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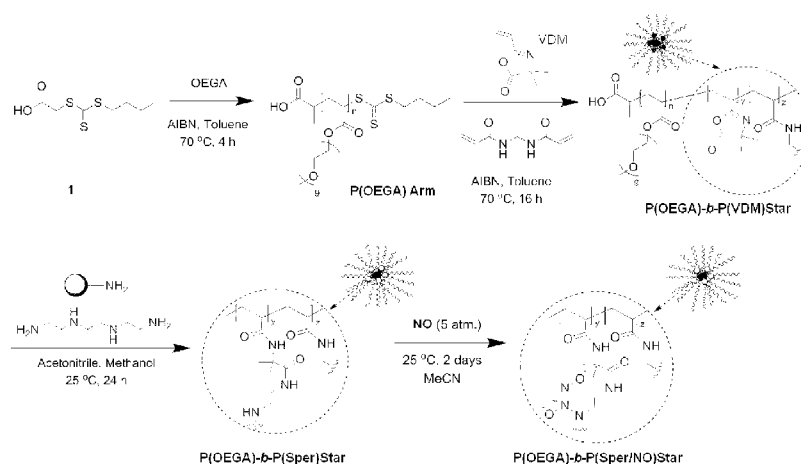
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(54) Title: ANTI-BIOFILM POLYMER

Figure 1



(57) Abstract: The invention relates to a polymer comprising a plurality of polymeric arms extending from a core, wherein on contact with an aqueous medium the polymer releases nitric oxide (NO) for an extended period of time. The invention also relates to the use of the polymer to promote dispersal of, or prevent or inhibit formation of, a biofilm.

ANTI-BIOFILM POLYMER

[0001] This application claims priority from Australian Provisional Patent Application No. 2014900998 filed on 21 March 2014 and Australian Provisional Patent Application No. 2014902080 filed on 30 May 2014. The disclosure of each of these provisional patent applications is incorporated herein in its entirety.

Field

[0002] The invention relates to a polymer, in particular to a nitric oxide (NO) releasing polymer. The invention also relates to the use of the polymer to promote dispersal of, or prevent or inhibit formation of, a biofilm.

Background

[0003] Despite intense efforts for the development of antimicrobial agents and many advances in the elucidation of disease mechanisms, infectious diseases still have a major detrimental impact on human health and the global economy. At present, infectious diseases are the second leading cause of death worldwide, accounting for about 15 million deaths each year. The main failure of antibiotic and antimicrobial agents at curing diseases often results from the development of drug resistance in pathogens and the adverse side effects associated with treatments. One key adaptive behavior adopted by bacteria is the formation of biofilms, which are highly-structured, usually surface-attached, multicellular communities of cells enclosed in a self-produced extracellular polymeric matrix. Bacteria embedded in biofilms exhibit upwards of 10-1,000-fold higher resistance to biocides and traditional antimicrobials than their planktonic counterparts, and they are less susceptible to host immune defense. Biofilms play a major role in human infectious diseases, forming on both living tissues and abiotic surfaces, such as indwelling biomedical devices or catheters. The inability to fully eradicate biofilms often forms the basis for chronic infections that can then lead to fatal outcomes. Not only are biofilms extremely important in a clinical context, but they can also be highly detrimental in industrial settings, for instance causing fouling of immersed marine surfaces, clogging of filtration membranes or corrosion of pipes as well as acting as a reservoir of pathogens in food and water processing. Therefore, novel efficient measures specifically aimed at inhibiting or preventing biofilms are urgently needed across a range of applications.

[0004] Recently, the signalling molecule nitric oxide (NO), a diatomic free radical, has been identified as a regulator of biofilm dispersal. NO is produced endogenously in late developmental stages of mature biofilms to induce dispersal events. Molecular analyses

revealed that NO triggers a signaling pathway involving the conserved intracellular second messenger cyclic di-GMP, which in turn activates a range of effectors leading to dispersal. Thus the use of NO represents one possible strategy for the control of biofilms in medical and industrial contexts. However, one major drawback with NO-based antibiofilm treatments is the high reactivity and short half-life of NO in biological systems, making it very challenging to administer treatments that can maintain over a period of time a level of NO effective to induce the signaling pathway.

Summary

[0005] In a first aspect, the present invention provides a polymer comprising a plurality of polymeric arms extending from a core, wherein on contact with an aqueous medium the polymer releases nitric oxide (NO) for an extended period of time.

[0006] In a second aspect, the present invention provides a polymer comprising a plurality of polymeric arms extending from a core, wherein the core comprises an NO-donor.

[0007] In a third aspect, the present invention provides a polymer comprising a plurality of polymeric arms extending from a core, wherein the core comprises an NO-donor precursor.

[0008] In a fourth aspect, the present invention provides a method for preparing the polymer of the first or second aspect, comprising exposing the polymer of the third aspect to an atmosphere of nitric oxide (NO).

[0009] In a fifth aspect, the present invention provides a polymer comprising a plurality of polymeric arms extending from a core, wherein the core comprises a moiety capable of binding to a compound comprising an NO-donor or NO-donor precursor.

[0010] In a sixth aspect, the present invention provides a method of preparing a polymer of the first or second aspect, comprising providing polymer chains comprising an arm portion and a core portion, wherein the core portion of the polymer chains are capable of undergoing a cross-linking reaction, and linking the core portion of the polymer chains by a cross-linking reaction to form a polymer comprising a plurality of polymeric arms extending from a core, wherein the core of the polymer comprises:

- a) an NO-donor; or
- b) an NO-donor precursor; or
- c) a moiety capable of binding to a compound comprising an NO-donor or NO-donor precursor.

[0011] In a seventh aspect, the present invention provides a method for promoting dispersal of, or preventing or inhibiting formation of, a biofilm, comprising exposing the biofilm, or a microorganism capable of forming the biofilm, to an effective amount of the polymer of the first or second aspect.

[0012] In an eighth aspect, the present invention provides a method of promoting dispersal of, or preventing or inhibiting formation of, a biofilm on a surface, the method comprising applying to the surface a polymer of the first or second aspect.

[0013] In a ninth aspect, the present invention provides a method of treatment and/or prevention of a condition associated with biofilm development, the method comprising administering to a subject in need thereof an effective amount of the polymer of the first or second aspect.

[0014] In a tenth aspect, the present invention provides a composition comprising the polymer of the first or second aspect and a carrier.

Brief description of drawings

[0015] The invention will be further described, by way of example only, with reference to the accompanying drawings, in which:

Figure 1 shows a synthetic scheme illustrating the synthesis of P(OEGA)-*b*-P(VDM) star polymer followed by spermine and NO donor conjugation as described in Example 1.

Figure 2 shows ¹H-NMR spectra of (A) P(OEGA) arm, (B) P(OEGA)-*b*-P(VDM) star and (C) P(OEGA)-*b*-P(Sper) star.

Figure 3 shows a size exclusion chromatography (SEC) trace of P(OEGA) arm and P(OEGA)-*b*-P(VDM) star before and after spermine conjugation.

Figure 4 shows (A) ATR-FTIR spectra of P(OEGA) arm, P(OEGA)-*b*-P(VDM) star and P(OEGA)-*b*-P(Sper) star; and (B) UV-Vis absorption spectra of P(OEGA)-*b*-P(VDM) star, P(OEGA)-*b*-P(Sper) star and P(OEGA)-*b*-P(Sper/NO) star. Figure 4B also shows the UV-Vis absorption spectra of the azo dye formed by the nitric oxide (NO) released from P(OEGA)-*b*-P(Sper/NO) star in water after the treatment with Griess reagent.

Figure 5 shows (A) number-weighted particle size distribution by dynamic light scattering (DLS) of P(OEGA) arm, P(OEGA)-*b*-P(VDM) star and P(OEGA)-*b*-P(Sper) star and (B) TEM images of P(OEGA)-*b*-P(VDM)star ("VDM star polymer") and P(OEGA)-*b*-P(Sper) star ("spermine star polymer").

Figure 6 shows a graph of zeta potential measurements of P(OEGA)-*b*-P(VDM)star, P(OEGA)-*b*-P(Sper) star and P(OEGA)-*b*-P(Sper) star after NO (nitric oxide) conjugation (i.e. P(OEGA)-*b*-P(Sper/NO) star).

Figure 7 shows (A) a calibration curve of the absorbance at 548 nm and (B) the UV-Vis absorption spectra of the azo dye formed by reaction of nitrite (formed under the assay conditions from released NO) and Griess reagent at different concentrations of nitric oxide.

Figure 8 shows a graph of *nirS* expression against time for assays conducted in the presence of P(OEGA)-*b*-P(Sper/NO) star (at 100 ppm and 400 ppm), sodium nitroprusside (SNP) at 200 μ M, P(OEGA)-*b*-P(Sper) star at 400 ppm (control) and untreated (control).

Figure 9 shows (A) a graph of planktonic biomass (OD₆₀₀) in the supernatant against time; and (B) a graph of biofilm biomass (crystal violet, OD₆₀₀) against time. These graphs show assay results obtained in the presence of P(OEGA)-*b*-P(Sper/NO) star (at 100 ppm and 400 ppm), sodium nitroprusside (SNP) at 20 μ M and 200 μ M and untreated (control). Error bars represent standard error (n = 2).

Figure 10 shows (A) a graph of biofilm or planktonic biomass (fold change vs. untreated). The graph shows results for assays conducted in the presence of P(OEGA)-*b*-P(Sper/NO) star (NO star) at various concentrations, P(OEGA)-*b*-P(Sper) star (sper star) at various concentrations, P(OEGA)-*b*-P(VDM)star (VDM star) at 400 ppm, spermine (sper) at 10 μ M and spermine NONOate (sper-NO) at 10 μ M. Error bars represent standard error (n = 2); and (B) pictures of stained biofilms treated with the indicated concentrations of NO star polymers.

Figure 11 shows (A) a graph of biofilm or planktonic biomass of *P. aeruginosa* from assays conducted in the presence of P(OEGA)-*b*-P(Sper/NO) star (NO star) at 100 ppm and 400 ppm, sodium nitroprusside (SNP) at 200 μ M and untreated (control). Error bars represent standard error (n = 2); and (B) pictures of stained biofilms treated or not with the indicated concentration of NO star or sodium nitroprusside (SNP).

Figure 12 shows (A) a graph of biofilm or planktonic biomass of wild type *P. aeruginosa* and mutant strains impaired in key components of the NO dispersal pathway, namely the phosphodiesterases *dipA* and *rbdA* that mediate decrease in intracellular levels of 2nd messenger c-di-GMP in response to NO, in the absence of P(OEGA)-*b*-P(Sper/NO) star or in the presence of P(OEGA)-*b*-P(Sper/NO) star (NO star) at 80 ppm and 133 ppm. Error bars represent standard error (n = 2); and (B) pictures of stained biofilms treated or not with NO star.

Figure 13 shows representative confocal images showing live bacterial cells stained with SYTO 9 Scale bars = 100 μ m (left hand side). The images show cells treated with P(OEGA)-*b*-P(Sper/NO) star ("NO-star") versus untreated cells. Figure 13 also shows representative pictures of stained biofilms treated or not with NO-star (right hand side).

Description of embodiments

[0016] A polymer comprising a plurality of polymeric arms extending from a core is sometimes referred to as a “star polymer” or “multi-arm polymer”. A star polymer is a macromolecule containing a single centre (core) from which linear chains (arms) emanate. Typically, the core of a star polymer is formed by cross-linking together linear polymeric chains. The core of a polymer comprising a plurality of polymeric arms extending from a core is generally more hydrophobic than the polymeric arms. The inventors have found that when a nitric oxide (NO) donor is incorporated into the core of such a polymer, the polymer, when in contact with an aqueous medium, such as a biological system, is able to release NO for an extended period of time. Such a polymer can be used to promote dispersal of, or prevent or inhibit the formation of, a biofilm.

[0017] In a first aspect, the present invention provides a polymer comprising a plurality of polymeric arms extending from a core, wherein on contact with an aqueous medium the polymer releases nitric oxide (NO) for an extended period of time.

[0018] In a second aspect, the present invention provides a polymer comprising a plurality of polymeric arms extending from a core, wherein the core comprises an NO-donor.

[0019] In a third aspect, the present invention provides a polymer comprising a plurality of polymeric arms extending from a core, wherein the core comprises a moiety capable of forming an NO-donor on exposure to NO.

[0020] Nitric oxide (NO) is a key regulator of biofilm dispersal. The polymer of the first or second aspect of the present invention is advantageously capable of releasing NO over an extended period of time when the polymer is in contact with an aqueous medium, that is, the polymer is capable of providing sustained release of NO in an aqueous medium. The release of NO over an extended period of time can be used to promote biofilm dispersal or prevent or inhibit biofilm formation over an extended period of time by continually providing a dispersal trigger. This may be useful, for example, for forming a coating on an implant to prevent biofilm formation on the implant prior to tissue integration of the implant.

[0021] The polymer of the present invention comprises a plurality of polymeric arms extending from a core. By a “plurality of polymeric arms” it is meant 3 or more polymeric arms. That is, the polymer of the present invention comprises 3 or more (e.g. 4 or more, 5 or more, 6 or more, etc) polymeric arms extending from the core of the polymer. Typically the polymer

comprises 8 to 12 polymeric arms.

[0022] A biofilm is a three-dimensional, matrix-encased microbial community displaying multicellular characteristics. As used herein, the term biofilm includes surface-associated biofilms as well as biofilms in suspension, such as flocs and granules. The surface may be a biological surface or a non-biological surface. Biological surfaces include surfaces both internal (such as a surface of a tissue or membrane) and external (such as the surface of skin, seeds or plant foliage) to an organism, including bacterial membranes and cell walls. Biological surfaces also include surfaces formed of a biological material such as wood or a plant fibre. Non-biological surfaces include man made surfaces of a non-biological material (e.g. a metal, plastic or glass surface) that can support the establishment and development of a biofilm. Such surfaces may be present in industrial plants and equipment. Further, the surface may be porous (such as a membrane) or non-porous, and may be rigid or flexible. Biofilms may comprise a single microbial species or may be mixed species complexes, and may include bacteria as well as fungi, algae, protozoa, or other microorganisms.

