BISMUTH-THIOLS AS ANTISEPTICS FOR AGRICULTURAL, INDUSTRIAL AND OTHER USES

CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of PCT Application No. PCT/US2011/023549 filed February 3, 2011, and of U.S. Provisional Application No. 61/373,188 filed August 12, 2010, each of which is incorporated herein by reference in its entirety.

BACKGROUND

Technical field

The presently disclosed invention embodiments relate to compositions and methods for the treatment of microbial infections. In particular, the present embodiments relate to improved treatments for managing bacterial infections in agricultural, industrial, manufacturing, clinical, personal healthcare, and other contexts, including treatment of bacterial biofilms and other conditions.

Description of the Related Art

The complex series of coordinated cellular and molecular interactions that contribute to responding to and resisting microbial infections and/or to healing or maintenance of plant and animal (including human) bodily tissues generally, may be adversely impacted by a variety of external factors, such as opportunistic and nosocomial infections (*e.g.*, clinical regimens that can increase the risk of infection), local or systemic administration of antibiotics (which may influence cell growth, migration or other functions and can also select for antibiotic-resistant microbes), and/or other factors.

Unfortunately, systemically or locally introduced antibiotics are often not effective for the treatment of many chronic infections, and are generally not used unless an acute bacterial infection is present. Current approaches include administration or application of antibiotics, but such remedies may promote the advent of antibiotic-resistant bacterial strains and/or may be ineffective against bacterial biofilms. It therefore may become especially important to use antiseptics when drug resistant bacteria (*e.g.*, methicillin resistant *Staphylococcus aureus*, or MRSA) are detected. There are many antiseptics widely in use, but bacterial populations or subpopulations that

are established may not respond to these agents, or to any other currently available treatments. Additionally, a number of antiseptics may be toxic to host cells at the concentrations that may be needed to be effective against an established bacterial infection, and hence such antiseptics are unsuitable. This problem may be particularly acute in the case of efforts to clear infections from natural surfaces, including surface features on commercially and/or agriculturally important plants such as many crop plants, and also including internal epithelial surfaces, such as respiratory (*e.g.*, airway, nasopharyngeal and laryngeal paths, tracheal, pulmonary, bronchi, bronchioles, alveoli, etc.) or gastrointestinal (*e.g.*, buccal, esophageal, gastric, intestinal, rectal, anal, etc.) tracts, or other epithelial surfaces.

Particularly problematic are infections composed of bacterial biofilms, a relatively recently recognized organization of bacteria by which free, single-celled ("planktonic") bacteria assemble by intercellular adhesion into organized, multi-cellular communities (biofilms) having markedly different patterns of behavior, gene expression, and susceptibility to environmental agents including antibiotics. Biofilms may deploy biological defense mechanisms not found in planktonic bacteria, which mechanisms can protect the biofilm community against antibiotics and host immune responses. Established biofilms can arrest the tissue-healing process.

Common microbiologic contaminants that underlie persistent and potentially deleterious infections include *S. aureus*, including MRSA (Methicillin Resistant *Staphylococcus aureus)*, *Enterococci*, *E. coli*, *P. aeruginosa*, *Streptococci*, and *Acinetobacter baumannii*. Some of these organisms exhibit an ability to survive on non-nutritive clinical surfaces for months. *S. aureus*, has been shown to be viable for four weeks on dry glass, and for between three and six months on dried blood and cotton fibers (Domenico et al., 1999 *Infect. Immun.* 67:664-669). Both *E. coli* and *P. aeruginosa* have been shown to survive even longer than *S. aureus* on dried blood and cotton fibers (*ibid*).

Microbial biofilms are associated with substantially increased resistance to both disinfectants and antibiotics. Biofilm morphology results when bacteria and/or fungi attach to surfaces. This attachment triggers an altered transcription of genes, resulting in the secretion of a remarkably resilient and difficult to penetrate polysaccharide matrix, protecting the microbes. Biofilms are very resistant to the mammalian immune system, in addition to their very substantial resistance to antibiotics. Biofilms are very difficult to eradicate once they become established, so preventing biofilm formation is a

very important clinical priority. Recent research has shown that open wounds can quickly become contaminated by biofilms. These microbial biofilms are thought to delay wound healing, and are very likely related to the establishment of serious wound infections.

Maintenance of intact, functioning skin and other epithelial tissues (*e.g.*, generally avascular epithelial surfaces that form barriers between an organism and its external environment, such as those found in skin and also found in the linings of respiratory and gastrointestinal tracts, glandular tissues, etc.) is significant to the health and survival of humans and other animals.

Bismuth Thiol- (BT) based Antiseptics

A number of natural products (*e.g.*, antibiotics) and synthetic chemicals having antimicrobial, and in particular antibacterial, properties are known in the art and have been at least partially characterized by chemical structures and by antimicrobial effects, such as ability to kill microbes ("cidal" effects such as bacteriocidal properties), ability to halt or impair microbial growth ("static" effects such as bacteriostatic properties), or ability to interfere with microbial functions such as colonizing or infecting a site, bacterial secretion of exopolysaccharides and/or conversion from planktonic to biofilm populations or expansion of biofilm formation. Antibiotics, disinfectants, antiseptics and the like (including bismuth-thiol or BT compounds) are discussed, for example, in U.S. 6,582,719, including factors that influence the selection and use of such compositions, including, *e.g.*, bacteriocidal or bacteriostatic potencies, effective concentrations, and risks of toxicity to host tissues.

Bismuth, a group V metal, is an element that (like silver) possesses antimicrobial properties. Bismuth by itself may not be therapeutically useful and may exhibit certain inappropriate properties, and so may instead be typically administered by means of delivery with a complexing agent, carrier, and/or other vehicle, the most common example of which is Pepto Bismol®, in which bismuth is combined (chelated) with subsalicylate. Previous research has determined that the combination of certain thiol- (-SH, sulfhydryl) containing compounds such as ethane dithiol with bismuth, to provide an exemplary bismuth thiol (BT) compound, improves the antimicrobial potency of bismuth, compared to other bismuth preparations currently available. There are many thiol compounds that may be used to produce BTs (disclosed, for example, in Domenico et al., 2001 *Antimicrob. Agent. Chemotherap.* 45(5):1417-1421, Domenico et al., 1997 *Antimicrob. Agent. Chemother.*

41(8):1697-1703, and in U.S. RE37,793, U.S. 6,248,371, U.S. 6,086,921, and U.S. 6,380,248; see also, *e.g.*, U.S. 6,582,719) and several of these preparations are able to inhibit biofilm formation.

BT compounds have proven activity against MRSA (methicillin resistant *S. aureus*), MRSE (methicillin resistant *S. epidermidis*), *Mycobacterium tuberculosis, Mycobacterium avium,* drug-resistant *P. aeruginosa,* enterotoxigenic *E. coli,* enterohemorrhagic *E. coli, Klebsiella pneumoniae, Clostridium difficile, Heliobacter pylori, Legionella pneumophila, Enterococcus faecalis, Enterobacter cloacae, Salmonella typhimurium, Proteus vulgaris, Yersinia enterocolitica, Vibrio cholerae,* and *Shigella Flexneri* (Domenico et al., 1997 *Antimicrob. Agents Chemother.* 41:1697-1703). There is also evidence of activity against cytomegalovirus, herpes simplex virus type 1 (HSV-1) and HSV-2, and yeasts and fungi, such as *Candida albicans.* BT roles have also been demonstrated in reducing bacterial pathogenicity, inhibiting or killing a broad spectrum of antibiotic-resistant microbes (gram-positive and gram-negative), preventing biofilm formation, preventing septic shock, treating sepsis, and increasing bacterial susceptibility to antibiotics to which they previously exhibited resistance (see, *e.g.*, Domenico et al., 2001 *Agents Chemother.* 45:1417-1421; Domenico et al., 2000 *Infect. Med.* 17:123-127; Domenico et al., 2003 *Res. Adv. In Antimicrob. Agents & Chemother.* 3:79-85; Domenico et al., 1997 *Antimicrob. Agents Chemother.* 41(8):1697-1703; Domenico et al., 1999 *Infect. Immun.* 67:664-669: Huang et al. 1999 *J Antimicrob. Chemother.* 44:601-605; Veloira et al., 2003 *J Antimicrob. Chemother.* 52:915-919; Wu et al., 2002 *Am J Respir Cell Mol Biol.* 26:731-738).

Despite the availability of BT compounds for well over a decade, effective selection of appropriate BT compounds for particular infectious disease indications has remained an elusive goal, where behavior of a particular BT against a particular microorganism cannot be predicted, where synergistic activity of a particular BT and a particular antibiotic against a particular microorganism cannot be predicted, where BT effects *in vitro* may not always predict BT effects *in vivo,* and where BT effects against planktonic (single-cell) microbial populations may not be predictive of BT effects against microbial communities, such as bacteria organized into a biofilm. Additionally, limitations in solubility, tissue permeability, bioavailability, biodistribution and the like may in the cases of some BT compounds hinder the ability to deliver clinical

benefit safely and effectively. The presently disclosed invention embodiments address these needs and offer other related advantages.

Protection of Plants and Agricultural Products: Description of the Related Art

In the agricultural and botanical arts there is a recognized need for formulations to reduce biofilms and disease in plants, and for methods of using such formulations on, *e.g.*, seeds, plants, fruits and flowers, soil, and on cut flowers, trees, fruits, leaves, stems and other plant parts.

In agriculture, every year billions of dollars of crops are lost due to the formation of biofilms. The problem of anthracnose and biofilm-related diseases in plants is well known despite numerous unsatisfactory approaches that have attempted to address it. Plant diseases also affect industries involved in transporting and preserving fruit, vegetables, cut flowers and trees, and other plant products, as the normal protective mechanisms employed by intact living plants are no longer operative in the harvested product.

It is therefore desirable for agricultural purposes to reduce the amount of microbial growth on the surfaces of leaves, stems, fruits and flowers in situ, in transit or at commercial venues while maintaining compliance with environmental regulations. At the same time, it is desirable to allow for the flow of water within cut flowers, plants and trees to maintain plant tissue turgidity, integrity and quality in order to enhance the desirable characteristics of these products.

Organisms that cause infectious disease in plants include fungi, bacteria, viruses, protozoa, nematodes and parasitic plants. Insects and other pests also affect plant health by consumption of plant tissues, and by exposure of plant tissues to microbes.

Biofilms occur when bacteria bind to a surface, typically in an aqueous milieu such as under aquatic conditions or in water droplets or other conditions of high humidity, and after binding the biofilm formers begin to excrete a sticky substance which can then bind to a variety of materials including metals, plastics, medical implants and tissues. These biofilms can cause many problems, including degradation of materials and clogging of pipes, in industrial and agricultural environments, and infection of surrounding tissue when occurring in a medical environment. The medical field is particularly susceptible to problems caused by biofilm formation; implanted medical

devices, catheters (urinary, venous, dialysis, cardiac) and slow-healing wounds are easily infiltrated by the bacteria present in biofilms. In agriculture, biofilms can cause mastitis, Pierce's disease, ring rot in potatoes, various crop blights and anthracnoses in many types of plants. Biofilms also reduce the quality and product life of cut flowers and trees.

Many plant diseases are caused by biofilm-producing bacteria indigenous to soil. Most microorganisms in the natural environment exist in multicellular aggregates generally described as biofilms. Cells adhere to surfaces and to each other through a complex matrix comprising a variety of extracellular polymeric substances (EPS) including exopolysaccharides, proteins and DNA. Plant-associated bacteria interact with host tissue surfaces during pathogenesis and symbiosis, and in commensal relationships. Observations of bacteria associated with plants increasingly reveal biofilm-type structures that vary from small clusters of cells to extensive biofilms. The surface properties of the plant tissue, nutrient and water availability, and the proclivities of the colonizing bacteria strongly influence the resulting biofilm structure (Ramey et al., 2004 *Curr Opinion Microbiol.* 7:602–9).

The terrestrial environment harbors abundant and diverse microbial populations that can compete for and modify resource pools. In this complex and competitive environment, plants offer protective oases of nutrient-rich tissues. Plants are colonized by bacteria on their leaves, roots, seeds and internal vasculature. Each tissue type has unique chemical and physical properties that represent challenges and opportunities for microbial colonists. Biofilms may form upon association or at later stages, with significant potential to direct or modulate the plant–microbe interaction. Additional temporal and spatial complexity arises as many microbes actively modify the colonized plant environment.

Surface-associated bacteria have a significant impact on agriculture. In developed countries, the losses caused by plant diseases reach up to 25% of crop yields, a percentage that is much higher in developing countries. Epiphytic populations constitute a reservoir and future source of

infection, and can be found on host and non-host plants. *Xylophylus ampelinus*, a bacterial pathogen of grapevines, forms thick biofilms in the vasculature of these plants (Grail & Manceau 2003). *Xylella fastidiosa* is the causal agent of Pierce's disease in grapevines. *X. fastidiosa* is able to form biofilms within xylem vessels of many economically important crops. The mechanisms of pathogenicity are largely due to occlusion of xylem vessels by aggregation of *X. fastidiosa* and biofilm formation. Vessel blockage is believed to be a major contributor to disease development, with xylem sap providing a natural medium that facilitates the virulence of Pierce's disease of grapevine and citrus variegated chlorosis (Zaini et al., 2009 *FEMS Microbiol LETT.* 295:129-34).

One of the most relevant plant pathogens, *Pseudomonas syringae*, causes brown spot disease on bean. It colonizes the leaf surface sparsely in solitary small groups (fewer than ten cells), while larger populations (more than 1000 cells) primarily develop near trichomes or veins with higher nutrient availability. Large aggregates survive desiccation stress better than solitary cells. *P. syringae* survives as an epiphyte (*i.e.*, colonizer of the aerial parts of plants) when not causing infections on host plant tissues (Monier et al. *PNAS* 2003;100:15977-82).

Pseudomonas putida can respond rapidly to the presence of root exudates in soils, converging at root colonization sites and establishing stable biofilms (Espinosa-Urgel et al. *Microbiol* 2002;148:341-3).

Xanthomonas campestris pv. *campestris* (Xcc) causes black rot on cruciferous plants, accessing the vasculature through wound sites in roots. Virulence involves degradative exoenzymes and the exopolysaccharide xanthan gum, which is necessary for virulence (Dow et al. *PNAS* 2003;100:10995-1000).

Xanthomonas smithii subsp. *citri* is responsible for the disease, citrus canker. This disease has been found in most continents of the world except Europe. The pathogen has been eradicated in many countries. *Xanthomonas smithii* forms canker lesions on fruit, leaves and twigs of citrus

plants. Wind-driven rain can spread the bacteria up to 15 km from the source to infect citrus trees via stomata or wounds (Sosnowski, et al. *Plant Pathol* 2009;**58**:621–35).

Pantoea stewartii subsp. *stewartii* causes Stewart's wilt disease in maize and is transmitted by the corn flea beetle. The bacteria reside primarily in the host xylem and produce large amounts of exopolysaccharide (von Bodman et al. *PNAS* 1998;95:7687-92).

Ralstonia solanacearum is a soil-borne pathogen that causes lethal wilt on many plants. Virulence depends on EPS and cell-wall-degrading enzymes controlled by a complex regulatory network (Kang et al. *Mol Microbiol* 2002; 46:427-37).

Clavibacter michiganensis subsp. *sepedonicus* is a Gram-positive phytopathogen that causes bacterial ring rot in potato. Marques and colleagues showed large bacterial, matrix-encased aggregates attached to the xylem vessels (Marques et al. *Phytopathol* 2003;93:S57).

Biofilm-producing *Erwinia chrysanthemi* causes soft-rot disease through rapid maceration of plant tissue. The production of pectic enzymes may be quorum-sensing (QS)-regulated, and therefore the inability to form bacterial aggregates may preclude pectinolytic enzyme secretion. *Erwinia amylovora*, a related plant pathogen, infects approximately 75 different species of plants, all in the family Rosaceae. Hosts for this bacterium include apple, pear, blackberry, cotoneaster, crabapple, firethorn (*Pyracantha*), hawthorn, Japanese or flowering quince, mountain-ash, pear, quince, raspberry, serviceberry, and spiraea. The cultivated apple, pear, and quince are the most seriously affected species. A single fire blight epidemic in Michigan in 2000 resulted in the death of over 220,000 trees with a total loss of $42 million. Annual losses to fire blight and cost of control in the U.S. are estimated at over $100 million (Norelli et al. *Plant Dis* 2003;87:26-32).

E. amylovora produces two exopolysaccharides, amylovoran and levan, which cause the characteristic fire blight wilting symptom in host plants (Koczan et al. *Phytopathol* 2009;99:1237-44). In addition, other genes, and

their encoded proteins, have been characterized as virulence factors of *E. amylovora* that encode enzymes facilitating sorbitol metabolism, proteolytic activity and iron harvesting (Oh & Beer. *FEMS Microbiology Lett* 2005;253:185–192).

No matter which part of the plant is attacked by a microbial plant pathogen such as a biofilm-former, the effect is usually to weaken or kill the plant. By infecting the leaves, the pathogen compromises the plant's ability to produce its food (*e.g.*, via photosynthesis). Some plant pathogens block the fluid transport vessels in the stems that supply the leaves, and when such pathogens attack the roots, the uptake of water and nutrients is reduced or stopped completely. Blockage of plant vasculature often involves biofilm-producing bacteria that clog the flow of water and nutrients, both in growing plants in soil and in cut plants in vase water.

When a plant is attacked by one of these microorganisms, the resulting damage provides an opportunity for additional microbial invasion of plant tissue and it is the combined onslaught that ultimately damages and destroys the plant. Plants that are under environmental stresses, such as drought or poor nutrition, are particularly e susceptible to microbial attack.

Sometimes the microbial 'infection' is symbiotic, where both organisms derive a benefit. A good example of this is the well known nitrogen fixing bacteria (Rhizobium) which reside in nodules on the roots of leguminous (pea family) plants-- the plant provides food and protection, while the bacteria take nitrogen from the air and convert it to a form usable by the host. As another example, the Mycorrhizae are a whole Order of fungi that have a symbiotic relationship with plant roots. In view of such mutually beneficial symbioses, preservation or protection of plants against harmful microbial pathogens may desirably employ antimicrobial agents that do not disrupt these symbiotic relationships, wherever possible.

Saprophytic fungi are essential in breaking down dead organic matter to produce the humus which is needed for good soil structure. They do not have any chlorophyll and so cannot use light to capture energy (*e.g.,* via photosynthesis); instead they derive their energy by breaking down plant and animal material - alive or dead. They can also live in a symbiotic relationship with certain plant species, *e.g.*, the micorrhizae in the fine roots of conifers, which cannot survive without them to take up vital nutrients. The widespread use of chemical agents to control harmful plant pathogens can damage the

balance of these beneficial fungi, and runs counter to the principals of organic management.

There are, however, other less welcome fungi, which attack living plants and weaken or kill them. Another category of microbial plant pathogens, viruses, may be resident within the cells of plant tissues and thus often cannot be treated with topically applied chemicals, such that affected plants must be destroyed. There are currently no antibiotics specifically developed for the treatment of plants (although some antibiotics developed for other purposes have found uses on plants), leaving a number of economically significant plant species vulnerable to pathogenic bacterial attacks. For instance, fireblight infestations of numerous plant species of the family Rosaceae have proven untreatable. Many harmful fungi, by contrast, can be killed with topically applied chemicals without damaging the plant host, because thefungal growth habitat is different, *i.e.,* a number of undesirable pathogenic fungi tend to grow on plant surfaces and not within plant tissues, using root-like structures to extract nourishment.

Because killing many plant pathogens is often difficult or impossible, a number of strategies for protecting plants against deleterious microbial pathogens adopt the philosophy that "prevention is better than cure". By observing good hygiene when propogating and growing plants, many microbial plant diseases can be prevented by blocking the opportunity for a microbial infection to be established. Often, significantly lower quantities of pesticides or microbicides can be effective when such agents are used prophylactically, rather than in response to an established infection.

Plants are also more susceptible to disease if they are not growing under optimal or near-optimal conditions, for example, due to poor soil quality (*e.g.*, dearth of nutrients) by itself or in combination with drought or excessive rainfall or flooding. Extremely wet conditions can, for instance, promote pathogenic fungal and/or bacterial growth. Quorum sensing in *P. syringae,* for example, is dictated by water availability on the leaf surface (Dulla & Lindow. *PNAS* 2008;105:3-082-7). Of course not all plant diseases can be prevented by good agricultural hygiene, insofar as some plant diseases are transmitted by insects and others are wind-borne. Aphids and other sap-sucking insects, for example, are the main vectors of viruses. Spores of fungal diseases are carried in the air, and in rain drops and splashes.

Biofilms on seeds and sprouts

Bacterial adherence to seeds is a process that strongly influences rhizosphere colonization. Seed suppliers often deliberately coat seed stocks with microbial biofilms to inoculate the developing rhizosphere. Conversely, biofilms on seeds and sprouts used for human consumption are often common sources of gastrointestinal infection. *P. putida* adheres effectively to seeds and will subsequently colonize the rhizosphere. Endophytic populations of nonpathogenic actinobacteria found in wheat tissues were derived from interior colonization by the actinobacteria of surface-sterilized seeds. Endophytic seed populations of beneficial nitrogen-fixing bacteria can help ensure future rhizosphere colonization. Other studies of seed colonization have reported rod shaped and coccal bacteria embedded within EPS in scanning electronmicrographs of alfalfa seeds and sprouts. Biofilms are notoriously resistant to washing and other common antibacterial treatments on seeds and sprouts. Fett et al. found that both *Escherichia coli* O157:H7 and Salmonella populations on alfalfa sprouts required treatments much harsher than simple water washing to reduce the numbers of adherent microbes, and full removal was never achieved. The surviving bacteria likely resided within biofilms (Ramey et al. *Curr Opinion Microbiol* 2004;7:602–9).

Cut flowers and trees

Vascular pathogens inhabit the xylem or phloem of plant hosts and generally depend on insect vectors or wounding for dissemination. Cutting flowers or trees is a similar type of wounding that is especially prone to vascular infection. Biofilm bacteria enter and clog the vasculature at the cut surface, and interfere with the flow of water, minerals and nutrients. Cut flower preservatives diluted in vase water often contain salicylate or aspirin to reduce biofilm formation (Domenico et al., *J Antimicrob Chemo* 1991;28:801-10; Salo et al., *Infection* 1995;23:371-7), and provide a low pH to prevent bacterial growth and disrupt biofilms.

Antimicrobial Agents in Agriculture. Eradication of plant pathogen incursions is very important for the protection of plant industries, managed gardens and natural environments worldwide. The consequence of a pathogen

becoming endemic can be serious, in some cases having an impact on the national economy. The current strategy for eradication of a pathogen relies on techniques for the treatment, removal and disposal of affected host plants. There are many examples where these techniques have been successful but many where they have not. Success relies on a sound understanding of the biology and epidemiology of the pathogen and its interaction with the host. In examining examples of dealing with plant pathogens and diseased host material around the world, particularly Australasia, various techniques including burning, burying, pruning, composting, soil- and biofumigation, solarization, steam sterilization and biological vector control have been used (Sosnowski, et al. *Plant Pathol* 2009; 58:621–35).

Antibiotics have also been used since the 1950s, to control certain bacterial diseases of high-value fruit, vegetable, and ornamental plants. Today, the antibiotics most commonly used on plants are oxytetracycline and streptomycin. In the USA, antibiotics applied to plants account for less than 0.5% of total antibiotic use. Resistance of plant pathogens to oxytetracycline is rare, but the emergence of streptomycin-resistant strains of *Erwinia amylovora*, *Pseudomonas* spp., and *Xanthomonas campestris* has impeded the control of several important diseases. Thus, the role of antibiotic use on plants in the antibiotic-resistance crisis in human medicine is the subject of debate (McManus et al. *Annu Rev Phytopathol* 2002;40:443–65).

The emergence of streptomycin-resistant (Sm^R) plant pathogens has complicated the control of bacterial diseases of plants. For example, in the United States, streptomycin is permitted on tomato and pepper for control of *X. campestris* pv. *vesicatoria*, but it is rarely used for this purpose because resistant strains are now widespread. Resistance in *E. amylovora*, the fire blight pathogen, has had widespread economic and political implications. Other phytopathogenic bacteria in which Sm^R has been reported include *Pectobacterium carotovora*, *Pseudomonas chichorii*, *Pseudomonas lachrymans*, *Pseudomonas syringae* pv. *papulans*, *Pseudomonas syringae* pv. *syringae*, and *Xanthomonas dieffenbachiae* (McManus et al. *Annu Rev Phytopathol* 2002;40:443–65). The emergence Sm^R *E. amylovora* has intensified fire blight epidemics in the western USA and Michigan.

Streptomycin and oxytetracycline have been assigned the lowest toxicity category by the U.S. Environmental Protection Agency (EPA), and carcinogenic or mutagenic activities have not been observed for either antibiotic.

Alternatives to antibiotics are available and, at least to some extent, practical. Indeed, bacterial disease management in most cropping systems is based on the integration of genetic resistance of the host, sanitation (avoidance or removal of inoculum), and cultural practices that create an environment unfavorable for disease development. Biocontrol of plants using various species of bacteria and fungi is of growing interest. Rhizobacteria are considered as efficient microbial competitors in the root zone. Representatives of many different bacterial genera have been introduced into soils, onto seeds, roots, tubers or other planting materials to improve crop growth. These bacterial genera include *Acinetobacter, Agrobacterium, Arthrobacter, Azospirillum, Bacillus, Bradyrhizobium, Frankia, Pseudomonas, Rhizobium, Serratia, Thiobacillus,* and many others. Certain species of *Bacillus*, for example, can induce systemic resistance in many plants (Choudhary &Johri. *Microbiol Res* 2009;164:493—513).

Application of copper compounds is effective in reducing populations of some bacterial plant pathogens, although several species have become resistant to copper (Cooksey *Annu Rev Phytopathol* 1990;28:201–14), and most tree-fruit crops are sensitive to copper injury.

A number of synthetic and natural remedies exist for various plant diseases. Natural remedies include apple cider vinegar for leafspot, mildew and scab; baking soda spray for anthracnose, early tomato blight, leaf blight, powdery mildew and as a general fungicide; neem oil; sulfur; garlic; hydrogen peroxide; compost teas, etc. Numerous synthetic chemicals are used to prevent or treat plant disease, and come in water-soluble or water-insoluble formulations. Microbicides include phenoxarsine or a phenarsazine, maleimide, isoindole dicarboximide, halogenated aryl alkanol, 4-thioxopyrimidine derivatives (US Patent 6384040), heterocyclic organosilyl compounds and

isothiazolinone. Many microbicides are combined with pyrithione derivatives to make synergistic compounds (*e.g.,* EP1468607). Certain isothiazolecarboxamides can be employed for the control of plant pests (*e.g.*, US 6552056; WO 2001/064644)

Recognizing the toxicity problem of microbicides in powder or crystalline form, U.S. Pat. No. Re. 29,409 teaches dissolving microbicides in liquid solvents, which may be added to the formulation mixture from which the end-use resin compositions are fabricated. Although liquid dispersions may be safely used at the site of preparing end-use resin compositions, careless use or disposal of the liquids may still pose environmental and health hazards. Alternatively, microbicides can also be administered in water-soluble thermoplastic resins. Microbicides can be added to rigid thermoplastic resin compositions and impart biocidal activity thereto so as to inhibit microbial growth on the surfaces thereof (US 5,229,124). This is a solid, melt-blended solution consisting essentially of a microbicide dissolved in a carrier resin that is a copolymer of vinyl alcohol and (alkyleneoxy) acrylate. Although a microbicide may be a highly toxic chemical, its low concentration in the end-use product and its retention by the resin composition ensures that the microbicide in the end-use product poses no hazard to humans or animals.

Isothiazolinones are often used as microbicides in agriculture, for example, N-alkylbenzenesulfonylcarbamoyl-5-chloroisothiazole derivatives (*e.g.*, US 5,045,555). This microbicide is widely useful in, for example, the paper industry, textile industry, for producing coatings and adhesives, in painting, metal processing, in the resin industry, wood industry, construction industry, agriculture, forestry, fisheries, food industry and petroleum industry as well as in medicine. It exhibits an intense microbicidal effect, and can be added, in an appropriate amount, to processing water, circulating water, a raw material or a product. Further, it may be employed for disinfecting or sterilizing facilities, plants, livestock barns or instruments as well as seeds, seedlings and raw materials. Other derivatives of isothiazolone are also known (U.S Pat. No. 3,523,121 and *J. Heterocyclic Chem.*, 8, 587 (1971)). However, every known

derivative compound is highly toxic to animals and fishes, which significantly restricts their application.

Sodium bicarbonate commonly has also been found to possess fungicidal properties when applied to plants, but typically requires frequent reapplication in order to realize efficacy.

The role of iron in plant host-parasite relationships has been elucidated in diseases as different as the soft rot and fire blight incited by *Erwinia chrysanthemi* and *E. amylovora*, respectively (*Expert. Annu Rev Phytopathol* 1999;37:307–34). Because of its unique position in biological systems, iron controls the activities of plant pathogens. The production of siderophores by pathogens not only represents a powerful strategy to acquire iron from host tissues but may also act as a protective agent against iron toxicity. The need of the host to bind and possibly sequester the metal during pathogenesis is another central issue. Antimicrobials that interfere with bacterial iron uptake and cell respiration may play an important role in plant disinfection.

Many natural products (*e.g.,* antibiotics) and synthetic chemicals with antimicrobial, antiseptic and in particular antibacterial, properties are known and have been at least partially characterized chemically and biologically. Exemplary characteristics include the ability to kill microbes (bactericidal effects), ability to halt or impair microbial growth (bacteriostatic effects), or ability to interfere with microbial functions such as colonizing or infecting a site, bacterial secretion of metabolites (some of which are malodorous), and/or conversion from planktonic to biofilm populations or expansion of biofilm formation (anti-biofilm effects). Antibiotics, disinfectants, antiseptics and the like (including bismuth-thiol or BT compounds) are discussed in U.S. 6,582,719, including factors that influence the selection and use of such compositions, including, *e.g.*, bactericidal, bacteriostatic, or anti-biofilm potencies, effective concentrations, and risks of toxicity to host tissues.

Bacterial microcolonies protected within the biofilm are typically resistant to antiseptics or disinfectants. Antibiotic doses that kill free-floating bacteria, for example, need to be increased as much as 1,500 times to kill biofilm bacteria. At this high concentration, some antimicrobials can be toxic.

Oxidizing brominated and chlorinated compounds, for example, are highly toxic and corrosive.

Suppression of the blossom-blight phase is a key to the management of fire blight. For blossom infection to occur, *Erwinia amylovora* to needs proliferate on stigmatic surfaces in an epiphytic phase. Rain is necessary for infection because it dilutes sugars on the hypanthium to osmotic potentials not inhibitory to *E. amylovora*. Rain is also important as an agent for redistribution of the bacterium from the stigmas to the hypanthium. These observations suggest that the optimal timing for use of antibiotic sprays is during this epiphytic phase, and after excessive rain (Johnson & Stockwell. *Annu Rev Phytopathol 1998;36:227–48*).

Other bacterial epiphytes also colonize stigmas where they can interact with and suppress epiphytic growth of the pathogen. A commercially available bacterial antagonist of *E. amylovora* (BlightBan, *Pseudomonas fluorescens* A506) can be included in antibiotic spray programs. Integration of bacterial antagonists with chemical methods suppresses populations of the pathogen and concomitantly, fills the ecological niche provided by the stigma with a nonpathogenic, competing microorganism (Johnson & Stockwell. *Annu Rev Phytopathol 1998;36:227–48*).

Pyrithione is the conjugate base derived from 2-mercaptopyridine-N-oxide (CAS# 1121-31-9), a derivative of pyridine-N-oxide. Its antifungal effect resides in its ability to disrupt membrane transport by blocking the proton pump that energizes the transport mechanism. Experiments have suggested that fungi are capable of inactivating pyrithione in low concentrations (Chandler & Segel. *Antimicrob. Agents Chemother* 1978;14:60–8). Zinc pyrithione is a coordination complex of zinc. This colorless solid is used as an antifungal and antibacterial agent. Due to its low solubility in water (8 ppm at neutral pH), zinc pyrithione is suitable for use in outdoor paints, cements and other products that provide protection against mildew and algae. It is an effective algaecide. It is chemically incompatible, however, with paints that rely on metal carboxylate curing agents. When used in latex paints comprising water that contains high amount of iron, a sequestering agent that will preferentially bind the iron ions is needed.

Particularly problematic in agriculture are infections composed of bacterial biofilms, a relatively recently recognized organization of bacteria by which free, single-celled ("planktonic") bacteria assemble by intercellular adhesion into organized, multi-cellular communities (biofilms) having markedly different patterns of behavior, gene expression, and susceptibility to environmental agents including antibiotics. Biofilms may deploy biological defense mechanisms not found in planktonic bacteria, which mechanisms can protect the biofilm community against antibiotics and host immune responses. Established biofilms can arrest growth, development or wound-healing processes in plants.

Microbial biofilms are associated with substantially increased resistance to both disinfectants and antibiotics. Biofilm morphology results when bacteria and/or fungi attach to surfaces. This attachment triggers an altered transcription of genes, resulting in the secretion of a remarkably resilient and difficult to penetrate polysaccharide matrix, protecting the microbes. Biofilms are very resistant to the plant immune defense mechanisms, in addition to their very substantial resistance to antibiotics. Biofilms are very difficult to eradicate once they become established, so preventing biofilm formation is a very important agricultural priority. Recent research has shown that open wounds can quickly become contaminated by biofilms. These microbial biofilms are thought to impair growth, development and/or wound healing, and are very likely related to the establishment of serious and often intractable infections.

Clearly there is a need for improved compositions and methods for treating and preventing microbial infections in and on plants, including microbial infections that occur as biofilms. Certain embodiments described herein address this need and provide other related advantages.

BRIEF SUMMARY

As disclosed herein, and without wishing to be bound by theory, according to certain embodiments described for the first time herein, bismuth-thiol (BT) compounds may be used as antiseptic agents for use in a wide variety of agricultural, industrial, manufacturing and other contexts, as well as in the treatment or prevention of infectious diseases and related conditions and in personal healthcare, while also decreasing the costs incurred for the treatment of such infections, including savings that are realized by prevention or prophylaxis mediated at least in part by BTs.

Also, in certain embodiments described herein there are contemplated formulations for treating plants or plant tissues (*e.g.,* a root, bulb, stem, leaf, branch, vine, runner, bud, flower or a part thereof, greentip, fruit, seed, seed pod, or the like) and animal tissues and/or natural and artificial surfaces that contain bacterial biofilms or bacteria related to biofilm formation (*e.g.,* bacteria that are capable of forming or otherwise promoting biofilms), which formulations comprise one or more BT compound and one or more antibiotic compound, as described herein, where according to non-limiting theory, appropriately selected combinations of BT compound(s) and antibiotic(s) based on the present disclosure provide heretofore unpredicted synergy in the antibacterial (including anti-biofilm) effects of such formulations, and/or unpredicted enhancing effects, for prevention, prophylaxis and/or therapeutically effective treatment against microbial infections including infections that contain bacterial biofilms.

Also provided herein for use in these and related embodiments are bismuth-thiol compositions that advantageously comprise substantially monodisperse microparticulate suspensions, and methods for their synthesis and use.

According to certain embodiments of the invention described herein there is thus provided a method for protecting a plant against a bacterial, fungal or viral pathogen, comprising contacting the plant or a part thereof (*e.g.,* all or part of a root, bulb, stem, leaf, branch, vine, runner, bud, flower or a part thereof, greentip, fruit, seed, seed pod, or the like) with an effective amount of a bismuth-thiol (BT) composition under conditions and for a time sufficient for one or more of: (i) prevention of infection of the plant by the bacterial, fungal or viral pathogen, (ii) inhibition of cell viability or cell growth of substantially all planktonic cells of the bacterial, fungal or viral pathogen, (iii) inhibition of biofilm formation by the bacterial, fungal or viral pathogen, and (iv) inhibition of biofilm viability or biofilm growth of substantially all biofilm-form cells of the bacterial, fungal or viral pathogen, wherein the BT composition comprises a substantially monodisperse suspension of microparticles that comprise a BT compound, said microparticles having a volumetric mean diameter of from about 0.4 μm to about 10 μm. In a further embodiment the bacterial pathogen comprises *Erwinia amylovora* cells. In another embodiment the bacterial pathogen is selected from *Erwinia amylovora*, *Xanthomonas campestris pv dieffenbachiae*, *Pseudomonas syringae, Xylella fastidiosa; Xylophylus ampelinus; Monilinia fructicola, Pantoea stewartii* subsp. *Stewartii, Ralstonia solanacearum, and*

Clavibacter michiganensis subsp. *sepedonicus*. In certain embodiments the bacterial pathogen exhibits antibiotic resistance. In certain embodiments the bacterial pathogen exhibits streptomycin resistance. In certain embodiments the plant is a food crop plant, which in certain further embodiments is a fruit tree. In certain still further embodiments the fruit tree is selected from an apple tree, a pear tree, a peach tree, a nectarine tree, a plum tree, an apricot tree. In certain other embodiments the food crop plant is a banana tree of genus *Musa*. In certain other embodiments the food crop plant is a plant selected from a tuberous plant, a leguminous plant, and a cereal grain plant. In certain further embodimenst the tuberous plant is selected from *Solanum tuberosum* (potato), and *Ipomoea batatas* (sweet potato). In certain embodiments of the above described method, the step of contacting is performed one or a plurality of times. In certain further embodiments at least one step of contacting comprises one of spraying, dipping, coating and painting the plant. In certain other further embodiments at least one step of contacting is performed at a flower blossom, green-tip or growth site of the plant. In certain embodiments at least one step of contacting is performed within 24, 48 or 72 hours of first flower blooming on the plant.

In certain embodiments of the above described method, the BT composition comprises one or more BT compounds selected from BisBAL, BisEDT, Bis-dimercaprol, Bis-DTT, Bis-2-mercaptoethanol, Bis-DTE, Bis-Pyr, Bis-Ery, Bis-Tol, Bis-BDT, Bis-PDT, Bis-Pyr/Bal, Bis-Pyr/BDT, Bis-Pyr/EDT, Bis-Pyr/PDT, Bis-Pyr/Tol, Bis-Pyr/Ery, bismuth-1-mercapto-2-propanol, and Bis-EDT/2-hydroxy-1-propanethiol. In certain embodiments the bacterial pathogen exhibits antibiotic resistance.

In certain further embodiments of the above described methods, the method comprises contacting the plant with a synergizing or enhancing antibiotic, simultaneously or sequentially and in any order with respect to the step of contacting the plant with the BT composition. In certain embodiments the synergizing or enhancing antibiotic comprises an antibiotic that is selected from an aminoglycoside antibiotic, a carbapenem antibiotic, a cephalosporin antibiotic, a fluoroquinolone antibiotic, a penicillinase-resistant penicillin antibiotic, and an aminopenicillin antibiotic. In certain embodiments the synergizing or enhancing antibiotic is an aminoglycoside antibiotic that is selected from amikacin, arbekacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, rhodostreptomycin, streptomycin, tobramycin and apramycin.

According to certain other embodiments there is provided a method for overcoming antibiotic resistance in a plant in or on which an antibiotic-resistant bacterial plant pathogen is present, comprising (a) contacting the plant with an effective amount of a BT composition under conditions and for a time sufficient for one or more of: (i) prevention of infection of the plant by the antibiotic-resistant bacterial pathogen, (ii) inhibition of cell viability or cell growth of substantially all planktonic cells of the antibiotic-resistant bacterial pathogen, (iii) inhibition of biofilm formation by the antibiotic-resistant bacterial pathogen, and (iv) inhibition of biofilm viability or biofilm growth of substantially all biofilm-form cells of the antibiotic-resistant bacterial pathogen, wherein the BT composition comprises a substantially monodisperse suspension of microparticles that comprise a BT compound, said microparticles having a volumetric mean diameter of from about 0.5 μm to about 10 μm; and (b) contacting the plant with a synergizing or enhancing antibiotic, simultaneously or sequentially and in any order with respect to the step of contacting the plant with the BT composition.

In certain embodiments of the above described methods, the bismuth-thiol composition comprises a plurality of microparticles that comprise a bismuth-thiol (BT) compound, substantially all of said microparticles having a volumetric mean diameter of from about 0.4 μm to about 5 μm and being formed by a process that comprises: (a) admixing, under conditions and for a time sufficient to obtain a solution that is substantially free of a solid precipitate, (i) an acidic aqueous solution that comprises a bismuth salt comprising bismuth at a concentration of at least 50 mM and that lacks a hydrophilic, polar or organic solubilizer, with (ii) ethanol in an amount sufficient to obtain an admixture that comprises about 25% ethanol by volume; and (b) adding to the admixture of (a) an ethanolic solution comprising a thiol-containing compound to obtain a reaction solution, wherein the thiol-containing compound is present in the reaction solution at a molar ratio of from about 1:3 to about 3:1 relative to the bismuth, under conditions and for a time sufficient for formation of a precipitate which comprises the microparticles comprising the BT compound.

In certain embodiments the bismuth salt is $Bi(NO_3)_3$. In certain embodiments the acidic aqueous solution comprises at least 5%, 10%, 15%, 20%, 22% or 22.5% bismuth by weight. In certain embodiments the acidic aqueous solution comprises at least 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5% or 5% nitric acid by weight. In certain embodiments the thiol-containing compound comprises one or more agents selected from 1,2-ethane dithiol, 2,3-

dimercaptopropanol, pyrithione, dithioerythritol, 3,4-dimercaptotoluene, 2,3-butanedithiol, 1,3-propanedithiol, 2-hydroxypropane thiol, 1-mercapto-2-propanol, dithioerythritol, alpha-lipoic acid, dithiothreitol, methanethiol (CH_3SH [m-mercaptan]), ethanethiol (C_2H_5SH [e- mercaptan]), 1-propanethiol (C_3H_7SH [n-P mercaptan]), 2-propanethiol ($CH_3CH(SH)CH_3$ [$2C_3$ mercaptan]), butanethiol (C_4H_9SH ([n-butyl mercaptan]), tert-butyl mercaptan ($C(CH_3)_3SH$ [t-butyl mercaptan]), pentanethiol ($C_5H_{11}SH$ [pentyl mercaptan]), coenzyme A, lipoamide, glutathione, cysteine, cystine, 2-mercaptoethanol, dithiothreitol, dithioerythritol, 2-mercaptoindole, transglutaminase, (11-mercaptoundecyl)hexa(ethylene glycol), (11-mercaptoundecyl)tetra(ethylene glycol), (11-mercaptoundecyl)tetra(ethylene glycol) functionalized gold nanoparticles, 1,1′,4′,1″-terphenyl-4-thiol, 1,11-undecanedithiol, 1,16-hexadecanedithiol, 1,2-ethanedithiol technical grade, 1,3-propanedithiol, 1,4-benzenedimethanethiol, 1,4-butanedithiol, 1,4-butanedithiol diacetate, 1,5-pentanedithiol, 1,6-hexanedithiol, 1,8-octanedithiol, 1,9-nonanedithiol, adamantanethiol, 1-butanethiol, 1-decanethiol, 1-dodecanethiol, 1-heptanethiol, 1-heptanethiol purum, 1-hexadecanethiol, 1-hexanethiol, 1-mercapto-(triethylene glycol), 1-mercapto-(triethylene glycol) methyl ether functionalized gold nanoparticles, 1-mercapto-2-propanol, 1-nonanethiol, 1-octadecanethiol, 1-octanethiol, 1-octanethiol, 1-pentadecanethiol, 1-pentanethiol, 1-propanethiol, 1-tetradecanethiol, 1-tetradecanethiol purum, 1-undecanethiol, 11-(1*H*-pyrrol-1-yl)undecane-1-thiol, 11-amino-1-undecanethiol hydrochloride, 11-bromo-1-undecanethiol, 11-mercapto-1-undecanol, 11-mercapto-1-undecanol, 11-mercaptoundecanoic acid, 11-mercaptoundecanoic acid, 11-mercaptoundecyl trifluoroacetate, 11-mercaptoundecylphosphoric acid, 12-mercaptododecanoic acid, 12-mercaptododecanoic acid, 15-mercaptopentadecanoic acid, 16-mercaptohexadecanoic acid, 16-mercaptohexadecanoic acid, 1*H*,1*H*,2*H*,2*H*-perfluorodecanethiol, 2,2′-(ethylenedioxy)diethanethiol, 2,3-butanedithiol, 2-butanethiol, 2-ethylhexanethiol, 2-methyl-1-propanethiol, 2-methyl-2-propanethiol, 2-phenylethanethiol, 3,3,4,4,5,5,6,6,6-nonafluoro-1-hexanethiol purum, 3-(dimethoxymethylsilyl)-1-propanethiol, 3-chloro-1-propanethiol, 3-mercapto-1-propanol, 3-mercapto-2-butanol, 3-mercapto-*N*-nonylpropionamide, 3-mercaptopropionic acid, 3-mercaptopropyl-functionalized silica gel, 3-methyl-1-butanethiol, 4,4′-bis(mercaptomethyl)biphenyl, 4,4′-dimercaptostilbene, 4-(6-mercaptohexyloxy)benzyl alcohol, 4-cyano-1-butanethiol, 4-mercapto-1-butanol, 6-(ferrocenyl)hexanethiol, 6-mercapto-1-hexanol, 6-mercaptohexanoic acid, 8-mercapto-1-octanol, 8-mercaptooctanoic acid, 9-mercapto-1-nonanol,

biphenyl-4,4′-dithiol, butyl 3-mercaptopropionate, copper(I) 1-butanethiolate, cyclohexanethiol, cyclopentanethiol, decanethiol functionalized silver nanoparticles, dodecanethiol functionalized gold nanoparticles, dodecanethiol functionalized silver nanoparticles, hexa(ethylene glycol)mono-11-(acetylthio)undecyl ether, mercaptosuccinic acid, methyl 3-mercaptopropionate, nanoTether BPA-HH, NanoThinks™ 18, NanoThinks™ 8, NanoThinks™ ACID11, NanoThinks™ ACID16, NanoThinks™ ALCO11, NanoThinks™ THIO8, octanethiol functionalized gold nanoparticles, PEG dithiol average M_n 8,000, PEG dithiol average mol wt 1,500, PEG dithiol average mol wt 3,400, *S*-(11-bromoundecyl)thioacetate, *S*-(4-cyanobutyl)thioacetate, thiophenol, triethylene glycol mono-11-mercaptoundecyl ether, trimethylolpropane tris(3-mercaptopropionate), [11-(methylcarbonylthio)undecyl]tetra(ethylene glycol), *m*-carborane-9-thiol, *p*-terphenyl-4,4″-dithiol, *tert*-dodecylmercaptan, and *tert*-nonyl mercaptan.

In certain embodiments the bacterial pathogen comprises at least one of: (i) one or more gram-negative bacteria; (ii) one or more gram-positive bacteria; (iii) one or more antibiotic-sensitive bacteria; (iv) one or more antibiotic-resistant bacteria; (v) a bacterial pathogen that is selected from *Staphylococcus aureus (S. aureus)*, MRSA (methicillin-resistant *S. aureus*), *Staphylococcus epidermidis*, MRSE (methicillin-resistant *S. epidermidis*), *Mycobacterium tuberculosis, Mycobacterium avium, Pseudomonas aeruginosa,* drug-resistant *P. aeruginosa, Escherichia coli,* enterotoxigenic *E. coli,* enterohemorrhagic *E. coli, Klebsiella pneumoniae, Clostridium difficile, Heliobacter pylori, Legionella pneumophila, Enterococcus faecalis,* methicillin-susceptible *Enterococcus faecalis, Enterobacter cloacae, Salmonella typhimurium, Proteus vulgaris, Yersinia enterocolitica, Vibrio cholera, Shigella flexneri,* vancomycin-resistant *Enterococcus* (VRE), *Burkholderia cepacia* complex, *Francisella tularensis, Bacillus anthracis, Yersinia pestis, Pseudomonas aeruginosa, Streptococcus pneumonia*, penicillin-resistant *Streptococcus pneumonia*, *Escherichia coli*, *Burkholderia cepacia*, *Bukholderia multivorans*, *Mycobacterium smegmatis* and *Acinetobacter baumannii.*

In certain embodiments the method comprises contacting the plant with at least one of (i) a synergizing antibiotic and (ii) a cooperative antimicrobial efficacy enhancing antibiotic, simultaneously or sequentially and in any order with respect to the step of contacting the surface with the BT composition. In certain further embodiments the synergizing antibiotic or the cooperative antimicrobial efficacy enhancing antibiotic comprises an antibiotic

that is selected from an aminoglycoside antibiotic, a carbapenem antibiotic, a cephalosporin antibiotic, a fluoroquinolone antibiotic, a glycopeptide antibiotic, a lincosamide antibiotic, a penicillinase-resistant penicillin antibiotic, and an aminopenicillin antibiotic. In certain further embodiments the synergizing antibiotic or the cooperative antimicrobial efficacy enhancing antibiotic is an aminoglycoside antibiotic that is selected from amikacin, arbekacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, rhodostreptomycin, streptomycin, tobramycin and apramycin.

