic soy agar. The DispersinBTM-FU combination coated catheters inhibited ≥ 99% gram negative and gram-positive bacterial biofilm formation (Figure 50), and it inhibited 80% *C. albicans* biofilm.

Example 47: Antibiofilm activity of DispersinBTM and sodium usnate (SU) combination-coated catheters against catheter-associated microorganisms

The antibiofilm activity of DispersinBTM and sodium usnate (SU) combination coated catheters against catheter-associated bacteria and yeast was determined. Catheters were coated with DispersinBTM (100 µg/ml)-SU (10 mg/ml). Catheter associated microorganisms such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *S. aureus* and *Candida albicans* were grown in TSB for 18 h. The coated and uncoated catheter segments were placed in 15 ml tubes separately containing 10 ml TSB inoculated with test organism. The tubes were incubated in a water bath at 37°C with gentle shaking. After 24 h incubation, catheter segments were washed, sonicated, vortexed and the serial dilutions were plated on tryptic soy agar. The DispersinBTM-SU combination coated catheters were more effective in inhibiting biofilm formation in gram-positive bacteria compared to that in gram-negative bacteria (Figure 51).

**Example 48: Antibiofilm activity of DispersinBTM and benzalkonium chloride (BKC)
combination-coated catheters against catheter-associated microorganisms**

The antibiofilm activity of DispersinBTM and benzalkonium chloride (BKC) combination coated catheters against catheter-associated bacteria and yeast was determined.

5 Catheters were coated with DispersinBTM (100 µg/ml)-BKC (100 mg/ml). Catheter-associated microorganisms such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *S. aureus* and *Candida albicans* were grown in TSB for 18 h. The coated and uncoated catheter segments were placed in 15 ml tubes separately containing 10 ml TSB inoculated with test organism. The tubes were incubated in a water bath at 37°C

10 with gentle shaking. After 24 h incubation catheter segments were washed, sonicated, vortexed and the serial dilutions were plated on tryptic soy agar. The DispersinBTM-BKC combination coated catheters completely inhibited biofilm formation in gram-negative as well as gram-positive bacteria and also in yeast (Figure 52).

**Example 49: Antibiofilm activity of DispersinBTM and chitosan combination-coated
catheters against catheter-associated microorganisms**

The antibiofilm activity of DispersinBTM and chitosan combination coated catheters against catheter-associated bacteria was determined. Catheters were coated with DispersinBTM (100 µg/ml)-chitosan (5 mg/ml). Catheter-associated microorganisms such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis* were grown in

20 TSB for 18 h. The coated and uncoated catheter segments were placed in 15 ml tubes separately containing 10 ml TSB inoculated with test organism. The tubes were incubated in a water bath at 37°C with gentle shaking. After 24 h incubation, catheter segments were washed, sonicated, vortexed and the serial dilutions were plated on tryptic soy agar. The DispersinBTM-chitosan combination coated catheters inhibited 67% *E. coli*, and > 99% *S.*

25 *epidermidis* biofilms (Figure 53).

Claims

1. A composition for inhibiting biofilm-embedded microorganisms comprising: (a) DispersinBTM, an active fragment or variant thereof that disperses a biofilm; and (b) an antimicrobial agent.
- 5 2. The composition of claim 1, wherein DispersinBTM is the mature DispersinBTM polypeptide.
3. The composition of claim 2, wherein DispersinBTM is in a concentration of about 1 µg/ml to about 500 µg/ml of the composition.
- 10 4. The composition any one of claims 1-3, wherein the antimicrobial agent comprises triclosan, rifampicin, cefamandole nafate, nitrofurazone, bismuth thiol, bismuth ethanedithiol (BisEDT), ciprofloxacin, ovotransferrin, lactoferrin, sodium usnate, 5-fluorouracil, sodium dodecyl sulfate (SDS), chlorhexidine, benzalkonium chloride, EDTA, silver nanopowder, silver compounds, glucose oxidase, lactose peroxidase, cadexomer iodine, methylene blue, gentian violet, medium-chain dextrans, sugar
15 alcohol, or mixtures thereof.
5. The composition of claim 4, wherein the antimicrobial agent is triclosan.
6. The composition of claim 5, wherein the triclosan is in a concentration of about 0.1 to about 100 mg/ml.
7. The composition of claim 4, wherein the antimicrobial agent is rifampicin.
- 20 8. The composition of claim 7, wherein the rifampicin is in a concentration of about 0.1 to about 1000 µg/ml.
9. The composition of claim 4, wherein the antimicrobial agent is cefamandole nafate.
10. The composition of claim 9, wherein the cefamandole nafate is in a concentration of about 0.05 to about 5 µg/ml.
- 25 11. The composition of claim 4, wherein the antimicrobial agent is nitrofurazone.
12. The composition of claim 11, wherein the nitrofurazone is in a concentration of about 0.01 to 1 mg/ml.

13. The composition of claim 4, wherein the antimicrobial agent is BisEDT.
14. The composition of claim 13, wherein the BisEDT is in a concentration of about 0.01 to about 2 mM.
15. The composition of claim 4, wherein the antimicrobial agent is ciprofloxacin.
- 5 16. The composition of claim 15, wherein the ciprofloxacin is in a concentration of about 0.01 to about 0.5 mg/ml.
17. The composition of claim 4, wherein the antimicrobial agent is ovotransferrin.
18. The composition of claim 17, wherein the ovotransferrin is in a concentration of about 10 to about 1000 mg/ml.
- 10 19. The composition of claim 4, wherein the antimicrobial agent is 5-fluorouracil.
20. The composition of claim 19, wherein the 5-fluorouracil is in a concentration of about 1 to about 1000 μ g/ml.
21. The composition of claim 4, wherein the antimicrobial agent is sodium dodecyl sulfate.
- 15 22. The composition of claim 21, wherein the sodium dodecyl sulfate is in a concentration of about 0.01 to about 10 mg/ml.
23. The composition of claim 4, wherein the antimicrobial agent is chlorhexidine.
24. The composition of claim 23, wherein the chlorhexidine is in a concentration of about 0.01 to about 10 μ g/ml.
- 20 25. The composition of claim 4, wherein the antimicrobial agent is benzalkonium chloride.
26. The composition of claim 25, wherein the benzalkonium chloride is in a concentration of about 0.01 to about 10 μ g/ml.
27. The composition of claim 4, wherein the antimicrobial agent is EDTA.

28. The composition of claim 27, wherein the EDTA is in a concentration of about 1 to about 1000 $\mu\text{g/ml}$.
29. The composition of claim 4, wherein the antimicrobial agent is silver nanopowder.
30. The composition of claim 29, wherein the silver nanopowder is in a concentration of about 0.01 to about 10 $\mu\text{g/ml}$.
- 5 31. The composition of any one of claims 1-30, further comprising one or more ingredients selected from the group consisting of: water; alcohol; polyethylene glycol; polypropylene glycol; a binding, bonding, or coupling agent; an antibiotic, and a pH adjuster.
- 10 32. A gel formulation comprising: (a) DispersinBTM, an active fragment or variant thereof that disperses a biofilm, and (b) a viscosity increasing agent, wherein said formulation inhibits biofilm growth.
33. The formulation of claim 31, wherein the DispersinBTM, active fragment or variant thereof, is about 0.001% to about 0.1% by weight.
- 15 34. The formulation of claim 31 or 32, wherein the viscosity increasing agent comprises a gelling agent.
35. The formulation of claim 31 or 32, wherein the viscosity increasing agent comprises an alginate-based material.
- 20 36. The formulation of claim 35, wherein the alginate-based material comprises sodium alginate.
37. The formulation of claim 36, wherein the sodium alginate is about 1% to 5% by weight.

38. A gel formulation comprising the combination of: (a) DispersinBTM, and active fragment or variant thereof, that disperses biofilms; and (b) an antimicrobial agent active against bacteria or fungi; and (c) a viscosity increasing agent.
- 5 39. The formulation of claim 38, wherein DispersinBTM, active fragment or variant thereof, is about 0.001% to 0.1% by weight.
- 10 40. The formulation of claim 38 or 39, wherein the antimicrobial agent is selected from a group consisting of triclosan, rifampicin, cefamendole nafate, nitrofurazone, bismuth thiol, bismuth ethanedithiol (BisEDT), ciprofloxacin, ovotransferrin, lactoferrin, sodium usnate, 5-fluorouracil, sodium dodecyl sulfate (SDS), chlorhexidine, benzalkonium chloride, EDTA, silver nanopowder, silver compounds, glucose oxidase, lactose peroxidase, cadexomer iodine, methylene blue, gentian violet, medium-chain dextrans, sugar alcohol, and mixtures thereof.
41. The formulation of claim 40, wherein the antimicrobial agent is triclosan.
- 15 42. The formulation of claim 41, wherein the triclosan is about 0.1% to about 10% by weight.
43. The formulation of any one of claims 38 to 42, further comprising polyethylene glycol (PEG).
44. The formulation of claim 43, further comprising ethanol.
45. The formulation of claim 44, wherein the ratio of PEG to ethanol is about 1:1.
- 20 46. The formulation of any one of claims 38 to 45, wherein the viscosity increasing agent comprises a gelling agent.
47. The formulation of any one of claims 38 to 46, wherein the viscosity increasing agent comprises an alginate-based material.

48. The formulation of claim 47, wherein the alginate-based material comprises sodium alginate.
49. The formulation of claim 48, wherein the sodium alginate is about 1% to 5% by weight.
- 5 50. The formulation of any one of claims 32 to 49, further comprising one or more ingredients selected from the group consisting of binders, wetting agents, odour absorbing agents, levelling agents, adherents, thickeners, coupling agents, antibiotics, and pH adjuster.
- 10 51. The formulation of any one of claims 32 to 50, wherein the formulation is in the form of a formable or pliable putty or flexible sheets that can be readily moulded and cut into shape.
52. A wound dressing or covering comprising the formulation of any one of claims 32 to 51.
- 15 53. A fibre comprising the composition of any one of claims 1-30 or the formulation of any one of claims 32 to 51.
54. The fibre of claim 53, wherein the fibre is a synthetic fibre.
55. The fibre of claim 54, wherein the synthetic fibre is selected from the group consisting of polyester, acrylic, polyamide, polyolefin, polyaramid, polyurethane, regenerated cellulose, and blends thereof.
- 20 56. A fabric comprising the fibre of any one of claims 53 to 55.
57. A method of inhibiting biofilm-embedded microorganisms comprising administering an effective amount DispersinBTM, an active fragment or variant thereof that disperses a biofilm; and (b) an effective amount of an antimicrobial agent.

58. The method of claim 57, wherein the antimicrobial agent comprises triclosan, rifampicin, cefamendole nafate, nitrofurazone, bismuth thiol, bismuth ethanedithiol (BisEDT), ciprofloxacin, ovotransferrin, lactoferrin, sodium usnate, 5-fluorouracil, sodium dodecyl sulfate (SDS), chlorhexidine, benzalkonium chloride, EDTA, silver nanopowder, silver compounds, glucose oxidase, lactose peroxidase, cadexomer iodine, methylene blue, gentian violet, medium-chain dextrans, sugar alcohol, or mixtures thereof.
59. The method of claim 57, wherein the DispersinBTM, an active fragment or variant thereof is administered prior to administration of the antimicrobial agent and wherein the antimicrobial agent comprises sodium dodecyl sulfate, chlorhexidine, or benzalkonium chloride.
60. A method of inhibiting biofilm-embedded microorganisms comprising administering an effective amount of the composition of any one of claims 1 to 31 or an effective amount of the formulation of any one of claims 32 to 51.
61. The method of any one of claims 57 to 60, wherein said biofilm-embedded microorganisms are associated with a wound.
62. The method of any one of claims 57 to 61, wherein the microorganisms comprise bacteria.
63. The method of 62, wherein the bacteria comprise gram-negative bacteria.
64. The method of claim 63, wherein the gram-negative bacteria comprise *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, *Providentia stuartii*, *Serratia marcescens*, *Bacteroides* spp., *Prevotella* spp., *Porphyromonas* spp., or mixtures thereof.
65. The method of 62, wherein the bacteria comprise gram-positive bacteria.
66. The method of claim 65, wherein the gram-positive bacteria comprise *Enterococcus faecalis*, Vancomycin Resistant *Enterococci* (VRE), *Peptostreptococcus* spp., *Corynebacterium* spp., *Clostridium* spp., *Streptococcus pyogenes*, *Streptococcus viridans*, *Micrococcus* sp., Beta-hemolytic streptococcus (group C), Beta-hemolytic

streptococcus (group B), *Bacillus* spp., *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Staphylococcus saprophyticus*, or mixtures thereof.

67. The method of any one of claims 57 to 61, wherein the microorganisms comprise a fungus.
- 5 68. The method of claim 67, wherein the fungus comprises *Candida albicans*, *Candida parapsilosis*, *Candida utilis*, or mixtures thereof.
69. The method of any one of claims 57 to 68, wherein biofilm is associated with a wound.
70. The method of claim 69, wherein the wound comprises a cutaneous abscess, surgical wound, sutured laceration, contaminated laceration, blister wound, soft tissue wound, partial thickness burn, full thickness burn, decubitus ulcer, stasis ulcer, foot ulcer, venous ulcer, diabetic ulcer, ischemic ulcer, pressure ulcer, or combinations thereof.
- 10 71. A method of any one of claims 57 to 60, wherein the biofilm embedded microorganisms comprise dental plaque.
- 15 72. A method of claim 71, wherein the dental plaque is subgingival dental plaque.
73. A method of any one of claims 57 to 60, wherein the biofilm embedded microorganisms are etiologic agents of periodontal disease.
74. A method of treating localized juvenile periodontitis comprising administering a composition of any one of claims 1-31.
- 20 75. A method of treating periodontal disease comprising administering a composition of any one of claims 1-31.
76. A method for preparing a wound care device comprising incorporating the composition of any one of claims 1-31 or the formulation of any one of claims 32 to 51 into bandage and wound dressing polymeric materials.
- 25 77. The method for preparing a wound care device according to claim 76, wherein the bandage and wound dressing polymeric material comprises silastic or other silicone-based material, Dacron[®], knitted Dacron[®], velour Dacron[®], nylon, silk, cotton,

polyethylene, polyurethane, polyvinyl chlorides silastic elastomer, silicone rubber, poly(methyl methacrylate), latex, polypropylene, polyolefin, cellulose, poly vinyl alcohol, polyhydroxyethyl methacrylate, poly glycolic acid, polyacrylonitrile, Teflon[®], copolymers thereof, and mixtures thereof.