[0023] The polymer of the first or second aspect of the present invention comprises a moiety capable of generating or releasing nitric oxide (NO) in an aqueous medium. The polymer of the first or second aspect of the present invention is capable, on contact with an aqueous medium, of releasing NO over an extended period of time. The extended period of time is a period of time longer than the period of release of NO from a similar NO-donor not associated with the core of a star polymer. For example, spermine NONOate has an NO donation half-life of about 30 minutes in aqueous media. For a polymer of the present invention comprising a spermine NONOate group in the core of the polymer, the NO may be released from the NONOate group over a period of hours, days or weeks. The polymer typically releases NO over a period of at least 12 hours after first contacting the polymer with an aqueous medium. Accordingly, in some embodiments, the extended period of time is at least 12 hours. In one embodiment, the extended period of time is about 12 hours to about 30 days, about 1 day to about 25 days, about 5 days to about 21 days, about 7 days to about 18 days or about 10 days to about 15 days. As used herein, a "day" is a 24 hour period of time. The release of NO from a polymer of the present invention may be detected by, for example, UV-Vis spectroscopy.

[0024] Preferably, the polymer is used in an amount such that the polymer provides an effective amount of NO over an extended period of time (e.g. over a period of at least 12 hours). As used herein, the term "effective amount of NO" is an amount of NO sufficient to prevent or

inhibit biofilm formation or promote biofilm dispersion. As used herein, the term "effective amount" in relation to the polymer of the first or second aspect of the present invention refers to an amount of the polymer sufficient to provide an effective amount of NO. The effective amount of NO is preferably a non-toxic amount of NO. The exact amount/concentration of the polymer required to provide an effective amount of NO will vary depending on factors such as the species of microorganism(s) being treated, the amount of microorganism present capable of biofilm formation, the extent, severity and/or age of a biofilm being treated, whether the biofilm is surface-associated or suspended, the particular polymer used, and so forth. For any given case, an appropriate effective amount of the polymer may be determined by one of ordinary skill in the art using only routine experimentation. In some embodiments, the polymer is used in an amount such that the polymer provides a concentration of NO between about 1 nM and 100 mM, e.g. between about 10 nM and about 50 mM, between about 100 nM and 10 mM, between about 200 nM and 1 mM, between 10 nM and 500 μ M, between about 10 nM and 1 μ M or between about 10 nM and 500 nM.

[0025] As used herein the term "non-toxic" as it pertains to concentrations or amounts of NO means concentrations or amounts of NO which do not have a direct toxic effect on cells, i.e. that do not kill individually free-living cells, but which may operate as a signal that triggers induction of differentiation processes in biofilms, which involve a programmed cell death response and which therefore may result in death of a subpopulation of cells, generation of dispersal cells and the dispersal of biofilms. For example, a non-toxic concentration or amount may comprise 100 mM nitric oxide, or less.

[0026] In one embodiment, the polymer, when in contact with an aqueous medium (e.g. water), releases nitric oxide (NO) at a rate of about 1 pmol(NO)/mg(polymer)/h to about 1 μ mol(NO)/mg(polymer)/h, about 100 pmol(NO)/mg(polymer)/h to about 50 nmol(NO)/mg(polymer)/h or about 500 pmol(NO)/mg(polymer)/h to about 10 nmol(NO)/mg(polymer)/h (that is, at a rate of about 1 nmol(NO)/g(polymer)/h to about 1 mmol(NO)/g(polymer)/h, about 100 nmol(NO)/g(polymer)/h to about 50 μ mol(NO)/g(polymer)/h or about 500 nmol(NO)/g(polymer)/h to about 10 μ mol(NO)/g(polymer)/h). The rate of release of nitric oxide (NO) from the polymer may be determined using the Griess assay, for example, conducted as described at [0119] below. The rate of release in moles of NO per gram of polymer per hour may be calculated from the concentration of NO determined using the Griess assay by calculating the amount of NO released (mole) per litre of the aqueous medium and dividing by the amount of polymer (grams) per litre used in the assay and dividing by the time (hours) over which NO is released.

Polymeric Core

[0027] The polymer of the present invention comprises a core. The core is typically overall more hydrophobic relative to the polymeric arms of the polymer. The core is the region of the polymer from which extend the plurality of polymeric arms.

[0028] In one embodiment, the core comprises an NO-donor. As used herein, the term “NO-donor” refers to a moiety capable of generating or releasing nitric oxide (NO) in an aqueous medium. As used herein, the term “NO-donor precursor” refers to a moiety which upon exposure to NO is able to form an NO-donor.

[0029] Conventional small-molecule NO-donors, including S-nitrosothiols (RSNOs) and *N*-diazoniumdiolates (NONOates), typically spontaneously release NO upon solvation. These compounds typically lack both stability and specificity and are not suitable for slow, sustained release of NO over time to revert bacterial attachment processes and confine bacteria in a planktonic mode of growth.

[0030] The inventors have found that when an NO-donor is incorporated into the core of a polymer comprising a plurality of polymeric arms extending from a core, the polymer is able to release NO over an extended period of time. Typically, the NO-donor is covalently bonded to the core of the polymer. Typically, an NO-donor will become activated for NO generation or release when solvated. Without wishing to be bound by theory, it is believed that the polymeric arms and the hydrophobicity of the core relative to the polymeric arms delay solvation of the NO-donor and thus increase the stability of the NO donor, which results in the release of NO from the polymer over an extended period of time.

[0031] The NO donor may be any moiety capable of generating or releasing NO on contact with an aqueous medium. Suitable NO donors may, for example, be selected from S-nitroso, O-nitroso, C-nitroso and N-nitroso groups and nitro derivatives thereof, including, for example, NONOates.

[0032] In one embodiment, the NO donor is a NONOate group. A NONOate may be formed by reaction of NO with an amine.

[0033] In one embodiment, the core of the polymer comprises an NO-donor precursor. An NO-donor precursor is capable of reacting with NO to form an NO-donor. The NO-donor precursor is preferably covalently bound to the core of the polymer. The NO-donor precursor

may be any suitable amine moiety. Preferably, the amine is a secondary amine, i.e. a disubstituted amine. NONOates formed from secondary amines demonstrate increased stability relative to NONOates formed from primary amines, and therefore advantageously extend the time for NO release.

[0034] An NO-donor precursor may be incorporated into the core of a star polymer by, for example, linking a polyamine comprising a secondary amine to the core of the polymer. The polyamine may be any polyamine comprising a primary amine moiety and a secondary amine moiety. In one embodiment, the polyamine comprising a secondary amine is selected from spermine, jeffamine, polyetheramines, polyethylenediamine and spermidine. A preferred polyamine comprising a secondary amine is spermine.

Polymeric arms

[0035] The polymer of the present invention comprises a plurality of polymeric arms extending from the core.

[0036] Preferably, the polymeric arms are hydrophilic. The hydrophilic polymeric arms assist in the solubilisation of the polymer. As biofilms typically form in aqueous environments, the polymer of the present invention is preferably soluble in water. It is believed that the polymeric arms assist in inhibiting solvation of the NO-donor and therefore assist in providing release of NO from the polymer over an extended period of time.

[0037] The polymeric arms are preferably overall hydrophilic relative to the core of the polymer. However, in some embodiments, the polymeric arms may include hydrophobic groups.

[0038] As used herein the term "monomer" refers to a compound capable of undergoing a polymerisation reaction to form a polymer. It will be appreciated that in forming the polymer a portion of the monomer will be included in the polymer chain and thus have different chemical properties, e.g. radical polymerisation of an olefin results in a polymer chain having saturated chemical character.

[0039] The polymeric arms may be prepared by any suitable technique known in the art, and may be formed from any monomer or combination of monomers able to undergo polymerisation.

[0040] The monomer may, for example, be selected from methyl methacrylate, ethyl methacrylate, propyl methacrylate (all isomers), butyl methacrylate (all isomers), 2-ethylhexyl methacrylate, isobomyl methacrylate, methacrylic acid, benzyl methacrylate, phenyl methacrylate, methacrylonitrile, alpha-methystyrene, methyl acrylate, ethyl acrylate, propyl acrylate (all isomers), butyl acrylate (all isomers), 2-ethylhexyl acrylate, isobornyl acrylate, acrylic acid, benzyl acrylate, phenyl acrylate, acrylonitrile, styrene, functional methacrylates, acrylates and styrenes selected from glycidyl methacrylate, 2-hydroxyethyl methacrylate, hydroxypropyl methacrylate (all isomers), hydroxybutyl methacrylate (all isomers), *N,N*-dimethylaminoethyl methacrylate, *N,N*-diethylaminoethyl methacrylate, triethyleneglycol methacrylate, itaconic anhydride, itaconic acid, glycidyl acrylate, 2-hydroxyethyl acrylate, hydroxypropyl acrylate (all isomers), hydroxybutyl acrylate (all isomers), *N,N*-dimethylaminoethyl acrylate, *N,N*-diethylaminoethyl acrylate, triethyleneglycol acrylate, methacrylamide, *N*-methylacrylamide, *N,N*-dimethylacrylamide, *N-tert*-butylmethacrylamide, *N-n*-butylmethacrylamide, *N*-methylolmethacrylamide, *N*-ethylolmethacrylamide, hydroxypropylmethacrylamide, *N-tert*-butylacrylamide, *N-n*-butylacrylamide, *N*-methylolacrylamide, *N*-ethylolacrylamide, vinyl benzoic acid (all isomers), diethylaminostyrene (all isomers), alpha-methylvinyl benzoic acid (all isomers), diethylamino alpha-methylstyrene (all isomers), *p*-vinylbenzene sulfonic acid, *p*-vinylbenzene sulfonic sodium salt, trimethoxysilylpropyl methacrylate, triethoxysilylpropyl methacrylate, tributoxysilylpropyl methacrylate, dimethoxymethylsilylpropyl methacrylate, diethoxymethylsilylpropylmethacrylate, dibutoxymethylsilylpropyl methacrylate, diisopropoxymethylsilylpropyl methacrylate, dimethoxysilylpropyl methacrylate, diethoxysilylpropyl methacrylate, dibutoxysilylpropyl methacrylate, diisopropoxysilylpropyl methacrylate, trimethoxysilylpropyl acrylate, triethoxysilylpropyl acrylate, tributoxysilylpropyl acrylate, dimethoxymethylsilylpropyl acrylate, diethoxymethylsilylpropyl acrylate, dibutoxymethylsilylpropyl acrylate, diisopropoxymethylsilylpropyl acrylate, dimethoxysilylpropyl acrylate, diethoxysilylpropyl acrylate, dibutoxysilylpropyl acrylate, diisopropoxysilylpropyl acrylate, oligoethylene methyl ester (meth)acrylate, hydroxypropyl (meth)acrylamide, vinyl acetate, vinyl butyrate, vinyl benzoate, vinyl chloride, vinyl fluoride, vinyl bromide, maleic anhydride, *N*-phenylmaleimide, *N*-butylmaleimide, *N*-vinylpyrrolidone, *N*-vinylcarbazole, butadiene, isoprene, chloroprene, ethylene, propylene, ethylene glycol methyl ether (meth)acrylate, hydropropyl (meth)acrylamide, *N,N*-isoprylacrylamide, hydroethyl acrylate, oligoethylene glycol (meth)acrylate, or a combination thereof. In some embodiments, the monomer is methyl methacrylate, hydroxypropylmethacrylamide, methacrylate, styrene, oligoethylene methyl ester (meth)acrylate or vinyl acetate, or a combination thereof.

[0041] In one embodiment, the polymeric arms comprise a co-polymer of two or more of the monomers described above. In another embodiment, the polymeric arms are block co-polymers comprising two or more blocks of oligomers. The oligomers may be short polymeric chains of any of the monomers described above.

[0042] In one embodiment, the polymeric arms comprise polymerised ethylene glycol methyl ether (meth)acrylate, hydropropyl (meth)acrylamide, *N,N*-isoprylacrylamide, hydroethyl acrylate, oligoethylene glycol (meth)acrylate or dimethylacrylamide, or a combination thereof.

[0043] Each polymeric arm of the plurality of polymeric arms may be the same or different. In some embodiments, the polymeric arms are the same, or comprise 2 or 3 different arm types.

[0044] The length of each arm may also vary. It will be appreciated that depending on the method selected to prepare the polymeric arms, the molecular weight of each arm may vary. In one embodiment, each arm has a molecular weight of about 2000 g/mol to about 250,000 g/mol, about 3000 g/mol to about 150,000 g/mol, about 4000 g/mol to about 100,000 g/mol, about 5,000 g/mol to about 75,000 g/mol, about 7,000 g/mol to about 50,000 g/mol, about 10,000 g/mol to about 25,000 g/mol or about 10,000 g/mol to about 20,000 g/mol.

[0045] In addition, the polydispersity of polymer arms may also vary depending on the method employed to prepare each arm. The polydispersity of the polymeric arms may be, for example, about 1.00 to about 3.00, about 1.01 to about 2.50, about 1.02 to about 2.00, about 1.03 to about 1.50 or about 1.04 to about 1.25.

Method for preparing the polymer

[0046] The polymer of the present invention is a star or multiarm polymer. The polymer of the present invention may be prepared by similar methods to methods known in the art for preparing other star or multiarm polymers. For example, a polymeric core may be prepared and then polymeric arms may be chain extended from, or grafted to, the core. Alternatively, an arm-first approach may be employed, where the arms are prepared and then linked together to form the core of the star or multiarm polymer.

[0047] In one embodiment, the polymer is prepared by an arm-first approach. Accordingly, in one embodiment, the invention provides a method for preparing the polymer of the present invention, comprising providing a polymer chain comprising an arm portion and a core portion,

and linking the core portion of the polymer chains by a cross-linking reaction. The core portion is the portion of the polymer chain that is able to form the core of the polymer of the present invention, and the arm portion is the portion of the polymer chain that is able to form the polymeric arms of the polymer of the present invention. The core portions of the polymer chains are capable of undergoing a cross-linking reaction to form a polymer comprising a plurality of polymeric arms extending from a core, wherein the core of the polymer comprises: a) an NO-donor; b) an NO-donor precursor; or c) a moiety capable of binding with a compound comprising an NO-donor or NO-donor precursor.

[0048] In one embodiment, the method comprises providing polymer chains comprising an arm portion and a core portion, wherein the core portion of the polymer chains comprise a) an NO-donor; b) an NO-donor precursor; or c) a moiety capable of binding to a compound comprising an NO-donor or NO-donor precursor, such that, when the core portions of the polymer chains are cross-linked, the core of the resultant polymer comprises a) an NO-donor; b) an NO-donor precursor; or c) a moiety that is capable of binding to a compound comprising an NO-donor or NO-donor precursor.