In certain other embodiments there is provided a method for overcoming antibiotic resistance in or on a plant where an antibiotic-resistant bacterial pathogen is present, comprising: contacting the plant simultaneously or sequentially and in any order with an effective amount of (1) at least one bismuth-thiol (BT) composition and (2) at least one antibiotic that is capable of enhancing or acting synergistically with the at least one BT composition, under conditions and for a time sufficient for one or more of: (i) prevention of infection of the plant by the bacterial pathogen, (ii) inhibition of cell viability or cell growth of substantially all planktonic cells of the bacterial pathogen, (iii) inhibition of biofilm formation by the bacterial pathogen, and (iv) inhibition of biofilm viability or biofilm growth of substantially all biofilm-form cells of the bacterial pathogen, wherein the BT composition comprises a plurality of microparticles that comprise a bismuth-thiol (BT) compound, substantially all of said microparticles having a volumetric mean diameter of from about 0.4 μm to about 5 μm; and thereby overcoming antibiotic resistance on the epithelial tissue surface. In certain further embodiments the bacterial pathogen exhibits resistance to an antibiotic that is selected from methicillin, vancomycin, naficilin, gentamicin, ampicillin, chloramphenicol, doxycycline, tobramycin, clindamicin and gatifloxacin. In certain other embodiments the BT composition comprises one or more BT compounds selected from BisBAL, BisEDT, Bis-dimercaprol, Bis-DTT, Bis-2-mercaptoethanol, Bis-DTE, Bis-Pyr, Bis-Ery, Bis-Tol, Bis-BDT, Bis-PDT, Bis-Pyr/Bal, Bis-Pyr/BDT, Bis-Pyr/EDT, Bis-Pyr/PDT, Bis-Pyr/Tol, Bis-Pyr/Ery, bismuth-1-mercapto-2-propanol, and Bis-EDT/2-hydroxy-1-propanethiol. In certain embodiments the synergizing or enhancing antibiotic comprises an antibiotic that is selected from clindamicin, gatifloxacin, an aminoglycoside antibiotic, a carbapenem antibiotic, a cephalosporin antibiotic, a fluoroquinolone antibiotic, a penicillinase-resistant penicillin antibiotic, and an aminopenicillin antibiotic. In certain further embodiments the synergizing or enhancing antibiotic is an aminoglycoside antibiotic that is selected from

amikacin, arbekacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, rhodostreptomycin, streptomycin, tobramycin and apramycin.

According to certain other embodiments there is provided a bismuth-thiol composition, comprising a plurality of microparticles that comprise a bismuth-thiol (BT) compound, substantially all of said microparticles having a volumetric mean diameter of from about 0.4 μm to about 5 μm, wherein the BT compound comprises bismuth or a bismuth salt and a thiol-containing compound. In another embodiment there is provided a bismuth-thiol composition, comprising a plurality of microparticles that comprise a bismuth-thiol (BT) compound, substantially all of said microparticles having a volumetric mean diameter of from about 0.4 μm to about 5 μm and being formed by a process that comprises (a) admixing, under conditions and for a time sufficient to obtain a solution that is substantially free of a solid precipitate, (i) an acidic aqueous solution that comprises a bismuth salt comprising bismuth at a concentration of at least 50 mM and that lacks a hydrophilic, polar or organic solubilizer, with (ii) ethanol in an amount sufficient to obtain an admixture that comprises at least about 5%, 10%, 15%, 20%, 25% or 30% ethanol by volume; and (b) adding to the admixture of (a) an ethanolic solution comprising a thiol-containing compound to obtain a reaction solution, wherein the thiol-containing compound is present in the reaction solution at a molar ratio of from about 1:3 to about 3:1 relative to the bismuth, under conditions and for a time sufficient for formation of a precipitate which comprises the microparticles comprising the BT compound. In certain embodiments the bismuth salt is $Bi(NO_3)_3$. In certain embodiments the acidic aqueous solution comprises at least 5%, 10%, 15%, 20%, 22% or 22.5% bismuth by weight. In certain embodiments the acidic aqueous solution comprises at least 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5% or 5% nitric acid by weight. In certain embodiments the thiol-containing compound comprises one or more agents selected from 1,2-ethane dithiol, 2,3-dimercaptopropanol, pyrithione, dithioerythritol, 3,4-dimercaptotoluene, 2,3-butanedithiol, 1,3-propanedithiol, 2-hydroxypropane thiol, 1-mercapto-2-propanol, dithioerythritol, alpha-lipoic acid and dithiothreitol.

In another embodiment there is provided a method for preparing a bismuth-thiol composition that comprises a plurality of microparticles that comprise a bismuth-thiol (BT) compound, substantially all of said microparticles having a volumetric mean diameter of from about 0.4 μm to about 5 μm, said method comprising the steps of (a) admixing, under conditions and for a time sufficient to obtain a solution that is substantially free of a solid precipitate, (i)

an acidic aqueous solution that comprises a bismuth salt comprising bismuth at a concentration of at least 50 mM and that lacks a hydrophilic, polar or organic solubilizer, with (ii) ethanol in an amount sufficient to obtain an admixture that comprises at least about 5%, 10%, 15%, 20%, 25% or 30% ethanol by volume; and (b) adding to the admixture of (a) an ethanolic solution comprising a thiol-containing compound to obtain a reaction solution, wherein the thiol-containing compound is present in the reaction solution at a molar ratio of from about 1:3 to about 3:1 relative to the bismuth, under conditions and for a time sufficient for formation of a precipitate which comprises the microparticles comprising the BT compound. In certain embodiments the method further comprises recovering the precipitate to remove impurities. In certain embodiments the bismuth salt is $Bi(NO_3)_3$. In certain embodiments the acidic aqueous solution comprises at least 5%, 10%, 15%, 20%, 22% or 22.5% bismuth by weight. In certain embodiments the acidic aqueous solution comprises at least 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5% or 5% nitric acid by weight. In certain embodiments the thiol-containing compound comprises one or more agents selected from the group consisting of 1,2-ethane dithiol, 2,3-dimercaptopropanol, pyrithione, dithioerythritol, 3,4-dimercaptotoluene, 2,3-butanedithiol, 1,3-propanedithiol, 2-hydroxypropane thiol, 1-mercapto-2-propanol, dithioerythritol, dithiothreitol, alpha-lipoic acid, methanethiol (CH_3SH [m-mercaptan]), ethanethiol (C_2H_5SH [e- mercaptan]), 1-propanethiol (C_3H_7SH [n-P mercaptan]), 2-propanethiol ($CH_3CH(SH)CH_3$ [$2C_3$ mercaptan]), butanethiol (C_4H_9SH ([n-butyl mercaptan]), tert-butyl mercaptan ($C(CH_3)_3SH$ [t-butyl mercaptan]), pentanethiols ($C_5H_{11}SH$ [pentyl mercaptan]), coenzyme A, lipoamide, glutathione, cysteine, cystine, 2-mercaptoethanol, dithiothreitol, dithioerythritol, 2-mercaptoindole, transglutaminase, (11-mercaptoundecyl)hexa(ethylene glycol), (11-mercaptoundecyl)tetra(ethylene glycol), (11-mercaptoundecyl)tetra(ethylene glycol) functionalized gold nanoparticles, 1,1′,4′,1″-terphenyl-4-thiol, 1,11-undecanedithiol, 1,16-hexadecanedithiol, 1,2-ethanedithiol technical grade, 1,3-propanedithiol, 1,4-benzenedimethanethiol, 1,4-butanedithiol, 1,4-butanedithiol diacetate, 1,5-pentanedithiol, 1,6-hexanedithiol, 1,8-octanedithiol, 1,9-nonanedithiol, adamantanethiol, 1-butanethiol, 1-decanethiol, 1-dodecanethiol, 1-heptanethiol, 1-heptanethiol purum, 1-hexadecanethiol, 1-hexanethiol, 1-mercapto-(triethylene glycol), 1-mercapto-(triethylene glycol) methyl ether functionalized gold nanoparticles, 1-mercapto-2-propanol, 1-nonanethiol, 1-octadecanethiol, 1-octanethiol, 1-octanethiol, 1-pentadecanethiol, 1-

pentanethiol, 1-propanethiol, 1-tetradecanethiol, 1-tetradecanethiol purum, 1-undecanethiol, 11-(1*H*-pyrrol-1-yl)undecane-1-thiol, 11-amino-1-undecanethiol hydrochloride, 11-bromo-1-undecanethiol, 11-mercapto-1-undecanol, 11-mercapto-1-undecanol, 11-mercaptoundecanoic acid, 11-mercaptoundecanoic acid, 11-mercaptoundecyl trifluoroacetate, 11-mercaptoundecylphosphoric acid, 12-mercaptododecanoic acid, 12-mercaptododecanoic acid, 15-mercaptopentadecanoic acid, 16-mercaptohexadecanoic acid, 16-mercaptohexadecanoic acid, 1*H*,1*H*,2*H*,2*H*-perfluorodecanethiol, 2,2′-(ethylenedioxy)diethanethiol, 2,3-butanedithiol, 2-butanethiol, 2-ethylhexanethiol, 2-methyl-1-propanethiol, 2-methyl-2-propanethiol, 2-phenylethanethiol, 3,3,4,4,5,5,6,6,6-nonafluoro-1-hexanethiol purum, 3-(dimethoxymethylsilyl)-1-propanethiol, 3-chloro-1-propanethiol, 3-mercapto-1-propanol, 3-mercapto-2-butanol, 3-mercapto-*N*-nonylpropionamide, 3-mercaptopropionic acid, 3-mercaptopropyl-functionalized silica gel, 3-methyl-1-butanethiol, 4,4′-bis(mercaptomethyl)biphenyl, 4,4′-dimercaptostilbene, 4-(6-mercaptohexyloxy)benzyl alcohol, 4-cyano-1-butanethiol, 4-mercapto-1-butanol, 6-(ferrocenyl)hexanethiol, 6-mercapto-1-hexanol, 6-mercaptohexanoic acid, 8-mercapto-1-octanol, 8-mercaptooctanoic acid, 9-mercapto-1-nonanol, biphenyl-4,4′-dithiol, butyl 3-mercaptopropionate, copper(I) 1-butanethiolate, cyclohexanethiol, cyclopentanethiol, decanethiol functionalized silver nanoparticles, dodecanethiol functionalized gold nanoparticles, dodecanethiol functionalized silver nanoparticles, hexa(ethylene glycol)mono-11-(acetylthio)undecyl ether, mercaptosuccinic acid, methyl 3-mercaptopropionate, nanoTether BPA-HH, NanoThinks™ 18, NanoThinks™ 8, NanoThinks™ ACID11, NanoThinks™ ACID16, NanoThinks™ ALCO11, NanoThinks™ THIO8, octanethiol functionalized gold nanoparticles, PEG dithiol average M_n 8,000, PEG dithiol average mol wt 1,500, PEG dithiol average mol wt 3,400, *S*-(11-bromoundecyl)thioacetate, *S*-(4-cyanobutyl)thioacetate, thiophenol, triethylene glycol mono-11-mercaptoundecyl ether, trimethylolpropane tris(3-mercaptopropionate), [11-(methylcarbonylthio)undecyl]tetra(ethylene glycol), *m*-carborane-9-thiol, *p*-terphenyl-4,4″-dithiol, *tert*-dodecylmercaptan, *tert*-nonyl mercaptan.

In another embodiment there is provided a method for protecting a natural or artificial surface, including a biological tissue surface such as a plant surface (*e.g.,* all or part of a surface of a root, bulb, stem, leaf, branch, vine, runner, bud, flower or a part thereof, greentip, fruit, seed, seed pod, or the like) or an epithelial tissue surface, against one or more of a bacterial pathogen,

a fungal pathogen and a viral pathogen, comprising contacting the epithelial tissue surface with an effective amount of a BT composition under conditions and for a time sufficient for one or more of (i) prevention of infection of the surface by the bacterial, fungal or viral pathogen, (ii) inhibition of cell viability or cell growth of substantially all planktonic cells of the bacterial, fungal or viral pathogen, (iii) inhibition of biofilm formation by the bacterial, fungal or viral pathogen, and (iv) inhibition of biofilm viability or biofilm growth of substantially all biofilm-form cells of the bacterial, fungal or viral pathogen, wherein the BT composition comprises a plurality of microparticles that comprise a bismuth-thiol (BT) compound, substantially all of said microparticles having a volumetric mean diameter of from about 0.4 μm to about 5 μm. In certain embodiments the bacterial pathogen comprises at least one of (i) one or more gram-negative bacteria; (ii) one or more gram-positive bacteria; (iii) one or more antibiotic-sensitive bacteria; (iv) one or more antibiotic-resistant bacteria; (v) a bacterial pathogen that is selected from *Staphylococcus aureus (S. aureus)*, MRSA (methicillin-resistant *S. aureus*), *Staphylococcus epidermidis* , MRSE (methicillin-resistant *S. epidermidis*), *Mycobacterium tuberculosis, Mycobacterium avium, Pseudomonas aeruginosa,* drug-resistant *P. aeruginosa, Escherichia coli,* enterotoxigenic *E. coli,* enterohemorrhagic *E. coli, Klebsiella pneumoniae, Clostridium difficile, Heliobacter pylori, Legionella pneumophila, Enterococcus faecalis,* methicillin-susceptible *Enterococcus faecalis, Enterobacter cloacae, Salmonella typhimurium, Proteus vulgaris, Yersinia enterocolitica, Vibrio cholera, Shigella flexneri,* vancomycin-resistant *Enterococcus* (VRE), *Burkholderia cepacia* complex, *Francisella tularensis, Bacillus anthracis, Yersinia pestis, Pseudomonas aeruginosa,* vancomycin-resistant enterococci, *Streptococcus pneumonia*, penicillin-resistant *Streptococcus pneumonia*, *Escherichia coli*, *Burkholderia cepacia*, *Bukholderia multivorans*, *Mycobacterium smegmatis* and *Acinetobacter baumannii.* In certain embodiments the bacterial pathogen exhibits antibiotic resistance. In certain embodiments the bacterial pathogen exhibits resistance to an antibiotic that is selected from methicillin, vancomycin, naficilin, gentamicin, ampicillin, chloramphenicol, doxycycline and tobramycin.

In certain embodiments the natural or artificial surface comprises an oral/buccal cavity surface, prosthetic device, ceramic, plastic, polymer, rubber, metal article of manufacture, painted surface, marine structure including ship hull, rudder, propeller, anchor, hold, ballast tank, dock, dry dock, pier, piling, bulkhead, or other natural or artificial surface.

In certain embodiments the surface comprises an epithelial tissue surface that comprises a tissue that is selected from epidermis, dermis, respiratory tract, gastrointestinal tract and glandular linings.

In certain embodiments the step of contacting is performed one or a plurality of times. In certain embodiments at least one step of contacting comprises one of spraying, irrigating, dipping and painting the natural or artifical surface. In certain embodiments at least one step of contacting comprises one of inhaling, ingesting and orally irrigating. In certain embodiments least one step of contacting comprises administering by a route that is selected from topically, intraperitoneally, orally, parenterally, intravenously, intraarterially, transdermally, sublingually, subcutaneously, intramuscularly, transbuccally, intranasally, via inhalation, intraoccularly, intraauricularly, intraventricularly, subcutaneously, intraadiposally, intraarticularly and intrathecally. In certain embodiments the BT composition comprises one or more BT compounds selected from the group consisting of BisBAL, BisEDT, Bis-dimercaprol, Bis-DTT, Bis-2-mercaptoethanol, Bis-DTE, Bis-Pyr, Bis-Ery, Bis-Tol, Bis-BDT, Bis-PDT, Bis-Pyr/Bal, Bis-Pyr/BDT, Bis-Pyr/EDT, Bis-Pyr/PDT, Bis-Pyr/Tol, Bis-Pyr/Ery, bismuth-1-mercapto-2-propanol, and Bis-EDT/2-hydroxy-1-propanethiol.

In certain embodiments the bacterial pathogen exhibits antibiotic resistance. In certain other embodiments the above described method further comprises contacting the natural or artificial surface with a synergizing antibiotic and/or with an enhancing antibiotic, simultaneously or sequentially and in any order with respect to the step of contacting the surface with the BT composition. In certain embodiments the synergizing and/or enhancing antibiotic comprises an antibiotic that is selected from an aminoglycoside antibiotic, a carbapenem antibiotic, a cephalosporin antibiotic, a fluoroquinolone antibiotic, a glycopeptide antibiotic, a lincosamide antibiotic, a penicillinase-resistant penicillin antibiotic, and an aminopenicillin antibiotic. In certain embodiments the synergizing and/or enhancing antibiotic is an aminoglycoside antibiotic that is selected from amikacin, arbekacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, rhodostreptomycin, streptomycin, tobramycin and apramycin.

In another embodiment of the invention described herein there is provided a method for overcoming antibiotic resistance (*e.g.,* for a bacterial pathogen that is resistant to at least one anti-bacterial effect of at least one antibiotic known to have an anti-bacterial effect against bacteria of the same bacterial species, rendering such a pathogen susceptible to an antibiotic) on a

natural or artificial surface where an antibiotic-resistant bacterial pathogen is present, comprising contacting the surface simultaneously or sequentially and in any order with an effective amount of (1) at least one bismuth-thiol (BT) composition and (2) at least one antibiotic that is enhanced by, and/or that is capable of acting synergistically with the at least one BT composition, under conditions and for a time sufficient for one or more of: (i) prevention of infection of the surface by the bacterial pathogen, (ii) inhibition of cell viability or cell growth of substantially all planktonic cells of the bacterial pathogen, (iii) inhibition of biofilm formation by the bacterial pathogen, and (iv) inhibition of biofilm viability or biofilm growth of substantially all biofilm-form cells of the bacterial pathogen, wherein the BT composition comprises a plurality of microparticles that comprise a bismuth-thiol (BT) compound, substantially all of said microparticles having a volumetric mean diameter of from about 0.4 μm to about 5 μm; and thereby overcoming antibiotic resistance on the epithelial tissue surface. In certain embodiments the bacterial pathogen comprises at least one of: (i) one or more gram-negative bacteria; (ii) one or more gram-positive bacteria; (iii) one or more antibiotic-sensitive bacteria; (iv) one or more antibiotic-resistant bacteria; (v) a bacterial pathogen that is selected from *Staphylococcus aureus (S. aureus)*, MRSA (methicillin-resistant *S. aureus*), *Staphylococcus epidermidis* , MRSE (methicillin-resistant *S. epidermidis*), *Mycobacterium tuberculosis, Mycobacterium avium, Pseudomonas aeruginosa,* drug-resistant *P. aeruginosa, Escherichia coli,* enterotoxigenic *E. coli,* enterohemorrhagic *E. coli, Klebsiella pneumoniae, Clostridium difficile, Heliobacter pylori, Legionella pneumophila, Enterococcus faecalis,* methicillin-susceptible *Enterococcus faecalis*, *Enterobacter cloacae, Salmonella typhimurium, Proteus vulgaris, Yersinia enterocolitica, Vibrio cholera, Shigella flexneri,* vancomycin-resistant *Enterococcus* (VRE), *Burkholderia cepacia* complex, *Francisella tularensis, Bacillus anthracis, Yersinia pestis, Pseudomonas aeruginosa,* vancomycin-resistant enterococci, *Streptococcus pneumonia*, penicillin-resistant *Streptococcus pneumonia*, *Escherichia coli*, *Burkholderia cepacia*, *Bukholderia multivorans*, *Mycobacterium smegmatis* and *Acinetobacter baumannii.*

In certain embodiments the bacterial pathogen exhibits resistance to an antibiotic that is selected from methicillin, vancomycin, naficilin, gentamicin, ampicillin, chloramphenicol, doxycycline, tobramycin, clindamicin and gatifloxacin.

In certain embodiments the natural or artificial surface comprises an oral/buccal cavity surface, prosthetic device, ceramic, plastic, polymer, rubber, metal article of manufacture, painted surface, marine structure including ship hull, rudder, propeller, anchor, hold, ballast tank, dock, dry dock, pier, piling, bulkhead, or other natural or artificial surface.

In certain embodiments the surface comprises a tissue that is selected from the group consisting of epidermis, dermis, respiratory tract, gastrointestinal tract and glandular linings. In certain embodiments the step of contacting is performed one or a plurality of times. In certain embodiments at least one step of contacting comprises one of spraying, irrigating, dipping and painting the surface. In certain other embodiments at least one step of contacting comprises one of inhaling, ingesting and orally irrigating. In certain embodiments at least one step of contacting comprises administering by a route that is selected from topically, intraperitoneally, orally, parenterally, intravenously, intraarterially, transdermally, sublingually, subcutaneously, intramuscularly, transbuccally, intranasally, via inhalation, intraoccularly, intraauricularly, intraventricularly, subcutaneously, intraadiposally, intraarticularly and intrathecally. In certain embodiments the BT composition comprises one or more BT compounds selected from BisBAL, BisEDT, Bis-dimercaprol, Bis-DTT, Bis-2-mercaptoethanol, Bis-DTE, Bis-Pyr, Bis-Ery, Bis-Tol, Bis-BDT, Bis-PDT, Bis-Pyr/Bal, Bis-Pyr/BDT, Bis-Pyr/EDT, Bis-Pyr/PDT, Bis-Pyr/Tol, Bis-Pyr/Ery, bismuth-1-mercapto-2-propanol, and Bis-EDT/2-hydroxy-1-propanethiol. In certain embodiments the synergizing and/or enhancing antibiotic comprises an antibiotic that is selected from clindamicin, gatifloxacin, an aminoglycoside antibiotic, a carbapenem antibiotic, a cephalosporin antibiotic, a fluoroquinolone antibiotic, a glycopeptide antibiotic, a lincosamide antibiotic, a penicillinase-resistant penicillin antibiotic, and an aminopenicillin antibiotic. In certain embodiments the synergizing and/or enhancing antibiotic is an aminoglycoside antibiotic that is selected from amikacin, arbekacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, rhodostreptomycin, streptomycin, tobramycin and apramycin.

Turning to another embodiment there is provided an antiseptic composition, comprising (a) at least one BT compound; (b) at least one antibiotic compound that is enhanced by and/or is capable of acting synergistically with the BT compound; and (c) a pharmaceutically acceptable excipient or carrier, including a carrier for topical use. In certain embodiments the BT compound is selected from BisBAL, BisEDT, Bis-dimercaprol, Bis-DTT,

Bis-2-mercaptoethanol, Bis-DTE, Bis-Pyr, Bis-Ery, Bis-Tol, Bis-BDT, Bis-PDT, Bis-Pyr/Bal, Bis-Pyr/BDT, Bis-Pyr/EDT, Bis-Pyr/PDT, Bis-Pyr/Tol, Bis-Pyr/Ery, bismuth-1-mercapto-2-propanol, and Bis-EDT/2-hydroxy-1-propanethiol. In certain embodiments the BT composition comprises a plurality of microparticles that comprise a bismuth-thiol (BT) compound, substantially all of said microparticles having a volumetric mean diameter of from about 0.4 μm to about 5 μm. In certain embodiments the BT compound is selected from BisEDT and BisBAL. In certain embodiments the antibiotic compound comprises an antibiotic that is selected from methicillin, vancomycin, naficilin, gentamicin, ampicillin, chloramphenicol, doxycycline, tobramycin, clindamicin, gatifloxacin and an aminoglycoside antibiotic. In certain embodiments the aminoglycoside antibiotic is selected from amikacin, arbekacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, rhodostreptomycin, streptomycin, tobramycin and apramycin. In certain embodiments the aminoglycoside antibiotic is amikacin.

In certain other embodiments there is provided a method for treating a natural or artificial surface that supports or contains bacterial biofilm, comprising (a) identifying a bacterial infection on or in the surface as comprising one of (i) gram positive bacteria, (ii) gram negative bacteria, and (iii) both (i) and (ii); (b) administering a formulation that comprises one or more bismuth thiol (BT) compositions to the surface, wherein (i) if the bacterial infection comprises gram positive bacteria, then the formulation comprises therapeutically effective amounts of at least one BT compound and at least one antibiotic that is rifamycin, (ii) if the bacterial infection comprises gram negative bacteria, then the formulation comprises therapeutically effective amounts of at least one BT compound and amikacin, (iii) if the bacterial infection comprises both gram positive and gram negative bacteria, then the formulation comprises therapeutically effective amounts of one or a plurality of BT compounds, rifamycin and amikacin, and thereby treating the surface.

In certain embodiments the biofilm comprises one or a plurality of antibiotic-resistant bacteria. In certain embodiments treating the surface comprises at least one of: (i) eradicating the bacterial biofilm, (ii) reducing the bacterial biofilm, and (iii) impairing growth of the bacterial biofilm. In certain embodiments the BT composition comprises a plurality of microparticles that comprise a bismuth-thiol (BT) compound, substantially all of said microparticles having a volumetric mean diameter of from about 0.4 μm to about 5 μm.

These and other aspects of the herein described invention embodiments will be evident upon reference to the following detailed description and attached drawings. All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet, including U.S. RE37,793, U.S. 6,248,371, U.S. 6,086,921, and U.S. 6,380,248, are incorporated herein by reference in their entirety, as if each was incorporated individually. Aspects and embodiments of the invention can be modified, if necessary, to employ concepts of the various patents, applications and publications to provide yet further embodiments.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figure 1 shows surviving numbers (log CFU; colony forming units) from *Pseudomonas aeruginosa* colony biofilms grown for 24 hours on 10% tryptic soy agar (TSA) at 37°C, followed with indicated treatment for 18 hours. Indicated antibiotic treatments are TOB, tobramycin 10X MIC; AMK, amikacin 100X MIC; IPM, imipenem 10X MIC; CEF, cefepime 10X MIC; CIP, ciprofloxacin 100X MIC; Cpd 2B, compound 2B (Bis-BAL, 1:1.5). (MIC; minimum inhibitory concentration, *e.g.*, lowest concentration that prevents bacterial growth).

Figure 2 shows surviving numbers (log CFU) from *Staphylococcus aureus* colony biofilms grown for 24 hours on 10% tryptic soy agar, followed by the indicated treatment. Indicated antibiotic treatments are Rifampicin, RIF 100X MIC; daptomycin, DAP 320X MIC; minocycline, MIN 100X MIC; ampicillin, AMC 10X MIC; vancomycin, VAN 10X MIC; Cpd 2B, compound 2B (Bis-BAL, 1:1.5), Cpd 8-2, compound 8-2 (Bis-Pyr/BDT (1:1/0.5).

Figure 3 shows scratch closure over time of keratinocytes exposed to biofilms. (*) Significantly different from control ($P<0.001$).

Figure 4A and 4B show the subinhibitory BisEDT reversing antibiotic-resistance to several antibiotics. Effects of antibiotics with and without BisEDT (0.05 μg/ml) on a lawn of MRSA (Methicillin-resistant *S. aureus*) is shown. Panel A shows standard antibiotic-soaked discs alone, and Panel B shows discs combined with a BisEDT (BE). [GM= gentamicin, CZ= cefazolin, FEP= cefepime, IPM= imipenim,SAM= ampicillin/ sulbactam, LVX= levofloxacin.

Figure 5 shows the effect of BisEDT and antibiotics on biofilm formation. *S. epidermidis* grown in TSB + 2% glucose in polystyrene plates for 48h at 37°C. Gatifloxacin (GF), clindamycin (CM), minocycline (MC), gentamicin (GM), vancomycin (VM), cefazolin (CZ), nafcillin (NC), and rifampicin (RP). Results were expressed as the mean change in the BPC (in serial 2-fold dilution steps) at 0.25 μM BisEDT (n=3).

Figure 6 shows the effect of BisEDT and antibiotics on growth of *S. epidermidis* grown in TSB plus 2% glucose for 48h at 37°C. Results are expressed as the mean change in MIC (dilution steps) with increasing BisEDT (n=3). See legend in Figure 5 for antibiotic definitions.

Figure 7 is a bar graph showing the mean *S. aureus* bacteria levels detected on the bone and hardware samples from open fractures in an *in vivo* rat model following treatment with three BT formulations, Bis-EDT , MB-11 and MB-8-2 with or without Cefazolin antibiotic treatment. Standard errors of the mean are shown as error bars. Animals euthanized early are not excluded from the analysis, however samples from one animal in group 2 have been excluded due to gross contamination.

DETAILED DESCRIPTION

Particular embodiments of the invention disclosed herein are based on the surprising discovery that certain bismuth-thiol (BT) compounds as provided herein (preferably including BT microparticles having a volumetric mean diameter of from about 0.4 μm to about 5 μm), but not certain other BT compounds (even if provided as microparticles), exhibited potent antiseptic, antibacterial and/or anti-biofilm activity against particular bacteria, including bacteria associated with a number of clinically significant infections including infections that can comprise bacterial biofilms.

Unexpectedly, not all BT compounds were uniformly effective against such bacteria in a predictable fashion, but instead exhibited different potencies depending on the target bacterial species. In particular and as described herein, certain BT compounds (preferably including BT microparticles having a volumetric mean diameter of from about 0.4 μm to about 5 μm) were found to exhibit higher potency against gram-negative bacteria, while certain other BT compounds (preferably including BT microparticles having a volumetric mean diameter of from about 0.4 μm to about 5 μm) were found to exhibit greater potency against gram-positive bacteria, in a manner that, according to non-limiting theory, may for the first time afford clinically relevant

strategies for the management of bacterial infections, including bacterial biofilm infections.

Additionally, and as described in greater detail below, certain embodiments of the invention described herein relate to surprising advantages that are provided by novel bismuth-thiol (BT) compositions that, as disclosed herein, can be made in preparations that comprise a plurality of BT microparticles that are substantially monodisperse with respect to particle size (*e.g.*, having volumetric mean diameter from about 0.4 μm to about 5 μm). In certain of these and related embodiments, the microparticulate BT is not provided as a component of a lipid vesicle or liposome such as a multilamellar phosphocholine-cholesterol liposome or other multilamellar or unilamellar liposomal vesicle.

As also disclosed herein, with respect to certain embodiments, it has been discovered that antibacterial and anti-biofilm efficacies of certain antibiotics, which antibiotics have previously been found to lack potent therapeutic effect against such bacterial infections, may be significantly enhanced (*e.g.,* increased in a statistically significant manner) by treating the infection (*e.g.,* by direct application on or in an infected site such as a natural or artificial surface) with one or more of these antibiotics in concert, simultaneously or sequentially and in either order, with a selected BT compound. In a manner that could not be predicted prior to the present disclosure, certain BT compounds can be combined with certain antibiotics to provide a synergizing or enhancing combination as provided herein with respect to antibacterial and/or anti-biofilm activity against certain bacterial species or bacterial strains. The unpredicted nature of such combinations, as described in greater detail below, is evidenced by the observations that while certain BT/antibiotic combinations acted synergistically or exhibited enhancement against certain bacteria, certain other BT/antibiotic combinations failed to exhibit such synergistic or enhanced antibacterial and/or anti-biofilm activity.

According to these and related embodiments, the antibiotic and the BT compound may be administered simultaneously or sequentially and in either order, and it is noteworthy that the specific synergizing or enhancing combinations of one or more antibiotic and one or more BT compound as disclosed herein for treatment of a particular infection (*e.g.*, a biofilm formed by gram-negative or gram-positive bacteria) did not exhibit predictable (*e.g.,* merely additive) activities but instead acted in an unexpectedly synergistic or

enhancing (*e.g.,* supra-additive) fashion, as a function of the selected antibiotic, the selected BT compound and the specifically identified target bacteria.

For example, by way of illustration and not limitation, disclosed herein in the context of a wide variety of actually or potentially microbially infected natural and/or artificial surfaces, and further in the context of improved substantially monodisperse microparticulate BT formulations, either or both of a particular antibiotic compound and a particular BT compound may exert limited antibacterial effects when used alone against a particular bacterial strain or species, but the combination of both the antibiotic compound and the BT compound exerts a potent antibacterial effect against the same bacterial strain or species, which effect is greater in magnitude (with statistical significance) than the simple sum of the effects of each compound when used alone, and is therefore believed according to non-limiting theory to reflect antibiotic-BT synergy (*e.g.,* FICI $\leq$ 0.5) or an enhancing effect (*e.g.*, 0.5 < FICI $\leq$ 1.0) of the BT on the antibiotic potency and/or of the antibiotic on the BT potency. Accordingly, not every BT compound may synergize with, or be enhancing for, every antibiotic, and not every antibiotic may synergize with, or be enhancing for, every BT compound, such that antibiotic-BT synergy and BT-antibiotic enhancement generally are not predictable. Instead, and according to certain embodiments as disclosed herein, specific combinations of synergizing or enhancing antibiotic and BT compounds surprisingly confer potent antibacterial effects against particular bacteria, including in particular environments such as natural and/or artificial surfaces as described herein, and further including in certain situations antibacterial effects against biofilms formed by the particular bacteria.

That is, certain BT-synergizing antibiotics are described herein, which includes an antibiotic that is capable of acting synergistically (FICI $\leq$ 0.5) with at least one BT composition that comprises at least one BT compound as provided herein, where such synergy manifests as a detectable effect that is greater (*i.e.*, in a statistically significant manner relative to an appropriate control condition) in magnitude than the effect that can be detected when the antibiotic is present but the BT compound is absent, and/or when the BT compound is present but the antibiotic is absent. Similarly, certain BT-antibiotic combinations exhibit enhancement (0.5 < FICI $\leq$ 1.0), where such enhancement manifests as a detectable effect that is greater (*i.e.*, in a statistically significant manner relative to an appropriate control condition) in magnitude than the effect

that can be detected when the antibiotic is present but the BT compound is absent, and/or when the BT compound is present but the antibiotic is absent.

Examples of such a detectable effect may in certain embodiments include (i) prevention of infection by a bacterial pathogen, (ii) inhibition of cell viability or cell growth of substantially all planktonic cells of a bacterial pathogen, (iii) inhibition of biofilm formation by a bacterial pathogen, and (iv) inhibition of biofilm viability or biofilm growth of substantially all biofilm-form cells of a bacterial pathogen, but the invention is not intended to be so limited, such that in other contemplated embodiments antibiotic-BT synergy may manifest as one or more detectable effects that may include alteration (*e.g.*, a statistically significant increase or decrease) of one or more other clinically significant parameters, for example, the degree of resistance or sensitivity of a bacterial pathogen to one or more antibiotics or other drugs or chemical agents, the degree of resistance or sensitivity of a bacterial pathogen to one or more chemical, physical or mechanical conditions (*e.g.*, pH, ionic strength, temperature, pressure), and/or the degree of resistance or sensitivity of a bacterial pathogen to one or more biological agents (*e.g.*, a virus, another bacterium, a biologically active polynucleotide, an immunocyte or an immunocyte product such as an antibody, cytokine, chemokine, enzyme including degradative enzymes, membrane-disrupting protein, a free radical such as a reactive oxygen species, or the like).

Persons familiar with the art will appreciate these and a variety of other criteria by which the effects of particular agents on the structure, function and/or activity of a bacterial population may be determined (*e.g.,* Coico et al. (Eds.), Current Protocols in Microbiology, 2008, John Wiley & Sons, Hoboken, NJ; Schwalbe et al., Antimicrobial Susceptibility Testing Protocols, 2007, CRC Press, Boca Raton, FL), for purposes of ascertaining antibiotic-BT synergy or enhancement which, as provided herein, is present when the effects of the synergizing or enhancing antibiotic-BT combination exceed the mere sum of the effects observed when one component of the combination is not present.

For example, in certain embodiments synergy may be determined by determining an antibacterial effect such as those described herein using various concentrations of candidate agents (*e.g.*, a BT and an antibiotic individually and in combination) to calculate a fractional inhibitory concentration index (FICI) and a fractional bactericidal concentration index (FBCI), according to Eliopoulos et al. (Eliopoulos and Moellering, (1996) Antimicrobial combinations. *In* Antibiotics in Laboratory Medicine (Lorian, V., Ed.), pp. 330–

96, Williams and Wilkins, Baltimore, MD, USA). Synergy may be defined as an FICI or FBCI index of ≤0.5, and antagonism at >4. (*e.g.*, Odds, FC (2003) Synergy, antagonism, and what the chequerboard puts between them. *Journal of Antimicrobial Chemotherapy* 52:1). Synergy may also be defined conventionally as ≥4-fold decrease in antibiotic concentration, or alternatively, using fractional inhibitory concentration (FIC) as described, *e.g.,* by Hollander et al. (1998 *Antimicrob. Agents Chemother.* 42:744). In certain embodiments, synergy may be defined as an effect that results from a combination of two drugs (*e.g.*, an antibiotic and a BT composition) wherein the effect of the combination is greater (*e.g.*, in a statistically significant manner) than it would be if the concentration of the second drug is replaced by the first drug.

Accordingly as described herein and in certain preferred embodiments, a combination of BT and antibiotic will be understood to synergize when a FICI value that is less than or equal to 0.5 is observed. (Odds, 2003). As also described herein, in certain other preferred embodiments and according to non-limiting theory, it is disclosed that certain BT-antibiotic combinations may exhibit a FICI value between 0.5 and 1.0 that signifies a high potential for such synergy, and which may be observed using non-optimal concentrations of at least one BT and at least one antibiotic that exhibit unilateral or mutually enhanced cooperative antimicrobial efficacy. Such an effect may also be referred to herein as "enhanced" antibiotic activity or "enhanced" BT activity.

Enhanced antibiotic and/or BT activity may be detected according to certain embodiments when the presence both (i) of at least one BT at a concentration that is less (in a statistically significant manner) than the characteristic minimum inhibitory concentration (MIC) for that BT for a given target microbe (*e.g.*, a given bacterial species or strain), and (ii) of at least one antibiotic at a concentration that is less (in a statistically significant manner) than the characteristic IC_{50} (concentration that inhibits the growth of 50% of a microbial population; *e.g.*, Soothill et al., 1992 *J Antimicrob Chemother* 29(2):137) and/or that is less than the biofilm-prevention concentration (BPC) of that antibiotic for the given target microbe, results in enhanced (in a statistically significant manner) antimicrobial efficacy of the BT-antibiotic combination relative to the antimicrobial effect that would be observed if either antimicrobial agent (*e.g.*, the BT or the antibiotic) were used at the same concentration in the absence of the other antimicrobial agent (*e.g.*, the antibiotic or the BT). In preferred embodiments, "enhanced" antibiotic and/or BT activity is present

when a FICI value that is less than or equal to 1.0, and greater than 0.5, is determined.

As will be appreciated by the skilled person based on the present disclosure, in certain embodiments synergistic or enhanced antibiotic and/or BT activity may be determined according to methods known in the art, such as using Loewe additivity-based models (*e.g.*, FIC index, Greco model), or Bliss independence based models (*e.g.*, non-parametric and semi-parametric models) or other methods described herein and known in the art (*e.g.*, Meletiadis *et al.*, 2005 *Medical Mycology* 43:133-152). Illustrative methods for determining synergy or enhanced antibiotic and/or BT activity are thus described, for instance, in Meletiadis *et al.*, 2005 *Medical Mycology* 43:133-152 and references cited therein (see also, Meletiadis *et al.*, 2002 *Rev Med Microbiol* 13:101-117; White *et al.*, 1996 *Antimicrob Agents Chemother* 40:1914-1918; Mouton et al., 1999 *Antimicrob Agents Chemother* 43:2473-2478).

Certain other embodiments contemplate specific combinations of one or more antibiotic and one or more BT compound as disclosed herein that may exhibit synergizing or enhancing effects *in vivo* for treatment of a particular infection (*e.g.*, a biofilm formed by gram-negative or gram-positive bacteria), even where the BT compound(s) and antibiotic(s) did not exhibit predictable (*e.g.,* merely additive) activities *in vivo* but instead acted in an unexpectedly synergistic or enhancing (*e.g.,* supra-additive; or conferring an effect when two or more such agents are present in combination that is greater (*e.g.*, in a statistically significant manner) than the effect that is obtained if the concentration of the second agent is replaced by the first agent) fashion, as a function of the selected antibiotic, the selected BT compound and one or more of the specifically identified target bacterial species of which the infection is comprised. It will therefore be appreciated, according to these and related embodiments, that in certain *in vivo* situations FICI or FBCI values (which are determined *in vitro*) may not be readily available, but that instead BT-antibiotic synergizing or enhancing effects may be determined in a manner afforded by the quantifiable metrics of the infection.

For example, in one embodiment, such as in the *in vivo* open fracture *Rattus norvegicus* femur critical defect model as described in Example 11, a statistically significant reduction in bacterial counts observed post-treatment for the BT-antibiotic combination as compared to the antibiotic treatment or BT compound alone, is an indication of synergizing or enhancing

effects. Statistical significance can be determined using methods well-known to the skilled person. In certain other embodiments, a reduction observed in this or other *in vivo* models by at least 5%, 10%, 20%, 30%, 40%, or 50% of bacterial counts observed in the injury post-treatment for the BT-antibiotic combination as compared to the antibiotic treatment or BT compound alone is considered an indication of synergizing or enhancing effects.

Other exemplary indicia of *in vivo* infections may be determined according to established methodologies that have been developed for quantification of the severity of the infection, such as a variety of wound scoring systems known to the skilled person (see *e.g.*, scoring systems reviewed in European Wound Management Association (EWMA), Position Document: *Identifying criteria for wound infection*. London: MEP Ltd, 2005). Illustrative wound scoring systems that may be used in assessing synergistic or enhancement activity of BT-antibiotic combinations as described herein include ASEPSIS (Wilson AP, *J Hosp Infect* 1995; 29(2): 81-86; Wilson et al., *Lancet* 1986; 1: 311-13), the Southampton Wound Assessment Scale (Bailey IS, Karran SE, Toyn K, et al. *BMJ* 1992; 304: 469-71). See also, Horan TC, Gaynes P, Martone WJ, *et al.* ,1992 *Infect Control Hosp Epidemiol* 1992; 13: 606-08. Additionally, recognized clinical indicia of wound healing known to the skilled clinician may also be measured in the presence or absence of BT compounds and/or antibiotics, such as wound size, depth, granulation tissue condition, infection, *etc.* Accordingly, and based on the present disclosure, the skilled person will readily appreciate a variety of methods for determining whether a BT composition –antibiotic combination alters (*e.g.,* increases or decreases in a statistically significant manner relative to appropriate controls) *in vivo* wound healing.

In view of these and related embodiments, there are provided herein a wide variety of methods for treating microbially infected natural and artificial surfaces such as surfaces that support or contain bacterial biofilms, with an effective amount (*e.g.*, in certain embodiments a therapeutically effective amount) of a composition or formulation that comprises one or more BT compounds and, optionally, one or more antibiotic compounds, such as one or more synergizing antibiotics, or one or more enhancing antibiotics, as provided herein. It will be appreciated that based on the present disclosure, certain antibiotics are now contemplated for use in the treatment of given types of infections, where such antibiotics had previously been viewed by persons familiar with the art as ineffective against infections of the same type.

Certain embodiments thus contemplate compositions that comprise one or more BT compounds for use as antiseptics. An antiseptic is a substance that kills or prevents the growth of microorganisms, and may be typically applied to living tissue, distinguishing the class from disinfectants, which are usually applied to inanimate objects (Goodman and Gilman's "*The Pharmacological Basis of Therapeutics* ", Seventh Edition, Gilman et al., editors, 1985, Macmillan Publishing Co., (hereafter, Goodman and Gilman") pp. 959-960). Common examples of antiseptics are ethyl alcohol and tincture of iodine. Germicides include antiseptics that kill microbes such as microbial pathogens.

Certain embodiments described herein may contemplate compositions that comprise one or more BT compounds and one or more antibiotic compound (*e.g.*, a synergizing antibiotic and/or an enhancing antibiotic as provided herein). Antibiotics are known in the art and typically comprise a drug made from a compound produced by one species of microorganism to kill another species of microorganism, or a synthetic product having an identical or similar chemical structure and mechanism of action, *e.g.*, a drug that destroys microorganisms within or on the body of a living organism, including such drug when applied topically. Among embodiments disclosed herein are those in which an antibiotic may belong to one of the following classes: aminoglycosides, carbapenems, cephalosporins, fluoroquinolones, glycopeptide antibiotics, lincosamides (*e.g.*, clindamycin), penicillinase-resistant penicillins, and aminopenicillins. Antibiotics thus may include, but need not be limited to, oxacillin, piperacillin, cefuroxime, cefotaxime, cefepime, imipenem, aztreonam, streptomycin, tobramycin, tetracycline, minocycline, ciprofloxacin, levofloxacin, erythromycin, linezolid, phosphomycin, capreomycin, isoniazid, ansamycin, carbacephem, monobactam, nitrofuran, penicillin, quinolone, sulfonamide, Clofazimine, Dapsone, Capreomycin, Cycloserine, Ethambutol, Ethionamide, Isoniazid, Pyrazinamide, Rifampicin, Rifampin, Rifabutin, Rifapentine, Streptomycin, Arsphenamine, Chloramphenicol, Fosfomycin, Fusidic acid, Linezolid, Metronidazole, Mupirocin, Platensimycin, Quinupristin, Dalfopristin, Rifaximin, Thiamphenicol, Tinidazole, aminoglycoside, beta-lactam, penicillin, cephalosporin, carbapenem, fluroquinolone, ketolide, lincosamide, macrolide, oxazolidinone, stretogramin, sulphonamide, tetracycline, glycylcycline, methicillin, vancomycin, naficilin, gentamicin, ampicillin , chloramphenicol, doxycycline, tobramycin, amikacin, arbekacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin,

rhodostreptomycin, streptomycin, tobramycin, apramycin, clindamicin, gatifloxacin, aminopenicillin, and others known to the art. Compendia of these and other clinically useful antibiotics are available and known to those familiar with the art (*e.g.*, Washington University School of Medicine, *The Washington Manual of Medical Therapeutics* (32nd Ed.), 2007 Lippincott, Williams and Wilkins, Philadelphia, PA; Hauser, AL, *Antibiotic Basics for Clinicians*, 2007 Lippincott, Williams and Wilkins, Philadelphia, PA).

An exemplary class of antibiotics for use with one or more BT compounds in certain herein disclosed embodiments is the aminoglycoside class of antibiotics, which are reviewed in Edson RS, Terrell CL. *The aminoglycosides. Mayo Clin Proc.* 1999 May; 74(5):519-28. This class of antibiotics inhibits bacterial growth by impairing bacterial protein synthesis, through binding and inactivation of bacterial ribosomal subunits. In addition to such bacteriostatic properties, aminoglycosides also exhibit bacteriocidal effects through disruption of cell walls in gram-negative bacteria.

Aminoglycoside antibiotics include gentamicin, amikacin, streptomycin, and others, and are generally regarded as useful in the treatment of gram-negative bacteria, mycobacteria and other microbial pathogens, although cases of resistant strains have been reported. The aminoglycosides are not absorbed through the digestive tract and so are not generally regarded as being amenable to oral formulations. Amikacin, for example, although often effective against gentamicin-resistant bacterial strains, is typically administered intravenously or intramuscularly, which can cause pain in the patient. Additionally, toxicities associated with aminoglycoside antibiotics such as amikacin can lead to kidney damage and/or irreversible hearing loss.

Despite these properties, certain embodiments disclosed herein contemplate oral administration of a synergizing BT/antibiotic combination (*e.g.,* where the antibiotic need not be limited to an aminoglycoside) for instance, for treatment of an epithelial tissue surface at one or more locations along the oral cavity, gastrointestinal tract/ alimentary canal. Also contemplated in certain other embodiments may be use of compositions and methods described herein as disinfectants, which refers to preparations that kill, or block the growth of, microbes on an external surface of an inanimate object.

