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DESCRIPTION

[0001] The invention relates to the field of antimicrobial materials, in particular to implantable and other medical devices, exhibiting antimicrobial activity. More specifically, the present invention is directed to a method for providing a substrate, e.g. a catheter or another medical device or implant, with an antimicrobial coating comprising surface-immobilized quaternary ammonium compounds, and coated substrates obtainable thereby. It also relates to methods for reducing microbial biofilm formation on the surface of a substrate and to methods for reducing or eliminating the incidence of an implant-associated infection.

[0002] Infections associated with medical implants represent a major health care problem. For example, 5% of patients admitted to an acute care facility develop a hospital acquired infection. Hospital acquired infections (nosocomial infections) are the 11th leading cause of death in the US and cost over \$2 billion annually. Nosocomial infections directly cause 19,000 deaths per year in the US and contribute to over 58,000 others.

[0003] The four most common causes of nosocomial infections are: urinary tract infection (28%); surgical site infection (19%); respiratory tract infection (17%); and bloodstream infection (16% and rising). A significant percentage of these infections are related to bacterial colonization of implanted medical implants such as Foley catheters (urinary tract infections), endotracheal and tracheostomy tubes (respiratory tract infections), and vascular infusion catheters (bloodstream infections). Although any infectious agent can infect medical implants, Gram positive bacteria such as Staphylococci (*S. aureus*, *S. epidermidis*, *S. pyogenes*), and Gram negative bacteria such as *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* are common pathogens. Once a medical implant becomes colonized by bacteria, it must frequently be replaced resulting in increased morbidity for the patient and increased cost to the healthcare system. Often the infected device serves as a source for a disseminated infection, which can lead to significant morbidity or even death.

[0004] Bacteria have a natural ability to attach themselves to surfaces, both natural and synthetic. Once attached, they often work cooperatively to form biofilms, thin layers of bacterial colonies that can cover the surface of a medical device and introduce the risk of infection. Due to their ability to produce a protective polysaccharide layer they become resistant against the immune system and administered antibiotics. As a result, orthopedic implants, catheters, and even contact lenses can become vehicles for infection.

[0005] The time course of infection development is not fully understood and varies greatly. However, it has been reported that approximately thirty percent of infections arise less than one month post-implant, another thirty-five percent occur between one month and twelve months post-implant, and the remainder appear more than a year post-implant.

[0006] Existing approaches against microbial adhesion use the release of antibiotics or silver from a coated surface of an implant. However, overuse of antibiotics can lead to bacterial

resistance and the silver coating can be toxic to blood, limiting the clinical implementation of these methods. Moreover, releasing system are exhausted within a short period.

[0007] Quaternary-ammonium-compounds (QACs) are potent cationic antimicrobials used in everyday consumer products. Surface-immobilized, quaternary-ammonium-compounds create an antimicrobial contact-killing coating. Antimicrobial efficacy of QACs remains preserved when QAC-molecules are immobilized on a surface. Such contact-killing coatings have potential in widely-varying applications, including but not limited to surgical equipment and protective apparel in hospitals, medical implants and wound dressings, water purification, food packaging materials and industrial equipment. Use of QACs has been popular since they are easily produced and can be conveniently incorporated in coating systems. QACs are also stable in the human body, poorly metabolized and mainly excreted in nonmetabolized form. However, QACs can be hemolytic when not immobilized on a surface and environmentally toxic.

[0008] Behlau et al. (Biomaterials. Dec 2011; 32(34): 8783-8796) describe the biocompatibility and antibacterial properties of N,N-hexyl,methyl-polyethylenimine (HMPEI) covalently attached to the Boston Keratoprosthesis materials. It was found that HMPEI-derivatized materials exert an inhibitory effect on biofilm formation by *Staphylococcus aureus* clinical isolates, as compared to the parent poly(methyl methacrylate) (PMMA) and titanium.

[0009] Park et al. (Biotechnol. Prog. 2006, 22, 584-589) developed hydrophobic polycationic systems based on QACs that can serve as such a "bactericidal paint". Disclosed is a single-step, general procedure akin to common painting. Glass or polyethylene slides were briefly dipped into organic solutions of certain optimally hydrophobic N-alkyl-PEI (where PEI stands for branched 750-kDa polyethylenimine) polycations, followed by solvent evaporation. The N-alkyl-PEI was physically absorbed to the surface. The resultant polycation coated slides were able to kill on contact all of the encountered bacterial cells, whether the Gram-positive human pathogen *Staphylococcus aureus* or its Gram-negative brethren *Escherichia coli*. See also US2013/0110237.

[0010] However, QACs immobilized on a surface only exert contact-killing when the positive charge density is above a well-defined threshold. Accordingly, a limitation of typical contact-killing coatings is that the contact area between an adhering organism and a coating is generally small and mainly depending on the deformability of the rigid bacterial cell surface.

[0011] Asri et al. (Adv. Funct. Mater. 2013) addressed this issue by developing a shape-adaptive, multi-layered coating on which multiple QAC-molecules can be covalently tethered which is able to kill bacteria by partially enveloping them. Briefly, the method comprises covalently grafting onto silanol groups of a glass surface a specific coupling agent comprising blocked isocyanate groups, covalently anchoring to the coupling agent a polyurea coating by allowing polymerization of a monomer on the surface, and providing the hyperbranched coating with QACs.

[0012] Even after extensive washing, coatings thus obtained caused high contact-killing of

Staphylococcus epidermidis, both in culture-based assays and through confocal-laser-scanning-microscopic examination of the membrane-damage of adhering bacteria. Staphylococcal adhesion forces to hyperbranched quaternary-ammonium coatings were extremely high, indicating that quaternary-ammonium-molecules on hyperbranched polyurea partially envelope adhering bacteria upon contact. However, whereas the potential of this shape-adaptive, flexible contact-killing coating for use in clinical applications is clear, the present inventors found that the glass coating procedure taught by Asri *et al.* did not yield satisfactory results when applied to materials that are typically used for medical implants, like titanium or medical grade polymers such as polydimethylsiloxane elastomer (PDMS). More in particular, adherence of the hyperbranched coating was found to be uneven and highly variable, with a highly irregular covering of the surface. They therefore set out to adapt the coating method such that it allows for providing medical implants that inherently resist microbial (e.g. bacterial) infection long-term.

[0013] It was surprisingly found that this goal can be achieved by allowing 'pre-oligomerization' of the monomers forming the hyperbranched polyurea to a low molecular weight polyurea prior to contacting the polyurea with the surface to be coated, rather than polymerization of the monomers on the surface. The coating obtained via pre-oligomerization was robust, reproducible and long-lasting, with a homogenous covering of the surface. The coating could be readily provided with antibacterial agents, including QACs.

[0014] Accordingly, the invention provides a method for providing a substrate with an antimicrobial coating by immobilizing a quaternary ammonium compound onto the surface of said substrate, comprising the steps of : (i) providing a surface comprising reactive hydroxyl groups; (ii) covalently grafting onto said reactive hydroxyl groups a siloxane coupling agent comprising a blocked isocyanate group; (iii) polycondensation of AB₂ monomers comprising a secondary amine as A-group and blocked isocyanates as B-groups to a low number average molecular weight (M_n) polyurea of at least 2500 Da, preferably at least 4000 Da; (iv) contacting said low molecular weight polyurea to the surface grafted with coupling agent to covalently anchor the polyurea, and continuing polycondensation by heating the surface, optionally in the presence of AB₂ monomers, to obtain a hyperbranched polyurea coating; followed by (v) immobilizing onto said hyperbranched polyurea coating a hydrophobic N-alkylated polyethylenimine (PEI) having antimicrobial properties.

[0015] A method provided herein is not taught or suggested in the prior art.

[0016] US5,908,808 relates to coating formulations useful for the formation of a back layer on heat-sensitive recording materials such as wax-type thermal transfer materials or sublimation-type thermal transfer materials. Disclosed is a back-side coating formulation comprising, in a liquid medium, a reaction product obtained by reacting (a) a polysiloxane compound containing at least one functional group reactive with an isocyanate group, (b) a silane coupling agent containing at least one functional group selected from a functional group reactive with an isocyanate group or an isocyanate group, and (c) a polyisocyanate. US5,908,808 is completely

silent about antimicrobial coatings, and moreover fails to disclose many of the essential aspects of the present invention, including the steps of the polycondensation of AB₂ monomers to a low molecular weight polyurea and the immobilization of N-alkylated PE onto the hyperbranched polyurea coating. Hence, US5,908,808 bears no relevance to the present invention.