- 5 78. The method for preparing wound care device according to claim 76 or 77, wherein the bandage or wound dressing comprises an outer fabric support layer and an inner layer with an absorbent body disposed there between and wherein the said inner layer and /or said absorbent body are fabricated from one or more polymeric materials having a
10 therapeutically effective amount of one or more materials including DispersinB[™] and triclosan, which method comprises incorporating therapeutically effective amounts of active compounds including DispersinB[™] and triclosan into a mixture of the polymeric materials prior to casting or spinning of the polymeric material.
- 15 79. A composition for inhibiting biofilm-embedded microorganisms comprising: (a) DispersinB[™], an active fragment or variant thereof that disperses a biofilm; and (b) a bacteriophage.
80. The composition of claim 79, wherein the composition comprises about 10⁸ bacteriophage.
- 20 81. The composition of claim 79 or 80, wherein the bacteriophage comprises more than one species of bacteriophage.
82. A composition for inhibiting biofilm-embedded microorganisms comprising a recombinant bacteriophage, wherein the recombinant bacteriophage displays DispersinB[™].
- 25 83. The composition of claim 82, wherein the displayed DispersinB[™] is fused to a phage coat protein.
84. The composition of claim 83, wherein the DispersinB[™] is fused to the major coat protein.
85. The composition of claim 83, wherein the DispersinB[™] is fused to the minor coat protein.

86. A fusion protein comprising at least a portion of a phage coat protein bonded to DispersinBTM.
87. A method of preparing a device comprising treating at least one surface of the device with the composition of any one of claims 1-31 or the formulation of any one of claims 32 to 52.
88. A method of preparing a device comprising incorporating the composition of any one of claims 1-31 into polymers, wherein said polymers are used to form the device.
89. A method of preparing a device comprising coating the composition of any one of claims 1-31 onto a surface of the device selected from the group consisting of an inner surface of the device, an outer surface of the device, and both an inner and an outer surface of the device.
90. The method according to any one of claims 87 to 89, wherein the composition comprises effective amounts of DispersinBTM and triclosan.
91. The method according to any one of claims 87 to 89, wherein the composition comprises effective amounts of DispersinBTM and rifampicin.
92. The method according to any one of claims 87 to 89, wherein the composition comprises effective amounts of DispersinBTM and cefamandole nafate.
93. The method according to any one of claims 87 to 89, wherein the composition comprises effective amounts of DispersinBTM and nitrofurazone.
94. The method according to any one of claims 87 to 93, wherein the device is a medical device.
95. The method as claimed in claim 94, wherein the medical device is a catheter.
96. The method of claim 95, wherein the catheter is an indwelling catheter.
97. The method of claim 96, wherein the indwelling catheter is selected from the group consisting of a central venous catheter, a peripheral intravenous catheter, an arterial catheter, a peritoneal catheter, a haemodialysis catheter, an umbilical catheter,

percutaneous nontunneled silicone catheter, a cuffed tunneled central venous catheter, an endotracheal tube, and a subcutaneous central venous port.

98. The method of claim 96, wherein the indwelling catheter is selected from the group consisting of urinary catheter, a peritoneal catheter, a peripheral intravenous catheter and central venous catheter.

99. The method according to claim 87 to 93, wherein the device is selected from the group consisting of catheters, pacemakers, prosthetic heart valves, prosthetic joints, voice prostheses, contact lenses, a stent, heart valve, penile implant, small or temporary joint replacement, urinary dilator, cannula, elastomer, and intrauterine devices.

100. The method according to any one of claims 87 to 93, wherein the device is selected from the group consisting of a catheter lock, a needle, a Leur-Lok® connector, a needleless connector, a clamp, a forcep, a scissor, a skin hook, a tubing, a needle, a retractor, a scaler, a drill, a chisel, a rasp, a surgical instrument, a dental instrument, a tube, an intravenous tube, a breathing tube, a dental water line, a dental drain tube, a feeding tube, a bandage, a wound dressing, an orthopedic implant, and a saw.

101. Use of the composition of any one of claims 1-31 or the formulation of any one of claims 34 to 53 for promoting detachment of bacterial or fungal cells from a biofilm.

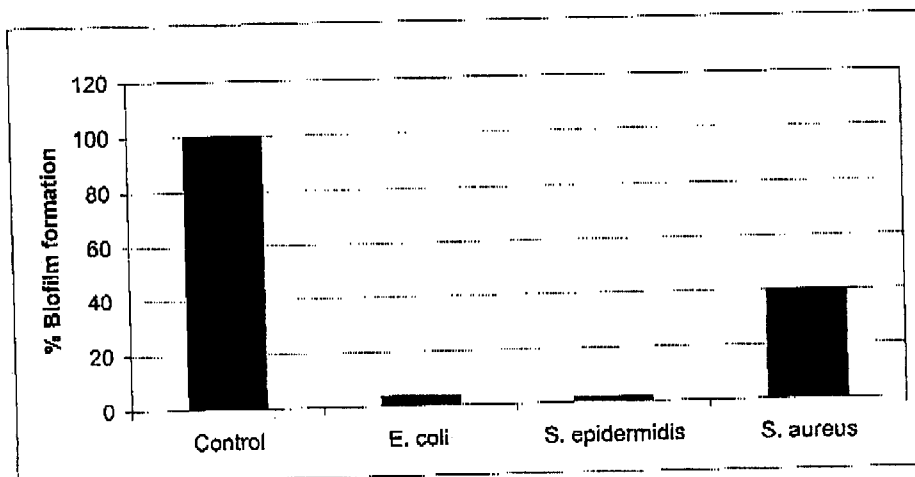
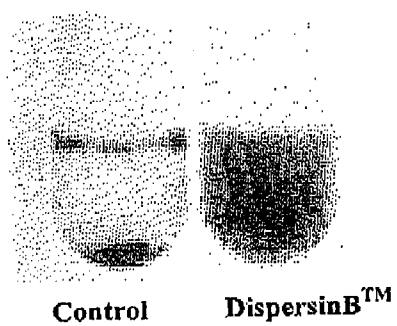
102. Use of the composition of any one of claims 1-31 in the preparation of a medical device for implantation in a mammal.

103. Use of the composition of any one of claims 1-31 for prevention of an infection caused by an implanted medical device in a mammal.

104. The use of claim 102 or 103, wherein the medical device is coated, incorporated, or treated with the composition.

105. The use of any one of claims 102 to 104, wherein the medical device is selected from the group consisting of catheters, pacemakers, prosthetic heart valves, prosthetic joints, voice prostheses, contact lenses, a stent, heart valve, penile implant, small or temporary joint replacement, urinary dilator, cannula, elastomer, and intrauterine devices.

106. The use of claim 105, wherein the catheter is an indwelling catheter.
107. The use of claim 106, wherein the indwelling catheter is selected from a group consisting of urinary catheter, a peritoneal catheter, and central venous catheter.
- 5 108. The use of claim 105, wherein the indwelling catheter is selected from a group consisting of a central venous catheter, a peripheral intravenous catheter, an arterial catheter, a peritoneal catheter, a haemodialysis catheter, an umbilical catheter, precutaneous nontunneled silicone catheter, a cuffed tunneled central venous catheter, an endotracheal tube, and a subcutaneous central venous port.
109. The use of claim 103, wherein the infection is a urinary tract infection.
- 10 110. The use of claims 103, wherein the infection is a vascular infection.

Figure 1**Figure 2**

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Figure 3

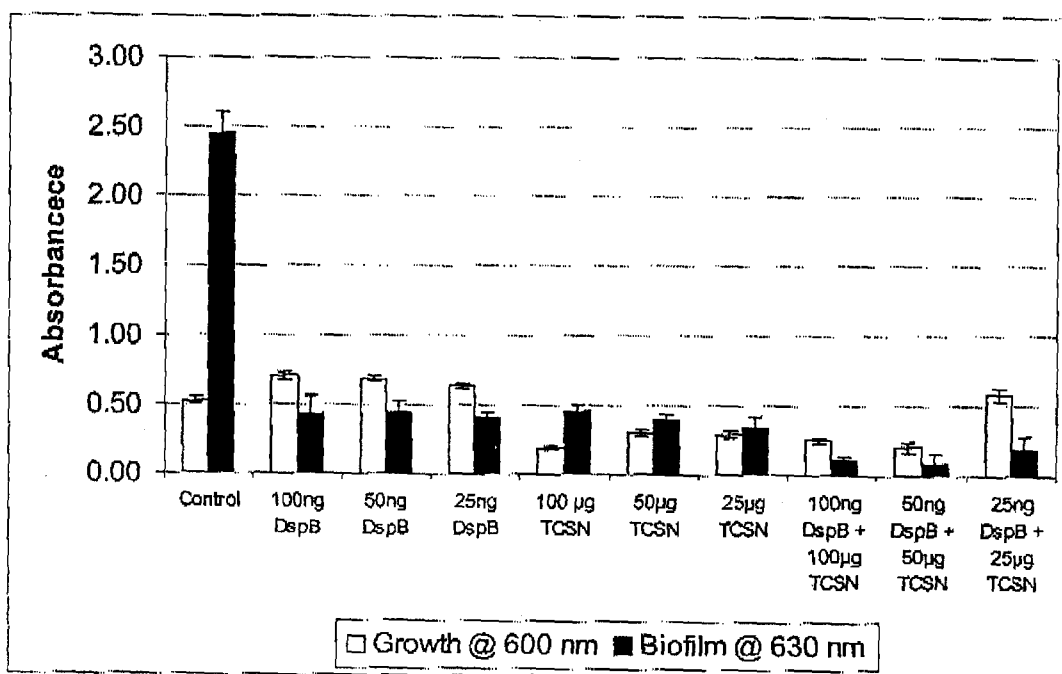
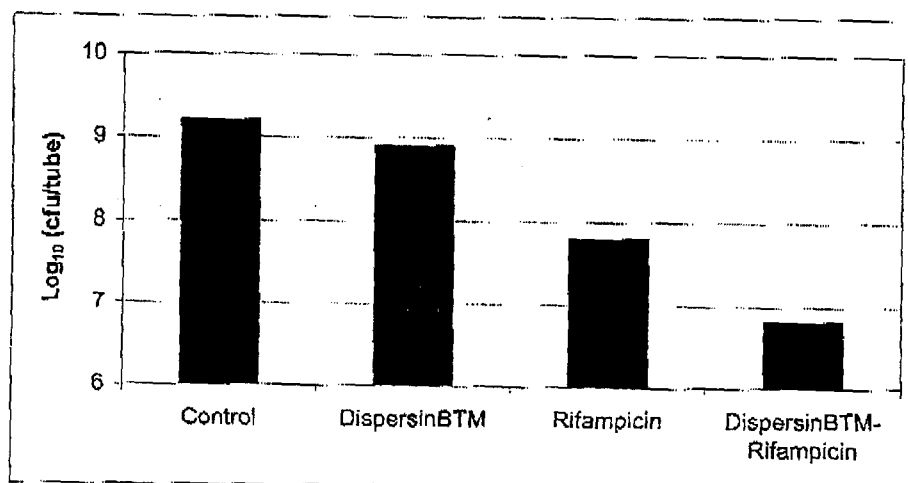


Figure 4



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Figure 5

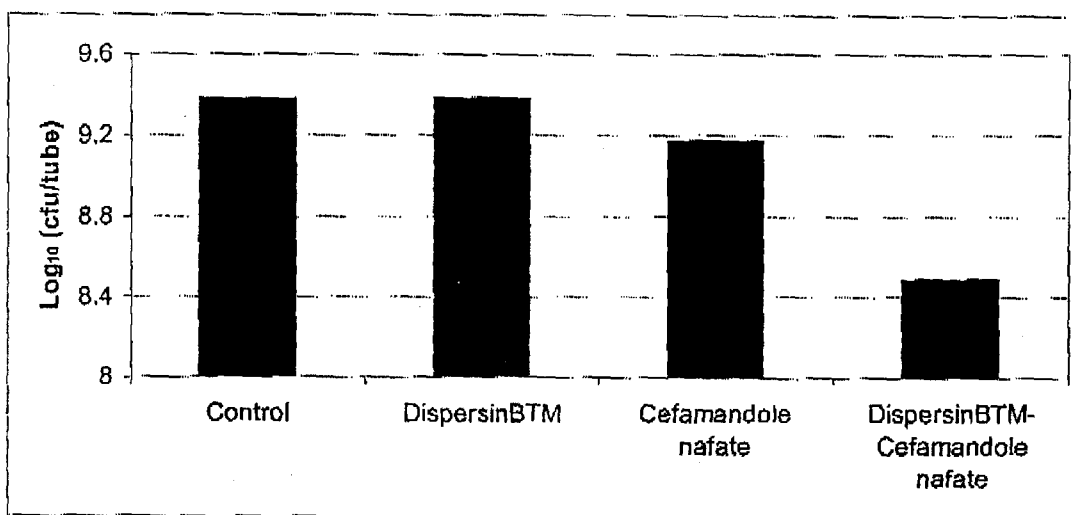
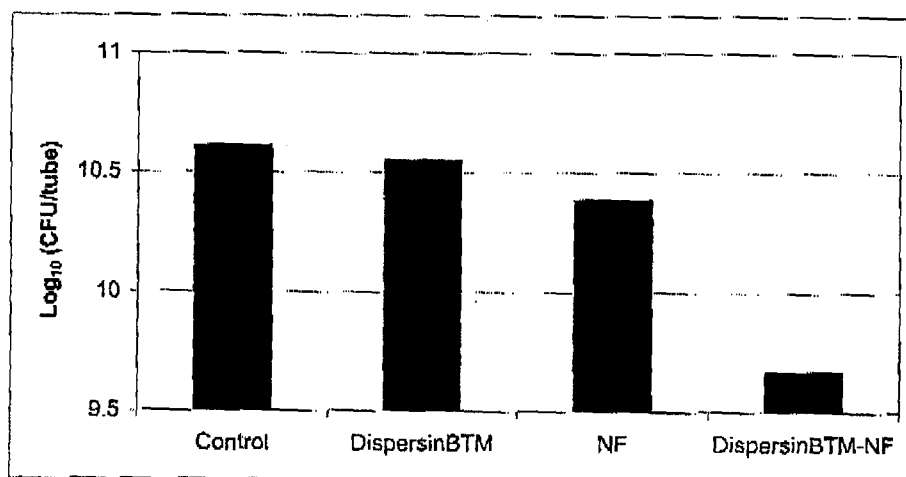