As also described elsewhere herein, a BT compound may be a composition that comprises bismuth or a bismuth salt and a thiol- (*e.g.,* -SH, or sulfhydryl) containing compound, including those that are described (including their methods of preparation) in Domenico et al., 1997 *Antimicrob. Agent.*

Chemother. 41(8):1697-1703, Domenico et al., 2001 *Antimicob.Agent. Chemother.* 45(5):1417-1421, and in U.S. RE37,793, U.S. 6,248,371, U.S. 6,086,921, and U.S. 6,380,248; see also, *e.g.*, U.S. 6,582,719. Certain embodiments are not so limited, however, and may contemplate other BT compounds that comprise bismuth or a bismuth salt and a thiol-containing compound. The thiol-containing compound may contain one, two, three, four, five, six or more thiol (*e.g.*, -SH) groups. In preferred embodiments the BT compound comprises bismuth in association with the thiol-containing compound via ionic bonding and/or as a coordination complex, while in some other embodiments bismuth may be associated with the thiol-containing compound via covalent bonding such as may be found in an organometallic compound. Certain contemplated embodiments, however, expressly exclude a BT compound that is an organometallic compound such as a compound in which bismuth is found in covalent linkage to an organic moiety.

Exemplary BT compounds are shown in Table 1:

TABLE 1
Exemplary BT Compounds*

1)	CPD 1B-1 Bis-EDT (1:1) $BiC_2H_4S_2$
2)	CPD 1B-2 Bis-EDT (1:1.5) $BiC_3H_6S_3$
3)	CPD 1B-3 Bis-EDT (1:1.5) $BiC_3H_6S_3$
4)	CPD 1C Bis-EDT (1:1.5) $BiC_3H_6S_3$
5)	CPD 2A Bis-Bal (1:1) $BiC_3H_6S_2O$
6)	CPD 2B Bis-Bal (1:1.5) $BiC_{4.5}H_9O_{1.5}S_3$
7)	CPD 3A Bis-Pyr (1:1.5) $BiC_{7.5}H_6N_{1.5}O_{1.5}S_{1.5}$
8)	CPD 3B Bis-Pyr (1:3) $BiC_{15}H_{12}N_3O_3S_3$
9)	CPD 4 Bis-Ery (1:1.5) $BiC_6H_{12}O_3S_3$
10)	CPD 5 Bis-Tol (1:1.5) $BiC_{10.5}H_9S_3$
11)	CPD 6 Bis-BDT (1:1.5) $BiC_6H_{12}S_3$
12)	CPD 7 Bis-PDT (1:1.5) $BiC_{4.5}H_9S_3$
13)	CPD 8-1 Bis-Pyr/BDT (1:1/1)
14)	CPD 8-2 Bis-Pyr/BDT (1:1/0.5)
15)	CPD 9 Bis-2hydroxy, propane thiol (1:3)
16)	CPD 10 Bis-Pyr/Bal (1:1/0.5)
17)	CPD 11 Bis-Pyr/EDT (1:1/0.5)

18)	CPD 12 Bis-Pyr/Tol (1:1/0.5)
19)	CPD 13 Bis-Pyr/PDT (1:1/0.5)
20)	CPD 14 Bis-Pyr/Ery (1:1/0.5)
21)	CPD 15 Bis-EDT/2hydroxy, propane thiol (1:1/1)

*Shown are atomic ratios relative to a single bismuth atom, for comparison, based on the stoichiometric ratios of the reactants used and the known propensity of bismuth to form trivalent complexes with sulfur containing compounds. Atomic ratios as shown may not be accurate molecular formulae for all species in a given preparation. The numbers in parenthesis are the ratios of bismuth to one (or more) thiol agents. (*e.g.* Bi:thiol1/thiol2) "CPD", compound.

BT compounds for use in certain of the presently disclosed embodiments may be prepared according to established procedures (*e.g.,* U.S. RE37,793, U.S. 6,248,371, U.S. 6,086,921, and U.S. 6,380,248; Domenico et al., 1997 *Antimicrob. Agent. Chemother.* 41(8):1697-1703, Domenico et al., 2001 *Antimicob.Agent. Chemother.* 45(5):1417-1421) and in certain other embodiments BT compounds may also be prepared according to methodologies described herein. Certain preferred embodiments thus contemplate the herein described synthetic methods for preparing BT compounds, and in particular for obtaining BT compounds in substantially monodisperse microparticulate form, in which an acidic aqueous bismuth solution that contains dissolved bismuth at a concentration of at least 50 mM, at least 100 mM, at least 150 mM, at least 200 mM, at least 250 mM, at least 300 mM, at least 350 mM, at least 400 mM, at least 500 mM, at least 600 mM, at least 700 mM, at least 800 mM, at least 900 mM or at least 1 M and that lacks a hydrophilic, polar or organic solubilizer is admixed with ethanol to obtain a first ethanolic solution, which is reacted with a second ethanolic solution comprising a thiol-containing compound to obtain a reaction solution, wherein the thiol-containing compound is present in the reaction solution at a molar ratio of from about 1:3 to about 3:1 relative to the bismuth, under conditions and for a time sufficient for formation of a precipitate which comprises the microparticles comprising the BT compound (such as the conditions of concentration, solvent strength, temperature, pH, mixing and/or pressure, and the like, as described herein and as will be appreciated by the skilled person based on the present disclosure).

Accordingly, exemplary BTs include compound 1B-1, Bis-EDT (bismuth-1,2-ethane dithiol, reactants at 1:1); compound 1B-2, Bis-EDT (1:1.5); compound 1B-3, Bis-EDT (1:1.5); compound 1C, Bis-EDT (soluble Bi

preparation, 1:1.5); compound 2A, Bis-Bal (bismuth-British anti-Lewisite (bismuth-dimercaprol, bismuth-2,3-dimercaptopropanol), 1:1); compound 2B, Bis-Bal (1:1.5); compound 3A Bis-Pyr (bismuth-pyrithione, 1:1.5); compound 3B Bis-Pyr (1:3); compound 4, Bis-Ery (bismuth-dithioerythritol, 1:1.5); compound 5, Bis-Tol (bismuth-3,4-dimercaptotoluene, 1:1.5); compound 6, Bis-BDT (bismuth-2,3-butanedithiol, 1:1.5); compound 7, Bis-PDT (bismuth-1,3-propanedithiol, 1:1.5); compound 8-1 Bis-Pyr/BDT (1:1/1); compound 8-2, Bis-Pyr/BDT (1:1/0.5); compound 9, Bis-2-hydroxy, propane thiol (bismuth-1-mercapto-2-propanol, 1:3); compound 10, Bis-Pyr/Bal (1:1/0.5); compound 11, Bis-Pyr/EDT (1:1/0.5); compound 12 Bis-Pyr/Tol (1:1/0.5); compound 13, Bis-Pyr/PDT (1:1/0.5); compound 14 Bis-Pyr/Ery (1:1/0.5); compound 15, Bis-EDT/2-hydroxy, propane thiol (1:1/1) (see, *e.g.,* Table 1).

Without wishing to be bound by theory, it is believed that the presently disclosed methods of preparing a BT compound, which in certain preferred embodiments may comprise preparing or obtaining an acidic aqueous liquid solution that comprises bismuth such as an aqueous nitric acid solution comprising bismuth nitrate, may desirably yield compositions comprising BT compounds where such compositions have one or more desirable properties, including ease of large-scale production, improved product purity, uniformity or consistency (including uniformity in particle size), or other properties useful in the preparation and/or administration of the present topical formulations.

In particular embodiments it has been discovered that BT compositions, prepared according to the methods described herein for the first time, exhibit an advantageous degree of homogeneity with respect to their occurrence as a substantially monodisperse suspension of microparticles each having a volumetric mean diameter (VMD) according to certain presently preferred embodiments of from about 0.4 μm to about 5 μm. Measures of particle size can be referred to as volumetric mean diameter (VMD), mass median diameter (MMD), or mass median aerodynamic diameter (MMAD). These measurements may be made, for example, by impaction (MMD and MMAD) or by laser (VMD) characterization. For liquid particles, VMD, MMD and MMAD may be the same if environmental conditions are maintained, *e.g.*, standard humidity. However, if humidity is not maintained, MMD and MMAD determinations will be smaller than VMD due to dehydration during impactor measurements. For the purposes of this description, VMD, MMD and MMAD measurements are considered to be under standard conditions such that descriptions of VMD, MMD and MMAD will be comparable. Similarly, dry

powder particle size determinations in MMD, and MMAD are also considered comparable.

As described herein, preferred embodiments relate to a substantially monodisperse suspension of BT-containing microparticles. Generation of a defined BT particle size with limited geometric standard deviation (GSD) may, for instance, optimize BT deposition, accessibility to desired target sites in or on a natural or artificial surface, and/or tolerability by a subject to whom the BT microparticles are administered. Narrow GSD limits the number of particles outside the desired VMD or MMAD size range.

In one embodiment, a liquid or aerosol suspension of microparticles containing one or more BT compounds disclosed herein is provided having a VMD from about 0.5 microns to about 5 microns. In another embodiment, a liquid or aerosol suspension having a VMD or MMAD from about 0.7 microns to about 4.0 microns is provided. In another embodiment, a liquid or aerosol suspension having aVMD or MMAD from about 1.0 micron to about 3.0 microns is provided. In certain other preferred embodiments there is provided a liquid suspension comprising one or a plurality of BT compound particles of from about 0.1 to about 5.0 microns VMD, or of from about 0.1, about 0.2, about 0.3, about 0.4, about 0.5, about 0.6, about 0.7, about 0.8 or about 0.9 microns to about 1.0, about 1.5, about 2.0, about 2.5, about 3.0, about 3.5, about 4.0, about 4.5, about 5.0, about 5.5, about 6.0, about 6.5, about 7.0, about 7.5 or about 8.0 microns, the particle comprising a BT compound prepared as described herein.

Accordingly and in certain preferred embodiments, a BT preparation described for the first time herein which is "substantially" monodisperse, for example, a BT composition that comprises a BT compound in microparticulate form wherein "substantially" all of the microparticles have a volumetric mean diameter (VMD) within a specified range (*e.g.*, from about 0.4 μm to about 5 μm), includes those compositions in which at least 80%, 85%, 90%, 91%, 92%, 93%, or 94%, more preferably at least 95%, 96%, 97%, 98%, 99% or more of the particles have a VMD that is within the recited size range.

These and related properties of BT compositions prepared according to the herein described synthetic methods offer unprecedented advantages over previously described BTs, including lower cost and ease of production, and uniformity within the composition that may permit its characterization in a manner that facilitates regulatory compliance according to one or more of pharmaceutical, formulary and cosmeceutical standards.

Additionally or alternatively, the herein described substantially monodisperse BT microparticles may advantageously be produced without the need for micronization, *i.e.,* without the expensive and labor-intensive milling or supercritical fluid processing or other equipment and procedures that are typically used to generate microparticles (*e.g.*, Martin et al. 2008 *Adv. Drug Deliv. Rev.* 60(3):339; Moribe et al., 2008 *Adv. Drug Deliv. Rev.* 60(3):328; Cape et al., 2008 *Pharm. Res.* 25(9):1967; Rasenack et al. 2004 *Pharm. Dev. Technol.* 9(1):1-13). Hence, the present embodiments offer beneficial effects of substantially uniform microparticulate preparations, including without limitation enhanced and substantially uniform solubilization properties, suitability for desired administration forms such as oral, inhaled or dermatological/ skin wound topical forms, increased bioavailability and other beneficial properties.

The BT compound microparticulate suspension can be administered as aqueous formulations, as suspensions or solutions in aqueous as well as organic solvents including halogenated hydrocarbon propellants, as dry powders, or in other forms as elaborated below, including preparations that contain wetting agents, surfactants, mineral oil or other ingredients or additives as may be known to those familiar with formulary, for example, to maintain individual microparticles in suspension. Aqueous formulations may be aerosolized by liquid nebulizers employing, for instance, either hydraulic or ultrasonic atomization. Propellant-based systems may use suitable pressurized dispensers. Dry powders may use dry powder dispersion devices, which are capable of dispersing the BT-containing microparticles effectively. A desired particle size and distribution may be obtained by choosing an appropriate device.

As also noted above, also provided herein according to certain embodiments is a method for preparing a bismuth-thiol (BT) composition that comprises a plurality of microparticles that comprise a BT compound, substantially all of such microparticles having a volumetric mean diameter (VMD) of from about 0.1 to about 8 microns, and in certain preferred embodiments from about 0.4 microns to about 5 microns.

In general terms, the method comprises the steps of (a) admixing, under conditions and for a time sufficient to obtain a solution that is substantially free of a solid precipitate, (i) an acidic aqueous solution that comprises a bismuth salt comprising bismuth at a concentration of at least 50 mM and that lacks a hydrophilic, polar or organic solubilizer, with (ii) ethanol in an amount sufficient to obtain an admixture that comprises at least about 5%,

10%, 15%, 20%, 25% or 30%, and preferably about 25% ethanol by volume; and (b) adding to the admixture of (a) an ethanolic solution comprising a thiol-containing compound to obtain a reaction solution, wherein the thiol-containing compound is present in the reaction solution at a molar ratio of from about 1:3 to about 3:1 relative to the bismuth, under conditions and for a time sufficient for formation of a precipitate which comprises the BT compound.

In certain preferred embodiments the bismuth salt may be $Bi(NO_3)_3$, but it will be appreciated according to the present disclosure that bismuth may also be provided in other forms. In certain embodiments the bismuth concentration in the acidic aqueous solution may be at least 100 mM, at least 150 mM, at least 200 mM, at least 250 mM, at least 300 mM, at least 350 mM, at least 400 mM, at least 500 mM, at least 600 mM, at least 700 mM, at least 800 mM, at least 900 mM or at least 1 M. In certain embodiments the acidic aqueous solution comprises at least 5%, 10%, 15%, 20%, 22% or 22.5% bismuth by weight. The acidic aqueous solution may in certain preferred embodiments comprise at least 5% or more nitric acid by weight, and in certain other embodiments the acidic aqueous solution may comprise at least 0.5%, at least 1%, at least 1.5%, at least 2%, at least 2.5%, at least 3%, at least 3.5%, at least 4%, at least 4.5% or at least 5% nitric acid by weight.

The thiol-containing compound may be any thiol-containing compound as described herein, and in certain embodiments may comprise one or more of 1,2-ethane dithiol, 2,3-dimercaptopropanol, pyrithione, dithioerythritol, 3,4-dimercaptotoluene, 2,3-butanedithiol, 1,3-propanedithiol, 2-hydroxypropane thiol, 1-mercapto-2-propanol, dithioerythritol and dithiothreitol. Other exemplary thiol-containing compounds include alpha-lipoic acid, methanethiol (CH_3SH [m-mercaptan]), ethanethiol (C_2H_5SH [e- mercaptan]), 1-propanethiol (C_3H_7SH [n-P mercaptan]), 2-Propanethiol ($CH_3CH(SH)CH_3$ [$2C_3$ mercaptan]), butanethiol (C_4H_9SH ([n-butyl mercaptan]), tert-butyl mercaptan ($C(CH_3)_3SH$ [t-butyl mercaptan]), pentanethiols ($C_5H_{11}SH$ [pentyl mercaptan]), coenzyme A, lipoamide, glutathione, cysteine, cystine, 2-mercaptoethanol, dithiothreitol, dithioerythritol, 2-mercaptoindole, transglutaminase and any of the following thiol compounds available from Sigma-Aldrich (St. Louis, MO): (11-mercaptoundecyl)hexa(ethylene glycol), (11-mercaptoundecyl)tetra(ethylene glycol), (11-mercaptoundecyl)tetra(ethylene glycol) functionalized gold nanoparticles, 1,1′,4′,1″-terphenyl-4-thiol, 1,11-undecanedithiol, 1,16-hexadecanedithiol, 1,2-ethanedithiol technical grade, 1,3-propanedithiol, 1,4-benzenedimethanethiol, 1,4-butanedithiol, 1,4-butanedithiol diacetate, 1,5-

pentanedithiol, 1,6-hexanedithiol, 1,8-octanedithiol, 1,9-nonanedithiol, adamantanethiol, 1-butanethiol, 1-decanethiol, 1-dodecanethiol, 1-heptanethiol, 1-heptanethiol purum, 1-hexadecanethiol, 1-hexanethiol, 1-mercapto-(triethylene glycol), 1-mercapto-(triethylene glycol) methyl ether functionalized gold nanoparticles, 1-mercapto-2-propanol, 1-nonanethiol, 1-octadecanethiol, 1-octanethiol, 1-octanethiol, 1-pentadecanethiol, 1-pentanethiol, 1-propanethiol, 1-tetradecanethiol, 1-tetradecanethiol purum, 1-undecanethiol, 11-(1*H*-pyrrol-1-yl)undecane-1-thiol, 11-amino-1-undecanethiol hydrochloride, 11-bromo-1-undecanethiol, 11-mercapto-1-undecanol, 11-mercapto-1-undecanol, 11-mercaptoundecanoic acid, 11-mercaptoundecanoic acid, 11-mercaptoundecyl trifluoroacetate, 11-mercaptoundecylphosphoric acid, 12-mercaptododecanoic acid, 12-mercaptododecanoic acid, 15-mercaptopentadecanoic acid, 16-mercaptohexadecanoic acid, 16-mercaptohexadecanoic acid, 1*H*,1*H*,2*H*,2*H*-perfluorodecanethiol, 2,2′-(ethylenedioxy)diethanethiol, 2,3-butanedithiol, 2-butanethiol, 2-ethylhexanethiol, 2-methyl-1-propanethiol, 2-methyl-2-propanethiol, 2-phenylethanethiol, 3,3,4,4,5,5,6,6,6-nonafluoro-1-hexanethiol purum, 3-(dimethoxymethylsilyl)-1-propanethiol, 3-chloro-1-propanethiol, 3-mercapto-1-propanol, 3-mercapto-2-butanol, 3-mercapto-*N*-nonylpropionamide, 3-mercaptopropionic acid, 3-mercaptopropyl-functionalized silica gel, 3-methyl-1-butanethiol, 4,4′-bis(mercaptomethyl)biphenyl, 4,4′-dimercaptostilbene, 4-(6-mercaptohexyloxy)benzyl alcohol, 4-cyano-1-butanethiol, 4-mercapto-1-butanol, 6-(ferrocenyl)hexanethiol, 6-mercapto-1-hexanol, 6-mercaptohexanoic acid, 8-mercapto-1-octanol, 8-mercaptooctanoic acid, 9-mercapto-1-nonanol, biphenyl-4,4′-dithiol, butyl 3-mercaptopropionate, copper(I) 1-butanethiolate, cyclohexanethiol, cyclopentanethiol, decanethiol functionalized silver nanoparticles, dodecanethiol functionalized gold nanoparticles, dodecanethiol functionalized silver nanoparticles, hexa(ethylene glycol)mono-11-(acetylthio)undecyl ether, mercaptosuccinic acid, methyl 3-mercaptopropionate, nanoTether BPA-HH, NanoThinks™ 18, NanoThinks™ 8, NanoThinks™ ACID11, NanoThinks™ ACID16, NanoThinks™ ALCO11, NanoThinks™ THIO8, octanethiol functionalized gold nanoparticles, PEG dithiol average M_n 8,000, PEG dithiol average mol wt 1,500, PEG dithiol average mol wt 3,400, *S*-(11-bromoundecyl)thioacetate, *S*-(4-cyanobutyl)thioacetate, thiophenol, triethylene glycol mono-11-mercaptoundecyl ether, trimethylolpropane tris(3-mercaptopropionate), [11-

(methylcarbonylthio)undecyl]tetra(ethylene glycol), *m*-carborane-9-thiol, *p*-terphenyl-4,4″-dithiol, *tert*-dodecylmercaptan, and *tert*-nonyl mercaptan.

Exemplary reaction conditions, including temperature, pH, reaction time, the use of stirring or agitation to dissolve solutes and procedures for collecting and washing precipitates, are described herein and employ techniques generally known in the art.

Unlike previously described methodologies for producing BT compounds, according to the present methods for preparing BT, BT products are provided as microparticulate suspensions having substantially all microparticles with VMD from about 0.4 to about 5 microns in certain preferred embodiments, and generally from about 0.1 microns to about 8 microns according to certain other embodiments. Further unlike previous approaches, according to the instant embodiments bismuth is provided in an acidic aqueous solution that comprises a bismuth salt at a concentration of from at least about 50 mM to about 1 M, and nitric acid in an amount from at least about 0.5% to about 5% (w/w), and preferably less than 5% (weight/weight), and that lacks a hydrophilic, polar or organic solubilizer.

In this regard the present methods offer surprising and unexpected advantages in view of generally accepted art teachings that bismuth is not water soluble at 50 μM (*e.g.,* U.S. RE37793), that bismuth is unstable in water (*e.g.*, Kuvshinova et al., 2009 *Russ. J Inorg. Chem* 54(11):1816), and that bismuth is unstable even in nitric acid solutions unless a hydrophilic, polar or organic solubilizer is present. For example, in all of the definitive descriptions of BT preparation methodologies (*e.g.,* Domenico et al., 1997 *Antimicrob. Agents. Chemother.* 41:1697; U.S. 6,380,248; U.S. RE37793; U.S. 6,248,371), the hydrophilic solubilizing agent propylene glycol is required to dissolve bismuth nitrate, and the bismuth concentration of solutions prepared for reaction with thiols is well below 15 mM, thereby limiting the available production modalities for BT compounds.

By contrast, according to the present disclosure there is no requirement for a hydrophilic, polar or organic solubilizer in order dissolve bismuth, yet higher concentrations are surprisingly achieved. Hydrophilic, polar or organic solubilizers include propylene glycol (PG) and ethylene glycol (EG) and may also include any of a large number of known solubility enhancers, including polar solvents such as dioxane and dimethylsulfoxide (DMSO), polyols (including, *e.g.,* PG and EG and also including polyethylene glycol (PEG), polypropyleneglycol (PPG), pentaerythritol and others), polyhydric

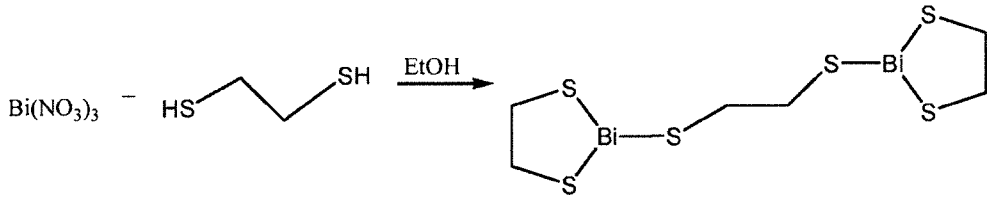
alchohols such as glycerol and mannitol, and other agents. Other water-miscible organic of high polarity include dimethylsulfoxide (DMSO), dimethylformamide (DMF) and NMP (N-methyl-2-pyrrolidone).

Thus, it will be appreciated by those familiar with the art that solvents, including those commonly used as hydrophilic, polar or organic solubilizers as provided herein, may be selected, for instance, based on the solvent polarity/ polarizability (SPP) scale value using the system of Catalan *et al.* (*e.g.,* 1995 *Liebigs Ann.* 241; see also Catalan, 2001 *In:* Handbook of Solvents, Wypych (Ed.), Andrew Publ., NY, and references cited therein), according to which, for example, water has a SPP value of 0.962, toluene a SPP value of 0.655, and 2-propanol a SPP value of 0.848. Methods for determining the SPP value of a solvent based on ultraviolet measurements of the 2-N,N-dimethyl-7-nitrofluorene/ 2-fluoro-7-nitrofluorene probe/ homomorph pair have been described (Catalan *et al.*, 1995).

Solvents with desired SPP values (whether as pure single-component solvents or as solvent mixtures of two, three, four or more solvents; for solvent miscibility see, *e.g.*, Godfrey 1972 *Chem. Technol.* 2:359) based on the solubility properties of a particular BT composition can be readily identified by those having familiarity with the art in view of the instant disclosure, although as noted above, according to certain preferred embodiments regarding the herein described synthetic method steps, no hydrophilic, polar or organic solubilizer is required in order dissolve bismuth.

Solubility parameters may also include the interaction parameter C, Hildebrand solubility parameter d, or partial (Hansen) solubility parameters: δp, δh and δd, describing the solvent's polarity, hydrogen bonding potential and dispersion force interaction potential, respectively. In certain embodiments, the highest value for a solubility parameter that describes a solvent or co-solvent system in which the bismuth salt comprising bismuth will dissolve may provide a limitation for the aqueous solution that comprises the bismuth salt, for instance, according to the presently described method for preparing a microparticulate BT composition. For example, higher δh values will have a greater hydrogen bonding ability and would therefore have a greater affinity for solvent molecules such as water. A higher value of maximum observed δh for a solvent may therefore be preferred for situations where a more hydrophilic environment is desired.

By way of non-limiting example, BisEDT having the structure shown below in formula **I** may be prepared according to the following reaction scheme:



(I)

Briefly, and as a non-limiting illustrative example, to an excess (11.4 L) of 5% aqueous HNO_3 at room temperature may be slowly added 0.331 L (about 0.575 moles) of an aqueous acidic bismuth solution such as a $Bi(NO_3)_3$ solution (*e.g.*, 43% $Bi(NO_3)_3$ (w/w), 5% nitric acid (w/w), 52% water (w/w), available from Shepherd Chemical Co., Cincinnati, OH) with stirring, followed by slow addition of absolute ethanol (4 L). An ethanolic solution (1.56 L) of a thiol compound such as 1,2-ethanedithiol [~0.55 M] may be separately prepared by adding, to 1.5 L of absolute ethanol, 72.19 mL (0.863 moles) of 1,2-ethanedithiol using a 60 mL syringe, and then stirring for five minutes. 1,2-ethanedithiol (CAS 540-63-6) and other thiol compounds are available from, *e.g.,* Sigma-Aldrich, St. Louis, MO. The ethanolic solution of the thiol compound may then be slowly added to the aqueous $Bi(NO_3)_3$ / HNO_3 solution with stirring overnight to form a reaction solution. The thiol-containing compound may be present in the reaction solution, according to certain preferred embodiments, at a molar ratio of from about 1:3 to about 3:1 relative to the bismuth. The formed product is allowed to settle as a precipitate comprising microparticles as described herein, which is then collected by filtration and washed sequentially with ethanol, water and acetone to obtain BisEDT as a yellow amorphous powdered solid. The crude product may be redissolved in absolute ethanol with stirring, then filtered and washed sequentially with ethanol several times followed by acetone several times. The washed powder may be triturated in 1M NaOH (500mL), filtered and washed sequentially with water, ethanol and acetone to afford purified microparticulate BisEDT.

According to non-limiting theory, bismuth inhibits the ability of bacteria to produce extracellular polymeric substances (EPS) such as bacterial exopolysaccharides, and this inhibition leads to impaired biofilm formation.

Bacteria are believed to employ the glue-like EPS for biofilm cohesion. Depending on the nature of an infection, biofilm formation and elaboration of EPS may contribute to bacterial pathogenicity such as interference with wound healing. However, bismuth alone is not therapeutically useful as an intervention agent, and is instead typically administered as part of a complex such as a BT. Bismuth-thiols (BTs) are thus a family of compositions that includes compounds that result from the chelation of bismuth with a thiol compound, and that exhibit dramatic improvement in the antimicrobial therapeutic efficacy of bismuth. BTs exhibit remarkable anti-infective, anti-biofilm, and immunomodulatory effects. Bismuth thiols are effective against a broad-spectrum of microorganisms, and are typically not affected by antibiotic-resistance. BTs prevent biofilm formation at remarkably low (sub-inhibitory) concentrations, prevent many pathogenic characteristics of common wound pathogens at those same sub-inhibitory levels, can prevent septic shock in animal models, and may be synergistic with many antibiotics.

As described herein, such synergy in the antibacterial effects of one or more specified BT when combined with one or more specified antibiotic compound is not readily predictable based on profiles of separate antibiotic and BT effects against a particular bacterial type, but surprisingly may result from selection of particular BT-antibiotic combinations in view of the specific bacterial population, including identification of whether gram-negative or gram-positive (or both) bacteria are present. For instance, as disclosed herein, antibiotics that synergize with certain BTs may include one or more of amikacin, ampicillin, aztreonam, cefazolin, cefepime, chloramphenicol, ciprofloxacin, clindamycin (or other lincosamide antibiotics), daptomycin (Cubicin®), doxycycline, gatifloxacin, gentamicin, imipenim, levofloxacin, linezolid (Zyvox®), minocycline, nafcilin, paromomycin, rifampin, sulphamethoxazole, tetracycline, tobramycin and vancomycin. In vitro studies showed, for example, that MRSA, which was poorly or not at all susceptible to gentamicin, cefazolin, cefepime, suphamethoxazole, imipenim or levofloxacin individually, exhibited marked sensitivity to any one of these antibiotics if exposed to the antibiotic in the presence of the BT compound BisEDT. Certain embodiments contemplated herein thus expressly contemplate compositions and/or methods in which may be included the combination of a BT compound and one or more antibiotics selected from amikacin, ampicillin, cefazolin, cefepime, chloramphenicol, ciprofloxacin, clindamycin (or another lincosamide antibiotic), daptomycin (Cubicin®), doxycycline, gatifloxacin, gentamicin, imipenim, levofloxacin,

linezolid (Zyvox®), minocycline, nafcilin, paromomycin, rifampin, sulphamethoxazole, tobramycin and vancomycin, whilst certain other embodiments contemplated herein contemplate compositions and/or methods in which may be included the combination of a BT compound and one or more antibiotics from which expressly excluded may be one or more antibiotic selected from amikacin, ampicillin, cefazolin, cefepime, chloramphenicol, ciprofloxacin, clindamycin (or other lincosamides), daptomycin (Cubicin®), doxycycline, gatifloxacin, gentamicin, imipenim, levofloxacin, linezolid (Zyvox®), minocycline, nafcilin, paromomycin, rifampin, sulphamethoxazole, tobramycin and vancomycin. It is noted in this context that gentamicin and tobramycin belong to the aminoglycoside class of antibiotics. Also expressly excluded from certain contemplated embodiments are certain compositions and methods described in Domenico et al., 2001 *Agents Chemother.* 45:1417-1421; Domenico et al., 2000 *Infect. Med.* 17:123-127; Domenico et al., 2003 *Res. Adv. In Antimicrob. Agents & Chemother.* 3:79-85; Domenico et al., 1997 *Antimicrob. Agents Chemother.* 41(8):1697-1703; Domenico et al., 1999 *Infect. Immun.* 67:664-669: Huang et al. 1999 *J Antimicrob. Chemother.* 44:601-605; Veloira et al., 2003 *J Antimicrob. Chemother.* 52:915-919; Wu et al., 2002 *Am J Respir Cell Mol Biol.* 26:731-738; Halwani et al., 2008 *Int. J Pharmaceut.* 358:278; Halwani et al., 2009 *Int. J. Pharmaceut.* 373:141-146; where it will be noted that none of these publications teach or suggest the mondisperse microparticulate BT compositions that are disclosed herein.

Accordingly and as described herein, in certain preferred embodiments there are provided compositions and methods for treating a plant, animal or human subject, or an article of manufacture, with a composition that comprises the herein described microparticulate BT and that optionally and in certain other embodiments also comprises a synergizing and/or an enhancing antibiotic. Persons familiar with the relevant art will, based on the present disclosure, recognize appropriate agricultural, clinical, commercial, industrial, manufacturing, domestic and other contexts and situations in which such treatment may be desired, criteria for which are established in the medical arts, including *inter alia*, *e.g.*, surgical, military surgical, dermatological, trauma medicine, gerontological, cardiovascular, metabolic diseases (*e.g.*, diabetes, obesity, etc.), infection and inflammation (including in the epithelial linings of the respiratory tract or the gastrointestinal tract, or other epithelial tissue surfaces such as in glandular tissues), and other relevant medical specialties and subspecialities.

Preferred compositions for treating a microbial infection on or in a natural or artificial surface for use according to the embodiments described herein, may include in certain embodiments compositions that comprise bismuth-thiol (BT) compounds as described herein, and which may in certain distinct but related embodiments also include other compounds that are known in the art such as one or more antibiotic compounds as described herein. BT compounds and methods for making them are disclosed herein and are also disclosed, for example, in Domenico et al. (1997 *Antimicrob. Agent. Chemother.* 41(8):1697-1703; 2001 *Antimicrob. Agent. Chemother.* 45(5)1417-1421) and in U.S. RE37,793, U.S. 6,248,371, U.S. 6,086,921, and U.S. 6,380,248. As also noted above, certain preferred BT compounds are those that contain bismuth or a bismuth salt ionically bonded to, or in a coordination complex with, a thiol-containing compound, such as a composition that comprises bismuth chelated to the thiol-containing compound, and certain other preferred BT compounds are those that contain bismuth or a bismuth salt in covalent bond linkage to the thiol-containing compound. Also preferred are substantially monodisperse microparticulate BT compositions as described herein. Neither from previous efforts to treat bacterial infections, nor from previous characterization in other contexts of any compounds described herein for the first time as having use in compositions and methods for promoting the herein described treatment of natural and/or artificial surfaces, could it be predicted that the present methods of using such compounds would have the herein described beneficial effects.

According to preferred embodiments there are thus provided methods for treating a natural or artificial surface, comprising administering to the surface at least one microparticulate BT compound as described herein. In certain embodiments the method further comprises administering, simultaneously or sequentially and in either order, at least one antibiotic compound, which in certain preferred embodiments may be a synergizing antibiotic as described herein, and which in certain other preferred embodiments may be an enhancing antibiotic as described herein. The antibiotic compound may be an aminoglycoside antibiotic, a carbapenem antibiotic, a cephalosporin antibiotic, a fluoroquinolone antibiotic, a glycopeptides antibiotic, a lincosamide antibiotic, a penicillinase-resistant penicillin antibiotic, or an aminopenicillin antibiotic. Clinically useful antibiotics are discussed elsewhere herein and are also described in, *e.g.*, Washington University School of Medicine, *The Washington Manual of Medical Therapeutics* (32nd Ed.), 2007 Lippincott, Williams and Wilkins, Philadelphia,

PA; and in Hauser, AL, *Antibiotic Basics for Clinicians*, 2007 Lippincott, Williams and Wilkins, Philadelphia, PA.

As described herein, certain embodiments derive from the unpredictable discovery that for a bacterial infection that comprises gram positive bacteria, a preferred therapeutically effective formulation may comprise a BT compound (*e.g.*, BisEDT, bismuth:1,2-ethanedithiol; BisPyr, bismuth:pyrithione; BisEDT/Pyr, bismuth:1,2-ethanedithiol/pyrithione) and rifamycin, or a BT compound and daptomycin (Cubicin®, Cubist Pharmaceuticals, Lexington, MA), or a BT compound and linezolid (Zyvox®, Pfizer, Inc., NY, NY), or a BT compound (*e.g.*, BisEDT, bismuth:1,2-ethanedithiol; BisPyr, bismuth:pyrithione; BisEDT/Pyr, bismuth:1,2-ethanedithiol/pyrithione) and one or more of ampicillin, cefazolin, cefepime, chloramphenicol, clindamycin (or another lincosamide antibiotic), daptomycin (Cubicin®), doxycycline, gatifloxacin, gentamicin, imipenim, levofloxacin, linezolid (Zyvox®), nafcilin, paromomycin, rifampin, sulphamethoxazole, tobramycin and vancomycin.

As also described herein, certain embodiments derive from the unpredictable discovery that for a bacterial infection that comprises gram negative bacteria, a preferred therapeutically effective formulation may comprise a BT compound and amikacin. Certain related embodiments contemplate treatment of an infection comprising gram negative bacteria with a BT compound and another antibiotic, such as another aminoglycoside antibiotic, which in certain embodiments is not gentamicin or tobramycin. Accordingly and in view of these embodiments, other related embodiments contemplate identifying one or more bacterial populations or subpopulations in or on a natural or artificial surface by the well known criterion of being gram positive or gram negative, according to methodologies that are familiar to those skilled in the medical microbiology art, as a step for selecting appropriate antibiotic compound(s) to include in a formulation to be administered according to the present methods.

The presently described compositions and methods may find use in the treatment of microbes (*e.g.*, bacteria, viruses, yeast, molds and other fungi, microbial parasites, etc.) in a wide variety of contexts, typically by application or administration of the herein described compounds (*e.g.*, one or more microparticulate BTs alone or in combination with one or more synergizing and/or enhancing antibiotics as disclosed herein) to a microbial site such as a microbial presence on or in a natural or artificial surface. Such

natural surfaces include but are not limited to surfaces found on plants (*e.g.,* all or a portion of a surface of a root, bulb, stem, leaf, branch, vine, runner, bud, flower or a part thereof, greentip, fruit, seed, seed pod, or the like), mammalian tissues (*e.g.,* epithelia including skin, scalp, gastrointestinal tract lining, buccal cavity, etc.; endothelia, cell and tissue membranes such as peritoneal membrane, pericardial membrane, pleural membrane, periosteal membrane, meningeal membranes, sarcolemal membranes, and the like; cornea, sclera, mucous membranes, etc.; and other mammalian tissues such as muscle, heart, lung, kidney, liver, spleen, gall bladder, pancreas, bladder, nerve, teeth, bone, joint, tendon, ligament, etc.) and can also include any site on an article of manufacture where a microbial presence may be found (*e.g.*, commercial, residential, industrial, educational, health care and other institutional building walls, windows, floors, crawlspaces, attics, basements, fences, roofs, ceilings, light and plumbing fixtures, vents, ducts, conduits, doorknobs, switches, sanitation systems, drains, cisterns, water lines; medical and dental devices, implants, tools, instruments, equipment and the like; metal, glass, plastic, wood, rubber and paper goods; transportation equipment including shipping containers, automobiles, railroad equipment, boats, ships (*e.g.*, exterior hull, rudder, anchor and/or propeller surfaces, interior holds and ballast tank and other interior surfaces), barges and other maritime equipment including docks, bulkheads, piers and the like; *etc.*).

The microparticulate antimicrobial agents described herein may be used to suppress microbial growth, reduce microbial infestation, treat products including natural and/or artificial surfaces to improve product resistance to microbial infestation, reduce biofilm, prevent conversion of bacteria to biofilm, prevent or inhibit microbial infection, prevent spoilage, and any other use described herein. These agents are also useful for a number of antiviral purposes, including prevention or inhibition of viral infection by herpes family viruses such as cytomegalovirus, herpes simplex virus Type 1, and herpes simplex virus Type 2, and/or infection by other viruses. In this regard, the agents are useful for the prevention or inhibition of viral infection by a variety of viruses, such as, single stranded RNA viruses, single stranded DNA viruses, Rous sarcoma virus (RSV), hepatitis A virus, hepatitis B virus (HBV), Hepatitis C (HCV), Influenza viruses, west nile virus (WNV), Epstein-Barr virus (EBV), eastern equine encephalitis virus (EEEV), severe acute respiratory virus (SARS), human immunodeficiency virus (HIV), human papilloma virus (HPV), and human T cell lymphoma virus (HTLV),and also including viruses that are

known as plant pathogens (*e.g.*, potato leaf roll virus; potato virus A, M, S, X, or Y; tomato spotted wilt virus; grapevine leaf roll-associated virus 3; plum pox virus; lettuce mosaic virus; pepino mosaic virus; pepper mild mottle virus; tomato mosaic virus; tobacco mosaic virus; Calibrachoa mottle virus; Impatiens necrotic spot virus; etc.).

Other internal and external pharmaceutical uses of the herein described antimicrobial agents include, but are not limited to, treatment or prevention of bacterial infection, of tuberculosis, of fungal infections such as yeast and mold infections (for example, *Candida* (*e.g.*, *Candida albicans, Candida glabrata, C. parapsilosis*, *C. tropicalis*, and *C. dubliniensis*) or *Cryptococcus* or other fungi), of *Helicobacter pylori* infection, and of peptic ulcer disease. In one embodiment, the agent is used at a dosage not generally lethal to bacteria but which is nonetheless sufficient to reduce protective polysaccharide coatings that would otherwise resist natural immune response. This technique is thus believed to aid immune system-mediated eradication of bacterial infection without harming human symbiotic microorganisms (*e.g.*, normal intestinal flora and the like) to the extent that may be the case with antibiotics. By way of illustration and not limitation, certain contemplated embodiments are now described.

<u>Microparticulate Bismuth-Thiols for Coating and Treating Water Lines.</u> In one embodiment, methods are provided herein for preventing and/or controlling (*i.e.*, slowing, retarding, inhibiting) biofilm development, disrupting a biofilm, or reducing the amount of biofilm on the interior or exterior surface of a water line (such as a water line used by dentists, dental hygienists, and other oral care specialists and caregivers), or other water delivery vehicle including a tube, pipe, faucet, water fountain, showerhead, or any other instrument or apparatus (*e.g.*, dental instruments including a high speed dental drill, air-water syringe, and cleaning apparatus or instrument (*e.g.*, Cavitron®)) that contacts or delivers water that is consumed by or applied to a human or non-human animal. These methods may also be useful for preventing, reducing, inhibiting, eliminating, or abrogating growth and division of bacteria, fungi, and/or protozoa in a water line or water delivery vehicle. These methods comprise applying, flushing, attaching, or adhering of microparticulate BT compound to a surface of a water line or water delivery vehicle.

Biofilms are microscopic communities that consist primarily of naturally occurring bacteria and fungi. The microorganisms form thin layers on surfaces, including dental water delivery systems and other water delivery

vehicles, such as showerheads, faucets and tubes. Water used as a coolant and irrigant during dental procedures can be heavily contaminated with microorganisms (*see, e.g.,* Environmental Protection Agency web site at epa.gov/safewater/mcl/html). Pathogenic microorganisms or opportunitistic pathogens that have been found in water from dental water lines and instruments include Actinomyces, Bacteroides, Bacillus, Cryptosporidium, *E. coli*, Flavobacterium, Klebsiella, Legionella, Moraxella, Mycobacterium, Peptostreptococcus, Pseudomonas, Staphylococcus, Streptococcus, and Veillonella. In addition, as a result of biofilm formation, Legionella spp. and protozoa can proliferate in the water line or water delivery vehicle. Bacteria from the biofilm and other microorganisms present in a water line or water delivery vehicle are continuously released as water flows through the line or vehicle. Patients and clinical staff are exposed to the microorganisms present in tiny droplets or fine mist sprayed out of the line or delivery vehicle.

For use and consumption of water in dental applications, the Center for Disease Control has recommended that the number of bacteria in water used as a coolant/irrigant for nonsurgical dental procedures should have an aerobic heterotrophic plate count (HPC) of ≤500 CFU/ml. The American Dental Association (ADA) has proposed a more stringent standard, recommending that water used in dental treatment contain a bacterial level of ≤200 CFU/ml. Measures taken to maintain low level of bacterial count in dental water systems include use of antimicrobial agents (*see, e.g.,* McDowell et al., *J. Am. Dent. Assoc.* 135:799-805 (2004)); hydrogen peroxide-based disinfectants (*see, e.g.,* Linger et al., *J. Am. Dent. Assoc.* 132:1287-91 (2001)); routine flushing of water lines before and after use; maintenance of water lines and delivery systems; use of filtering systems; use of chemicals as disinfectants (*e.g.,* diluted bleach 1:10, glutaraldehyde, food grade ethyl, alcohol, chlorhexidine-based products); thermal eradication; copper-silver ionization; chlorine dioxide; ultraviolet light; ozone; disinfectant combinations (*e.g.,* Adec® ICX (Adex, Newburg, OR): sodium percarbonate, silver nitrate, and cationic surfactants and silver ion catalyst.

An alternative antimicrobial that may be used for preventing and/or controlling (*i.e.,* slowing, retarding, inhibiting) biofilm development, disrupting a biofilm, or reducing the amount of biofilm on the interior or exterior surface of a water line or water delivery vehicle include the microparticulate BT compounds (or compositions comprising at least one microparticulate BT compound) described herein. Microparticulate BT compounds may be

introduced into water lines, water conduit systems, and water delivery vehicles manually or automatically as gels, sprays, pastes, liquids, or powders or other forms known to a person skilled in the art. In particular embodiments, a microparticulate BT compound, either in powder or liquid form is mixed with at least one or more additional ingredients, which may include at least one additional biologically active ingredient and/or a biologically inactive excipient, to formulate the product, which is delivered or injected periodically into the water line, water delivery vehicle, or water conduit system. Compositions may be prepared by a person skilled in the art using any number of methods known in the art. By way of example, a microparticulate BT compound in an anti-microbial effective amount may be combined with DMSO may be used. With routine use, a level of microparticulate BT compound that is sufficient to prevent biofilm formation is desired. However, in other embodiments, the level of microparticulate BT compound may be higher for reducing, removing, disrupting, or eliminating existing biofilms present in a water line, water delivery vehicle, or water conduit system.

A microparticulate BT compound may also be formulated to release slowly from the composition comprising the microparticulate BT compound applied to the water line, water delivery vehicle, or water conduit system. A microparticulate BT compound can also be incorporated into a coating, which can be applied to, adfixed to, adhered to, or in some manner placed into contact with the interior surface of a waterline, vehicle, or system. The composition comprising a microparticulate BT compound may be a gel (*e.g.,* a hydrogel, thiomer, aerogel, or organogel) or liquid. An organogel may comprise an organic solvent, lipoic acid, vegetable oil, or mineral oil. A slow-release composition may deliver an antimicrobially effective amount of microparticulate BT compound for 1, 2, 3, 4, 5, 6, or 7 (a week) days or for 2, 3, 4, 5, 6, 7 weeks, or 1, 2, 3, 4, 5, or 6 months.

The microparticulate BT compound (or a composition comprising the microparticulate BT compound) may be combined with at least one other antimicrobial agent (*i.e.,* a second, third, fourth, etc. antimicrobial agent) that when administered in combination have enhanced or synergistic antimicrobial effects as described herein. By way of example, an enhanced antimicrobial effect may be observed when microparticulate BT compound is administered together with an antimicrobial agent that chelates iron. A microparticulate BT compound may be combined with at least one of an oxidizing agent, microbicide, or disinfectant. Microparticulate BT compounds that are prepared

with hydrophobic thiols (*e.g.,* thiochlorophenol) may be used and which may exhibit greater capability than less hydrophobic BT compounds to adhere to surfaces of water lines and water delivery vehicles and systems. BT compounds that have a net negative charge, such as those having a 1:2 molar ratio (bismuth to thiol) may also have favorable adhesive properties.

A microparticulate BT compound (and compositions comprising microparticulate BT compound) may be combined with baking soda or another alkaline compound or substance. Because of the chemical and physical properties of baking soda, it has wide range of applications, including cleaning, deodorizing, and buffering. Baking soda neutralizes odors chemically, rather than masking or absorbing them. Baking soda can be combined with microparticulate BT compound either as a mixture of powders, or dissolved or suspended in a powder, spray, gel, paste, or liquid described herein. In other embodiments, microparticulate BT compound can be combined with other alkali metal bicarbonate or carbonate substances (*e.g.,* potassium bicarbonate or calcium carbonate) that help maintain a desired alkaline pH and that also possess cleansing and deodorizing properties.

By way of an additional example, a microparticulate BT compound (or a composition comprising microparticulate BT compound) may be combined with one or more of the following. Antimicrobial agents: for example, chlorhexidine; sanguinarine extract; metronidazole; quaternary ammonium compounds (such as cetylpyridinium chloride); bis-guanides (*e.g.,* chlorhexidine digluconate, hexetidine, octenidine, alexidine); halogenated bisphenolic compounds (*e.g.,* 2,2' methylenebis-(4-chloro-6-bromophenol) or other phenolic antibacterial compounds; alkylhydroxybenzoate; cationic antimicrobial peptides; aminoglycosides; quinolones; lincosamides; penicillins; cephalosporins, macrolides; tetracyclines; other antibiotics known in the art; *Coleus forskohlii* essential oil; silver or colloidal silver antimicrobials; tin- or copper-based antimicrobials; Manuka oil; oregano; thyme; rosemary; or other herbal extracts; and grapefruit seed extract. Anti-caries agents: for example, sodium- and stannous fluoride, aminefluorides, sodium monofluorophosphate, sodium trimetaphosphate, zinc citrate or other zinc agents, and casein. Plaque buffers: for example, urea, calcium lactate, calcium glycerophosphate, and strontium polyacrylates. Vitamins: for example, Vitamins A, C and E. Plant extracts. Anti-calculus agents: for example, alkali-metal pyrophosphates, hypophosphite-containing polymers, organic phosphonates and phosphocitrates etc. Biomolecules: for example, bacteriocins. Preservatives. Opacifying agents.

pH-adjusting agents. Sweetening agents. Surfactants: for example, anionic, nonionic, cationic and zwitterionic or amphoteric surfactants, saponins from plant materials (*see, e.g.,* U.S. Patent No. 6,485,711). Particulate abrasive materials: for example, silicas, aluminas, calcium carbonates, dicalcium phosphates, calcium pyrophosphates, hydroxyapatites, trimetaphosphates, insoluble hexametaphosphates, agglomerated particulate abrasive materials, chalk, fine ground natural chalk and the like. Humectants: for example, glycerol, sorbitol, propyleneglycol, xylitol, lactitol etc. Binders and thickeners: for example, sodium carboxy methyl cellulose, hydroxyethyl cellulose (Natrosol®), xanthan gum, gum arabic, synthetic polymers (*e.g.,* polyacrylates and carboxyvinyl polymers such as Carbopol®). Polymeric compounds that enhance the delivery of active ingredients such as antimicrobial agents. Buffers and salts to buffer the pH and ionic strength of the oral care composition. Bleaching agents: for example, peroxy compounds (*e.g.,* potassium peroxydiphosphate). Effervescing systems: for example, sodium bicarbonate/citric acid systems.