[0017] The method of the present invention finds its use in, among others, a method of manufacturing an implantable or insertable medical device being resistant to microbial growth and/or biofilm formation.

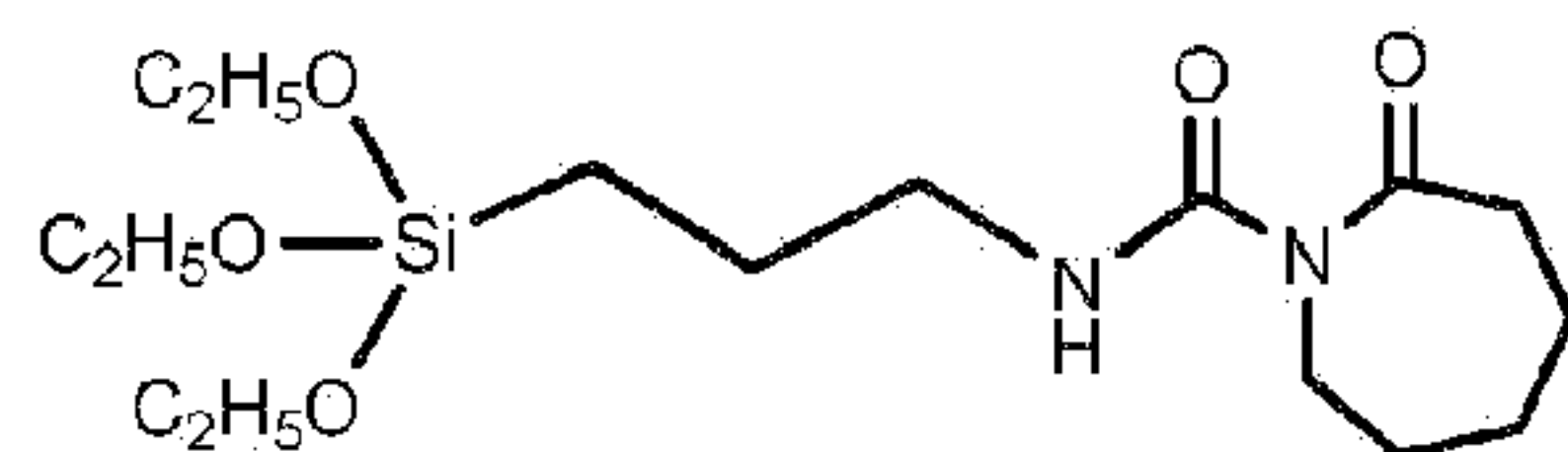
[0018] Any device prepared from a biocompatible material having appropriate mechanical properties for the particular application can be used when practicing the present invention. For example, suitable materials include titanium, titanium alloys (such as nickel titanium), nickel alloys, stainless steel, stainless steel alloys, platinum, platinum alloys and mixtures thereof. In other embodiments, suitable materials may include polymeric materials such as polyurethanes, polyamides, polyetheretherketones (PEEK), polyether block amides (PEBA), polytetrafluoroethylene (PTFE), silicones, and mixtures thereof. Polydimethylsiloxane (PDMS) belongs to a group of polymeric organosilicon compounds that are commonly referred to as silicones. PDMS is the most widely used silicon-based organic polymer. PDMS is in general, inert, nontoxic, and non-flammable.

[0019] As used herein, the term reactive hydroxyl group refers to any hydroxyl moiety being covalently attached to the surface which can react with a siloxane compound comprising blocked isocyanate groups (hereinafter referred to as "coupling agent"). A surface comprising reactive hydroxyl groups can be provided according to procedures known in the art depending on the substrate used. For example, plasma treatment of PDMS has been described by Whitesides et al. (Langmuir 1991, 7, 1013-1025) to provide PDMS surfaces with hydroxyl groups. Another suitable method to introduce hydroxyl groups on the surface is by using piranha solutions (H₂SO₄/H₂O₂, Kai-Seng Koh, et al, Micromachines 2012, 3, 427-441) Surface hydrophilicity of samples, changes of surface chemistry, surface morphologies of samples, and structural analysis of formed hydroxyapatite can be investigated by contact angle to water, X-ray photoelectron spectrometer (XPS), scanning electron microscopy (SEM), Fourier transform infrared (FTIR) and X-ray diffraction (XRD).

[0020] In another embodiment, reactive hydroxyl groups are silanol groups. A silanol is a functional group in silicon chemistry with the connectivity Si-O-H. For example, a silicone rubber substrate like PDMS can be treated with oxygen plasma to alter the surface chemistry, creating silanol (SiOH) groups to the surface using previously described methods. Atmospheric air plasma and argon plasma will work for this application. See for instance Olander et al (Biomacromolecules 2002, 3, 505-510) disclosing argon microwave plasma treatment and subsequent hydrosilylation Alternatively, surfaces can be treated with piranha solutions to obtain hydroxyl groups. See for example Kai-Seng et al. Micromachines 2012, 3, 427-441; doi:10.3390/mi3020427. In step (ii) of a method according to the invention, a siloxane compound comprising blocked isocyanate groups is covalently grafted onto the reactive

hydroxyl to serve as coupling agent between the surface and the hyperbranched polyurea coating. Suitable siloxane compounds include 3-isocyanato-propyl trialkoxy silane, 3-mercapto-propyl trialkoxy silane and 3-amino-propyl trialkoxy silane.

[0021] In a preferred embodiment, the siloxane coupling agent is of the formula



(2-oxo-N(3-triethoxysilyl)propyl)azepane-1-carboxamide). It is readily obtained from γ -amino propyl trialkoxy silane and carbonyl biscalpitolactam.

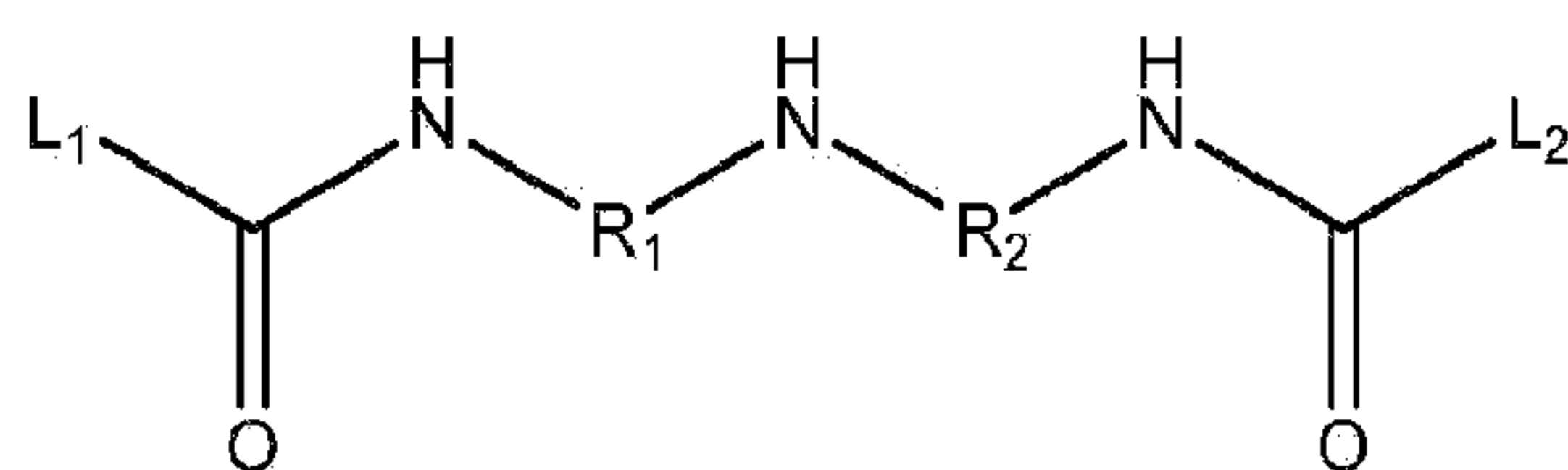
[0022] The coupling agent can simply be reacted with the reactive groups by contacting (e.g. by immersion) at least part of the surface with a solution of coupling agent, followed by continued reaction at elevated temperature. For example, an activated surface is immersed for 5-15 minutes in an alcohol solution and thereafter maintained at about 100-120°C for at least 1 hour, preferably at least 2 hours, to allow for covalent binding of coupling agent. Unreacted coupling agent is preferably removed by washing, e.g. in ethanol, under sonication.