[0049] In another embodiment, the cross-linking reaction is carried out using a cross-linking agent where the cross-linking agent comprises a) an NO-donor; b) an NO-donor precursor; or c) a moiety capable of binding to a compound comprising an NO-donor or NO-donor precursor, wherein the cross-linking agent is incorporated into the core of the polymer during the cross-linking reaction such that the core of the polymer comprises a) an NO-donor; b) an NO-donor precursor; or c) a moiety that is capable of binding to a compound comprising an NO-donor or NO-donor precursor.

[0050] In a further embodiment, the cross-linking reaction is carried out in the presence of a compound which comprises a) an NO-donor; b) an NO-donor precursor; or c) a moiety capable of binding to a compound comprising an NO-donor or NO-donor precursor, wherein the compound is incorporated into the core of the polymer during the cross-linking reaction such that the core of the polymer comprises a) an NO-donor; b) an NO-donor precursor; or c) a moiety that is capable of binding to a compound comprising an NO-donor or NO-donor precursor.

[0051] Any polymerisation technique known in the art may be used to form the polymer chains. The polymerisation technique may, for example, be a radical polymerisation, preferably radical addition fragmentation chain transfer (RAFT) polymerisation. RAFT polymerisation typically involves the polymerisation of a monomer or combination of monomers in solution in

the presence of a radical initiator, chain transfer agent and heat. Some suitable RAFT conditions are described in the reviews Moad, G.; Rizzardo, E.; Thang, S. H. *Accounts of Chemical Research* 2008, 41, 1133 and Moad, G.; Rizzardo, E.; Thang, S. H. *Polymer* 2008, 49, 1079. The person skilled in the art will be able to determine appropriate conditions, including solvent selection, temperature for reaction and combination of radical initiator and chain transfer agent depending on the monomer or combination of monomers selected.

[0052] The core portion may be formed at one end of the polymer chain. Alternatively, the core portion may be formed in the middle of the polymer chain, such that the polymer chain comprises two arm portions on either end of the core portion.

[0053] Cross-linking the core portion of the polymer chains provides a star or multi-arm polymer. Cross-linking the core portions of the polymer chains may be achieved by reaction with a cross-linking agent. The cross-linking agent may be any monomer comprising a moiety able to form a cross-link between two or more polymer chains or may be any compound able to form covalent bonds between two or more polymer chains to form the core of the polymer described herein.

[0054] In one embodiment, the cross-linking agent is a monomer comprising a moiety capable of undergoing a cross-linking reaction and link together two or more polymer chains. The cross-linking agent may, for example, be selected from a bisacrylamide, such as methylenebisacrylamide; divinyl benzene; dimethacrylate and diacrylate compounds, such as dimethacrylate hexyl; or a combination thereof. In one embodiment, the cross-linking monomer is methylenebisacrylamide.

[0055] In another embodiment, the cross-linking agent is a compound able to form covalent bonds between two or more polymer chains and the core portion of the polymer chain comprises a moiety that is able to undergo cross-linking by reaction with the cross-linking agent.

[0056] In one embodiment, the invention provides a method for preparing a polymer described herein comprising providing a polymer chain comprising an arm portion and a core portion, wherein the core portion comprises a) an NO-donor; b) an NO-donor precursor; or c) a moiety capable of binding to a compound comprising an NO-donor or NO-donor precursor. Such a polymer chain may be prepared by preparing a polymer chain, e.g. a polymer of ethylene glycol monomethyl ether, and then extending the polymer chain by a polymerisation reaction with a monomer comprising a) an NO-donor; b) an NO-donor precursor; or c) a moiety capable of binding to a compound comprising an NO-donor or NO-donor precursor, to form a polymer chain comprising a core portion, wherein the core portion comprises a) an NO-donor; b)

an NO-donor precursor; or c) a moiety capable of binding to a compound comprising an NO-donor or NO-donor precursor. Cross-linking the core portions of the polymer chains forms a star polymer having a core comprising a) an NO-donor; b) an NO-donor precursor; or c) a moiety capable of binding to a compound comprising an NO-donor or NO-donor precursor.

[0057] In one embodiment, the cross-linking reaction occurs under the same conditions as the polymerisation reaction with the monomer comprising a) an NO-donor; b) an NO-donor precursor; or c) a moiety capable of binding to a compound comprising an NO-donor or NO-donor precursor. In such embodiments, the polymerisation with the monomer comprising a) an NO-donor; b) an NO-donor precursor; or c) a moiety capable of binding to a compound comprising an NO-donor or NO-donor precursor, and the cross-linking reaction, can be carried out at the same time (concurrently) in the same vessel. In such embodiments, the A) cross-linking agent and B) the monomer comprising a) an NO-donor; b) an NO-donor precursor; or c) a moiety capable of binding to a compound comprising an NO-donor or NO-donor precursor, may, for example, be used in a molar ratio of A:B of about 10:1 to about 1:10, about 5:1 to about 1:5, about 1:1 to about 1:4, or about 1:1 to about 1:3, preferably about 1:2.

[0058] When the core of the polymer comprises a moiety that is capable of binding to a compound comprising an NO-donor or NO-donor precursor, the polymer may be exposed to conditions effective for the moiety to bind to the compound to form a polymer in which the core comprises an NO-donor or NO-donor precursor. Accordingly, the method of the present invention may comprise the further step of contacting the polymer with the compound comprising an NO-donor or NO-donor precursor under conditions effective for the moiety to bind to the compound to produce a polymer comprising an NO-donor or NO-donor precursor. Preferably the moiety that is capable of binding to a compound comprising an NO-donor or NO-donor precursor is a moiety capable of forming a covalent bond with a compound comprising an NO-donor or NO-donor precursor.

[0059] The moiety capable of binding to a compound comprising an NO-donor or NO-donor precursor may be, for example, an activated ester, such as a pentafluorophenyl ester, a *N*-hydroxosuccinamide ester, a nitrophenyl ester or a tetraphenyl ester; an activated "trapped" ester, such as a 4,4-dimethyl-5-oxalone moiety; a carboxylic acid (which may be activated *in situ* with an activating agent, such as thionyl chloride or oxalyl chloride, or a coupling agent, such as *N,N*-dicyclohexylcarbodiimide (DCC) with or without a catalyst such as 4-dimethylaminopyridine (DMAP)); an isocyanate moiety; an epoxy moiety, such as a glycidyl moiety; or an aldehyde, and the compound comprising an NO-donor or NO-donor precursor may, for example, be selected from a polyamine comprising a secondary amine, such as

spermine or spermidine.

[0060] When the core of the polymer comprises an NO-donor precursor, the polymer may be exposed to an atmosphere containing NO to form a polymer in which the core comprises an NO-donor. Accordingly, the method of the present invention may comprise the further step of contacting the polymer with an atmosphere of NO (that is, an atmosphere containing NO) to form a polymer comprising an NO-donor.

[0061] In some embodiments, the NO-donor or NO-donor precursor may be in a protected form during the cross-linking of the core portion of the polymer chains to form a star polymer and de-protected after formation of the star polymer comprising the protected NO-donor or NO-donor precursor. For example, in order to form a polymer of the present invention comprising a polyamine NONOate group (an NO-donor), the core portion of the polymer chain may comprise:

- a) a polyamine NONOate; or
- b) a polyamine moiety (contacting a polyamine moiety with NO forms a polyamine NONOate group); or
- c) a protected form of a) or b); or
- d) a moiety, e.g. a 4,4-dimethyl-5-oxalone moiety, that is capable of binding to a polyamine comprising a secondary amine.

[0062] In one embodiment, the core portion of the polymer chain is derived from a cross-linking monomer and a monomer comprising a moiety capable of binding to a compound comprising an NO-donor or NO-donor precursor. After cross-linking the core portion of the polymer chains to form a star polymer, exposing the star polymer to the compound comprising an NO-donor or NO-donor precursor, e.g. spermine, under conditions effective for the moiety to bind to the compound, produces a star polymer comprising an NO-donor or NO-donor precursor within the core of the polymer.

[0063] In one embodiment, the method comprises forming a polymer chain comprising an arm portion, for example, via RAFT polymerisation. The polymer chain may then be extended to form a core portion at one end, the core portion being derived from a cross-linking monomer and a monomer comprising an NO-donor or an NO-donor precursor.

[0064] In another embodiment, the method comprises forming a polymer chain comprising an arm portion, for example, via RAFT polymerisation. The polymer chain may then be extended to form a core portion at one end, the core portion being derived from a cross-linking monomer and a monomer comprising a moiety capable of binding to a compound comprising an

NO-donor or NO-donor precursor. Preferably, the cross-linking monomer undergoes a cross-linking reaction under the polymerisation conditions employed to extend the polymer chain. Exposing the cross-linked polymer (a star polymer) to the compound comprising an NO-donor or NO-donor precursor under conditions effective for the moiety to bind to the compound provides a polymer of the first, second or third aspect of the present invention.

[0065] In one embodiment, the core portion of the polymer chain is derived from A) a cross-linking monomer and B) a monomer comprising a moiety capable of binding to a compound comprising an NO-donor or NO-donor precursor, in a ratio of A:B of about 10:1 to about 1:10, e.g. about 5:1 to about 1:5, about 1:1 to about 1:4, or about 1:1 to about 1:3, preferably about 1:2.

[0066] In another embodiment, the method comprises forming a polymer chain comprising an arm portion, for example, via RAFT polymerisation. The polymer chain may then be extended to form a core portion at one end, the core portion comprising a co-polymer of a monomer comprising a moiety that is able to undergo cross-linking by reaction with a cross-linking agent and a monomer comprising a moiety capable of binding to a compound comprising an NO-donor or NO-donor precursor. Cross-linking the polymer chains together may be promoted by exposing the polymer chains to the cross-linking agent. Exposing the cross-linked polymer (a star polymer) to the compound comprising an NO-donor or NO-donor precursor under conditions effective for the moiety to bind to the compound provides a polymer of the first, second or third aspect of the present invention.

[0067] In one embodiment, the core portion of the polymer chain is derived from A) a monomer comprising a moiety that is able to undergo cross-linking by reaction with a cross-linking agent and B) a monomer comprising a moiety capable of binding to a compound comprising an NO-donor or an NO-donor precursor, in a ratio of A:B of about 10:1 to about 1:10, about 5:1 to about 1:5, about 1:1 to about 1:4, or about 1:1 to about 1:3, preferably about 1:2.

[0068] In one embodiment, the core portion of the polymer chain comprises a block co-polymer comprising a block derived from a cross-linking monomer and a block derived from a monomer comprising a moiety capable of binding to a compound comprising an NO-donor or an NO-donor precursor.

[0069] The moiety capable of binding to a compound comprising an NO-donor or an NO-donor precursor is typically a moiety capable of reacting with the compound comprising an NO-

donor or an NO-donor precursor to form a covalent bond with the compound. The monomer comprising a moiety capable of binding to a compound comprising an NO-donor or an NO-donor precursor may be, for example, an activated ester containing monomer, such as pentafluorophenyl ester acrylate, pentafluorophenyl ester methacrylate, N-hydroxysuccinimide (NHS) acrylate, N-hydroxysuccinimide (NHS) methacrylate, nitrophenyl ester acrylate, nitrophenyl ester methacrylate, tetraphenyl methacrylate, tetraphenyl acrylate and acrylic acid; an activated "trapped" ester monomer, such as 2-vinyl-4,4-dimethyl-5-oxazolone; an isocyanate-containing monomer, such as 3-isopropenyl- α,α -dimethylbenzyl isocyanate or 2-isocyanatoethyl methacrylate; an epoxy-containing monomer, such as glycidyl methacrylate; an aldehyde-containing monomer, such as vinyl benzyl aldehyde; or a combination thereof. For example, the 4,4-dimethyl-5-oxazolone moiety of 2-vinyl-4,4-dimethyl-5-oxazolone can be reacted with, for example, a polyamine comprising a secondary amine, such as spermine, to form a covalent bond with the polyamine.

[0070] In one aspect, the present invention provides a method for preparing the polymer of the first or second aspect of the present invention, comprising exposing a polymer comprising a plurality of polymeric arms extending from a core, wherein the core comprises an NO-donor precursor, to an atmosphere of nitric oxide (NO). Exposure of the polymer to an atmosphere of NO advantageously causes the formation of an NO-donor. Preferably the atmosphere of NO, consists of, or consists essentially of, NO.

[0071] The atmosphere of NO may be at elevated pressure. For example, the NO atmosphere may be at about 1 atm to about 10 atm. In one embodiment, the step of exposing the polymer to the atmosphere of NO is conducted in a Parr Hydrogenator apparatus which is typically capable of pressures of about 1 atm up to about 5 atm.

[0072] In another embodiment, the NO-donor may be formed by exposing a polymer comprising an NO-donor precursor to a nitrosating agent. The nitrosating agent is any agent capable of nitrosating a moiety capable of forming an NO-donor (NO-donor precursor).

[0073] The core portion of the polymer chain may comprise a further monomer selected from the group of monomers described above.

Biofilm dispersal

[0074] In one aspect, the present invention provides a method for promoting dispersal of, or preventing or inhibiting formation of, a biofilm, comprising exposing the biofilm, or a

microorganism capable of forming a biofilm, to an effective amount of a polymer of the first or second aspect of the present invention.

[0075] In one embodiment, the biofilm, or microorganism capable of forming a biofilm, is exposed to the polymer of the first or second aspect of the present invention at a concentration of about 1 ppm to about 1000 ppm, e.g. about 10 ppm to about 500 ppm or about 20 ppm to about 200 ppm.

[0076] The biofilm may be surface-associated or suspended. The suspended biofilm may be in the form of flocs or granules.

[0077] The microorganism or microorganisms present in the biofilm or capable of forming a biofilm may be of a single species or of multiple species.

[0078] The microorganisms within the biofilm or capable of forming a biofilm may comprise bacterial or fungal species or both, and may comprise one or more species selected from, for example, *Candida* spp., *Hormoconis* spp., *Pseudomonas* spp., *Pseudoalteromonas* spp., *Staphylococcus* spp., *Streptococcus* spp., *Shigella* spp., *Mycobacterium* spp., *Enterococcus* spp., *Escherichia* spp., *Salmonella* spp., *Legionella* spp., *Haemophilus* spp., *Bacillus* spp., *Desulfovibrio* spp., *Shewanella* spp., *Geobacter* spp., *Klebsiella* spp., *Proteus* spp., *Aeromonas* spp., *Arthrobacter* spp., *Micrococcus* spp., *Serratia* spp., *Porphyromonas* spp., *Fusobacterium* spp. and *Vibrio* spp., representative examples of such species being *Candida albicans*, *P. aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Bacillus licheniformis*, *Serratia marcescens*, *Fusobacterium nucleatum*, and *Vibrio Cholerae*. Any biofilm sensitive to non-toxic amounts of NO may be dispersed by exposure to an effective amount of a polymer of the first or second aspect of the present invention.