Figure 6



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Figure 7

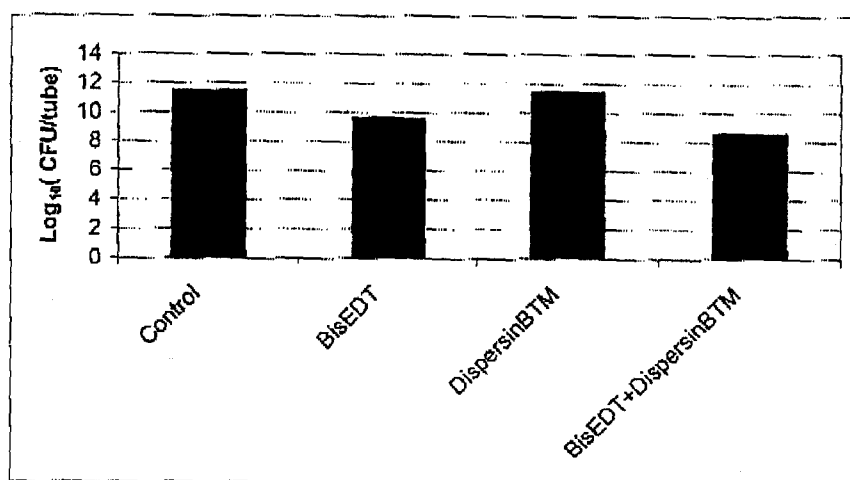
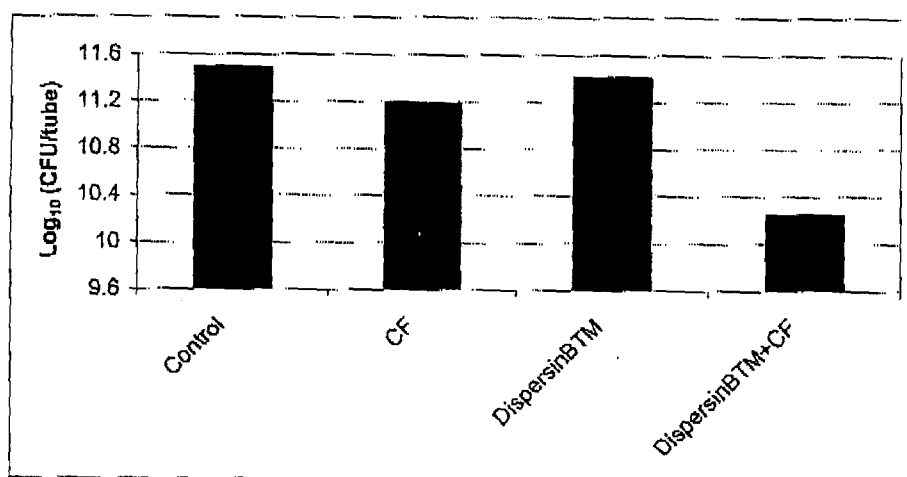


Figure 8



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Figure 9

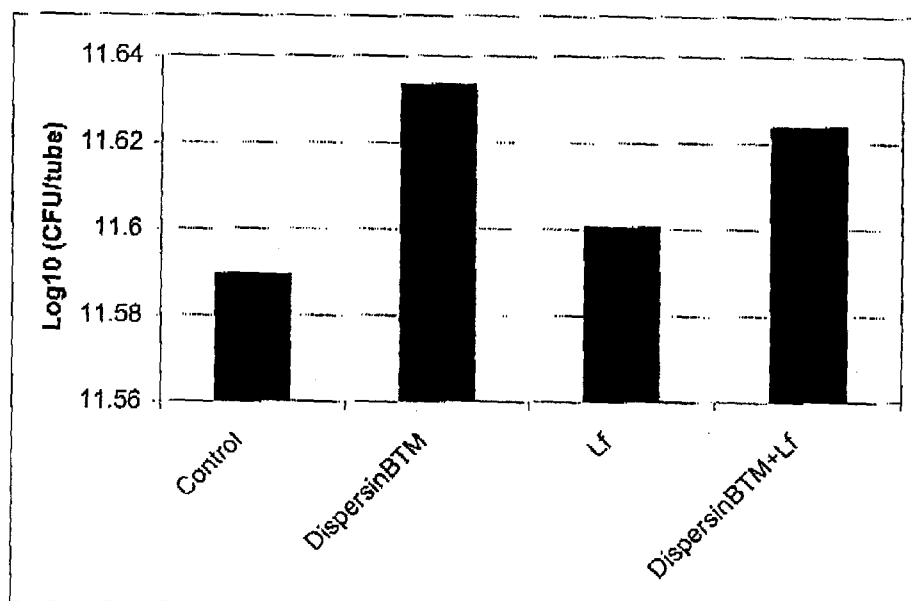
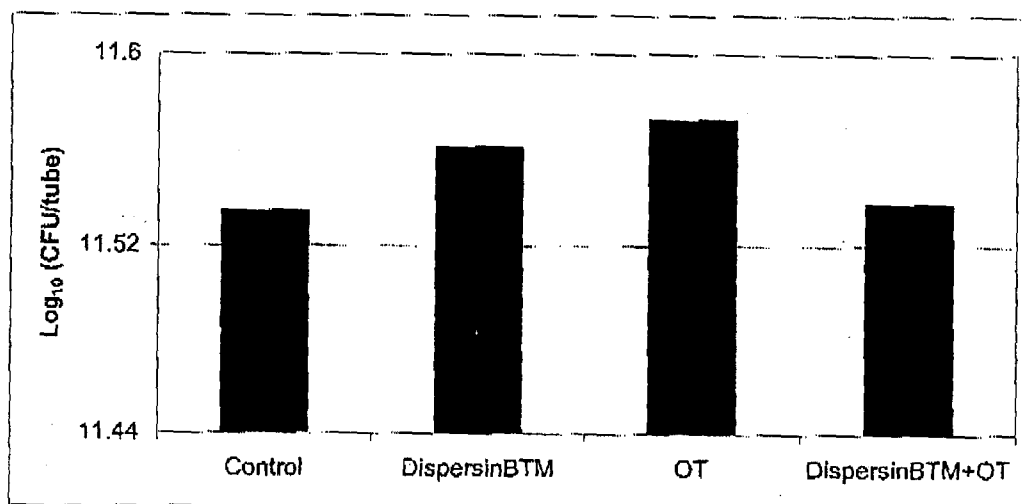


Figure 10



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Figure 11

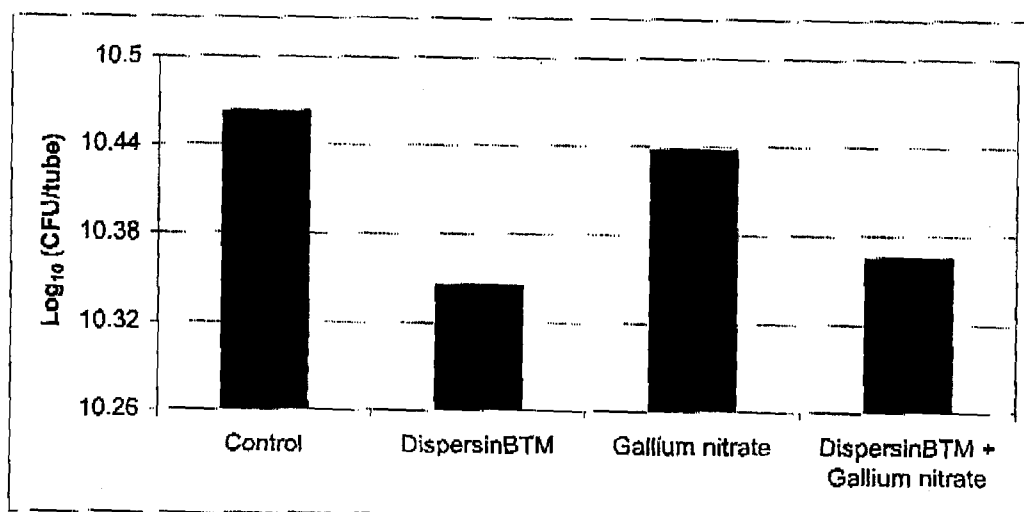
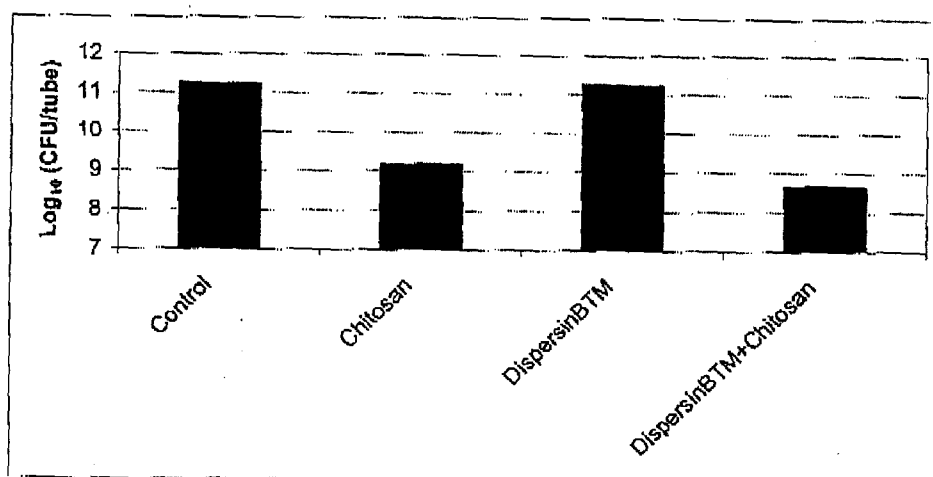


Figure 12



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Figure 13

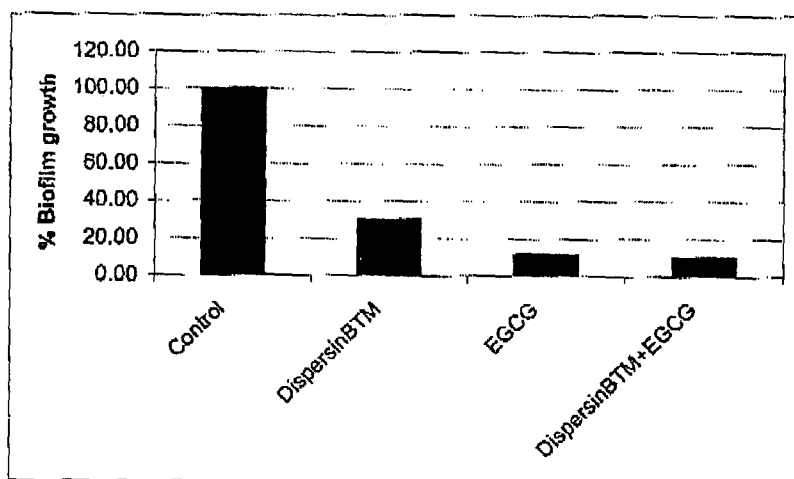
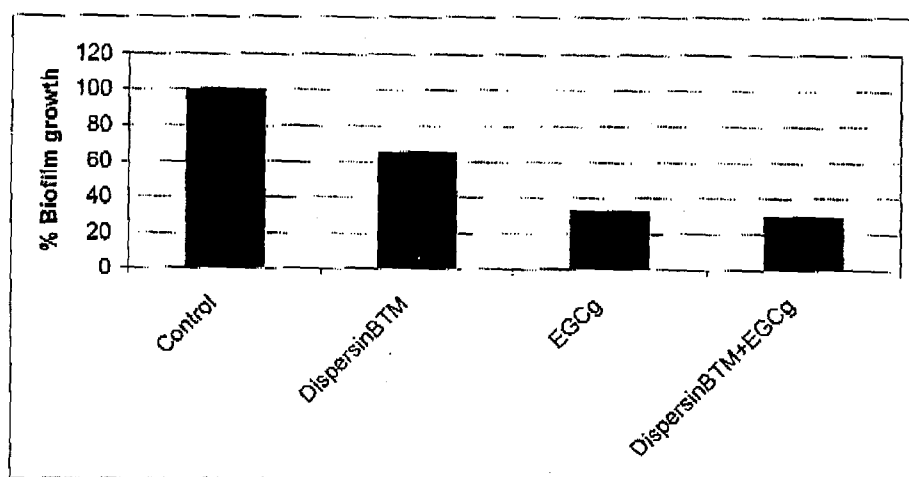


Figure 14



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Figure 15

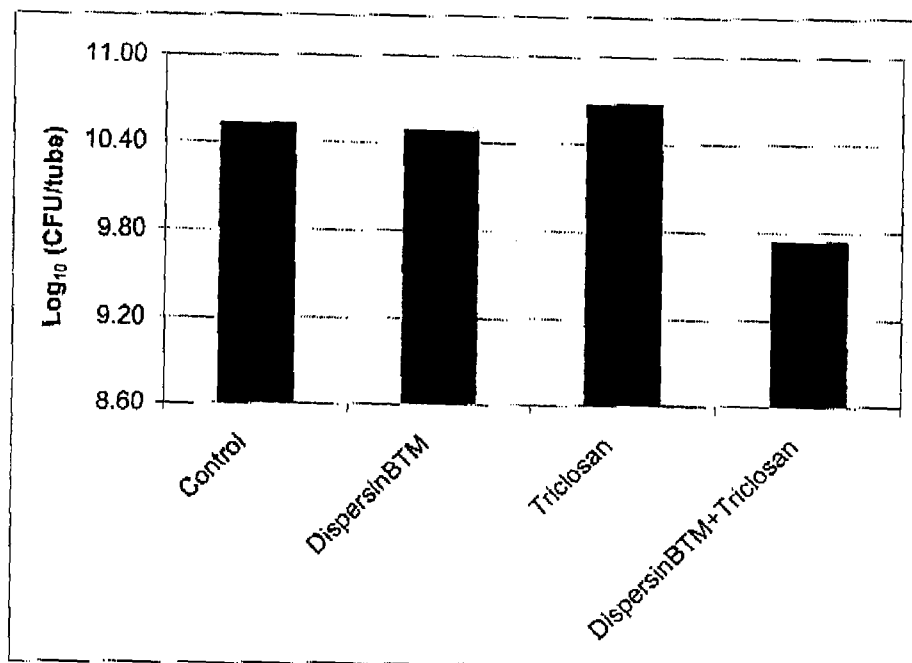
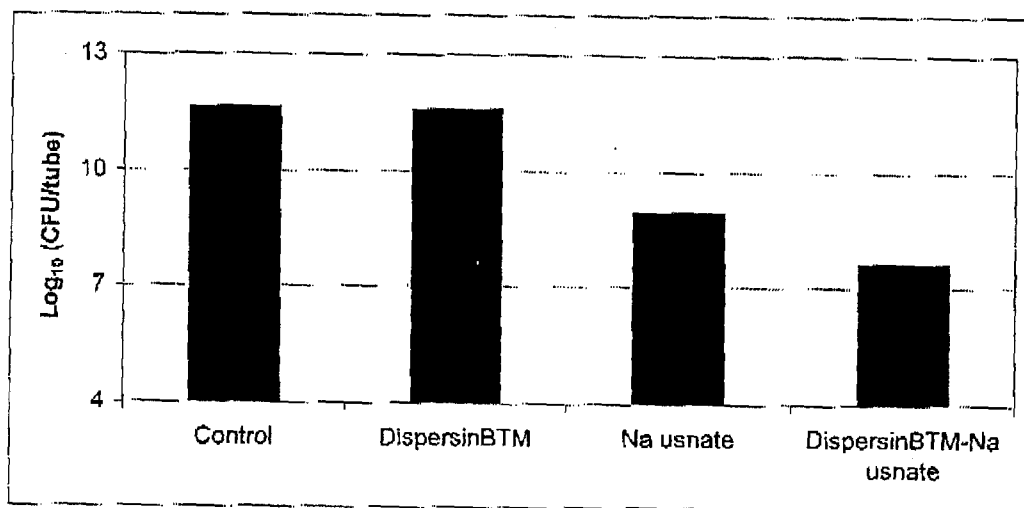