[0023] As described above, a method of the invention is among others characterized in that it comprises the pre-oligomerization of AB₂ monomers comprising a secondary amine as A-group and blocked isocyanates as B-groups to a low number average molecular weight polyurea of at least 2500 Da.

[0024] The polymerization can be done in bulk as well as in a appropriate solvent such as dimethyl formamide (DMF). By heating the system above 125 °C, e.g. 145°C, the polymerization proceeds. The molecular weight depends on the polymerization time. The molecular weight is suitably determined by size exclusion chromatography using methods known in the art, for example as described in Macromol. Chem. Phys. 2012, 213, 1841-1850.

[0025] Preferably, oligomerization is performed to obtain polyurea having a M_n of at least 3000 Da, more preferably at least 3500 Da, like 4000 Da or even more. The best results coating results are obtained with polyurea oligomers having a molecular weight up to about 5000 Da. In one embodiment, the molecular weight is in the range of 2500 to 5000 Da, preferably 3000-5000 Da like around 3500, 4000, 4200, 4500 or 4700 Da. Any AB₂ monomer comprising a secondary amine as A-group and blocked isocyanates as flanking B-groups (i.e. forming the structure B-A-B) and capable of forming a hyperbranched structure via a ureido unit can be used to obtain a hyperbranched polyurea coating in a method of the invention.

[0026] In a preferred embodiment, the AB₂ monomer is of the general formula



Wherein R₁ and R₂ are aliphatic chains (CH₂)_m and (CH₂)_n wherein m and n are an integer in

the range of 3 to 15, preferably 3 to 8, and wherein. L_1 and L_2 are blocking groups for isocyanates, including but not limiting to caprolactam, phenol, oxime, triazole and malonic esters. Caprolactam is most preferred.

M and n can be the same or they can be different. In one embodiment, m and n are the same. In a specific aspect, m and n are 3, 4, 5 or 6.

AB_2 monomers can be synthesized using methods known in the art. For example, an AB_2 monomer of the general formula herein above is readily obtained by reacting carbonyl biscaprolactam with the appropriate triamine, like bishexamethylene triamine (m and n are 6) or bispentamethylene triamine (m and n are 5).

[0027] Following pre-oligomerization, the low molecular weight polyurea is contacted with the surface grafted with coupling agent to covalently anchor the polyurea, and polycondensation is continued by heating, optionally in the presence of AB_2 monomers, to obtain a hyperbranched polyurea coating. Heating is typically performed at a temperature of about 100-150°C, depending on the blocking group L_1 and L_2 . for a period of at least 1 hour, preferably at least 1.5 hours. In one embodiment, polymerization is performed at around 145°C for 1-3 hours, preferably under nitrogen. Unreacted compounds can be removed, for example by sequential sonication in ethanol, extraction in DMF overnight, and a second sonication in ethanol.

[0028] Depending on the desired thickness of the coating, several rounds of subsequent polycondensation can be performed. For example, a (cross-linked) coating comprising several layers of covalently bound polyurea can be obtained which is robust, reproducible and long-lasting. In one embodiment, step (iv) of a method of the invention comprises at least two, preferably at least three, sequential rounds of continued polycondensation e.g. to obtain a (cross-linked) coating with a thickness of up to several micrometers.

[0029] To obtain a coating having antimicrobial properties, an antibacterial functionality is coupled to the hyperbranched coating. More specifically, step (v) of a method of the invention comprises immobilizing onto the hyperbranched polyurea coating a hydrophobic N-alkylated polyamines, such as polyethylenimine (PEI) having antimicrobial properties. N-alkylation comprises alkylation with linear, cyclic or branched C_5 - C_{15} alkyl chains, or aromatic compounds, such as benzyl, preferable method is N-hexylation, N-dodecylation, and N-methylation.

[0030] Polyethylenimine based biocides are effective in inhibiting the growth of many microorganisms. As used herein, microorganisms includes single-cell and multi-cell bacteria, fungi, parasites, protozoans, archaea, protests, amoeba, viruses, diatoms, and algae. Microorganisms whose growth may be inhibited by polyethylenimine based biocides of the invention include *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus faecalis*, *Bacillus subtilis*, *Salmonella chloraesius*, *Salmonella typhosa*, *Escherichia coli*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Aerobacter aerogenes* *Saccharomyces cerevisiae*, *Candida albicans*, *Aspergillus niger*, *Aspergillus flares*, *Aspergillus terreus*, *Aspergillus verrucaria*, *Aureobasidium pullulans*, *Chaetomium globosum*, *Penicillium funiculosum*,

Trichophyton interdigital, *Pullularia pullulans*, *Trichoderm sp. madison P-42*, and *Cephaldascus fragans*; *Chrysophyta*, *Oscillatoria bometi*, *Anabaena cylindrical*, *Selenastrum gracile*, *Pleurococcus sp.*, *Gonium sp.*, *Volvox sp.*, *Klebsiella pneumoniae*, *Pseudomonas fluorescens*, *Proteus mirabilis*, *Enterobacteriaceae*, *Acinetobacter spp.*, *Pseudomonas spp.*, *Candida spp.*, *Candida tropicalis*, *Streptococcus salivarius*, *Rothia dentocariosa*, *Micrococcus luteus*, *Sarcina lutea*, *Salmonella typhimurium*, *Serratia marcescens*, *Candida utilis*, *Hansenula anomala*, *Kluyveromyces marxianus*, *Listeria monocytogenes*, *Serratia liquefaciens*, *Micrococcus lysodeikticus*, *Alicyclobacillus acidoterrestris*, *MRSA*, *Bacillus megaterium*, *Desulfovibrio sulfuricans*, *Streptococcus mutans*, *Cobetia marina*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Proteus vulgaris*, *Proteus mirabilis*, *Lactobacillus plantarum*, *Halomonas pacifica*, *Ulva linza*, and *Clostridium difficile*. Polyethylenimine based biocides may inhibit the growth of small colonies of microorganisms, as well as biofilms.

[0031] There are various ways by which the coating can be provided with N-alkylated PEI. In one embodiment, it comprises tethering PEI to the hyperbranched polyurea coating followed by a two-step N-alkylation of PEI to form quaternary amino groups. This procedure is known per se in the art and typically comprises sequential N-alkylation using halogenated alkanes, for example N-hexylation of PEI with bromohexane followed by N-methylation of N-hexyl-PEI using iodomethane. See for example Lin et al. (Biotechnology and Bioengineering, Vol. 83, 168-172; Behlau et al. (2011, Biomaterials 32(34):8783-8796) or Asri *et al.*

[0032] In another embodiment, it comprises N-alkylation of PEI prior to tethering N-alkylated PEI to the hyperbranched polyurea coating. This has the advantage that the substrate surface, e.g. an implant surface, does not need to be exposed to reaction conditions used for alkylation. Prior N-alkylation may comprise the traditional two-step alkylation. In a preferred embodiment, it comprises a one-stage reaction wherein PEI is N-alkylated simultaneously with two different alkyls.

[0033] The present inventors observed that triethylamine, commonly used as base in the PEI N-alkylation reaction, causes an undesirable side-reaction with halogenated alkane, e.g. hexylbromide, and moreover yields a coating having a rough surface. It was surprisingly found that these disadvantages can be overcome by preparing N-alkylated PEI by alkylating PEI in the presence of a proton sponge, including 1,6-di-*t*-butyl pyridine, 1,6-dimethyl pyridine and N,N,N',N'-tetramethylnaphthalene-1,8-diamine (1,8-Bis(dimethylamino)naphthalene, which can react with a proton but not with halogenated alkane.

[0034] A method of the invention is advantageously used to modify a substrate having, at least in part, a medical grade polymer surface, preferably selected from the group consisting of plasma-treated silicone rubber, such as plasma-treated medical grade polydimethylsiloxane elastomer (PDMS), polyurethane, polyvinylchloride (PVC).

[0035] In another embodiment, the surface is a metal which is biocompatible with the mammalian body, preferably selected from the group consisting of titanium, titanium-alloy, tantalum and tantalum-alloy.

