Title: SYNERGISTIC ANTIMICROBIAL COMPOSITIONS AND METHODS FOR REDUCING BIOFILM FORMATION

Abstract: The present invention provides compositions comprising: (a) a thiol-specific reagent and a cationic polypeptide; (b) a thiol-specific reagent and an iron-sequestering glycoprotein; or (c) a thiol-specific reagent and a quaternary ammonium compound; and uses thereof for the preparation of devices, and in particular medical devices, susceptible to colonization by biofilm forming bacteria. Compositions comprising a thiol-specific reagent and a cationic polypeptide, iron-sequestering glycoprotein, or quaternary ammonium compound have significant synergistic antibiofilm activity.
TITLE:
SYNERGISTIC ANTIMICROBIAL COMPOSITIONS AND METHODS FOR REDUCING BIOFILM FORMATION

FIELD OF THE INVENTION

This invention relates to synergistic antimicrobial compositions, which inhibit biofilm formation on or in devices, and in particular medical devices such as catheters.

BACKGROUND OF THE INVENTION

Biofilms are medically and industrially important because they can accumulate on a wide variety of substrates and are resistant to antimicrobial agents and detergents. Microbial biofilms develop when microorganisms adhere to a surface and produce extracellular polymers that facilitate adhesion and provide a structural matrix. Therefore inhibiting adhesion to surfaces is important. This surface may be inert, non-living material or living tissue.

Biofilm-associated microorganisms behave differently from planktonic (freely suspended) organisms with respect to growth rates and ability to resist antimicrobial treatments and therefore pose a public health problem. Many chronic infections that are difficult or impossible to eliminate with conventional antibiotic therapies are known to involve biofilms. A partial list of the infections that involve biofilms includes: otitis media, prostatitis, vascular endocarditis, cystic fibrosis pneumonia, meliodosis, necrotising faciitis, osteomyelitis, periodontitis, biliary tract infection, struvite kidney stone and host of nosocomial infections (Costerton, J.W., et al., Science, 284:1318-1322, 1999).

Biofilms on indwelling medical devices may be composed of gram-positive or gram-negative bacteria or yeasts. Bacteria commonly isolated from these devices include the gram-positive Enterococcus faecalis (E. faecalis), Staphylococcus epidermidis (S. epidermidis), Staphylococcus aureus (S. aureus), Streptococcus viridans (St. viridans); and the gram-negative Escherichia coli (E.coli), Klebsiella
pneumoniae (K. pneumoniae), Proteus mirabilis (P. mirabilis) and Pseudomonas aeruginosa (P. aeruginosa) (Donlan, R.M., Emerging Infectious Diseases, 7:277-281, 2001). The organisms most commonly isolated from urinary catheter biofilms are Staphylococcus epidermidis, Enterococcus faecalis, E. coli, Proteus mirabilis, Pseudomonas aeruginosa and Klebsiella pneumoniae. In the case of vascular catheters, Staphylococcus aureus and Staphylococcus epidermidis account for almost 70-80% of all infectious organisms, with Staphylococcus epidermidis being the most common organism. Candida albicans accounts for about 10-15% of catheter infections. Gram-negative bacilli account for almost 60-70%, enterococci for about 25% and Candida albicans for about 10% of cases of urinary tract infections. Catheter-associated urinary tract infection is the most common nosocomial infection. Each year, about 1 million patients in US hospitals acquire such an infection. It is the second most common cause of nosocomial infections (Maki, D.G. and P.A. Tambyah, Emerging Infectious Diseases, 7:1-6, 2001).

In recent years, there have been numerous efforts to sequester antimicrobials and antibiotics on the surface of or within devices that are then placed in the vasculature or urinary tract as a means of reducing the incidence of device-related infections. These antimicrobial agents are of varying chemical composition and cationic polypeptides (protamine, polylysine, lysozyme, etc.), surfactants (SDS, Tween-80, surfactin, etc.), quaternary ammonium compounds (benzalkonium chloride, tridodecyl methyl ammonium chloride, didecyl dimethyl ammonium chloride, etc.). The iron-sequestering glycoproteins such as lactoferrin from milk and ovotransferrin (conalbumin) from egg white are iron-binding glycoproteins, which inhibit the growth of certain bacteria by making iron unavailable for bacterial metabolism (Bezkorovainy, A., Adv. Exp. Med. Biol. 135:139-154, 1981).

The main methods of antimicrobial catheter preparation include immersion or flushing, coating, drug-polymer conjugate and impregnating (Tunny, M.M., et al., Rev. Med. Microbiol., 74: 195-205, 1996). In a clinical setting, suitable catheters can be treated by immersion immediately prior to placement, which offers flexibility and
control to clinicians in certain situations. Several studies have examined the clinical
efficacy of catheters coated with antimicrobial agents. Minocycline and rifampin
coatings have been shown to significantly reduce the risk of catheter-associated
onto urethral catheters has been shown to provide some protection against
Johnson, et al., described substantial in vitro antimicrobial activity of a commercially
available nitrofurazone coated silicone catheter in comparison with commercial
silver-hydrogel coated catheter (Antimicrob. Agents. Chemother. 43: 2,990-2, 995,
1999). The antibacterial activity of silver-containing compounds as antimicrobial
coatings for medical devices has been widely investigated. Silver-sulfadiazine used
in combination with chlorhexidine has received particular interest as a central venous
catheter coating (Stickler, D.J., Curr. Opin. Infect. Dis., 13:389-393, 2000; Darouiche,
efficacy of liposomal hydrogel coated urinary catheters for the prevention of bacterial

The loading of antimicrobial agents into medical devices by immersion or
coating technologies has the advantage of being relatively simple. However, the
limited mass of drug that can be incorporated may be insufficient for a prolonged
 antimicrobial effect, and the release of the drug following clinical insertion of the
device is rapid and relatively uncontrolled. A means of reducing these problems is by
direct incorporation of the antimicrobial agent into the polymeric matrix of the medical
device at the polymer synthesis stage or at the device manufacture stage. Rifampicin
has been incorporated into silicone in an attempt to prevent infection of
cerebrospinal fluid shunts with some success (Schierholz, J.M., et al., Biomaterials,
18: 839-844,1997). Iodine has also been incorporated into medical device
biomaterials. Coronary stents have been modified to have antithrombogenic and
antibacterial activity by covalent attachment of heparin to silicone with subsequent
entrainment of antibiotics in cross-linked collagen bound to the heparinised surface
Welle, C.J., et al., in US Patent No. 6,187,768 disclosed the method of preparing a kit for flushing a medical device. The kit includes a solution containing an antibiotic, an anticoagulant (protamine sulfate) and an antithrombotic agent or chelating agent useful for preventing infections caused by bacterial growth in catheters. Budny, J.A. et al., discloses various antimicrobial agents for anchoring to biofilms (US Patent Application No. 20020037260). Raad, et al., in US Patent No. 5,362,754 disclosed that pharmaceutical compositions of a mixture of minocycline and EDTA were useful in maintaining the patency of a catheter port. US Patent No. 6,187,768 to Welle et al. teaches the use of several anticoagulants for use in medical devices, including protamine sulfate.

In medical devices, various techniques have been described that incorporate potentially toxic metal ions in the form of metal salts into materials that make up the medical devices. The protection against biofilm formation lasts only as long as the coating remains on the device. A method of long-term prevention from biofilm formation that acts at the level of prevention of biofilm formation is needed. Also needed is a composition that allows for low quantities of the composition to be used effectively, thus reducing toxicity or other side effects to the user or patient without sacrificing effectiveness against biofilm formation. There is also a need for compositions that are environmentally friendly, medically acceptable, effective at lower concentrations and relatively economical to manufacture on a commercial scale for reducing biofilm formation in biomedical devices.

A few recent studies have demonstrated the antimicrobial activity of thiol-specific reagents, such as, N-substituted maleimides and thiosulfinates (Cechinel Filho, V., et al., Farmaco. 49: 675-677, 1994; Yoshida, H., et al., Biosci. Biotechnol. Biochem. 63: 591-594, 1999 and Zentz, F., et al., Farmaco. 57: 21-426, 2002). Wu, et al., in US Patent No. 5,466,707, disclosed the use of thione maleimides and compositions containing them as antimicrobial and marine antifouling agents. Thiol-specific reagents react with thiol groups of various enzymes, such as, thioredoxin reductase, coenzyme A disulfide reductase and glucosamine-1-phosphate

**SUMMARY OF THE INVENTION**

The invention provides a composition for inhibiting bacterial biofilms on a device comprising: (a) a thiol-specific reagent and a cationic polypeptide, or (b) a thiol-specific reagent and an iron-sequestering glycoprotein.

In an embodiment of the invention, the composition is effective against biofilms produced by gram-negative bacterial species selected from a group consisting of *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*.

In another embodiment of the invention, the composition is effective against biofilms produced by gram-positive bacterial species selected from a group consisting of *Enterococcus faecalis* and *Staphylococcus epidermidis*.
In yet another embodiment of the invention, the composition is effective against biofilms produced by bacterial species selected from a group consisting of *Staphylococcus epidermidis, Enterococcus faecalis, E. coli, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus, Streptococcus viridans, Klebsiella oxytoca, Staphylococcus saprophyticus, Providencia stuartii* and *Serratia marcescens*.