Figure 16



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Figure 17

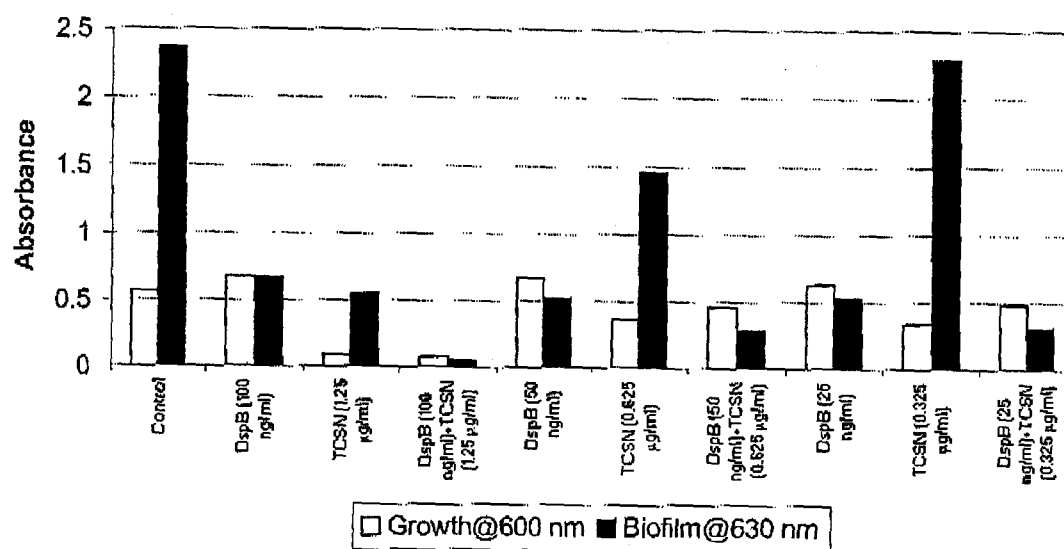
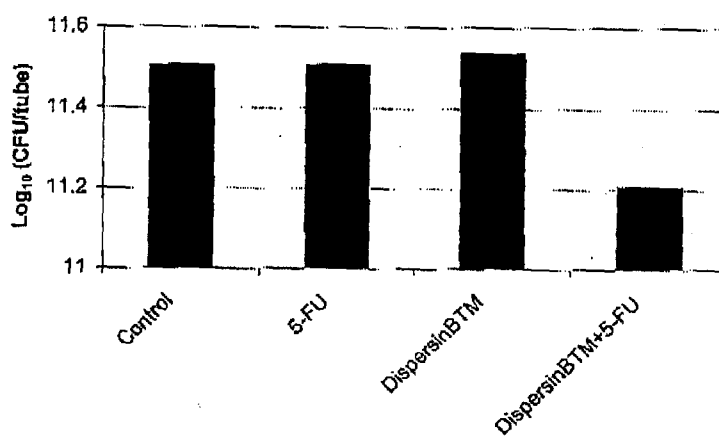


Figure 18



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Figure 19

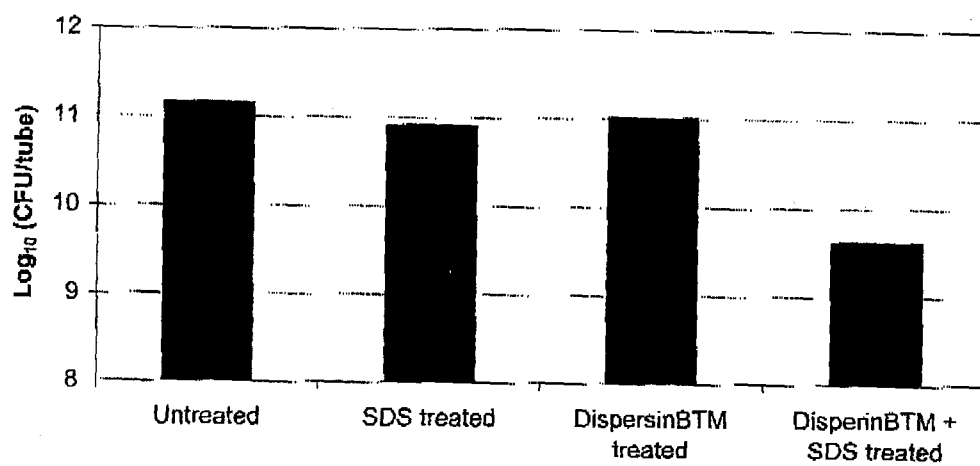
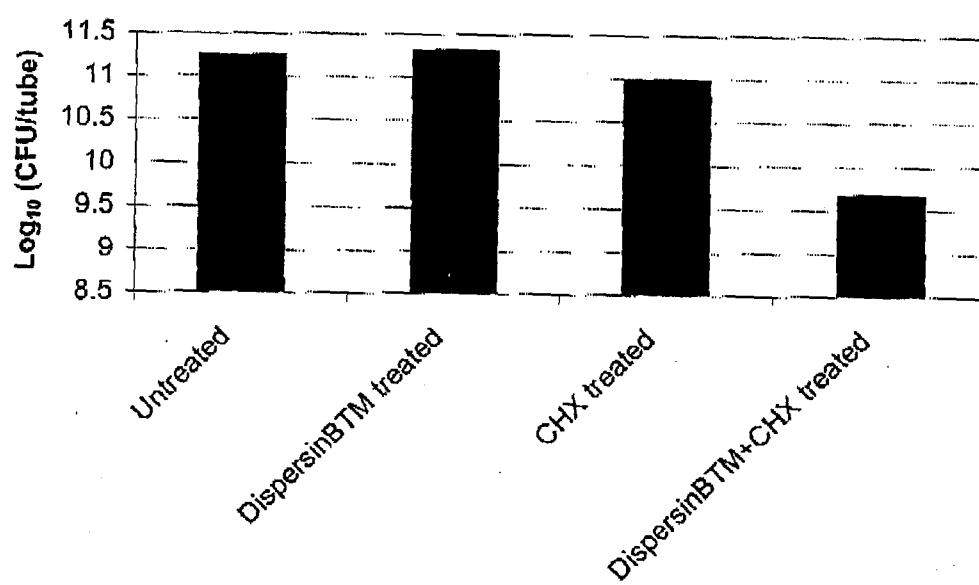