[0036] Also provided herein is a substrate coated with an antimicrobial coating obtainable by a method of the invention. Encompassed are continuous coatings covering the entire substrate but also discontinuous local coatings or combinations of local coatings and continuous top coatings. A substrate of the invention is characterized by a strongly adhered antimicrobial coating which displays a high mechanical strength. A further aspect relates to a method for reducing microbial biofilm formation on the surface of a medical device or implant, comprising the step of providing at least part of the surface of said device or implant with an antimicrobial coating employing a method according to the invention.

[0037] The substrate is for example a medical device or implant, for instance a catheter or a prosthesis. In one embodiment, the implant is an orthopedic implant or a cardiovascular implant, for example selected from the group consisting of cardiac valves, alloplastic vessel wall supports, and total artificial heart implants. In another embodiment, the implant is selected from the group consisting of ear tubes, endotracheal tubes, ventilation tubes, cochlear implants and bone anchored hearing devices.

In a specific aspect, the invention provided a coated voice prosthesis. Total laryngectomy, a surgical treatment for extensive cancer of larynx, which alters swallowing and respiration in patients, is typically followed up with a surgical voice restoration procedure comprising tracheoesophageal puncture techniques with insertion of a "voice prosthesis" to improve successful voice rehabilitation. However, microbial or fungal colonization is a major drawback of these devices. Antimicrobials are usually used to prevent the colonization of (silicone rubber) voice prostheses by microorganisms. However, long-term medication induces the development of resistant strains with all associated risks and the development of alternative prophylactic and therapeutic agents, including probiotics and biosurfactants, have been suggested. A voice prosthesis provided with a coating of the present invention is highly effective in inhibiting microbial growth on surfaces of voice prostheses. Herewith, the frequency of replacing voice prostheses in laryngectomized patients can be reduced through microbial biofilm retardation.

[0038] In another specific aspect, the substrate is a catheter. The catheter can be a vascular catheter, chronic dwelling gastrointestinal catheter, dialysis catheter, hemodialysis catheter, or chronic dwelling genitourinary catheter. In one embodiment, it is a central venous catheter ("central line", "CVC", "central venous line" or "central venous access catheter"), which is typically placed into a large vein in the neck (internal jugular vein), chest (subclavian vein or axillary vein) or groin (femoral vein). It is used to administer medication or fluids, obtain blood tests, and measure central venous pressure. All catheters can introduce bacteria into the bloodstream, but CVCs are known for occasionally causing *Staphylococcus aureus* and *Staphylococcus epidermidis* sepsis. The problem of central line-associated bloodstream infections (CLABSI) has gained increasing attention in recent years. They cause a great deal of morbidity and deaths, and increase health care costs. The present invention provides unique tools to provide a catheter with an antimicrobial coating, thus reducing the risk of bacterial infections in the bloodstream.

[0039] With urinary tract infections as the most frequent implant related hospital acquired

infections (HAI) in developed countries, orthopedic implant infections is another major sub-populations within the multifactorial group of HAI (together with infections related to cardiovascular, neurological and gastrointestinal interventions). Infections due to implantation of total hip and total knee prostheses account for about 2% of the HAI, without taking trauma implants into account. Trauma implants or implants for fracture fixation and stabilization, like plates, screws and stabilizing frames, have been described to have an even higher risk for infection, mainly due to the fact that they are used to repair complex injuries and open fractures. Infection together with the eventual loosening of an orthopedic implant explains the limited lifespan of an orthopedic device (generally up to 15 years for an artificial joint).

[0040] Since the discovery of antibiotics, (implant) infections have been reduced and implant infections have become less lethal and can even be cured in most cases. However, sometimes the prosthesis has to be removed to allow the patient to recover. Moreover, the extensive use of antibiotics has resulted in an increasing amount of resistant bacterial strains, which makes infections caused by those pathogens challenging. Medical device implantation remains troublesome also in the case of orthopedic implants. Hence, in one embodiment the substrate is an orthopedic implant. Implants in the field of orthopedic and trauma surgery have been used for a long time to restore joint function, reduce pain or stabilize fractures. Both prevention of infection as well as integration of the implant with the host-tissue (mostly bone) are factors of concern. The most frequently used implants for these purposes are made of metallic alloys (for plates and nails for fracture repair and for total joint arthroplasties) and polyethylene (PE) (used in the articulating parts of a prosthesis).

[0041] Infection of orthopedic implants and prostheses is a medical issue that has intrigued people since their very first use over two-and-a-half millennia ago. Since that early beginning, lack of fixation and infection has been the major problem with such medical devices. In many cases, it may result in serious discomfort, limb amputation, illness and in many cases it may have even resulted in death of the patient. Thus, postoperative infection associated with implants remains a serious complication in orthopedic surgery. Implant surface treatment according to the present invention is a highly attractive means of reducing the incidence of implant-associated infections. Accordingly, also provided is a method for reducing or eliminating the incidence of an implant-associated infection, comprising implanting a medical device or implant coated with an antimicrobial coating obtainable by according to a method of the invention.

[0042] The implant may comprise metallic material, metal oxides, synthetic or natural, cross-linked or non-crosslinked, polymeric materials, ceramics, porcelain, allogenic or xenogenic bone or bone matrix; genetically engineered bone; and combinations thereof. The metallic material is preferably selected from the group consisting of NiTi alloys, stainless steel, titanium or alloys thereof.

[0043] Preferably, the polymeric material is non-degradable. For example, the polymeric material is selected from the group consisting of polydimethyl siloxane (PDMS), polypropylene (PP), polyethylene (PE), polyacetylene, cross-linked or non-crosslinked polystyrene, styrene-

butadiene rubber, TEFLON®, poly(vinyl chloride) (PVC), polyolefin copolymers, poly(methyl methacrylate), polyphenylene, polyacrylonitrile, carbon fiber, and blends or copolymers thereof.

[0044] The coated substrate may comprise at least one therapeutic agent and/or at least one further antimicrobial, e.g. antibacterial, agent. The therapeutic agent can be selected from the group consisting of immunosuppressants, anti-inflammatories, anti-proliferatives, anti-migratory agents, anti-fibrotic agents, cell permeabilizers, proapoptotics, calcium channel blockers, antineoplastics, antibodies, anti-thrombotics, anti-platelet agents, IIb/IIIa blockers, antiviral agents, anti-cancer agents, chemotherapeutics, thrombolytics, vasodilators, antibiotics, growth factor antagonists, free radical scavengers, radiopaque agents, anti-angiogenesis agents, angiogenesis drugs, cyclooxygenase inhibitors, phosphodiesterase inhibitors, cytokine inhibitors, nitrogen oxide donors, cytokine activators, antioxidants, radioactive compounds, steroids, and non-steroidal antiinflammatory drugs and derivatives and analogues thereof. In one embodiment, the coated substrate comprises a cell permeabilizer capable of increasing the permeability of the outer membrane (OM) of a target Gram-negative bacterium such that the activity of the immobilized quaternary ammonium compound is enhanced. Exemplary cell permeabilizers include chelating agents like EDTA, nitrilotriacetic acid (NTA) and succimer (meso-2,3-dimercaptosuccinic acid).

LEGEND TO THE FIGURES

[0045]

Figure 1: Microscopic images of glass coating with 5wt% oligomer (20x zoom) according to the invention. Panels A and B show representative sections.

Figure 2: Microscopic images of glass coating with 5wt% monomer (20x zoom) according to the prior art. Panels A and B show representative sections.

EXPERIMENTAL SECTION

Materials

[0046] Glass slides were purchased from Waldemar Knittel® (Braunschweig, Germany). Polydimethyl siloxane (PDMS) sheets were kindly provided by Atos Medical (Hörby, Sweden). Bishexamethylene triamine, (3-aminopropyl) triethoxysilane, polyethyleneimine (750 kDa, 50 wt% in water), iodomethane, 2-methyl-2-butanol, fluorescein disodium salt, 1-bromohexane and cetyltrimethylammonium chloride were all purchased from Sigma-Aldrich. Potassium

hydroxide and dimethylformamide were purchased from Acros organic. Sulfuric acid, hydrogen peroxide and ethanol were obtained from Merck. Methanol and toluene were obtained from Lab-Scan. Carbonyl biscaprolactam (CBC; >99%) was kindly provided by DSM innovation center, ALLINCO® (The Netherlands). All chemicals were used as received.