[0079] The method may further comprise exposing the biofilm or the microorganism capable of forming a biofilm, to at least one antimicrobial agent.

[0080] Any suitable antimicrobial agent may be used, for example, antibiotics, detergents, surfactants, agents that induce oxidative stress, bacteriocins and antimicrobial enzymes, peptides and phage. The antimicrobial agent may be natural or synthetic. The antimicrobial agent employed may be selected for the particular application of the invention on a case-by-case basis, and those skilled in the art will appreciate that the scope of the present invention is not limited by the nature or identity of the particular antimicrobial agent. By way of example only, suitable antibiotics may be selected from p-lactams, monopenems, carboxypenems,

aminoglycosides, quinolones, macrolides, lincosamides, tetracyclines, streptogramins, glycopeptides, rifamycins, sulphonamides chloramphenicol, nalidixic acid, azole-containing compounds and peptide antibiotics. Antimicrobial enzymes include, but are not limited to, lipases, pronases, lyases (e.g. alginate lyases) and various other proteolytic enzymes and nucleases.

[0081] The biofilm or microorganism capable of forming a biofilm may be exposed to the polymer of the first or second aspect of the present invention by any means which bring the biofilm or microorganism into contact with NO released from the polymer. For example, the polymer of the present invention may be dispersed or dissolved into a solution comprising the microorganisms. Alternatively, the polymer may be incorporated into a coating on a surface that is in contact with the microorganism or which may come into contact with the microorganism.

[0082] The present invention also relates to a method of treatment and/or prevention of a condition associated with biofilm development, comprising administering to a subject in need thereof an effective amount of the polymer of the first or second aspect of the present invention. In one embodiment, the polymer is administered as a coating on an implant or implantable device. In another embodiment, the polymer is administered topically, e.g. to the skin or to a mucosal membrane. The method of treatment and/or prevention of a condition associated with biofilm development may further comprise administration of an antimicrobial agent. The antimicrobial agent and the polymer of the first or second aspect of the invention may be administered by concurrent, sequential or separate administration.

[0083] The present invention also relates to a polymer of the first or second aspect of the present invention for use in treating and/or preventing a condition associated with biofilm development.

[0084] The present invention also relates to use of a polymer of the first or second aspect of the present invention for the preparation of a medicament for treating and/or preventing a condition associated with biofilm development.

[0085] The polymer of the first or second aspect of the present invention and/or the antimicrobial agent may, for example, be coated onto or be impregnated in or incorporated in the surface of a surgical or implantable medical device such as a catheter, stent or prosthesis.

[0086] The polymer of the first or second aspect of the present invention and/or the antimicrobial agent may, for example, be topically administered. In one embodiment, the polymer of the first or second aspect of the present invention and/or the antimicrobial agent are

topically applied to a wound, e.g., a wound susceptible to microbial infection, or infected by one or more microbes.

[0087] The present invention also relates to compositions for promoting dispersal of microorganisms from a biofilm, or for inhibiting biofilm formation and/or development.

[0088] Thus, according to another aspect of the invention, there is provided a composition for promoting dispersal of, or preventing formation of, a microbial biofilm over an extended period of time, the composition comprising the polymer of the first or second aspect of the present invention and a carrier. Suitable carriers are compatible with the other ingredients of the composition and, in the case of pharmaceutical compositions, not deleterious to the recipient of the composition. The carrier may be liquid or solid. In the case of liquid carriers, the carrier may be an aqueous or non-aqueous solvent. For anti-fouling for industrial applications, the composition may, for example, be in the form of a paint or other surface coating.

[0089] In one embodiment, the composition further comprises an antimicrobial agent. Any suitable antimicrobial agent, e.g. those described above, may be used.

[0090] In one embodiment, the present invention provides a pharmaceutical composition comprising the polymer of the first or second aspect of the present invention and a carrier. In some embodiments, the pharmaceutical composition further comprises an antimicrobial agent. The pharmaceutical composition typically comprises an amount of the antimicrobial agent effective to kill, or to inhibit the growth or reproduction of, a microorganism. The effective amount will vary depending on the particular antimicrobial agent selected and may be determined according to general principles known in the art. The pharmaceutical compositions of the present invention may be formulated according to techniques such as those well known in the art of pharmaceutical formulation (See, for example, Remington: *The Science and Practice of Pharmacy*, 21st Ed., 2005, Lippincott Williams & Wilkins).

[0091] In one embodiment, the pharmaceutical composition is administered topically to the subject. Modes of topical administration include administration by aerosol, gel, solution, suspension, emulsion, ointment, tincture, powder, cream or lotion, or by way of a bandage.

[0092] An aerosol may be prepared, for example, by means of a metering atomising spray pump. Another aerosol formulation includes the polymer of the first or second aspect of the invention in a pressurised pack with a suitable propellant such as a chlorofluorocarbon (CFC) for example dichlorodifluoromethane, trichlorofluoromethane, or dichlorotetrafluoroethane, carbon dioxide, or other suitable gas. The aerosol may conveniently also contain a surfactant

such as lecithin.

[0093] Solutions or suspensions may be administered to the skin or to a membrane by conventional means, for example with a dropper, pipette or spray. The formulations may be provided in single or multidose form. In the latter case of a dropper or pipette, this may be achieved by the patient administering an appropriate, predetermined volume of the solution or suspension.

[0094] Emulsions may be oil-in-water or water-in-oil emulsions.

[0095] Ointments and creams may, for example, be formulated with an aqueous or oily base with the addition of suitable thickening and/or gelling agents.

[0096] Lotions may be formulated with an aqueous or oily base and will in general also contain one or more emulsifying agents, stabilising agents, dispersing agents, suspending agents, thickening agents, or colouring agents.

[0097] Alternatively a pharmaceutical composition for topical application may be provided in the form of a dry powder, for example a powder mix of the polymer of the first or second aspect of the present invention in a suitable powder base such as lactose, starch, starch derivatives such as hydroxypropylmethyl cellulose and polyvinylpyrrolidone (PVP).

[0098] The polymer of the first or second aspect of the present invention, optionally together with an antimicrobial agent, may be impregnated in or incorporated in a bandage or a portion thereof. Bandages include all forms of bandage, such as cloth, pressure, spray-on and adhesive bandages. In one embodiment, a coating comprising the polymer of the first or second aspect of the present invention is applied to the bandage or a portion thereof.

Examples

[0099] The invention will be further described by way of the following non-limiting example. It will be understood to persons skilled in the art of the invention that many modifications may be made without departing from the spirit and scope of the invention.

[0100] Example 1

[0101] In Example 1, a NONOate conjugated star polymer that is able to maintain a slow release of NO in the presence of the model organism *Pseudomonas aeruginosa* and completely inhibit bacterial attachment and biofilm formation over time in a non-growth-inhibitory fashion is

described. The star polymer confines growth of the bacterial population to the suspended liquid, while keeping the surface free of biofilm.

[0102] **Materials**

[0103] The monomer 2-vinyl-4,4-dimethyl-5-oxazolone was synthesized according to the method reported in Levere, M.E., et al., *Stable azlactone-functionalized nanoparticles prepared from thermoresponsive copolymers synthesized by RAFT polymerization*. Polymer Chemistry, 2011. 2(12): p. 2878-2887. 2-(((Butylsulfanyl)carbonothioyl)sulfanyl)propanoic acid was synthesized according to the method reported in Ferguson et al., *Ab initio emulsion polymerization by RAFT-controlled self-assembly*. Macromolecules, 2005. 38(6): p. 2191-2204. Oligo(ethylene glycol) methyl ether acrylate ($M_n = 480 \text{ g mol}^{-1}$) was used as received. Dialysis membranes with a molecular weight cut-off of 3500 Da were sourced from Fisher Biotec (Cellu Sep-T4, regenerated cellulose-Tubular membrane). Deuterated solvents, CDCl_3 - d and $\text{DMSO}-d_6$ were obtained from Cambridge Isotope Laboratories, Inc. High purity N_2 (Linde gases) was used for degassing. Ultrapure deionized water ($17.8 \text{ m}\Omega \text{ cm}$) was obtained using a MilliQ purification system. All other chemicals were purchased from Sigma-Aldrich. 2,2-azobisisobutyronitrile (162 g mol^{-1} , AIBN) which was purchased from Sigma-Aldrich was recrystallised from methanol before use. For the measurement of nitric oxide release, a Nitrate/Nitrite Colorimetric Assay Kit was purchased from Cayman Chemicals. A LIVE/DEAD BacLight Bacterial Viability Kit for was purchased from Life Technologies for fluorescence microscopy of treated biofilms.

[0104] **Characterization methods**

[0105] NMR Spectroscopy. ^1H NMR spectra were recorded using a Bruker Avance 300 (300 MHz) spectrometer. d_3 -Acetonitrile and d_6 -DMSO were used as solvents. All chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS), referenced to residual the residual solvent frequencies ^1H NMR: d_3 -Acetonitrile = 1.94, d_6 -DMSO = 2.50 and D_2O = 4.79 ppm. The monomer (i.e. OEGA) conversion was calculated according to equation 1 to give ~90% conversion, where $I^{5.9 \text{ ppm}}$ and $I^{4.1 \text{ ppm}}$ correspond to the integral of the vinyl signal from the monomer at 5.9 ppm and the ester signal from the monomer/polymer at 4.1 ppm, respectively.

$$\alpha (\text{OEGA Conversion, \%}) = \left[1 - \frac{I^{5.9 \text{ ppm}}}{\frac{I^{4.1 \text{ ppm}}}{2}} \right] \times 100\% \quad \text{Equation 1}$$

[0106] The theoretical molecular weight was calculated according to equation 2, which

resulted in 11,400 g/mol:

$$M_n(\text{Theoretical}) = \left(\left(\frac{[M]_0}{[CTA]_0} \right) \times \alpha \times MW_M \right) + MW_{CTA} \quad \text{Equation 2}$$

wherein:

$[M]_0$: Initial monomer concentration

$[CTA]_0$: Initial RAFT agent concentration

α : Conversion of monomer

MW_M : Molecular weight of monomer

MW_{CTA} : Molecular weight of the RAFT agent

[0107] Size Exclusion Chromatography (SEC). Size exclusion chromatography or Gel Permeation Chromatography (GPC) was performed using a Shimadzu modular system comprised of a DGU-12A degasser, a LC-10AT pump, a SIL-10AD automatic injector, a CTO-10A column oven, a RID-10A refractive index detector, and a SPD-10A Shimadzu UV/vis detector. A 50 x 7.8 mm guard column and four 300 x 7.8 mm linear columns (500, 10^3 , 10^4 , and 10^5 Å pore size, 5 µm particle size) were used for the analyses. *N,N'*-dimethylacetamide (DMAc, HPLC grade, 0.05% w/v 2,6-dibutyl-4-methylphenol (BHT), 0.03% w/v LiBr) with a flow rate of 1 mL min⁻¹ and a constant temperature of 50 °C was used as the eluent with an injection volume of 50 µL. Prior to injection, the samples were filtered through 0.45 µm filters. The unit was calibrated using commercially available linear poly(methyl methacrylates) standard. Chromatograms were processed using Cirrus 2.0 software (Polymer Laboratories). The arm incorporation was calculated according to equation 3 with A^{arm} and A^{star} represent the area of the chromatogram of P(OEGA) arm and P(OEGA)-*b*-P(VDM)star, respectively.

$$\text{Arm Incorporation (\%)} = \frac{A^{star}}{(A^{arm} + A^{star})} \times 100\% \quad \text{Equation 3}$$

[0108] Infrared Spectroscopy. ATR-FTIR spectra of the star polymer samples were obtained using a Bruker Spectrum BX FTIR system using diffuse reflectance sampling accessories. The spectrophotometer was equipped with a tungsten halogen lamp and Si/Ca beam splitter. Spectra were obtained at regular time intervals in the MIR region of 4000 – 500 cm⁻¹ at a resolution of 4 cm⁻¹ (128 scans) and analysed using OPUS software.

[0109] UV-Vis Spectroscopy. UV-vis measurements were performed on a CARY 300 spectrophotometer (Bruker) using a quartz cuvette.

[0110] Dynamic Light Scattering (DLS). DLS measurements were carried out on a Malvern Zetasizer Nano Series running DTS software (laser, 4 mW, $\lambda = 633$ nm; angle 173°). Polymer samples were dissolved in MilliQ grade water (1 mg/mL) and filtered using $0.45\mu\text{m}$ syringe filter prior to analysis. The samples were transferred to their respective disposable cuvettes for analysis.

[0111] Transmission Electron Microscopy (TEM). The sizes of the star polymers were observed using a JEOL 1400 transmission electron microscope. It was operated at an acceleration voltage of 80 kV. The samples were prepared by casting the polymer solution (1 mg mL^{-1}) onto a Formvar-coated copper grid. No staining was applied.

[0112] Zeta Potential. Zeta potential (ζ) of the star polymer samples in solution (1 mg/mL, water) were measured *via* electrophoretic mobility using Malvern Instruments Zetasizer.

[0113] **Synthesis of core cross-linked star polymers**

[0114] Synthesis of P(OEGA): OEG- A_{480} (10.033 g, 0.021 mol), CTA (0.1909 g, 0.0008 mol) and AIBN (0.0258 g, 1.57×10^{-4} mol) were placed into a round bottom flask equipped with a magnetic stirrer. The reactants were dissolved in Toluene (25 mL) and the reaction mixture was degassed with N_2 under ice (0°C) for 30 min. The reaction mixture was then placed into an oil bath preheated to 70°C and the polymerisation was run for 4 h. Upon completion, the reaction was quenched in an ice bath for 15 min. The polymer was purified via 3 repetitions of precipitating with petroleum ether and centrifuging (5 min, 8000 rpm). The purified polymer (sometimes referred to below and in the figures as “P(OEGA) arm”) was then placed into a vacuum oven overnight to remove remaining solvent.