In another embodiment, a microparticulate BT compound described herein (or composition comprising the microparticulate BT compound) may be combined with at least one or more anti-biofilm agents for controlling biofilm development, disrupting a biofilm, or reducing the amount of biofilm. As understood in the art, interspecies quorum sensing is related to biofilm formation. Certain agents that increase LuxS-dependent pathway or interspecies quorum sensing signal (*see, e.g.,* U.S. Patent No. 7,427,408) contribute to controlling development and/or proliferation of a biofilm. Exemplary agents include, by way of example, N-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) blocking compounds and N-butyryl-L-homoserine lactone (BHL) analogs, either in combination or separately (*see, e.g.,* U.S. Patent No. 6,455,031). An oral hygiene composition comprising a microparticulate BT compound and at least one anti-biofilm agent can be delivered locally for disruption and inhibition of bacterial biofilm and for treatment of periodontal disease (*see, e.g.,* U.S. Patent No.6,726,898).

The effectiveness of a microparticulate BT compound as an anti-biofilm agent may be enhanced by heating the water line, water delivery vehicle, or water conduit system to which the microparticulate BT compound is applied by heating the line, vehicle, or system. In certain embodiments, the line, vehicle, or system is heated to between about 37° C to about 60° C or to about 37° C to about 100° C. In other embodiments, the line, vehicle, or

system is heated to between about 45° C to about 50° C; to between about 50° C to about 55° C; between about 55° C to about 60° C; to between about 60° C to about 70° C; to between about 70° C to about 80° C; to between about 80° C to about 90° C; or to between about 90° C to about 100° C. In particular embodiments, the line, vehicle, or system is heated to about 37° C. In another particular embodiment, the line, vehicle, or system is heated to about 55° C. As would be understood by a person skilled in the art, the length of time that the line, vehicle, or system, is heating may vary depending on the temperature applied. For example, the length of time required to achieve the same antimicrobial effect will be longer when the line, vehicle, or system is heated to a lower temperature than needed when heated to the higher temperatures. Determining the appropriate length of time for exposure of the line, vehicle, or system at each temperature may readily be determined by a person skilled in the art.

A microparticulate BT compound (or compositions comprising a microparticulate BT compound) can be employed in conjunction with other modalities to reduce or prevent development of biofilm. By way of example, microparticulate BT compounds may be combined with oxidative chemicals, descaling compounds, biofilm disruptors, or flushing systems, which are described herein and used in the art.

Compositions Comprising Microparticulate Bismuth-Thiols and Uses for Dental Restoration. In another embodiment provided herein are compositions comprising a microparticulate BT compound and dental amalgam and microparticulate BT compound and dental composites for use in prevention and/or treatment of dental caries. Currently, the only treatment for carious lesions is tooth restoration by placement of an inert material that acts as a block to further decay. Dental amalgam and dental composites are most commonly used for restoration of teeth affected by dental caries.

Recurrent marginal decay is an important contributor to restoration failure, particularly when dental composites are used for restoration. The presence of bacteria located at the interface between a composite material and dental tissues may an important factor in restoration failure (*see, e.g.,* Hansel et al, *J. Dent. Res.* 77:60-67 (1998)). In a study in Portugal (Casa Pia Study, 1986-1989), 1,748 posterior restorations were placed and 177 (10.1%) of them failed during the course of the study. Recurrent marginal decay was the main reason for failure in both amalgam and composite restorations, accounting for 66% (32/48) and 88% (113/129) failure, respectively (*see*

Bernardo et al. *JADA* 2007;138:775-83). Polymerization shrinkage, which is the shrinkage that occurs during the composite curing process, has been implicated as the primary reason for postoperative marginal leakage (*see, e.g.,* Estefan et al., *Gen. Dent.* 2003;51:506-509).

Incorporation of antimicrobial compounds and agents into restoration materials, such as dentin bonding systems (DBS), have been attempted but with limited success. Development of composites and amalgam and other restoration materials that have antimicrobial properties may contribute to prevention of secondary dental caries (*see, e.g.,* Imazato, *Dent. Materials* 19:449 (2003)). The present embodiments contemplate replacement of antimicrobials formulated with restoration compositions described herein, which are described in the art, with the presently described microparticulate BT compounds to provide the advantages disclosed herein, including the range of antimicrobial activities, solubility and bioavailability, anti-biofilm effects, non-toxicity, enhancement of antibiotic efficacies, and other properties as described herein.

In certain embodiments, a composition is provided comprising a microparticulate BT compound and a dental composite. Dental composites typically contain a polymerizable resin base containing ceramic filler. A microparticulate BT compound may be combined with any one of the dental composites known in the art using methods practiced in the art (*see, e.g.,* O'Brien, *Dental Materials and Their Selection* (Chicago: Quintessence Publishing Co.) (2002); Powers et al., *Dental Materials: Properties and Manipulation* (New York: Mosby) (2007); Roeters et al., *J. Dent.* 32:371-77 (1998)).

In other embodiments, a composition is provided comprising a microparticulate BT compound and amalgam. An amalgam is an alloy of mercury with one or more other metals. Most dental amalgams are called silver amalgams because silver is the principal constituent that reacts with mercury. The kinetics of reactions between mercury and silver are not appropriate for clinical use, so that the silver is provided as an alloy with other elements. This alloy is often referred to as a dental amalgam alloy or, collectively, the alloys are known as 'alloys for dental amalgam' (*see, e.g.,* International Standars Organization Standard ISO 1559, *Dental Materials – Alloys for Dental Amalgam* (1995)). Several types of dental amalgam alloy are known, and all include tin and most have some copper and, to a lesser extent, zinc. Some of the dental amalgam alloys themselves contain a little mercury to facilitate the

amalgamation reaction. A conventional dental amalgam alloy will contain between 67% and 74% silver, with 25-28% tin, and up to 6% copper, 2% zinc and 3% mercury. The so-called dispersion type amalgam alloys have about 70% silver, 16% tin and 13% copper. A different group of amalgam alloys may contain up to 30% copper, which are known as high-copper content amalgam alloys. The amalgam alloys are mixed with mercury before clinical placement at a 1 to 1 weight ratio. The mercury content of a finished dental amalgam restoration is therefore approximately 50% by weight. In the conventional dental amalgam alloys, the ratio of silver to tin results in a crystal structure that is essentially the intermetallic compound Ag_3Sn, referred to as the gamma (γ) phase. The exact percentage of this phase controls the kinetics of the amalgamation reaction and many properties of the resulting amalgam structure. With the higher copper dispersion alloys, the microstructure is usually a mixture of the gamma phase with the eutectic silver-copper phase. Different manufacturers present the amalgam alloy in different formats, although they are usually made available as fine particles, either spherical or irregular in shape, with particle sizes around 25-35 microns. (See Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), European Commission: Directorate-General, Health & Consumer Protection, May 6, 2008 at Internet site: ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_016.pdf.)

A microparticulate BT compound may also be used for preventing or treating caries and/or inflammation (*i.e.,* reducing the likelihood of occurrence or recurrence of caries and/or inflammation, respectively) by administering the microparticulate BT compound to the surface of the teeth, amalgam, or composite. A composition comprising a microparticulate BT compound may be a mucoadhesive composition that is applied to the surface of a tooth and/or gum or oral mucous membrane may be in any form that adheres to some extent to a surface or that delivers a pharmaceutically effective amount of the active ingredient(s) to the desired surface. A microparticulate BT compound can also be formulated to release slowly from the composition applied to the tooth. For example, the composition may be a gel (*e.g.,* a hydrogel, thiomer, aerogel, or organogel) or liquid. An organogel may comprise an organic solvent, lipoic acid, vegetable oil, or mineral oil. Such gel or liquid coating formulations may be applied interior or exterior to an amalgam or composite or other restorative composition. A slow-release composition may deliver a pharmaceutically effective amount of microparticulate BT compound for 1, 2, 3,

4, 5, 6, or 7 (a week) days or for 2, 3, 4, 5, 6, 7 weeks, or 1, 2, 3, 4, 5, or 6 months. Such compositions can be prepared by a person skilled in the art using any number of methods known in the art.

Compositions comprising a microparticulate BT compound that are useful for dental restoration may comprise glass ionomer cements; giomers (formed by reacting fluoride containing glass and a liquid polyacid); compomers (a polymerizable dimethacrylate resin and ion-leachable glass filler particles). Compomers may further comprise fluoride.

Compositions comprising a microparticulate BT compound that are applied to the surface of the teeth, amalgam, or composite may further comprise one or more other surface active agents that enhance the antimicrobial effect. Exemplary antimicrobial agents for use in the compositions comprising a microparticulate BT compound include, for example, chlorhexidine, sanguinarine extract, metronidazole, quaternary ammonium compounds, such as cetylpyridinium chloride; bis-guanides, such as chlorhexidine digluconate, hexetidine, octenidine, alexidine; and halogenated bisphenolic compounds, such as 2,2' methylenebis-(4-chloro-6-bromophenol), or other phenolic antibacterial compounds, alkylhydroxybenzoate, cationic antimicrobial peptides, aminoglycosides, quinolones, lincosamides, penicillins, cephalosporins, macrolides, tetracyclines, and other antibiotics, taurolidine or taurultam, A-dec ICX, *Coleus forskohlii* essential oil, silver or colloidal silver antimicrobials, tin- or copper-based antimicrobials, chlorine or bromine oxidants, Manuka oil, oregano, thyme, rosemary or other herbal extracts, and grapefruit seed extract; anti-inflammatory or antioxidant agents such as ibuprofen, flurbiprofen, aspirin, indomethacin, aloe vera, turmeric, olive leaf extract, cloves, panthenol, retinol, omega-3 fatty acids, gamma-linolenic acid (GLA), green tea, ginger, grape seed, etc.

The compositions may also further comprise one or more pharmaceutically acceptable carriers, such as, starch, sucrose, water or water/alcohol systems, DMSO, etc. The compositions may also include a surfactant, such as an anionic, nonionic, cationic and zwitterionic or amphoteric surfactants, or may include saponins from plant materials (*see, e.g.,* U.S. Patent No. 6,485,711). Buffers and salts to buffer the pH and ionic strength of composition for oral use my also be included. Other optional ingredients that may be included are bleaching agents such as peroxy compounds; potassium peroxydiphosphate; effervescing systems such as sodium bicarbonate/citric acid systems, and the like.

Compositions Comprising Microparticulate Bismuth-Thiols and Uses for Oral Hygiene and for Treating Inflammation and Infection of the Mouth. In another embodiment, compositions comprising microparticulate BT compounds are formulated for oral use and may be used in methods for preventing or reducing microbial growth in the mouth and for preventing and/or treating microbial infections and inflammation of the oral cavity. These compositions are therefore useful for preventing or treating (*i.e.,* reducing or inhibiting development of, reducing the likelihood of occurrence or recurrence of) dental plaque, halitosis, periodontal disease, gingivitis, and other infections of the mouth. The oral compositions comprising microparticulate BT compound may also be useful for preventing and/or controlling (*i.e.,* slowing, retarding, inhibiting) biofilm development, disrupting a biofilm, or reducing the amount of biofilm present on an oral surface, particularly a tooth or gums.

Trapped food particles, poor oral hygiene and poor oral health, and improper cleaning of dentures can promote microbial growth between teeth, around the gums, and on the tongue. Continued microbial growth and the presence of dental caries may result in halitosis, dental plaque (*i.e.,* a biofilm formed by colonization of microorganisms), gingivitis, and inflammation. In the absence of proper oral care (*e.g.,* tooth brushing, flossing), more serious infections, such as periodontal disease and infections of the jaw, may ensue.

Good oral hygiene is important not only for oral health, but for prevention of several chronic conditions. Controlling bacterial growth in the mouth may help lower risk of heart disease, preserve memory, and reduce the risk of infection and inflammation in other areas of the body. People with diabetes are at greater risk for developing severe gum problems, and reducing the risk of gingivitis by maintaining good oral health may help control blood sugar. Pregnant women may be more likely to experience gingivitis, and some research suggests a relationship between gum disease in pregnant women and delivery of preterm, low-birth-weight infants.

Bacteria are the primary etiologic agents in periodontal disease. More than 500 bacterial strains may be found in dental plaque (Kroes et al., *Proc. Natl. Acad. Sci. USA* 96:14547-52 (1999)). Bacteria have evolved to survive in the environment of the tooth surface, gingival epithelium, and oral cavity as biofilms, which contributes to the difficulty in treating periodontitis. Bactericidal agents as well as antibiotics that are currently used to treat such infections often do not kill all of offending organisms. Use of an agent that is ineffective against certain bacteria species may result in proliferation of

resistant bacterial species. Moreover, these agents may cause unpleasant side effects, such allergic reactions, inflammation, and tooth discoloration.

Dental bacterial plaque is a biofilm that adheres tenaciously to tooth surfaces, restorations, and prosthetic appliances. The primary means to control biofilms in the mouth is through mechanical cleaning (*i.e.,* tootbrushing, flossing, etc.). Within the first two days after which no such cleaning has been undertaken, the tooth's surface is colonized predominantly by gram-positive facultative cocci, which are primarily streptococci species. The bacteria excrete an extracellular slime layer that helps anchor the bacteria to the surface and provides protection for the attached bacteria. Microcolony formation begins once the surface of the tooth has been covered with attached bacteria. The biofilm grows primarily through cell division of adherent bacteria, rather than through the attachment of new bacteria. Doubling times of bacteria forming plaque are rapid in early development and slower in more mature biofilms.

Coaggregation occurs when bacterial colonizers subsequently adhere to bacteria already attached to the pellicle. The result of coaggregation is the formation of a complex array of different bacteria linked to one another. After a few days of undisturbed plaque formation, the gingival margin becomes inflamed and swollen. Inflammation may result in creation of a deepened gingival sulcus. The biofilm extends into this subgingival region and flourishes in this protected environment, resulting in the formation of a mature subgingival plaque biofilm. Gingival inflammation does not appear until the biofilm changes from one composed largely of gram-positive bacteria to one containing gram-negative anaerobes. A subgingival bacterial microcolony, composed predominantly of gram-negative anaerobic bacteria, becomes established in the gingival sulcus between 3 and 12 weeks after the beginning of supragingival plaque formation. Most bacterial species currently suspected of being periodontal pathogens are anaerobic, gram-negative bacteria.

Bacterial microcolonies protected within the biofilm are typically resistant to antibiotics (administered systemically), antiseptics or disinfectants (administered locally), and immune defenses. Antibiotic doses that kill free-floating bacteria, for example, need to be increased as much as 1,500 times to kill biofilm bacteria. At this high concentration, these antimicrobials tend to be toxic to the patient as well (*see, e.g.,* Coghlan 1996, *New Scientist* 2045:32-6; Elder et al., 1995, *Eye* 9:102-9).

Diligent and frequent physical removal of bacterial plaque biofilms is the most effective means of eliminating and controlling plaque. However,

subgingival plaque within pockets cannot be reached by brushes, floss, or oral rinses. Therefore, frequent periodontal debridement of subgingival root surfaces by a dental hygienist or dentist is an essential component in prevention and treatment of periodontitis.

In certain embodiments, a microparticulate BT compound may be incorporated into oral hygiene compositions and onto (such as a coating) or into devices, such as but not limited to, toothpaste, mouthwash (*i.e.,* mouth rinse), oral gels, dentifrice powders, oral sprays (including a spray dispersed by an oral inhaler), edible film, chewing gum, oral slurry, denture liquid cleaners, denture storage liquids, and dental floss, which may be routinely used by any subject. A microparticulate BT compound may be incorporated into oral hygiene compositions and onto devices that are used primarily by dental care professions, including for example, fluoride liquid treatments, cleaning compositions, buffing compositions, oral rinses, dental floss, and cleaning tools. The present embodiments contemplate replacement of antimicrobials formulated with oral hygiene compositions and/or coated onto devices, which are described in the art, with the presently described microparticulate BT compounds to provide the advantages disclosed herein, including the range of antimicrobial activities, solubility and bioavailability, anti-biofilm effects, non-toxicity, enhancement of antibiotic efficacies, and other properties as described herein.

A microparticulate BT compound may also be used for preventing or treating caries and/or inflammation (*i.e.,* reducing the likelihood of occurrence or recurrence of caries and/or inflammation, respectively) by administering the microparticulate BT compound to the surface of the teeth. A composition comprising a microparticulate BT compound may be a mucoadhesive composition that is applied to the surface of a tooth and/or gum or oral mucous membrane may be in any form that adheres to some extent to a surface or that delivers a pharmaceutically effective amount of the active ingredient(s) to the desired surface. A microparticulate BT compound can also be formulated to release slowly from the composition applied to the tooth. For example, the composition may be a gel (*e.g.,* a hydrogel, thiomer, aerogel, or organogel) or liquid. An organogel may comprise an organic solvent, lipoic acid, vegetable oil, or mineral oil. Such gel or liquid coating formulations may be applied interior or exterior to an amalgam or composite or other restorative composition. A slow-release composition may deliver a pharmaceutically effective amount of microparticulate BT compound for 1, 2, 3, 4, 5, 6, or 7 (a week) days or for 2, 3,

4, 5, 6, 7 weeks, or 1, 2, 3, 4, 5, or 6 months. Such compositions can be prepared by a person skilled in the art using any number of methods known in the art.

In certain other embodiments, and as described herein, antimicrobial compositions are provided for oral use that comprise microparticulate BT compound and one or more additional antimicrobial compounds or agents. Particularly useful are the compositions comprising s and a second antimicrobial agent that when administered in combination have enhanced or synergistic antimicrobial effects, as described herein. By way of example, an enhanced antimicrobial effect may be observed when a microparticulate BT compound is administered together with an antimicrobial agent that chelates iron. In other particular embodiments, a microparticulate BT compound is formulated with an anti-inflammatory agent, compound, small molecule, or macromolecule (such as a peptide or polypeptide).

Any of the microparticulate BT compounds described herein may be formulated for oral use. In certain embodiments, microparticulate BT compounds that are prepared with hydrophobic thiols (*e.g.,* thiochlorophenol) may be used and which may exhibit greater capability than less hydrophobic BT compounds to adhere to teeth and tissues of the mouth. BT compounds that have a net negative charge, such as those having a 1:2 molar ratio (bismuth to thiol) may also have favorable adhesive properties.

The oral hygiene compositions comprising a microparticulate BT compound may further comprise one or more active ingredients and/or one or more orally suitable excipients or carriers. In one embodiment, the oral hygiene compositions may further comprise baking soda or another alkaline compound or substance. Because of the chemical and physical properties of baking soda, it has wide range of applications, including cleaning, deodorizing, and buffering. Baking soda neutralizes odors chemically, rather than masking or absorbing them. Baking soda can be combined with a microparticulate BT compound either as a mixture of powders, or dissolved or suspended in any one of the dentifrice powders, gels, pastes, and liquids described herein. In other embodiments, a microparticulate BT compound can be combined with other alkali metal bicarbonate or carbonate substances (*e.g.,* potassium bicarbonate or calcium carbonate) that help maintain a desired alkaline pH and that also possess cleansing and deodorizing properties.

Oral hygiene compositions comprising a microparticulate BT compound may further comprise one or more of the following ingredients.

Antimicrobial agents: for example, chlorhexidine; sanguinarine extract; metronidazole; quaternary ammonium compounds (such as cetylpyridinium chloride); bis-guanides (*e.g.,* chlorhexidine digluconate, hexetidine, octenidine, alexidine); halogenated bisphenolic compounds (*e.g.,* 2,2' methylenebis-(4-chloro-6-bromophenol) or other phenolic antibacterial compounds; alkylhydroxybenzoate; cationic antimicrobial peptides; aminoglycosides; quinolones; lincosamides; penicillins; cephalosporins, macrolides; tetracyclines; other antibiotics known in the art; *Coleus forskohlii* essential oil; silver or colloidal silver antimicrobials; tin- or copper-based antimicrobials; Manuka oil; oregano; thyme; rosemary; or other herbal extracts; and grapefruit seed extract. Anti-inflammatory or antioxidant agents: for example, ibuprofen, flurbiprofen, aspirin, indomethacin, aloe vera, turmeric, olive leaf extract, cloves, panthenol, retinol, omega-3 fatty acids, gamma-linolenic acid (GLA), green tea, ginger, grape seed, etc. Anti-caries agents: for example, sodium- and stannous fluoride, aminefluorides, sodium monofluorophosphate, sodium trimetaphosphate, zinc citrate or other zinc agents, and casein. Plaque buffers: for example, urea, calcium lactate, calcium glycerophosphate, and strontium polyacrylates. Vitamins: for example, Vitamins A, C and E. Plant extracts. Desensitizing agents: for example, potassium citrate, potassium chloride, potassium tartrate, potassium bicarbonate, potassium oxalate, potassium nitrate, and strontium salts. Anti-calculus agents: for example, alkali-metal pyrophosphates, hypophosphite-containing polymers, organic phosphonates and phosphocitrates etc. Biomolecules: for example, bacteriocins, bacteriophages, antibodies, enzymes, etc. Flavors: for example, peppermint and spearmint oils, fennel, cinnamon, etc. Proteinaceous materials: for example, collagen. Preservatives. Opacifying agents. Coloring agents. pH-adjusting agents. Sweetening agents. Pharmaceutically acceptable carriers: for example, starch, sucrose, water or water/alcohol systems etc. Surfactants: for example, anionic, nonionic, cationic and zwitterionic or amphoteric surfactants, saponins from plant materials (*see, e.g.,* U.S. Patent No. 6,485,711). Particulate abrasive materials: for example, silicas, aluminas, calcium carbonates, dicalcium phosphates, calcium pyrophosphates, hydroxyapatites, trimetaphosphates, insoluble hexametaphosphates, agglomerated particulate abrasive materials, chalk, fine ground natural chalk and the like. Humectants: for example, glycerol, sorbitol, propyleneglycol, xylitol, lactitol etc. Binders and thickeners: for example, sodium carboxy methyl cellulose, hydroxyethyl cellulose (Natrosol®), xanthan gum, gum arabic,

synthetic polymers (*e.g.*, polyacrylates and carboxyvinyl polymers such as Carbopol®). Polymeric compounds that enhance the delivery of active ingredients such as antimicrobial agents. Buffers and salts to buffer the pH and ionic strength of the oral care composition. Bleaching agents: for example, peroxy compounds (*e.g.*, potassium peroxydiphosphate). Effervescing systems: for example, sodium bicarbonate/citric acid systems. Color change systems. In particular embodiments, an abrasive is silica or fine ground natural chalk.

The oral hygiene compositions comprising a microparticulate BT compound that are formulated for use as a toothpaste may further comprise a humectant (for example, glycerol or sorbitol), a surface-active agent, binding agent, and/or a flavoring agent. The toothpastes may also include a sweetening agent, whitening agent, preservative, and antimicrobial agent. The pH of a toothpaste and other compositions for oral use is typically between pH 5.5 and 8.5. In certain embodiments, oral hygiene compositions, including toothpaste, have a pH between 7 and 7.5, between 7.5 and 8, between 8 and 8.5, or between 8.5 and 9, which may enhance the antimicrobial activity of the microparticulate BT compound. The toothpaste compositions described herein may include one or more of chalk, dicalcium phosphate dihydrate, sorbitol, water, hydrated aluminum oxide, precipitated silica, sodium lauryl sulfate, sodium carboxymethyl cellulose, flavoring, sorbitan monooleate, sodium saccharin, tetrasodium pyrophosphate, methyl paraben, propyl paraben. One or more coloring agents, for example, FD&C Blue, can be employed if desired. Other suitable ingredients that may be including in a toothpaste formulation are described in the art, for example, in U.S. Pat. No. 5,560,517.

In one particular embodiment, the oral hygiene composition is a mouthspray and comprises a microparticulate BT compound, an alkaline buffer (*e.g.*, potassium bicarbonate), an alcohol, a sweetener component, and a flavor system. The flavor system may also have or more of the following: a flavorant, a humectant, a surfactant, a sweetener, and a colorant agent (*see, e.g.*, U.S. Patent No. 6,579,513). Surfactants described herein and known in the art for use in oral hygiene compositions may be anionic, nonionic, or amphoteric.

In another embodiment, the microparticulate BT-containing oral hygiene composition may be combined with additional active ingredients such as taurolidine and taurultam, which have been described in the art as useful for including in toothpastes, tooth gels, and mouthwashes for treating treat serious infections (*see, e.g.*, United Kingdom Patent Application No., GB 1557163, U.S. Patent No. 6,488,912). As described herein, microparticulate BT can also be

combined with one or more additional antimicrobial agents that when combined with microparticulate BT, the combination has additive or synergistic effects.

In yet another particular embodiment, an oral hygiene composition described herein may further comprise at least one or more anti-biofilm agents for controlling biofilm development, disrupting a biofilm, or reducing the amount of biofilm. As understood in the art, interspecies quorum sensing is related to biofilm formation. Certain agents that increase LuxS-dependent pathway or interspecies quorum sensing signal (*see, e.g.,* U.S. Patent No. 7,427,408) contribute to controlling development and/or proliferation of a biofilm. Exemplary agents include, by way of example, N-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) blocking compounds and N-butyryl-L-homoserine lactone (BHL) analogs, either in combination or separately (*see, e.g.,* U.S. Patent No. 6455031). An oral hygiene composition comprising a microparticulate BT compound and at least one anti-biofilm agent can be delivered locally for disruption and inhibition of bacterial biofilm and for treatment of periodontal disease (*see, e.g.,* U.S. Patent No.6,726,898).

An oral hygiene composition described herein may contain a sufficient amount of a microparticulate BT compound that effects substantial antimicrobial action during the time required for a normal tooth brushing, mouth rinsing, or flossing. As described herein a microparticulate BT compound may be retained on oral surfaces (such as tooth, amalgam, composite, mucous membrane, gums). A microparticulate BT compound retained on the teeth and gums after completion of brushing, rinsing, flossing, for example, may continue to provide extended anti-biofilm and anti-inflammatory action.

In other embodiments, microparticulate BT compounds are slowly released from muco-adhesive polymers or other agents that contribute to retention of microparticulate BT compound on mucosal, tooth, and restoration surfaces. Microparticulate BTcompounds may be added to stable, viscous, mucoadhesive aqueous compositions, which may also be used for the prevention and treatment of ulcerative, inflammatory, and/or erosive disorders of mucous membranes and/or the delivery of pharmaceutically active compounds to mucosal surfaces for topical treatment or transfer to the systemic circulation (*see, e.g.,* U.S. Patent No. 7,547,433).

In another embodiment, oral hygiene compositions comprising a microparticulate BT compound further comprise olive oil, which may enhance plaque removal. The use of olive oil in a product intended for oral hygiene, such as a toothpaste, a mouthwash, a spray, oral inhaler, or chewing gum, may

contribute to elimination or reduction (a decrease) of bacterial plaque and/or to elimination or reduction (decrease of) in the numbers of bacteria present in the buccal cavity, thereby achieving a reduction in the occurrence of dental diseases (*e.g.,* tooth decay, periodontal disease) and halitosis (*see, e.g.,* U.S. Patent No. 7,074,391).

In other embodiments, an oral hygiene composition comprising a microparticulate BT compound may further comprise a mucosal disinfectant preparation for topical application in the mouth. An oral hygiene composition may further comprise an aqueous slurry useful for cleaning the tongue and throat (*see, e.g.,* U.S. Patent No. 6,861,049). In still another embodiment, an oral hygiene composition comprising a microparticulate BT compound may further comprise at least one mint that is used for preventing (*i.e.,* reducing the likelihood of occurrence) formation of a cavity (dental caries) or reducing the number of cavities. One such mint, called CaviStat® (Ortek Therapeutics, Inc., Roslyn Heights, NY), contains arginine and calcium, which helps neutralize acid pH and promotes adherence of calcium to enamel surfaces. The inclusion of mint in an oral hygiene composition comprising a microparticulate BT compound may thus increase pH and enhance adherence of a microparticulate BT compound to oral surfaces.

<u>Adhesive Compositions Comprising Microparticulate Bismuth-Thiols Formulated for Dental and Orthopedic Use.</u> In another embodiment, compositions comprising a microparticulate BT compound are formulated for use in methods for preventing or reducing microbial growth on a bone or joint prosthesis or of the tissue and skeletal structure adjacent to the bone or joint prosthesis. In a particular embodiment, methods are provided for using compositions comprising a microparticulate BT compound for preventing and/or treating microbial infections and inflammation resulting from an orthopedic procedure (*e.g.,* orthopedic surgery, orthopedic therapy, arthroplasty (including two-step arthoplasty), orthodontic therapy). In certain embodiments, the compositions comprise a microparticulate BT compound and bone cement, and in other certain embodiments, comprise a microparticulate BT compound and dental cement. These compositions are therefore useful for preventing and/or treating (*i.e.,* reducing or inhibiting development of, reducing the likelihood of occurrence or recurrence of) microbial infections of the skeleton and supporting structure (*i.e.,* bones, joints, muscles, ligaments, tendons) such as osteomyelitis. The compositions described herein comprising a microparticulate BT compound and a bone cement or dental cement may also

be useful for preventing and/or controlling (*i.e.,* slowing, retarding, inhibiting) biofilm development, disrupting a biofilm, or reducing the amount of biofilm present in a joint or on a surface, such as the surface of a joint, bone, ligament, tendon, or tooth or a replacement joint, bone (partial or total), ligament, tendon, or tooth.

A cement as described herein and known in the art is a binder substance that binds materials together and that is capable of hardening. Such a substance is capable of binding tissues together or capable of binding a prosthetic or artificial device (*e.g.,* prosthetic joint, bone, or tooth) to the adjacent tissue. Bone cements include, for example, polymethyl methacrylate (PMMA), magnesium phosphate, and calcium phosphate. Forms of calcium phosphate are used as "replacement bone" for treating fissures and breaks in bone that may not heal sufficiently quickly and/or properly without an implanted material. The compositions that comprise a bone cement (*e.g.,* calcium phosphate) and a microparticulate BT compound may also be used for treating cancellous bone defects by providing mechanical integrity to the cancellous bone. Cements may be resorbed or may remain at the implantation site.

In particular embodiments, the compositions described herein that are useful as bone cements comprise a BT compound or microparticulate BT compound and a preparation of calcium phosphate or magnesium phosphate suitable for use as a bone cement. A preparation of calcium phosphate or magnesium sulfate may also be called herein a calcium phosphate-containing bone cement or calcium phosphate bone cement or magnesium phosphate-containing bone cement or magnesium phosphate bone cement, respectively. Calcium phosphate may be included in the compositions in any one of several forms known and used in the art and include, by way of non-limiting example, hydroxyapatite ($Ca_{10}(PO_4)_6(OH)2$); brushite ($CaHPO_4 * 2H_2O$); monetite ($CaHPO_4$); calcium deficient hydroxyapatite (CDHA, $Ca_9(PO_4)_5HPO_4OH$); calcium sulfate/phosphate (CSPC) (*see, e.g.,* Hu et al., *J. Mater. Sci. Mater. Med.* 2009 October 13, e-publication ahead of print) cements. A magnesium phosphate used in the art is also called struvite ($MgNH_4PO_4 * 6H_2O$) cement (*see, e.g.,* Grosshardt et al., *Tissue Eng. Part A*, 2010 Jul 30, e-pub ahead of print; *see also, e.g.,* Bohner et al., *J. Pharm. Sci.* 86:565-72; (1997); Fulmer et al., 3:299-305 (1992); Lobenhoffer et al., *J. Orthopaedic Trauma* 16:143-49 (2002); Lee et al., *J. Carniofac. Surg.* 21:1084-88 (2010)). In a particular embodiment, the compositions described herein comprising a microparticulate BT compound and a calcium phosphate-containing bone cement comprise

calcium sulfate/phosphate (CSPC) as the form of calcium phosphate (*see, e.g.,* Hu et al., *J. Mater. Sci. Mater. Med.* 2009 October 13, e-publication ahead of print). In certain other embodiments, the compositions comprising a microparticulate BT compound and calcium phosphate or magnesium phosphate cement may further comprise chitosan (biopolymer from crustacean cells); at least one or more antibiotics or antimicrobial agents; and/or at least one or more anti-inflammatory agents.

Bone cements have been used in the art for release of drugs and agents. In certain particular embodiments, a calcium phosphate cement may be in the form, at least in part, as a hydroxyapatite microsphere that encapsulates an agent (such as an antimicrobial agent) for therapeutic use (*see, e.g.,* U.S. Patent No. 6,730,324). Such cements that include microspheres are useful for slow release of the agent included within the microsphere. Contemplated herein are compositions comprising calcium phosphate microspheres that comprise a microparticulate BT compound.

Also provided herein are compositions comprising a microparticulate BT compound and a PMMA bone cement. The PMMA bone cement may be formulated with a microparticulate BT compound according to methods described in the art for formulating PMMA with other agents having antimicrobial activity (see, for example, European Patent Application No. EP1649874).

Also provided herein are compositions comprising a microparticulate BT compound and a dental cement (*i.e.,* dental adhesive), which compositions may be used for inhibiting, preventing, or treating a microbial infection of the tooth or gums. Dental cements may comprise any one of the following compounds or compositions: zinc phosphate, glass ionomers, alpha-tricalcium phosphate (α-TCP), alkyl methacrylate (*see, e.g.,* U.S. Patent No. 6,071,528); bismuth oxide (*see, e.g.,* Bueno et al., *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* 107:e65-69 (2009)); and mineral trioxide aggregate (MTA) (*see, e.g.,* Hwang et al., *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* 107:e96-102 (2009)).

The present embodiments contemplate replacement of antimicrobials formulated with dental cement or bone cement, which are described in the art, with the presently described microparticulate BT compounds to provide the advantages disclosed herein, including the range of antimicrobial activities, solubility and bioavailability, anti-biofilm effects, non-toxicity, enhancement of antibiotic efficacies, and other properties as described

herein. Bone and dental cements may be formulated with a microparticulate BT compound and one or more additional antibiotics according to methods described in the art (*see, e.g.*, U.S. Patent Application Publication No. 2006/0205838; Alt et al., *Antimicrob. Agents Chemother.* 48:4-84-88 (2004); Bohner et al., *supra*; Bueno et al., *supra*; Chuard et al., *Antimicrob. Agents Chemother.* 37:625-32 (1993); *J. Orthopaed. Res.* 27:1008-15 (2009); De Lalla, *J. Chemother.* 13:48-53 (2001); Domenico et al., *Peptides* 25:2047-53 (2004); Widmer et al., *Antimicrob. Agents Chemother.* 35:741-46 (1991)).

The amount of a BT compound used in a microparticulate BT-containing composition comprising bone cement or dental cement may range from between about 10-500 μg BT per gram of the respective cement. The microparticulate BT compounds, alone or in combination with at least one additional antibiotic, provide advantages as described herein over presently used antibiotics in bone and dental cements. The compositions described herein that comprise a microparticulate BT compound and a bone cement (*e.g.*, calcium phosphate) or dental cement may further comprise one or more additional antimicrobial compounds or agents. Particularly useful are the compositions comprising a microparticulate BT compound and a second antimicrobial agent that when administered in combination have enhanced or synergistic antimicrobial effects, as described herein. By way of an additional example, an enhanced antimicrobial effect may be observed when a microparticulate BT compound is administered together with an antimicrobial agent that chelates iron. In other particular embodiments, a microparticulate BT compound is formulated with an anti-inflammatory agent, compound, small molecule, or macromolecule (such as a peptide or polypeptide).

Compositions comprising a microparticulate BT compound and a bone cement as described herein may also be used for coating hardware (for example, screws, plates, staples, pins, and wires and the like) that is used to attach, stabilize, or fixate a fracture, fusion, osteotomy, or replacement joint. Compositions comprising a microparticulate BT compound and a dental cement as described herein may be used for coating tooth pulp, a tooth cap, a liner, a tooth, or a dental filling or restoration composition within a tooth, or the like. These compositions may be formulated into a coating that can be applied to, adfixed to, adhered to, or in some manner placed into contact with the surface of bone and/or joint related hardware. In particular embodiments, the coating comprises a microparticulate BT compound and a calcium phosphate or magnesium phosphate bone cement. The microparticulate BT compound and

calcium phosphate or magnesium phosphate are formulated together for application to bone hardware according to methods practiced in the art. For example, a composition comprising a microparticulate BT compound and a bone cement (*e.g.,* calcium phosphate or magnesium phosphate bone cement) may be in the form of a liquid, gel, paste, or spray (*e.g.,* a thermal spray, which includes a plasma spray) for application to the hardware. The composition comprising a microparticulate BT compound and a bone cement may be a gel (*e.g.,* a hydrogel, thiomer, aerogel, or organogel) or liquid. An organogel may comprise an organic solvent, lipoic acid, vegetable oil, or mineral oil. A slow-release composition may deliver an antimicrobially effective amount of microparticulate BT compound for 1, 2, 3, 4, 5, 6, or 7 (a week) days or for 2, 3, 4, 5, 6, 7 weeks, or 1, 2, 3, 4, 5, or 6 months. The rate of release may be controlled, at least in part, according to the porosity of the cement (*see, e.g.,* Bohner et al., *supra*).

Compositions comprising a microparticulate BT compound and a bone cement or dental cement may be combined with at least one other antimicrobial agent (*i.e.,* a second, third, fourth, etc. antimicrobial agent) that when administered in combination have enhanced or synergistic antimicrobial effects (*i.e.,* greater than an additive effect). By way of example, an enhanced antimicrobial effect may be observed when a microparticulate BT compound is administered together with an antimicrobial agent that chelates iron. In particular embodiments, compositions comprising a microparticulate BT compound and a bone cement or dental cement may be combined with at least one other antimicrobial agent and/or anti-inflammatory agent selected from the following: <u>Antimicrobial agents</u>: for example, chlorhexidine; sanguinarine extract; metronidazole; quaternary ammonium compounds (such as cetylpyridinium chloride); bis-guanides (*e.g.,* chlorhexidine digluconate, hexetidine, octenidine, alexidine); halogenated bisphenolic compounds (*e.g.,* 2,2' methylenebis-(4-chloro-6-bromophenol) or other phenolic antibacterial compounds; alkylhydroxybenzoate; cationic antimicrobial peptides; aminoglycosides; quinolones; lincosamides; penicillins; cephalosporins, macrolides; tetracyclines; other antibiotics known in the art; *Coleus forskohlii* essential oil; silver or colloidal silver antimicrobials; tin- or copper-based antimicrobials; Manuka oil; oregano; thyme; rosemary; or other herbal extracts; and grapefruit seed extract. <u>Anti-inflammatory or antioxidant agents</u>: for example, ibuprofen, flurbiprofen, aspirin, indomethacin, aloe vera, turmeric, olive leaf extract, cloves, panthenol, retinol, omega-3 fatty acids, gamma-

linolenic acid (GLA), green tea, ginger, grape seed, etc. In particular embodiments, the compositions comprising microparticulate BT compound and a bone cement or dental cement may further comprise an antibiotic selected from clindamycin, vancomycin, daptomycin, cefazolin, gentamicin, tobramycin, metronidazole, cefaclor, ciprofloxacin, or other antimicrobial such as a quaternary ammonium compound (*e.g.,* benzalkonium chloride, cetyl pyridinium chloride), an anti-microbial zeolite, alkali metal hydroxide, or an alkaline earth metal oxide. The compositions may optionally comprise one or more pharmaceutically suitable carriers (*i.e.,* excipients), surfactants, buffers, diluents, and salts, and bleaching agents, which are described herein. Antimicrobial agents may be formulated with dental cements and bone cements as described herein and in the art (*see, e.g.,* Akashi et al., *Biomaterials* 22:2713- 17 (2001); U.S. Patent No. 6,071,528; Alt et al., *supra*).

Animal models of foreign body infection may be used to characterize the antimicrobial activity of the compositions comprising a microparticulate BT compound and a dental cement or bone cement (*see, e.g.,* Chuard et al. *Antimicrob. Agents Chemother.* 1993;37:625–32). In vivo efficacy of antibiotics in these models correlates with the ability of antimicrobials to kill stationary-phase microorganisms and those that are adherent to foreign material (*see, e.g.,* Widmer et al. *J. Infect. Dis.* 1990;162:96–102; Widmer et al. *Antimicrob Agents Chemother* 1991;35:741–6; *see also, e.g.,* Karchmer. *Clin. Infect. Dis.* 1998;27:714–6).

By way of non-limiting example and for illustration purposes only, a bone cement may comprise a microparticulate BT compound in 75% (2/2) methyl methacrylate styrene copolymer, 15% polymethylmethacrylate (to assist handling of the composition), and 10% barium sulfate (for radio-opaqueness), and from about 10 to about 500 μg of a microparticulate BT compound per gram of cement powder (i.e., 0.001 – 0.05% w/w). In other particular embodiments, at least one additional antimicrobial agent may be added.

<u>Compositions Comprising Microparticulate Bismuth-Thiols Formulated with Paints and Paint Coatings.</u> Certain other embodiments contemplate incorporation of the microparticulate BT compounds described herein into paints or onto paints as paint coatings for reducing biofouling and preventing and/or controlling (*i.e.,* slowing, retarding, inhibiting) biofilm development, disrupting a biofilm, or reducing the amount of biofilm present on a painted surface. The compositions described herein comprising a microparticulate BT compound may be formulated with a paint or paint coating

that is applied to any one of numerous articles of manufacture, including but not limited to, medical devices, orthopedic devices, dental devices, industrial devices, electronic devices, walls, floors, ceilings, roofs, pilings, docks, piers, pipes, pipelines and piping structures (*e.g.,* intake screens, cooling towers), heat exchangers, dams, and textiles, and other surfaces, such as those present in and on vehicles of all types, including automobiles, trains, planes, and water vessels such as ships, boats, submarines, and other water vessels.

In a particular embodiment, the compositions and methods described herein are useful for preventing and/or reducing biofouling or biofilms that form on articles of manufacture that are exposed to water. The formation of biofilm on surfaces in the marine environment is believed to be an important factor contributing to the colonization and recruitment of some sessile invertebrate communities on marine structures (*see, e.g.,* Siboni et al., *FEMS Microbiol Lett* 2007;274:24-9). Subsequent interactions of macrobiota with these microbial films lead within days or weeks to the attachment and growth of invertebrates and algae, which account for most of the hydrodynamic drag associated with biofouling (*see, e.g.,* Schultz, *Biofouling* 2007;23:331–41). Old biofilms on surfaces supported barnacle larval attachment, irrespective of the type of substrata (*see, e.g.,* Hunga et al., *J Exptl Marine Biol Ecol* 2008;361:36-41). Biofilms also significantly increased adhesion strength in the ascidian *Phallusia nigra*, the polychaete tubeworm *Hydroides elegans*, and the barnacle *Balanus amphitrite* at one or more developmental stages (*see, e.g.,* Zardus et al., *Biol Bull* 2008;214:91-8). Biofilms can also enhance attachment of Zebra mussels (*Dressena polymorpha*) to some artificial surfaces (*see, e.g.,* Kavouras & Maki. *Inverteb Biol* 2005;122:138-51), which has resulted in millions if not billions of dollars in lost revenues and costs to the seafood, power generation, and manufacturing industries and to water and wastewater treatment facilities and has caused significant damage to ecosystems into which the mussel is introduced.

In marine, brackish, and freshwater environments, organisms collect, settle, attach, and grow on submerged structures and vessels. Such organisms include algae, fungi and other microorganisms, and aquatic animals, such as tunicates, hydroids, bivalves, bryozoans, polychaete worms, sponges, and barnacles. The presence of these organisms, known as the "fouling" of a structure, can be detrimental, for example, by adding to the weight of the structure and/or hampering its hydrodynamics thereby reducing its operating

efficiency, increasing susceptibility to corrosion, and degrading or fracturing the structure.

Certain paints and coatings used to date to prevent or reduce biofouling and biofilm production include toxic components that while inhibiting biofouling and biofilm formation may be toxic to desired and beneficial flora and fauna. Exemplary biocides and chemical toxins include copper and copper containing compounds (*e.g.,* cuprous oxide), mercury, arsenic, tributyltin oxide (TBT), organotins (*i.e.,* tin with one or more carbon groups attached), hexio two-part bisphenol-A-(epichlrohydrin expoxy compounds, difunctional glycidyl ether epoxy resin, glycidyl ether expoxy, and barium metaborate epoxy.

The presently described microparticulate BT compounds provide a non-toxic alternative and provide the advantages disclosed herein, including the range of antimicrobial activities, solubility and bioavailability, anti-biofilm effects, enhancement of antibiotic efficacies, and other properties as described herein. The microparticulate BT compounds be substituted for other antimicrobial agents in paints and paint coatings and may be incorporated into these paints and paint coatings by integration of the microparticulate BT compounds and methods described herein, with processes that are known for producing paints and paint coating that include biocidal agents (*see, e.g.,* U.S. Patent Nos. 4,596,724; 4,410,642; 4,788,302; 5,470,586; 6,162,487; 5,384,176; U.S. Patent Application Publication Nos. 2007/125703 and 2009/0197003; Gerhart et al., *J. Chem. Ecol.* 14:1905-17 (1988); Sears et al., *J. Chem. Ecol.* 16:791-99 (1990); Ganguli et al., *Smart Mater. Struct.* 18:104027 (2009); Cao et al., *ACS Applied Materials Interfaces* 1:494 (2009); Kumar et al., *Nature Materials* 7:236-41 (2008)). Paints into which microparticulate BT compounds may be incorporated include epoxy, silicone, or acrylic based paints. In more particular embodiments, microparticulate BT compounds may be incorporated into paints formulated for marine use and exposure to seawater and which include, for example, alkyd resin based, Bitumen based, Gilsonite based paints, chlorinated rubber based, and epoxy resin based paints.

Antimicrobial agents may be released in a controlled manner by incorporating the agents into paint coatings. Methods of enhancing drug release rate from a composite material are known in the art. Composite material can include a natural or synthetic, bioabsorbable polymer matrix and a drug particle phase dispersed therein (*see, e.g.,* U.S. Patent Nos. 7,419,681 and 5,028,664; *see also, e.g.,* U.S. Patent Application No. 2009/0043388). By way of example, a drug eluting pain coating composition may comprise at least one

microparticulate BT compound dispersed in a modified, biologically active binders.

A microparticulate BT compound may also be formulated to release slowly from the composition comprising the microparticulate BT compound applied to a painted surface. A microparticulate BT compound can also be incorporated into a coating (*e.g.,* an epoxy coating), which can be applied to, adfixed to, adhered to, or in some manner placed into contact with a surface of a painted structure or article of manufacture. A microparticulate BT compound may be slowly released from such compositions. A slow-release composition comprising a microparticulate BT compound may be a gel (*e.g.,* a hydrogel, thiomer, aerogel, or organogel) or liquid. An organogel may comprise an organic solvent, lipoic acid, vegetable oil, or mineral oil. A slow-release composition may deliver an antimicrobially effective amount of microparticulate BT compound for 1, 2, 3, 4, 5, 6, or 7 (a week) days or for 2, 3, 4, 5, 6, 7 weeks, or 1, 2, 3, 4, 5, or 6 months.

Other coatings used in the art and with which the microparticulate BT compounds described herein may be formulated include polysaccharides including a polysaccharide matrix reversibly cross-linked with polyvalent metal cations (*see, e.g.,* U.S. Patent Application Publication No. 2009/0202610); titania nanotubes; nanostructured surfaces; biocompatible dextran-coated nanoceria with pH-dependent antioxidant properties; polysulfone block polymers; and other biodegradable coatings (see also, e.g., U.S. Patent No. 6,162,487). Other coatings contemplated herein are formulating microparticulate BT compounds with anti-corrosion and antifouling antiseptic coatings used in industry, and include by way of non-limiting example, Carnauba wax fluoropolymer, Xylan®, PTFE, and moly materials.