Example 1: Synthesis of 2-oxo-N(3-triethoxysilyl)propyl)azepane-1-carboxamide (Coupling agent)

[0047] A three neck flask provided with a reflux condenser, a nitrogen inlet and a connector to a vacuum pump, was three times evacuated and flushed with nitrogen, to remove all oxygen. CBC (11.34 g, 45 mmol) and (3-aminopropyl) triethoxysilane (9.95 g, 45 mmol) were dissolved in 40 mL toluene. The reaction was carried out under nitrogen at 80°C overnight. After the solution was cooled down to room temperature toluene was removed under reduced pressure. The obtained coupling agent was stored under nitrogen. The liberated caprolactam was not removed. According to the NMR spectrum, the yield was more than 98%. ¹H-NMR (300 MHz, CDCl₃) δ = 0.60 (2H, m, Si-CH₂), 1.19 (9H, m, Si-O-CH₂-CH₃), 1.65-1.74 (2H, m, Si-CH₂-CH₂ and 4H CO-N-CH₂-(CH₂)₃ (ring)), 2.67 (2H, m, N-CO-CH₂(ring)), 3.28 (2H, m, NH-CH₂), 3.79 (6H, m, Si-O-CH₂), 3.97 (2H, m, CO-N-CH₂, ring), 9.27 (1H, br, NH-CO-N).

Example 2: Application coupling agent

[0048] Glass slides (76 mm x 26 mm) were sonicated for 20 min at RT in dichloromethane. Subsequently, they were provided with silanol groups (Si-OH) by using an oxygen plasma cleaner (Diener electronic, Femto) for 60 seconds under stable vacuum (~2mTorr).

[0049] PDMS sheets were cut into small pieces (2.0 x 1.5 cm) and immersed in toluene for 24 h at room temperature with stirring. Next, the pieces were transferred into hexane and stirred for 24 h at room temperature. The samples were dried overnight at 60°C. The PDMS samples were provided with silanol groups (Si-OH) by using an oxygen plasma cleaner (Diener electronic, Femto) for 60 seconds under stable vacuum (~2mTorr).

[0050] The obtained hydrophilic slides were immersed in the 3v/v% solution of coupling agent (see Example 1) in ethanol for 10 min at RT and then placed in vacuum oven and heated at 110°C for 2 hours under vacuum. The unreacted coupling agent was removed by washing the slides in ethanol for 20 minutes in sonic bath at RT and then dried under nitrogen.

Example 3: Synthesis of AB₂ monomer

[0051] A three necked flask equipped with an inlet and outlet was flushed with nitrogen.

Carbonyl biscalprolactam (23.22 g, 92 mmol) and bishexamethylene triamine (9.98 g, 46 mmol) were dissolved in 40 mL toluene and stirred at 80 °C for 20 h in a nitrogen atmosphere. The toluene solution was cooled down to room temperature (RT) and extracted five times with an aqueous solution containing 5 wt% CaCl₂. After the aqueous layer had been removed, the organic layer was concentrated to half its volume using a rotary evaporator. Toluene was removed under reduced pressure and the product was dried in a vacuum oven at 40 °C overnight (Yield 80%). ¹H-NMR of AB₂ monomer (400 MHz, CDCl₃): δ 1.33-1.47 (m, 16H, CH₂(CH₂)₄CH₂), 1.70 (m, 12H, CH₂(CH₂)₃CH₂, ring), 2.56 (m, 4H, CH₂NH), 2.67 (m, 4H, NCOCH₂, ring), 3.25 (q, 4H, CONHCH₂), 3.96 (t, 4H, CH₂N, ring), 9.23 (br, 2H, CONH).

Example 4: Condensation of AB₂ monomers to a low average number molecular weight polyurea

[0052] A three-neck flask, provided with a reflux condenser, a nitrogen inlet and a connector to a vacuum pump, was three times evacuated and flushed with nitrogen, to remove all oxygen. To a solution of bishexamethylene triamine (BHTA) (8.81 g, 40.90 mmol) in dry DMF (50 mL) a solution of carbonyl biscalprolactam (20.64 g, 81.81 mmol) in DMF (60 mL) was added and the resulting mixture was stirred at 80 °C overnight under a nitrogen atmosphere. Subsequently, the temperature was raised to 145 °C after which the polymerization started. A number of oligomers with various molecular weights were prepared by heating the monomers during various polymerization times (table 1). The polymer solutions were precipitated in cold water (500 mL). The precipitates were isolated, washed with diethyl ether and dried in the vacuum oven at 40 °C for 48 h. ¹H-NMR (300 MHz, DMSO) δ = 1.07 to 1.57 (8H, m, (CH₂)₄), 1.61 (6H, m (CH₂)₃, ring), 2.65 (2H, m, NCOCH₂), 2.90 to 3.20 (2H, t, CON(CH₂)₂, 2H, m, CONHCH₂), 3.86 (2H, m, CH₂NCO), 6.06 (1H, m, CH₂NHCON(CH₂)₂), 9.13 (1H, t, CONH).

Table 1: Molecular weights at various residence times

Residence time (h)	M _n (Da)	M _w /M _n	BI end-groups
0.65	3,500	2.0	9
2	4,900	2.2	12
4	6,700	2.3	14
6	8,000	2.5	18
9	9,300	3.6	25
10	9,500	4.1	33
15	11,000	7.2	38

Example 5: Hyperbranched coatings on glass slides

[0053]

1. A. 100 pL of a solution of low molecular weight polyurea ($M_n = 4,900\text{Da}$; 5 wt% in ethanol) of Example 4 was dropped onto glass slides of Example 2 and spin-coated (2,000 rpm, 60 s). After evaporation of the ethanol the polymerization was continued at 145°C for 2 h under nitrogen. Unreacted compounds were removed by sonication in ethanol for 20 min followed by extraction in DMF at 115°C overnight and sonication again in 100 mL of ethanol for 20 min at RT. The coated glass slides were dried and stored under nitrogen. The microscopic images are shown in Figure 1. The roughness of coated samples was $0.156 \pm 0.027 \mu\text{m}$, measured by the optical profilometer operating in non-contact mode using white light interferometry (STIL, France). A surface roughness below about $1 \mu\text{m}$ is generally considered too rough and hence unsuitable for application as medical device. Not only causes a rough surface undesired friction e.g. when inserting it in the body, rougher surfaces also increase the rate of bacterial attachment.
2. B. As a comparative example, glass slides were coated with AB_2 monomers as has been described in the prior art. Briefly, a solution of AB_2 monomers (5 wt% concentrations in ethanol, 100 pL) was dropped and spin-coated onto glass slides of example 2 (2,000 rpm, 60 s). After evaporation of the ethanol a bulk polymerization was carried out at 145°C for 2 h under nitrogen. Unreacted compounds were removed by sonication in ethanol for 20 min followed by extraction in DMF at 115°C overnight and sonication again in 100 mL of ethanol for 20 min at RT. The coated glass slides were dried and stored under nitrogen. The microscopic images of the coated samples is shown in Figure 2. The roughness was $1.14 \pm 0.35 \mu\text{m}$, measured by the optical profilometer operating in non-contact mode using white light interferometry (STIL, France).

[0054] These data show that pre-oligomerization approach of the present invention resulted in a much lower roughness ($0.156 \pm 0.027 \mu\text{m}$) than a coating obtained via the surface polymerization of monomers ($1.14 \pm 0.35 \mu\text{m}$)

Example 6: Hyperbranched coatings on PDMS slides

[0055]

1. A. 100 pL of a solution of low molecular weight polyurea ($M_n = 4,900\text{Da}$; 5 wt% in ethanol) was dropped onto PDMS slides of example 2 and spin-coated (2,000 rpm, 60 s). After evaporation of the ethanol the polymerization was continued at 145°C for 2 h under nitrogen. Unreacted compounds were removed by sonication in ethanol for 20 min followed by extraction in DMF at 115°C overnight and sonication again in 100 mL of ethanol for 20 min at RT. The coated glass slides were dried and stored under nitrogen. The surface topography (roughness) of coated samples was $0.79 \pm 0.15 \mu\text{m}$, measured by the optical profilometer operating in non-contact mode using white light interferometry

(STIL, France).

2. B. As a comparative example, PDMS slides were also coated with AB₂ monomers.

100 μ L of a solution of the AB₂ monomer (5 wt% in ethanol) was dropped onto PDMS slides and spin-coated (2,000 rpm, 60 s). After evaporation of the ethanol the bulk polymerization was carried out at 145°C for 2 h under nitrogen. Unreacted compounds were removed by sonication in ethanol for 20 min followed by extraction in DMF at 115°C overnight and sonication again in 100 mL of ethanol for 20 min at RT. The coated glass slides were dried and stored under nitrogen. The surface topography (roughness) of coated samples was $2.35 \pm 0.32 \mu\text{m}$, measured by the optical profilometer operating in non-contact mode using white light interferometry (STIL, France).

