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(54) **Title**: PLASMA ACTIVATION OF BIOLOGICAL MATERIALS FOR SURFACE MODIFICATION

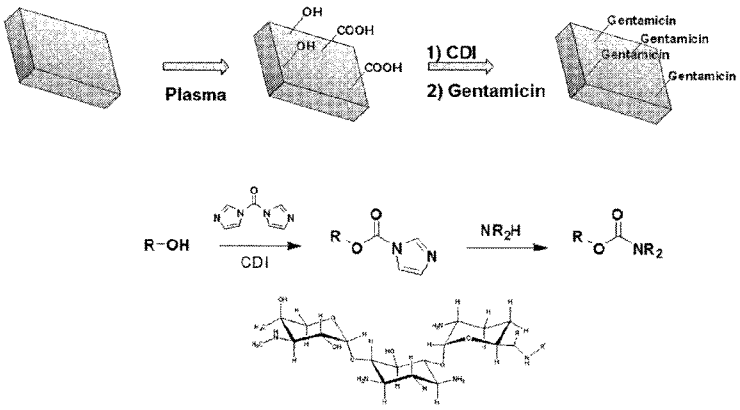

Figure 1

(57) **Abstract**: Disclosed in certain embodiments is a composition comprising a biological materia! activated by oxygen plasma and a ligand bound to a surface of the biological material

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PLASMA ACTIVATION OF BIOLOGICAL MATERIALS FOR SURFACE MODIFICATION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. provisional application number 61/479,627 filed April 27, 2011, the disclosure of which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0002] The presently disclosed subject matter includes compositions comprising biological material with pharmacologically active agent(s) bound to a surface thereof, either directly or through intermediary layers, and methods for producing said compositions.

BACKGROUND

[0003] It is desirable to alter the surface chemistry of tissue and other biological materials in order to control the body's interaction with it after implantation or grafting. One reason is to prevent infection. Another is to reduce inflammatory response. Yet another is to prevent the rejection of implanted biological material.

[0004] In some cases, alteration of surface chemistry can be achieved by soaking the material in a compatible solution of a pharmacological agent. A number of issues arise from this approach. Once implanted, the pharmacological solution will diffuse from the material into the patient. Generally speaking, a higher concentration that is necessary for local efficacy must be used due to this diffusion effect. The surgeon must therefore balance the total dosage of pharmacological agent with the necessary amount required to have the desired local effect. In some cases, concentration level required may cause undesirable side effects in the patient.

[0005] In addition, once implanted, it is impossible to control the rapid elution rate of the bioactive agent form the implanted tissue into the implant site and from there, into the patient. It is generally desirable for the bioactive agent to remain within the implanted or grafted tissue for a certain amount of time. Further, depending on the pharmacological agent, the amount required in solution for local efficacy may make the implantation or grafting procedure prohibitively expensive.

[0006] Accordingly, there remains a need in the art for biological materials suitable for implantation or grafting into living mammals, and methods of creating the same.

SUMMARY OF THE INVENTION

[0007] It is an object of certain embodiments of the present invention to provide biological materials with surface modifications to allow for conjugation with a ligand.

[0008] It is an object of certain embodiments of the present invention to provide biological materials conjugated with an antimicrobial agent.

[0009] It is an object of certain embodiments of the present invention to provide biological materials that have reduced incidence of infection upon implantation or grafting into a patient.

[0010] It is an object of certain embodiments of the present invention to provide biological materials that have reduced incidence of rejection upon implantation or grafting into a patient.

[0011] It is an object of certain embodiments of the present invention to provide biological materials that have improved stability and integrity upon implantation or grafting into a patient.

[0012] It is an object of certain embodiments of the present invention to provide methods of manufacturing modified biological materials as disclosed herein.

[0013] It is an object of certain embodiments of the present invention to provide methods of dehydrating biological materials.

[0014] The above objects of the invention, and others, may be achieved by the present invention which in certain embodiments is directed to a composition comprising a biological material and a ligand bound to a surface of the biological material. The biological material can be, e.g., collagen, tissue, or bone. In certain embodiments, the tissue is acellular dermal tissue.