[0115] Synthesis of star polymer: P(OEG-A) $_{Mn=12,000\text{g/mol}}$ (1.212 g, 1×10^{-4} mol), 2-vinyl-4,4-dimethylazlactone (223mg, 1.6×10^{-3} mol), *N,N*-methylenebisacrylamide (125 mg, 8×10^{-4} mol) and AIBN (5 mg, 3×10^{-5} mol) was dissolved in Toluene (6 mL) and was transferred into a Cospak bottle equipped with a magnetic stirrer. The reaction mixture was degassed for 30 min at 0°C with N_2 . The above composition resulted in a [macroCTA]:[M]:[X-linker]:[I] of 1:16:8:0.3. The reaction was also repeated at a composition of 1:16:8:0.3. The degassed and sealed reaction vessels were placed into an oil bath preheated to 70°C and the polymerisation was run for 24h. The reaction was then quenched in an ice bath for 15 min. The purification involved precipitation in diethyl ether to remove unreacted macroCTA arms and VDM monomer. The precipitation was repeated 3 times then the reaction mixture was dissolved and dialysed against methanol for 48 h to removed unreacted cross-linker. The purified star polymers (referred to in

the figures as “P(OEGA)-*b*-P(VDM) star” or “VDM star”) were then placed in a vacuum oven overnight to remove remaining solvent.

[0116] Synthesis of spermine star polymers: 100 mg star polymer was dissolved in acetonitrile (5mL). Separate from the star polymer, spermine (5 mg, 2.47×10^{-5} mol) was dissolved in 500 μ L methanol and then the 2 solutions were transferred into a Cospak bottle. The reaction mixture was then placed into an incubator (25 °C, 80 rpm) for 24 h. After 24 h, a 100 μ L aliquot was dried and checked via FTIR to determine conversion of the VDM functional groups. The reaction mixture was then returned to the incubator to achieve full conversion of the VDM functional groups. The fully reacted star polymers (referred to in the figures as “P(OEGA)-*b*-P(Sper)star” or “sper star”) were purified by dialysis against water for 48 h, with the water being changed twice per day. The dialysed polymer was then freeze dried overnight.

[0117] Synthesis of NO star polymers: A solution of the desired polymer sample in acetonitrile and methanol (1:1) mixture was placed in a Parr apparatus and clamped. The apparatus was purged and evacuated with nitrogen three times, followed by charging with excess nitric oxide gas (25 °C, 5 atm) for 48 h. After 48 h, the excess NO was vented by purging with nitrogen and the solvent removed via evaporation under nitrogen to yield the desired polymer-diazeniumdiolate sample (referred to in the figures as “P(OEGA)-*b*-P(Sper/NO)star” or “NO star”).

[0118] **Nitric oxide release and biofilm inhibition study**

[0119] Determination of released nitric oxide (NO): Griess Assay. NO release was determined using the two-step process of the Cayman Chemical Nitrate/Nitrite Assay Kit. Diazeniumdiolates readily release NO upon contact with water at physiological pH. Typically, 20 mg NO-containing polymer was dissolved in 240 μ L of sterile MilliQ water. To this, 50 μ L each of nitrate reductase and cofactor were added and the solution was left to incubate on the bench top for 3 h. Following the incubation period wherein the nitrates were converted into the assayable nitrite, 150 μ L of each Griess reagent (A & B) were added and the solution left to incubate for a further 30 min. Nitrite concentrations were then measured using an UV-vis spectrometer; the absorbance at 548 nm was used to calculate the NO content from the assay's calibration curve.

[0120] NO Reporter Bioassays. Overnight cultures of *P. aeruginosa nirS::gfp* reporter strain (NSGFP) were diluted to an OD₆₀₀ of 0.2 in fresh LB medium and grown with shaking at 37 °C to an OD₆₀₀ of 0.4. Treatments were added to 3 mL aliquots of the cultures in 15 mL Falcon

tubes (BD), in duplicate, and the bacteria were incubated further for up to 3 h. After compound exposure cells were washed once in phosphate-buffered saline (PBS) and resuspended in 0.5 mL PBS. Two hundred microliter-aliquots were transferred to a microtiter plate for fluorescence measurements (excitation, 485 nm; emission, 535 nm; Wallac Victor², Perkin-Elmer).

[0121] Biofilm Prevention Assays. The laboratory strain *P. aeruginosa* PAO1 was mainly used to characterise the effects of NO star polymers on biofilm formation. PAO1 mutant strains containing a transposon Tn5-derived insertion element in key genes in mediating dispersal in response to NO, *dipA* (PA5017) and *rbdA* (PA0861), were obtained from the University of Washington *P. aeruginosa* mutant two-allele library: strains PW9424 *dipA*-A01::IS*phoA*/hah and PW2569 *rbdA*-F02::IS*lacZ*/hah, respectively. Biofilms were grown as previously described in Yepuri, N.R., et al., *Synthesis of cephalosporin-3'-diazoniumdiolates: biofilm dispersing NO-donor prodrugs activated by β -lactamase*. Chem. Commun., 2013. 49(42): p. 4791-4793 with some modifications. Briefly in all assays, overnight cultures in Luria Bertani medium were diluted to an OD₆₀₀ of 0.005 in 1 mL M9 minimal medium (containing 48 mM Na₂HPO₄, 22 mM KH₂PO₄, 9 mM NaCl, 19 mM NH₄Cl, 2 mM MgSO₄, 20 mM glucose, 100 μ M CaCl₂, pH 7.0) or Mueller Hinton broth (Oxoid; containing 30% beef extracts, 1.75% casein hydrolysate and 0.15% starch) in tissue-culture treated 24-well plates (BD). Prior to incubation, the bacterial medium was inoculated with NO-releasing compounds at final concentrations of 57 to 400 ppm, as indicated, whilst control wells were left untreated. Treatments were added to the wells, each from a 10 μ L aliquot of a stock solution at the appropriate concentration previously sterilized by passing through a 0.22 μ m pore size filter: NO star polymer, spermine star polymer or star polymer, all dissolved in 10 mM NaOH. Commercial NO donors sodium nitroprusside (Sigma) freshly dissolved in deionized water, and spermine NONOate or DETA NONOate (Cayman chemicals) in 10 mM NaOH were also used. The plates were incubated at 37 °C with shaking at 180 rpm and the biofilms were allowed to grow for up to 7.5 h. After incubation, the planktonic biomass was quantified by removing the supernatant and measuring its OD₆₀₀. The remaining biofilm was washed once with PBS (1 mL), before adding 0.03% crystal violet stain made from a 1:10 dilution of Gram Crystal Violet (BD) in PBS. The plates were incubated on the bench for 20 min before washing the wells twice with PBS. Photographs of the stained biofilms were obtained using a digital camera. The amount of remaining crystal violet stained biofilm was quantified by adding 1 mL 100% ethanol and measuring OD₅₅₀ of the homogenized suspension. OD measurements of control wells where no bacteria were added at the beginning of the experiment were subtracted from all values (i.e. OD₆₀₀ = 0.03, and OD₅₅₀ = 0.10).

[0122] Confocal Microscopy. For microscopy analysis, *P. aeruginosa* PAO1 biofilms were

grown in glass-bottom 24-well plates (MatTek Corporation, Ashland MA, USA) with or without NO star polymer from the beginning of growth as described above. After 6 h incubation, biofilms were rinsed twice with PBS before being stained with LIVE/DEAD *BacLight* bacterial viability kit reagents (Molecular Probes) according to the manufacturer's procedure. One microliter of each of the two components were mixed thoroughly in 1 ml of PBS, then 0.5 ml of this solution was trapped between the sample and the glass microscopy slide and allowed to incubate at room temperature in the dark for 20 min. The samples were observed with an Olympus FV1000 Confocal Inverted Microscope, and imaged with Leica DFC 480 camera. For bacterial adhesion, images from 15 representative areas on each of triplicate samples for each surface were taken. Cells that were stained green were considered to be viable, those that stained red were considered to be dead as were those that stained both green and red.

[0123] **Results and discussion**

[0124] Core cross-linked star polymers were synthesized using an 'arm-first' approach. Reversible addition fragmentation transfer (RAFT) polymerization was employed to synthesize P(OEGA) arms using chain transfer agent (Figure 1, compound **1**; RAFT **1**, *n*-butyltrithiocarbonate isopropionate) and AIBN (2,2'-azobisisobutyronitrile) as radical initiator with the ratio of OEGA:RAFT **1**:AIBN = 25:1:0.1 in toluene at 70°C.

[0125] After 4 h of reaction, ~90% OEGA conversion was achieved and the resultant polymer arm was purified through dialysis. After purification, P(OEGA) was then characterized using SEC and ¹H-NMR. The molecular weight distribution based on SEC showed a $M_{n, SEC}$ of 14,500 g/mol with a polydispersity index of 1.11 (**Figure 3**), which was slightly higher than the theoretical molecular weight by ¹H-NMR ($M_{n, NMR}$ of 11,500 g/mol). This slight difference is due to the difference of hydrodynamic volume in the SEC between the P(MMA) calibration and P(OEGA) polymer. Chain extension from P(OEGA) arm was carried out in the presence of 2-vinyl-4,4-dimethyl-5-oxazolone monomer (VDM) and a cross-linker, methylenebisacrylamide. The molar ratio between the P(OEGA) arm, VDM and the cross-linker was set to 1:16:8 followed by the addition of AIBN to this mixture in toluene. After the reaction at 70°C, the reaction mixture was purified by precipitation in diethyl ether to remove the unreacted P(OEGA) arm. The result from SEC showed a shift in the molecular weight giving $M_{n, SEC}$ of 177,000 g/mol with a polydispersity index of 1.39. The ratio between the area of the chromatogram of star polymer and its precursor, P(OEGA) arm was calculated to provide an arm incorporation of ~71%. The incorporation of VDM monomer to the P(OEGA) arm was confirmed by ¹H-NMR via a signal at 1.3 ppm from the VDM and the ester group from OEGA at 4.1 ppm (**Figure 2**). In addition, ATR-FTIR has been employed to confirm the presence of oxazolone group at 1820 cm⁻¹

(**Figure 4A**). The peak at 1630 cm^{-1} , 1720 cm^{-1} and 1100 cm^{-1} represents the cross-linking amide, OEGA ester and ether groups, respectively, confirming the incorporation of arm to the star polymer. When spermine was reacted with VDM star polymer followed by purification by dialysis, the formation of star conjugated with spermine was confirmed by the disappearance of the characteristic azalactone peak at 1820 cm^{-1} in the FTIR spectra, indicating the oxazolone ring opening *via* amidation. A broad peak at around 3500 cm^{-1} was observed characteristic to the secondary amines from the conjugated spermine. The formation of the core cross-linked star polymer was confirmed by the DLS showing the number-weighted particle size of $\sim 25\text{ nm}$ (**Figure 5**), which was corroborated by the TEM results. Zeta potential measurement of initial star polymer in water revealed a neutral surface charge due to the P(OEGA) hydrophilic layers (**Figure 6**). After conjugation of spermine, the zeta potential of star polymer shifted to $+24\text{ mV}$ indicative of the presence of quaternary amine (NH_3^+ or NH_2^+). The effect of spermine conjugation was observed in the UV-Vis spectra through the disappearance of a peak at around 310 nm (**Figure 4B**), which is attributed to aminolysis of the trithiocarbonate RAFT group. In order to conjugate nitric oxide to the nanoparticles, spermine conjugated star polymer was dissolved in acetonitrile and the solution was transferred to a Parr hydrogenation apparatus, purged with nitrogen and then stirred with NO gas for 48 h at 80 psi (5 bar) (**Figure 1**). NO was reacted to the secondary amine of spermine to yield *N*-diazoniumdiolate (or NONOate) moieties, which was stabilized inside the core of the polymeric nanoparticles. This was also confirmed by the decreasing zeta-potential of the resultant NO star polymer ($+10\text{ mV}$) due to the formation of the *N*-diazoniumdiolate. When the NO star polymer was dispersed in water, NO gas was released, in particularly the release will be accelerated at the pH lower than 7.0. This was characterized by the presence of characteristic signal at around 240 nm observed by UV-Vis spectroscopy attributed to NO in water (**Figure 4B**).

[0126] Qualitative and quantitative analysis of the released NO from star polymer was performed using Griess assay. Briefly, NO star polymer dissolved in deionized water was incubated with nitrate reductase and its cofactor to detect NO-derived nitrate and nitrite. Following the reaction protocol, the sample was incubated for 3 h that allowed a maximum release of NO and its conversion to nitrite in the aqueous medium. Subsequent addition of a Griess reagent to the nitrite sample formed a diazenium salt that was converted instantaneously to an azo dye (**Figure 4B**). This was characterized by the presence of pink colour and UV-Vis absorption at 548 nm . Based on a calibration curve (**Figure 7**), the concentration of NO released from NO star polymer was quantified as $192\text{ }\mu\text{M}$. Using the concentration of NO in the nanoparticle or star polymer the biological activity of this sample was tested on its inhibition of biofilm formation. Spermine conjugated star polymer sample was used as a negative control.

First the ability of NO release from the polymers to induce a response in bacteria was assessed by using an engineered bacterial reporter strain that fluoresces in the presence of low doses of NO. A transcriptional fusion reporter strain, NSGFP, which co-expresses downstream green fluorescent protein (GFP) when the NO-responsive anaerobic gene *nirS* is expressed was exposed to NO star polymer at 100-400 ppm (based on NO concentration) and the spontaneous NO donor sodium nitroprusside (SNP) at 200 μ M. Exposure to NO star polymer was able to induce a GFP-response and the response gradually increased over a 3 h time period (**Figure 8**). In contrast, the spontaneous SNP induced a rapid increase in GFP, which did not increase further after 2 h exposure. These results suggest that NO star polymer is able to induce a sustained release of NO in the presence of bacteria. When using a NO specific electrode (Apollo, World Precision Instrument), no release of NO from 400 ppm NO star polymer in a buffered aqueous solution could be observed, suggesting that the release is too slow to accumulate detectable NO (limit $\sim$ 10 nM) (data not shown). The data presented in **Figure 2** suggest NO star polymer induces a slow release of NO available to bacteria. Cultures of the NO reporter mutant train, NSGFP, which express GFP under control of the NO responsive *nirS* promoter, were exposed to NO star polymer, spontaneous NO donor SNP or negative control spermine star polymers for up to 3 h before fluorescence measurement.