In a further embodiment of the invention, the composition is effective against biofilms produced by bacterial species selected from a group consisting of *Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterococcus faecalis* and *Staphylococcus epidermidis*.

In a still further embodiment of the invention, the thiol-specific reagent is between about 12.5 mg/L and about 100 mg/L of the composition.

In yet a still further embodiment of the invention, the cationic polypeptide is between about 12.5 mg/L and about 100 mg/L of the composition.

In another embodiment of the invention, the iron-sequestering glycoprotein is between about 125 mg/L and about 1000 mg/L of the composition.

In yet another embodiment of the invention, the thiol-specific reagent is selected from a group consisting of N-ethylmaleimide (NEM), 5,5-dithiobis-(2-nitrobenzoic acid)(DTNB), 2-nitro-5-thiocyanobenzoic acid (NTCB), p-hydroxymercuribenzoic acid (pHMB), iodoacetamide (IA), N-phenylmaleimide (PheM), N-(1-pyrenyl) maleimide (PyrM), naphthalene-1,5-dimaleimide (NDM), N,N'-1,2-phenylene) dimaleimide (oPDM), N,N'-1,4-phenylene dimaleimide (pPDM), N,N'-1,3-phenylene dimaleimide (mPDM), 1,1-(methylene-4,1-phenylene)bismaleimide (BM), 4-(N-maleimidophenyl)trimethylammonium (MPTM), N,N'-bis(3-maleimidopropionyl)-2-hydroxy-1,3-propanediamine (BMP), p-chloromercuribenzenesulphonic acid and thiosulfinates.
In a further embodiment of the invention, the cationic polypeptide is selected from a group consisting of protamine sulfate, polylysine, defensin, lactoperoxidase and lysozyme.

In a still further embodiment, the iron-sequestering glycoprotein is selected from a group consisting of ovotransferrin, lactoferrin and serotransferrin.

In yet a further embodiment of the invention, the compositions further comprises one or more ingredients selected from a group consisting of: an organic solvent, a binding or bonding or coupling agent, a surfactant, a quaternary ammonium compound and an antibiotic.

In another aspect, the invention provides a method of preparing a device comprising treating at least a surface of the device with a composition of the invention.

In a further aspect of the invention provides a method of preparing a device comprising coating a device with a composition of the invention.

In an embodiment of the invention, the method further comprises treating the device with a composition comprising hydrogel.

In another embodiment of the invention, the hydrogel is selected from the group consisting of polyvinylpyrrolidone-hydrogel, polyvinyl alcohol-hydrogel and polyethylene glycol-hydrogel.

In yet another embodiment of the invention, the treated device is a surface medical device.

In a still further embodiment of the invention, the device is a catheter.

The catheter may be an indwelling catheter.
The catheter may be selected from a group consisting of a urinary catheter, a suction catheter, a mucous extraction catheter, an umbilical catheter and a peritoneal catheter.

In another embodiment of the invention, the device is selected from a group consisting of catheters, contact lenses, intrauterine devices, intravaginal and intraintestinal devices, endotracheal tubes, umbilical cannula, bronchoscopes, dental prostheses and orthodontic devices. The device may be selected from the group consisting of ulcer, burn, and granulation tissue dressings or healing devices and occlusive patches.

In yet another embodiment of the invention, the device is selected from a group consisting of electrodes, external prostheses, fixation tapes, compression bandages, and monitors of various types.

In a still further embodiment of the invention, the device is selected from a group consisting of pipes, heat exchangers and computer chips.

In another aspect, the invention provides a method of preparing a device comprising incorporating a composition of the invention into polymers, which are used to form the device.

In yet another aspect, the invention provides a method of preparing a device comprising impregnating a composition of the invention into the device.

20 **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is a bar graph showing the effects of N,N'-(1,2-phenylene) dimaleimide (oPDM), protamine sulfate (PS) and ovotransferrin (OT) alone and in combinations (oPDM+PS and oPDM+OT) on biofilm formation in *E. coli* P18;
Figure 2 is a bar graph showing the effects of N,N'-(1,2-phenylene) dimaleimide (oPDM), protamine sulfate (PS) and ovotransferrin (OT) alone and in combinations (oPDM+PS and oPDM+OT) on biofilm formation in *Proteus mirabilis*;

Figure 3 is a bar graph showing the effects of N,N'-(1,2-phenylene) dimaleimide (oPDM), protamine sulfate (PS) and ovotransferrin (OT) alone and in combinations (oPDM+PS and oPDM+OT) on biofilm formation in *Klebsiella pneumoniae*;

Figure 4 is a bar graph showing the effects of N,N'-(1,2-phenylene) dimaleimide (oPDM), protamine sulfate (PS) and ovotransferrin (OT) alone and in combinations (oPDM+PS and oPDM+OT) on biofilm formation in *Pseudomonas aeruginosa*;

Figure 5 is a bar graph showing the effects of N,N'-(1,2-phenylene) dimaleimide (oPDM), protamine sulfate (PS) and ovotransferrin (OT) alone and in combinations (oPDM+PS and oPDM+OT) on biofilm formation in *Enterococcus faecalis*;

Figure 6 is a bar graph showing the effects of N,N'-(1,2-phenylene) dimaleimide (oPDM), protamine sulfate (PS) and ovotransferrin (OT) alone and in combinations (oPDM+PS and oPDM+OT) on biofilm formation in *Staph. epidermidis*;

Figure 7 is a bar graph showing the effects of N-(1-pyrenyl) maleimide (PyrM), protamine sulfate (PS) and ovotransferrin (OT) alone and in combinations (PyrM+PS and PyrM+OT) on biofilm formation in *E. coli* P18;

Figure 8 is a bar graph showing the effects of N-(1-pyrenyl) maleimide (PyrM), protamine sulfate (PS) and ovotransferrin (OT) alone and in combinations (PyrM+PS and PyrM+OT) on biofilm formation in *Proteus mirabilis*;
Figure 9 is a bar graph showing the effects of N-(1-pyrenyl) maleimide (PyrM), protamine sulfate (PS) and ovotransferrin (OT) alone and in combinations (PyrM+PS and PyrM+OT) on biofilm formation in *Klebsiella pneumoniae*;

Figure 10 is a bar graph showing the effects of N-(1-pyrenyl) maleimide (PyrM), protamine sulfate (PS) and ovotransferrin (OT) alone and in combinations (PyrM+PS and PyrM+OT) on biofilm formation in *Pseudomonas aeruginosa*;

Figure 11 is a bar graph showing the effects of N-(1-pyrenyl) maleimide (PyrM), protamine sulfate (PS) and ovotransferrin (OT) alone and in combinations (PyrM+PS and PyrM+OT) on biofilm formation in *Enterococcus faecalis*;

Figure 12 is a bar graph showing the effects of N-(1-pyrenyl) maleimide (PyrM), protamine sulfate (PS) and ovotransferrin (OT) alone and in combinations (PyrM+PS and PyrM+OT) on biofilm formation in *Staph. epidermidis*;

Figure 13 is a bar graph showing the combined effects of N,N’-(1,2-phenylene) dimaleimide and protamine sulfate on biofilm formation in *E. coli* P18;

Figure 14 is a bar graph showing the combined effects of N,N’-(1,2-phenylene) dimaleimide and protamine sulfate on biofilm formation in *Proteus mirabilis*;

Figure 15 is a bar graph showing the combined effects of N,N’-(1,2-phenylene) dimaleimide and protamine sulfate on biofilm formation in *Klebsiella pneumoniae*;

Figure 16 is a bar graph showing the combined effects of N,N’-(1,2-phenylene) dimaleimide and protamine sulfate on biofilm formation in *Pseudomonas aeruginosa*;
Figure 17 is a bar graph showing the combined effects of N,N'-(1,2-phenylene) dimaleimide and protamine sulfate on biofilm formation in *Enterococcus faecalis*;

Figure 18 is a bar graph showing the combined effects of N,N'-(1,2-phenylene) dimaleimide and protamine sulfate on biofilm formation in *Staphylococcus epidermidis*;

Figure 19 is a bar graph showing the combined effects of N,N'-(1,2-phenylene) dimaleimide and ovotransferrin on biofilm formation in *E. coli* P18;

Figure 20 is a bar graph showing the combined effects of N,N'-(1,2-phenylene) dimaleimide and ovotransferrin on biofilm formation in *Proteus mirabilis*;

Figure 21 is a bar graph showing the combined effects of N,N'-(1,2-phenylene) dimaleimide and ovotransferrin on biofilm formation in *Klebsiella pneumoniae*;

Figure 22 is a bar graph showing the combined effects of N,N'-(1,2-phenylene) dimaleimide and ovotransferrin on biofilm formation in *Pseudomonas aeruginosa*;

Figure 23 is a bar graph showing the combined effects of N,N'-(1,2-phenylene) dimaleimide and ovotransferrin on biofilm formation in *Enterococcus faecalis*;

Figure 24 is a bar graph showing the combined effects of N,N'-(1,2-phenylene) dimaleimide and ovotransferrin on biofilm formation in *Staphylococcus epidermidis*;