Figure 20



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Figure 21

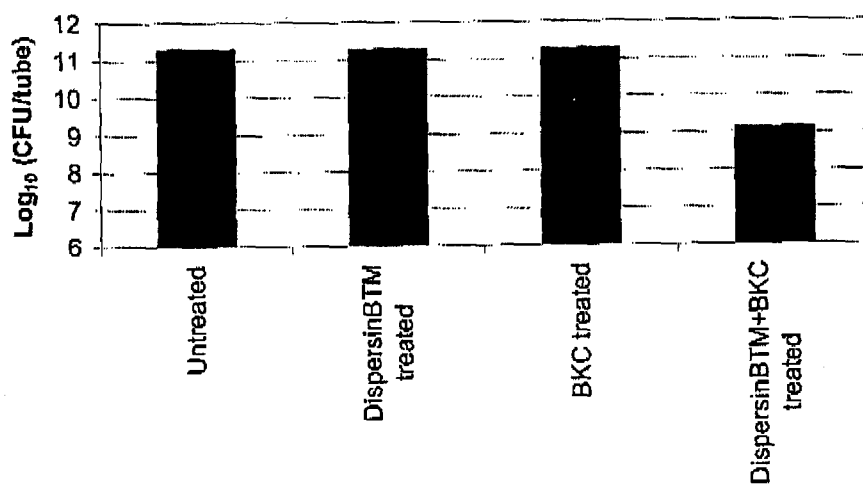


Figure 22

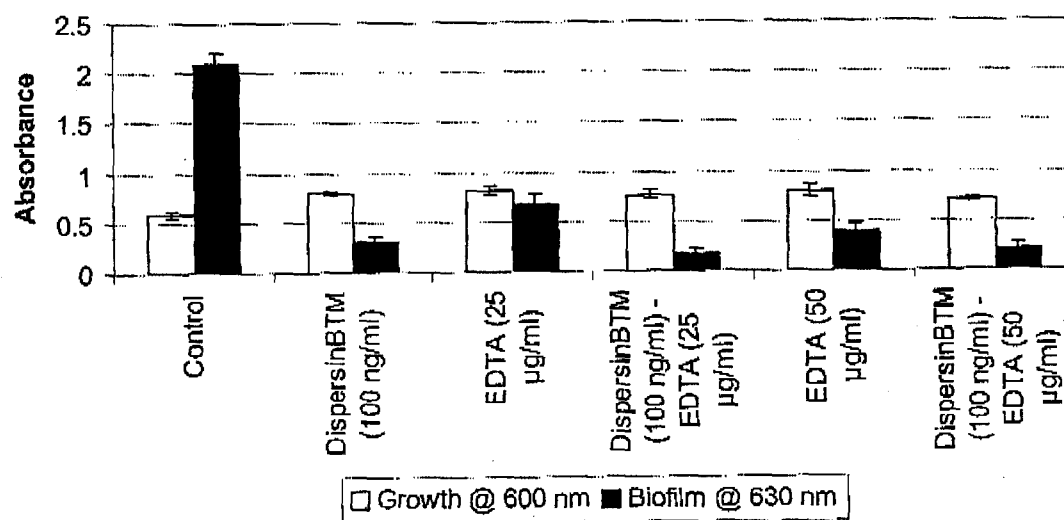


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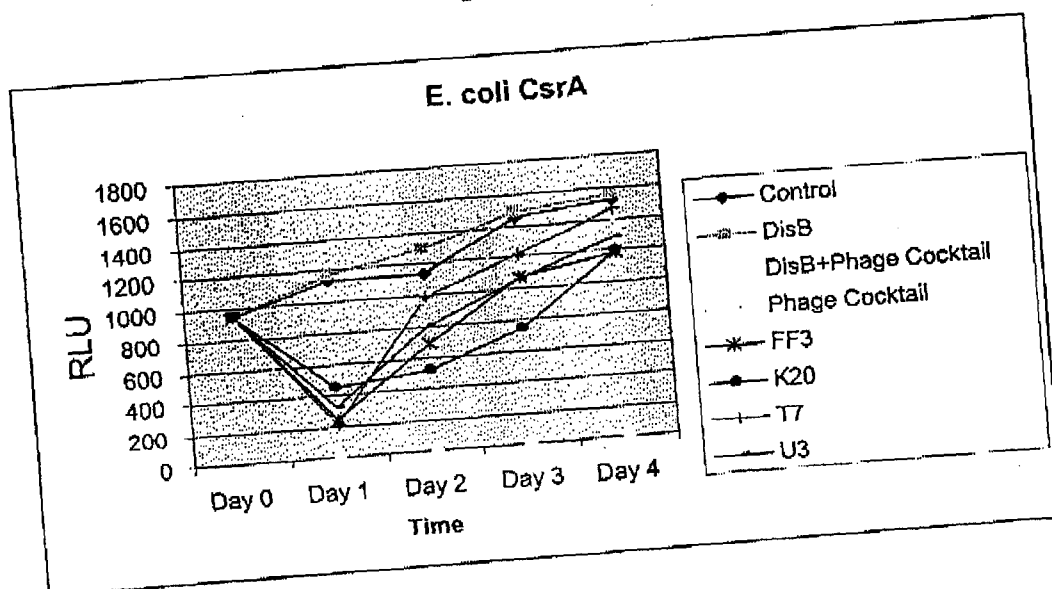
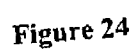
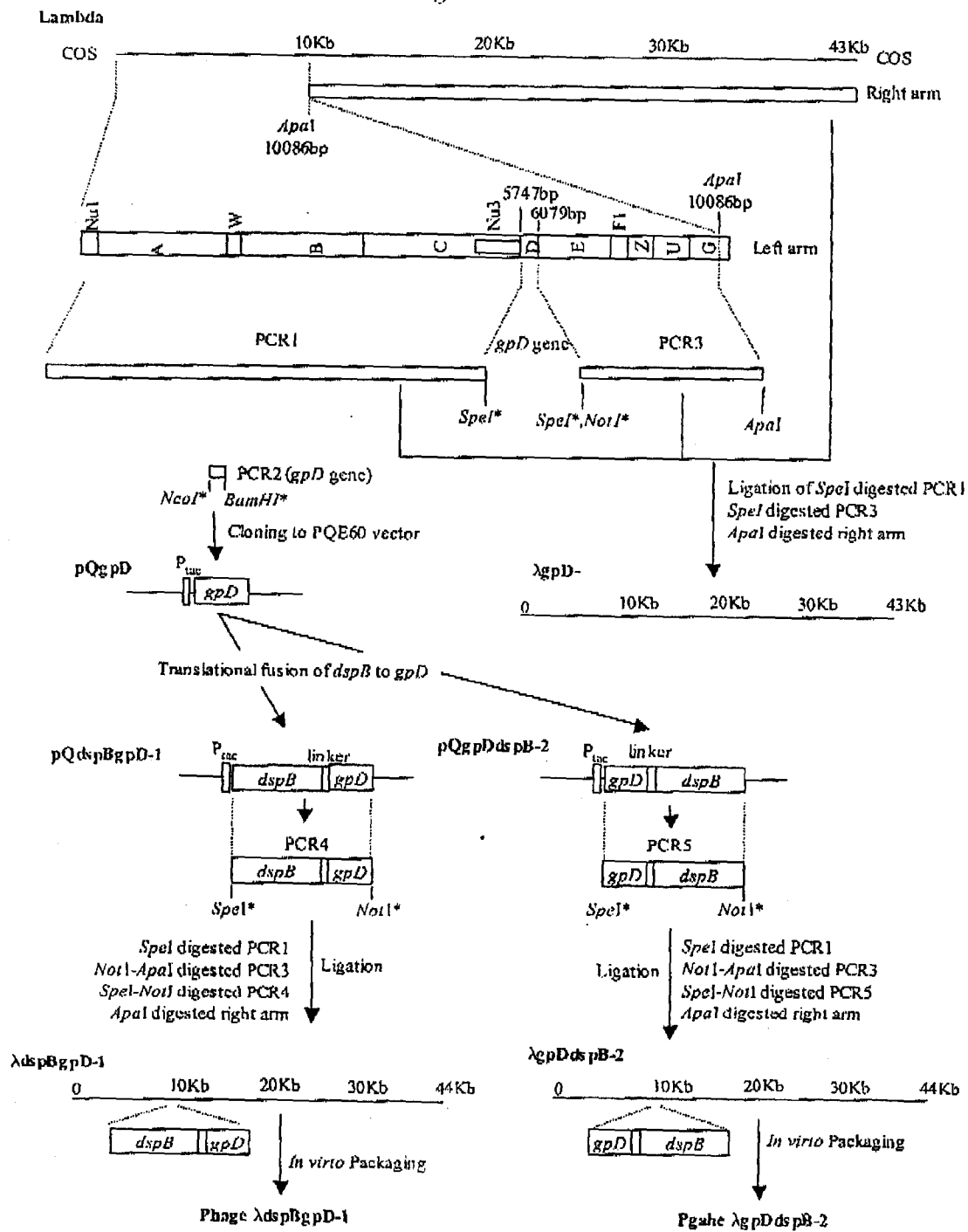
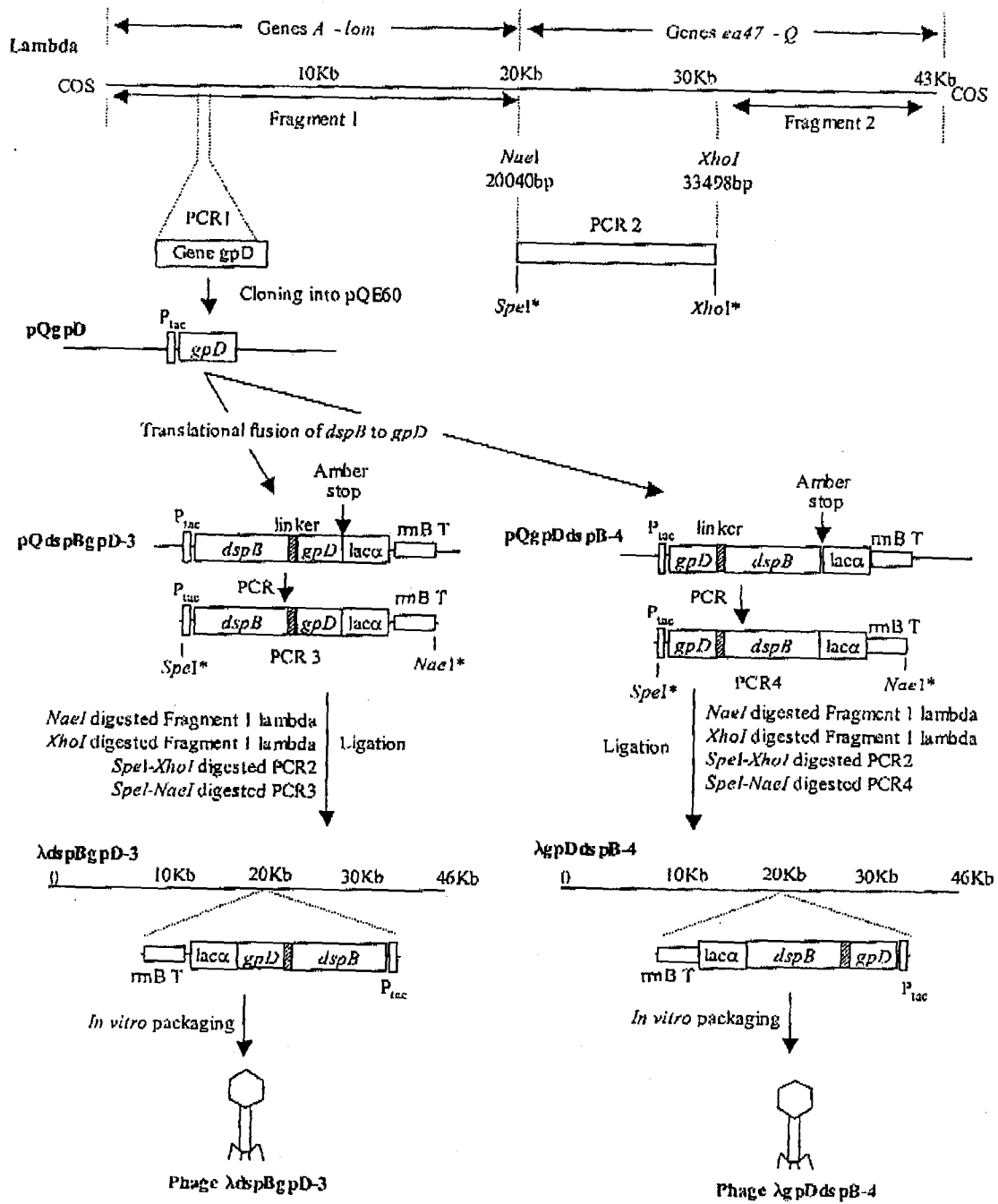


Figure 25



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Figure 26



* The new endonuclease restriction sites introduced by PCR primers

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Figure 27

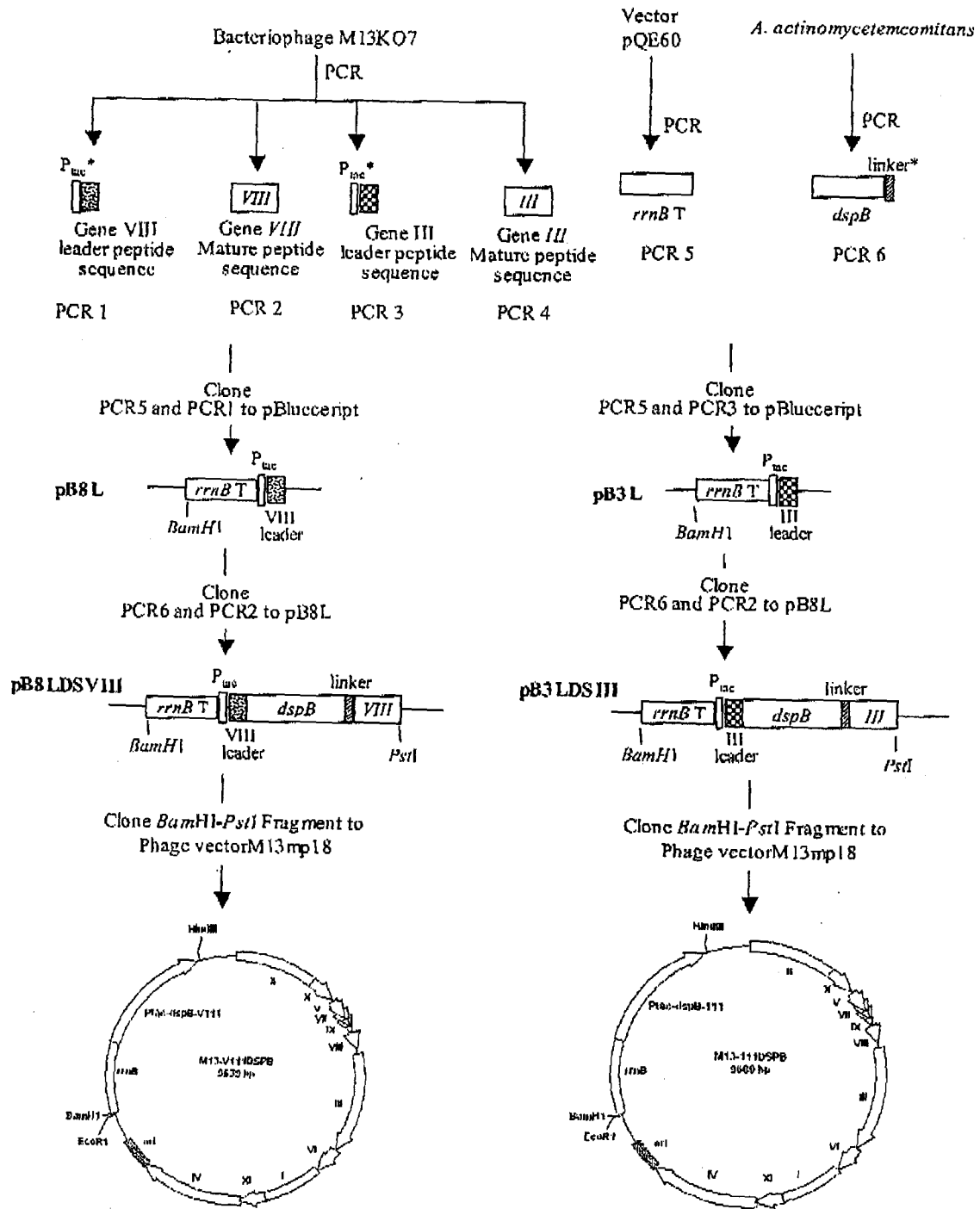
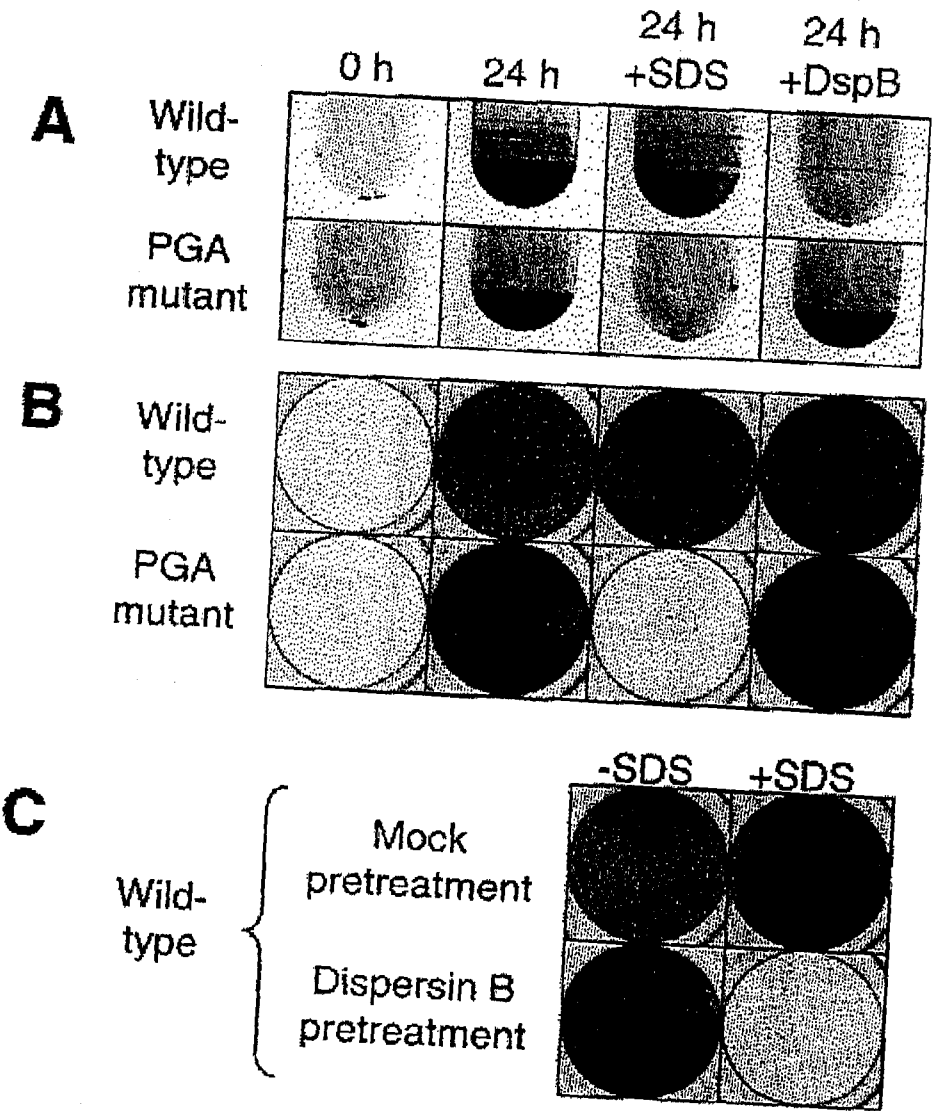