[0127] The effect of NO star polymer on biofilm formation was assessed. *P. aeruginosa* biofilms grown in minimal M9 medium in the presence of 100 ppm and 400 ppm NO star were strongly inhibited over the incubation period, with respectively a 90% and 95% reduction in biofilm biomass after 7.5 h compared to untreated biofilms. Concomitantly, the number of planktonic cells increased in culture wells treated with 100 ppm NO star, resulting in 32% more suspended biomass after 7.5 h compared to untreated wells (**Figure 9**), a result that is consistent with a non-toxic effect of NO star polymer inducing a switch from the biofilm to the planktonic mode of growth over sustained periods of time. At 400 ppm, NO star polymer induced a slight decrease in planktonic growth by 20% compared to untreated wells, suggesting that at this concentration of NO star and under these growth conditions, NO may have been released at levels showing some toxicity. The spontaneous NO donor SNP was also able to reduce biofilm formation in this system, although to a lesser extent compared to NO star polymer, by only 35-50% vs. untreated controls, while at the same time increasing planktonic growth (**Figure 9**).

[0128] *P. aeruginosa* biofilms were grown in multiwell plates in M9 for 6 h in the presence or absence of 57-200 ppm (based on NO concentration) NO star polymer, or negative controls spermine star and VDM star polymers, or spermine before analysing biofilms by crystal violet

staining. Treatment with fast NO releasing NONOate donor spermine-NONOate (sper-NO) did not prevent biofilm formation over this incubation time. These results are presented in **Figure 10A**.

[0129] When testing a range of NO star polymer concentrations, the star polymer was found to be active even at low concentrations, with 57 ppm inducing a 73% decrease in biofilm formation compared to untreated controls after 6 h, concomitant with a 55% increase in planktonic growth (**Figure 10**). In contrast, spermine conjugated stars (used as a control) were unable to prevent formation of *P. aeruginosa* biofilms. A fast NO-releasing NONOate donor, (Z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino]diazen-1-ium-1,2-diolate (spermine NONOate; $t_{1/2} = 40$ min at pH 7.4, 37 °C) used at 10 μ M a concentration that store similar amounts of NO compared to NO star polymer 100 ppm as detected by Griess assay, was also unable to inhibit biofilm formation. Other NONOate donors with more stable NO release in buffered system compared to spermine NONOate, such as (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA NONOate; $t_{1/2} = 20$ h at pH 7.4, 37 °C) or (Z)-1-[N-(3-aminopropyl)-N-(3-ammoniopropyl)amino]diazen-1-ium-1,2-diolate (DPTA NONOate; $t_{1/2} = 3$ h at pH 7.4, 37 °C) were also tested. At concentrations that were non-toxic to bacterial growth none of these compounds showed any sustained biofilm prevention effect (data not shown). NO star was also tested on biofilms grown in complex medium Mueller Hinton broth, which showed a strong inhibition effect reducing biofilm by 80% at 400 ppm with no reduction in planktonic growth (**Figure 11**). Under these growth conditions NO donor SNP was unable to prevent biofilm formation over a 6 h incubation period.

[0130] To better understand the effects of NO star polymer on biofilms, it was tested against biofilms of mutant strains impaired in key components of the NO dispersal pathway, namely the phosphodiesterases *dipA* and *rbdA* that mediate decrease in intracellular levels of 2nd messenger c-di-GMP in response to NO. *dipA* mutant biofilms were not affected, while *rbdA* mutant biofilms showed only 50% reduction with 400 ppm NO star polymer compared to 99% reduction obtained with the wild type (**Figure 12**). These results strongly suggest that NO star polymer inhibits the biofilm switch in planktonic cells in contact with a surface by inducing a cellular response that continuously stimulates phosphodiesterase activity and maintains low intracellular levels of c-di-GMP in the growing bacterial population, thus confining growth to an unattached free-swimming mode.

[0131] Confocal microscopy was used to evaluate the ability of the NO star polymers to prevent colonisation and biofilm dispersal of *P. aeruginosa*. The areas of the surfaces covered

by bacteria and the relative proportions of live and dead bacteria (stained green and red, respectively) for each surface were evaluated by image analysis and representative images are shown in **Figure 13**. After treatment with NO star polymer at 400 ppm at 2 hour intervals over a 6 hour period, there is a significant reduction in biofilm biovolume and increased biofilm dispersal compared to the untreated control.

[0132] In summary, the polymer of example 1 can release NO sustainably in the presence of bacteria with demonstrated efficacy in preventing formation of *P. aeruginosa* biofilms via a non-toxic mechanism over time. The present compound appears far superior compared to existing NO donors for the long term prevention of biofilms and is likely to be useful for combating biofilm infections in clinical settings.

[0133] It is to be understood that, if any prior art publication is referred to herein, such reference does not constitute an admission that the publication forms a part of the common general knowledge in the art, in Australia or any other country.

[0134] In the claims which follow and in the preceding description of the invention, except where the context requires otherwise due to express language or necessary implication, the word “comprise” or variations such as “comprises” or “comprising” is used in an inclusive sense, i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention.

Claims

1. A polymer comprising a plurality of polymeric arms extending from a core, wherein on contact with an aqueous medium the polymer releases nitric oxide (NO) for an extended period of time.
2. The polymer of claim 1, wherein the core comprises a nitric oxide-donor (NO-donor) selected from S-nitroso, O-nitroso, C-nitroso and N-nitroso group and nitro derivatives thereof.
3. The polymer of claim 1 or 2, wherein the core comprises a nitric oxide-donor (NO-donor) that is a NONOate.
4. The polymer of claim 3, wherein the NONOate is a NONOate derived from a secondary amine.
5. The polymer of any one of claims 1 to 4, wherein each of the plurality of polymeric arms has a molecular weight of about 2000 g/mol to about 250000 g/mol.
6. The polymer of any one of claims 1 to 5, wherein each of the plurality of polymeric arms comprises polymerised ethylene glycol monomethyl ether.
7. The polymer of any one of claims 1 to 6, wherein the polymer releases nitric oxide (NO) in an amount effective to prevent or inhibit biofilm formation or to promote biofilm dispersal.
8. The polymer of any one of claims 1 to 7, wherein nitric oxide (NO) is released at a rate of about 1 nmol(NO)/g(polymer)/h to about 1 mmol(NO)/g(polymer)/h.
9. The polymer of any one of claims 1 to 8, wherein the extended period of time is about 12 hours to about 30 days.
10. A polymer comprising a plurality of polymeric arms extending from a core, wherein the core comprises an NO-donor.
11. The polymer of claim 10, wherein the NO-donor is selected from S-nitroso, O-nitroso, C-nitroso and N-nitroso group and nitro derivatives thereof.
12. A polymer comprising a plurality of polymeric arms extending from a core, wherein

the core comprises an NO-donor precursor.

13. The polymer of claim 12, wherein the NO-donor precursor is a moiety comprising a secondary amine.

14. A method of preparing the polymer of claim 1, comprising exposing the polymer of claim 12 or 13 to an atmosphere of nitric oxide (NO).

15. A polymer comprising a plurality of polymeric arms extending from a core, wherein the core comprises a moiety capable of binding to a compound comprising an NO-donor or NO-donor precursor.

16. The polymer of claim 15, wherein the compound comprising an NO-donor precursor is a polyamine comprising a secondary amine.

17. A method of preparing a polymer of any one of claims 1 to 13, 15 or 16, comprising providing polymer chains comprising an arm portion and a core portion, wherein the core portion of the polymer chains are capable of undergoing a cross-linking reaction, and linking the core portion of the polymer chains by a cross-linking reaction to form a polymer comprising a plurality of polymeric arms extending from a core, wherein the core of the polymer comprises:

- a) an NO-donor; or
- b) an NO-donor precursor; or
- c) a moiety capable of binding to a compound comprising an NO-donor or NO-donor precursor.

18. The method according to claim 17, wherein the core portion of the polymer chain comprises an NO-donor, an NO-donor precursor or a moiety capable of binding to a compound comprising an NO-donor or NO-donor precursor.

19. The method according to claim 17, wherein the cross-linking reaction is carried out in the presence of a compound comprising:

- a) an NO-donor; or
- b) an NO-donor precursor; or
- c) a moiety capable of binding to a compound comprising an NO-donor or NO-donor precursor.

20. The method of any one of claims 17 to 19, wherein the compound capable of binding to a compound comprising an NO-donor or NO-donor precursor is a monomer comprising a 4,4-dimethyl-5-oxalone moiety.
21. The method of any one of claims 17 to 20, wherein the cross-linking reaction involves a cross-linking reaction with methylenebisacrylamide.
22. A method for promoting dispersal of, or preventing or inhibiting formation of, a biofilm, the method comprising exposing the biofilm, or a microorganism capable of forming the biofilm, to an effective amount of the polymer of any one of claims 1 to 11.
23. A method of promoting dispersal of, or preventing or inhibiting formation of, a biofilm on a surface, the method comprising applying to the surface a polymer of any one of claims 1 to 11.
24. A method of treatment and/or prevention of a condition associated with biofilm development, the method comprising administering to a subject in need thereof an effective amount of the polymer of any one of claims 1 to 11.
25. A composition comprising the polymer of any one of claims 1 to 11 and a carrier.
26. A composition according to claim 25 further comprising an antimicrobial agent.

Figure 1

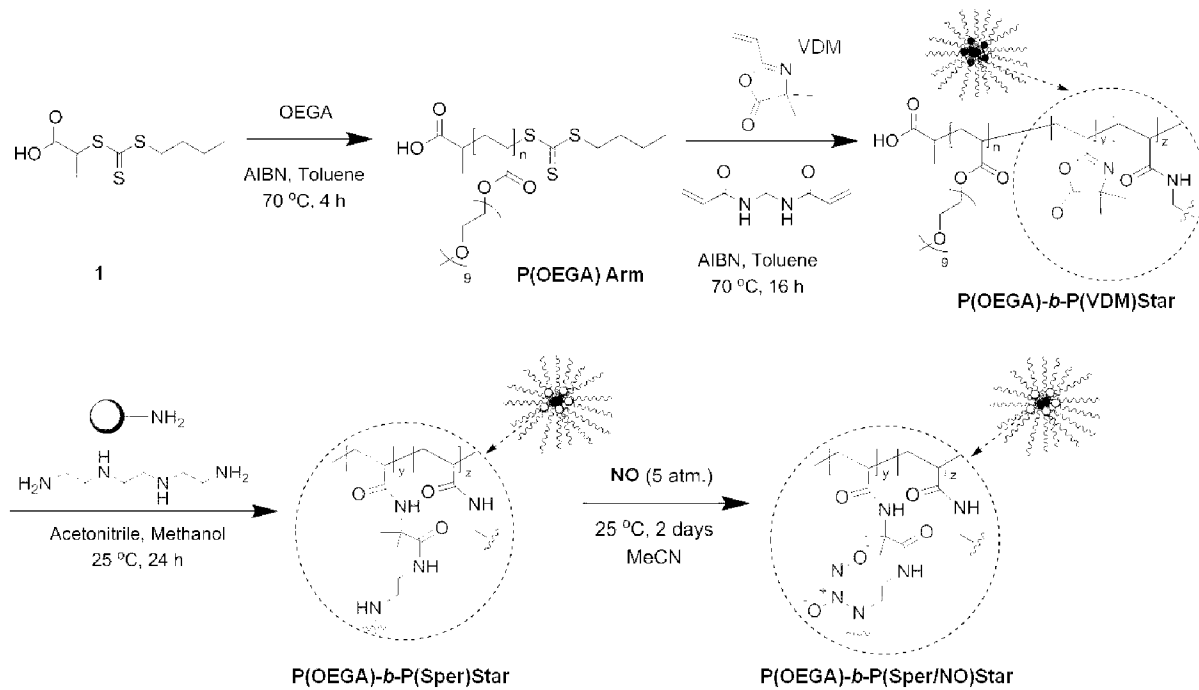
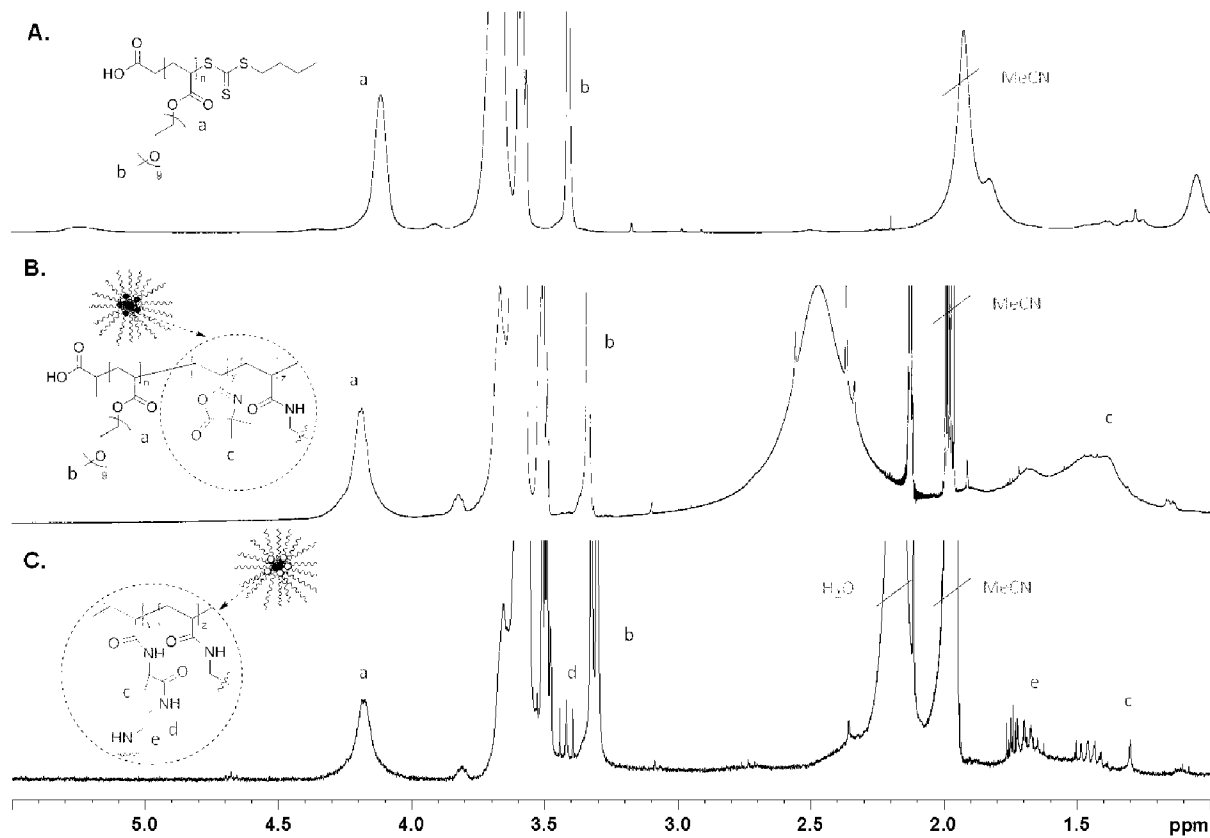