Figure 25 is a bar graph showing the combined effects of N-(1-pyrenyl) maleimide and protamine sulfate on biofilm formation in *E. coli* P18;
Figure 26 is a bar graph showing the combined effects of N-(1-pyrenyl) maleimide and protamine sulfate on biofilm formation in *Proteus mirabilis*;

Figure 27 is a bar graph showing the combined effects of N-(1-pyrenyl) maleimide and protamine sulfate on biofilm formation in *Klebsiella pneumoniae*;

Figure 28 is a bar graph showing the combined effects of N-(1-pyrenyl) maleimide and protamine sulfate on biofilm formation in *Pseudomonas aeruginosa*;

Figure 29 is a bar graph showing the combined effects of N-(1-pyrenyl) maleimide and protamine sulfate on biofilm formation in *Enterococcus faecalis*;

Figure 30 is a bar graph showing the combined effects of N-(1-pyrenyl) maleimide and protamine sulfate on biofilm formation in *Staphylococcus epidermidis*;

Figure 31 is a bar graph showing the combined effects of N-(1-pyrenyl) maleimide and ovotransferrin on biofilm formation in *E. coli* P18;

Figure 32 is a bar graph showing the combined effects of N-(1-pyrenyl) maleimide and ovotransferrin on biofilm formation in *Proteus mirabilis*;

Figure 33 is a bar graph showing the combined effects of N-(1-pyrenyl) maleimide and ovotransferrin on biofilm formation in *Klebsiella pneumoniae*;

Figure 34 is a bar graph showing the combined effects of N-(1-pyrenyl) maleimide and ovotransferrin on biofilm formation in *Pseudomonas aeruginosa*;

Figure 35 is a bar graph showing the combined effects of N-(1-pyrenyl) maleimide and ovotransferrin on biofilm formation in *Enterococcus faecalis*; and

Figure 36 is a bar graph showing the combined effects of N-(1-pyrenyl) maleimide and ovotransferrin on biofilm formation in *Staphylococcus epidermidis*. 
DETAILED DESCRIPTION

The present inventor has found that compositions, which contain at least one thiol-specific reagent and at least one cationic polypeptide or an iron-sequestering glycoprotein, have significant synergistic antibiofilm activity. The synergistic antibiofilm activity is evidenced by the small quantities of each of these compounds that need to be used to produce an effective antimicrobial composition. The necessary overall amount of the compounds is less than that which would be required if any of the compounds were to be used on their own. In particular, it is possible to use small amounts of thiol-specific reagents with small amounts of cationic polypeptides.

Accordingly, the present invention provides compositions for inhibiting bacterial biofilms comprising: (a) a thiol-specific reagent and a cationic polypeptide or (b) a thiol-specific reagent and an iron-sequestering glycoprotein. The synergistic antimicrobial compositions of the invention require remarkably small amounts of active ingredients (compared to that which has been used in the past) to be effective. These compositions have properties that include those of the separate compounds but go beyond them in efficacy and scope of application. The extremely low levels, and hence increased efficacy, of the active compounds or ingredients, make this invention very desirable and relatively economical to manufacture. A further advantage of using these compositions is that they have a moderate effect on the viability of bacterial cells, but alter their ability to form biofilms significantly. Thus, bacterial resistances that are typical for antibiotics that kill or inhibit growth may not develop.

Examples of thiol-specific compounds useful for preparing compositions of the present invention include, but are not limited to: N-ethylmaleimide (NEM), 5,5'-dithiobis-(2-nitrobenzoic acid)(DTNB), 2-nitro-5-thiocyanobenzoic acid (NTCB), P-hydroxymercuribenzoic acid (pHMB), iodoacetamide (IA), N-phenylmaleimide (PheM), N-(1-pyrenyl) maleimide (PyrM), naphthalene-1,5-dimaleimide (NDM),
N,N'-{(1,2-phenylene) dimaleimide (oPDM), N,N'-1,4-phenylene dimaleimide (pPDM),
N,N'-1,3-phenylene dimaleimide (mPDM), 1,1-(methylenedi-4,1-phenylene)
bismaleimide(BM), 4-(N-aleimido) phenyltrimethylammonium (MPTM), N,N'-bis(3-
maleimidopropionyl)-2-hydroxy-1,3-propanediamine (BMP), p-chloromercuribenzenesulphonic acid and thiosulfinates.

The amount of thiol-specific reagent included in the composition is preferably
between 12.5 to 200 mg/L. The higher end of this stated range can be used to
prepare a concentrated product that would be diluted prior to use. For non-
concentrated products, the amount of thiol-specific reagent to be used in this
invention is preferably between about 12.5 to 100 mg/L.

Examples of cationic polypeptides useful for preparing compositions of the
present invention include, but are not limited to: protamine sulfate, polylysine,
defensin, lactoperoxidase and lysozyme.

The amount of cationic polypeptide included in the composition is preferably
between about 12.5 to 200 mg/L. The higher end of this stated range can be used to
prepare a concentrated product. For non-concentrated products, the amount of
cationic polypeptide to be used in this invention is preferably between about 12.5-
100 mg/L.

Examples of iron-sequestering glycoproteins useful for preparing
compositions of the present invention include, but are not limited to: ovotransferrin,
lactoferrin and serotransferrin.

The amount of iron-sequestering glycoprotein included in the composition is
preferably between about 125 to 2000 mg/L. The higher end of this stated range can
be used to prepare a concentrated product. For non-concentrated products, the
amount of iron-sequestering glycoprotein to be used in this invention is preferably
between about 125-1000 mg/L.
Higher concentrations of these compounds can be used if it is desired for certain applications and will depend on the bacteria targeted and the device to be treated. Suitable working concentrations can easily be determined using methods known in the art.

Compositions of the invention can be prepared using methods known in the art. Generally, the components comprising the composition are dissolved in a suitable solvent. Thiol-specific reagents, such as, N,N’-(1,2-phenylene) dimaleimide (oPDM) or N-(1-pyrenyl) maleimide (PyrM) are not soluble in water, but are soluble in organic solvents, such as, DMSO, methanol, acetone and acetonitrile.

Compositions comprising a thiol-specific reagent and a cationic polypeptide can be prepared in DMSO if the cationic polypeptide is first dissolved in water before adding to DMSO.

Compositions comprising a thiol-specific reagent and an iron-sequestering glycoprotein can also be prepared in DMSO. While protamine sulfate is sparingly soluble in water, it needs to be dissolved in water before adding to the composition in DMSO.

Compositions of the invention can further comprise additional antibiofilm ingredients such as quaternary ammonium compounds and surfactants. In a preferred embodiment of the invention, the composition comprises: (a) at least one thiol-specific reagent; and (b) at least one compound from the group consisting of a quaternary ammonium compounds and/or a surfactant, wherein, the amount of each of components (a) and (b) is sufficient to form, in combination, an antibiofilm composition.

The compositions of the invention may include any number of active components and base materials known to persons skilled in the art.
While the active components discussed herein may be 100% of the composition of the invention, preferably, the composition contains from at least about 1% to about 50% of the active components by weight based upon the total weight of the composition of the invention being employed. In a preferred embodiment, the composition comprises from at least about 0.5% to about 25% (by weight) active components.

Other possible components of the composition include, but are not limited to, buffer solutions, phosphate buffered saline, saline, water, polyvinyl, polyethylene, polyurethane, polypropylene, silicone (e.g., silicone elastomers and silicone adhesives), polycarboxylic acids, (e.g., polyacrylic acid, polymethacrylic acid, polymaleic acid, poly-(maleic acid monoester), polyaspartic acid, polyglutamic acid, aginic acid or pectimic acid), polycarboxylic acid anhydrides (e.g., polymaleic anhydride, polymethacrylic anhydride or polyacrylic acid anhydride), polyamines, polyamine ions (e.g., polyethylene imine, polyvinylamine, polylysine, poly-(dialkylamineoethyl methacrylate), poly-(dialkylaminomethyl styrene) or poly-(vinylpyridine)), polyammonium ions (e.g., poly-(2-methacryloxyethyl trialkyl ammonium ion), poly-(vinylbenzyl trialkyl ammonium ions), poly-(N,N,-alklypyridinium ion) or poly-(dialkyloctamethylene ammonium ion) and polysulfonates (e.g. poly-(vinyl sulfonate) or poly-(styrene sulfonate)), collodion, nylon, rubber, plastic, polyesters, DacronTM. (polyethylene tetraphthalate), teflon (polytetrafluoroethylene), latex, and derivatives thereof, elastomers and Dacron (sealed with gelatin, collagen or albumin, cyanoacrylates, methacrylates, papers with porous barrier films, adhesives, e.g., hot melt adhesives, solvent based adhesives, and adhesive hydrogels, fabrics, and crosslinked and non-crosslinked hydrogels, and any other polymeric materials which facilitate dispersion of the active components and adhesion of the biofilm penetrating coating to at least one surface of the medical device. Linear copolymers, cross-linked copolymers, graft polymers, and block polymers, containing monomers as constituents of the above-exemplified polymers may also be used.
The present invention also provides methods for preparing devices, and in particular, medical devices using a composition according to the invention.