The microparticulate BT compound concentration (by weight) within the paint or paint coating may, for example, vary from as low as about 0.001% to about 0.1%, depending on the intended use and desired properties of the paint or paint coating. The microparticulate BT compound (or a composition comprising the microparticulate BT compound) incorporated into a paint or paint coating may be combined with at least one other antimicrobial agent (*i.e.,* a second, third, fourth, etc. antimicrobial agent) that when administered in combination have enhanced or synergistic antimicrobial effects as described herein. By way of non-limiting example, an antimicrobial agent that may be included in a composition comprising a microparticulate BT compound includes chlorhexidine; sanguinarine extract; metronidazole;

quaternary ammonium compounds (such as cetylpyridinium chloride); bis-guanides (*e.g.*, chlorhexidine digluconate, hexetidine, octenidine, alexidine); halogenated bisphenolic compounds (*e.g.*, 2,2' methylenebis-(4-chloro-6-bromophenol) or other phenolic antibacterial compounds; alkylhydroxybenzoate; cationic antimicrobial peptides; aminoglycosides; quinolones; lincosamides; penicillins; cephalosporins, macrolides; tetracyclines; other antibiotics known in the art; *Coleus forskohlii* essential oil; silver or colloidal silver antimicrobials; tin- or copper-based antimicrobials; Manuka oil; oregano; thyme; rosemary; or other herbal extracts; and grapefruit seed extract. The compositions may also further optionally comprise a surfactant, diluent or carrier, buffer, and/or bleaching agent, which are described above and herein.

Compositions Comprising Microparticulate Bismuth-Thiols Formulated with Concrete and Cement Compounds. Certain other embodiments contemplate incorporation of the microparticulate BT compounds described herein in industrial cements and in or on concrete, mortar, and grout, including coating of concrete, mortar, and grout for preventing and/or controlling (*i.e.*, slowing, retarding, inhibiting) biofilm development, disrupting a biofilm, or reducing the amount of biofilm present on a concrete surface. Microorganisms that grow on and within concrete structures reduce the useful life of the product and can pose health hazards to animals and humans who are exposed to microorganisms present on a concrete surface (*see, e.g.*, Idachaba et al., *Waste Manag. Res.* 19:284-91 (2001); Idachaba et al., *J. Hazard. Mater.* 90:279-95 (2002); Tazaki, *Canadian Mineralogist* 30:431-34 (1992)).

As used herein and in the art, cement refers to the dry powder substance (typically limestone that may also contain additional substances) that is used to bind the aggregate materials of concrete. Exemplary cements that are described in the art are called Ordinary Portland Cement, Portland blast furnace cement, masonry cements, slag-lime cements, and calcium aluminate cements. Upon the addition of water and/or additives the cement mixture is referred to as concrete, especially if aggregates have been added. Concrete is a composite material consisting of aggregate (*e.g.*, gravel and sand), cement, and water. Cements used in construction are characterized as hydraulic or non-hydraulic. Hydraulic cements are typically used for finishing brick buildings in wet climates; for masonry construction of harbor works and the like that are in contact with seawater; and development of strong concretes.

The compositions described herein that comprise microparticulate BT compounds may be used to coat or may be mixed with cement that is used

for concrete structures including, for example, bridges, buildings, pipes, elevated highways, tunnels, parking garages, offshore oil platforms, piers, dam walls, water systems and pipelines, floors, counter tops, sidewalks, driveways, loading docks, skate park structures, and radioactive waste holding structures. The microparticulate BT compounds described herein may be incorporated into cements as described in the art (*see, e.g.,* U.S. Patent No. 7,507,281). The alkalinity of the cement or concrete may also enhance the anti-microbial effect of the microparticulate BT compounds.

Cement can also be degraded by acidifying bacteria, such as *Thiobacillus thiooxidans*. As non-limiting examples by way of illustration and not limitation, a bismuth thiol compound, BisEDT (but not a presently described microparticulate BT compound), was shown to retard the growth of *T. thiooxidans* in concrete used for waste and nuclear disposal systems. The effective antibacterial range of BisEDT in concrete was shown to be 10-500μg/g, or 0.001-0.05%. Higher BisEDT levels interfered with concrete strength. Other compounds, such as BisPYR, may be useful for inhibiting fouling and biofilm development by molds and algae. The present embodiments contemplate replacement of bismuth thiol compounds and other antimicrobials with the presently described microparticulate BT compounds to provide the advantages disclosed herein, including the range of antimicrobial activities, solubility and bioavailability, anti-biofilm effects, non-toxicity, enhancement of antibiotic efficacies, and other properties as described herein.

Microparticulate BT compounds may be introduced onto a concrete surface manually or automatically as a gel, spray, paste, liquid, or powder or other forms known to a person skilled in the art. In particular embodiments, a microparticulate BT compound, either in powder or liquid form is mixed with at least one or more additional ingredients, which may include at least one additional biologically active ingredient and/or a biologically inactive excipient, to formulate the product, which is delivered or injected periodically into or onto the concrete structure (*i.e.,* onto a surface of the concrete structure that is exposed, particularly a surface exposed to water). Compositions may be prepared by a person skilled in the art using any number of methods known in the art. By way of example, a microparticulate BT compound in an anti-microbial effective amount combined with DMSO may be used (*e.g.,* 1 mg/ml microparticulate BT compound in DMSO). With routine use, a level of microparticulate BT compound that is sufficient to prevent biofilm formation is desired. However, in other embodiments, the level of microparticulate BT

compound may be higher for reducing, removing, disrupting, or eliminating existing biofilms present on a concrete surface.

A microparticulate BT compound may also be formulated to release slowly from the composition comprising the microparticulate BT compound applied to a surface of a concrete structure. A microparticulate BT compound can also be incorporated into a coating (*e.g.,* an epoxy coating), which can be applied to, adfixed to, adhered to, or in some manner placed into contact with a surface of a concrete structure. A microparticulate BT compound may be slowly released from such compositions. A slow-release composition comprising a microparticulate BT compound may be a gel (*e.g.,* a hydrogel, thiomer, aerogel, or organogel) or liquid. An organogel may comprise an organic solvent, lipoic acid, vegetable oil, or mineral oil. A slow-release composition may deliver an antimicrobially effective amount of microparticulate BT compound for 1, 2, 3, 4, 5, 6, or 7 (a week) days or for 2, 3, 4, 5, 6, 7 weeks, or 1, 2, 3, 4, 5, or 6 months.

The microparticulate BT compound (or a composition comprising the microparticulate BT compound) may be combined with at least one other antimicrobial agent (*i.e.,* a second, third, fourth, etc. antimicrobial agent) that when administered in combination have enhanced or synergistic antimicrobial effects as described herein. By way of example, an enhanced or synergistic antimicrobial effect may be observed when a microparticulate BT compound is administered together with an antimicrobial agent that chelates iron. A microparticulate BT compound described herein may be combined with at least one other antimicrobial agent, including a fungicide or an algicide. By way of non-limiting example, an antimicrobial agent that may be included in a composition comprising a microparticulate BT compound includes chlorhexidine; sanguinarine extract; metronidazole; quaternary ammonium compounds (such as cetylpyridinium chloride); bis-guanides (*e.g.,* chlorhexidine digluconate, hexetidine, octenidine, alexidine); halogenated bisphenolic compounds (*e.g.,* 2,2' methylenebis-(4-chloro-6-bromophenol) or other phenolic antibacterial compounds; alkylhydroxybenzoate; cationic antimicrobial peptides; aminoglycosides; quinolones; lincosamides; penicillins; cephalosporins, macrolides; tetracyclines; other antibiotics known in the art; *Coleus forskohlii* essential oil; silver or colloidal silver antimicrobials; tin- or copper-based antimicrobials; Manuka oil; oregano; thyme; rosemary; or other herbal extracts; and grapefruit seed extract. The compositions may also further optionally

comprise a surfactant, diluent or carrier, buffer, and/or bleaching agent, which are described above and herein.

Microparticulate BT compounds that are prepared with hydrophobic thiols (*e.g.,* thiochlorophenol) may be used and may exhibit greater capability than less hydrophobic BT compounds to adhere to concrete surfaces, particularly those exposed to water. BT compounds that have a net negative charge, such as those having a 1:2 molar ratio (bismuth to thiol) may also have favorable adhesive properties.

Microparticulate BTs in Rubber, Silicone and Plastic Products. Certain embodiments contemplate incorporation of the herein described microparticulate BT compounds in or on artificial surfaces that comprise fabricated natural and synthetic rubber and/or rubber coatings, including silicone and silicone coatings, to reduce biofilms and biofouling of such rubber surfaces, for example, in medical devices (*e.g.,* catheters, stents, Foley catheters and other urological catheters, gastrostomy tubes, feeding tubes, etc.), orthopedic devices, dental devices, industrial devices, electronic devices, surfaces, such as those present in and on vehicles of all types, including automobiles, tires, door and window profiles, hoses, belts, matting, flooring and dampeners (anti-vibration mounts), trains, planes, ships, boats, submarines, pilings, pipes, pipelines, tubing and textiles, plumbing/water fixtures, houseware products, flooring materials, footwear products, athletic apparatus, mobile phones, computer equipment and compounds that use organic fillers, outdoor products including decking, awnings, tarps, roofing membranes, and swimming pool liners, and also including disinfection products and systems for food and beverage preservation, pharmaceuticals manufacturing, and chemical and water disinfection.

The presently described microparticulate BT compounds may be incorporated into these and other natural and artificial rubber products by integration of the BT compositions and methods described herein, with fabrication processes that are known for these categories of articles of manufacture. As non-limiting examples by way of illustration and not limitation, BTs (but not the presently described microparticulate BTs) have been incorporated into hydrogel-coated polyurethane rods and Dacron grafts (Domenico et al. *Antimicrob Agents Chemother* 2001;45:1417-1421; Domenico et al., *Peptides* 2004;25:2047-53). WO/2002/077095 and Japanese Patent Application 1997-342076 describe pre-vulcanized and/or vulcanized raw rubber formulations containing silver-based compounds to provide antimicrobial

characteristics; U.S. Pat. Nos. 6,448,306, 6,555,599, 6,638,993, 6,848,871, 6,852,782, 6,943,205, and 7,060,739 teach the use of silver-based antimicrobial agents in a rubber matrix. Drug eluting silicone compositions may comprise an antimicrobial agent dispersed in modified, biologically active binders that can be applied to medical devices or other surfaces without using inert polymer carriers (US Application Pub. No. 2009/0043388).

Silicone oils generally have molecular weights in the range of 2,000 to 30,000 with viscosities ranging from 20 to 1,000 centistokes. Silicone rubbers generally have molecular weights in the range of 40,000 to 100,000 with viscosities ranging from 10 to 1,000 stokes. Silicone is used in a variety of materials that are typically subject to microbial fouling. These include sealants, caulk, grease, oil, spray, rubber, hose and implants. Silicone-based antifouling and other antimicrobial coatings have been described but suffer from shortcomings associated with poor efficacy, poor durability, poor biocompatibility, loss of antimicrobial activity, short useful lifetime, high cost of materials and other issues (*e.g.*, Schultz *J Fluids Eng* 2004;126:1039-47; US Patent 4,025,693; Yan & Li. *Ophthalmologica* 2008;222:245-8; US Patent 6,221,498; US Patent 7,381,751; European Patent Application EP0506113; Sawada et al. *JPRAS* 1990;43:78-82; Tiller et al. *Surface Coatings International Part B: Coatings Transactions* 2005;88:1-82; Juhni & Newby *Proceedings Annual Meeting Adhesion Society* 2005;28:179-181; Ozdamar et al. *Retina* 1999;19:122-6; Piccirillo et al. *J Mater Chem* 2009;19:6167; US Pub. 2009/0215924; Bayston et al. *Biomaterials* 2009;30:3167-73; Gottenbos et al. *Biomaterials* 2002;23:1417-23; Millsap et al. *Antonie Van Leeuwenhoek* 2001;79:337-43). While these publications describe methods for the incorporation of antimicrobial materials into rubber articles of manufacture, none of the products or processes that they describe offer the advantages provided by the herein described microparticulate BTs.

The present embodiments thus contemplate substitution of the herein described microparticulate BTs in these and similar rubber (including silicone) products and processes, as well as in plastics and polymer fabrication methodologies such as those to which reference is made below. In each of these and other known fabrication contexts, the presently described microparticulate BTs may be incorporated based on the disclosure herein, in place of other antimicrobial agents, to afford the herein disclosed advantages as provided by these microparticulate BTs, including the range of antimicrobial

activities, solubility and bioavailability, anti-biofilm effects, non-toxicity, enhancement of antibiotic efficacies, and other properties as described herein.

BT compounds can also be formulated, for instance, at low concentrations that do not interfere with the rubber fabrication process, into products for reducing biofilms and preventing fouling in or on silicone products. The microparticulate BT concentration (by weight) within the silicone may, for example, vary from as low as about 0.0001% to about 0.1%, depending on the intended uses and properties of the silicone rubber product. The herein described microparticulate BTs similarly may be incorporated as coatings on silicone, or in silicone gels or oils, to prevent or treat biofilms on silicone surfaces for extended time periods. Silicone rubber injection port valves are described in WO/2008/064173 that exude silicone oil periodically, such that the presence in such exudates of effective antimicrobial levels of the herein described microparticulate BT confers anti-biofilm and/or anti-fouling capabilities on manufactured articles containing such valves or similarly configured silicone rubber devices. The erodible oil spreads across any surface in the vicinity of the valve, providing a renewable source of protection for extended time periods. This configuration may, for instance, be built into the under-surfaces of ship hulls, or into other surfaces exposed to water or humidity.

For enhanced retention of BT on rubber surfaces, the herein described microparticulate BTs may be selected to possess greater hydrophobicity by virtue of the particular thiol moiety, for example by using a hydrophobic thiol (*e.g.*, thiochlorophenol), which may have enhanced adhesive properties, and/or by including BTs that are made to have a net negative charge (*e.g.,* 1:2 molar ratio of bismuth to thiol) which may also possess enhanced adhesive properties. Silicone materials can, for example, be assembled in the presence of appropriate concentrations of the herein described microparticulate BTs at temperatures of 100 °C or below. Bioerodible materials can also be produced to allow gradual release of such BTs at levels that thwart biofilm formation, for example, around 1-2 ppm. In other embodiments, rubber and/or plastic components are contemplated that are fabricated from materials which slowly elute microparticulate BT compounds and which can be replaced regularly, to prevent biofouling in various industrial systems or medical devices.

In certain other embodiments, and in a manner analogous to that described above for compositions and methods that relate to BT incorporation

into rubber (including silicone) items, the presently described microparticulate BT compounds may also be incorporated into these and other plastic and polymeric products by integration of the BT compositions and methods described herein, with fabrication processes that are known for these categories of articles of manufacture.

Non-limiting examples of uses for such microparticulate BT-containing plastic products include plastics and plastic coatings in medical devices, orthopedic devices, dental devices, industrial devices, electronic devices, walls, floors, ceilings, roofs, and other surfaces, such as those present in and on vehicles of all types, including automobiles, trains, planes, ships, boats, submarines, pilings, pipes, pipelines, and textiles, sprinkler heads, hair care products, plumbing/water fixtures, houseware products, footwear products, athletic apparatus, mobile phones, compounds that use organic fillers, outdoor products that include decking, awnings, tarps, roofing membranes, and swimming pool liners, and other products that include those used in food and beverage preservation, and in pharmaceutical, chemical and water disinfection.

Modern plastic materials have been in use since the 1930s. Plastics are typically made of polymers and, usually, additives. Typical polymers include: synthetic resins, styrenes, polyolefins, polyamides, fluoropolymers, vinyls, acrylics, polyurethanes, cellulosics, imides, acetals, polycarbonates, and polysulfphones. In order to improve physical characteristics of polymers, additives such as plasticizers are often used, which serve as a source of nutrients for microorganisms. Examples of such modern plasticizers include phthalates, adipates, and other esters. These and other plasticizers may be particularly susceptible to bacteria and fungi, especially in high moisture areas, leading to microbial surface growth and development of spores, which may result in one or more of infections in humans and animals, allergic reactions, unpleasant odors, staining, embrittlement of the plastic, premature product failure and other undesirable consequences.

Modifying plastic products during or after the fabrication process by the introduction of antifouling and other antimicrobial coatings has been described, but typically suffers from shortcomings associated with poor efficacy, poor durability, poor biocompatibility, loss of antimicrobial activity, short useful lifetime, high cost of materials and other issues (*e.g.,* U.S. Pat. Nos. 3,624,062; 4,086,297; 4,663,077; 3,755,224; 3,890,270; 6,495,613; 4,348,308; 5,654,330; 5,281,677; 6,120,790; 5,906,825; 7,419,681, 5,028,664; 6,162,487; Markarian, *Plastics, Additives and Compounding* 2009, 11:18-22; EP 927 222 B1; JP 08-

157641; CN 1528470 A; Masatoshi et al. 2006;51:18-23; U.S. Pub. Nos. 2008/0071229, 2009/0202610 and 2009/0043388); none of the existing approaches offers the advantages provided by the herein described microparticulate BTs. Nevertheless, generally known to the artisan will be incorporation of an antimicrobial agent into or onto a plastic product according to a strategy such as (a) adsorption of the agent on the polymer surface (passively or via surfactants); (b) introduction into a polymer of an antimicrobial coating which is applied on the surface of a molding device; (c) incorporation into the bulk phase of the polymeric substrate material; (d) covalent bonding of the agent to the polymer surface; and/or (e) mixing an antimicrobial agent with a polymer-forming (*e.g.,* polyurethane) component prior to the polymerization reaction, to give the finished polymer.

For example, the herein described microparticulate BTs can be introduced into these and similar systems manually or automatically, as gels, sprays, liquids or powders. In one embodiment, for instance, the microparticulate BT in powder or liquid form is mixed with the ingredients for plastic fabrication, including active components (*e.g.*, polymeric precursors, catalysts, reaction initiators, crosslinkers, etc.) and excipients (*e.g.*, carrier solvents, mold-releasing agents, dyes or colorants, plasticizers, etc.), involved in the production mixture, which is injected periodically into the fabrication system. For example, a 1 mg/ml solution or suspension of microparticulate BT in DMSO may be injected periodically into the polymer-forming reaction liquor, or sprayed into the working parts of a molding unit, to achieve desired anti-biofilm concentrations in the finished product.

Accordingly, these and certain of the related herein disclosed embodiments contemplate inclusion in such products and processes of the presently disclosed microparticulate BT compositions, which may include one or more microparticulate BT, and which may also optionally further include an antibiotic such as a synergizing or an enhancing antibiotic as described herein.

Non-limiting examples of bacteria against which the herein described compositions and methods may find beneficial use, according to certain embodiments as described herein, include *Staphylococcus aureus (S. aureus)*, MRSA (methicillin-resistant *S. aureus*), *Staphylococcus epidermidis* , MRSE (methicillin-resistant *S. epidermidis*), *Mycobacterium tuberculosis, Mycobacterium avium, Pseudomonas aeruginosa,* drug-resistant *P. aeruginosa, Escherichia coli,* enterotoxigenic *E. coli,* enterohemorrhagic *E. coli, Klebsiella pneumoniae, Clostridium difficile, Heliobacter pylori, Legionella pneumophila,*,

Enterococcus faecalis, methicillin-susceptible *Enterococcus faecalis, Enterobacter cloacae, Salmonella typhimurium, Proteus vulgaris, Yersinia enterocolitica, Vibrio cholera, Shigella flexneri,* vancomycin-resistant *Enterococcus* (VRE), *Burkholderia cepacia* complex, *Francisella tularensis, Bacillus anthracis, Yersinia pestis, Pseudomonas aeruginosa,* vancomycin–sensitive and vancomycin-resistant enterococci (*e.g., E. faecalis, E. faecium*), methicillin-sensitive and methicillin-resistant staphylococci (*e.g., S. aureus , S. epidermidis*) and *Acinetobacter baumannii, Staphylococcus haemolyticus, Staphylococcus hominis, Enterococcus faecium, Streptococcus pyogenes, Streptococcus agalactiae, Bacillus anthracis, Klebsiella pneumonia, Proteus mirabilis, Proteus vulgaris, Yersinia enterocolytica, Stenotrophomonas maltophilia, Streptococcus pneumonia*, penicillin-resistant *Streptococcus pneumonia*, *Burkholderia cepacia*, *Bukholderia multivorans*, *Mycobacterium smegmatis* and *E. cloacae.*

The practice of certain embodiments of the present invention will employ, unless indicated specifically to the contrary, conventional methods of microbiology, molecular biology, biochemistry, cell biology, virology and immunology techniques that are within the skill of the art, and reference to several of which is made below for the purpose of illustration. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, et al. *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); Maniatis et al. *Molecular Cloning: A Laboratory Manual* (1982); *DNA Cloning: A Practical Approach*, vol. I & II (D. Glover, ed.); *Oligonucleotide Synthesis* (N. Gait, ed., 1984); *Nucleic Acid Hybridization* (B. Hames & S. Higgins, eds., 1985); *Transcription and Translation* (B. Hames & S. Higgins, eds., 1984); *Animal Cell Culture* (R. Freshney, ed., 1986); Perbal, *A Practical Guide to Molecular Cloning* (1984).

Unless the context requires otherwise, throughout the present specification and claims, the word "comprise" and variations thereof, such as, "comprises" and "comprising" are to be construed in an open, inclusive sense, that is as "including, but not limited to".

Reference throughout this specification to "one embodiment" or "an embodiment" or "an aspect" means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, the appearances of the phrases "in one embodiment" or "in an embodiment" in various places throughout this specification are not necessarily all referring to the same

embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments.

As noted above, certain invention embodiments described herein relate to agricultural, industrial, manufacturing and other formulations of the described BT compounds (*e.g.*, BisEDT and/or BisBAL), which formulations may in certain further embodiments comprise one or more antibiotic compounds as described herein, for instance, amikacin, ampicillin, cefazolin, cefepime, chloramphenicol, ciprofloxacin, clindamycin (or another lincosamide antibiotic), daptomycin (Cubicin®), doxycycline, gatifloxacin, gentamicin, imipenim, levofloxacin, linezolid (Zyvox®), minocycline, nafcilin, paromomycin, rifampin, sulphamethoxazole, tobramycin and vancomycin; or a carbapenem antibiotic, a cephalosporin antibiotic, a fluoroquinolone antibiotic, a glycopeptide antibiotic, a lincosamide antibiotic, a penicillinase-resistant penicillin antibiotic, and/or an aminopenicillin antibiotic, and/or an aminoglycoside antibiotic such as amikacin, arbekacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, rhodostreptomycin, streptomycin, tobramycin or apramycin, and/or a lipopeptide antibiotic such as daptomycin (Cubicin®), or an oxazolidinone antibiotic such as linezolid (Zyvox®). These and related formulations may comprise the BT compound(s) (and optionally one or more antibiotics) in a suitable carrier, excipient or diluent and in an effective amount, as disclosed herein, when administered to a plant or animal or applied to a natural or artificial surface, such as a plant, animal or article of manufacture in or on which is present a bacterial infection which may be biofilm-related (*e.g.*, in which bacteria capable of promoting biofilm formation may be present but a biofilm is not yet detectable) or that contains a bacterial infection such as a biofilm or other bacterial presence.

Administration or incorporation of the BT compounds described herein, or their salts, in pure form or in an appropriate agricultural, manufacturing or other industrial composition, can be carried out via any of the accepted modes of administration or incorporation of agents for serving similar utilities. Application, incorporation or administration of a composition includes, in preferred embodiments, directly contacting the composition with the subject plant or animal or article of manufacture undergoing treatment, which may be at one or more localized or widely distributed surface sites and which may generally refer to contacting the topical formulation with an acute or chronic infection site (*e.g.*, a wound site on a plant surface) that is surrounded by intact tissue but need not be so limited; for instance, certain embodiments

contemplate as a topical application the administration of a topical formulation described herein to injured, abraded or damaged natural or artificial surfaces.

The formulations (*e.g.*, agricultural compositions) may be prepared by combining the described BT compound (*e.g.*, comprising a compound described in U.S. RE37,793, U.S. 6,248,371, U.S. 6,086,921, and/or U.S. 6,380,248 and/or prepared according to the present disclosure such as the herein described microparticulate BT suspensions), and in certain related embodiments as described herein by combining one or more desired antibiotics (*e.g.*, an aminoglycoside antibiotic such as amikacin) separately or together with the BT compound, with an appropriate vehicle, dispersant, carrier, diluent or excipient for use in preparation of the formulation as may vary depending upon the intended use, and may be formulated into preparations in solid, semi-solid, gel, cream, colloid, suspension or liquid or other topically applied forms, such as powders, granules, ointments, solutions, washes, gels, pastes, plasters, paints, bioadhesives, microsphere suspensions, and aerosol sprays.

Compositions of these and related embodiments are formulated so as to allow the active ingredients contained therein, and in particularly preferred embodiments the herein described BT compound(s) alone or in combination with one or more desired antibiotics (*e.g.*, a carbapenem antibiotic, a cephalosporin antibiotic, a fluoroquinolone antibiotic, a glycopeptide antibiotic, a lincosamide antibiotic, a penicillinase-resistant penicillin antibiotic, and an aminopenicillin antibiotic, or an aminoglycoside antibiotic such as amikacin, or rifamycin) which may be applied simultaneously or sequentially and in either order, to be bioavailable upon administration of the formulation containing the BT compound(s) and/or antibiotic composition(s) to a desired site and optionally to surrounding natural or artificial surfaces of a plant or animal (including human) subject or of an article of manufacture. Certain embodiments disclosed herein contemplate administration to and/or incorporation into such a subject or article of a BT compound and of an antibiotic, including administration that may be simultaneous or sequential and in either order, but the invention is not intended to be so limited and in other embodiments expressly contemplates a distinct route of administration for the BT compound relative to the route of administration of the antibiotic. Thus, the antibiotic may be administered by any route of administration as described herein, while the BT compound may be administered by a route that is independent of the route used for the antibiotic.

The formulations described herein deliver an effective amount of the antiseptic agent(s) (and optionally the antibiotic(s)) to the desired site, such

as an infection site or a site where it is desired to prevent infection or biofilm formation.

As noted above, the present formulations may take any of a wide variety of forms, and include, for example, liquids, suspensions, plasters, creams, lotions, solutions, sprays, gels, ointments, pastes or the like, and/or may be prepared so as to contain liposomes, micelles, and/or microspheres. See, *e.g.,* U.S. Patent No. 7,205,003. For instance, creams, as is well known in the arts of pharmaceutical and cosmeceutical formulation, are viscous liquids or semisolid emulsions, either oil-in-water or water-in-oil. Cream bases are water-washable, and contain an oil phase, an emulsifier, and an aqueous phase. The oil phase, also called the "internal" phase, is generally comprised of petrolatum and a fatty alcohol such as cetyl or stearyl alcohol. The aqueous phase usually, although not necessarily, exceeds the oil phase in volume, and generally contains a humectant. The emulsifier in a cream formulation is generally a nonionic, anionic, cationic or amphoteric surfactant.

Solutions are homogeneous mixtures prepared by dissolving one or more chemical substances (solutes) in a liquid such that the molecules of the dissolved substance are dispersed among those of the solvent. The solution may contain other chemicals to buffer, stabilize or preserve the solute. Common examples of solvents used in preparing solutions are ethanol, water, propylene glycol or any other vehicle.

Gels are semisolid, suspension-type systems. Single-phase gels contain organic macromolecules distributed substantially uniformly throughout the carrier liquid, which is typically aqueous, but also, preferably, contain an alcohol, and, optionally, an oil. Preferred "organic macromolecules," *i.e.,* gelling agents, may be chemically crosslinked polymers such as crosslinked acrylic acid polymers, for instance, the "carbomer" family of polymers, *e.g.,* carboxypolyalkylenes, that may be obtained commercially under the Carbopol® trademark. Also preferred in certain embodiments may be hydrophilic polymers such as polyethylene oxides, polyoxyethylene-polyoxypropylene copolymers and polyvinylalcohol; cellulosic polymers such as hydroxypropyl cellulose, hydroxyethyl cellulose, hydroxypropyl methylcellulose, hydroxypropyl methylcellulose phthalate, and methyl cellulose; gums such as tragacanth and xanthan gum; sodium alginate; and gelatin. In order to prepare a uniform gel, dispersing agents such as alcohol or glycerin can be added, or the gelling agent can be dispersed by trituration, mechanical mixing or stirring, or combinations thereof.

Ointments, as also well known in the art, are semisolid preparations that are typically based on petrolatum or other petroleum derivatives. The specific ointment base to be used, as will be appreciated by those skilled in the art, is one that will provide for a number of desirable characteristics, *e.g.*, emolliency or the like. As with other carriers or vehicles, an ointment base should be inert, stable, nonirritating, and nonsensitizing. As explained in Remington: The Science and Practice of Pharmacy, 19th Ed. (Easton, Pa.: Mack Publishing Co., 1995), at pages 1399-1404, ointment bases may be grouped in four classes: oleaginous bases; emulsifiable bases; emulsion bases; and water-soluble bases. Oleaginous ointment bases include, for example, vegetable oils, fats obtained from animals, and semisolid hydrocarbons obtained from petroleum. Emulsifiable ointment bases, also known as absorbent ointment bases, contain little or no water and include, for example, hydroxystearin sulfate, anhydrous lanolin, and hydrophilic petrolatum. Emulsion ointment bases are either water-in-oil (W/O) emulsions or oil-in-water (O/W) emulsions, and include, for example, cetyl alcohol, glyceryl monostearate, lanolin, and stearic acid. Preferred water-soluble ointment bases are prepared from polyethylene glycols of varying molecular weight (*see, e.g.,* Remington, *Id.*).

Pastes are semisolid dosage forms in which the active agent is suspended in a suitable base. Depending on the nature of the base, pastes are divided between fatty pastes or those made from single-phase aqueous gels. The base in a fatty paste is generally petrolatum or hydrophilic petrolatum or the like. The pastes made from single-phase aqueous gels generally incorporate carboxymethylcellulose or the like as a base.

Formulations may also be prepared with liposomes, micelles, and microspheres. Liposomes are microscopic vesicles having one (unilamellar) or a plurality (multilamellar) of lipid walls comprising a lipid bilayer, and, in the present context, may encapsulate and/or have adsorbed to their lipid membranous surfaces one or more components of the formulations herein described, such as the antiseptic, or certain carriers or excipients. Liposomal preparations herein include cationic (positively charged), anionic (negatively charged), and neutral preparations. Cationic liposomes are readily available. For example, N[1-2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA) liposomes are available under the tradename Lipofectin® (GIBCO BRL, Grand Island, N.Y.). Similarly, anionic and neutral liposomes are readily available as well, *e.g.,* from Avanti Polar Lipids (Birmingham, AL), or can be easily prepared

using readily available materials. Such materials include phosphatidyl choline, cholesterol, phosphatidyl ethanolamine, dioleoylphosphatidyl choline (DOPC), dioleoylphosphatidyl glycerol (DOPG), and dioleoylphoshatidyl ethanolamine (DOPE), among others. These materials can also be mixed with DOTMA in appropriate ratios. Methods for making liposomes using these materials are well known in the art.

Micelles are known in the art as comprised of surfactant molecules arranged so that their polar headgroups form an outer spherical shell, while the hydrophobic, hydrocarbon chains are oriented towards the center of the sphere, forming a core. Micelles form in an aqueous solution containing surfactant at a high enough concentration so that micelles naturally result. Surfactants useful for forming micelles include, but are not limited to, potassium laurate, sodium octane sulfonate, sodium decane sulfonate, sodium dodecane sulfonate, sodium lauryl sulfate, docusate sodium, decyltrimethylammonium bromide, dodecyltrimethylammonium bromide, tetradecyltrimethylammonium bromide, tetradecyltrimethyl-ammonium chloride, dodecylammonium chloride, polyoxyl-8 dodecyl ether, polyoxyl-12 dodecyl ether, nonoxynol 10, and nonoxynol 30.

Microspheres, similarly, may be incorporated into the presently described topical formulations. Like liposomes and micelles, microspheres essentially encapsulate one or more components of the present formulations. They are generally, but not necessarily, formed from lipids, preferably charged lipids such as phospholipids. Preparation of lipidic microspheres is well known in the art.

Various additives, as known to those skilled in the art, may also be included in the formulations. For example, solvents, including relatively small amounts of alcohol, may be used to solubilize certain formulation components. Examples of suitable enhancers include, but are not limited to, ethers such as diethylene glycol monoethyl ether (available commercially as Transcutol®) and diethylene glycol monomethyl ether; surfactants such as sodium laurate, sodium lauryl sulfate, cetyltrimethylammonium bromide, benzalkonium chloride, Poloxamer® (231, 182, 184), Tween® (20, 40, 60, 80), and lecithin (U.S. Pat. No. 4,783,450); alcohols such as ethanol, propanol, octanol, benzyl alcohol, and the like; polyethylene glycol and esters thereof such as polyethylene glycol monolaurate (PEGML; see, *e.g.*, U.S. Pat. No. 4,568,343); amides and other nitrogenous compounds such as urea, dimethylacetamide (DMA), dimethylformamide (DMF), 2-pyrrolidone, 1 -methyl-

2-pyrrolidone, ethanolamine, diethanolamine, and triethanolamine; terpenes; alkanones; and organic acids, particularly citric acid and succinic acid. Azone® and sulfoxides such as DMSO and C_{10}MSO may also be used, but are less preferred.

Certain permeation enhancers may include those lipophilic co-enhancers typically referred to as "plasticizing" enhancers, *i.e.,* enhancers that have a molecular weight in the range of about 150 to 1000 daltons, an aqueous solubility of less than about 1 wt %, preferably less than about 0.5 wt %, and most preferably less than about 0.2 wt %. The Hildebrand solubility parameter of plasticizing enhancers is in the range of about 2.5 to about 10, preferably in the range of about 5 to about 10. Preferred lipophilic enhancers are fatty esters, fatty alcohols, and fatty ethers. Examples of specific and most preferred fatty acid esters include methyl laurate, ethyl oleate, propylene glycol monolaurate, propylene glycerol dilaurate, glycerol monolaurate, glycerol monooleate, isopropyl n-decanoate, and octyldodecyl myristate. Fatty alcohols include, for example, stearyl alcohol and oleyl alcohol, while fatty ethers include compounds wherein a diol or triol, preferably a C_2-C_4 alkane diol or triol, are substituted with one or two fatty ether substituents. Additional permeation enhancers will be known to those of ordinary skill in the art of topical drug delivery, and/or are described in the relevant literature. See, *e.g.,* Percutaneous Penetration Enhancers, eds. Smith et al. (CRC Press, Boca Raton, FL, 1995).

Various other additives may be included in the topical formulations according to certain embodiments of the present invention, in addition to those identified above. These include, but are not limited to, antioxidants, astringents, perfumes, preservatives, emollients, pigments, dyes, humectants, propellants, and sunscreen agents, as well as other classes of materials whose presence may be cosmetically, medicinally or otherwise desirable. Typical examples of optional additives for inclusion in the formulations of certain embodiments of the invention are as follows: preservatives such as sorbate; solvents such as isopropanol and propylene glycol; astringents such as menthol and ethanol; emollients such as polyalkylene methyl glucosides; humectants such as glycerine; emulsifiers such as glycerol stearate, PEG-100 stearate, polyglyceryl-3 hydroxylauryl ether, and polysorbate 60; sorbitol and other polyhydroxyalcohols such as polyethylene glycol; sunscreen agents such as octyl methoxyl cinnamate (available commercially as Parsol MCX) and butyl methoxy benzoylmethane (available

under the tradename Parsol 1789); antioxidants such as ascorbic acid (vitamin C), α-tocopherol (Vitamin E), β-tocopherol , γ-tocopherol, δ-tocopherol, ε-tocopherol , ζ_1-tocopherol, ζ_2-tocopherol, η-tocopherol , and retinol (vitamin A); essential oils, ceramides, essential fatty acids, mineral oils, wetting agents and other surfactants such as the PLURONIC® series of hydrophilic polymers available from BASF (Mt. Olive, NJ), vegetable oils (*e.g.,* soy bean oil, palm oil, liquid fraction of shea butter, sunflower oil), animal oils (*e.g.*, perhydrosqualene), mineral oils, synthetic oils, silicone oils or waxes (*e.g.*, cyclomethicone and dimethicone), fluorinated oils (generally perfluoropolyethers), fatty alcohols (*e.g.*, cetyl alcohol), and waxes (*e.g.,* beeswax, carnauba wax, and paraffin wax); skin-feel modifiers; and thickeners and structurants such as swelling clays and cross-linked carboxypolyalkylenes that may be obtained commercially under the Carbopol® trademark.

Other additives include agents such as, by way of example, pyrrolidine carboxylic acid and amino acids; organic antimicrobial agents such as 2,4,4'-trichloro-2-hydroxy diphenyl ether (triclosan) and benzoic acid; anti-inflammatory agents such as acetylsalicylic acid and glycyrrhetinic acid; anti-seborrhoeic agents such as retinoic acid; vasodilators such as nicotinic acid; inhibitors of melanogenesis such as kojic acid; and mixtures thereof. Other advantageously included active agents may be present, for example, α-hydroxyacids, α-ketoacids, polymeric hydroxyacids, moisturizers, collagen, marine extracts, and antioxidants such as ascorbic acid (vitamin C), α-tocopherol (Vitamin E) or other tocopherols such as those described above, and retinol (vitamin A), and/or suitable salts, esters, amides, or other derivatives thereof. Additional agents include those that are capable of improving oxygen supply in living tissue, as described, for example, in WO 94/00098 and WO 94/00109. Sunscreens may also be included.

The formulations of certain embodiments of the invention may also include conventional additives such as opacifiers, fragrance, colorant, gelling agents, thickening agents, stabilizers, surfactants, and the like. Other agents may also be added, such as antimicrobial agents, to prevent spoilage upon storage, *i.e.*, to inhibit growth of microbes such as yeasts and molds. Suitable antimicrobial agents are typically selected from methyl and propyl esters of *p*-hydroxybenzoic acid (*e.g.*, methyl and propyl paraben), sodium benzoate, sorbic acid, imidurea, and combinations thereof.

The topical formulations may also contain, in addition to the BT compound, (*e.g.*, as substantially homogeneous microparticles as provided

herein, and optionally in combination with one or more synergizing antibiotics as described herein), an effective amount of one or more additional active agents suitable for a particular mode of administration or incorporation.

A pharmacologically acceptable carrier may also be incorporated in the topical formulation of certain present embodiments and may be any carrier conventionally used in the art. Examples include water, lower alcohols, higher alcohols, honey, polyhydric alcohols, monosaccharides, disaccharides, polysaccharides, sugar alcohols such as, for example, glycols (2-carbon), glycerols (3-carbon), erythritols and threitols (4-carbon), arabitols, xylitols and ribitols (5-carbon), mannitols, sorbitols, dulcitols and iditols (6-carbon), isomaltols, maltitols, lactitols and polyglycitols, hydrocarbon oils, fats and oils, waxes, fatty acids, silicone oils, nonionic surfactants, ionic surfactants, silicone surfactants, and water-based mixtures and emulsion-based mixtures of such carriers.

Topical formulation embodiments of the present invention may be applied regularly to whatever natural (*e.g.*, plant or animal, including human) or artificial (*e.g.,* article of manufacture) surface requires treatment with the frequency and in the amount necessary to achieve the desired results. The frequency of treatment depends on the nature of the application, the strength of the active ingredients (*e.g.*, BT compound and optionally one or more additional active ingredients, such as an antibiotic, *e.g.*, amikacin or other antibiotic) in the particular embodiment, the effectiveness of the vehicle used to deliver the active ingredients, and the ease with which the formula is removed by environmental factors (*e.g.*, physical contact with other materials or objects, precipitation, wind, temperature).

Typical concentrations of active substances such as the BT compound in the compositions described herein can range, for example, from about 0.001-30% by weight based on the total weight of the composition, to about 0.01-5.0%, and more preferably to about 0.1-2.0%. As one representative example, compositions of these embodiments of the present invention may be applied to a natural or artificial surface at a rate equal to from about 1.0 mg/cm^2 to about 20.0 mg/cm^2. Representative examples of topical formulations include, but are not limited to, aerosols, alcohols, anhydrous bases, aqeuous solutions, creams, emulsions (including either water-in-oil or oil-in-water emulsions), fats, foams, gels, hydro-alcoholic solutions, liposomes, lotions, microemulsions, ointments, oils, organic solvents, polyols, polymers, powders, salts, silicone derivatives, and waxes. The formulations may include,

for example, chelating agents, conditioning agents, emollients, excipients, humectants, protective agents, thickening agents, or UV absorbing agents. One skilled in the art will appreciate that formulations other than those listed may be used in embodiments of the present invention.

Chelating agents may be optionally included in certain formulations, and may be selected from any natural or synthetic chemical agent which has the ability to bind divalent cationic metals such as Ca^{2+}, Mn^{2+}, or Mg^{2+}. Examples of chelating agents include, but are not limited to EDTA, disodium EDTA, EGTA, citric acid, and dicarboxylic acids.

Conditioning agents may also be optionally included in certain formulations. Examples of conditioning agents include, but are not limited to, acetyl cysteine, N-acetyl dihydrosphingosine, acrylates/behenyl acrylate/dimethicone acrylate copolymer, adenosine, adenosine cyclic phosphate, adensosine phosphate, adenosine triphosphate, alanine, albumen, algae extract, allantoin and deriviatives, aloe barbadensis extracts, aluminum PCA, amyloglucosidase, arbutin, arginine, azulene, bromelain, buttermilk powder, butylene glycol, caffeine, calcium gluconate, capsaicin, carbocysteine, carnosine, beta-carotene, casein, catalase, cephalins, ceramides, chamomilla recutita (matricaria) flower extract, cholecalciferol, cholesteryl esters, coco-betaine, coenzyme A, corn starch modified, crystallins, cycloethoxymethicone, cysteine DNA, cytochrome C, darutoside, dextran sulfate, dimethicone copolyols, dimethylsilanol hyaluronate, DNA, elastin, elastin amino acids, epidermal growth factor, ergocalciferol, ergosterol, ethylhexyl PCA, fibronectin, folic acid, gelatin, gliadin, beta-glucan, glucose, glycine, glycogen, glycolipids, glycoproteins, glycosaminoglycans, glycosphingolipids, horseradish peroxidase, hydrogenated proteins, hydrolyzed proteins, jojoba oil, keratin, keratin amino acids, and kinetin, lactoferrin, lanosterol, lauryl PCA, lecithin, linoleic acid, linolenic acid, lipase, lysine, lysozyme, malt extract, maltodextrin, melanin, methionine, mineral salts, niacin, niacinamide, oat amino acids, oryzanol, palmitoyl hydrolyzed proteins, pancreatin, papain, PEG, pepsin, phospholipids, phytosterols, placental enzymes, placental lipids, pyridoxal 5-phosphate, quercetin, resorcinol acetate, riboflavin, RNA, saccharomyces lysate extract, silk amino acids, sphingolipids, stearamidopropyl betaine, stearyl palmitate, tocopherol, tocopheryl acetate, tocopheryl linoleate, ubiquinone, *vitis vinifera* (grape) seed oil, wheat amino acids, xanthan gum, and zinc gluconate. Conditioning agents other than those listed above may be combined with a

disclosed composition or preparation provided thereby, as can be readily appreciated by one skilled in the art.

In certain embodiments the herein described formulations may also optionally include one or more emollients, examples of which include, but are not limited to, acetylated lanolin, acetylated lanolin alcohol, acrylates/C_{10-30} alkyl acrylate crosspolymer, acrylates copolymer, alanine, algae extract, aloe barbadensis extract or gel, althea officinalis extract, aluminum starch octenylsuccinate, aluminum stearate, apricot (prunus armeniaca) kernel oil, arginine, arginine aspartate, arnica montana extract, ascorbic acid, ascorbyl palmitate, aspartic acid, avocado (persea gratissima) oil, barium sulfate, barrier sphingolipids, butyl alcohol, beeswax, behenyl alcohol, beta-sitosterol, BHT, birch (betula alba) bark extract, borage (borago officinalis) extract, 2-bromo-2-nitropropane-1,3-diol, butcherbroom (ruscus aculeatus) extract, butylene glycol, calendula officinalis extract, calendula officinalis oil, candelilla (euphorbia cerifera) wax, canola oil, caprylic/capric triglyceride, cardamon (elettaria cardamomum) oil, carnauba (copernicia cerifera) wax, carrageenan (chondrus crispus), carrot (daucus carota sativa) oil, castor (ricinus communis) oil, ceramides, ceresin, ceteareth-5, ceteareth-12, ceteareth-20, cetearyl octanoate, ceteth-20, ceteth-24, cetyl acetate, cetyl octanoate, cetyl palmitate, chamomile (anthemis nobilis) oil, cholesterol, cholesterol esters, cholesteryl hydroxystearate, citric acid, clary (salvia sclarea) oil, cocoa (theobroma cacao) butter, coco-caprylate/caprate, coconut (cocos nucifera) oil, collagen, collagen amino acids, corn (zea mays) oil, fatty acids, decyl oleate, dextrin, diazolidinyl urea, dimethicone copolyol, dimethiconol, dioctyl adipate, dioctyl succinate, dipentaerythrityl hexacaprylate/hexacaprate, DMDM hydantoin, DNA, erythritol, ethoxydiglycol, ethyl linoleate, eucalyptus globulus oil, evening primrose (oenothera biennis) oil, fatty acids, tructose, gelatin, geranium maculatum oil, glucosamine, glucose glutamate, glutamic acid, glycereth-26, glycerin, glycerol, glyceryl distearate, glyceryl hydroxystearate, glyceryl laurate, glyceryl linoleate, glyceryl myristate, glyceryl oleate, glyceryl stearate, glyceryl stearate SE, glycine, glycol stearate, glycol stearate SE, glycosaminoglycans, grape (vitis vinifera) seed oil, hazel (corylus americana) nut oil, hazel (corylus avellana) nut oil, hexylene glycol, honey, hyaluronic acid, hybrid safflower (carthamus tinctorius) oil, hydrogenated castor oil, hydrogenated coco-glycerides, hydrogenated coconut oil, hydrogenated lanolin, hydrogenated lecithin, hydrogenated palm glyceride, hydrogenated palm kernel oil, hydrogenated soybean oil, hydrogenated tallow glyceride, hydrogenated vegetable oil,

hydrolyzed collagen, hydrolyzed elastin, hydrolyzed glycosaminoglycans, hydrolyzed keratin, hydrolyzed soy protein, hydroxylated lanolin, hydroxyproline, imidazolidinyl urea, iodopropynyl butylcarbamate, isocetyl stearate, isocetyl stearoyl stearate, isodecyl oleate, isopropyl isostearate, isopropyl lanolate, isopropyl myristate, isopropyl palmitate, isopropyl stearate, isostearamide DEA, isostearic acid, isostearyl lactate, isostearyl neopentanoate, jasmine (jasminum officinale) oil, jojoba (buxus chinensis) oil, kelp, kukui (aleurites moluccana) nut oil, lactamide MEA, laneth-16, laneth-10 acetate, lanolin, lanolin acid, lanolin alcohol, lanolin oil, lanolin wax, lavender (lavandula angustifolia) oil, lecithin, lemon (citrus medica limonum) oil, linoleic acid, linolenic acid, macadamia ternifolia nut oil, magnesium stearate, magnesium sulfate, maltitol, matricaria (chamomilla recutita) oil, methyl glucose sesquistearate, methylsilanol PCA, microcrystalline wax, mineral oil, mink oil, mortierella oil, myristyl lactate, myristyl myristate, myristyl propionate, neopentyl glycol dicaprylate/dicaprate, octyldodecanol, octyldodecyl myristate, octyldodecyl stearoyl stearate, octyl hydroxystearate, octyl palmitate, octyl salicylate, octyl stearate, oleic acid, olive (olea europaea) oil, orange (citrus aurantium dulcis) oil, palm (elaeis guineensis) oil, palmitic acid, pantethine, panthenol, panthenyl ethyl ether, paraffin, PCA, peach (prunus persica) kernel oil, peanut (arachis hypogaea) oil, PEG-8 C12 18 ester, PEG-15 cocamine, PEG-150 distearate, PEG-60 glyceryl isostearate, PEG-5 glyceryl stearate, PEG-30 glyceryl stearate, PEG-7 hydrogenated castor oil, PEG-40 hydrogenated castor oil, PEG-60 hydrogenated castor oil, PEG-20 methyl glucose sesquistearate, PEG-40 sorbitan peroleate, PEG-5 soy sterol, PEG-10 soy sterol, PEG-2 stearate, PEG-8 stearate, PEG-20 stearate, PEG-32 stearate, PEG-40 stearate, PEG-50 stearate, PEG-100 stearate, PEG-150 stearate, pentadecalactone, peppermint (mentha piperita) oil, petrolatum, phospholipids, polyamino sugar condensate, polyglyceryl-3 diisostearate, polyquaternium-24, polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 80, polysorbate 85, potassium myristate, potassium palmitate, potassium sorbate, potassium stearate, propylene glycol, propylene glycol dicaprylate/dicaprate, propylene glycol dioctanoate, propylene glycol dipelargonate, propylene glycol laurate, propylene glycol stearate, propylene glycol stearate SE, PVP, pyridoxine dipalmitate, quaternium-15, quaternium-18 hectorite, quaternium-22, retinol, retinyl palmitate, rice (oryza sativa) bran oil, RNA, rosemary (rosmarinus officinalis) oil, rose oil, safflower (carthamus tinctorius) oil, sage (salvia officinalis) oil, salicylic acid, sandalwood (santalum

album) oil, serine, serum protein, sesame (sesamum indicum) oil, shea butter (butyrospermum parkii), silk powder, sodium chondroitin sulfate, sodium DNA, sodium hyaluronate, sodium lactate, sodium palmitate, sodium PCA, sodium polyglutamate, sodium stearate, soluble collagen, sorbic acid, sorbitan laurate, sorbitan oleate, sorbitan palmitate, sorbitan sesquioleate, sorbitan stearate, sorbitol, soybean (glycine soja) oil, sphingolipids, squalane, squalene, stearamide MEA-stearate, stearic acid, stearoxy dimethicone, stearoxytrimethylsilane, stearyl alcohol, stearyl glycyrrhetinate, stearyl heptanoate, stearyl stearate, sunflower (helianthus annuus) seed oil, sweet almond (prunus amygdalus dulcis) oil, synthetic beeswax, tocopherol, tocopheryl acetate, tocopheryl linoleate, tribehenin, tridecyl neopentanoate, tridecyl stearate, triethanolamine, tristearin, urea, vegetable oil, water, waxes, wheat (triticum vulgare) germ oil, and ylang ylang (cananga odorata) oil.