[0015] In certain embodiments, the present invention is directed to a composition comprising a biological material activated by oxygen plasma. Optionally, a ligand is bound to the activated biological material.

[0016] In certain embodiments, the present invention is directed to a composition comprising acellular tissue; a coupling agent bound to the acellular tissue; and

[0017] a pharmacological agent bound to the coupling agent.

[0018] In certain embodiments, the present invention is directed to a method of treating biological material comprising: contacting biological material with oxygen plasma to form activated biological material. In other embodiments, a ligand can be bound to the activated biological material.

[0019] Where bonding is referenced herein, such bonding can be achieved through any type of chemical bond, including, without limitation, covalent bonding, polar covalent bonding, ionic bonding, hydrogen bonding, van der Waals forces and a combination thereof.

[0020] In certain embodiments, the present invention is directed to a process for dehydrating a biological material (e.g., an acellular biological material).

[0021] In certain embodiments, the present invention is directed to the implantation or grafting of a modified biological material as disclosed herein in a patient in need thereof.

[0022] In certain embodiments, the present invention is directed to a method of performing reconstructive surgery in a patient in need thereof comprising implanting or grafting a modified biological material as disclosed herein.

[0023] In certain embodiments, the present invention is directed to a method of administering a drug to a patient in need thereof comprising implanting or grafting a biological material conjugated with pharmacological agent or bioactive agent as disclosed herein.

[0024] In describing the present invention, the following terms are to be used as indicated below. As used herein, the singular forms "a," "an," and "the" include plural references unless the context clearly indicates otherwise. Thus, for example, reference to "a pharmacological agent" includes a single pharmacological agent as well as a mixture of two or more different pharmacological agents.

[0025] As used herein, "biological material" means any material derived in whole or in part from an organism, including, without limitation, soft tissue sources such connective and non-connective tissue. Examples of connective tissue includes, without limitation, tendons, ligaments, fascia, dermal tissue, fat, dura, pericardia, fibrous tissues and synovial membranes. Examples of non-connective tissue includes, without limitation, muscles, blood vessels and nerves. "Biological material" also includes hard tissue sources such as bone and cartilage. In certain embodiments, such materials may have been harvested from a living organism and then submitted to further processing and/or chemical treatment. The living organism could be comprised of eukaryotic or prokaryotic

cells. Recombinant proteins, which can be derived from bacteria such as *E. coli* and are produced from recombinant DNA, can also be modified with the present invention.

[0026] "Acellular biological material" refers to biological material from which all, or substantially all, viable cells and detectable subcellular components and/or debris from cell death have been removed.

[0027] In some embodiments, the acellular biological material utilized in the present invention has a concentration of viable cells that is less that about 5%, less than about 3%, less than about 1% or less than about 0.5% of the concentration in the original biological material from which the acellular biological material was derived. In other embodiments, the acellular biological material has an amount of viable cells that is less that about 3%, less than about 1%, less than about 0.5% or less than about 0.2% of the total weight of the acellular material.

[0028] In some embodiments, the acellular biological materials utilized in the present invention comprise less than about 40%, less than about 25%, less than about 10%, or less than about 5% of nucleic acid that was present in the original cellularized biological material from which the acellular biological material was derived. In other embodiments, the acellular biological material has an amount of nucleic acid that is less than about 25%, less than about 10%, less than about 5%, or less than about 2% of the total weight of the acellular material

[0029] "Activated biological material" refers to biological material that has been contacted with an activating agent (e.g., oxygen plasma) to provide reactive functional groups on the surface of the material.

[0030] "Pharmacological agent" or "bioactive agent" means any agent that can be bound to activated biological material. Examples of bioactive or pharmacological agents include, without limitation, (i) those of an anti-infective nature, such as antimicrobials, antibiotics, antifungals, antiseptics, disinfectants, and preservatives; (ii)

immunosuppressant drugs such as glucocorticoids, antibodies, ciclosporin, tacrolimus, calcineurin inhibitors, and sirolimus; and (iii) agents to mediate and induce cellular/tissue growth such as bone morphogenic proteins (BMP), epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF-I and II), TGF-D, and vascular endothelial growth factor (VEGF).