Figure 28



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Figure 29

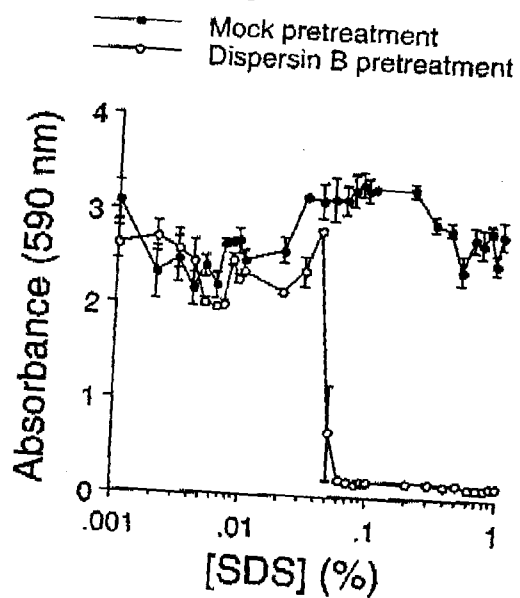
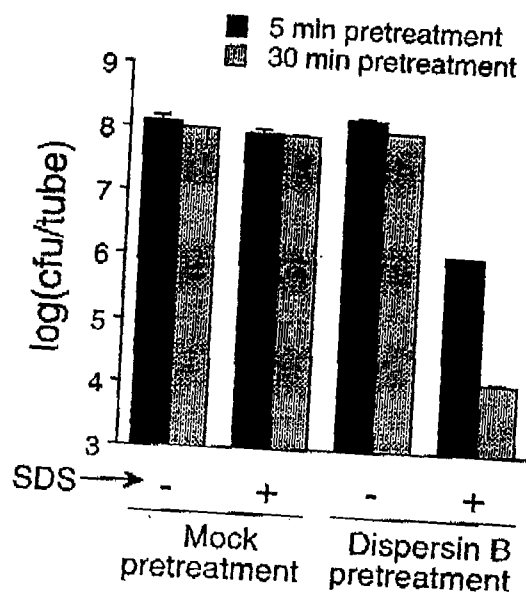


Figure 30



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Figure 31

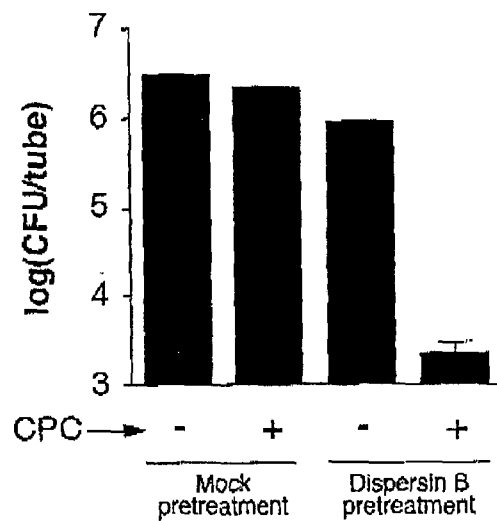
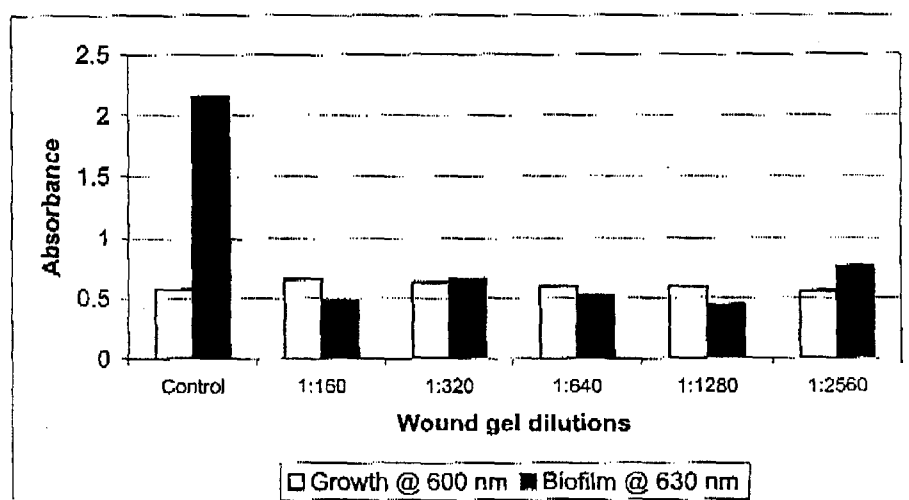


Figure 32



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Figure 33

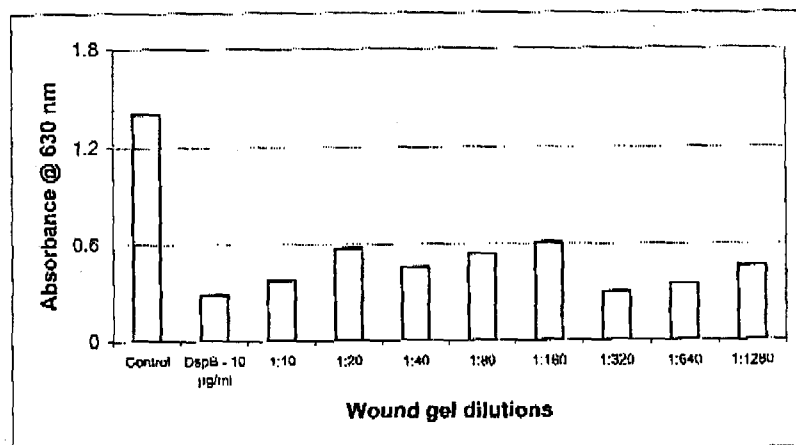
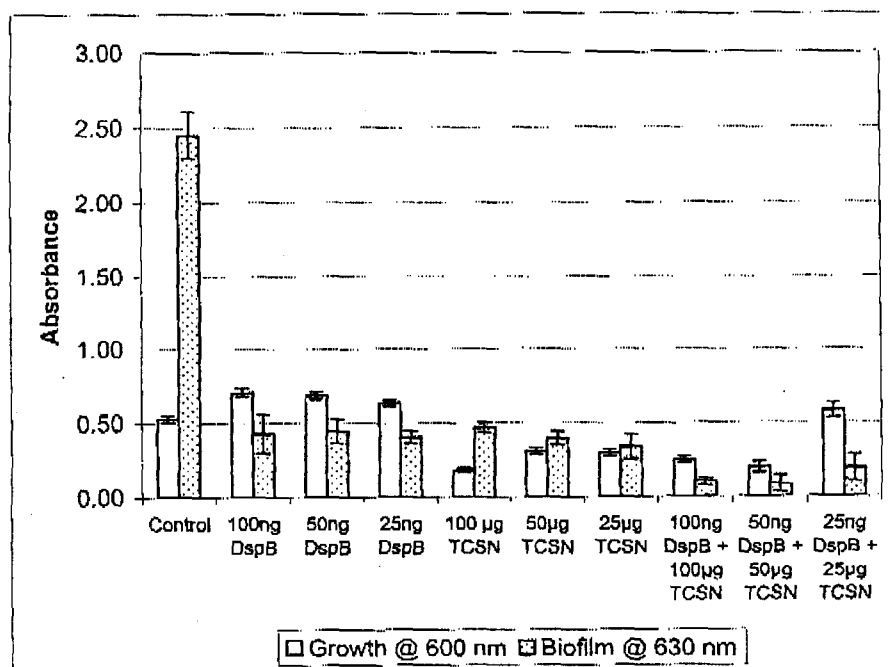


Figure 34



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Figure 35

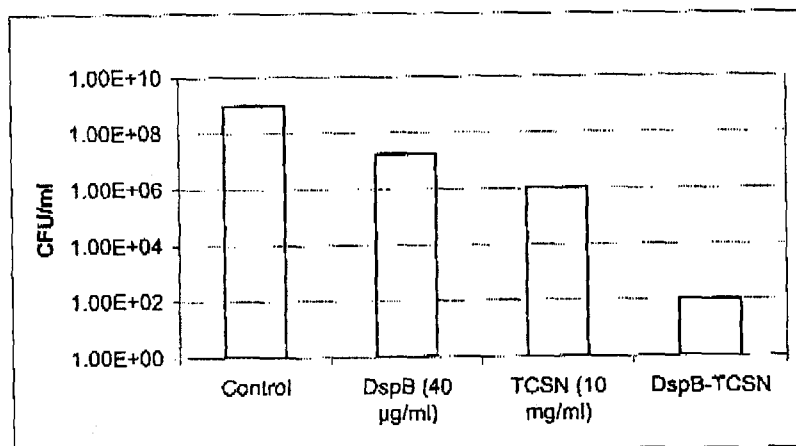
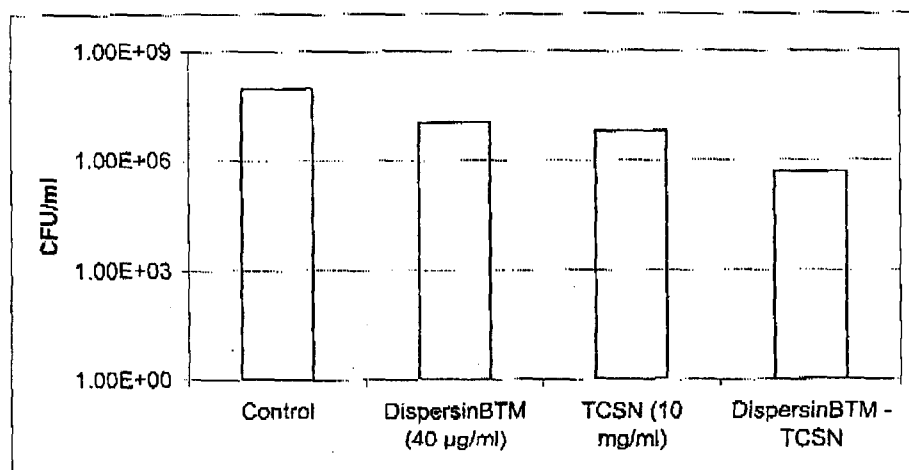


Figure 36



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Figure 37

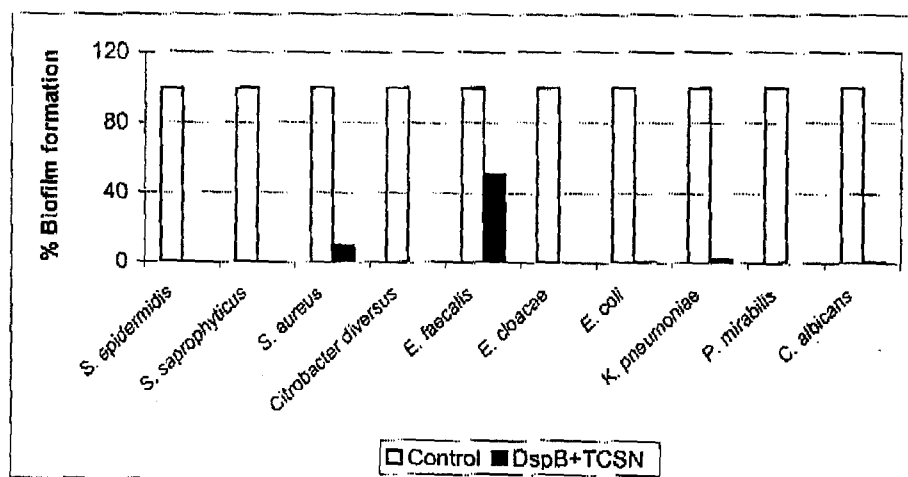
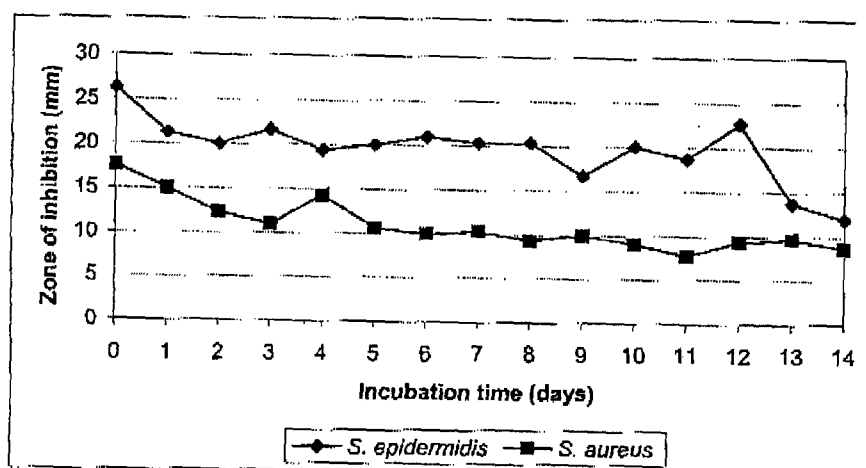


Figure 38



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Figure 39

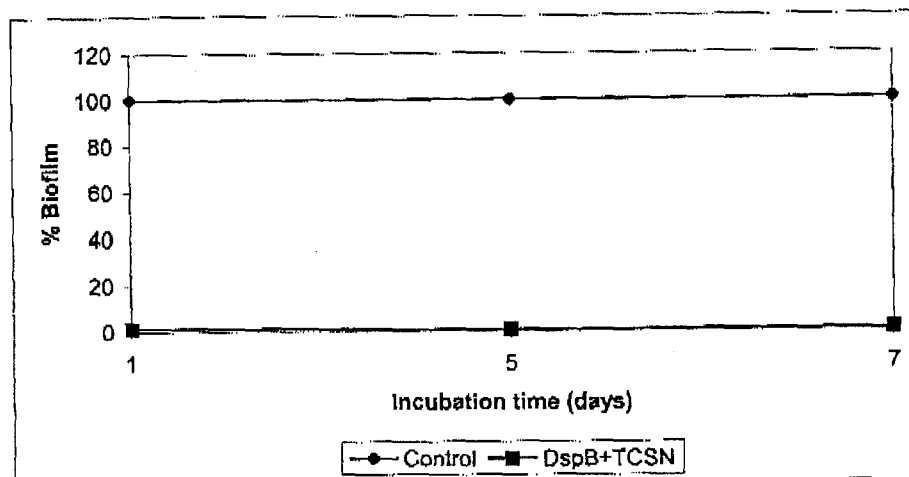
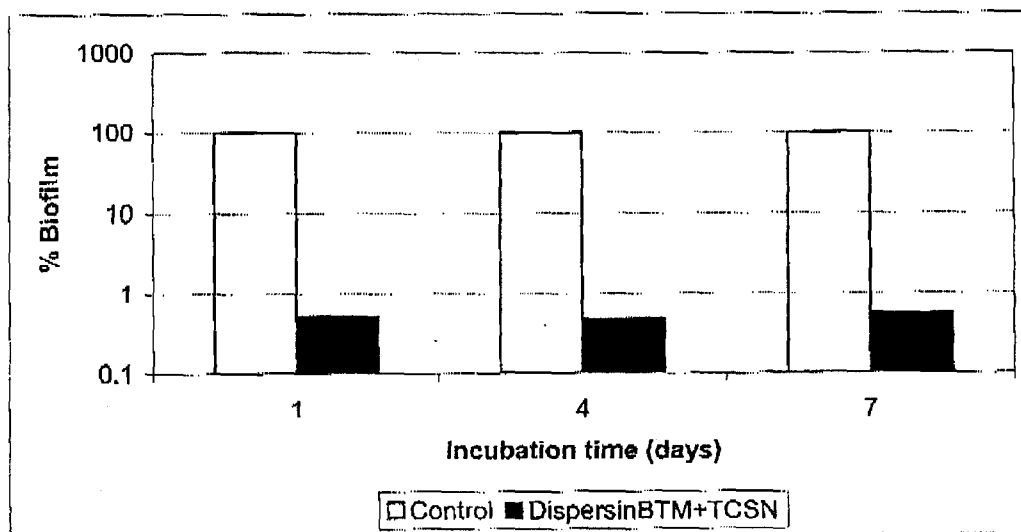