The present invention includes a method of preparing a device comprising the step of adding an effective amount of composition to at least one surface of a device. The term "effective" is herein defined as a sufficient amount of the active components to substantially reduce the growth or proliferation or colonization of biofilm microorganisms on the at least one surface of the medical device in the case of the composition of the invention being a coating; and as a sufficient amount of the active components to substantially penetrate, or break-up, the biofilm on the at least one surface of the medical device, thereby facilitating access of the active components, antimicrobial agents, and/or antifungal agents to the microorganisms embedded in the biofilm, and thus, inhibition of colonization of substantially all of the microorganisms from at least one surface of the medical device in the case of the composition of the invention being a solution. The amount will vary for each of the active components and upon known factors such as pharmaceutical characteristics; the type of medical device; the degree of biofilm microorganism contamination; and the use and length of use.

Coating the device prevents the formation of biofilm on the surface, while showing moderate effect on the viability of microbes. The moderate effect on the viability of microbes can be attributed to non-lethal compounds in the composition, which include thiol-specific reagent and protamine sulfate. Lethal compounds such as silver or antibiotics often create selective pressure to increase the likelihood of amplifying silver-resistant or antibiotic resistant strains, thus rendering the antibiofilm agents useless. This is an important consideration when the object to be coated is a medical device that will be implanted in the body, where resident bacteria exist.

Examples of bacteria that produce biofilms (biofilm bacteria), which can be inhibited by the present invention, include bacteria such as Staphylococcus epidermidis, Enterococcus faecalis, E. coli, Proteus mirabilis, Pseudomonas
aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus, and Streptococcus viridans. These bacteria are commonly found associated with medical devices including catheters. Other bacteria producing biofilms, which may be inhibited by the compositions of the present invention include Klebsiella oxytoca, Staphylococcus saprophyticus, Providencia stuartii, Citrobacter freundii and Serratia marcescens.

Examples of devices that can be protected using the compositions of the invention include tubings and other surface medical devices, such as urinary catheter, mucous extraction catheter, suction catheter, umbilical cannula, contact lenses, intrauterine devices, intravaginal and intraintestinal devices, endotracheal tubes, bronchoscopes, dental prostheses and orthodontic devices, surgical instruments, dental instruments, tubings, dental water lines, dental drain tubes, fabrics, paper, indicator strips (e.g., paper indicator strips or plastic indicator strips), adhesives (e.g., hydrogel adhesives, hot-melt adhesives, or solvent-based adhesives), bandages, tissue dressings or healing devices and occlusive patches, and any other surface devices used in the medical field. The devices may include electrodes, external prostheses, fixation tapes, compression bandages, and monitors of various types. Medical devices also include any device which may be placed at the insertion or implantation site such as the skin near the insertion or implantation site, and which include at least one surface which is susceptible to colonization by biofilm embedded microorganisms. In one specific embodiment, the composition of the invention is integrated into an adhesive, such as tape, thereby providing an adhesive, which may prevent growth or proliferation of biofilm embedded microorganisms on at least one surface of the adhesive. Medical devices for the present invention include surfaces of equipment in operating rooms, emergency rooms, hospital rooms, clinics, and bathrooms.

Insertable medical devices include catheters, which can be inspected without invasive techniques such as endoscopy. The medical devices may be formed of any suitable metallic materials or non-metallic materials known to persons skilled in the art. Examples of metallic materials include, but are not limited to, tivanium, titanium,
and stainless steel, and derivatives or combinations thereof. Examples of non-metallic materials include, but are not limited to, thermoplastic or polymeric materials such as rubber, plastic, polyesters, polypropylene, polycarbonate, polyvinyl chloride, nylon, polyethylene, polyurethane, silicone, Dacron.RTM. (polyethylene terephthalate), teflon (polytetrafluoroethylene), latex, elastomers and Dacron.RTM. sealed with gelatin, collagen or albumin, and derivatives or combinations thereof. The medical devices include at least one surface for applying the composition of the invention. Preferably, the composition of the invention is applied to the entire medical device. The compositions can also be incorporated into polymers, which are used to form the devices such as catheters by impregnating or by drug-polymer conjugation.

In another aspect, the invention provides a method for coating a medical device. Broadly, the method for coating a medical device includes the steps of providing a medical device, providing, or forming, a composition coating, and applying the composition coating to at least one surface of the medical device in an amount sufficient to substantially reduce the growth or proliferation or colonization of biofilm microorganisms on at least one surface of the medical device.

In one specific embodiment, the method for coating a medical device includes the steps of forming a composition of the invention of an effective concentration for activating the active components, and thus substantially reducing the growth or proliferation or colonization of microorganisms on at least one surface of the medical device, wherein the composition of the invention is formed by combining a active components and a base material. At least one surface of the medical device is then contacted with the composition of the invention under conditions wherein the composition of the invention covers at least one surface of the medical device. "Contacting" includes, but is not limited to, impregnating, compounding, mixing, integrating, coating, spraying and dipping.

In another aspect, the invention provides a method for reducing biofilm microorganisms from at least one surface of the medical device. In one specific
embodiment, the method of reducing biofilm formation from at least one surface of the medical device includes the steps of providing a medical device having at least one surface, the at least one surface having biofilm attached thereto, and contacting the medical device with a composition as described in greater detail above. "Contacting" further includes, but is not limited to, soaking, rinsing, flushing, submerging, and washing. The medical device should be contacted with the composition for a period of time sufficient to reduce substantially all of the biofilm from the at least one surface of the medical device. In one specific embodiment, the medical device is submerged in the composition for at least 5 minutes. Alternatively, the medical device may be flushed with the composition. In the case of the medical device being a tubing, such as dental drain tubing (dental water line), the composition may be poured into the dental drain tubing and both ends of the tubing clamped such that the composition is retained within the lumen of the tubing. The tubing is then allowed to remain filled with the composition for a period of time sufficient to remove substantially all of the microorganisms from at least one surface of the medical device, generally, for at least about 1 minute to about 48 hours. Alternatively, the tubing may be flushed by pouring the composition into the lumen of the tubing for an amount of time sufficient to reduce substantially all biofilm growth.

The concentration of active components in the compositions may vary as desired or necessary to decrease the amount of time the composition of the invention is in contact with the medical device. Persons skilled in the art easily determine these variations in the concentrations of active components.

In specific embodiments of the method for coating devices and the methods for inhibiting biofilm on at least one surface of the medical devices, the step of forming a composition of the invention may also include any one or all of the steps of adding an organic solvent, a medical device material penetrating agent, or adding an alkalinizing agent to the composition, to enhance the reactivity of the surface of the medical device with the composition. In the case of the method for coating medical devices, the organic solvent, medical device material penetrating agent, and/or
alkalinizing agent preferably facilitate adhesion of the composition to at least one surface of the medical device.

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

The device is preferably placed in contact with the composition by dipping the medical device in the composition for a period of time ranging from about 30 minutes to about 120 minutes at a temperature ranging from about 35°C to about 65°C. Preferably, the device is placed in contact with the composition by dipping the medical device in the composition for about 120 minutes at a temperature of about 45°C. The device is then removed from the composition and the composition is allowed to dry. The medical device may be placed in an oven, or other heated environment for a period of time sufficient for the composition to dry.

Although one layer, or coating, of the composition is believed to provide the desired composition coating, multiple layers are preferred. The multiple layers of the composition are preferably applied to the at least one surface of the medical device by repeating the steps discussed above. Preferably, the medical device is contacted with the composition three times, allowing the composition to dry on at least one surface of the medical device prior to contacting the medical device with the composition for each subsequent layer. In other words, the medical device preferably includes three coats, or layers, of the composition on at least one surface of the medical device.
In another embodiment, the method for coating medical devices with a composition coating includes the steps of forming a composition coating of an effective concentration to substantially reduce the growth or proliferation or colonization of biofilm microorganisms on at least one surface of the medical device by dissolving the active components in an organic solvent, combining a medical device material penetrating agent to the active components and organic solvent, and combining an alkalinizing agent to improve the reactivity of the material of the medical device. The composition is then heated to a temperature ranging from about 35°C to about 65°C to enhance the adherence of the composition coating to at least one surface of the device. The composition coating is applied to at least one surface of the medical device, preferably by contacting the composition coating to the at least one surface of the medical device for a sufficient period of time for the composition coating to adhere to at least one surface of the medical device. The medical device is removed from the composition coating and allowed to dry for at least 8 hours, and preferably, overnight, at room temperature. The medical device may then be rinsed with a liquid, such as water and allowed to dry for at least 2 hours, and preferably 8 hours, before being sterilized. To facilitate drying of the composition of the invention onto the surface of the medical device, the medical device may be placed into a heated environment such as an oven.

In another embodiment, the method for coating the medical devices with a composition includes the steps of forming the composition and incorporating the composition into the material forming the medical device during the formation of the medical device. For example, the composition may be combined with the material forming the medical device, e.g., silicone, polyurethane, polyvinyl chloride, polyethylene, polytetrafluoroethylene (teflon), polyethylene terephthalate and/or polypropylene, and extruded with the material forming the medical device, thereby incorporating the composition into material forming the medical device. In this embodiment, the composition may be incorporated in a septum or adhesive, which is placed at the medical device insertion or implantation site. An example of a coated medical device having a composition incorporated into the material forming the
medical device in accordance with this embodiment is the catheter insertion seal having an adhesive layer described below in greater detail.