[0031] "Anti-infective" refers to anything that is capable of destroying or inhibiting the microorganism growth, including, without limitation, antimicrobial agents such as antibacterial and antifungal agents.

[0032] "Coupling agent" means an agent capable of forming a bond between the surface of an activated biological material and a ligand (such as a pharmacological or bioactive agent). This bond may be achieved by first forming a bond between the surface of the activated biological material and the coupling agent, and then forming a bond between the coupling agent and the ligand. In other embodiments, the ligand can be bonded to the coupling agent, and the resultant conjugate is bound to the biological material. Alternatively, the coupling agent may facilitate bonding directly between the surface of an activated biological material and the ligand.

[0033] The term "oxygen plasma" means an oxygen source having a portion of the molecules ionized.

BRIEF DESCRIPTION OF THE FIGURES

[0034] **FIGURE 1** depicts a schematic diagram of one embodiment of the present invention.

[0035] **FIGURE 2** depicts retention of gentamicin under conditions of soaking in an infinite sink in PBS for 28 days versus untreated control. The Y-axis is nanograms per sample core. The X-axis is time.

[0036] **FIGURE 3** depicts the results of elution studies of (i) samples treated according to the method of the present invention, (ii) untreated samples, (iii) samples not treated with plasma but soaked in gentamicin, and (iv) samples dehydrated and exposed to plasma and then soaked in gentamicin. Figure 3 demonstrates that soaked samples eluted practically all of their gentamicin by the end of the first day and thereafter were equivalent to untreated samples. Samples treated with plasma according to the present invention showed a burst elution of unbound material followed by a steady state of gentamicin over the 14 days of the experiment. Drug retention was measured by soaking the test samples in buffer of sufficient volume to be considered an infinite sink.

[0037] **FIGURE 4** depicts the long-term efficacy of gentamicin in samples treated according to the method of the present invention. Figure 4 demonstrates that explanted tissue retains antimicrobial activity even after almost 7 days of implantation time

[0038] **FIGURE 5** depicts a graph that demonstrates gentamicin load is significantly improved after introducing short vacuum during the CDI activation step of the method of the present invention.

[0039] **FIGURE 6** depicts the antimicrobial efficacy of samples in which a short vacuum has been introduced during the CDI activation step of the method of the present invention versus samples prepared without vacuum.

[0040] **FIGURE 7** depicts bacterial outgrowth results. N=3 per group, per timepoint. Error bars represent the standard deviation.

[0041] **FIGURE 8** depicts results from a fibroblast assay. N=4 per group. Error bars represent the standard deviation.

[0042] **FIGURE 9** depicts cell viability results using an MTS assay. Relative cell number was extrapolated from a standard curve produced from dermal fibroblasts grown on TCPS. N=4 for all groups. Error bars represent the standard deviation.

[0043] **FIGURE 10** depicts the results of a gentamicin quantification assay using an ELISA kit.

[0044] **FIGURE 11** depicts the results of killing assays for samples treated according to a method of the present invention using 4 different bacteria. For all groups, n=3 and error bars represent the standard deviation.

DETAILED DESCRIPTION

[0045] Harvested biological material is often utilized for implantation or grafting in a host organism for a variety of reasons such as reconstructive surgery (e.g., hernia repair or external burn treatment). A common source of biological material is dermal tissue. The source of the dermal tissue can be from another area of the patient's body, called an autograft, obtained from another person (e.g., donor skin from cadavers called an allograft, or from an animal (e.g., porcine or bovine source), called a xenograft.

[0046] Implanted or grafted biological material is susceptible to complications (e.g., infection) which can lead to failure of the procedure and issues to the patient. Accordingly, it is desirable to have the biological material functionally modified (e.g., conjugated or bound to an anti-infective agent) prior to implantation or grafting.