Surfactants may also desirably be included in certain formulations contemplated herein, and can be selected from any natural or synthetic surfactants suitable for use in cosmetic compositions, such as cationic, anionic, zwitterionic, or non-ionic surfactants, or mixtures thereof. (See Rosen, M., "Surfactants and Interfacial Phenomena," Second Edition, John Wiley & Sons, New York, 1988, Chapter 1, pages 4 31). Examples of cationic surfactants may include, but are not limited to, DMDAO or other amine oxides, long-chain primary amines, diamines and polyamines and their salts, quaternary ammonium salts, polyoxyethylenated long-chain amines, and quaternized polyoxyethylenated long-chain amines. Examples of anionic surfactants may include, but are not limited to, SDS; salts of carboxylic acids (*e.g.*, soaps); salts of sulfonic acids, salts of sulfuric acid, phosphoric and polyphosphoric acid esters; alkylphosphates; monoalkyl phosphate (MAP); and salts of perfluorocarboxylic acids. Examples of zwitterionic surfactants may include, but are not limited to, cocoamidopropyl hydroxysultaine (CAPHS) and others which are pH-sensitive and require special care in designing the appropriate pH of the formula (*i.e.,* alkylaminopropionic acids, imidazoline carboxylates, and betaines) or those which are not pH-sensitive (*e.g.*, sulfobetaines, sultaines). Examples of non-ionic surfactants may include, but are not limited to, alkylphenol ethoxylates, alcohol ethoxylates, polyoxyethylenated polyoxypropylene glycols, polyoxyethylenated mercaptans, long-chain carboxylic acid esters, alkonolamides, tertiary acetylenic glycols, polyoxyethylenated silicones, N-alkylpyrrolidones, and alkylpolyglycosidases. Wetting agents, mineral oil or other surfactants such as non-ionic detergents or agents such as one or more

members of the PLURONICS® series (BASF, Mt. Olive, NJ) may also be included, for example and according to non-limiting theory, to discourage aggregation of BT microparticles within the microparticulate suspension. Any combination of surfactants is acceptable. Certain embodiments may include at least one anionic and one cationic surfactant, or at least one cationic and one zwitterionic surfactant which are compatible, *i.e.*, do not form complexes which precipitate appreciably when mixed.

Examples of thickening agents that may also be present in certain topical formulations include, but are not limited to, acrylamides copolymer, agarose, amylopectin, bentonite, calcium alginate, calcium carboxymethyl cellulose, carbomer, carboxymethyl chitin, cellulose gum, dextrin, gelatin, hydrogenated tallow, hydroxytheylcellulose, hydroxypropylcellulose, hydroxpropyl starch, magnesium alginate, methylcellulose, microcrystalline cellulose, pectin, various PEG's, polyacrylic acid, polymethacrylic acid, polyvinyl alcohol, various PPG's, sodium acrylates copolymer, sodium carrageenan, xanthan gum, and yeast beta-glucan. Thickening agents other than those listed above may also be used in embodiments of this invention.

According to certain embodiments contemplated herein, a BT formulation may comprise one or more sunscreening or UV absorbing agents. Where ultraviolet light- (UVA and UVB) absorbing properties are desired, such agents may include, for example, benzophenone, benzophenone-1, benzophenone-2, benzophenone-3, benzophenone-4, benzophenone-5, benzophenone-6, benzophenone-7, benzophenone-8, benzophenone-9, benzophenone-10, benzophenone-11, benzophenone-12, benzyl salicylate, butyl PABA, cinnamate esters, cinoxate, DEA-methoxycinnamate, diisopropyl methyl cinnamate, ethyl dihydroxypropyl PABA, ethyl diisopropylcinnamate, ethyl methoxycinnamate, ethyl PABA, ethyl urocanate, glyceryl octanoate dimethoxycinnamate, glyceryl PABA, glycol salicylate, homosalate, isoamyl p-methoxycinnamate, oxides of titanium, zinc, zirconium, silicon, manganese, and cerium, PABA, PABA esters, Parsol 1789, and isopropylbenzyl salicylate, and mixtures thereof. One skilled in the art will appreciate that sunscreening and UV absorbing or protective agents other than those listed may be used in certain embodiments of the present invention.

The BT formulations disclosed herein are typically effective at pH values between about 2.5 and about 10.0. Preferably, the pH of the composition is at or about the following pH ranges: about pH 5.5 to about pH 8.5, about pH 5 to about pH 10, about pH 5 to about pH 9, about pH 5 to about

pH 8, about pH 3 to about pH 10, about pH 3 to about pH 9, about pH 3 to about pH 8, and about pH 3 to about pH 8.5. Most preferably, the pH is about pH 7 to about pH 8. One of ordinary skill in the art may add appropriate pH adjusting ingredients to the compositions of the present invention to adjust the pH to an acceptable range. "About" a specified pH is understood by those familiar with the art to include formulations in which at any given time the actual measured pH may be less or more than the specified value by no more than 0.7, 0.6, 0.5, 0.4., 0.3, 0.2 or 0.1 pH units, where it is recognized that formulation composition and storage conditions may result in drifting of pH from an original value.

A cream, lotion, gel, ointment, paste or the like may be spread on the affected surface and gently rubbed in. A solution may be applied in the same way, but more typically will be applied with a dropper, swab, or the like, and carefully applied to the affected areas. The application regimen will depend on a number of factors that may readily be determined, such as the severity of the infection and its responsiveness to initial treatment. One of ordinary skill may readily determine the optimum amount of the formulation to be administered, administration methodologies and repetition rates. In general, it is contemplated that the formulations of these and related embodiments of the invention will be applied in the range of once or twice or more weekly up to once, twice, thrice, four times or more daily.

As also discussed above, the BT formulations useful herein thus also may contain an acceptable carrier, including any suitable diluent or excipient, which includes any agent that does not itself harm the subject (*e.g.*, plant or animal including a human) or article of manufacture receiving the composition, and which may be administered without undue toxicity. Acceptable carriers may include, but are not limited to, liquids, such as water, saline, glycerol and ethanol, and the like, and may also include viscosity enhancers (*e.g.*, balsam fir resin) or film-formers such as colloidion or nitrocellulose solutions. A thorough discussion of pharmaceutically acceptable carriers, diluents, and other excipients is presented in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J. current edition).

The BT formulation may include an agent that binds to the BT compound and thereby assists in its delivery to or retention at a desired site on a subject or article of manufacture. Suitable agents that may act in this capacity include clathrating agents such as cyclodextrins; other agents may include a protein or a liposome.

The BT formulations,are administered, applied or incorporated in an effective amount, which will vary depending upon a variety of factors including the nature of the delivery site (where relevant), the activity of the specific BT compound employed (including the inclusion or absence from the formulation of an antibiotic, such as an aminoglycoside antibiotic, *e.g.*, amikacin); the metabolic stability and length of action of the compound; the condition of the (plant or animal, including a human) subject or article of manufacture; the mode and time of administration; the rate of loss of the BT compound in the ordinay course of activities undertaken by the subject or article; and other factors. Generally, a therapeutically effective daily dose is (for a 70 kg mammal) from about 0.001 mg/kg (*i.e.*, 0.07 mg) to about 100 mg/kg (*i.e.*, 7.0 g); preferably a therapeutically effective dose is (for a 70 kg mammal) from about 0.01 mg/kg (*i.e.*, 7 mg) to about 50 mg/kg (*i.e.*, 3.5 g); more preferably a therapeutically effective dose is (for a 70 kg mammal) from about 1 mg/kg (*i.e.*, 70 mg) to about 25 mg/kg (*i.e.*, 1.75 g). Effective doses for plants may be expected to be lower by about 10, 20, 50 or 75 percent or more.

The ranges of effective doses provided herein are not intended to be limiting and represent preferred dose ranges. However, the most preferred dosage will be tailored to the individual subject, as is understood and determinable by one skilled in the relevant arts. (see, *e.g.*, Berkow et al., eds., The Merck Manual, 16th edition, Merck and Co., Rahway, N.J., 1992; Goodman et al., eds., Goodman and Gilman's The Pharmacological Basis of Therapeutics, 10th edition, Pergamon Press, Inc., Elmsford, N.Y., (2001); Avery's Drug Treatment: Principles and Practice of Clinical Pharmacology and Therapeutics, 3rd edition, ADIS Press, Ltd., Williams and Wilkins, Baltimore, MD. (1987); Ebadi, Pharmacology, Little, Brown and Co., Boston, (1985); Osolci al., eds., Remington's Pharmaceutical Sciences, 18th edition, Mack Publishing Co., Easton, PA (1990); Katzung, Basic and Clinical Pharmacology, Appleton and Lange, Norwalk, CT (1992)).

The total dose required for each treatment can be administered by multiple doses or in a single dose over the course of the day, if desired. Certain preferred embodiments contemplate a single application of the BT formulation per day, per week, per 10 days, per 14 days or per longer time periods. Generally, and in distinct embodiments, treatment may be initiated with smaller dosages, which are less than the optimum dose of the compound. Thereafter, the dosage is increased by small increments until the optimum effect under the circumstances is reached.

Bismuth-Thiols for Protection of Plants and Agricultural Products

Certain herein disclosed embodiments relate to compositions and methods for protecting plants and flowers from microbial infections and infestations including biofilms, to reduce blight and increase product life.

According to certain herein described embodiments, including those summarized above, there is provided a method for protecting a plant against a bacterial, fungal or viral pathogen, comprising contacting the plant with an effective amount of a BT composition under conditions and for a time sufficient for one or more of (i) prevention of infection of the plant by the bacterial, fungal or viral pathogen, (ii) inhibition of cell viability or cell growth of substantially all planktonic cells of the bacterial, fungal or viral pathogen, (iii) inhibition of biofilm formation by the bacterial, fungal or viral pathogen, and (iv) inhibition of biofilm viability or biofilm growth of substantially all biofilm-form cells of the bacterial, fungal or viral pathogen, wherein the BT composition comprises a substantially monodisperse suspension of microparticles that comprise a BT compound, said microparticles having a volumetric mean diameter of from about 0.5 μm to about 10 μm.

In certain embodiments the bacterial pathogen comprises *Erwinia amylovora* cells and in certain embodiments the bacterial pathogen is selected from *Erwinia amylovora, Xanthomonas campestris pv dieffenbachiae, Pseudomonas syringae, Xylella fastidiosa; Xylophylus ampelinus; Monilinia fructicola, Pantoea stewartii* subsp. *Stewartii, Ralstonia solanacearum, and Clavibacter michiganensis* subsp. *sepedonicus.* In certain embodiments the bacterial pathogen exhibits antibiotic resistance and in certain other embodiments the bacterial pathogen exhibits streptomycin resistance. In certain embodiments the plant is a food crop plant, which in certain further embodiments is a fruit tree that in certain still further embodiments is selected from an apple tree, a pear tree, a peach tree, a nectarine tree, a plum tree, and an apricot tree. In certain embodiments the food crop plant is a banana tree of genus *Musa*. In certain other embodiments the food crop plant is a plant selected from a tuberous plant, a leguminous plant, and a cereal grain plant. In certain further embodiments the tuberous plant is selected from *Solanum tuberosum* (potato), and *Ipomoea batatas* (sweet potato).

In certain embodiments the step of contacting is performed one or a plurality of times. In certain embodiments at least one step of contacting comprises one of spraying, dipping, coating and painting the plant. In certain embodiments at least one step of contacting is performed at a flower blossom,

green-tip or growth site of the plant, or on, at or in other plant parts such as a root, bulb, stem, leaf, branch, vine, runner, bud, flower or a part thereof, greentip, fruit, seed, seed pod, or the like. In certain embodiments at least one step of contacting is performed within 24, 48 or 72 hours of first flower blooming on the plant. In certain embodiments the BT composition comprises one or more BT compounds selected from BisBAL, BisEDT, Bis-dimercaprol, Bis-DTT, Bis-2-mercaptoethanol, Bis-DTE, Bis-Pyr, Bis-Ery, Bis-Tol, Bis-BDT, Bis-PDT, Bis-Pyr/Bal, Bis-Pyr/BDT, Bis-Pyr/EDT, Bis-Pyr/PDT, Bis-Pyr/Tol, Bis-Pyr/Ery, bismuth-1-mercapto-2-propanol, and Bis-EDT/2-hydroxy-1-propanethiol. In certain embodiments the bacterial pathogen exhibits antibiotic resistance.

In certain embodiments of the above described methods, the method further comprises contacting the plant with a synergizing or enhancing antibiotic, simultaneously or sequentially and in any order with respect to the step of contacting the plant with the BT composition. In certain further embodiments the synergizing or enhancing antibiotic comprises an antibiotic that is selected from an aminoglycoside antibiotic, a carbapenem antibiotic, a cephalosporin antibiotic, a fluoroquinolone antibiotic, a penicillinase-resistant penicillin antibiotic, and an aminopenicillin antibiotic. In certain embodiments the synergizing or enhancing antibiotic is an aminoglycoside antibiotic that is selected from amikacin, arbekacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, rhodostreptomycin, streptomycin, tobramycin and apramycin.

In another embodiment there is provided a method for overcoming antibiotic resistance in a plant in or on which an antibiotic-resistant bacterial plant pathogen is present, comprising (a) contacting the plant with an effective amount of a BT composition under conditions and for a time sufficient for one or more of (i) prevention of infection of the plant by the antibiotic-resistant bacterial pathogen, (ii) inhibition of cell viability or cell growth of substantially all planktonic cells of the antibiotic-resistant bacterial pathogen, (iii) inhibition of biofilm formation by the antibiotic-resistant bacterial pathogen, and (iv) inhibition of biofilm viability or biofilm growth of substantially all biofilm-form cells of the antibiotic-resistant bacterial pathogen, wherein the BT composition comprises a substantially monodisperse suspension of microparticles that comprise a BT compound, said microparticles having a volumetric mean diameter of from about 0.5 μm to about 10 μm; and (b) contacting the plant with a synergizing or enhancing antibiotic, simultaneously or sequentially and in any order with respect to the step of contacting the plant with the BT composition.

Bismuth Thiol- (BT) based Antiseptics

As also noted above, a number of natural products (*e.g.*, antibiotics) and synthetic chemicals having antimicrobial (*e.g.*, antibacterial, antiviral, antifungal), and in particular antibacterial, properties are known in the art and have been at least partially characterized by chemical structures and by antimicrobial effects, such as ability to kill microbes ("cidal" effects such as bacteriocidal properties), ability to halt or impair microbial growth ("static" effects such as bacteriostatic properties), or ability to interfere with microbial functions such as colonizing or infecting a site, bacterial secretion of exopolysaccharides and/or conversion from planktonic to biofilm populations or expansion of biofilm formation. Antibiotics, disinfectants, antiseptics and the like (including bismuth-thiol or BT compounds) are discussed herein above and, for example, in U.S. 6,582,719, including factors that influence the selection and use of such compositions, *e.g.*, bacteriocidal or bacteriostatic potencies, effective concentrations, and risks of toxicity to host tissues.

Bismuth thiols (BTs), and related thiol compounds having a different group V metal (*e.g.*, arsenic, antimony) substituting for the bismuth, are discussed above. Also discussed herein are compositions and methods directed to advantageous microparticulate BT compositions microparticles having a volumetric mean diameter of from about 0.5 μm to about 10 μm. Certain exemplary embodiments thus pertain to the use of herein described antimicrobial, including antibiofilm, agents to treat or prevent infections and biofilms in plants, said agents typically present in compositions that contain one or more microparticulate bismuth thiols at a concentration that is between 0.0001% and 0.001% by weight, preferably in alkaline form. The compositions may comprise BTs and one or more carriers or excipients, and/or may further comprise other ingredients such as other compatible germicides, which in certain preferred embodiments comprise synergizing or enhancing antibiotics as described herein.

Target crops to be protected within certain contemplated but non-limiting embodiments include, for example, the following species of plants: cereals (*e.g.*, wheat, barley, rye, oats, rice, sorghum and related crops), beets (*e.g.*, sugar beet and fodder beet), pomes, drupes and softfruit (*e.g.*, apples, pears, plums, peaches, almonds, cherries, strawberries, raspberries and blackberries), leguminous plants, (*e.g.*, beans, lentils, peas, soybeans), oil plants (*e.g.*, rapeseed, mustard, poppy, olives, sunflowers, coconut, castor oil plants, cocoa beans, ground nuts), cucumber plants (*e.g.*, cucumber, marrows,

melons), fiber plants (*e.g.,* cotton, flax, hemp, jute), citrus fruit (*e.g.,* oranges, lemons, grapefruit, mandarins), vegetables (*e.g.,* spinach, lettuce, asparagus, cabbages, carrots, onions, tomatoes, potatoes, paprika), lauraceae (*e.g.,* avocados, cinnamon, camphor), and other plants such as maize, tobacco, nuts, coffee, sugar cane, tea, vines, hops, bananas and natural rubber plants, as well as ornamentals (composites) including flowering plants and harvested cut flowers therefrom. Certain embodiments thus contemplate extending the product lifetime (*e.g.*, prolonging the period of time during which the item is commercially, nutritionally and/or aesthetically useful, in a statistically significant manner relative to a control group that is not contacted with the presently described microparticulate BT) of a harvested target crop item such as a cut flower or a target-crop derived foodstuff (*e.g.*, fruit, vegetable, grain, seed, etc.) by contacting the crop item with a composition that comprises one or more of the microparticulate BT compounds as provided herein.

Effective concentrations of microparticulate BTs as described herein, for use in these and related embodiments, will depend on many factors, including the choice of BT, pH, temperature, molar ratio of BT components, and the offending microorganisms. Effectiveness also depends on whether prevention of an infection or treatment of an existing infection (*e.g.*, a biofilm) is the goal of a particular application. A preventive dose will suffice in most instances. The effective sustained concentration of BTs is likely to be around the MIC of the most resistant organism. This concentration is likely to be in the range of 1-2 μg/ml, but may go up to 8 μg/ml or beyond, depending on the specific microparticulate BT compound(s). In one exemplary embodiment, microparticulate BisPyrithione (BisPyr) is provided at a 5:1 molar ratio (bismuth to pyrithione) for application to plants. In another embodiment, a dual bismuth thiol in microparticulate form, BisPyr/Ery (Bis-pyrithione/ dithioerythritol) may be provided as a broad-spectrum antimicrobial. In yet another embodiment, microparticulate BTs may be combined with specific antibiotics as provided herein, preferably a synergizing or an enhancing antibiotic, to provide targeted and potent protection against microbial infections for plants and cut flowers/trees. Based on observed synergy between BisEDT and gentamicin, this BT-antibiotic combination is preferred in certain embodiments for agricultural applications.

In other embodiments, the addition to a microparticulate BT formulation of baking soda (sodium bicarbonate) or other alkaline substance(s) (*e.g.,* potassium bicarbonate, calcium carbonate) may add to or enhance the

antimicrobial effects of the BT. Other ingredients in the microparticulate BT formulations for agricultural uses may include surface-active agents and other antimicrobial agents, *e.g.,* chlorhexidine, sanguinarine extract, metronidazole, quaternary ammonium compounds, such as cetylpyridinium chloride; bis-guanides, such as chlorhexidine digluconate, hexetidine, octenidine, alexidine; and halogenated bisphenolic compounds, such as 2,2' methylenebis-(4-chloro-6-bromophenol), or other phenolic antibacterial compounds, alkylhydroxybenzoate, cationic antimicrobial peptides, aminoglycosides, quinolones, lincosamides, penicillins, cephalosporins, macrolides, tetracyclines, and other antibiotics, taurolidine or taurultam, A-dec ICX, *Coleus forskohlii* essential oil, silver or colloidal silver antimicrobials, tin- or copper-based antimicrobials, chlorine or bromine oxidants, Manuka oil, oregano, thyme, rosemary or other herbal extracts, grapefruit seed extract; anti-inflammatory or antioxidant agents such as ibuprofen, flurbiprofen, aspirin, indomethacin, aloe vera, turmeric, olive leaf extract, cloves, panthenol, retinol, omega-3 fatty acids, gamma-linolenic acid (GLA), green tea, ginger, grape seed, etc.; pharmaceutically acceptable carriers, *e.g.,* starch, sucrose, water or water/alcohol systems, DMSO, etc.; surfactants, such as anionic, nonionic, cationic and zwitterionic or amphoteric surfactants, or saponins from plant materials (*e.g.*, U.S. Patent 6,485,711); buffers and salts; and other optional ingredients that may be included, *e.g.*, bleaching agents such as peroxy compounds, potassium peroxydiphosphate, effervescing systems such as sodium bicarbonate/citric acid systems, and the like.

Microparticulate BT compositions for agricultural use and use on plants can, in certain embodiments, also be combined with these and optionally other agents that produce additive, enhancing or synergistic effects as described herein, or in liposomal or nanoparticle form to enhance activity and delivery. Certain embodiments expressly exclude microparticulate BT formulations that comprise liposomes such as phospholipid (*e.g.,* phosphocholine) and/or cholesterol-containing liposomes, while certain other embodiments are not so limited and may include these and other liposomes. Specific formulations of microparticulate BTs can also be made that contain carriers, excipients or other additives that promote adherence of the formulation to surfaces (*e.g.,* glucose, starch, citric acid, carrier oils, emulsions, dispersants, surfactants, and the like, etc.).

In other contemplated embodiments, microparticulate BT formulations for use as anti-biofilm agents on plants or agricultural crops can be

combined with other agents for controlling biofilm development. It is known, for example, that interspecies quorum sensing is related to biofilm formation. Certain agents that increase LuxS-dependent pathway or interspecies quorum sensing signal (*e.g.*, U.S. Pat. Nos. 7,427,408 and 6,455,031) help control biofilms, such as N-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) blocking compounds and/or N-butyryl-L-homoserine lactone (BHL) analogs. These anti-biofilm agents combined with the herein described microparticulate BTs may be delivered in foliar sprays for inhibition of bacterial biofilm development or for treatment of pre-formed biofilms. In another embodiment, these anti-biofilm agents are contained within a biodegradable microparticle for controlled release, and/or in liposomal form with other antimicrobial agents.

The presently described microparticulate BTs thus may, according to certain embodiments, be used with other existing technologies to improve anti-biofilm effects. The present microparticulate BTs may synergize or enhance the activity against certain plant pathogens of the antibiotics streptomycin and/or gentamicin. Streptomycin does not kill bacteria but instead inhibits their multiplication and thus reduces the rate at which flower stigmata are colonized, thereby diminishing the subsequent multiplication of the bacteria within the nectarthodes. (See, *e.g.,* Domenico et al. *J Antimicrob Chemo* 1991;28:801-10; Domenico et al. *Research Advances in Antimicrob Agents Chemother* 2003;3:79-85). Further benefits may accrue through the use of an activator-type spray adjuvant (*e.g.,* Regulaid™) that improves the coverage and penetration of streptomycin enough to allow reduced amounts of this antibiotic to be used safely.

The present microparticulate BTs may be combined with any of the active ingredients currently in use for combating agricultural and plant microbial pathogens, including those having antibiofilm activity, such as oxidizing agents, chelating agents (*e.g.*, iron chelators), germicides and disinfectants. Preferred combinations may be additive, or may be enhancing or synergistic according to the present disclosure, with regard to their anti-biofilm effects. Certain embodiments contemplate microparticulate BT compositions that are formulated to be hydrophobic in order to enhance retention of the BT on surfaces, for example by using hydrophobic thiols (*e.g.,* thiochlorophenol) that confer enhanced adhesive properties. BTs with a net negative charge (*e.g.,* 1:2 molar ratio of bismuth to thiol) may also possess enhanced adhesive properties.

The BT compound microparticulate suspension can be administered as aqueous formulations, as suspensions or solutions in organic solvents including halogenated hydrocarbon propellants, dispersion oils, or as dry powders. Aqueous formulations may be aerosolized by liquid nebulizers employing either hydraulic or ultrasonic atomization. Propellant-based systems may use suitable pressurized dispensers. Dry powders may use dry powder dispersion devices, which are capable of dispersing the BT-containing microparticles effectively. A desired particle size and distribution may be obtained by choosing an appropriate device.

Throughout this specification, unless the context requires otherwise, the words "comprise", "comprises" and "comprising" will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of." Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that no other elements are required and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

In this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise. As used herein, in particular embodiments, the terms "about" or "approximately" when preceding a numerical value indicates the value plus or minus a range of 5%, 6%, 7%, 8% or 9%. In other embodiments, the terms "about" or "approximately" when preceding a numerical value indicates the value plus or minus a range of 10%, 11%, 12%, 13% or 14%. In yet other embodiments, the terms "about" or "approximately" when preceding a numerical value indicates the value plus or minus a range of 15%, 16%, 17%, 18%, 19% or 20%.

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The following Examples are presented by way of illustration and not limitation.

EXAMPLES

EXAMPLE 1

PREPARATION OF BT COMPOUNDS

The following BT compounds were prepared either according to the methods of Domenico et al. (U.S. RE37,793, U.S. 6,248,371, U.S. 6,086,921, U.S. 6,380,248) or as microparticles according to the synthetic protocol described below for BisEDT. Shown are atomic ratios relative to a single bismuth atom, for comparison, based on the stoichiometric ratios of the reactants used and the known propensity of bismuth to form trivalent complexes with sulfur containing compounds. The numbers in parenthesis are the ratios of bismuth to one (or more) thiol agents (*e.g.* Bi:thiol1/thiol2; see also Table 1).

1) CPD 1B-1 Bis-EDT (1:1) BiC2H4S2
2) CPD 1B-2 Bis-EDT (1:1.5) $BiC_3H_6S_3$
3) CPD 1B-3 Bis-EDT (1:1.5) $BiC_3H_6S_3$
4) CPD 1C Bis-EDT (soluble Bi prep.) (1:1.5) $BiC_3H_6S_3$
5) CPD 2A Bis-Bal (1:1) $BiC_3H_6S_2O$
6) CPD 2B Bis-Bal (1:1.5) $BiC_{4.5}H_9O_{1.5}S_3$
7) CPD 3A Bis-Pyr (1:1.5) $BiC_{7.5}H_6N_{1.5}O_{1.5}S_{1.5}$
8) CPD 3B Bis-Pyr (1:3) $BiC_{15}H_{12}N_3O_3S_3$
9) CPD 4 Bis-Ery (1:1.5) $BiC_6H_{12}O_3S_3$
10) CPD 5 Bis-Tol (1:1.5) $BiC_{10.5}H_9S_3$
11) CPD 6 Bis-BDT (1:1.5) $BiC_6H_{12}S_3$
12) CPD 7 Bis-PDT (1:1.5) $BiC_{4.5}H_9S_3$
13) CPD 8-1 Bis-Pyr/BDT (1:1/1)
14) CPD 8-2 Bis-Pyr/BDT (1:1/0.5)
15) CPD 9 Bis-2hydroxy, propane thiol (1:3)
16) CPD 10 Bis-Pyr/Bal (1:1/0.5)
17) CPD 11 Bis-Pyr/EDT (1:1/0.5)
18) CPD 12 Bis-Pyr/Tol (1:1/0.5)
19) CPD 13 Bis-Pyr/PDT (1:1/0.5)
20) CPD 14 Bis-Pyr/Ery (1:1/0.5)
21) CPD 15 Bis-EDT/2hydroxy, propane thiol (1:1/1)

Microparticulate bismuth-1,2-ethanedithiol (Bis-EDT, soluble bismuth preparation) was prepared as follows:

To an excess (11.4 L) of 5% aqueous HNO_3 at room temperature in a 15 L polypropylene carboy was slowly added by dropwise addition 0.331 L (~0.575 moles) of an aqueous $Bi(NO_3)_3$ solution (43% $Bi(NO_3)_3$ (w/w), 5% nitric acid (w/w), 52% water (w/w), Shepherd Chemical Co., Cincinnati, OH, product no. 2362; δ ~1.6 g/mL) with stirring, followed by slow addition of absolute ethanol (4 L). Some white precipitate formed but was dissolved by continued stirring. An ethanolic solution (~1.56 L, ~0.55 M) of 1,2-ethanedithiol (CAS 540-63-6) was separately prepared by adding, to 1.5 L of absolute ethanol, 72.19 mL (0.863 moles) of 1,2-ethanedithiol using a 60 mL syringe, and then stirring for five minutes. The 1,2-ethanedithiol/ EtOH reagent was then slowly added by dropwise addition over the course of five hours to the aqueous $Bi(NO_3)_3$ / HNO_3 solution, with continued stirring overnight. The formed product was allowed to settle as a precipitate for approximately 15 minutes, after which the filtrate was removed at 300 mL/min using a peristaltic pump. The product was then collected by filtration on fine filter paper in a 15-cm diameter Buchner funnel, and washed sequentially with three, 500-mL volumes each of ethanol, USP water, and acetone to obtain BisEDT (694.51 gm/ mole) as a yellow amorphous powdered solid. The product was placed in a 500 mL amber glass bottle and dried over $CaCl_2$ under high vacuum for 48 hours. Recovered material (yield ~200 g) gave off a thiol-characteristic odor. The crude product was redissolved in 750 mL of absolute ethanol, stirred for 30 min, then filtered and washed sequentially with 3 x 50 mL ethanol, 2 x 50 mL acetone, and washed again with 500 mL of acetone. The rewashed powder was triturated in 1M NaOH (500 mL), filtered and washed with 3 x 220 mL water, 2 x 50 mL ethanol, and 1 x 400 mL acetone to afford 156.74 gm of purified BisEDT. Subsequent batches prepared in essentially the same manner resulted in yields of about 78-91%.

The product was characterized as having the structure shown above in formula **I** by analysis of data from ^1H and ^{13}C nuclear magnetic resonance (NMR), infrared spectroscopy (IR), ultraviolet spectroscopy (UV), mass spectrometry (MS) and elemental analysis. An HPLC method was developed to determine chemical purity of BisEDT whereby the sample was prepared in DMSO (0.5mg/mL). The λ_{max} was determined by scanning a solution of BisEDT in DMSO between 190 and 600nm. Isocratic HPLC elution at 1 mL/min was performed at ambient temperature in a mobile phase of 0.1%

formic acid in acetonitrile:water (9:1) on a Waters (Millipore Corp., Milford, MA) model 2695 chromatograph with UV detector monitoring at 265 nm (λ_{max}), 2 μL injection volume, equipped with a YMC Pack PVC Sil NP, 5μm, 250X4.6 mm inner diameter analytical column (Waters) and a single peak was detected, reflecting chemical purity of 100 ±0.1%. Elemental analysis was consistent with the structure of formula (I).

The dried particulate matter was characterized to assess the particle size properties. Briefly, microparticles were resuspended in 2% Pluronic® F-68 (BASF, Mt. Olive, NJ) and the suspension was sonicated for 10 minutes in a water bath sonicator at standard setting prior to analysis using a Nanosizer/Zetasizer Nano-S particle analyzer (model ZEN1600 (without zeta-potential measuring capacity), Malvern Instruments, Worcestershire, UK) according to the manufacturer's recommendations. From compiled data of two measurements, microparticles exhibited a unimodal distribution with all detectable events between about 0.6 microns and 4 microns in volumetric mean diameter (VMD) and having a peak VMD at about 1.3 microns. By contrast, when BisEDT was prepared by prior methods (Domenico et al., 1997 *Antimicrob. Agents Chemother.* 41(8):1697-1703) the majority of particles were heterodisperse and of significantly larger size, precluding their characterization on the basis of VMD.

EXAMPLE 2

COLONY BIOFILM MODEL OF CHRONIC WOUND INFECTION: INHIBITION BY BT COMPOUNDS

Because bacteria that exist in chronic wounds adopt a biofilm lifestyle, BTs were tested against biofilms for effects on bacterial cell survival using biofilms prepared essentially according to described methods (Anderl et al., 2003 *Antimicrob Agents Chemother* 47:1251-56; Walters et al., 2003 Antimicrob Agents Chemother 47:317; Wentland et al., 1996 *Biotchnol. Prog.* 12:316; Zheng et al., 2002 *Antimicrob Agents Chemother* 46:900).

Briefly, colony biofilms were grown on 10% tryptic soy agar for 24 hours, and transferred to Mueller Hinton plates containing treatments. After treatment the biofilms were dispersed into peptone water containing 2% w/v glutathione (neutralizes the BT), and serially diluted into peptone water before being spotted onto plates for counting. Two bacteria isolated from chronic wounds were used separately in the production of colony biofilms for testing.

These were *Pseudomonas aeruginosa*, a gram negative bacterial strain, and Methicillin Resistant *Staphylococcus aureus* (MRSA), which is gram positive.

Bacterial biofilm colonies were grown on top of micro porous membranes resting on an agar plate essentially as described (Anderl et al., 2003 *Antimicrob Agents Chemother* 47:1251-56; Walters et al., 2003 *Antimicrob Agents Chemother* 47:317; Wentland et al., 1996 *Biotchnol. Prog.* 12:316; Zheng et al., 2002 *Antimicrob Agents Chemother* 46:900) The colony biofilms exhibited many of the familiar features of other biofilm models, *e.g.*, they consisted of cells densely aggregated in a highly hydrated matrix. As also reported by others (Brown et al., *J Surg Res* 56:562; Millward et al, 1989 *Microbios* 58:155; Sutch et al., 1995 *J Pharm Pharmacol* 47:1094; Thrower et al., 1997 *J Med Microbiol* 46:425) it was observed that bacteria in colony biofilms exhibited the same profoundly reduced anti-microbial susceptibility that has been quantified in more sophisticated in vitro biofilm reactors. Colony biofilms were readily and reproducibly generated in large numbers. According to non-limiting theory, this colony biofilm model shared some of the features of an infected wound: bacteria grew at an air interface with nutrients supplied from beneath the biofilm and minimal fluid flow. A variety of nutrients sources was used to cultivate colony biofilms, including blood agar, which is believed to mimic in vivo nutrient conditions.

Colony biofilms were prepared by inoculating 5 µl spots of planktonic bacterial liquid cultures onto a 25 mm diameter polycarbonate filter membrane. The membranes were sterilized prior to inoculation, by exposure to ultraviolet light for 10 min per side. The inocula were grown overnight in bacterial medium at 37°C and diluted in fresh medium to an optical density of 0.1 at 600 nm prior to deposition on the membrane. The membranes were then placed on the agar plate containing growth medium. The plates were then covered and placed, inverted, in an incubator at 37°C. Every 24 h, the membrane and colony biofilm were transferred, using sterile forceps, to a fresh plate. Colony biofilms were typically used for experimentation after 48 hours of growth, at which time there were approximately 10^9 bacteria per membrane. The colony biofilm method was successfully employed to culture a wide variety of single species and mixed species biofilms.

To measure susceptibility to antimicrobial agents (*e.g.*, BT compounds including combinations of BT compounds; antibiotics; and BT compound-antibiotic combinations), colony biofilms were transferred to agar plates supplemented with the candidate antimicrobial treatment agent(s).

Where the duration of exposure to antimicrobial treatment exceeded 24 hours, the colony biofilms were moved to fresh treatment plates daily. At the end of the treatment period, the colony biofilms were placed in tubes containing 10 ml of buffer and vortexed for 1-2 min to disperse the biofilm. In some cases, it was necessary to briefly process the sample with a tissue homogenizer to break up cell aggregates. The resulting cell suspensions were then serially diluted and plated to enumerate surviving bacteria, which were reported as colony forming units (CFU) per unit area. Survival data were analyzed using $\log_{10}$ transformation.

For each type of bacterial biofilm colony cultures (*Pseudomonas aeruginosa*, PA; methicilin resistant *Staphylococcus aureus*, MRSA or SA) five antibiotics and thirteen BT compounds were tested. Antimicrobial agents tested against PA included the BTs referred to herein as BisEDT and Compounds 2B, 4, 5, 6, 8-2, 9, 10, 11 and 15 (see Table 1), and the antibiotics tobramycin, amikacin, imipenim, cefazolin, and ciprofloxacin. Antimicrobial agents tested against SA included the BTs referred to herein as BisEDT and Compounds 2B, 4, 5, 6, 8-2, 9, 10 and 11 (see Table 1), and the antibiotics rifampicin, daptomycin, minocycline, ampicillin, and vancomycin. As described above under "brief descriptions of the drawings", antibiotics were tested at concentrations of approximately 10-400 times the minimum inhibitory concentrations (MIC) according to established microbiological methodologies.

Seven BT compounds exhibited pronounced effects on PA bacterial survival at the concentrations tested, and two BT compounds demonstrated pronounced effects on MRSA survival at the concentrations tested; representative results showing BT effects on bacterial survival are presented in Figure 1 for BisEDT and BT compound 2B (tested against PA) and in Figure 2 for BT compounds 2B and 8-2 (tested against SA), in both cases, relative to the effects of the indicated antibiotics. As also shown in Figures 1 and 2, inclusion of the indicated BT compounds in combination with the indicated antibiotics resulted in a synergistic effect whereby the potency of reducing bacterial survival was enhanced relative to the anti-bacterial effects of either the antibiotic alone or the BT compound alone. In the PA survival assay, compound 15 (Bis-EDT/2hydroxy, propane thiol (1:1/1)) at a concentration of 80 μg/mL exhibited an effect (not shown) that was comparable to the effect obtained using the combination of 1600 μg/mL AMK plus 80 μg/mL BisEDT (Fig. 1).

EXAMPLE 3

DRIP FLOW BIOFILM MODEL OF CHRONIC WOUND INFECTION: INHIBITION BY BT COMPOUNDS

Drip flow biofilms represent an art accepted authentic model for forming, and testing the effect of candidate anti-bacterial compounds against, bacterial biofilms. Drip flow biofilms are produced on coupons (substrates) placed in the channels of a drip flow reactor. Many different types of materials can be used as the substrate for bacterial biofilm formation, including frosted glass microscope slides. Nutritive liquid media enters the drip flow bioreactor cell chamber by dripping into the chamber near the top, and then flows the length of a coupon down a 10 degree slope.

Biofilms are grown in drip flow bioreactors and exposed to BT compounds individually or in combinations and/or to antibiotic compounds individually or in combinations with other antibacterial agents, including BT compounds, or to other conventional or candidate treatments for chronic wounds. BT compounds are thus characterized for their effects on bacterial biofilms in the drip-flow reactor. Biofilms in the drip-flow reactor are prepared according to established methodologies (*e.g.,* Stewart et al., 2001 *J Appl Microbiol.* 91:525; Xu et al., 1998 *Appl. Environ. Microbiol.* 64:4035). This design involves cultivating biofilms on inclined polystyrene coupons in a covered chamber. An exemplary culture medium contains 1 g/l glucose, 0.5 g/l NH_4NO_3, 0.25g/l KCl, 0.25 g/l KH_2PO_4, 0.25 g/l $MgSO_4$-$7H_2O$, supplemented with 5% v/v adult donor bovine serum (ph 6.8) that mimics serum protein-rich, iron limited conditions that are similar to biofilm growth conditions in vivo, such as in chronic wounds. This medium flows drop-wise (50ml/h) over four coupons contained in four separate parallel chambers, each of which measures 10cm x 1.9cm by 1.9cm deep. The chambered reactor is fabricated from polysulfone plastic. Each of the chambers is fitted with an individual removable plastic lid that can be tightly sealed. The biofilm reactor is contained in an incubator at 37° C, and bacterial cell culture medium is warmed by passing it through an aluminum heat sink kept in the incubator. This method reproduces the antibiotic tolerant phenotype observed in certain biofilms, mimics the low fluid shear environment and proximity to an air interface characteristic of a chronic wound while providing continual replenishment of nutrients, and is compatible with a number of analytical methods for characterizing and monitoring the effects of introduced candidate antibacterial regimens. The drip-flow reactor has been successfully employed to culture a wide variety of pure and mixed-

species biofilms. Biofilms are typically grown for two to five days prior to application of antimicrobial agents.

To measure the effects of anti-biofilm agents on biofilms grown in drip-flow reactors, the fluid stream passing over the biofilm is amended or supplemented with the desired treatment formulation (*e.g.,* one or more BT compounds and/or one or more antibiotics, or controls, and/or other candidate agents). Flow is continued for the specified treatment period. The treated biofilm coupon is then briefly removed from the reactor and the biofilm is scraped into a beaker containing 10 ml of buffer. This sample is briefly processed (typically 30s to 1 min) with a tissue homogenizer to disperse bacterial aggregates. The suspension is serially diluted and plated to enumerate surviving microorganisms according to standard microbiological methodologies.

EXAMPLE 4

WOUND BIOFILM INHIBITION OF KERATINOCYTE SCRATCH REPAIR: BIOFILM SUPPRESSION BY BT COMPOUNDS

This Example describes a modification of established in vitro keratinocyte scratch models of wound healing, to arrive at a model having relevance to biofilm-associated wound pathology and wound healing, and in particular to acute or chronic wounds or wounds containing biofilms as described herein. According to the keratinocyte scratch model of the effects of chronic wound biofilms, cultivation of mammalian (*e.g.,* human) keratinocytes and bacterial biofilm populations proceeds in separate chambers that are in fluid contact with one another, to permit assessment of the effects of conditions that influence the effects, of soluble components elaborated by biofilms, on keratinocyte wound healing events.

Newborn human foreskin cells are cultured as monolayers in treated plastic dishes, in which monolayers a controlled "wound" or scratch is formed by mechanical means (*e.g.,* through physical disruption of the monolayer such as by scraping an essentially linear cell-free zone between regions of the monolayer with a suitable implement such as a sterile scalpel, razor, cell scraper, forceps or other tool). In vitro keratinocyte monolayer model systems are known to undergo cellular structural and functional process in response to the wounding event, in a manner that simulates wound healing in vivo. According to the herein disclosed embodiments, the influence of the presence of bacterial biofilms on such processes, for instance, on the healing

time of the scratch, is observed, and in these and related embodiments the effects are also assessed of the presence of selected candidate antimicrobial (*e.g.*, antibacterial and antibiofilm) treatments.

Wounded keratinocyte monolayers cultured in the presence of biofilms are examined according to morphological, biochemical, molecular genetic, cell physiologic and other parameters to determine whether introduction of BT comopunds alters (*e.g.*, increases or decreases in a statistically significant manner relative to appropriate controls) the damaging effects of the biofilms. Wounds are first exposed to each BT compound alone, and to contemplated combinations of BT compounds, in order to test the toxicity of each BT compound treatment prior to assessing the effects of such treatments on biofilm influences toward the model wound healing process.

In a representative embodiment, a three-day biofilm is cultured on a membrane (*e.g.*, a TransWell membrane insert or the like) that is maintained in a tissue culture well above, and in fluid communication with, a keratinocyte monolayer that is scratched to initiate the wound healing process. Biofilms cultured out of authentic acute or chronic wounds are contemplated for use in these and related embodiments.

Thus, an in vitro system has been developed for evaluating soluble biofilm component effects on migration and proliferation of human keratinocytes. The system separates the biofilm and keratinocytes using a dialysis membrane. Keratinocytes are cultured from newborn foreskin as previously described (Fleckman et al., 1997 *J Invest. Dermatol.* 109:36; Piepkorn et al., 1987 *J Invest. Dermatol.* 88:215-219) and grown as confluent monolayers on glass cover slips. The keratinocyte monolayers can then be scratched to yield "wounds" with a uniform width, followed by monitoring cellular repair processes (*e.g.*, Tao et al., 2007 *PLoS ONE* 2:e697; Buth et al. 2007 *Eur. J Cell Biol.* 86:747; Phan et al. 2000 *Ann. Acad. Med. Singapore* 29:27). The artificial wounds are then placed in the bottom of a sterile double-sided chamber and the chamber is assembled using aseptic technique. Both sides of the chamber are filled with keratinocyte growth medium (EpiLife) with or without antibiotics and/or bismuth-thiols. Uninoculated systems are used as controls.

The system is inoculated with wound-isolated bacteria and incubated in static conditions for two hours to enable bacterial attachment to surfaces in the upper chambers. Following the attachment period, liquid medium flow is initiated in the upper chamber to remove unattached cells. Flow of medium is then continued at a rate that minimizes the growth of planktonic

cells within the upper chamber, by washout of unattached cells. After incubation periods ranging from 6 to 48 hours, the systems (keratinocyte monolayers on coverslips and bacterial biofilm on membrane substrate) are disassembled and the cover slips removed and analyzed. In related embodiments, mature biofilms are grown in the upper chamber prior to assembling the chamber. In other related embodiments, the separate co-culturing of biofilms and scratch-wounded keratinocyte monolayers is conducted in the absence and presence of one or more BT compounds, optionally with the inclusion or exclusion of one or more antibiotics, in order to determine effects of candidate agents such as BT compounds, or of potentially synergizing BT compound-plus-antibiotic combinations (*e.g.,* a BT compound as provided herein such as a BT that is provided in microparticulate form, and one or more of amikacin, ampicillin, cefazolin, cefepime, chloramphenicol, ciprofloxacin, clindamycin (or another lincoasamide antibiotic), daptomycin (Cubicin®), doxycycline, gatifloxacin, gentamicin, imipenim, levofloxacin, linezolid (Zyvox®), minocycline, nafcilin, paromomycin, rifampin, sulphamethoxazole, tobramycin and vancomycin), on keratinocyte repair of the scratch wound, *e.g.,* to identify an agent or combination of agents that alters (*e.g.,* increases or decreases in a statistically significant manner relative to appropriate controls) at least one indicator of scratch wound healing, such as the time elapsing for wound repair to take place or other wound-repair indicia (*e.g.*, Tao et al., 2007 *PLoS ONE* 2:e697; Buth et al. 2007 *Eur. J Cell Biol.* 86:747; Phan et al. 2000 *Ann. Acad. Med. Singapore* 29:27).

EXAMPLE 5

WOUND BIOFILM INHIBITION OF KERATINOCYTE SCRATCH REPAIR

Isolated human keratinocytes were cultured on glass coverslips and scratch-wounded according to methodologies described above in Example 4. Wounded cultures were maintained under culture conditions alone or in the presence of a co-cultured biofilm on a membrane support in fluid communication with the keratinocyte culture. The scratch closure time interval during which keratinocyte cell growth and/or migration reestablishes the keratinocyte monolayer over the scratch zone was then determined. Figure 3 illustrates the effect that the presence in fluid communication (but without direct contact) of biofilms had on the healing time of scratched keratinocyte monolayers.