Figure 40



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Figure 41

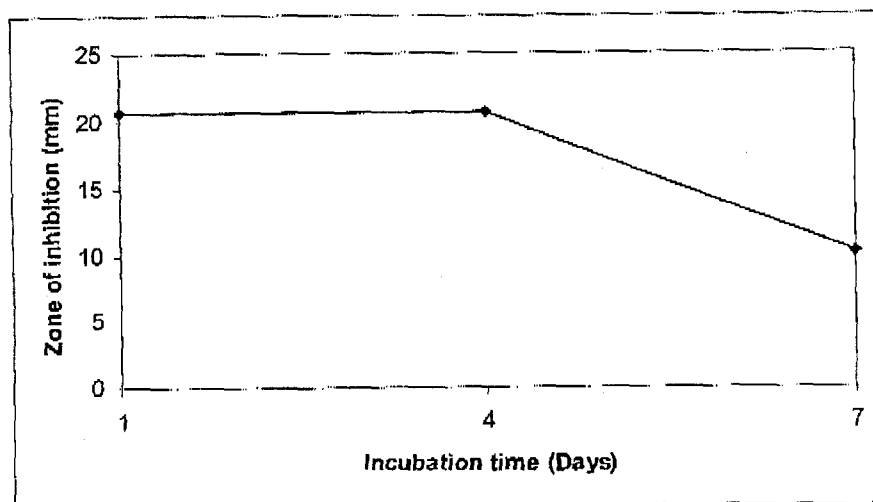
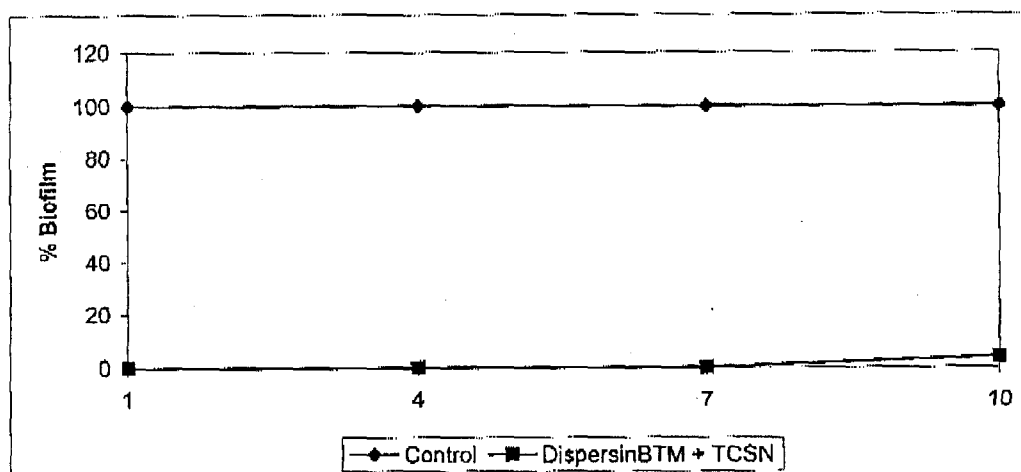


Figure 42



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Figure 43

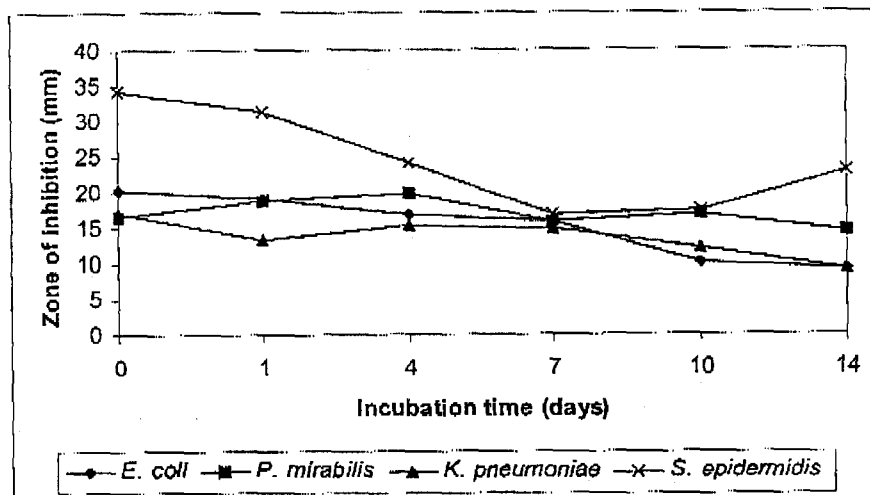
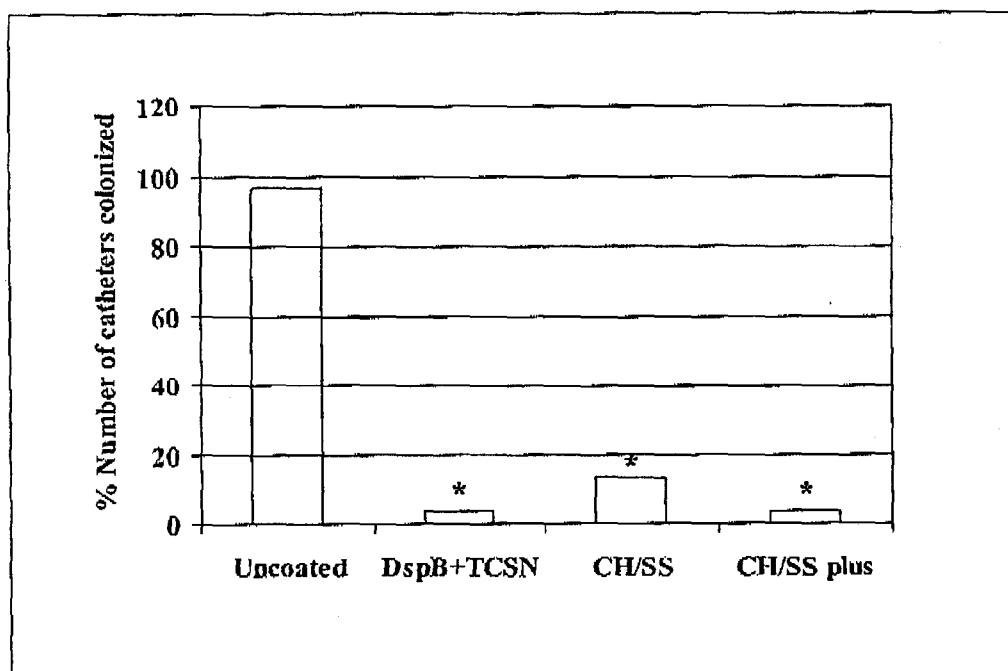


Figure 44



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Figure 45

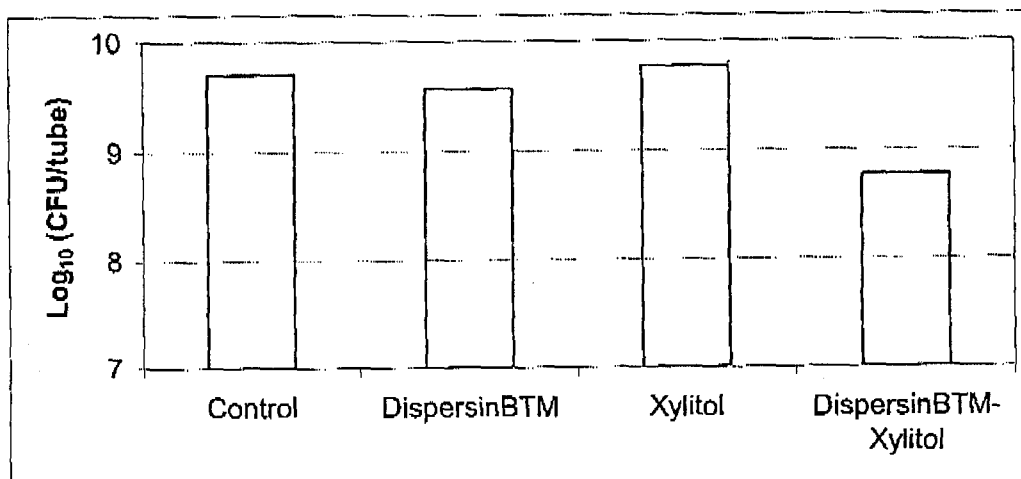
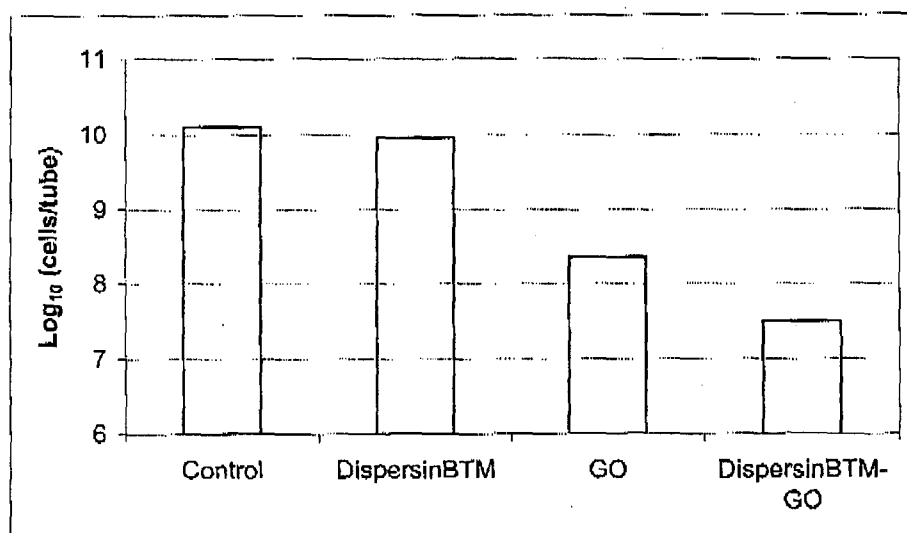


Figure 46



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Figure 47

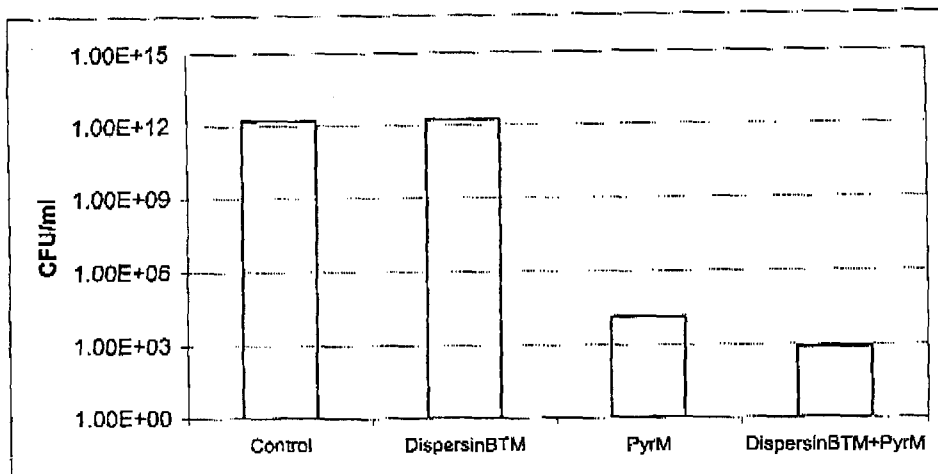
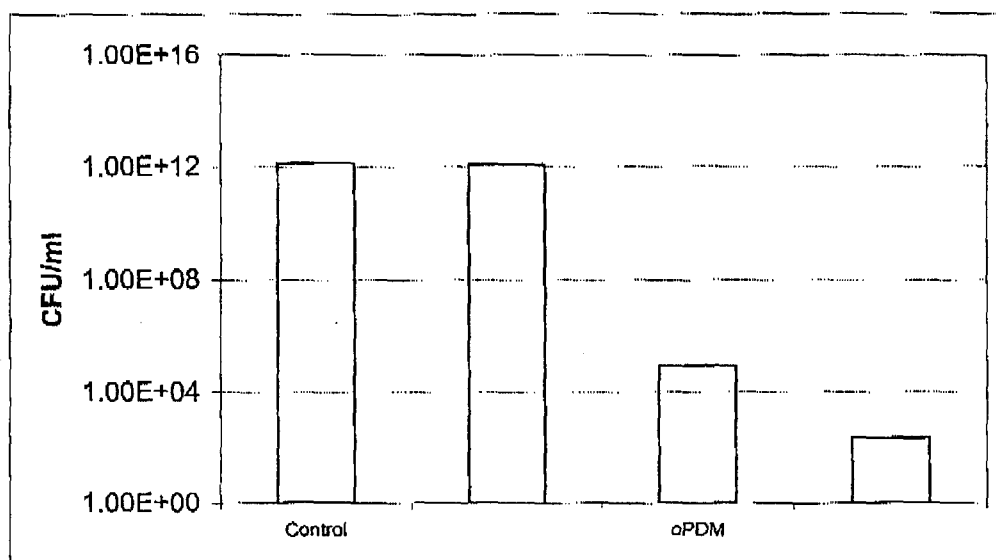