Figure 2



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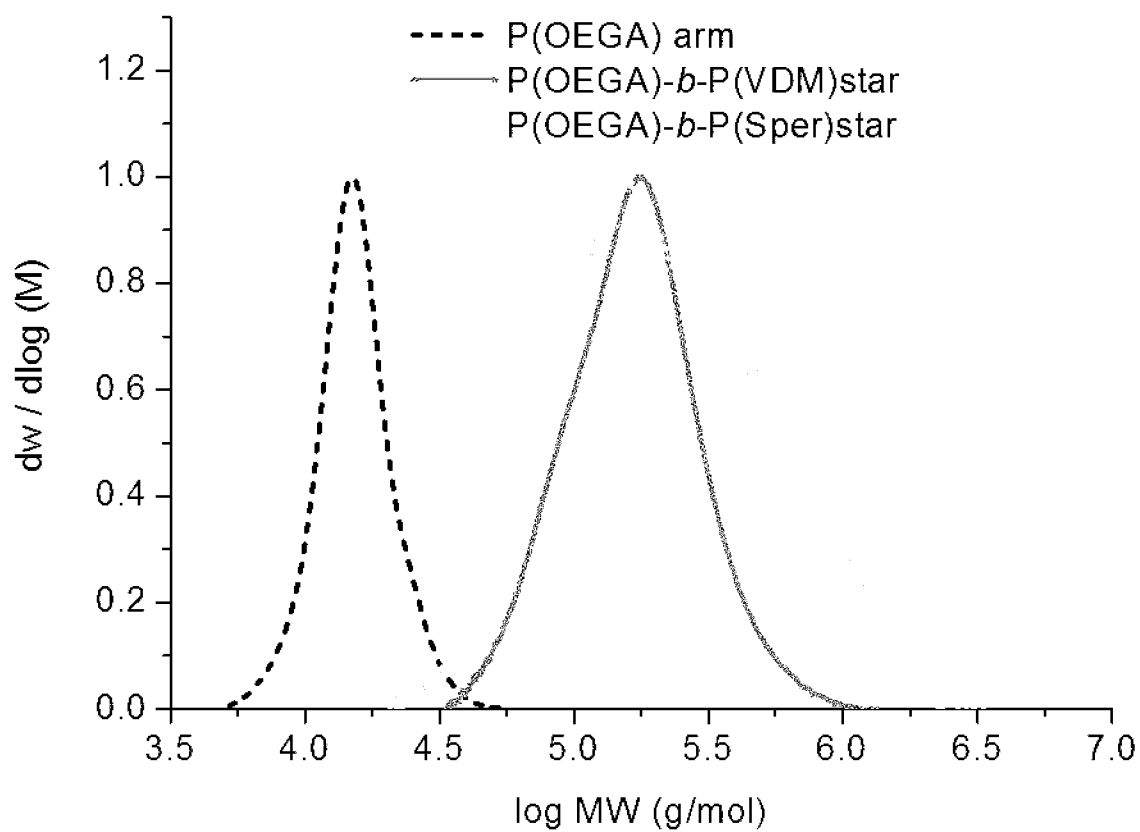
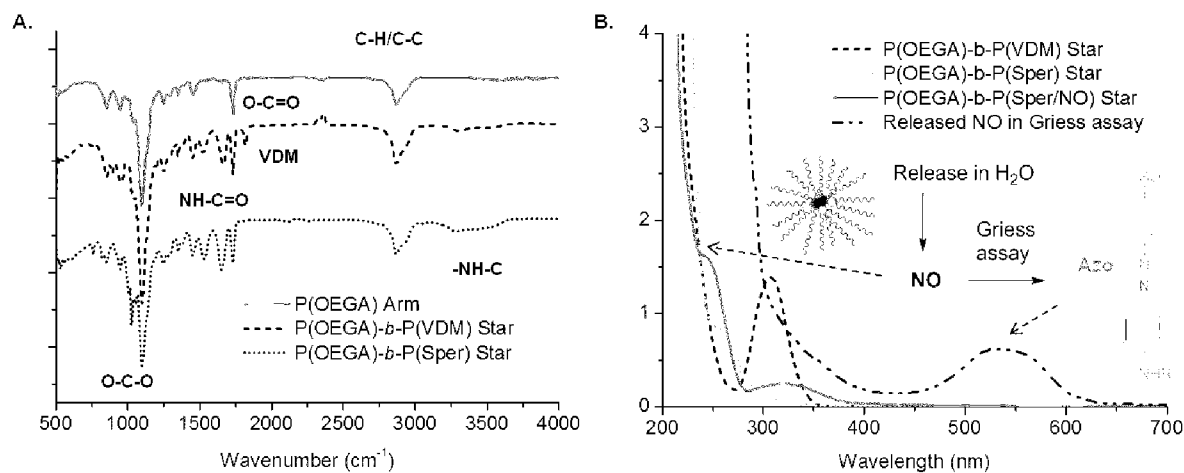
Figure 3**Figure 4**