Example 7: Modification of hyperbranched coating with polyethyleneimines

[0056] A solution of poly(ethyleneimine) (PEI) in water (50 wt%) was freeze dried overnight ($M_w=750$ kDa) and the residue was dissolved in methanol in 20 wt% concentration. 300 μ L of the PEI solution was dropped on coated glass slides of Example 6A and spin casted (2000 rpm, 60 s). The anchoring reactions were carried out at 125°C for 24 h under nitrogen. Unreacted PEI was removed with methanol using ultrasonic bath for 45 min at RT and dried under nitrogen.

Alkylation of PEI functionalized hyperbranched coating

[0057] In a round bottom flask provided with a reflux condenser, coated slides comprising tethered PEI were immersed in 75 mL 1-bromohexane and heated under nitrogen at 90°C overnight. Next 1.07 g of 1,8-bis(dimethylamino)naphthalene (proton sponge) in 25 mL of tert-amyl alcohol was added. The reaction was continued for another 6 hours at 90°C. Afterwards, the coatings were three times sonicated in methanol for 20 min at RT and dried under nitrogen. A second alkylation step was done in a round bottom flask fitted with a reflux condenser. Samples were immersed in a solution of 10 mL iodomethane in 75 mL tert-amyl alcohol. The alkylation reaction was carried out at 42°C overnight; the samples were subsequently sonicated in methanol for 20 min at RT and followed by extraction in methanol at 65°C for 1 day and another sonication in methanol for 20 min at RT. The obtained coatings were dried and stored under nitrogen.

Example 8: Antibacterial properties of the coated surfaces

Bacterial culture preparation

[0058] Bacteria were grown aerobically overnight at 37°C on a blood agar plate from a frozen stock solution. Plates were kept at 4°C and used no longer than for 2 weeks. A single bacterial colony from a blood agar plate was inoculated in 10 mL trypton soya broth (TSB, OXOID, England) in case of Gram-positive bacteria or 10 mL Todd Hewitt broth (THB, OXOID, England) in case of Gram-negative bacteria, and incubated at 37°C for 24 h. This pre-culture was used to inoculate 200 mL of TSB or THB and incubated for 16 h. Bacteria were harvested by centrifugation at 5000 × g for 5 min and washed twice with phosphate buffered saline (PBS buffer, 10 mM potassium phosphate, 150 mM NaCl, pH 7). Bacteria were sonicated on ice for 3 × 10 s at 30 W (Vibra Cell model 375, Sonics and Materials, USA) to break up aggregates if needed. This established procedure did not cause cell lysis.

Petrifilm assay

[0059] The Petrifilm assay (Petrifilm™ Aerobic Countplate, 3M, USA) was used to determine killing bacteria on contact under nutrient rich conditions. Bacterial concentrations of 10⁶, 10⁵ and 10⁴ per mL in PBS buffer were used. The bottom of the Petrifilm containing gelling agent was first swelled with 1 mL sterile demi water for 30 min. Next, 10 µL of bacterial suspensions at the appropriate concentration was dropped on coated and un-coated samples that were placed on the bottom film of the Petrifilm. After the top-lid was placed on the coated samples of example 6 the Petrifilms were incubated at 37°C for 48h. Bacterial suspension without a sample in between the films (10 µL) was used as a control. After the incubation time numbers of the CFU per cm² were counted. The results with various bacterial strains is given in Table 2. Table 2. Antibacterial properties of coated substrate against various bacterial strains and at various concentrations.

Bacterial strain	Control* 10 ²	10 ²	10 ³	10 ⁴	10 ⁵
<i>Staphylococcus epidermidis</i> ATCC12228	74±5	0	0	0	0
<i>Staphylococcus epidermidis</i> 3081	58±9	0	0	3±1	-
<i>Escherichia coli</i> ATCC 1110	80±7	0	6±2	43±4	-
<i>Escherichia coli</i> Hu734	77±4	12±7	140±16	-	-
*In the control experiment a calculated number of 100 bacteria were suspended on the substrate to check the viability.					

[0060] These data show that the antibacterial coating of example 6 kills >99% of the *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus epidermidis* 3081, *Escherichia coli* ATCC 1110 and about 85% of *Escherichia coli* Hu734.

Example 9: Bactericidal properties against Gram negative bacteria in the presence of EDTA.

[0061] Coated PDMS slides described in example 7 (PDMS-PEI⁺) were evaluated according the procedure of example 8 for their bactericidal effect against Gram negative bacteria (strains ATCC 15597 and Hu 734), in the absence or presence of EDTA. The results (Table 3) show that a permeabilisor, such as EDTA, has a profound influence on the antibacterial properties of said coatings against Gram negative bacteria. EDTA as such has no antibacterial properties at all.

Table 3

Sample	Bacterial challenge per sample (5 cm ²)		
	10 ²	10 ³	10 ⁴
<i>E.coli</i> ATCC 15597 (triplicates)			
Control (viability check)	79 ± 3	-	-
PDMS uncoated	65 ± 6	TMTC	TMTC
PDMS-PEI ⁺	6 ± 3	65 ± 8	TMTC
control + 0.1 mM EDTA	76 ± 4	-	-
control + 1 mM EDTA	78 ± 2.5	-	-
PDMS-PEI ⁺ + 0.1 mM EDTA	0	3 ± 1.5	41 ± 4.5
PDMS-PEI ⁺ + 1 mM EDTA	0	0	25 ± 6
<i>E. coli</i> Hu 734 (triplicates)			
Control (viability check)	78 ± 4	-	-
PDMS uncoated	73 ± 5	TMTC	TMTC
PDMS-PEI ⁺	12 ± 7	140 ± 16	TMTC
control + 0.1 mM EDTA	76 ± 8	-	-
control + 1 mM EDTA	75 ± 6	-	-
PDMS-PEI ⁺ + 0.1 mM EDTA	2 ± 1.5	12 ± 4	38 ± 8
PDMS-PEI ⁺ + 1 mM EDTA	0	4 ± 2.5	26 ± 6.5

REFERENCES CITED IN THE DESCRIPTION

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Patentkrav

1. Fremgangsmåde til tilvejebringelse af et substrat med en antimikrobiel coating ved immobilisering af en kvaternær ammoniumforbindelse på substratets overflade, hvilken

5 fremgangsmåde omfatter følgende trin:

(i) tilvejebringelse af en overflade omfattende reaktive hydroxylgrupper;

(ii) kovalent podning på de reaktive hydroxylgrupper med et siloxankoblingsmiddel omfattende en blokeret isocyanatgruppe;

(iii) polykondensering af AB₂-monomerer omfattende et sekundært amin som A-gruppe

10 og blokerede isocyanater som B-grupper for at opnå polyurea med en lav antalsmiddelmolekylvægt (M_n) på mindst 2500 Da;

(iv) etablering af kontakt mellem polyurea med lav molekylvægt og overfladen podet med koblingsmidlet for kovalent at forankre polyurea, og fortsættelse af polykondensering ved opvarmning, eventuelt i tilstedeværelse af AB₂-monomerer, for at opnå en hyperforgrenet