Accordingly there are contemplated in certain embodiments a method of identifying an agent for treating a chronic wound, comprising culturing a scratch-wounded cell (*e.g.,* keratinocyte or fibroblast) monolayer in the presence of a bacterial biofilm with and without a candidate anti-biofilm agent being present; and assessing an indicator of healing of the scratch-wounded cell monolayer in the absence and presence of the candidate anti-biofilm agent, wherein an agent (*e.g.*, a BT compound such as a substantially monodisperse BT microparticle suspension as described herein, alone or in synergizing combination with an antibiotic, such as one or more of amikacin, ampicillin, cefazolin, cefepime, chloramphenicol, ciprofloxacin, clindamycin, daptomycin (Cubicin®),_doxycycline, gatifloxacin, gentamicin, imipenim, levofloxacin, linezolid (Zyvox®), minocycline, nafcilin, paromomycin, rifampin, sulphamethoxazole, tobramycin and vancomycin) that promotes at least one indicator of healing is identified as a suitable agent for treating an acute or chronic wound or a wound that contains a biofilm.

EXAMPLE 6

SYNERGIZING BISMUTH-THIOL (BT)-ANTIBIOTIC COMBINATIONS

This example shows instances of demonstrated synergizing effects by combinations of one or more bismuth-thiol compounds and one or more antibiotics against a variety of bacterial species and bacterial strains, including several antibiotic-resistant bacteria.

Materials & Methods. Susceptibility studies were performed by broth dilution in 96-well tissue culture plates (Nalge Nunc International, Denmark) in accordance with NCCLS protocols (National Committee for Clinical Laboratory Standards. (1997). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically: Approved Standard M7-A2 and Informational Supplement M100-S10. NCCLS, Wayne, PA, USA).

Briefly, overnight bacterial cultures were used to prepare 0.5 McFarland standard suspensions, which were further diluted 1:50 (~2×10^6 cfu/mL) in cation-adjusted Mueller–Hinton broth medium (BBL, Cockeysville, MD, USA). BTs (prepared as described above) and antibiotics were added at incremental concentrations, keeping the final volume constant at 0.2 mL. Cultures were incubated for 24 h at 37°C and turbidity was assessed by absorption at 630 nm using an ELISA plate reader (Biotek Instruments, Winooski, VT, USA) according to the manufacturer's recommendations. The Minimum Inhibitory Concentration (MIC) was expressed as the lowest drug

concentration inhibiting growth for 24 h. Viable bacterial counts (cfu/mL) were determined by standard plating on nutrient agar. The Minimal Bactericidal Concentrations (MBC) was expressed as the concentration of drug that reduced initial viability by 99.9% at 24 h of incubation.

The checkerboard method was used to assess the activity of antimicrobial combinations. The fractional inhibitory concentration index (FICI) and the fractional bactericidal concentration index (FBCI) were calculated, according to Eliopoulos et al. (Eliopoulos and Moellering, (1996) Antimicrobial combinations. *In* Antibiotics in Laboratory Medicine (Lorian, V., Ed.), pp. 330–96, Williams and Wilkins, Baltimore, MD, USA). Synergy was defined as an FICI or FBCI index of ≤0.5, no interaction at >0.5–4 and antagonism at >4 (Odds, FC (2003) Synergy, antagonism, and what the chequerboard puts between them. *Journal of Antimicrobial Chemotherapy* 52:1). Synergy was also defined conventionally as ≥4-fold decrease in antibiotic concentration.

Results are presented in Tables 2-17.

TABLE 2
S. aureus Nafcilin resistant

Strain	NAF MIC (μg/ml)	NAF/BE MIC (μg/ml)	Δ	Synergy
60187-2	10.00	0.6	16.7	+
52446-3	175.00	40.0	4.4	+
M1978	140.00	50.0	2.8	-
W54793	130.00	33.3	3.9	-
S24341	210.00	65.0	3.2	-
H7544	28.33	15.0	1.9	-
H72751	145.00	43.3	3.3	-
W71630	131.67	46.7	2.8	-
X22831	178.33	75.0	2.4	-
X23660	123.33	43.3	2.8	-
O36466	191.67	93.3	2.1	

BE = 0.2 μg/ml BisEDT; Bacterial strains were obtained from the Clinical Microbiology Laboratory at Winthrop-University Hospital, Mineola, NY. Nafcillin was obtained from Sigma (St. Louis, MO).

TABLE 3

S. aureus Nafcilin resistant

Strain	**GM MIC** (µg/ml)	**GM/BE MIC** (µg/ml)	**Δ**	**Synergy**
60187-2	0.233	0.004	58.3	+
52446-3	10.667	1.500	7.1	+
M1978	32.500	4.000	8.1	+
W54793	0.250	0.080	3.1	-
S24341	0.250	0.058	4.3	+
H7544	0.383	0.093	4.1	+
H72751	0.200	0.072	2.8	-
W71630	17.667	3.800	4.6	+
X22831	-	0.085		
X23660	22.500	4.000	5.6	+
O36466	0.267	0.043	6.2	+

BE = 0.2 µg/ml BisEDT; Bacterial strains were obtained from the Clinical Microbiology Laboratory at Winthrop-University Hospital, Mineola, NY. Nafcillin was obtained from Sigma.

TABLE 4

S. aureus

Rifampin/Neomycin/Paromomycin

ATCC 25923	**MIC** (µg/ml)	**MIC + BE** (µg/ml)	**Δ**	**Synergy**
RIF	0.033	0.003	13.0	+
NEO	0.500	0.200	2.5	-
PARO	1.080	0.188	5.7	+
MRSA S2446-3				
RIF	2.500	2.500	1.0	-
NEO	13.400	8.500	1.6	-
PARO	335.000	183.300	1.8	-

BE = 0.2 µg/ml BisEDT; Strain S2446-3 was obtained from the Clinical Microbiology Laboratory at Winthrop-University Hospital, Mineola, NY. Antibiotics were obtained from Sigma.

TABLE 5

S. epidermidis - GM resistant

	strain ATCC 35984		**strain S2400-1**	
BisEDT (µg/ml)	**MIC** (µg/ml GM)	**MBC** (µg/ml GM)	**MIC** (µg/ml GM)	**MBC** (µg/ml GM)
0	53.3	384.0	85.3	426.7
0.005	20.0	96.0	96.0	512.0
0.01	37.3	117.3	64.0	256.0
0.02	21.3	**26.7**	28.0	128.0
0.04	**2.0**	**16.0**	**2.0**	128.0
0.08	**2.0**	**10.7**	**2.0**	**53.3**
0.16 (MIC)		**3.0**		**10.0**
0.32		**2.0**		**4.0**

GM = gentamicin; Strain S2400-1 was obtained from the Clinical Microbiology Laboratory at Winthrop-University Hospital, Mineola, NY. Gentamicin was obtained from the Pharmacy Department at Winthrop; synergy in bold

TABLE 6

S. epidermidis - S2400-1

Biofilm Prevention

	BisEDT (µg/ml)			**Δ**	
Antibiotic	**0**	**0.05**	**0.1**	**(0.05 BE)**	**Synergy**
cefazolin	28	10	1	2.8	-
vancomycin	3.2	0.9	0.1	3.6	-
gatifloxacin	1.6	0.1	0.1	16.0	++
rifampicin	0.03	0.04	0.04	0.7	-
nafcillin	48	64	8	0.8	-
clindamycin	1195	48	12	24.9	++++
gentamicin	555	144	12	3.9	borderline
minocycline	0.85	0.73	0.08	1.2	-

Data in µg/ml; Strain S2400-1 was obtained from the Clinical Microbiology Laboratory at Winthrop-University Hospital, Mineola, NY. Antibiotics were obtained from the Pharmacy Department at Winthrop.

TABLE 7
S. epidermidis - S2400-1
MIC

Antibiotic	BisEDT (µg/ml) 0	0.05	0.1	Δ (0.05 BE)	Synergy
cefazolin	32	8	1	4.00	+
vancomycin	3.2	2.3	0.3	1.40	-
gatifloxacin	1.7	0.8	0.3	2.13	-
rifampicin	0.03	0.04	0.04	0.75	-
nafcillin	171	192	68	0.89	-
clindamycin	2048	768	24	2.67	-
gentamicin	2048	320	80	6.40	+
minocycline	1.13	0.43	0.10	2.63	-

Data in µg/ml; Strain S2400-1 was obtained from the Clinical Microbiology Laboratory at Winthrop-University Hospital, Mineola, NY. Antibiotics were obtained from the Pharmacy Department at Winthrop.

TABLE 8
S. epidermidis - S2400-1
MBC

Antibiotic	BisEDT (µg/ml) 0.0	0.1	Δ (0.1 BE)	Synergy
cefazolin	48	10	4.80	+
vancomycin	5.4	1.4	3.86	borderline
gatifloxacin	2.8	1.4	2.00	-
rifampicin	0.03	0.07	0.43	-
nafcillin	256	128	2.00	-
clindamycin	2048	768	2.67	-
gentamicin	1536	256	6.00	+
minocycline	1.20	1.20	1.00	-

Data in µg/ml; Strain S2400-1 was obtained from the Clinical Microbiology Laboratory at Winthrop-University Hospital, Mineola, NY. Antibiotics were obtained from the Pharmacy Department at Winthrop.

TABLE 9

S. epidermidis

ATCC 35984

MIC

Antibiotic	BisEDT (µg/ml) 0.0	0.05	Δ	Synergy
Nafcillin	16.00	5.00	3.2	-
Clindamycin	2048.00	1024.00	2	-
Gentamicin	213.33	16.00	13.3	++
Minocycline	0.13	0.04	3.3	-
Rifampicin	0.021	0.014	1.5	-

Data in µg/ml; Antibiotics were obtained from the Pharmacy Department at Winthrop-University Hospital, Mineola, NY.

TABLE 10

E. coli - Ampicillin/Chloramphenicol resistant

Strain	MIC AB (µg/ml)	MIC AB/BE (µg/ml AB)	Δ	Synergy	MIC BE (µg/ml)
MC4100/TN9 (CM)	220	12.7	17.4	+	0.6
MC4100/P9 (AM)	285	49	5.8	+	0.5
MC4100 (AM)	141.7	35	4.0	+	0.6

AB = antibiotic; CM = chloramphenicol; AM = ampicillin; BE = BisEDT at 0.3 µg/ml; Strains were obtained from the laboratory of Dr. MJ Casadaban, Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, IL. Antibiotics were obtained from the Pharmacy Department at Winthrop-University Hospital, Mineola, NY.

TABLE 11

E. coli - Tetracycline-resistant:

Doxycycline + BisEDT

Strain	DOX MIC (µg/ml)	DOX/BE MIC (µg/ml DOX)	Δ	Synergy	BE MIC (µg/ml)
TET M	16.50	4.50	4.0	+	0.85
TET D	20.50	0.03	820.0	++++	0.85
TET A	15.00	10.00	1.5	-	0.40
TET B	20.13	10.33	2.0	-	0.60

DOX = doxycycline; BE = BisEDT at 0.3 µg/ml; Strains were obtained from the laboratory of Dr. I Chopra, Department of Bacteriology, The University of Bristol, Bristol, UK. Antibiotics were obtained from the Pharmacy Department at Winthrop-University Hospital, Mineola, NY.

TABLE 12

P. aeruginosa - Tobramycin-resistant:

BisEDT Synergy

Strain	NN (µg/ml)	NN+BE (µg/ml NN)	Δ	Synergy	BE MIC (µg/ml)
Xen5	0.32	0.19	1.68	-	0.9
Agr PA E	115	70	1.64	-	0.9
Agr PA I	200	73	2.74	-	1
Agr PA K	4.8	3	1.60	-	0.82
Agr PA O	130	20.5	6.34	+	0.98

Agr =aminoglycoside resistant; NN = tobramycin; PA = Pseudomonas aeruginosa; BE = BisEDT, 0.3 µg/ml; Strains were obtained from the laboratory of Dr. K. Poole, Department of Microbiology and Immunology, Queens University, Ontario, CN. Tobramycin was obtained from the Pharmacy Department at Winthrop-University Hospital, Mineola, NY.

TABLE 13

B. cepacia

Tobramycin+BE Synergy

MIC

Strain	NN (µg/ml)	NN+BE (µg/ml NN)	Δ	Synergy	BE MIC (µg/ml)
13945	200	50	4	+	2.4
25416	125	10	12.5	++	1.2
HI 2229	64	8	8	+	0.8
AU 0267	128	2	64	++++	0.8
AU 0259	1024	256	4	+	1.6
HI 2255	64	8	8	+	1.6
AU 0273	512	32	16	++	1.6
HI 2253	64	16	4	+	1.6
HI 2147	512	8	64	++++	1.6

NN = Tobramycin; BE = BisEDT, 0.4 µg/ml; Strains were obtained from the laboratory of Dr. J.J. LiPuma, Department of Pediatrics and Communicable Diseases, University of Michigan, Ann Arbor, MI; also Veloira et al. 2003. Tobramycin was obtained from the Pharmacy Department at Winthrop-University Hospital, Mineola, NY.

TABLE 14

B. cepacia

Tobramycin+BE Synergy

MBC

Strain	NN (µg/ml)	NN+BE (µg/ml NN)	Δ	Synergy	BE MIC (µg/ml)
HI 2249	256	8	**32**	++	3.2
HI 2229	128	32	4	+	6.4
AU 0267	256	32	8	+	6.4
AU 0259	1024	1024	1	-	12.8
HI 2255	128	32	4	+	12.8
HI 2711	512	8	64	++++	6.4
AU 0284	1024	64	16	++	0.8
AU 0273	512	32	16	++	1.6
HI 2253	128	64	2	-	3.2
HI 2147	512	128	4	+	6.4

NN = Tobramycin; BE = BisEDT, 0.4 µg/ml; Strains were obtained from the laboratory of Dr. J.J. LiPuma, Department of Pediatrics and Communicable Diseases, University of Michigan, Ann Arbor, MI; also Veloira et al. 2003. Tobramycin was obtained from the Pharmacy Department at Winthrop-University Hospital, Mineola, NY.

TABLE 15

Tobramycin Resistant Strains

MIC

Strain	NN (µg/ml)	NN+BE (µg/ml NN)	Δ	Synergy	Lipo-BE-NN (µg/ml NN)
M13637	512	32	16	++	0.25
M13642R	128	64	2	-	0.25
PA-48913	1024	256	4	+	0.25
PA-48912-2	64	8	8	+	0.25
PA-10145	1	4	0.25	-	0.25
SA-29213	2	1	2	-	0.25

NN = Tobramycin; BE = BisEDT, 0.8 µg/ml; Lipo-BE-NN = liposomal BE-NN; Strains were obtained from the laboratory of Dr. A. Omri, Department of Chemistry and Biochemistry, Laurentian University, Ontario, CN; (M strains are mucoid B. cepacia; PA=P. aeruginosa; SA=S. aureus). Tobramycin was obtained from the Pharmacy Department at Winthrop-University Hospital, Mineola, NY.

TABLE 16
Tobramycin Resistant Strains
MBC

Strain	NN (µg/ml)	NN+BE (µg/ml NN)	Δ	Synergy	Lipo-BE-NN (µg/ml NN)
M13637	1024	64	16	++	8
M13642R	256	128	2	-	16
PA-48913	4096	512	8	+	4
PA-48912-2	128	32	4	+	0.5
PA-10145	1	8	0.125	-	4
SA-29213	2	1	2	-	0.25

NN = Tobramycin; BE = BisEDT, 0.8 µg/ml; Lipo-BE-NN = liposomal BE-NN; Strains were obtained from the laboratory of Dr. A. Omri, Department of Chemistry and Biochemistry, Laurentian University, Ontario, CN; (M strains are mucoid *B. cepacia*; PA=*P. aeruginosa*; SA=*S. aureus*). Tobramycin was obtained from the Pharmacy Department at Winthrop-University Hospital, Mineola, NY.

TABLE 17
BisEDT-Pyrithione Synergy

NaPYR (ug/ml)	*P. aeruginosa* ATCC 27853 (µg/ml BE)	*E. coli* ATCC 25922 (µg/ml BE)	*S. aureus* ATCC 25923 (µg/ml BE)
0	0.25	0.1	0.25
0.025		0.1	0.125
0.05		**0.025**	**0.063**
0.1	0.125	**0.0125**	**0.063**
0.2	0.125	**0.0125**	**0.031**
0.4		**0.00625**	**0**
0.8	0.125	**0.00625**	
1.6 (MIC)	0.063	**0.00625**	
3.2	0.063	**0**	
6.4	0.063		
12.8	0		

BE = BisEDT; NaPYR = sodium pyrithione; Chemicals were obtained from Sigma-Aldrich; synergy in bold. Indicated bacterial strains were from American Type Culture Collection (ATCC, Manassas, VA).

EXAMPLE 7

COMPARATIVE BISMUTH-THIOL (BT) AND ANTIBIOTIC EFFECTS AGAINST GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA INCLUDING ANTIBIOTIC-RESISTANT BACTERIAL STRAINS

In this example the *in vitro* activities of BisEDT and comparator agents were assessed against multiple clinical isolates of Gram-positive and –negative bacteria that are responsible for skin and soft tissue infections.

Materials and Methods. Test compounds and test concentration ranges were as follows: BisEDT (Domenico et al., 1997; Domenico et al., *Antimicrob. Agents Chemother.* 45(5):1417-1421. and Example 1), 16-0.015 µg/mL; linezolid (ChemPacifica Inc., #35710), 64-0.06 µg/mL; Daptomycin (Cubist Pharmaceuticals #MCB2007), 32-0.03 µg/mL and 16-0.015 µg/mL; vancomycin (Sigma-Aldrich, St. Louis, MO, # V2002), 64-0.06 µg/mL; ceftazidime, (Sigma #C3809), 64-0.06 µg/mL and 32-0.03 µg/mL; imipenem (United States Pharmacopeia, NJ, #1337809) 16-0.015 µg/mL and 8-0.008 µg/mL; ciprofloxacin (United States Pharmacopeia, # IOC265), 32-0.03 µg/mL and 4-0.004 µg/mL; gentamicin (Sigma #G3632) 32-0.03 µg/mL and 16-0.015 µg/mL. All test articles, except gentamicin, were dissolved in DMSO; gentamicin was dissolved in water. Stock solutions were prepared at 40-fold the highest concentration in the test plate. The final concentration of DMSO in the test system was 2.5%.

Organisms. The test organisms were obtained from clinical laboratories as follows: CHP, Clarian Health Partners, Indianapolis, IN; UCLA, University of California Los Angeles Medical Center, Los Angeles, CA; GR Micro, London, UK; PHRI TB Center, Public Health Research Institute Tuberculosis Center, New York, NY; ATCC, American Type Culture Collection, Manassas, VA; Mt Sinai Hosp., Mount Sinai Hospital, New York, NY; UCSF, University of California San Francisco General Hospital, San Francisco, CA; Bronson Hospital, Bronson Methodist Hospital, Kalamazoo, MI; quality control isolates were from the American Type Culture Collection (ATCC, Manassas, VA). Organisms were streaked for isolation on agar medium appropriate to each organism. Colonies were picked by swab from the isolation plates and put into suspension in appropriate broth containing a cryoprotectant. The suspensions were aliquoted into cryogenic vials and maintained at -80°C. Abbreviations are: BisEDT, bismuth-1,2-ethanedithiol; LZD, linezolid; DAP, daptomycin; VA, vancomycin; CAZ, ceftazidime; IPM, imipenem; CIP, ciprofloxacin; GM, gentamicin; MSSA, methicillin-susceptible *Staphylococcus*

aureus; CLSI QC, Clinical and Laboratory Standards Institute quality control strain; MRSA, methicillin-resistant *Staphylococcus aureus*; CA-MRSA, community-acquired methicillin-resistant *Staphylococcus aureus*; MSSE, methicillin-susceptible *Staphylococcus epidermidis*; MRSE, methicillin-resistant *Staphylococcus epidermidis*; VSE, vancomycin-susceptible *Enterococcus.*

The isolates were streaked from the frozen vials onto appropriate medium: Trypticase Soy Agar (Becton-Dickinson, Sparks, MD) for most organisms or Trypticase Soy Agar plus 5% sheep blood (Cleveland Scientific, Bath, OH) for streptococci. The plates were incubated overnight at 35°C. Quality control organisms were included. The medium employed for the MIC assay was Mueller Hinton II Broth (MHB II- Becton Dickinson, # 212322) for most of the organisms. MHB II was supplemented with 2% lysed horse blood (Cleveland Scientific Lot # H13913) to accommodate the growth of *Streptococcus pyogenes* and *Streptococcus agalactiae*. The media were prepared at 102.5% normal weight to offset the dilution created by the addition of 5 µL drug solution to each well of the microdilution panels. In addition, for tests with daptomycin, the medium was supplemented with an additional 25mg/L Ca^{2+}.

The MIC assay method followed the procedure described by the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Seventh Edition.* Clinical and Laboratory Standards Institute document M7-A7 [ISBN 1-56238-587-9]. Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2006) and employed automated liquid handlers to conduct serial dilutions and liquid transfers. Automated liquid handlers included the Multidrop 384 (Labsystems, Helsinki, Finland), Biomek 2000 and Multimek 96 (Beckman Coulter, Fullerton CA). The wells of Columns 2-12 of standard 96-well microdilution plates (Falcon 3918) were filled with 150µL of DMSO or water for gentamicin on the Multidrop 384. The drugs (300 µL) were dispensed into Column 1 of the appropriate row in these plates. These would become the mother plates from which the test plates (daughter plates) were prepared. The Biomek 2000 completed serial transfers through Column 11 in the mother plates. The wells of Column 12 contained no drug and were the organism growth control wells in the daughter plates. The daughter plates were loaded with 185 µL of the appropriate test media (described above) using the Multidrop 384. The daughter plates were prepared

on the Multimek 96 instrument which transferred 5 µL of drug solution from each well of a mother plate to each corresponding well of each daughter plate in a single step.

Standardized inoculum of each organism was prepared per CLSI methods (ISBN 1-56238-587-9, cited *supra*). Suspensions were prepared in MHB to equal the turbidity of a 0.5 McFarland standard. The suspensions were diluted 1:9 in broth appropriate to the organism. The inoculum for each organism was dispensed into sterile reservoirs divided by length (Beckman Coulter), and the Biomek 2000 was used to inoculate the plates. Daughter plates were placed on the Biomek 2000 work surface reversed so that inoculation took place from low to high drug concentration. The Biomek 2000 delivered 10 µL of standardized inoculum into each well. This yielded a final cell concentration in the daughter plates of approximately 5 x 105 colony-forming-units/mL. Thus, the wells of the daughter plates ultimately contained 185 µL of broth, 5 µL of drug solution, and 10 µL of bacterial inoculum. Plates were stacked 3 high, covered with a lid on the top plate, placed in plastic bags, and incubated at 35°C for approximately 18 hours for most of the isolates. The *Streptococcus* plates were read after 20 hours incubation. The microplates were viewed from the bottom using a plate viewer. For each of the test media, an uninoculated solubility control plate was observed for evidence of drug precipitation. The MIC was read and recorded as the lowest concentration of drug that inhibited visible growth of the organism.

Results. All marketed drugs were soluble at all of the test concentrations in both media. BisEDT exhibited a trace precipitate at 32 µg/mL, but MIC readings were not affected as the inhibitory concentrations for all organisms tested were well below that concentration. On each assay day, an appropriate quality control strain(s) was included in the MIC assays. The MIC values derived for these strains were compared to the published quality control ranges (Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing; Eighteenth Informational Supplement.* CLSI document M100-S18 [ISBN 1-56238-653-0]. Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2008) for each agent, as appropriate.

On each assay day, an appropriate quality control strain(s) was included in the MIC assays. The MIC values derived for these strains were compared to the published quality control ranges (Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility*

Testing; Eighteenth Informational Supplement. CLSI document M100-S18 [ISBN 1-56238-653-0]) for each agent, as appropriate. Of 141 values for quality control strains where quality control ranges are published, 140(99.3%) were within the specified ranges. The one exception was imipenem versus *S. aureus* 29213 which yielded one value on a single run (≤ 0.008 μg/mL) that was one dilution below the published QC range. All other quality control results on that run were within the specified quality control ranges.

BisEDT demonstrated potent activity against both methicillin-susceptible *Staphylococcus aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), and community-acquired MRSA (CA-MRSA), inhibiting all strains tested at 1 μg/mL or less with an MIC90 values of 0.5 μg/mL for all three organism groups. BisEDT exhibited activity greater than that of linezolid and vancomycin and equivalent to that of daptomycin. Imipenem was more potent than BisEDT against MSSA (MIC90 = 0.03 μg/mL). However, MRSA and CAMRSA were resistant to imipenem while BisEDT demonstrated activity equivalent to that shown for MSSA. BisEDT was highly-active against methicillin-susceptible and methicillin–resistant *Staphylococcus epidermidis* (MSSE and MRSE), with MIC90 values of 0.12 and 0.25 μg/mL, respectively. BisEDT was more active against MSSE than any of the other agents tested except imipenem. BisEDT was the most active agent tested against MRSE.

BisEDT demonstrated activity equivalent to that of daptomycin, vancomycin, and imipenem against vancomycin-susceptible *Enterococcus faecalis* (VSEfc) with an MIC90 value of 2μg/mL. Significantly, BisEDT was the most active agent tested against vancomycin-resistant *Enterococcus faecalis* (VREfc) with an MIC90 value of 1 μg/mL.

BisEDT was very active against vancomycin-susceptible *Enterococcus faecium* (VSEfm) with an MIC90 value of 2 μg/mL; its activity was equivalent to that or similar to that of daptomycin and one-dilution higher than that of vancomycin. BisEDT and linezolid were the most active agents tested against vancomycin-resistant *Enterococcus faecium* (VREfm), each demonstrating an MIC90 value of 2 μg/mL. The activity of BisEDT against *Streptococcus pyogenes* (MIC90 value of 0.5 μg/mL) was equivalent to that of vancomycin, greater than that of linezolid, and slightly less than that of daptomycin and ceftazidime. The compound inhibited all strains tested at 0.5 μg/mL or less. In these studies, the species that was least sensitive to BisEDT was *Streptococcus agalactiae* where the observed MIC90 value was 16 μg/mL. BisEDT was less active than all of the agents tested except gentamicin.

The activity of BisEDT and comparators against Gram-negative bacteria included demonstrated BisEDT potency against *Acinetobacter baumanii* (MIC90 value of 2 μg/mL) making BisEDT the most active compound tested. Elevated MICs for a significant number of test isolates for the comparator agents resulted in off-scale MIC90 values for these agents. BisEDT was a potent inhibitor of *Escherichia coli*, inhibiting all strains at 2 μg/mL or less (MIC90 = 2 μg/mL). The compound was less active than imipenem, but more active than ceftazidime, ciprofloxacin, and gentamicin. BisEDT also demonstrated activity against *Klebsiella pneumoniae* with an MIC90 value of 8 μg/mL which was equivalent to that of imipenem. The relatively high MIC90 values exhibited by imipenem, ceftazidime, ciprofloxacin, and gentamicin indicated that this was a highly antibiotic-resistant group of organisms. BisEDT was the most active compound tested against *Pseudomonas aeruginosa* with an MIC90 value of 4 μg/mL. There was a high level of resistance to the comparator agents for this group of test isolates.

In summary, BisEDT demonstrated broad-spectrum potency against multiple clinical isolates representing multiple species, including species commonly involved in acute and chronic skin and skin structure infections in humans. The activity of BisEDT and key comparator agents was evaluated against 723 clinical isolates of Gram-positive and Gram–negative bacteria. The BT compound demonstrated broad spectrum activity, and for a number of the test organisms in this study, BisEDT was the most active compound tested in terms of anti-bacterial activity. BisEDT was most active against MSSA, MRSA, CA-MRSA, MSSE, MRSE, and *S. pyogenes,* where the MIC90 value was 0.5 μg/mL or less. Potent activity was also demonstrated for VSEfc, VREfc,VSEfm, VREfm, *A. baumanii*, *E. coli*, and *P. aeruginosa* where the MIC90 value was in the range of 1 - 4 μg/mL. MIC90 values observed were, for *K. pneumoniae* (MIC90 = 8 μg/mL), and for *S. agalactiae* (MIC90= 16 μg/mL).

EXAMPLE 8

MICROPARTICULATE BT-ANTIBIOTIC ENHANCING AND SYNERGIZING ACTIVITIES

This example shows that microparticulate bismuth thiols (BTs) promote antibiotic activity through enhancing and/or synergizing interactions.

A major complicating factor in treating infections is the emerging resistance of bacteria to antibiotics. Methicillin resistance in *S. epidermidis* (MRSE) and *S. aureus* (MRSA) actually reflects multiple drug resistance, making these pathogens very difficult to eradicate. However, no staphylococci

from hundreds of strains tested showed resistance to BTs. Furthermore, BTs at subinhibitory (subMIC) concentrations reduced resistance to several important antibiotics.

Staphylococcus aureus. A graphic demonstration of the antibiotic-resensitizing effects of subMIC bismuth ethanedithiol (BisEDT) against MRSA is provided (Figure 4) showing enhanced antibiotic action of several classes of antibiotics, including gentamicin, cefazolin, cefepime, imipenim, sulphamethoxazole, and levofloxacin. Thus, BisEDT nonspecifically enhanced the activity of most antibiotics.

Broth dilution antimicrobial susceptibility studies were performed against 12 MRSA strains using several antibiotics combined with subMIC levels of BisEDT (Table 18). Both the biofilm-prevention concentration (BPC) and the minimum inhibitory concentration (MIC) were determined in a special biofilm culture medium (BHIG/X). The MIC and BPC for gentamicin and cefazolin were reduced by subMIC BisEDT (BisEDT MIC, 0.2-0.4 μg/ml), but not below the breakpoint for sensitivity. subMIC BisEDT enhanced the sensitivity of MRSA to gatifloxacin and cefepime close to the breakpoint for sensitivity. These strains were already sensitive to vancomycin, but were made considerably moreso in the presence of subMIC BisEDT. Generally, the MIC and BPC were reduced 2- to 5-fold with subMIC BisEDT.

TABLE 18.
Antimicrobial Activity of BT-Antibiotic Combinations against MRSA

Antibiotic	BisEDT (μg/mL)				MIC Standards (μg/ml)	
	0	0.025	0.05	0.1	S	R
Gentamicin						
BPC	81±41	63±30	53±31	33±25		
MIC	81±40	60±27	58±30	48±31	≤4	≥16
Cefazolin						
BPC	109±86	76±86	76±105	34±28		
MIC	93±75	99±76	90±60	45±32	≤8	≥32
Gatifloxacin						
BPC	3.6±2.6	2.6±0.9	2.4±1.1	0.9±0.8		
MIC	3.6±2.6	4.0±2.8	4.0±2.8	2.4±1.1	≤2	≥8
Vancomycin						
BPC	2.5±1.7	1.5±0.6	1.3±0.5	0.7±0.4		
MIC	2.5±1.7	2.5±1.7	1.5±0.6	1.3±0.5	≤4	≥32
Cefepime						
BPC	24±37	27±28	18±16	5.0±7.3		
MIC	45±32	32±28	37±24	9.3±6.1	≤8	≥32

12 MRSA clinical isolates were grown in BHIG/X and exposed to serial dilutions of antibiotics in the presence of 0-0.1 µg/ml BisEDT. The MIC and BPC, calculated in µg/ml, are the means± standard deviations from at least three trials. The right hand column lists the Standard MIC for antibiotic senstivity (S) and resistance (R)

A broth dilution study of cefepime-resistant MRSA isolates is shown in Table 19. BisEDT at 0.1 µg/ml significantly enhanced the inhibitory activity of cefepime in 11 of 12 isolates. In this particular study, the data indicated synergy between BisEDT and cefepime (FIC < 0.5), with many of the isolates at the breakpoint for sensitivity.

TABLE 19
Cefepime-resistant MRSA Sensitized by BisEDT

	MIC for Cefepime (ug/mL) in subMIC BisEDT		
	BE 0 µg/mL	BE 0.05 µg/mL	BE 0.1 µg/mL
MRSA	MIC	MIC	MIC
Strain #			
4	256	256	16
6	256	256	32
7	128	256	32
10	128	32	16
18	256	128	8
24	256	64	8
28	256	128	8
35	256	256	8
37	128	128	8
41	128	256	8
46	256	256	256
47	32	8	8

Twelve cefepime-resistant MRSA were tested in BHIG/X medium in polystyrene plates for sensitivity to cefepime combined with subMIC BisEDT at 37°C for 48h.

Results for combination studies with nafcillin or gentamicin are shown in Table 20. Combined with nafcillin, BisEDT (0.2 µg/ml) reduced the MIC90 for nafcillin by over 4-fold against MRSA (FIC, 0.74). Combined with gentamicin, BisEDT reduced the MIC90 for gentamicin over 10-fold against MRSA (FIC, 0.6). BTs reversed the resistance of all four gentamicin-resistant isolates tested to clinically relevant concentrations [Domenico et al., 2002]. The

MICs for these antimicrobial agents was reduced substantially, especially for gentamicin. The broth used in these studies was Trypticase Soy Broth (TSB) with 2% glucose, which showed results similar to that seen in Mueller-Hinton II broth fortified with 1% sheep's blood.

TABLE 20

MRSA: Nafcillin or Gentamicin + BisEDT Synergy

Strain	NAF MIC	NAF+BE MIC	Δ		GM MIC	GM+BE MIC	Δ
60187-2	10.00	0.60	16.67		0.23	0.00	58.33
52446-3	175.00	40.00	4.38		10.67	1.50	7.11
M1978	140.00	50.00	2.80		32.50	4.00	8.13
W54793	130.00	33.33	3.90		0.25	0.08	3.13
S24341	210.00	65.00	3.23		0.25	0.06	4.29
H7544	28.33	15.00	1.89		0.38	0.09	4.11
H72751	145.00	43.33	3.35		0.20	0.07	2.79
W71630	131.67	46.67	2.82		17.67	3.80	4.65
X22831	178.33	75.00	2.38				
X23660	123.33	43.33	2.85		22.50	4.00	5.63
O36466	191.67	93.33	2.05		0.27	0.04	6.15
		AVG Δ	4.21			AVG Δ	10.43

NAF or GM in µg/ml; BE at 0.2 µg/ml

Staphylococcus epidermidis. The activities of most classes of antibiotic were promoted in the presence of BisEDT. With regard to the BPC, clindamycin and gatifloxacin showed significantly more antibiofilm activity against *S. epidermidis* when combined with BisEDT (Figure 5). Stated in different terms, the BPC for clindamycin, gatifloxacin and gentamicin were reduced 50-fold, 10-fold and 4-fold, respectively, in the presence of subMIC BisEDT.

Only modest decreases in the biofilm prevention concentration (BPC) were noted for minocycline, vancomycin, and cefazolin, while rifampicn and nafcillin remained unaffected at 0.05 µg/ml BisEDT. At 0.1 µg/ml BisEDT no biofilm was detected, regardless of antibiotic employed, signifying that no antagonism occurred. This BisEDT concentration was close to the MIC for *S. epidermidis* [Domenico et al., 2003] (See Figure 5).

With regard to growth inhibition, seven of eight antibiotics tested were significantly enhanced in the presence of 0.1 μg/ml (0.5 μM) BisEDT against *S. epidermidis* (Figure 6). The MIC change was most pronounced for clindamycin and gentamicin, followed by vancomycin, cefazolin, minocycline, gatifloxacin and nafcillin, with rifampicin unaffected. Of the antibiotics this strain was resistant to (NC, CZ, GM, CM), only cefazolin resistance was reversed to clinically relevant levels by BisEDT.

Minimum bactericidal concentration (MBC) for most antibiotics tested against *S. epidermidis* decreased slightly with subMIC BisEDT. Gentamicin showed the greatest reduction in MBC (4- to 16-fold), followed by cefazolin (4- to 5-fold), vancomycin and nafcillin (3- to 4-fold), minocycline and gatifloxacin (2- to 3-fold), while clindamycin and rifampicin MBC remained largely unaffected. Clindamycin is a bacteriostatic agent, which explains its lack of bactericidal activity. Cefazolin resistance was reversed with respect to the MBC [Domenico et al., 2003]. These effects were additive.

The potentiation of antimicrobial agents was also demonstrated in vivo in a graft infection rat model (Table 21). BisEDT levels as low as 0.1 μg/ml were able to promote the prevention of resistant *S. epidermidis* biofilm for 7 days.

As summarized in Table 21, implants impregnated with 0.1 μg/ml BisEDT, 10 μg/ml RIP and 10 μg/ml rifampin, alone or combined were implanted s.c. into rats. Physiological solution (1 ml) containing the MS and MR strains at 2×10^7 cfu/ml was inoculated onto the graft surface using a tuberculin syringe. All grafts were explanted at 7 days following implantation and sonicated for 5 minutes in sterile saline solution to remove the adherent bacteria. Quantitation of viable bacteria was obtained by culturing dilutions on blood agar plates. The limit of detection was approximately 10 cfu/cm^2.

TABLE 21

RIP, BTs, and rifampin against S. epidermidis in a graft infection model

Group[a]	Graft-bonded drug[b]	Quantitative graft culture (cfu/cm^2)	
No MSSE	-	<10	
Untreated MSSE	-	$5.0 \times 10^7 \pm 7.7 \times 10^6$	
MS1[c]	RIP	$4.3 \times 10^2 \pm 1.2 \times 10^2$	
MS2[c]	BTs	$5.8 \times 10^2 \pm 0.9 \times 10^2$	

Group[a]	Graft-bonded drug[b]	Quantitative graft culture (cfu/cm^2)	
MS3[c]	Rifampin	$5.9 \times 10^3 \pm 1.8 \times 10^3$	
MS[cd]	RIP plus BTs	<10	
MS5[cd]	RIP plus rifampin	$2.0 \times 10^1 \pm 0.6 \times 10^1$	
MS6[cd]	BTs plus rifampin	$1.9 \times 10^1 \pm 0.4 \times 10^1$	
No MRSE	-	<10	
Untreated MRSE	-	$7.8 \times 10^7 \pm 2.0 \times 10^7$	
MR1[c]	RIP	$6.7 \times 10^2 \pm 2.1 \times 10^2$	
MR2[c]	BTs	$6.2 \times 10^2 \pm 2.3 \times 10^2$	
MR3[c]	Rifampin	$7.6 \times 10^4 \pm 2.1 \times 10^4$	
MR4[ce]	RIP plus BTs	<10	
MR5[c]	RIP plus rifampin	$4.3 \times 10^1 \pm 1.1 \times 10^1$	
MR6[c]	BTs plus rifampin	$3.0 \times 10^1 \pm 1.1 \times 10^1$	

[a] Each group had 15 animals; MS, methicillin-susceptible *S. epidermidis*; MR, methicillin-resistant *S. epidermidis*

[b] Dacron graft segments impregnated with 0.1 mg/l of BTs, 10 mg/l of RIP, 10 mg/l of rifampin

[c] Statistically significant when compared with control groups MS and MR

[d] Statistically significant when compared with MS3 group

[e] Statistically significant when compared with MR1, MR2, and MR3 groups

Gram-negative Bacteria. Tobramycin activity against resistant *Pseudomonas aeruginosa* was enhanced several-fold with subMIC BisEDT (Table 22). In these trials, the MIC was defined more precisely as the IC_{24}.

TABLE 22

Tobramycin-resistant *P. aeruginosa*: BisEDT Effect

Strain	NN MIC (µg/ml)	BE MIC (µg/ml)	NN+BE MIC (µg/ml)	Δ
PA Xen5	0.3	0.9	0.2	1.7
Agr PA E	115.0	0.9	70.0	1.6
Agr PA I	200.0	1.0	73.0	2.7
Agr PA K	4.8	0.86	3.0	1.6
Agr PA O	130.0	0.98	20.5	6.3

Resistant strains of *P. aeruginosa* were cultured in Mueller-Hinton II broth at 37°C in the presence of tobramycin (NN) and BisEDT (BE; 0.33 µg/ml). The MIC was determined as the antibiotic concentration that inhibited growth for 24±1 h.

Against tobramycin-resistant *Burkholderia cepacia*, 0.4 µg/ml BisEDT rendered seven of 10 isolates tobramycin sensitive (mean FIC; 0.48), and reduced the MIC_{90} by 10-fold (Table 23). Both the MIC and MBC of tobramycin were reduced significantly to achievable levels against 50 clinical *Burkholderia cepacia* isolates with subMIC BisEDT [Veloira et al., 2003]. BisEDT and tobramycin in liposomal form have proven highly synergistic against *P. aeruginosa*. (Halwani et al., 2008; Halwani et al., 2009).

TABLE 23

Tobramycin and BisEDT versus *B. cepacia*

	MIC for			
Strain	Tobramycin (μg/ml)	BisEDT (μg/ml)	Tobramycin (BisEDT at 0.4 μg/ml)	FIC Index
B. multivorans				
HI 2249	256	0.4	a	a
HI 2229	64	0.8	8	0.63
AU 0267	128	0.8	2	0.52
AU 0259	1024	1.6	256	0.50
HI 2255	64	1.6	8	0.38
B. cenocepacia				
HI 2711	256	0.4	a	a
AU 0284	512	0.4	a	a
AU 0273	512	1.6	32	0.31
HI2253	64	1.6	16	0.50
HI 2147	512	1.6	8	0.27

[a] The three strains inhibited by BisEDT at 0.4 μg/ml were excluded from further study.

FIC Index ≤ 0.5 indicates synergy: FICI >0.5 and <1.0 indicates enhancement.

Chloramphenicol and ampicillin resistant *Escherichia coli* were made sensitive to these drugs by the addition of subMIC BisEDT (Table 24).

TABLE 24

Chloramphenicol/Ampicillin Resistant *E. coli*: BisEDT Effect

Strain	Drug	Drug MIC (μg/ml)	BE MIC (μg/ml)	Drug + BE MIC (μg/ml)	Δ
MC4100/TN9	CM	220.0	0.6	12.7	17.4
MC4100/P9	AMP	285.0	0.5	49.0	5.8
MC4100	AMP	141.7	0.6	35.0	4.0

Resistant strains of *E.coli* were cultured in Mueller-Hinton II broth at 37°C in the presence of chloramphenicol (CM) or ampicillin (AMP) and BisEDT alone or in combination (BE; 0.33 μg/ml). The MIC was determined as the antibiotic concentration that inhibited growth for 24±1 h.

Tetracycline resistant *Escherichia coli* were made sensitive to doxycycline by the addition of subMIC BisEDT (Table 25). The combination

exhibited synergy against the TET M and TET D strains (FIC ≤ 0.5), with additive effects against the TET A and TET B strains.

TABLE 25
Tetracycline Resistant E. Coli: BisEDT Effect

Strain	DOX MIC (μg/ml)	BE MIC (μg/ml)	DOX+BE MIC (μg/ml)	Δ
TET M	16.5 ± 1.3	0.85	4.5 ± 2.7	4.0
TET D	20.5 ± 1.1	0.85	0.03 ± 0.0	820.0
TET A	15.0 ± 1.8	0.40	10.0 ± 1.0	1.5
TET B	20.1 ± 2.4	0.60	10.3 ± 3.2	2.0

Resistant strains of *E.coli* were cultured in Mueller-Hinton II broth at 37°C in the presence of doxycycline (DOX) and BisEDT alone or in combination (BE; 0.33 μg/ml). The MIC was determined as the antibiotic concentration that inhibited growth for 24±1 h.

References

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Synergy of tobramycin and bismuth thiols against *Burkholderia cepacia*. *J Antimicrob Chemother* 52:915-919.

EXAMPLE 9

MICROPARTICULATE BT-ANTIBIOTIC ENHANCING AND SYNERGIZING ACTIVITIES

This example shows that the microparticulate bismuth thiol BisEDT promotes antibiotic activity through enhancing and/or synergizing interactions with specific antibiotics against specific microbial target organisms. Single-point data for each indicated combination in Table 26 were generated essentially according to the methods used in Example 8.

TABLE 26

FICI Values for single-point BisEDT-antibiotic combinations

Anti biotic	SA 100	MRSA 773	E Fc 3121	SP 1195	PRSP 5348	EC 102	EC 2232	KP 1231	PA 1380	Bcep 1756	Bmult 5665	Abau 2594	Msmeg 817
Oxacillin	1.28	2.28	0.92		1.03								
Piperacillin	0.57	1.28	1.11		1.11	0.87	1.29	2.23	0.67	1.12	1.12	1.12	
Cefuroxime	1.11	4.23	1.11		1.03								
Cefotaxime	1.11	2.23	0.73	1.11	1.11	1.37	1.29	0.61	0.64	1.29	1.11	1.29	
Cefepime						0.87	0.96	1.11	0.62	1.34	0.96	0.71	
Imipenem	0.67	1.48	0.73	0.92	0.43	1.11	1.29	1.23	1.12	0.73	1.23	0.81	
Aztreonam						0.74	1.29	0.73	0.55	0.67	0.96	0.87	
Streptomycin		0.95	0.61		0.66		1.29	1.04	1.98	1.37	1.12	2.62	1.13
Tobramycin	0.73	0.78	0.47		0.57		0.96	0.87	1.29	0.91	0.67	1.12	
Tetracycline	0.89	1.23	0.92	1.23	0.34	0.62	0.79	1.29	1.29	1.96	1.12	1.12	
Minocycline	1.09	1.23	1.11		0.46	1.37	1.04	1.29	0.99	2.23	1.12	1.29	
Ciprofloxacin						1.14	1.29	1.29	2.75	2.23	2.29	1.04	
Levofloxacin	1.23	1.11	1.08	0.95	0.70								
Erythromycin	1.28	0.67	0.92	0.78	1.03								
Linezolid	1.23	1.23	1.23	1.01	1.11								
Phosphomycin		0.61	1.23		1.45		1.96	1.02	1.86	1.29	1.23	1.12	
Capreomycin													0.75
Isoniazid													0.88

SA, Staphylococcus aureus; MRSA, methicillin-resistant Staphylococcus aureus; E Fc, Enterococcus faecalis; SP, Streptococcus pneumoniae; PRSP, penicillin-resistant Streptococcus pneumoniae; EC, Escherichia coli; KP, Klebsiella pneumoniae; PA, Pseudomonas aeruginosa; Bcep, Burkholderia cepacia; Bmult, Bukholderia multivorans; Abau, Acinetobacter baumanii; Msmeg, Mycobacterium smegmatis.

EXAMPLE 10

MICROPARTICULATE BT-ANTIBIOTIC ENHANCING AND SYNERGIZING ACTIVITIES

The effects of combinations of microparticulate Bis-EDT and four Bis-EDT analogs prepared as described above, and other agents against representative strains of several Gram-negative pathogenic bacteria were tested. A modification of a common laboratory method was used to determine synergism (FICI $\leq$ 0.5), enhancement (0.5 < FICI $\leq$ 1.0), antagonism (FICI > 4.0) and indifference (1.0 < FICI $\leq$ 4.0) used fractional inhibitory concentrations (FICs) and FIC indices (FICI) (Eliopoulos G and R Moellering. 1991. Antimicrobial combinations. In *Antibiotics in Laboratory Medicine, Third Edition,* edited by V Lorian. Williams and Wilkins, Baltimore, MD, pp. 432-492; Odds, 2003 *J. Antimicrob. Chemother.* 52(1):1). The checkerboard technique was used to determine FIC indices and was employed in this study.

TABLE 27
Test Components

Test Cpd	Lot No.	Solvent	FIC Highest Stock Concentration (µg/mL)	Conc. Range Tested in FIC (µg/mL)
Bis-EDT	MB-1B-3	DMSO	320	0.12-8
Bis-EDT (analog)	MB-2B	DMSO	320	0.12-8
Bis-EDT (analog)	MB-8-2	DMSO	320	0.12-8
Bis-EDT (analog)	MB-11	DMSO	320	0.12-8
Bis-EDT (analog)	MB-15	DMSO	320	0.12-8
Aztreonam	095K1324 (Sigma)	10% DMSO	2,560	0.06-64
Cefepime HCl	GOD116 (USP)	dH_2O	2,560	0.06-64
Cefotaxime	084K0674 (Sigma)	dH_2O	640	0.015-16
Piperacillin	014K1362 (Sigma)	dH_2O	2,560	0.06-64

Stock solutions of all test articles were prepared at 40X the final target concentration in the appropriate solvent. All test articles were in solution under these conditions. The final drug concentrations in the FIC assay plates were set to bracket the MIC value of each agent for each test organism, unless the strain was totally resistant to the test agent. The concentration ranges tested are displayed in Table 27. The test organisms were originally received from clinical sources, or from the American Type Culture Collection. Upon receipt, the isolates were streaked onto Tryptic Soy Agar II (TSA). Colonies were harvested from these plates and a cell suspension was prepared in an appropriate broth growth medium containing cryoprotectant. Aliquots were then frozen at -80°C. The frozen seeds of the organisms to be tested in a given assay were thawed, streaked for isolation onto TSA plates, and incubated at 35°C. All organisms were tested in Mueller Hinton II Broth (Becton Dickinson, Lot No.9044411). The broth was prepared at 1.05X normal weight/volume to offset the 5 % volume of the drugs in the final test plates.