In still another aspect, the invention is directed to coated medical devices. Broadly, the coated medical devices include a composition coating applied to at least one surface of the medical device. Suitable medical devices and compositions are described above in greater detail. The composition may be applied to at least one surface of the medical devices in any suitable manner. For example, the composition may be applied to the medical devices following any of the methods described above in greater detail.

Although the invention has been described with reference to illustrative embodiments, it is understood that the invention is not limited to these precise embodiments and that such various changes and modifications may be effected therein by one skilled in the art. All changes and modifications are intended to be encompassed in the appended claims.

**EXAMPLE 1** - Synergistic effects of N,N’-(1,2-phenylene) dimaleimide (oPDM) + protamine sulfate (PS), PDM + ovotransferrin (OT), N-(1-pyrenyl) maleimide (PyrM) + PS and PyrM + OT on biofilm formation in catheter-associated bacteria

Catheter-associated bacterial strains used: E. coli P18, Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterococcus faecalis and Staphylococcus epidermidis. Concentrations of oPDM, PyrM, PS and OT alone and in combinations used for screening are given in Table 1.
Table 1: Concentrations of oPDM, PyrM, PS and OT alone and in combinations used for screening (µg/ml of DMSO)¹

<table>
<thead>
<tr>
<th>Compound</th>
<th>E. coli</th>
<th>P. mirabilis</th>
<th>K. pneumoniae</th>
<th>P. aeruginosa</th>
<th>E. faecalis</th>
<th>Staph. epidermidis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>10</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>oPDM</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
<td>100</td>
<td>12.5</td>
<td>50</td>
</tr>
<tr>
<td>PS</td>
<td>12.5</td>
<td>25</td>
<td>12.5</td>
<td>100</td>
<td>12.5</td>
<td>50</td>
</tr>
<tr>
<td>oPDM+PS</td>
<td>12.5+12.5</td>
<td>25+25</td>
<td>12.5+12.5</td>
<td>100+100</td>
<td>12.5+12.5</td>
<td>50+50</td>
</tr>
<tr>
<td>oPDM+OT</td>
<td>12.5</td>
<td>100</td>
<td>12.5</td>
<td>125</td>
<td>125</td>
<td>250</td>
</tr>
<tr>
<td>PyrM+OT</td>
<td>12.5+125</td>
<td>100+1000</td>
<td>12.5+125</td>
<td>100+1000</td>
<td>12.5+125</td>
<td>25+250</td>
</tr>
<tr>
<td>PyrM</td>
<td>12.5</td>
<td>25</td>
<td>25</td>
<td>50</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>PyrM+PS</td>
<td>12.5+12.5</td>
<td>25+25</td>
<td>25+25</td>
<td>50+50</td>
<td>100+100</td>
<td>25+25</td>
</tr>
<tr>
<td>PyrM+OT</td>
<td>12.5</td>
<td>25</td>
<td>12.5</td>
<td>50</td>
<td>125</td>
<td>125</td>
</tr>
<tr>
<td>PyrM+OT</td>
<td>12.5+125</td>
<td>25+250</td>
<td>12.5+125</td>
<td>50+500</td>
<td>12.5+125</td>
<td>12.5+125</td>
</tr>
</tbody>
</table>

¹Concentrations were based on the data from previous studies (data not shown).

Synergistic effects of combinations such as oPDM+PS, oPDM+OT, PyrM+PS and PyrM+OT on biofilm formation in catheter-associated bacteria were determined following the procedure described by Jackson, et al. (J. Bacteriol. 184: 290-301).

Bacteria were routinely cultured at 37°C in Luria-Bertani (LB) or Tryptic Soy Broth (TSB). Biofilm assays were generally carried out in colony-forming antigen (CFA) medium at 26°C. However, biofilm assays for Enterococcus faecalis and Staphylococcus epidermidis were carried out in TSB at 37°C.

Method: Overnight cultures were inoculated 5:100 into fresh medium. In the microtiter plate assay, inoculated cultures were grown in a 96-well polystyrene microtiter plate. N,N'-(1,2-phenylene) dimaleimide, N-(1-pyrenyl) maleimide and ovotransferrin were dissolved in DMSO. Protamine Sulfate was dissolved in water first by stirring and then added to solutions of N,N'- (1,2-phenylene) dimaleimide or N-(1-pyrenyl) maleimide (in DMSO) and appropriate volume of each one was added to microtiter plate wells in replicates. Concentrations of four compounds, N,N'- (1,2-phenylene) dimaleimide, N-(1-pyrenyl) maleimide, protamine sulfate and ovotransferrin ranged from 0-100 µg/ml, 0-100 µg/ml, 0-100 µg/ml and 0-1000 µg/ml, respectively. Growth of planktonic cells was determined by absorbance at 600 nm using Labsystems Multiskan Ascent microplate reader. Biofilm was measured by discarding the medium; rinsing the wells with water (three times) and staining bound
cells with crystal violet. The dye was solubilized with 33% acetic acid, and absorbance at 630 nm was determined using a microtiter plate reader. For each experiment, background staining was corrected by subtracting the crystal violet bound to uninoculated control.

Results: Figures 1-12 show the effects of N,N′-(1,2-phenylene) dimaleimide (oPDM), N-(1-pyrenyl) maleimide (PyrM), protamine sulfate (PS) and ovotransferrin (OT) alone and in combinations on biofilm formation in catheter-associated bacteria. Values represent the Mean ± Standard Deviation of eight replicates for each concentration. Two compositions consisting of oPDM+PS and oPDM+OT, respectively showed appreciable synergistic inhibitory effects on biofilm formation in *Proteus mirabilis* and *Staph. epidermidis* (Figures 2 and 6). Furthermore, oPDM +PS combination showed marginal synergistic inhibitory effect on biofilm formation in *Enterococcus faecalis* (Figure 5). Both PyrM+PS and PyrM+OT combinations had significant synergistic inhibitory effects on biofilm formation in *Klebsiella pneumoniae* (Figure 9) and *Staph. epidermidis* (Figure 12). Synergistic antibiofilm activity of PyrM+PS combination against *Proteus mirabilis* was also observed (Figure 8). In some cases, the combinations were less effective as compared to individual compounds in reducing biofilm formation (Figures 1, 4, 5, 7, 10 and 11). This might suggest that interaction between two compounds in combinations could render the combinations less effective against some biofilm bacteria.

**EXAMPLE 2 - Effects of oPDM)+PS, oPDM + OT, PyrM+PS and PyrM+OT combinations on biofilm formation in catheter-associated bacteria**

Catheter-associated bacterial strains used: E. coli P18, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterococcus faecalis and Staphylococcus epidermidis.
Method: Base Formulas for N,N’-(1,2-phenylene) dimaleimide (oPDM), N-(1-
pyrenyl) maleimide (PyrM), Protamine Sulfate (PS) and Ovotransferrin (OT) were
prepared as described in Table 2.

Table 2: Base Formulas for Screening (μg/ml of DMSO)

<table>
<thead>
<tr>
<th>Compound</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>oPDM+PS</td>
<td>12.5+12.5</td>
<td>25+25</td>
<td>50+50</td>
<td>100+100</td>
</tr>
<tr>
<td>oPDM+OT</td>
<td>12.5+12.5</td>
<td>25+25</td>
<td>50+600</td>
<td>100+1000</td>
</tr>
<tr>
<td>PyrM+PS</td>
<td>12.5+12.5</td>
<td>25+25</td>
<td>50+50</td>
<td>100+100</td>
</tr>
<tr>
<td>PyrM+OT</td>
<td>12.5+12.5</td>
<td>25+25</td>
<td>50+500</td>
<td>100+1000</td>
</tr>
</tbody>
</table>

Studies were conducted to test biofilm formation in microtiter plate wells.
Quantitative biofilm assay for catheter-associated bacteria was standardized
following the procedure described by Jackson, et al. (J. Bacteriol. 184: 290-301).
Bacteria were routinely cultured at 37°C in Luria-Bertani (LB) or Tryptic Soy Broth
(TSB). Biofilm assays were generally carried out in colony-forming antigen (CFA)
medium at 26°C. However, biofilm assays for Enterococcus faecalis and
Staphylococcus epidermidis were carried out in TSB at 37°C (see Example 1 for
detailed procedure).

Results: Figures 13-36 show biofilm formation by the above catheter-
associated bacterial strains in the wells of microtiter plate in the presence and
absence of N,N’-(1,2-phenylene) dimaleimide (oPDM), N-(1-pyrenyl) maleimide
(PyrM), Protamine Sulfate (PS) and Ovotransferrin (OT) in combinations at different
concentrations. Values represent the Mean ± Standard Deviation of eight replicates
for each concentration. The combinations such as oPDM+OT and PyrM+OT
showed significant inhibitory effects on biofilm formation in E. coli (Figures 19 & 31),
Enterococcus faecalis (Figures 23 & 35) and Staphylococcus epidermidis (Figures
24 & 36). The combinations were also had considerable inhibitory effects on biofilm
formation in Proteus mirabilis (Figures 20 & 32) and Klebsiella pneumoniae (Figures
21 & 33). However, they had minimal effect on Pseudomonas aeruginosa biofilm
formation (Figures 22 and 34). The oPDM+PS and PyrM+PS combinations had
significant inhibitory effects on biofilm formation in all the above bacterial strains tested (Figures 13,14,15,16,18,25,26,27,28 and 30), except Enterococcus faecalis (Figures 17 and 29).