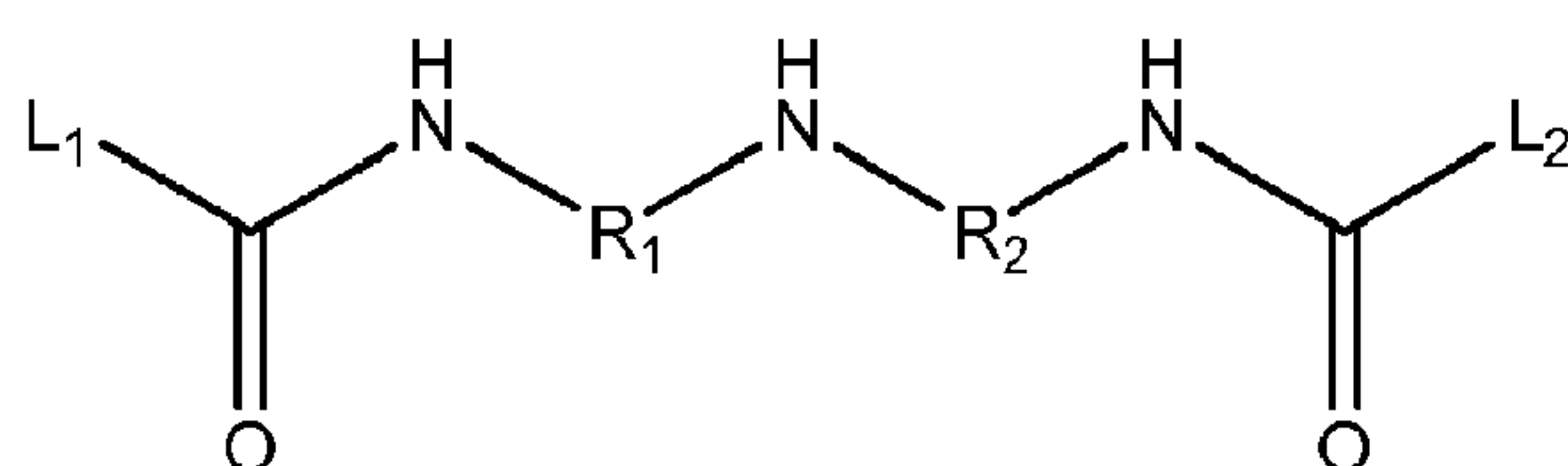
15 polyureacoating; efterfulgt af

(v) immobilisering på den hyperforgrenede polyureacoating af et hydrofobt N-alkyleret polyethylenimin (PEI) med antimikrobielle egenskaber.

2. Fremgangsmåde ifølge krav 1, hvor koblingsmidlet er (2-oxo-N(3-triethoxysilyl)propyl)azepan-1-carboxamid.

20

3. Fremgangsmåde ifølge krav 1 eller 2, hvor AB₂-monomererne har den almene formel



hvor

25 R₁ og R₂ er alifatiske kæder (CH₂)_m og (CH₂)_n, hvor m og n er et heltal i intervallet fra 3 til 15, fortrinsvis 3 til 8, og

L₁ og L₂ er blokeringsgrupper, fortrinsvis udvalgt fra caprolactam, phenol, oxim, triazol og malonsyreestere.

30 4. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor trin (iii) omfatter polykondensering af AB₂-monomerer til en antalsmiddelmolekylvægt i intervallet fra 2500 til 5000 Da, fortrinsvis 3000-5000 Da.

5. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor trin (v) omfatter fremstilling af N-alkyleret PEI ved alkylering af PEI i tilstedeværelse af 1,8-bis(dimethylamino)naphthalen, 1,6-di-t-butylpyridin eller 1,6-dimethylpyridin.

5

6. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor trin (v) omfatter immobilisering af PEI til den hyperforgrenede polyureacoating efterfulgt af N-alkylering af PEI.

7. Fremgangsmåde ifølge et hvilket som helst af kravene 1-5, hvor trin (v) omfatter N-alkylering af PEI før immobilisering af N-alkyleret PEI til den hyperforgrenede polyureacoating.

10

8. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor N-alkylering omfatter alkylering med en lineær eller forgrenet C₅-C₁₅-alkylkæde, fortrinsvis N-hexylering eller N-dodecylering og N-methylering.

15

9. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor N-alkylering omfatter en tottrinsalkylering af PEI, fortrinsvis N-hexylering efterfulgt af N-methylering af N-hexyl-PEI.

10. Fremgangsmåde ifølge et hvilket som helst af kravene 1-8, hvor N-alkylering omfatter en ettrinsalkylering af PEI.

20

11. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor overfladen er en polymer af medicinsk kvalitet, fortrinsvis udvalgt fra gruppen bestående af plasmabehandlet silikonegummi, såsom plasmabehandlet polydimethylsiloxanelastomer (PDMS), polyurethan og polyvinylchlorid (PVC) af medicinsk kvalitet.

25

12. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 10, hvor overfladen er et metal, der er biokompatibelt med pattedyrelegemet, fortrinsvis udvalgt fra gruppen bestående af titan, titanlegering, tantal og tantallegering.

30

13. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor substratet er en medicinsk anordning eller et implantat.

14. Fremgangsmåde ifølge krav 13, hvor substratet er udvalgt fra gruppen bestående af et kateter, en protese, et ortopædisk implantat og et kardiovaskulært implantat.

15. Substrat coatet med en antimikrobiel coating, der kan opnås ved en fremgangsmåde ifølge et hvilket som helst af kravene 1-14.

16. Coatet substrat ifølge krav 15, der omfatter mindst ét terapeutisk middel og/eller et yderligere antimikrobielt middel.

17. Coatet substrat ifølge krav 15 eller 16, der er en medicinsk anordning eller et implantat, fortrinsvis udvalgt fra gruppen bestående af et kateter, en protese, et ortopædisk implantat og et kardiovaskulært implantat.

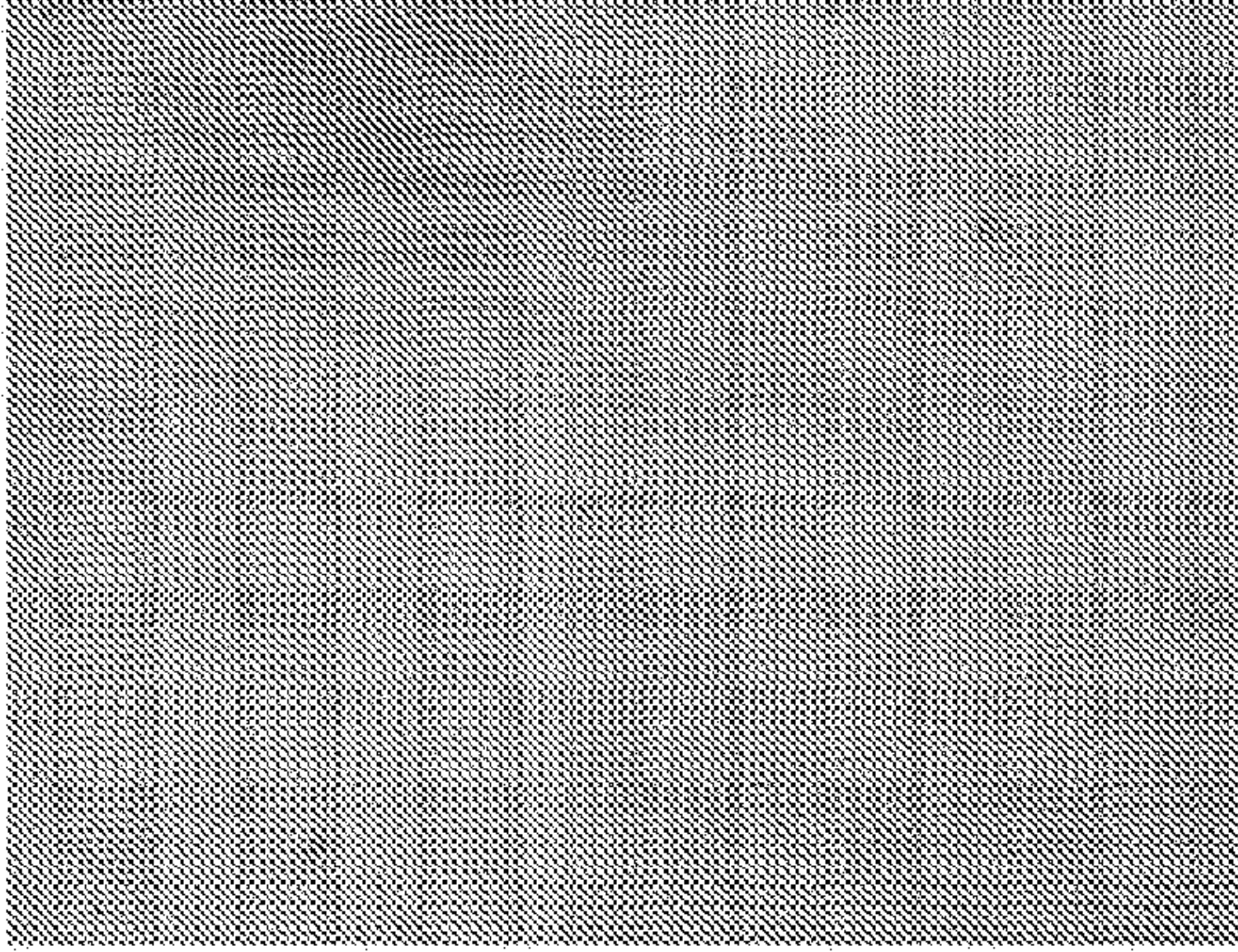
18. Fremgangsmåde til reduktion af mikrobiel biofilmdannelse på overfladen af et substrat, fortrinsvis en medicinsk anordning eller et implantat, omfattende trinnet med tilvejebringelse af mindst en del af overfladen af substratet med en antimikrobiel coating, der kan opnås ved en fremgangsmåde ifølge et hvilket som helst af kravene 1-14.