Figure 48



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Figure 49

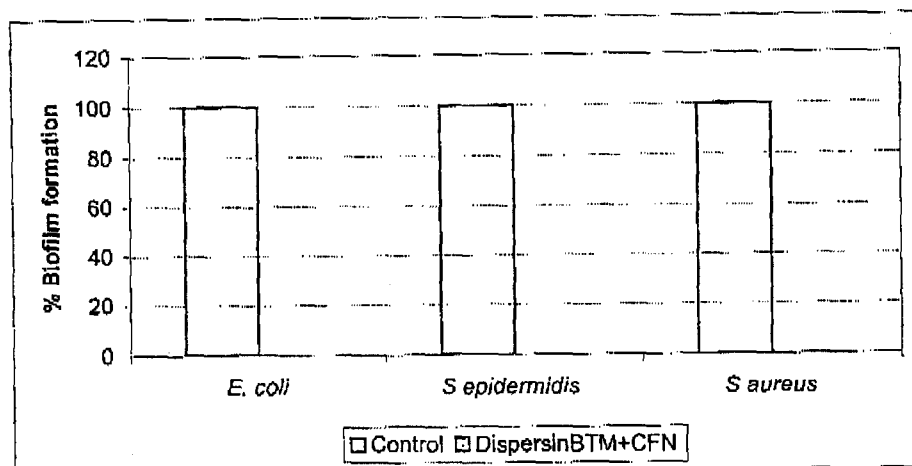
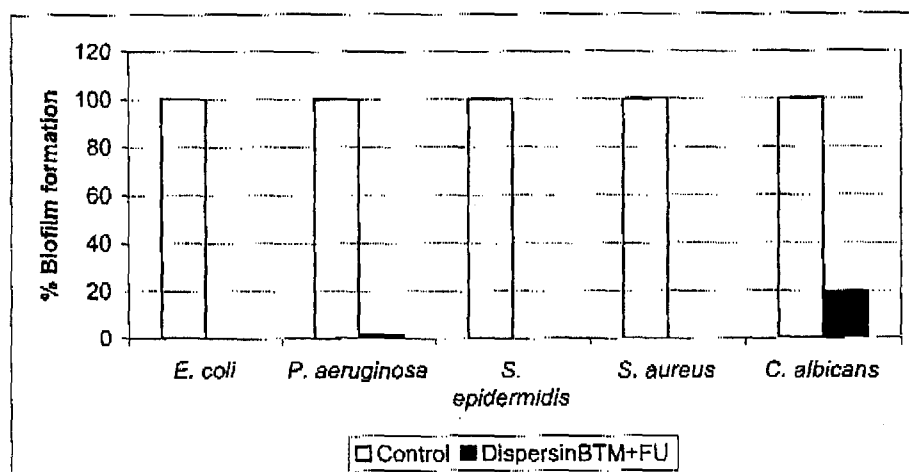


Figure 50



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Figure 51

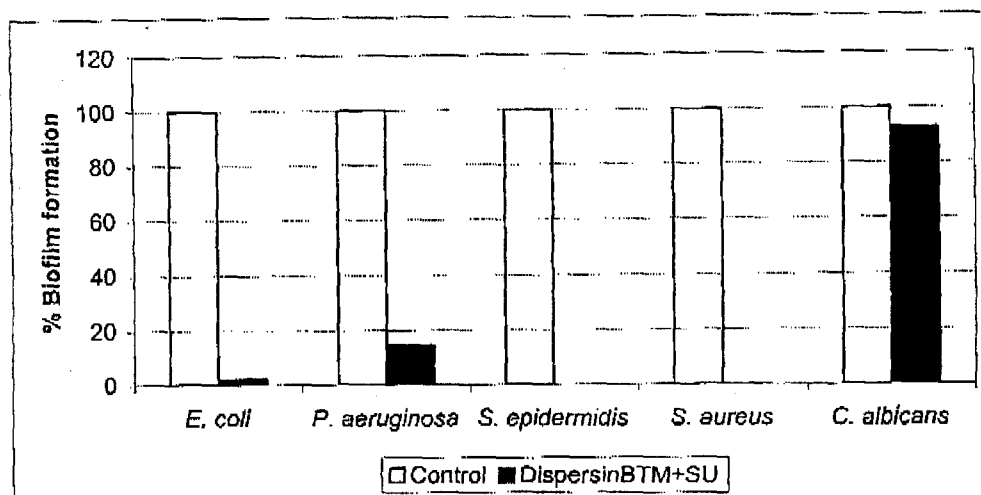


Figure 52

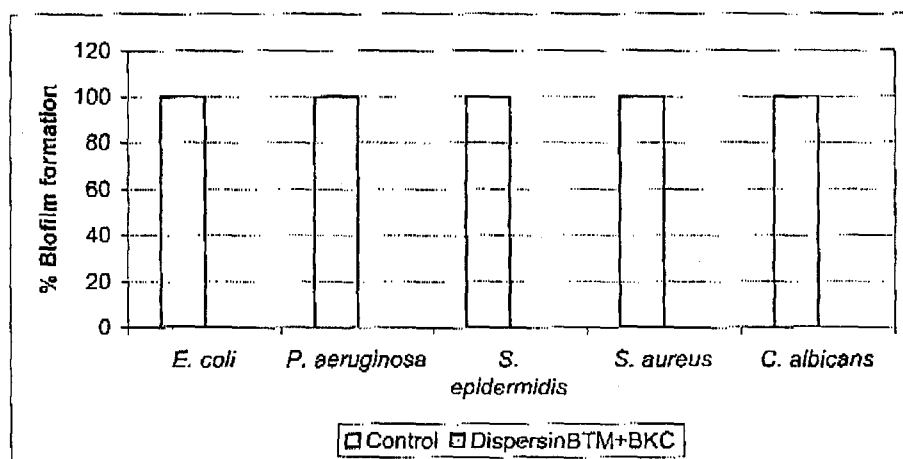
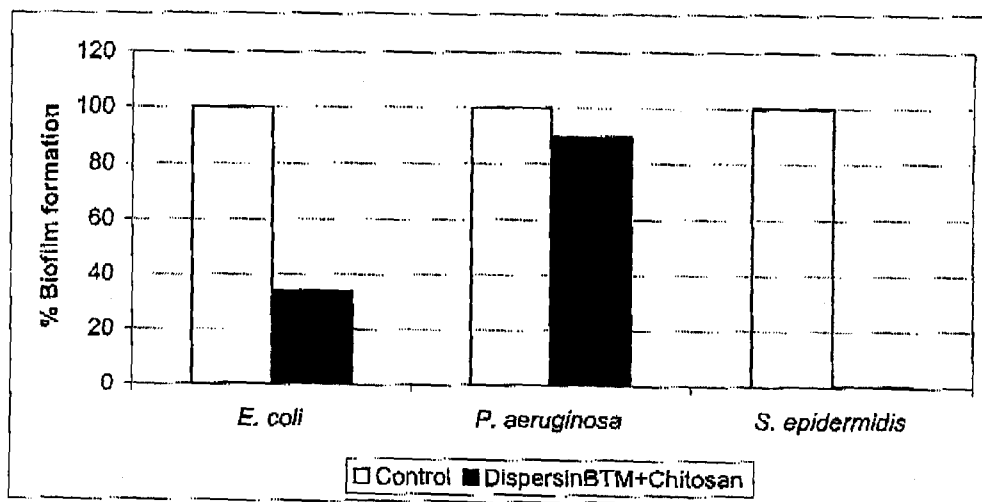


Figure 53



INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2007/001807

A. CLASSIFICATION OF SUBJECT MATTER IPC: C07K 19/00 (2006.01) , A01N 25/04 (2006.01) , A01N 31/16 (2006.01) , A01N 63/02 (2006.01) , A01P 1/00 (2006.01) , A61K 31/09 (2006.01) (more IPCs on the last page) According to International Patent Classification (IPC) or to both national classification and IPC														
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC: C07K 19/00 (2006.01) , A01N 25/04 (2006.01) , A01N 31/16 (2006.01) , A01N 63/02 (2006.01) , A01P 1/00 (2006.01) , A61K 31/09 (2006.01) (more IPCs on the last page) Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used) Scopus, PubMed, Delphion, Canadian Patent Database (Keywords: dspB, N-acetylglucosaminidase, biofilm, disperse, antibiotic, bacteriophage, antimicrobial, anti-infective and similar terms)														
C. DOCUMENTS CONSIDERED TO BE RELEVANT <table border="1"> <thead> <tr> <th>Category*</th> <th>Citation of document, with indication, where appropriate, of the relevant passages</th> <th>Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td>X</td> <td>WO 04/061117 A2 (UNIVERSITY OF MEDICINE AND DENTISTRY OF NEW JERSEY). 22 July 2004 page 3, lines 27 to 30; page 31, lines 12 to 18; page 33, line 3 to page 34, line 5</td> <td>1 - 78, 87 - 110</td> </tr> <tr> <td>X</td> <td>WO 06/137847 A2 (TRUSTEES OF BOSTON UNIVERSITY and MASSACHUSETTS INSTITUTE OF TECHNOLOGY). 28 December 2006 page 3, line 17 to page 4, line 5; page 22, line 23</td> <td>79 - 86</td> </tr> </tbody> </table>			Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	X	WO 04/061117 A2 (UNIVERSITY OF MEDICINE AND DENTISTRY OF NEW JERSEY). 22 July 2004 page 3, lines 27 to 30; page 31, lines 12 to 18; page 33, line 3 to page 34, line 5	1 - 78, 87 - 110	X	WO 06/137847 A2 (TRUSTEES OF BOSTON UNIVERSITY and MASSACHUSETTS INSTITUTE OF TECHNOLOGY). 28 December 2006 page 3, line 17 to page 4, line 5; page 22, line 23	79 - 86			
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.												
X	WO 04/061117 A2 (UNIVERSITY OF MEDICINE AND DENTISTRY OF NEW JERSEY). 22 July 2004 page 3, lines 27 to 30; page 31, lines 12 to 18; page 33, line 3 to page 34, line 5	1 - 78, 87 - 110												
X	WO 06/137847 A2 (TRUSTEES OF BOSTON UNIVERSITY and MASSACHUSETTS INSTITUTE OF TECHNOLOGY). 28 December 2006 page 3, line 17 to page 4, line 5; page 22, line 23	79 - 86												
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex. <table border="1"> <tbody> <tr> <td>* Special categories of cited documents :</td> <td>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"A" document defining the general state of the art which is not considered to be of particular relevance</td> <td>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"E" earlier application or patent but published on or after the international filing date</td> <td>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"&" document member of the same patent family</td> </tr> <tr> <td>"O" document referring to an oral disclosure, use, exhibition or other means</td> <td></td> </tr> <tr> <td>"P" document published prior to the international filing date but later than the priority date claimed</td> <td></td> </tr> </tbody> </table>			* Special categories of cited documents :	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family	"O" document referring to an oral disclosure, use, exhibition or other means		"P" document published prior to the international filing date but later than the priority date claimed	
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"P" document published prior to the international filing date but later than the priority date claimed														
Date of the actual completion of the international search 3 January 2008 (03-01-2008)		Date of mailing of the international search report 13 February 2008 (13-02-2008)												
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476		Authorized officer Antonio Candeliere 819- 934-7935												

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. ☒ Claim Nos. : 57 - 75

because they relate to subject matter not required to be searched by this Authority, namely :

Claims 57 - 75, are directed to a method for treatment of the human or animal body by surgery or therapy which the International Search Authority is not required to search. Regardless, this Authority has carried out a search based on the alleged effects or purposes/uses of the product defined in claims 57 - 75.

2. ☐ Claim Nos. :

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

3. ☐ Claim Nos. :

because they are dependant claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

The claims are directed to a plurality of inventive concepts as follows:

Group A - Claims 1 - 31, 38 - 78 and 87 - 110 are directed to a composition and use of a composition comprising DispersinB and an antimicrobial agent. This group should be subdivided further into separate DispersinB and antimicrobial agent combinations.

Group B - Claims 32 - 37 are directed to a gel formulation composition comprising DispersinB and a viscosity increasing agent.

Group C - Claims 79 - 86 are directed to a composition and fusion protein comprising DispersinB and a bacteriophage.

The claims must be limited to one inventive concept as set out in Rule 13 of the PCT.

An a posteriori analysis has concluded that WO 04/061117 A2 disclosed methods for enhancing efficacy of antibiotic therapy against bacterial infections by administration of DispersinBTM protein and an antibiotic (page 33, lines 3 to 10). The addition of suitable thickening and/or gelling agents is also disclosed (page 31, line 12 to 18). Thus, the combination of DispersinB with specific antimicrobial agents, specific viscosity increasing agents or specific bacteriophages each constitutes an inventive concept.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ROHDE H ET AL.: “Pathogenesis of staphylococcal device-related infections: from basic science to new diagnostic, therapeutic and prophylactic approaches.” REVIEWS IN MEDICAL MICROBIOLOGY. April 2006, vol. 17(2) ISSN: 0954-139X page 52, column 1, lines 47 to 58	1 - 31, 57 - 78, 87 - 110
X, P	DONELLI G ET AL. “Synergistic activity of Dispersin B and Cefamandole Nafate in inhibition of staphylococcal biofilm growth on polyurethanes.” ANTIMICROBIAL AGENTS AND CHEMOTHERAPY. epub 4 June 2007 ISSN: 0066-4804 whole document	1 - 78, 87 - 110
X, P	IZANO EA ET AL.: “Poly-N-acetylglucosamine mediates biofilm formation and antibiotic resistance in Actinobacillus pleuropneumoniae.” MICROBIAL PATHOGENESIS. epub 4 March 2007 ISSN: 0882-4010 figures 4 and 5.	1 - 31, 57 - 78

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2007/001807

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO 2004061117A2	22-07-2004	AU 2003284385A1	29-07-2004
		CA 2511103A1	22-07-2004
		EP 1585809A2	19-10-2005
		EP 1585809A4	02-08-2006
		JP 2006511226T	06-04-2006
		US 7294497B2	13-11-2007
		US 2006246049A1	02-11-2006
		WO 2004061117A3	30-06-2005
WO 2006137847A2	28-12-2006	WO 2006137847A3	18-05-2007

A61K 35/76 (2006.01) , *A61K 38/16* (2006.01) , *A61K 47/10* (2006.01) , *A61K 47/34* (2006.01) ,
A61K 47/36 (2006.01) , *A61K 47/48* (2006.01) , *A61L 15/46* (2006.01) , *A61L 2/16* (2006.01) ,
A61L 27/54 (2006.01) , *A61L 29/16* (2006.01) , *A61L 31/16* (2006.01) , *A61P 1/02* (2006.01) ,
A61P 17/02 (2006.01) , *A61P 31/00* (2006.01) , *C07K 14/005* (2006.01) , *C12N 9/24* (2006.01)