[0047] In certain embodiments, the biological material is animal tissue. Animal tissue is mainly made of Type I collagen, which contains a very limited number of functional groups for bioconjugation (approximately 5%). In instances where it is desirable for these tissues to be processed, altered or derivitized for implantation or grafting into an organism, this lack of functional groups can present challenges. For example, it may be desirable to treat implanted tissues with antibiotic agents prior to implantation to prevent

infection at the surgery site. However, the lack of functional groups to which these agents can bind makes it difficult for the tissue to retain the agents long enough for the agents to impart the desired effect - i.e., preventing infection.

[0048] The present invention provides a solution to this problem by creating functional groups on the surface of biological material after exposure to an activating agent. Preferably, the activating agent is oxygen plasma. By virtue of the present invention, biological material (e.g., tissue) with surface functional groups such as hydroxyl and carboxyl are created after contact with oxygen plasma. This creation of functional groups creates a surface on the biological material for the binding of ligands such as antimicrobial and antibiotic agents. The plasma-generated surface reactive hydroxyl and carboxyl groups may be formed by breaking hydrocarbon bonds on the surface of the biological material through bombardment of oxygen plasma. Preferably, the treatment does not disrupt the integrity of the bulk tissue underneath, presenting minimal challenges to post-implant integration.

[0049] One method of the present invention involves contacting a biological material with oxygen plasma to, for example, remove the water shell that is tightly associated with the biological material and activate the exposed surface to generate more functional groups for chemical modification. Ligands may then be bound to these functional groups. Ligands may include coupling agents and pharmacological or bioactive agents. In some embodiments, a coupling agent is used to facilitate bonding (e.g., covalent bonding) between the activated biological material and a pharmacological or bioactive agent (such as an antibiotic). For example, plasma-generated surface reactive groups are bound to a coupling agent. Ligands are then directly conjugated onto the coupling agent biological material conjugate. In a certain embodiment, a coupling agent such as 1,1'-carbonyldiimidazole can be used to create a bond between the activated surface groups and free amine groups and the antibiotic gentamicin. Another coupling agent is a mixture of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and N-Hydroxysuccinimide (NHS). In certain embodiments, the biological material is dehydrated prior to activation and/or ligand binding.

[0050] Intermediary layers (e.g., coupling agents) that may be used to create a series of bonds between the plasma-activated tissue surface and the further ligand preferably have one or more of the following qualities: (i) in its final processed state it is biocompatible; (ii) it is reactive with the activated tissue substrate upon application; (iii) after application, the intermediary layer is reactive with the bioactive agent or subsequent intermediary layer; and (iv) in its final processed state, it is substantially as flexible as its underlying substrate, which may be achieved, for example, through the thinness of the layer.

[0051] Certain embodiments of the present invention include a composition comprising a biological material and a ligand bound (e.g., via covalent bonding) to a surface of the biological material. The biological material may be, for example, soft tissue, collagen, bone, dermal tissue, or any other suitable tissue. In certain embodiments, the biological material is acellular. The ligand can, for example, be a pharmacological agent, a biologically active molecule, or a coupling agent.

[0052] In certain embodiments, the present invention includes a composition comprising acellular tissue, a coupling agent bound to the acellular tissue, and pharmacological agent bound to the coupling agent.

[0053] The biological material can be derived from any suitable biological source, including, without limitation, mammalian, avian, reptilian, amphibian and bacteria. In certain embodiments, the mammalian source is selected from humans, primates (e.g., monkeys, chimpanzees, gorillas, gibbons and orangutan), livestock (e.g., pigs, cows, horses, goats, sheep), dogs, cats, rabbits, guinea pigs, gerbils, hamsters, rats, and mice. In certain embodiments, the mammalian source is a human cadaver. In other embodiments, the mammalian source is a living human. Examples of avian sources include chicken, turkey, duck and goose.

[0054] The biological material is preferably acellular as to avoid the rejection of the biological material from the host. One source of acellular tissue that can be modified according to the present invention is FlexHD® commercially available from Ethicon. Further examples of acellular biological materials are described in U.S. Patent Application Publication No. 2006/0275377; International Application No. PCT/US08/52882; International Application No. PCT/US08/52884, and International Application No. PCT/US08/52885, which are incorporated herein by reference in their entireties.