19. Fremgangsmåde til reduktion eller eliminering af forekomsten af en implantatforbundet infektion, omfattende implantering af en coatet medicinsk anordning eller et implantat ifølge krav 17 hos en person med behov derfor.

DRAWINGS

Figure 1

A



B

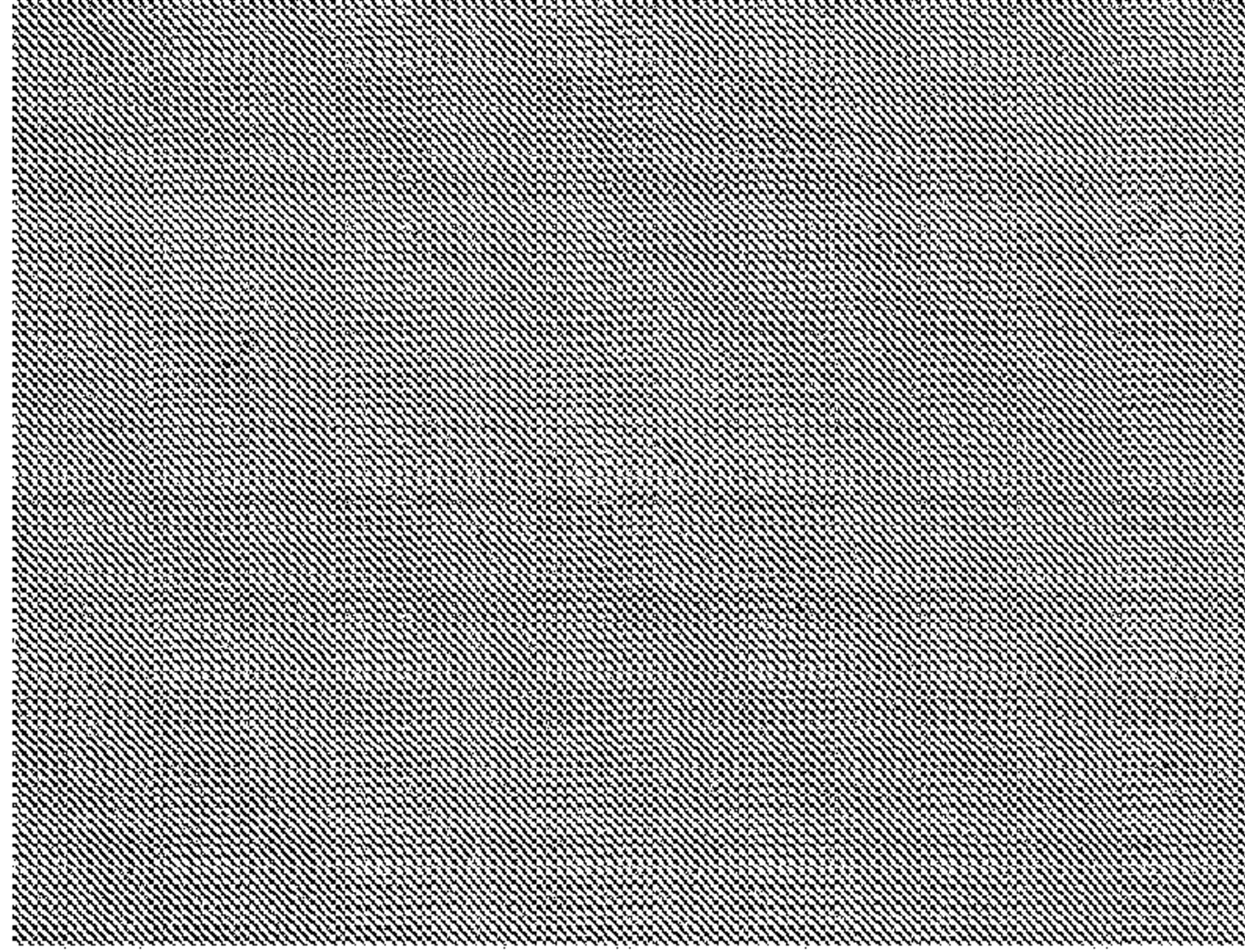
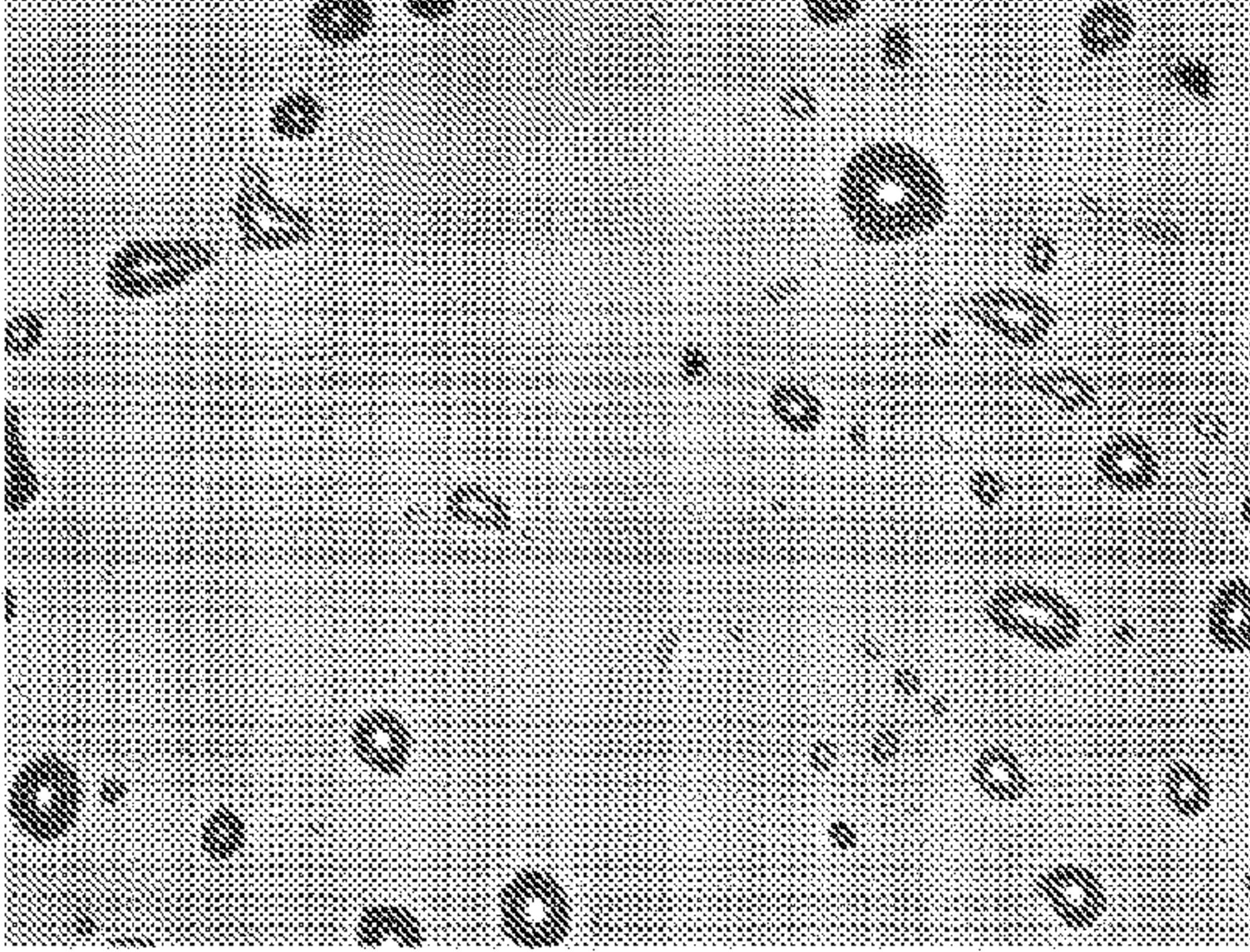


Figure 2

A



B