Figure 5

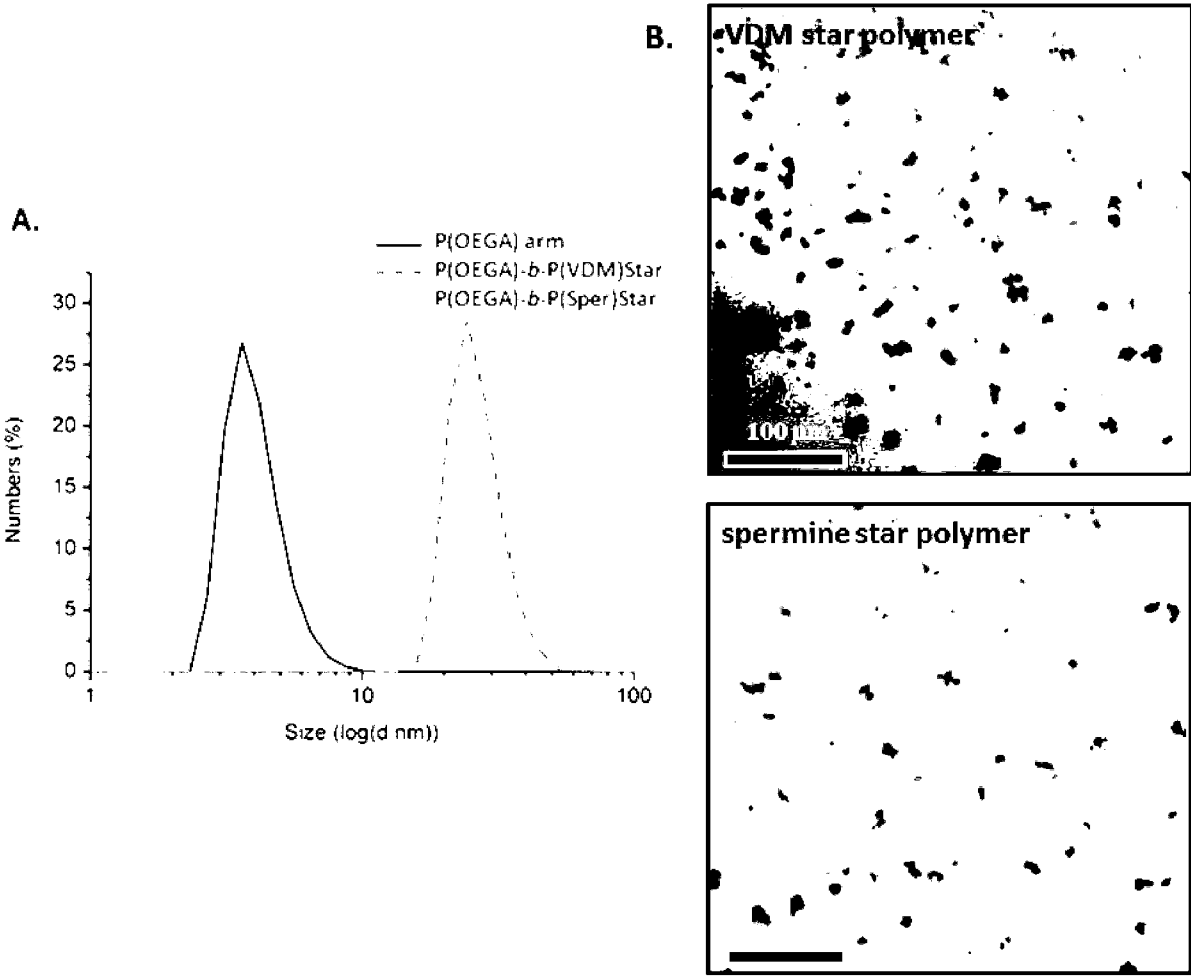


Figure 6

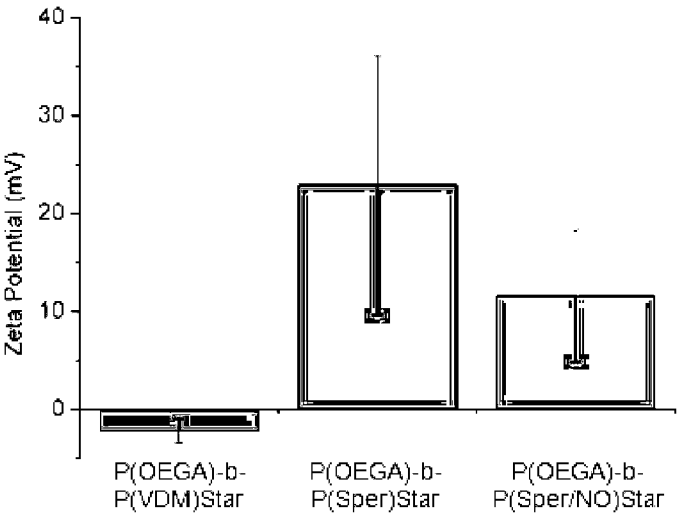


Figure 7

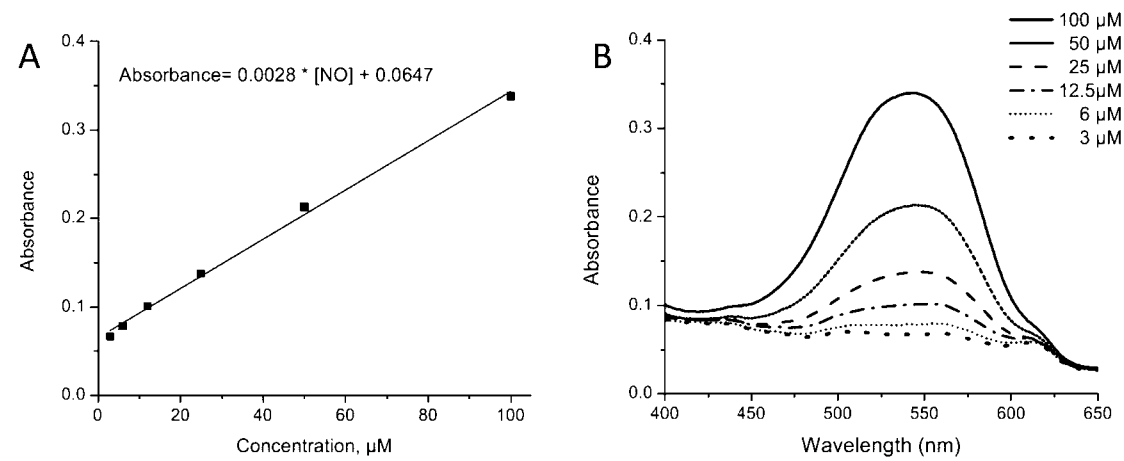


Figure 8

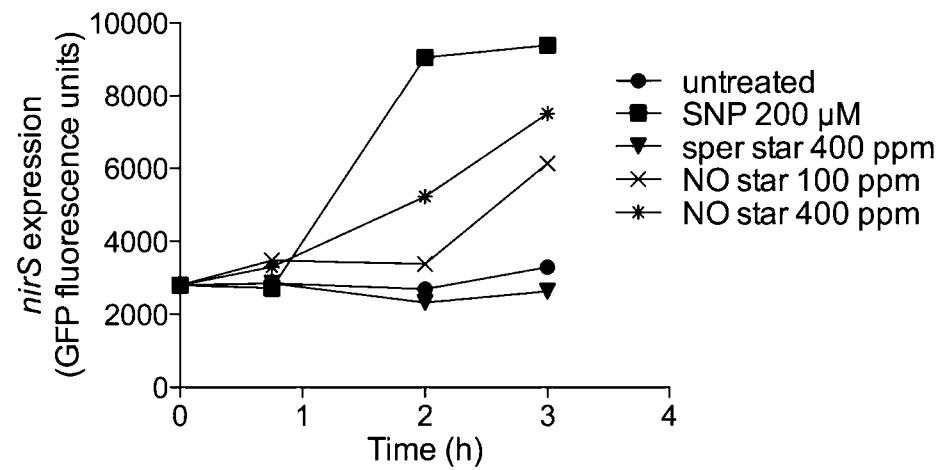


Figure 9

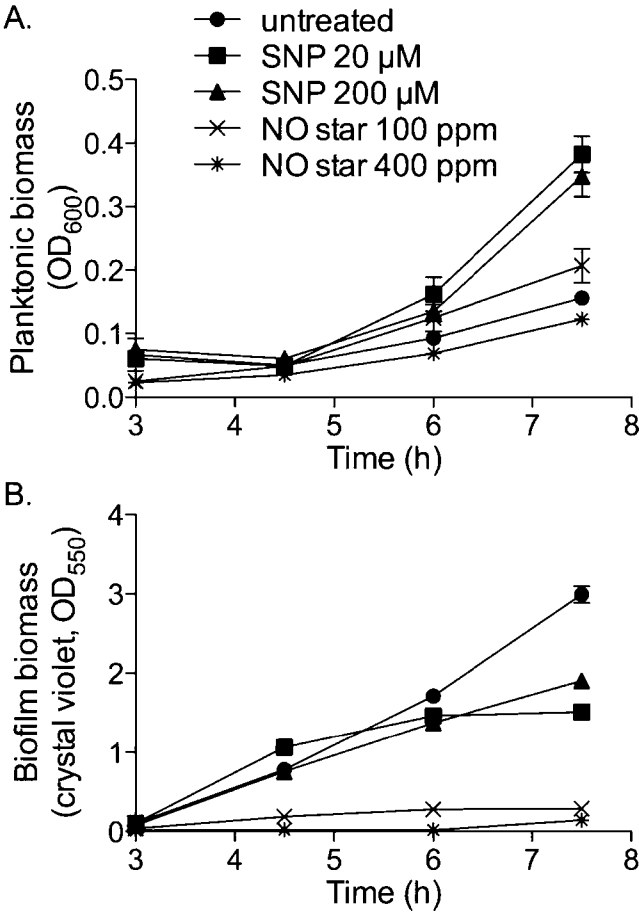


Figure 10

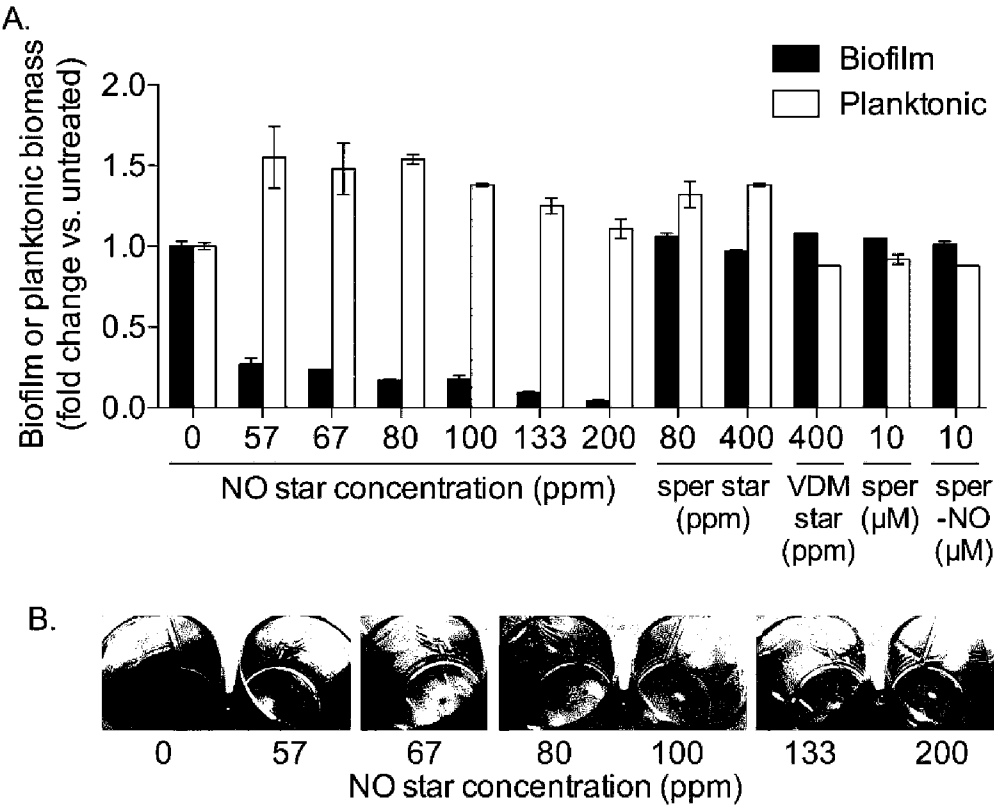


Figure 11

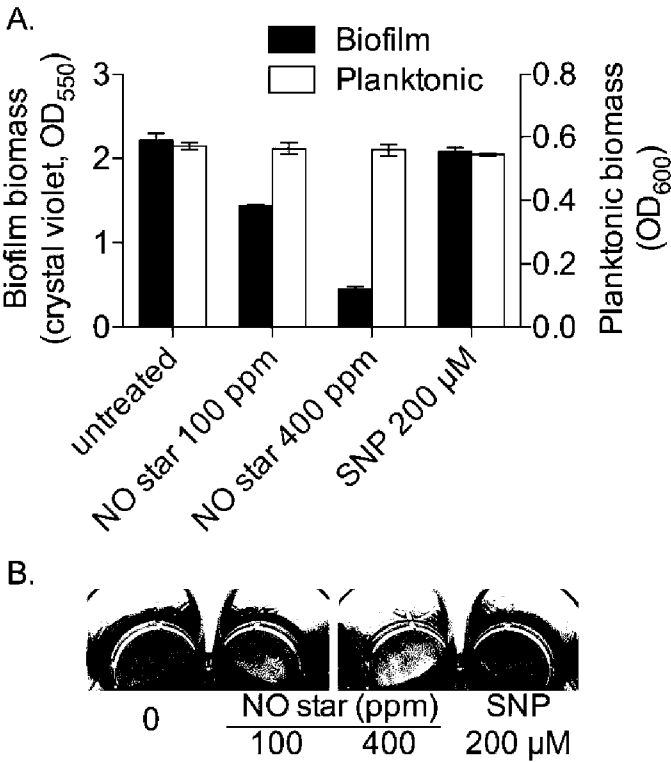


Figure 12

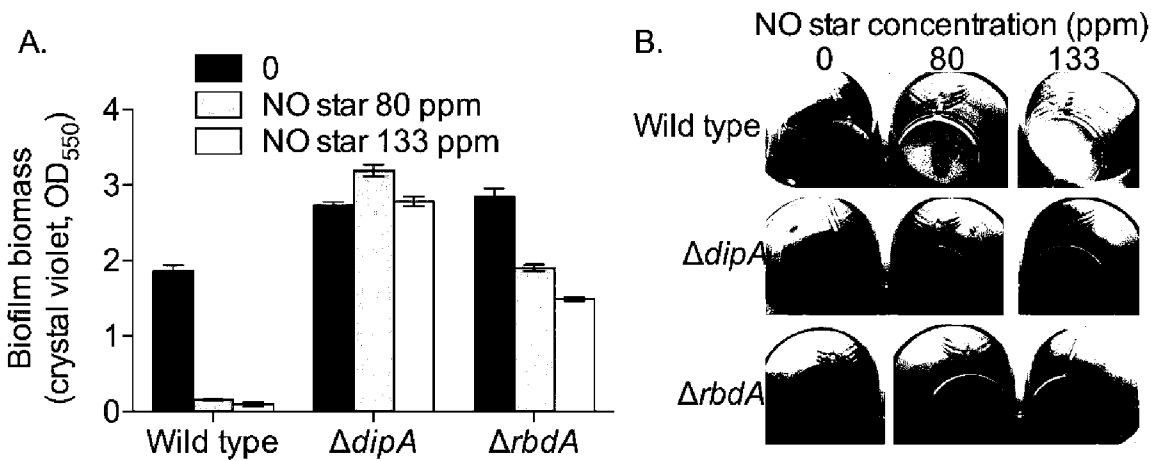
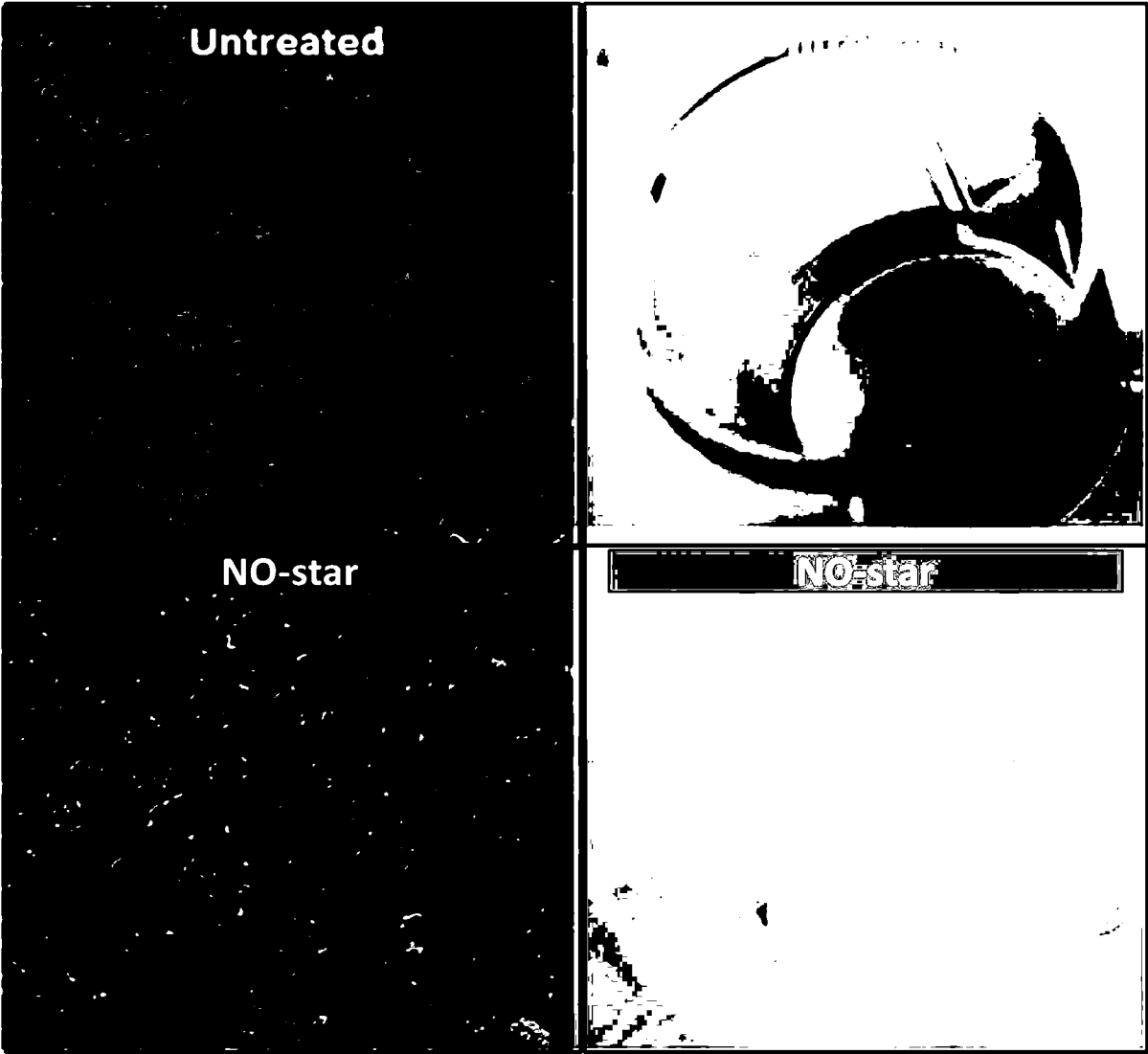


Figure 13



INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2015/000162

A. CLASSIFICATION OF SUBJECT MATTER

**A61K 47/48 (2006.01) A61K 31/04 (2006.01) A61K 31/74 (2006.01) A01N 33/16 (2006.01) A01N 33/18 (2006.01)
A61L 101/46 (2006.01)**

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)

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAPLUS, CAS REG, WPIAP, EPODOC, Google, Google Patents (Advanced), Google Scholar, Espacenet (Advanced)

Keywords: dendrimer, dendritic polymer, star polymer, nitric oxide, nitroso, nonoate, arm, branch, mikto

Registry number / component registry number: 10102-43-9 (nitric oxide)

Google Patents and Google Scholar: Applicant/Inventor search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search 4 June 2015		Date of mailing of the international search report 04 June 2015	
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaaustralia.gov.au		Authorised officer Andrew Davis AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. 0262104067	

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/AU2015/000162
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TAITE, L. J. <i>et al.</i> , 'Poly(ethylene glycol)-lysine dendrimers for targeted delivery of nitric oxide', J. Biomater. Sci. Polymer Edn., 2006, vol. 17, no. 10, pages 1159-1172. page 1163, paragraph 3; page 1165, paragraph 4; Figure 3	1-5, 9, 25
P,X	WORLEY, B. V. <i>et al.</i> , 'Nitric Oxide-Releasing Quaternary Ammonium-Modified Poly(amidoamine) Dendrimers as Dual Action Antibacterial Agents', Bioconjugate Chemistry, 2014, vol. 25, pages 918-927. Published on 05 May 2014. Scheme 1, Table 1, Figure 1	1-9, 22-26
A	WORLEY, B. V. <i>et al.</i> , 'Nitric Oxide-Releasing Quaternary Ammonium-Modified Poly(amidoamine) Dendrimers as Dual Action Antibacterial Agents', Bioconjugate Chemistry, 2014, vol. 25, pages 918-927. Published on 05 May 2014. Scheme 1, Table 1, Figure 1	8
A	DUONG, H. T. T. <i>et al.</i> , 'Intracellular nitric oxide delivery from stable NO-polymeric nanoparticle carriers', Chem. Commun., 2013, vol. 49, pages 4190-4192. Scheme 1	1-26

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2015/000162

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

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

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

See Supplemental Box for Details

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

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Supplemental Box**Continuation of: Box III**

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

This Authority has found that there are different inventions based on the following features that separate the claims into distinct groups:

- Claims 1-9 (in full) and 22-26 (in part) are directed to a polymer comprising a plurality of polymeric arms extending from a core, wherein on contact with an aqueous medium the polymer releases nitric oxide (NO) for an extended period of time. The feature of on contact with an aqueous medium the polymer releases nitric oxide (NO) for an extended period of time is specific to this group of claims.
- Claims 10-21 (in full) and 22-26 (in part) are directed to a polymer comprising a plurality of polymeric arms extending from a core, wherein the core comprises an NO-donor, NO-donor precursor, or a moiety capable of binding to a compound comprising an NO-donor or NO-donor precursor. The feature of the core of the polymer comprising either a NO-donor, NO-donor precursor, or a moiety capable of binding to a compound comprising an NO-donor or NO-donor precursor is specific to this group of claims.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

When there is no special technical feature common to all the claimed inventions there is no unity of invention.

In the above groups of claims, the identified features may have the potential to make a contribution over the prior art but are not common to all the claimed inventions and therefore cannot provide the required technical relationship. The only feature common to all of the claimed inventions and which provides a technical relationship among them is :

a polymer comprising a plurality of polymeric arms extending from a core

However this feature does not make a contribution over the prior art because it is disclosed in:

D1 (*cf.* Figure 2)

Therefore in the light of this document this common feature cannot be a special technical feature. Therefore there is no special technical feature common to all the claimed inventions and the requirements for unity of invention are consequently not satisfied *a posteriori*.

INTERNATIONAL SEARCH REPORT Information on patent family members		International application No. PCT/AU2015/000162
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.		
Patent Document/s Cited in Search Report		Patent Family Member/s
Publication Number	Publication Date	Publication Number Publication Date
End of Annex		
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. Form PCT/ISA/210 (Family Annex)(July 2009)		