[0055] In certain embodiments, the present invention is directed to dehydrated biological materials and methods of preparing the same. In certain embodiments, the dehydrated biological material can optionally be activated and/or ligand bound as disclosed herein. The dehydration process can include, for example, lyophilization or a solvent exchange process. In some embodiments, the biological material has been dehydrated via a solvent exchange process comprising soaking the biological material in a solvent. In further embodiments, the biological material has been dehydrated via a solvent exchange process comprising soaking the biological material in a hydrophilic solvent followed by soaking the biological material in an organic solvent. In some embodiments, the biological material has been dehydrated via a solvent exchange process comprising soaking the biological material in a hydrophilic solvent followed by soaking the biological material in an organic solvent under vacuum.

[0056] In a particular embodiment, the solvent exchange process comprises soaking the biological material in a solvent miscible with or capable of forming an azeotrope with water. The biological material can then be optionally placed under a vacuum with or without heat.

[0057] In another embodiment, the solvent exchange process comprises soaking the biological material in a solvent miscible with or capable of forming an azeotrope with water followed by soaking the biological material in a volatile organic solvent. Preferably, the boiling point of the organic solvent at atmospheric pressure is less than or

equal to about 80° C, less than or equal to about 70° C, or less than or equal to about 80° C. The biological material can then be optionally placed under a vacuum with or without heat.

[0058] An exemplary solvent exchange process comprises soaking the biological material in an organic hydrophilic solvent miscible with water, such as ethanol, iso-propanol, or any other azeotrope-forming solvent to extract the water from the biological material. This can be performed for one or more cycles (e.g., 2 or 3 cycles). The tissue can then be soaked in organic solvents with lower boiling points, such as dichloromethane or tetrahydrofuran, for one or more cycles (e.g., 2 or 3 cycles) to replace the previous solvent. After draining out the solvent, the biological material may be dried. In one embodiment, the sample is placed under vacuum with or without gentle heating, to remove any residual solvent that remains.

[0059] The solvent for a solvent exchange process used to prepare the biological material of the present invention can be, for example, one or more of ethanol, n-propanol, iso-propanol, n-butanol, sec-butanol, iso-butanol, tert-butanol, allyl alcohol, benzyl alcohol, furfuryl alcohol, cyclohexanol, benzyl alcohol, tetrahydrofuran, chloroform, methyl ethyl ketone, benzene, ethyl acetate, cyclohexane, benzene, carbon tetrachloride, ethylene chloride, acetonitrile, toluene, n-hexane, n-heptane, carbon disulfide, diethyl ketone, n-propyl acetate, methanol, acetone, aqueous mixtures thereof, and combinations thereof. In certain embodiments, the solvent is an organic solvent and can be, for example, one or more of dichloromethane, tetrahydrofuran ethyl ether, methyl t-butyl ether, pentane, hexane, aqueous mixtures thereof, and combinations thereof.

[0060] In embodiments that subject the biological material to a solvent exchange process, the biological material may be placed under vacuum at room temperature or with heat.

[0061] In certain embodiments of the present invention, the ligand that is bound to the biological material is a coupling agent. The coupling agent can, for example, be 1,1'-

carbonyldiimidazole, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride, N,N'-Disuccinimidyl carbonate, N-hydroxysuccimimidyl chloroformate, isocyanate, Benzotriazol-1-yloxy)tris(dimethylamino) phosphonium hexafluorophosphate, (Benzotriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate, *O*-(7-Azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate, *O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate, *O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate, (7-Azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate, 1-Hydroxybenzotriazole, *N,N'*-Dicyclohexylcarbodiimide, *N,N'*-Diisopropylcarbodiimide, Diethyl azodicarboxylate or N,N'-Di-tert-butylcarbodiimide, salts thereof, derivatives thereof and combinations thereof.