**EXAMPLE 3 - Effect of oPDM + PS coating on the adherence of catheter-associated bacteria to urinary catheter**

Catheter-associated bacterial strains used: *E. coli* P18, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Staphylococcus epidermidis*.

Method: All-silicone foley catheter segments (3 cm sections of tubing) that had been preheated (incubated in sterile water at 65°C overnight and air dried) were immersed in oPDM+OT solution (50 μg oPDM + 50 μg OT per ml of DMSO) at 45°C for two hours and air dried under aseptic conditions. The control catheter segments were immersed in DMSO at 45°C for two hours and air dried under aseptic conditions. Both control and treated segments were immersed in tryptic soy broth cultures of *E. coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Staphylococcus epidermidis* (1%) for 3 hours at 37°C. The catheter segments were washed 3 times in 3 changes of sterile saline and rolled on tryptic soy agar plates. The plates were incubated overnight at 37°C and the colonies were counted.

Results: N, N-(1,2-phenylene) dimaleimide in combination with protamine sulfate showed significant inhibitory effects on the adherence of catheter-associated bacteria to urinary catheter. Inhibition of *E. coli* P18, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Staphylococcus epidermidis* adherence to urinary catheter varied from 45 to 65% (Table 3).

Table 3: Effect of oPDM+PS coating on the adherence of catheter-associated bacteria to all-silicone urinary catheter

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Viable Counts (CFU/mm)</th>
<th>%Inhibition</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Control</th>
<th>Coated</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> P18</td>
<td>1.59±0.16</td>
<td>0.72±0.40</td>
<td>55</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>26.94±1.89</td>
<td>14.94±0.76</td>
<td>46</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>10.54±0.19</td>
<td>4.37±0.47</td>
<td>59</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>8.02±0.45</td>
<td>2.80±0.66</td>
<td>65</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>22.67±1.13</td>
<td>12.49±1.53</td>
<td>45</td>
</tr>
<tr>
<td><em>Staph. epidermidis</em></td>
<td>9.50±0.04</td>
<td>4.75±0.07</td>
<td>50</td>
</tr>
</tbody>
</table>

1 Colony Forming Units (CFU) per millimeter of catheter tubing (Mean ± SD)
2 Control is coated with DMSO; 3 Coated with oPDM + PS in DMSO

**EXAMPLE 4 - Effect of oPDM+OT coating on the adherence of catheter-associated bacteria to urinary catheter**

Catheter-associated bacterial strains used: *E. coli*, *Proteus mirabilis*,
*Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and
*Staphylococcus epidermidis*.

Method: The effects of coating all-silicone urinary catheter with oPDM+OT on
the adherence of catheter-associated bacteria were determined using an *in vitro*
assay method as described previously (see Example 3).

Results: N, N-(1,2-phenylene) dimaleimide in combination with ovotransferrin
inhibited the adherence of all six catheter-associated bacterial strains to urinary
catheter. Inhibition of *E. coli* P18, *Proteus mirabilis*, *Klebsiella pneumoniae*,
*Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Staphylococcus epidermidis*
adherence to urinary catheter varied from 40 to 73% (Table 4).

Table 4: Effect of oPDM+OT coating on the adherence of catheter-associated
bacteria to all-silicone urinary catheter
<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Viable Counts (CFU/mm)</th>
<th>%Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control$^2$</td>
<td>Coated$^3$</td>
</tr>
<tr>
<td><strong>E. coli P18</strong></td>
<td>9.53±1.32</td>
<td>4.87±0.80</td>
</tr>
<tr>
<td><strong>Proteus mirabilis</strong></td>
<td>27.45±0.02</td>
<td>13.40±0.28</td>
</tr>
<tr>
<td><strong>Klebsiella pneumoniae</strong></td>
<td>10.92±1.06</td>
<td>2.93±0.94</td>
</tr>
<tr>
<td><strong>Pseudomonas aeruginosa</strong></td>
<td>16.53±1.51</td>
<td>9.93±1.23</td>
</tr>
<tr>
<td><strong>Enterococcus faecalis</strong></td>
<td>14.14±0.76</td>
<td>5.80±1.17</td>
</tr>
<tr>
<td><strong>Staph. epidermidis</strong></td>
<td>5.60±1.08</td>
<td>2.53±0.00</td>
</tr>
</tbody>
</table>

$^1$ Colony Forming Units (CFU) per millimeter of catheter tubing (Mean ± SD)  
$^2$ Control is coated with DMSO; $^3$ Coated with oPDM + OT in DMSO

5 **EXAMPLE 5 - In vitro inhibitory activity of oPDM+PS, oPDM+OT, PyrM+PS, PyrM+OT and oPDM+PyrM combinations against catheter-associated bacteria**

Catheter-associated bacterial strains used: *E. coli P18, Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterococcus faecalis and Staphylococcus epidermidis.*

10 Method: A simple disc-diffusion assay was used to determine the susceptibility of catheter-associated bacteria to oPDM+PS, oPDM+OT, PyrM+PS, PyrM+OT and oPDM+PyrM combinations. Disc diffusion assay was carried out on Tryptic Soy Agar (TSA) plates seeded with each bacterial strain separately. While solutions of oPDM and PyrM were prepared in DMSO, the solutions of PS and OT were prepared in sterile water. Filter paper discs (6.5 mm diameter) were impregnated with the above compounds in combinations at different concentrations (oPDM+PS= 50µg+50µg, oPDM+OT= 50µg+200µg, PyrM+PS= 50µg+50µg, PyrM+OT= 50µg+200µg and oPDM+PyrM= 50µg+50µg per disc; 10 µl of oPDM/PyrM solution in DMSO plus 10 µl of PS/OT solution in water/disc) and dried discs were placed on the seeded agar surface. Controls included DMSO (20 µl/disc)
and DMSO+H₂O (10µl+10µl/disc). Plates were incubated for 24 hours at 37°C. The diameter of inhibition zone was measured in millimeters with a ruler.

Results: Table 5 summarizes the results of the zones of inhibition produced by the oPDM+PS, oPDM+OT, PyrM+PS, PyrM+OT and oPDM+PyrM combinations.

While the oPDM+PS, oPDM+OT and oPDM+PyrM combinations showed appreciable antibacterial activity against all the catheter-associated bacteria tested, PyrM+PS and PyrM+OT combinations inhibited the growth of only gram-positive bacterial strains. Thus, the PyrM+PS and PyrM+OT combinations seem to attenuate the growth of gram-negative bacteria without producing a clear zone of inhibition.

Table 5: Inhibitory effects of oPDM+PS, oPDM+OT, PyrM+PS, PyrM+OT and oPDM+PyrM combinations on catheter-associated bacteria

<table>
<thead>
<tr>
<th>Compound</th>
<th>E. coli</th>
<th>P. mirabilis</th>
<th>K. pneumoniae</th>
<th>P. aeruginosa</th>
<th>E. faecalis</th>
<th>S. epidermidis</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>NGI</td>
<td>NGI</td>
<td>NGI</td>
<td>NGI</td>
<td>NGI</td>
<td>NGI</td>
</tr>
<tr>
<td>DMSO+H₂O</td>
<td>NGI</td>
<td>NGI</td>
<td>NGI</td>
<td>NGI</td>
<td>NGI</td>
<td>NGI</td>
</tr>
<tr>
<td>oPDM+PS</td>
<td>9.5±0.71</td>
<td>9.0±0.0</td>
<td>10.0±1.41</td>
<td>9.5±0.71</td>
<td>11.5±0.71</td>
<td>10.0±1.41</td>
</tr>
<tr>
<td>oPDM+OT</td>
<td>9.0±1.41</td>
<td>8.0±0.0</td>
<td>9.0±0.0</td>
<td>9.5±0.71</td>
<td>10.5±0.71</td>
<td>9.0±0.0</td>
</tr>
<tr>
<td>PyrM+PS</td>
<td>NGI</td>
<td>NGI</td>
<td>NGI</td>
<td>NGI</td>
<td>8.0±0.0</td>
<td>8.0±0.0</td>
</tr>
<tr>
<td>PyrM+OT</td>
<td>NGI</td>
<td>NGI</td>
<td>NGI</td>
<td>NGI</td>
<td>8.0±0.0</td>
<td>7.5±0.71</td>
</tr>
<tr>
<td>oPDM+PyrM</td>
<td>9.0±0.0</td>
<td>8.0±0.0</td>
<td>10.5±0.71</td>
<td>9.0±0.0</td>
<td>11.5±2.12</td>
<td>11.0±0.0</td>
</tr>
</tbody>
</table>

1°PDM+PS= 50µg+50µg, oPDM+OT= 50µg+200µg, PyrM+PS= 50µg+50µg, PyrM+OT= 50µg+200µg and oPDM+PyrM= 50µg+50µg per disc (10 µl of oPDM/PyrM solution in DMSO plus 10 µl of PS/OT solution in water/disc)

2°Mean ± SD
NGI, No Growth Inhibition
REFERENCES


Donlan, "Biofilms and device-associated infections", Emerging Infectious Diseases, 7:277-281, 2001


Maki and Tambyah, "Engineering out the risk of infection with urinary catheters", Emerging Infectious Diseases, 7:1-6, 2001


What is claimed is:

1. A composition for inhibiting bacterial biofilms on devices comprising a composition selected from the group consisting of:

   5. (a) a thiol-specific reagent and a cationic polypeptide;
   (b) a thiol-specific reagent and an iron-sequestering glycoprotein; and
   (c) a thiol-specific reagent and a quaternary ammonium compound.