Minimal Inhibitory Concentration (MIC) values were previously determined using the broth microdilution method for aerobic bacteria (Clinical and Laboratory Standards Institute (CLSI). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Eighth Edition.* CLSI document M07-A8 [ISBN 1-56238-689-1]. Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2009.).

FIC values were determined using a broth microdilution method previously described (Sweeney et al., 2003 *Antimicrob. Agents Chemother.* 47(6):1902-1906). To prepare the test plates, automated liquid handlers (Multidrop 384, Labsystems, Helsinki, Finland; Biomek 2000 and Multimek 96, Beckman Coulter, Fullerton CA) were used to conduct serial dilutions and liquid transfers.

The appropriate wells of standard 96-well microdilution plates (Falcon 3918) were filled with 150 µL of the appropriate solvent in columns 2-12 using the Multidrop 384. Three hundred microliters of each secondary test drug was added to each well in Column 1 of the plates. These plates were used to prepare the drug "mother plates" which provided the serial drug dilutions for the drug combination plates. The Biomek 2000 was used to transfer 150 µL of each secondary drug solution (40X) from the wells in Column 1 of the mother plate and to make eleven 2-fold serial dilutions. Mother plates of Bis-EDT (and

analogs) were serial diluted top to bottom by hand, using a multichannel pipette. Two mother plates, one for each secondary drug and one for Bis-EDT (or analogs), were combined to form a "checkerboard" pattern by transfer of equal volumes (using a multi-channel pipette) to the drug combination plate. Row H and Column 12 each contained serial dilutions of one of the agents alone for determination of the MIC.

The "daughter plates" were loaded with 180 µL of test medium using the Multidrop 384. Then, the Multimek 96 was used to transfer 10 µL of drug solution from each well of the drug combination mother plate to each corresponding well of the daughter plate in a single step. Finally, the daughter plates were inoculated with test organism. Standardized inoculum of each organism was prepared per published guidelines (CLSI, 2009). For all isolates, the inoculum for each organism was dispensed into sterile reservoirs divided by length (Beckman Coulter), and the Biomek 2000 was used to inoculate the plates. The instrument delivered 10 µL of standardized inoculum into each well to yield a final cell concentration in the daughter plates of approximately 5×10^5 colony-forming-units/mL.

The test format resulted in the creation of an 8 x 12 checkerboard where each compound was tested alone (Column 12 and Row H) and in combination at varying ratios of drug concentration. All organism plates were stacked three high, covered with a lid on the top plate, placed in plastic bags, and incubated at 35°C for approximately 20 hours. Following incubation, the microplates were removed from the incubators and viewed from the bottom using a ScienceWare plate viewer. Prepared reading sheets were marked for the MIC of drug 1 (row H), the MIC of drug 2 (column 12) and the wells of the growth-no growth interface.

An Excel program was used to determine the FIC according to the formula: (MIC of Compound 1 in combination/MIC of Compound 1 alone) + (MIC of Compound 2 in combination/MIC of Compound 2 alone). The FICI for the checkerboard was calculated from the individual FICs by the formula: $(FIC_1 + FIC_2 + \ldots FIC_n)/n$, where n = number of individual wells per plate for which FICs were calculated. In instances where an agent alone yielded an off-scale MIC result, the next highest concentration was used as the MIC value in the FIC calculation.

Microparticulate Bis-EDT, the four microparticulate BT analogs, and all of the other agents (and combinations of agents) were soluble at all final

test concentrations. The MIC and FICI values that were determined are presented in the Tables below.

TABLE 28

Summary of Minimum Inhibitory Concentration and Fractional Inhibitory Concentration Results for MB-1B-3 and Piperacillin

Organism[1]	Compound 1		Compound 2		FICI[2]
	Name	MIC[1] (µg/mL) Alone	Name	MIC (µg/mL) Alone	
P. aeruginosa 1381	MB-1B-3	1	Piperacillin	>64	0.83
P. aeruginosa 1384		1		8	0.96
P. aeruginosa 1474		1		8	0.71
P. aeruginosa 1479		0.5		8	1.12
P. aeruginosa 2566		0.5		32	1.37
P. aeruginosa 2568		1		8	0.71
P. aeruginosa 103		1		8	0.79

[1]MIC, Minimum Inhibitory Concentration
[2]FICI, Fractional Inhibitory Concentration Index

TABLE 29

Summary of Minimum Inhibitory Concentration and Fractional Inhibitory Concentration Results for MB-1B-3 and Aztreonam

Organism[1]	Compound 1		Compound 2		FICI[2]
	Name	MIC[1] (µg/mL) Alone	Name	MIC (µg/mL) Alone	
P. aeruginosa 1381	MB-1B-3	1	Aztreonam	32	1.04
P. aeruginosa 1384		1		8	0.71
P. aeruginosa 1474		1		8	0.71
P. aeruginosa 1479		0.5		8	0.87
P. aeruginosa 2566		0.5		16	1.37
P. aeruginosa 2568		1		8	0.71
P. aeruginosa 103		1		4	1.29

[1]MIC, Minimum Inhibitory Concentration
[2]FICI, Fractional Inhibitory Concentration Index

TABLE 30

Summary of Minimum Inhibitory Concentration and Fractional Inhibitory Concentration Results for MB-15 and Piperacillin

Organism[1]	Compound 1		Compound 2		FICI[2]
	Name	MIC[1] (µg/mL) Alone	Name	MIC (µg/mL) Alone	
P. aeruginosa 1381	MB-15	1	Piperacillin	>64	1.29
P. aeruginosa 1384		1		16	0.71
P. aeruginosa 1474		1		8	1.12
P. aeruginosa 1479		1		8	1.29
P. aeruginosa 2566		1		32	1.04
P. aeruginosa 2568		1		8	1.12
P. aeruginosa 103		2		8	0.73

[1]MIC, Minimum Inhibitory Concentration
[2]FICI, Fractional Inhibitory Concentration Index

TABLE 31

Summary of Minimum Inhibitory Concentration and Fractional Inhibitory Concentration Results for MB-15 and Aztreonam

Organism[1]	Compound 1		Compound 2		FICI[2]
	Name	MIC[1] (µg/mL) Alone	Name	MIC (µg/mL) Alone	
P. aeruginosa 1381	MB-15	2	Aztreonam	32	1.11
P. aeruginosa 1384		1		8	0.79
P. aeruginosa 1474		1		8	0.71
P. aeruginosa 1479		2		8	0.67
P. aeruginosa 2566		0.5		16	1.12
P. aeruginosa 2568		1		8	0.79
P. aeruginosa 103		2		4	1.23

[1]MIC, Minimum Inhibitory Concentration
[2]FICI, Fractional Inhibitory Concentration Index

TABLE 32

Summary of Minimum Inhibitory Concentration and Fractional Inhibitory Concentration Results for MB-8-2 and Piperacillin

Organism[1]	Compound 1		Compound 2		FICI[2]
	Name	MIC[1] (µg/mL) Alone	Name	MIC (µg/mL) Alone	
P. aeruginosa 1381	MB-8-2	2	Piperacillin	>64	1.23
P. aeruginosa 1384		2		16	0.73
P. aeruginosa 1474		2		8	1.23
P. aeruginosa 1479		2		8	1.23
P. aeruginosa 2566		2		32	1.23
P. aeruginosa 2568		2		8	0.98
P. aeruginosa 103		4		8	1.19

[1]MIC, Minimum Inhibitory Concentration
[2]FICI, Fractional Inhibitory Concentration Index

TABLE33

Summary of Minimum Inhibitory Concentration and Fractional Inhibitory Concentration Results for MB-8-2 and Aztreonam

Organism[1]	Compound 1		Compound 2		FICI[2]
	Name	MIC[1] (µg/mL) Alone	Name	MIC (µg/mL) Alone	
P. aeruginosa 1381	MB-8-2	2	Aztreonam	32	1.11
P. aeruginosa 1384		2		8	1.11
P. aeruginosa 1474		2		8	0.73
P. aeruginosa 1479		2		8	0.98
P. aeruginosa 2566		2		16	1.23
P. aeruginosa 2568		2		8	0.98
P. aeruginosa 103		4		8	1.19

[1]MIC, Minimum Inhibitory Concentration
[2]FICI, Fractional Inhibitory Concentration Index

TABLE 34

Summary of Minimum Inhibitory Concentration and Fractional Inhibitory Concentration Results for MB-11 and Piperacillin

Organism[1]	Compound 1		Compound 2		FICI[2]
	Name	MIC[1] (µg/mL) Alone	Name	MIC (µg/mL) Alone	
P. aeruginosa 1381	MB-11	1	Piperacillin	>64	1.12
P. aeruginosa 1384		1		16	0.71
P. aeruginosa 1474		1		8	1.12
P. aeruginosa 1479		1		8	1.29
P. aeruginosa 2566		0.5		32	1.12
P. aeruginosa 2568		1		8	1.12
P. aeruginosa 103		2		8	1.11

[1]MIC, Minimum Inhibitory Concentration
[2]FICI, Fractional Inhibitory Concentration Index

TABLE 35

Summary of Minimum Inhibitory Concentration and Fractional Inhibitory Concentration Results for MB-11 and Aztreonam

Organism[1]	Compound 1		Compound 2		FICI[2]
	Name	MIC[1] (µg/mL) Alone	Name	MIC (µg/mL) Alone	
P. aeruginosa 1381	MB-11	2	Aztreonam	32	0.92
P. aeruginosa 1384		1		8	0.96
P. aeruginosa 1474		1		8	0.71
P. aeruginosa 1479		1		8	0.79
P. aeruginosa 2566		0.5		16	1.12
P. aeruginosa 2568		1		8	0.96
P. aeruginosa 103		2		8	1.11

[1]MIC, Minimum Inhibitory Concentration
[2]FICI, Fractional Inhibitory Concentration Index

TABLE 36

Summary of Minimum Inhibitory Concentration and Fractional Inhibitory Concentration Results for MB-2B and Piperacillin

Organism[1]	Compound 1		Compound 2		FICI[2]
	Name	MIC[1] (µg/mL) Alone	Name	MIC (µg/mL) Alone	
P. aeruginosa 1381	MB-2B	2	Piperacillin	>64	1.02
P. aeruginosa 1384		8		16	0.79
P. aeruginosa 1474		8		8	0.91
P. aeruginosa 1479		8		8	1.08
P. aeruginosa 2566		8		32	1.04
P. aeruginosa 2568		8		8	0.97
P. aeruginosa 103		8		8	1.16

[1]MIC, Minimum Inhibitory Concentration
[2]FICI, Fractional Inhibitory Concentration Index

TABLE 37

Summary of Minimum Inhibitory Concentration and Fractional Inhibitory Concentration Results for MB-2B and Aztreonam

Organism[1]	Compound 1		Compound 2		FICI[2]
	Name	MIC[1] (µg/mL) Alone	Name	MIC (µg/mL) Alone	
P. aeruginosa 1381	MB-2B	8	Aztreonam	64	0.89
P. aeruginosa 1384		8		8	0.91
P. aeruginosa 1474		8		8	0.54
P. aeruginosa 1479		8		8	0.87
P. aeruginosa 2566		8		16	0.91
P. aeruginosa 2568		8		8	0.87
P. aeruginosa 103		8		8	1.08

[1]MIC, Minimum Inhibitory Concentration
[2]FICI, Fractional Inhibitory Concentration Index

TABLE 38

Summary of Minimum Inhibitory Concentration and Fractional Inhibitory Concentration Results for MB-1B-3 and Cefotaxime

Organism[1]	Compound 1		Compound 2		FICI[2]
	Name	MIC[1] (µg/mL) Alone	Name	MIC (µg/mL) Alone	
K. pneumoniae 1346	MB-1B-3	2	Cefotaxime	0.06	1.23
K. pneumoniae 1355		1		0.06	2.29
K. pneumoniae 2238		1		16	1.29
K. pneumoniae 2541		2		0.12	1.23
K. pneumoniae 2546		1		0.25	1.12
K. pneumoniae 2549		1		0.12	0.79
P. aeruginosa 103		1		16	0.96

[1]MIC, Minimum Inhibitory Concentration
[2]FICI, Fractional Inhibitory Concentration Index

TABLE 39

Summary of Minimum Inhibitory Concentration and Fractional Inhibitory Concentration Results for MB-1B-3 and Cefepime

Organism[1]	Compound 1		Compound 2		FICI[2]
	Name	MIC[1] (µg/mL) Alone	Name	MIC (µg/mL) Alone	
P. aeruginosa 1381	MB-1B-3	1	Cefepime	32	1.29
P. aeruginosa 1384		1		2	0.79
P. aeruginosa 1474		1		2	0.79
P. aeruginosa 1479		1		4	1.12
P. aeruginosa 2566		0.5		8	1.37
P. aeruginosa 2568		1		2	0.79
P. aeruginosa 103		1		2	0.71

[1]MIC, Minimum Inhibitory Concentration
[2]FICI, Fractional Inhibitory Concentration Index

TABLE 40

Summary of Minimum Inhibitory Concentration and Fractional Inhibitory Concentration Results for MB-15 and Cefotaxime

Organism[1]	Compound 1		Compound 2		FICI[2]
	Name	MIC[1] (µg/mL) Alone	Name	MIC (µg/mL) Alone	
K. pneumoniae 1346	MB-15	2	Cefotaxime	0.06	1.23
K. pneumoniae 1355		1		0.12	2.37
K. pneumoniae 2238		2		16	1.23
K. pneumoniae 2541		2		0.12	1.23
K. pneumoniae 2546		2		0.25	0.97
K. pneumoniae 2549		2		0.06	1.23
P. aeruginosa 103		1		16	0.96

[1]MIC, Minimum Inhibitory Concentration
[2]FICI, Fractional Inhibitory Concentration Index

TABLE 41

Summary of Minimum Inhibitory Concentration and Fractional Inhibitory Concentration Results for MB-15 and Cefepime

Organism[1]	Compound 1		Compound 2		FICI[2]
	Name	MIC[1] (µg/mL) Alone	Name	MIC (µg/mL) Alone	
P. aeruginosa 1381	MB-15	1	Cefepime	32	1.29
P. aeruginosa 1384		1		2	0.79
P. aeruginosa 1474		1		2	1.12
P. aeruginosa 1479		1		4	1.12
P. aeruginosa 2566		0.5		8	1.37
P. aeruginosa 2568		1		2	1.12
P. aeruginosa 103		1		1	1.12

[1]MIC, Minimum Inhibitory Concentration
[2]FICI, Fractional Inhibitory Concentration Index

TABLE 42

Summary of Minimum Inhibitory Concentration and Fractional Inhibitory Concentration Results for MB-8-2 and Cefotaxime

Organism[1]	Compound 1		Compound 2		FICI[2]
	Name	MIC[1] (µg/mL) Alone	Name	MIC (µg/mL) Alone	
K. pneumoniae 1346	MB-8-2	0.5	Cefotaxime	0.06	1.37
K. pneumoniae 1355		0.5		0.06	1.37
K. pneumoniae 2238		0.5		16	1.37
K. pneumoniae 2541		1		0.12	1.12
K. pneumoniae 2546		1		0.25	1.29
K. pneumoniae 2549		1		0.06	1.12
P. aeruginosa 103		2		16	1.11

[1]MIC, Minimum Inhibitory Concentration
[2]FICI, Fractional Inhibitory Concentration Index

TABLE 43

Summary of Minimum Inhibitory Concentration and Fractional Inhibitory Concentration Results for MB-8-2 and Cefepime

Organism[1]	Compound 1		Compound 2		FICI[2]
	Name	MIC[1] (µg/mL) Alone	Name	MIC (µg/mL) Alone	
P. aeruginosa 1381	MB-8-2	2	Cefepime	32	1.23
P. aeruginosa 1384		2		2	0.80
P. aeruginosa 1474		2		2	1.11
P. aeruginosa 1479		2		4	1.23
P. aeruginosa 2566		2		8	1.23
P. aeruginosa 2568		2		2	0.98
P. aeruginosa 103		2		1	1.11

[1]MIC, Minimum Inhibitory Concentration
[2]FICI, Fractional Inhibitory Concentration Index

TABLE 44

Summary of Minimum Inhibitory Concentration and Fractional Inhibitory Concentration Results for MB-11 and Cefotaxime

Organism[1]	Compound 1		Compound 2		FICI[2]
	Name	MIC[1] (µg/mL) Alone	Name	MIC (µg/mL) Alone	
K. pneumoniae 1346	MB-11	0.5	Cefotaxime	0.06	1.37
K. pneumoniae 1355		0.5		0.06	1.87
K. pneumoniae 2238		0.5		8	1.37
K. pneumoniae 2541		0.5		0.25	0.73
K. pneumoniae 2546		0.5		0.25	1.37
K. pneumoniae 2549		0.5		0.06	1.37
P. aeruginosa 103		1		16	1.12

[1]MIC, Minimum Inhibitory Concentration
[2]FICI, Fractional Inhibitory Concentration Index

TABLE 45

Summary of Minimum Inhibitory Concentration and Fractional Inhibitory Concentration Results for MB-11 and Cefepime

Organism[1]	Compound 1		Compound 2		FICI[2]
	Name	MIC[1] (µg/mL) Alone	Name	MIC (µg/mL) Alone	
P. aeruginosa 1381	MB-11	1	Cefepime	32	1.12
P. aeruginosa 1384		1		2	1.12
P. aeruginosa 1474		0.5		2	1.12
P. aeruginosa 1479		0.5		8	0.87
P. aeruginosa 2566		0.5		16	0.93
P. aeruginosa 2568		0.5		2	0.87
P. aeruginosa 103		1		1	0.12

[1]MIC, Minimum Inhibitory Concentration
[2]FICI, Fractional Inhibitory Concentration Index

TABLE 46

Summary of Minimum Inhibitory Concentration and Fractional Inhibitory Concentration Results for MB-2B and Cefotaxime

Organism[1]	Compound 1		Compound 2		FICI[2]
	Name	MIC[1] (µg/mL) Alone	Name	MIC (µg/mL) Alone	
K. pneumoniae 1346	MB-2B	4	Cefotaxime	0.06	1.19
K. pneumoniae 1355		4		0.06	1.19
K. pneumoniae 2238		4		8	1.64
K. pneumoniae 2541		8		0.25	0.64
K. pneumoniae 2546		8		0.25	1.16
K. pneumoniae 2549		8		0.12	0.83
P. aeruginosa 103		2		16	1.11

[1]MIC, Minimum Inhibitory Concentration
[2]FICI, Fractional Inhibitory Concentration Index

TABLE 47

Summary of Minimum Inhibitory Concentration and Fractional Inhibitory Concentration Results for MB-2B and Cefepime

Organism[1]	Compound 1		Compound 2		FICI[2]
	Name	MIC[1] (µg/mL) Alone	Name	MIC (µg/mL) Alone	
P. aeruginosa 1381	MB-2B	4	Cefepime	32	1.09
P. aeruginosa 1384		4		2	0.94
P. aeruginosa 1474		2		2	0.98
P. aeruginosa 1479		2		4	1.11
P. aeruginosa 2566		2		8	1.23
P. aeruginosa 2568		2		2	1.11
P. aeruginosa 103		2		2	0.61

[1]MIC, Minimum Inhibitory Concentration
[2]FICI, Fractional Inhibitory Concentration Index

EXAMPLE 11

THE EFFECT OF BISMUTH THIOLS ON INFECTION IN A RATTUS NORVEGICUS FEMUR CRITICAL DEFECT

The current standard of care for open fractures is irrigation, debridement and antibiotics; this is intended to reduce the bacterial load in the wound to the point that infection does not occur. Despite these treatments, infections still complicate up to 75% of severe combat open tibia fractures. Interestingly, even though early infections are often caused by gram negative bacteria, late infections that are implicated in healing problems and amputation are due to gram positive infections, frequently Staphylococci species (Johnson 2007).

One of the reasons that *S. aureus* are resistant to standard treatment is their ability to form a biofilm. Bacteria in biofilms are able to resist concentrations of antimicrobial compounds which would kill similar organisms in a culture medium (Costerton 1987).

The aim of this study was to determine whether BTs will reduce infection in a contaminated open fracture model either on their own or with antibiotics. The contaminated rat femur critical defect model is a well-accepted model and was used for the experiments described in this Example. This model offers a standardized model for comparing various possible treatments and their effects on reducing infection and/or improving healing.

Compounds (CPD) CPD-8-2 (bismuth pyrithione/ butanedithiol; Table 1) and CPD-11 (bismuth pyrithione/ ethanedithiol; Table 1) are two analogues of BIS-Bis that have shown potential against Biofilm secreting bacteria *in vitro*, though with a different spectrum of activity than Bis-EDT.

The three BT formulations, Bis-EDT, CPD-11 and CPD-8-2 (see Table 1) demonstrated inhibitory effects on *S. aureus* strains *in vitro* when used with and without Tobramicin and Vancomycin in a Poly Methyl Methacrylate (PMMA) cement bead vehicle. Three formulations of microparticulate BTs were produced in a clinically useful hydrogel gel form as described herein. These BTs were tested suspended in a gel at a concentration of $5mg/ml^{-1}$ as has been found to be an appropriate concentration for gel delivery. The gel formulations conformed to the wound contours, and did not require removal following application.

Two treatment arms were used: in the first arm, BT was used singularly; in the second arm BT was used in conjunction with a systemic antibiotic (ABx).

(a) BT Singularly.

Six hours after inoculation with *S. aureus*, the wound was debrided, irrigated with saline and 1ml of BT gel inserted within the defect.

(b) BT with Systemic Antibiotics (ABx).

Six hours after inoculation with *S. aureus*, the wound was debrided, irrigated with saline and 1ml of BT gel added inserted within the defect. The antibiotic used was Cefazolin at a dose equivalent to $5mgKg^{-1}$ delivered via sub-cutaneous injection twice daily for a total of 3-days following the injury. The first dose was administered immediately prior to debridement. Previous data suggested that this dose would result in a reduction in bacteria levels from $\approx 10^6$ to $\approx 10^4$ and therefore still allow the relative effect of different BTs to be measured.

(c) Control

Six hours after inoculation with *S. aureus*, the wound was debrided and irrigated with saline. The control animals were also treated with Cefazolin as per the regime described above.

PROCEDURE:

The procedure for the in vivo rat injury model was performed as described by Chen *et al.* (2002 *J. Orthop. Res.* 20:142; 2005 *J. Orthop. Res.* 23:816; 2006 *J. Bone Joint Surg. Am.* 88:1510; 2007 *J. Orthop. Trauma* 21:693). The rats were anesthetized and prepped for surgery. The anterolateral aspect of the femoral shaft was exposed through a 3-cm incision. The periosteum and attached muscle was stripped from the bone. A polyacetyl plate (27 x 4 x 4 mm) was placed on the anterolateral surface of the femur. The plates were predrilled to accept 0.9-mm diameter threaded Kirschner wires. The bases of these plates were formed to fit the contour of the femoral shaft. Pilot holes were drilled through both cortices of the femur using the plate as a template and threaded Kirschner wire was inserted through the plate and femur. The notches that were 6 mm apart on the plate served as a guide for bone removal. A small oscillating saw was used to create the defect while the tissue was cooled by continuous irrigation in an effort to prevent thermal damage.

Several groups of 10 animals each were inoculated with $1x10^5$ CFU of *S. aureus* and treated with BT alone or in combination with antibiotics 6 hours post-inoculation as described above. The groups were as follows: Bis-EDT gel; MB-11 gel; MB-8-2 gel; Bis-EDT gel & Abx; MB-11 gel & Abx; MB-8-2 gel & Abx; Control (Abx alone).

Animals were euthanized 14 days after surgery and bone and hardware sent for microbiological analysis, the results of which are shown in Figure 7.

Based on the power analysis, 10 animals per group will give a power of 80% to detect a 25% difference between the treatment and control groups. This is with an expected standard deviation of 35% and alpha of 0.05.

As shown in Figure 7, in combination with Bis-EDT, MB-11 and MB-8-2, Cefazolin antibiotic activity was enhanced as compared to Cefazolin or any of the Bis compounds alone to reduce *S. aureus* infection of injured bone. Cefazolin in combination with MB-11 and MB-8-2 showed enhanced antibiotic activity as compared to Cefazolin alone to reduce *S. aureus* infection detected on hardware. Bis-EDT did not appear to affect Cefazolin activity in this capacity.

EXAMPLE 12

ACTIVITY OF BISMUTH CONTAINING COMPOUNDS AGAINST MARINE ORGANISMS

This example describes the antimicrobial activity of bismuth containing compounds. The MIC values of three bismuth containing compounds, bismuth dimercaprol (BisBAL), bismuth dimercaptotoluene (BisTOL), and bismuth ethanedithiol (BisEDT), against three different marine bacteria were determined using methods routinely practiced by persons skilled in the art. The data are presented in the following table.

BT Compound (μg/ml)	BisBAL	BisTOL	BisEDT
V. alginolyticus	3.1	1	0.1
H. marina	17.5	7.2	2.6
M. hydrocarbonoclasicus	2	0.4	.28

EXAMPLE 13

EFFECT OF BISMUTH CONTAINING COMPOUNDS ON BARNACLE SETTLEMENT BEHAVIOR

Compounds, BisBAL and BisTOL were included in an assay to determine the inhibitory activity of each compound on barnacle larvae settlement behavior. Methods were performed according to techniques practiced in the art. BisBAL had an EC_{50} (the concentration at which 50%

settlement inhibition occurs) of 1.6 ppm, and BisTOL had an EC_{50} of 15.4 ppm. In another experiment, BisEDT was dissolved either directly in natural seawater or first dissolved in DMSO and then diluted in natural seawater. The EC_{50} measurements were not statistically different. BisEDT had an EC_{50} of 1.5 ppm when dissolved directly in seawater and had an EC_{50} of 2.1 ppm when first dissolved in DMSO. The EC_{50} of the commercial biocide, SEANINE 211, was 0.5 ppm.

EXAMPLE 14

EFFECT OF BISMUTH CONTAINING COMPOUNDS ON SETTLEMENT OF ALGAE

The effect of three bismuth containing compounds, bismuth dimercaprol (BisBAL), bismuth dimercaptotoluene (BisTOL), and bismuth ethanedithiol (BisEDT), on the settlement of algae was determined, particularly the ability of each compound to inhibit germination of Enteromorpha spores. Each compound was tested at 0.001, 0.01, 0.1, 1.0, and 10.0 µg/ml. BisEDT was the most effective compound; at 1 µg/ml BisEDT, germination of approximately 50% of the algae spore population was inhibited, and at 10 µg/ml, germination of approximately 75% algae spores was inhibited. Up to ten micrograms per ml of BisBAL and BisTOL had no inhibitory effect on germination of the spores of this particular algae species.

EXAMPLE 15

EFFECT OF BISMUTH CONTAINING COMPOUNDS ON SETTLEMENT OF ALGAE

The effect of three bismuth containing compounds, bismuth dimercaprol (BisBAL), bismuth dimercaptotoluene (BisTOL), and bismuth ethanedithiol (BisEDT), on growth of a marine diatom was determined according to techniques practiced in the art. Settlement of marine diatoms (diatoms per fov) was inhibited by increasing concentrations of each of the three compounds (0.001, 0.01, 0.1, 1.0, and 10.0 µg/ml). Each compound exhibited inhibitory activity at 0.1 µg/ml; BisEDT was the most active, demonstrating nearly 100% inhibition. Each of BisTOL and BisBAL exhibited approximately 30% of marine diatom settlement at 0.1 µg/ml.

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The various embodiments described above can be combined to provide further embodiments. All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet are incorporated herein by reference, in their entirety. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, applications and publications to provide yet further embodiments.

These and other changes can be made to the embodiments in light of the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

STATEMENTS OF INVENTION

1. A method for protecting a plant against a bacterial, fungal or viral pathogen, comprising:

contacting the plant with an effective amount of a bismuth-thiol (BT) composition under conditions and for a time sufficient for one or more of:

(i) prevention of infection of the plant by the bacterial, fungal or viral pathogen,

(ii) inhibition of cell viability or cell growth of substantially all planktonic cells of the bacterial, fungal or viral pathogen,

(iii) inhibition of biofilm formation by the bacterial, fungal or viral pathogen, and

(iv) inhibition of biofilm viability or biofilm growth of substantially all biofilm-form cells of the bacterial, fungal or viral pathogen,

wherein the BT composition comprises a substantially monodisperse suspension of microparticles that comprise a BT compound, said microparticles having a volumetric mean diameter of from about 0.4 μm to about 10 μm.

2. The method of claim 1 wherein the bacterial pathogen comprises *Erwinia amylovora* cells.

3. The method of claim 1 wherein the bacterial pathogen is selected from the group consisting of *Erwinia amylovora*, *Xanthomonas campestris pv dieffenbachiae*, *Pseudomonas syringae, Xylella fastidiosa; Xylophylus ampelinus; Monilinia fructicola, Pantoea stewartii* subsp. *Stewartii, Ralstonia solanacearum, and Clavibacter michiganensis* subsp. *sepedonicus.*

4. The method of claim 1 wherein the bacterial pathogen exhibits antibiotic resistance.

5. The method of claim 1 wherein the bacterial pathogen exhibits streptomycin resistance.

6. The method of claim 1 wherein the plant is a food crop plant.

7. The method of claim 6 wherein the food crop plant is a fruit tree.

8. The method of claim 7 wherein the fruit tree is selected from the group consisting of an apple tree, a pear tree, a peach tree, a nectarine tree, a plum tree, an apricot tree.

9. The method of claim 6 wherein the food crop plant is a banana tree of genus *Musa*.

10. The method of claim 6 wherein the food crop plant is a plant selected from a tuberous plant, a leguminous plant, and a cereal grain plant.

11. The method of claim 10 wherein the tuberous plant is selected from the group consisting of *Solanum tuberosum* (potato), and *Ipomoea batatas* (sweet potato).

12. The method of claim 1 in which the step of contacting is performed one or a plurality of times.

13. The method of claim 12 in which at least one step of contacting comprises one of spraying, dipping, coating and painting the plant.

14. The method of claim 12 in which at least one step of contacting is performed at a flower blossom, green-tip or growth site of the plant.

15. The method of claim 12 in which at least one step of contacting is performed within 24, 48 or 72 hours of first flower blooming on the plant.

16. The method of claim 1 wherein the BT composition comprises one or more BT compounds selected from the group consisting of BisBAL, BisEDT, Bis-dimercaprol, Bis-DTT, Bis-2-mercaptoethanol, Bis-DTE, Bis-Pyr, Bis-Ery, Bis-Tol, Bis-BDT, Bis-PDT, Bis-Pyr/Bal, Bis-Pyr/BDT, Bis-Pyr/EDT, Bis-Pyr/PDT, Bis-Pyr/Tol, Bis-Pyr/Ery, bismuth-1-mercapto-2-propanol, and Bis-EDT/2-hydroxy-1-propanethiol.

17. The method of claim 1 wherein the bacterial pathogen exhibits antibiotic resistance.

18. The method of any one of claims 1-17 which further comprises contacting the plant with a synergizing or enhancing antibiotic, simultaneously or sequentially and in any order with respect to the step of contacting the plant with the BT composition.

19. The method of claim 18 wherein the synergizing or enhancing antibiotic comprises an antibiotic that is selected from the group consisting of an aminoglycoside antibiotic, a carbapenem antibiotic, a cephalosporin antibiotic, a fluoroquinolone antibiotic, a penicillinase-resistant penicillin antibiotic, and an aminopenicillin antibiotic.

20. The method of claim 19 wherein the synergizing or enhancing antibiotic is an aminoglycoside antibiotic that is selected from the group consisting of amikacin, arbekacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, rhodostreptomycin, streptomycin, tobramycin and apramycin.

21. A method for overcoming antibiotic resistance in a plant in or on which an antibiotic-resistant bacterial plant pathogen is present, comprising

(a) contacting the plant with an effective amount of a BT composition under conditions and for a time sufficient for one or more of:

(i) prevention of infection of the plant by the antibiotic-resistant bacterial pathogen,

(ii) inhibition of cell viability or cell growth of substantially all planktonic cells of the antibiotic-resistant bacterial pathogen,

(iii) inhibition of biofilm formation by the antibiotic-resistant bacterial pathogen, and

(iv) inhibition of biofilm viability or biofilm growth of substantially all biofilm-form cells of the antibiotic-resistant bacterial pathogen,

wherein the BT composition comprises a substantially monodisperse suspension of microparticles that comprise a BT compound, said microparticles having a volumetric mean diameter of from about 0.5 μm to about 10 μm; and

(b) contacting the plant with a synergizing or enhancing antibiotic, simultaneously or sequentially and in any order with respect to the step of contacting the plant with the BT composition.

22. The method of any one of claims 1-21 wherein the bismuth-thiol composition comprises a plurality of microparticles that comprise a bismuth-thiol (BT) compound, substantially all of said microparticles having a volumetric mean diameter of from about 0.4 μm to about 5 μm and being formed by a process that comprises:

(a) admixing, under conditions and for a time sufficient to obtain a solution that is substantially free of a solid precipitate, (i) an acidic aqueous solution that comprises a bismuth salt comprising bismuth at a

concentration of at least 50 mM and that lacks a hydrophilic, polar or organic solubilizer, with (ii) ethanol in an amount sufficient to obtain an admixture that comprises about 25% ethanol by volume; and

(b) adding to the admixture of (a) an ethanolic solution comprising a thiol-containing compound to obtain a reaction solution, wherein the thiol-containing compound is present in the reaction solution at a molar ratio of from about 1:3 to about 3:1 relative to the bismuth, under conditions and for a time sufficient for formation of a precipitate which comprises the microparticles comprising the BT compound.

23. The method of claim 22 wherein the bismuth salt is $Bi(NO_3)_3$.

24. The method of claim 22 wherein the acidic aqueous solution comprises at least 5%, 10%, 15%, 20%, 22% or 22.5% bismuth by weight.

25. The method of claim 22 wherein the acidic aqueous solution comprises at least 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5% or 5% nitric acid by weight.

26. The method of claim 22 wherein the thiol-containing compound comprises one or more agents selected from the group consisting of 1,2-ethane dithiol, 2,3-dimercaptopropanol, pyrithione, dithioerythritol, 3,4-dimercaptotoluene, 2,3-butanedithiol, 1,3-propanedithiol, 2-hydroxypropane thiol, 1-mercapto-2-propanol, dithioerythritol, alpha-lipoic acid, dithiothreitol, methanethiol (CH_3SH [m-mercaptan]), ethanethiol (C_2H_5SH [e- mercaptan]), 1-propanethiol (C_3H_7SH [n-P mercaptan]), 2-propanethiol ($CH_3CH(SH)CH_3$ [$2C_3$ mercaptan]), butanethiol (C_4H_9SH ([n-butyl mercaptan]), tert-butyl mercaptan ($C(CH_3)_3SH$ [t-butyl mercaptan]), pentanethiol ($C_5H_{11}SH$ [pentyl mercaptan]), coenzyme A, lipoamide, glutathione, cysteine, cystine, 2-mercaptoethanol, dithiothreitol, dithioerythritol, 2-mercaptoindole, transglutaminase, (11-mercaptoundecyl)hexa(ethylene glycol), (11-mercaptoundecyl)tetra(ethylene glycol), (11-mercaptoundecyl)tetra(ethylene glycol) functionalized gold nanoparticles, 1,1′,4′,1″-terphenyl-4-thiol, 1,11-undecanedithiol, 1,16-hexadecanedithiol, 1,2-ethanedithiol technical grade, 1,3-propanedithiol, 1,4-benzenedimethanethiol, 1,4-butanedithiol, 1,4-butanedithiol diacetate, 1,5-

pentanedithiol, 1,6-hexanedithiol, 1,8-octanedithiol, 1,9-nonanedithiol, adamantanethiol, 1-butanethiol, 1-decanethiol, 1-dodecanethiol, 1-heptanethiol, 1-heptanethiol purum, 1-hexadecanethiol, 1-hexanethiol, 1-mercapto-(triethylene glycol), 1-mercapto-(triethylene glycol) methyl ether functionalized gold nanoparticles, 1-mercapto-2-propanol, 1-nonanethiol, 1-octadecanethiol, 1-octanethiol, 1-octanethiol, 1-pentadecanethiol, 1-pentanethiol, 1-propanethiol, 1-tetradecanethiol, 1-tetradecanethiol purum, 1-undecanethiol, 11-(1*H*-pyrrol-1-yl)undecane-1-thiol, 11-amino-1-undecanethiol hydrochloride, 11-bromo-1-undecanethiol, 11-mercapto-1-undecanol, 11-mercapto-1-undecanol, 11-mercaptoundecanoic acid, 11-mercaptoundecanoic acid, 11-mercaptoundecyl trifluoroacetate, 11-mercaptoundecylphosphoric acid, 12-mercaptododecanoic acid, 12-mercaptododecanoic acid, 15-mercaptopentadecanoic acid, 16-mercaptohexadecanoic acid, 16-mercaptohexadecanoic acid, 1*H*,1*H*,2*H*,2*H*-perfluorodecanethiol, 2,2'-(ethylenedioxy)diethanethiol, 2,3-butanedithiol, 2-butanethiol, 2-ethylhexanethiol, 2-methyl-1-propanethiol, 2-methyl-2-propanethiol, 2-phenylethanethiol, 3,3,4,4,5,5,6,6,6-nonafluoro-1-hexanethiol purum, 3-(dimethoxymethylsilyl)-1-propanethiol, 3-chloro-1-propanethiol, 3-mercapto-1-propanol, 3-mercapto-2-butanol, 3-mercapto-*N*-nonylpropionamide, 3-mercaptopropionic acid, 3-mercaptopropyl-functionalized silica gel, 3-methyl-1-butanethiol, 4,4'-bis(mercaptomethyl)biphenyl, 4,4'-dimercaptostilbene, 4-(6-mercaptohexyloxy)benzyl alcohol, 4-cyano-1-butanethiol, 4-mercapto-1-butanol, 6-(ferrocenyl)hexanethiol, 6-mercapto-1-hexanol, 6-mercaptohexanoic acid, 8-mercapto-1-octanol, 8-mercaptooctanoic acid, 9-mercapto-1-nonanol, biphenyl-4,4'-dithiol, butyl 3-mercaptopropionate, copper(I) 1-butanethiolate, cyclohexanethiol, cyclopentanethiol, decanethiol functionalized silver nanoparticles, dodecanethiol functionalized gold nanoparticles, dodecanethiol functionalized silver nanoparticles, hexa(ethylene glycol)mono-11-(acetylthio)undecyl ether, mercaptosuccinic acid, methyl 3-mercaptopropionate, nanoTether BPA-HH, NanoThinks™ 18, NanoThinks™ 8, NanoThinks™ ACID11, NanoThinks™ ACID16, NanoThinks™ ALCO11, NanoThinks™ THIO8, octanethiol functionalized gold nanoparticles, PEG dithiol average M_n 8,000, PEG dithiol average mol wt 1,500, PEG dithiol average mol wt 3,400, *S*-(11-bromoundecyl)thioacetate, *S*-(4-cyanobutyl)thioacetate, thiophenol, triethylene glycol mono-11-mercaptoundecyl ether, trimethylolpropane tris(3-mercaptopropionate), [11-

(methylcarbonylthio)undecyl]tetra(ethylene glycol), *m*-carborane-9-thiol, *p*-terphenyl-4,4″-dithiol, *tert*-dodecylmercaptan, and *tert*-nonyl mercaptan.

27. The method of any one of claims 1-21 wherein the bacterial pathogen comprises at least one of:

(i) one or more gram-negative bacteria;

(ii) one or more gram-positive bacteria;

(iii) one or more antibiotic-sensitive bacteria;

(iv) one or more antibiotic-resistant bacteria;

(v) a bacterial pathogen that is selected from the group consisting of *Staphylococcus aureus (S. aureus)*, MRSA (methicillin-resistant *S. aureus*), *Staphylococcus epidermidis* , MRSE (methicillin-resistant *S. epidermidis*), *Mycobacterium tuberculosis, Mycobacterium avium, Pseudomonas aeruginosa,* drug-resistant *P. aeruginosa, Escherichia coli,* enterotoxigenic *E. coli,* enterohemorrhagic *E. coli, Klebsiella pneumoniae, Clostridium difficile, Heliobacter pylori, Legionella pneumophila*, *Enterococcus faecalis,* methicillin-susceptible *Enterococcus faecalis*, *Enterobacter cloacae, Salmonella typhimurium, Proteus vulgaris, Yersinia enterocolitica, Vibrio cholera, Shigella flexneri,* vancomycin-resistant *Enterococcus* (VRE), *Burkholderia cepacia* complex, *Francisella tularensis, Bacillus anthracis, Yersinia pestis, Pseudomonas aeruginosa, Streptococcus pneumonia*, penicillin-resistant *Streptococcus pneumonia*, *Escherichia coli*, *Burkholderia cepacia*, *Bukholderia multivorans*, *Mycobacterium smegmatis* and *Acinetobacter baumannii.*

28. The method of any one of claims 1-21 which comprises contacting the plant with at least one of (i) a synergizing antibiotic and (ii) a cooperative antimicrobial efficacy enhancing antibiotic, simultaneously or sequentially and in any order with respect to the step of contacting the surface with the BT composition.

29. The method of claim 28 wherein the synergizing antibiotic or the cooperative antimicrobial efficacy enhancing antibiotic comprises an antibiotic that is selected from the group consisting of an aminoglycoside antibiotic, a carbapenem antibiotic, a cephalosporin antibiotic, a fluoroquinolone antibiotic, a glycopeptide antibiotic, a lincosamide antibiotic, a penicillinase-resistant penicillin antibiotic, and an aminopenicillin antibiotic.

30. The method of claim 29 wherein the synergizing antibiotic or the cooperative antimicrobial efficacy enhancing antibiotic is an aminoglycoside antibiotic that is selected from the group consisting of amikacin, arbekacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, rhodostreptomycin, streptomycin, tobramycin and apramycin.

31. A method for overcoming antibiotic resistance in or on a plant where an antibiotic-resistant bacterial pathogen is present, comprising:

contacting the plant simultaneously or sequentially and in any order with an effective amount of (1) at least one bismuth-thiol (BT) composition and (2) at least one antibiotic that is capable of enhancing or acting synergistically with the at least one BT composition, under conditions and for a time sufficient for one or more of:

(i) prevention of infection of the plant by the bacterial pathogen,

(ii) inhibition of cell viability or cell growth of substantially all planktonic cells of the bacterial pathogen,

(iii) inhibition of biofilm formation by the bacterial pathogen, and

(iv) inhibition of biofilm viability or biofilm growth of substantially all biofilm-form cells of the bacterial pathogen,

wherein the BT composition comprises a plurality of microparticles that comprise a bismuth-thiol (BT) compound, substantially all of said microparticles having a volumetric mean diameter of from about 0.4 μm to about 5 μm; and thereby overcoming antibiotic resistance on the epithelial tissue surface.

32. The method of claim 31 wherein the bacterial pathogen exhibits resistance to an antibiotic that is selected from the group consisting of methicillin, vancomycin, naficilin, gentamicin, ampicillin, chloramphenicol, doxycycline, tobramycin, clindamicin and gatifloxacin.

33. The method of claim 31 wherein the BT composition comprises one or more BT compounds selected from the group consisting of BisBAL, BisEDT, Bis-dimercaprol, Bis-DTT, Bis-2-mercaptoethanol, Bis-DTE, Bis-Pyr, Bis-Ery, Bis-Tol, Bis-BDT, Bis-PDT, Bis-Pyr/Bal, Bis-Pyr/BDT, Bis-

Pyr/EDT, Bis-Pyr/PDT, Bis-Pyr/Tol, Bis-Pyr/Ery, bismuth-1-mercapto-2-propanol, and Bis-EDT/2-hydroxy-1-propanethiol.

34. The method of claim 33 wherein the synergizing or enhancing antibiotic comprises an antibiotic that is selected from the group consisting of clindamicin, gatifloxacin, an aminoglycoside antibiotic, a carbapenem antibiotic, a cephalosporin antibiotic, a fluoroquinolone antibiotic, a penicillinase-resistant penicillin antibiotic, and an aminopenicillin antibiotic.

35. The method of claim 34 wherein the synergizing or enhancing antibiotic is an aminoglycoside antibiotic that is selected from the group consisting of amikacin, arbekacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, rhodostreptomycin, streptomycin, tobramycin and apramycin.

CLAIMS

1. A method for protecting a surface of an inanimate object against a bacterial, fungal or viral pathogen, comprising:

contacting the surface of the inanimate object with an effective amount of a disinfectant comprising a bismuth-thiol (BT) composition under conditions and for a time sufficient for one or more of:

(i) prevention of infection of the surface of the inanimate object by the bacterial, fungal or viral pathogen,

(ii) inhibition of cell viability or cell growth of substantially all planktonic cells of the bacterial, fungal or viral pathogen,

(iii) inhibition of biofilm formation by the bacterial, fungal or viral pathogen, and

(iv) inhibition of biofilm viability or biofilm growth of substantially all biofilm-form cells of the bacterial, fungal or viral pathogen,

wherein the BT composition comprises a substantially monodisperse suspension of microparticles that comprise a BT compound, said microparticles having a volumetric mean diameter of from about 0.4 μm to about 10 μm.

2. A method for protecting an artificial surface against a bacterial, fungal or viral pathogen, comprising:

contacting the artificial surface with an effective amount of a disinfectant comprising a bismuth-thiol (BT) composition under conditions and for a time sufficient for one or more of:

(i) prevention of infection of the artificial surface by the bacterial, fungal or viral pathogen,

(ii) inhibition of cell viability or cell growth of substantially all planktonic cells of the bacterial, fungal or viral pathogen,

(iii) inhibition of biofilm formation by the bacterial, fungal or viral pathogen, and

(iv) inhibition of biofilm viability or biofilm growth of substantially all biofilm-form cells of the bacterial, fungal or viral pathogen,

wherein the BT composition comprises a substantially monodisperse suspension of microparticles that comprise a BT compound, said microparticles having a volumetric mean diameter of from about 0.4 μm to about 10 μm.

3. The method of claim 1 or claim 2 wherein the surface is exposed to aquatic conditions, water droplets, or conditions of high humidity that are permissive for biofilm formation.

4. The method of claim 3 wherein the surface is present as part of a pipe, ship hull, rudder, propeller, anchor, hold, ballast tank, dock, dry dock, pier, piling, bulkhead, or other natural or artificial surface.

5. A method for protecting an article of manufacture against a bacterial, fungal or viral pathogen, comprising:

contacting the article of manufacture with an effective amount of a disinfectant comprising a bismuth-thiol (BT) composition under conditions and for a time sufficient for one or more of:

(i) prevention of infection of the article of manufacture by the bacterial, fungal or viral pathogen,

(ii) inhibition of cell viability or cell growth of substantially all planktonic cells of the bacterial, fungal or viral pathogen,

(iii) inhibition of biofilm formation by the bacterial, fungal or viral pathogen, and

(iv) inhibition of biofilm viability or biofilm growth of substantially all biofilm-form cells of the bacterial, fungal or viral pathogen,

wherein the BT composition comprises a substantially monodisperse suspension of microparticles that comprise a BT compound, said microparticles having a volumetric mean diameter of from about 0.4 μm to about 10 μm.

6. The method of any one of claims 1, 2 or 6, wherein the surface of the inanimate object, the artificial surface, or the article of manufacture comprises one or more of:

(i) a plumbing fixture, a vent, a duct, a conduit, a sanitation system, a water disinfection system, a drain, a cistern, a water delivery vehicle, or a water line;

(ii) a piling, a dock, a pier, a pipe, a pipeline, a piping structure intake screen, a cooling tower, a heat exchanger, or a dam; or

(iii) a cement surface, a concrete surface, a rubber surface, a silicone surface, a plastic surface, a painted surface, or a coated surface.