2. The compositions of claim 1, wherein the thiol-specific reagent is between about 12.5 mg/L and about 100 mg/L of the composition.

3. The compositions of any one of claims 1 and 2, wherein the cationic polypeptide is between about 12.5 mg/L and about 100 mg/L of the composition.

4. The compositions of any one of claims 1 to 3, wherein the iron-sequestering glycoprotein is between about 125 mg/L and about 1000 mg/L of the composition.

5. The composition of any one of claims 1 to 4, wherein the composition is effective against biofilms produced by gram-negative bacterial species selected from the group consisting of Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae and Pseudomonas aeruginosa.

6. The composition of any one of claims 1 to 4, wherein the composition is effective against biofilms produced by gram-positive bacterial species selected from the group consisting of Enterococcus faecalis and Staphylococcus epidermidis.

7. The composition of any one of claims 1 to 6, wherein the thiol-specific reagent is selected from the group consisting of N-ethylmaleimide (NEM), 5,5-dithiobis-(2-nitrobenzoic acid)(DTNB), 2-nitro-5-thiocyanobenzoic acid (NTCB), P-hydroxymercuribenzoic acid (pHMB), iodoacetamide (IA), N-phenylmaleimide.
(PheM), N-(1-pyrenyl) maleimide (PyrM), naphthalene-1,5-dimaleimide (NDM), N,N’-(1,2-phenylene) dimaleimide (oPDM), N,N’-1,4-phenylene dimaleimide (pPDM), N,N’-1,3-phenylene dimaleimide (mPDM), 1,1-(methylenedi-4,1-phenylene)bismaleimide(BM), 4-(N-aleimido) phenyltrimethylammonium (MPTM), N,N’-bis(3-maleimidopropionyl)-2-hydroxy-1,3-propanediamine (BMP), p-chloromercuribenzenzene sulphonylic acid and thiosulfinates.

8. The composition of any one of claims 1 to 7, wherein the cationic polypeptide is selected from the group consisting of protamine sulfate, polylysine, defensin, lactoperoxidase and lysozyme.

9. The composition of any one of claims 1 to 8, wherein the iron sequestering glycoprotein is selected from the group consisting of ovotransferrin, lactoferrin and serotransferrin.

10. The composition of any one of claims 1 to 9, further comprising one or more ingredients selected from the group consisting of: an organic solvent, a binding or bonding or coupling agent, a surfactant, a quaternary ammonium compound and an antibiotic.

11. The composition of any one of claims 1 to 10, wherein the thiol-specific reagent is N,N’-(1,2-phenylene) dimaleimide or N-(1-pyrenyl) maleimide and the cationic polypeptide is protamine sulfate.

12. The compositions of claim 11, wherein the N,N’-(1,2-phenylene) dimaleimide or N-(1-pyrenyl) maleimide is present as about 0.1 mg/ml and the protamine sulfate is present as about 0.1 mg/ml.

13. The composition of any one of claims 1 to 10, wherein the thiol-specific reagent is N,N’-(1,2-phenylene) dimaleimide or N-(1-pyrenyl) maleimide and the glycoprotein is ovotransferrin.
14. The compositions of claim 13, wherein the N,N'-(1,2-phenylene) dimaleimide or N-(1-pyrenyl) maleimide is present as about 0.1 mg/ml and or the ovotransferrin is present as about 1 mg/ml.

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15. The composition of any one of claims 1 to 14, further comprising dimethyl sulfoxide or methanol.

16. A method of preparing a device comprising treating at least one surface of the device with the composition of any one of claims 1 to 15.

17. The method of preparing a device comprising coating a device with the composition of any one of claims 1 to 15.

18. The method as claimed in any one of claims 16 and 17, wherein the composition comprises an effective amount of N,N'-(1,2-phenylene) dimaleimide or N-(1-pyrenyl) maleimide and protamine sulfate.

19. The method as claimed in any one of claims 16 and 17, wherein the composition comprises an effective amount of N,N'-(1,2-phenylene) dimaleimide or N-(1-pyrenyl) maleimide and ovotransferrin.

20. The method as claimed in any one of claims 16 and 17, wherein the method comprises treating the device with a quaternary ammonium compound before coating or treating the device with the composition.

21. The method as claimed in claim 20, wherein the quaternary ammonium compound is selected from the group consisting of tridodecylmethyl ammonium chloride and benzalkonium chloride.
22. The method as claimed in any one of claims 16 to 21, wherein the composition further comprises a hydrogel.

23. The method as claimed in any one of claims 16 to 21, further comprising coating the device with a hydrogel selected from the group consisting of polyvinylpyrrolidone-hydrogel, polyvinyl alcohol-hydrogel and polyethylene glycol-hydrogel.

24. The method as claimed in any one of claims 16 to 23, wherein the device is a medical device.

25. The method as claimed in claim 24, wherein the medical device is a catheter.

26. The method of claim 25, wherein the catheter is an indwelling catheter.

27. The method of claim 26, wherein the indwelling catheter is selected from the group consisting of a urinary catheter, a peritoneal catheter, an umbilical catheter, a suction catheter and a mucous extraction catheter.

28. The method as claimed in any one of claims 16 to 23, wherein the device is selected from the group consisting of catheters, contact lenses, intrauterine devices, dental prostheses, orthodontic devices, stomach tubes, endotracheal tubes, dental water lines, compression bandages, tissue dressings, wound dressings, surgical tapes, occlusive patches and external prostheses.

29. The method as claimed in any one of claims 16 to 23, wherein the device selected from the group consisting of pipes, heat exchangers and computer chips.

30. A method of preparing a device comprising incorporating the composition of any one of claims 1 to 15 into polymers, which are used to form the device.
31. The method of preparing a device comprising impregnating the composition of any one of claims 1 to 15 into the device.

32. The method as claimed in any one of claims 16 to 19 and 22 to 31, wherein the quaternary ammonium compound is selected from the group consisting of benzalkonium chloride, tridodecyl methyl ammonium chloride, and didecyl dimethyl ammonium chloride.

33. The composition as claimed in any one of claims 1 to 15, wherein the quaternary ammonium compound is selected from the group consisting of benzalkonium chloride, tridodecyl methyl ammonium chloride, and didecyl dimethyl ammonium chloride.
Figure 1: Effects of N,N'-(1,2-phenylene) dimaleimide (oPDM), protamine sulfate (PS) and ovotransferrin (OT) alone and in combinations (oPDM+PS and oPDM+OT) on biofilm formation in *E. coli* P18

![Graph showing absorbance values for different treatments](image)

Figure 2: Effects of N,N'-(1,2-phenylene) dimaleimide (oPDM), protamine sulfate (PS) and ovotransferrin (OT) alone and in combinations (oPDM+PS and oPDM+OT) on biofilm formation in *Proteus mirabilis*

![Graph showing absorbance values for different treatments](image)
**Figure 3:** Effects of N,N'-[(1,2-phenylene) dimaleimide (oPDM), protamine sulfate (PS) and ovaltransferrin (OT) alone and in combinations (oPDM+PS and oPDM+OT) on biofilm formation in *Klebsiella pneumoniae*.

**Figure 4:** Effects of N,N'-[(1,2-phenylene) dimaleimide (oPDM), protamine sulfate (PS) and ovaltransferrin (OT) alone and in combinations (oPDM+PS and oPDM+OT) on biofilm formation in *Pseudomonas aeruginosa*. 
Figure 5: Effects of N,N'- (1,2-phenylene) dimaleimide (oPDM), protamine sulfate (PS) and ovotransferrin (OT) alone and in combinations (oPDM+PS and oPDM+OT) on biofilm formation in Enterococcus faecalis

![Graph showing absorbance at different concentrations of oPDM, PS, OT, and combinations]

Figure 6: Effects of N,N'- (1,2-phenylene) dimaleimide (oPDM), protamine sulfate (PS) and ovotransferrin (OT) alone and in combinations (oPDM+PS and oPDM+OT) on biofilm formation in Staph. Epidermidis

![Graph showing absorbance at different concentrations of oPDM, PS, OT, and combinations]
Figure 7: Effects of N-(1-pyrenyl) maleimide (PyrM), protamine sulfate (PS) and ovotransferrin (OT) alone and in combinations (PyrM+PS and PyrM+OT) on biofilm formation in *E. coli* P18

![Graph showing effects of PyrM + PS and PyrM + OT on biofilm formation in *E. coli* P18](image)

Figure 8: Effects of N-(1-pyrenyl) maleimide (PyrM), protamine sulfate (PS) and ovotransferrin (OT) alone and in combinations (PyrM+PS and PyrM+OT) on biofilm formation in *Proteus mirabilis*

![Graph showing effects of PyrM + PS and PyrM + OT on biofilm formation in *P. mirabilis*](image)
**Figure 9**: Effects of N-(1-pyrenyl) maleimide (PyrM), protamine sulfate (PS) and ovotransferrin (OT) alone and in combinations (PyrM+PS and PyrM+OT) on biofilm formation in *Klebsiella pneumoniae*

![Graph showing effects of PyrM + PS and PyrM + OT on biofilm formation in *Klebsiella pneumoniae*]

**Figure 10**: Effects of N-(1-pyrenyl) maleimide (PyrM), protamine sulfate (PS) and ovotransferrin (OT) alone and in combinations (PyrM+PS and PyrM+OT) on biofilm formation in *Pseudomonas aeruginosa*

![Graph showing effects of PyrM + PS and PyrM + OT on biofilm formation in *Pseudomonas aeruginosa*]
**Figure 11:** Effects of N-(1-pyrenyl) maleimide (PyrM), protamine sulfate (PS) and ovotransferrin (OT) alone and in combinations (PyrM+PS and PyrM+OT) on biofilm formation in *Enterococcus faecalis*

![Graph showing effects of PyrM + PS and PyrM + OT on biofilm formation in E. faecalis](image)

**Figure 12:** Effects of N-(1-pyrenyl) maleimide (PyrM), protamine sulfate (PS) and ovotransferrin (OT) alone and in combinations (PyrM+PS and PyrM+OT) on biofilm formation in *Staph. Epidermidis*

![Graph showing effects of PyrM + PS and PyrM + OT on biofilm formation in S. epidermidis](image)
Figure 13: Combined effects of N,N’-(1,2-phenylene) dimaleimide and protamine sulfate on biofilm formation in E. coli.

![Graph showing the effect of N,N’-(1,2-phenylene) dimaleimide and protamine sulfate on biofilm formation in E. coli.]

Figure 14: Combined effects of N,N’-(1,2-phenylene) dimaleimide and protamine sulfate on biofilm formation in Proteus mirabilis.

![Graph showing the effect of N,N’-(1,2-phenylene) dimaleimide and protamine sulfate on biofilm formation in P. mirabilis.]

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Figure 15: Combined effects of N,N’-(1,2-phenylene) dimaleimide and protamine sulfate on biofilm formation in *Klebsiella pneumoniae*.

![Graph showing absorbance of biofilm formation and planktonic growth](image)

Figure 16: Combined effects of N,N’-(1,2-phenylene) dimaleimide and protamine sulfate on biofilm formation in *P. aeruginosa*.

![Graph showing absorbance of biofilm formation and planktonic growth](image)
Figure 17: Combined effects of N,N'-(1,2-phenylene) dimaleimide and protamine sulfate on biofilm formation in Enterococcus faecalis.

![Graph showing absorbance vs. concentrations of N,N'-(1,2-phenylene) dimaleimide and protamine sulfate in E. faecalis](image)

- Planktonic growth @ 600nm
- Biofilm formation @ 630nm

N,N'-(1,2-phenylene) dimaleimide + Protamine Sulfate (μg/ml)

0+0 | 12.5+12.5 | 25+25 | 50+50 | 100+100

Figure 18: Combined effects of N,N'-(1,2-phenylene) dimaleimide and protamine sulfate on biofilm formation in Staph. epidermidis.

![Graph showing absorbance vs. concentrations of N,N'-(1,2-phenylene) dimaleimide and protamine sulfate in Staph. epidermidis](image)

- Planktonic growth @ 600nm
- Biofilm formation @ 630nm

N,N'-(1,2-phenylene) dimaleimide + Protamine Sulfate (μg/ml)

0+0 | 12.5+12.5 | 25+25 | 50+50 | 100+100
Figure 19: Combined effects of N,N'-(1,2-phenylene) dimaleimide and ovotransferrin on biofilm formation in *E. coli* P18.

Figure 20: Combined effects of N,N'-(1,2-phenylene) dimaleimide and ovotransferrin on biofilm formation in *Proteus mirabilis*.
Figure 21: Combined effects of N,N'-((1,2-phenylene) dimaleimide and ovotransferrin on biofilm formation in *Klebsiella pneumoniae*.

![Graph showing effect of N,N'-((1,2-phenylene) dimaleimide and ovotransferrin combination on biofilm formation in *K. pneumoniae*.

Figure 22: Combined effects of N,N'-((1,2-phenylene) dimaleimide and ovotransferrin on biofilm formation in *P. aeruginosa*.

![Graph showing effect of N,N'-((1,2-phenylene) dimaleimide and ovotransferrin combination on biofilm formation in *P. aeruginosa*.

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Figure 23: Combined effects of N,N’-(1,2-phenylene) dimaleimide and ovotransferrin on biofilm formation in *Enterococcus faecalis*

![Graph showing the effect of N,N’-(1,2-phenylene) dimaleimide and ovotransferrin on biofilm formation in E. faecalis.](image)

Figure 24: Combined effects of N,N’-(1,2-phenylene) dimaleimide and ovotransferrin on biofilm formation in *Staph. Epidermidis* 

![Graph showing the effect of N,N’-(1,2-phenylene) dimaleimide and ovotransferrin on biofilm formation in Staph. epidermidis.](image)
**Figure 25**: Combined effects of N-(1-pyrenyl)maleimide and protamine sulfate on biofilm formation in *E. coli* P18.

**Figure 26**: Combined effects of N-(1-pyrenyl)maleimide and protamine sulfate on biofilm formation in *Proteus mirabilis*
Figure 27: Combined effects of N-(1-pyrenyl)maleimide and protamine sulfate on biofilm formation in *Klebsiella pneumoniae*.

![Diagram showing effect of N-(1-pyrenyl)maleimide and protamine sulfate combination on biofilm formation in *K. pneumoniae*.](image)

Figure 28: Combined effects of N-(1-pyrenyl)maleimide and protamine sulfate on biofilm formation in *Pseudomonas aeruginosa*.

![Diagram showing effect of N-(1-pyrenyl)maleimide and protamine sulfate combination on biofilm formation in *P. aeruginosa*.](image)
Figure 29: Combined effects of N-(1-pyrenyl)maleimide and protamine sulfate on biofilm formation in *Enterococcus faecalis*.

![Graph showing the effect of N-(1-pyrenyl)maleimide and protamine sulfate combination on biofilm formation in *Enterococcus faecalis*](image)

Figure 30: Combined effects of N-(1-pyrenyl)maleimide and protamine sulfate on biofilm formation in *Staph. epidermidis*.

![Graph showing the effect of N-(1-pyrenyl)maleimide and protamine sulfate combination on biofilm formation in *Staph. epidermidis*](image)
Figure 31: Combined effects of N-(1-pyrenyl)maleimide and ovotransferrin on biofilm formation in *E. coli P18*.

![Graph showing the effect of N-(1-pyrenyl)maleimide and ovotransferrin on biofilm formation in *E. coli P18*.](image)

Figure 32: Combined effects of N-(1-pyrenyl) maleimide and protamine sulfate on biofilm formation in *Proteus mirabilis*.

![Graph showing the effect of N-(1-pyrenyl)maleimide and ovotransferrin on biofilm formation in *P. mirabilis*.](image)
Figure 33: Combined effects of N-(1-pyrenyl)maleimide and ovotransferrin on biofilm formation in Klebsiella pneumoniae.

Figure 34: Combined effects of N-(1-pyrenyl)maleimide and ovotransferrin on biofilm formation in Pseudomonas aeruginosa.
**Figure 35:** Combined effects of N-(1-pyrenyl)maleimide and ovotransferrin on biofilm formation in *Enterococcus faecalis*.

![Graph showing the effect of N-(1-pyrenyl)maleimide and ovotransferrin combination on biofilm formation in *E. faecalis*.](image1)

**Figure 36:** Combined effects of N-(1-pyrenyl)maleimide and ovotransferrin on biofilm formation in *Staph. Epidermidis*.

![Graph showing the effect of N-(1-pyrenyl)maleimide and ovotransferrin combination on biofilm formation in *Staph. epidermidis*.](image2)
### INTERNATIONAL SEARCH REPORT

**A. CLASSIFICATION OF SUBJECT MATTER**
- IPC(7): A01N 37/00, A61L 29/04, A61L 29/14, A01N 37/02, A01N 37/10, A01N 33/12, A01N 33/24

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
- IPC(7): A01N 37/00, A61L 29/04, A61L 29/14, A01N 37/02, A01N 37/10, A01N 33/12, A01N 33/24

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
- Canadian Patent Database, Delphion, STN

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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[ ] Further documents are listed in the continuation of Box C.  
[X] See patent family annex.

**Date of the actual completion of the international search**

**Date of mailing of the international search report**

**Name and mailing address of the ISA/CA**
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  - Place du Portage, C114 - 1st Floor, Box PCT  
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  - Facsimile No.: 001(819)953-2476
- **Charles Barabé** (819) 997-2751
# INTERNATIONAL SEARCH REPORT

Information on patent family members

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