[0062] In some embodiments, a second ligand is bound to the composition of the present invention. The second ligand can, for example, be a pharmacological agent that is bound (e.g., covalently bound) to the biological material via the first ligand. The pharmacological agent can be, for example, an antimicrobial agent such as an antibiotic. In certain embodiments, the antibiotic has an available nucleophilic group.

[0063] In certain embodiments, the antimicrobial agent is selected from one or more of amikacin, gentamicin, kanamycin, neomycin, netilmicin, tobramycin, paromomycin, geldanamycin, herbimycin, loracarbef, ertapenem, doripenem, imipenem/cilastatin, meropenem, cefadroxil, cefazolin, cefalotin, cefalexin, cefaclor, cefamandole, cefoxitin, cefprozil, cefuroxime, cefditoren, cefoperazone, cefotaxime, cefpodoxime, ceftazidime, ceftibuten, ceftizoxime, ceftriaxone, cefepime, ceftaroline fosamil, ceftobiprole, teicoplanin, vancomycin, telavancin, clindamycin, lincomycin, daptomycin, azithromycin, clarithromycin, dirithromycin, erythromycin, roxithromycin, troleandomycin, telithromycin, spectinomycin, spiramycin, aztreonam, furazolidone, nitrofurantoin, amoxicillin, ampicillin, azlocillin, carbenicillin, cloxacillin, dicloxacillin, flucloxacillin, mezlocillin, methicillin, nafcillin, oxacillin, penicillin G, penicillin V, piperacillin, temocillin, ticarcillin, amoxicillin/clavulanate, ampicillin/sulbactam, piperacillin/tazobactam, ticarcillin/clavulanate, bacitracin, colistin, polymyxin B,

ciprofloxacin, enoxacin, gatifloxacin, levofloxacin, lomefloxacin, moxifloxacin, nalidixic acid, norfloxacin, ofloxacin, trovafloxacin, grepafloxacin, sparfloxacin, temafloxacin, mafenide, sulfonamidochrysoidine, sulfacetamide, sulfadiazine, silver sulfadiazine, sulfamethizole, sulfamethoxazole, sulfanilimide, sulfasalazine, sulfisoxazole, trimethoprim, trimethoprim-sulfamethoxazole, demeclocycline, doxycycline, minocycline, oxytetracycline, tetracycline, clofazimine, dapsone, capreomycin, cycloserine, ethambutol, ethionamide, isoniazid, pyrazinamide, rifampicin, rifabutin, rifapentine, streptomycin, arsphenamine, chloramphenicol, fosfomycin, fusidic acid, linezolid, metronidazole, mupirocin, platensimycin, quinupristin/dalfopristin, rifaximin, thiamphenicol, tigecycline, tinidazole, salts thereof, derivatives thereof, and combinations thereof.

[0064] In certain embodiments, the antimicrobial agent of the present invention is selected from one or more of chlorhexidine, biguanides, quaternary ammonium compounds, salts thereof, derivatives thereof, and combinations thereof.

[0065] The attachment of the ligand can be performed, e.g., by contact with an aqueous, non-aqueous or partial aqueous solution of the ligand. The solution can contain the ligand, e.g., in an amount from about 0.0001% to about 99% w/w. In particular embodiments, the ligand solution (e.g., gentamycin or 1,1'-carbonyldiimidazole) comprises from about 0.0001% to about 50%, from about 0.001 to about 25%, from about 0.01 to about 10% or from about 0.1 to about 5% ligand.

[0066] In certain embodiments, the source of the oxygen plasma can be O_2, air, or a combination thereof. In other embodiments, the source of oxygen plasma is any gaseous mixture that has a minimum percent of oxygen to provide a suitable surface of functional groups on the biological material after contact to allow for further chemical modification

[0067] In certain embodiments, a ligand such as an antimicrobial or antibiotic agent is directly bound to the surface functional groups of the biological material without the use of intermediary layers such as coupling agents.

[0068] In certain embodiments, the compositions, biological materials, or activated biological materials are sterilized, i.e., they are substantially free of living microorganisms such as bacteria and viruses.

[0069] In certain embodiments of the inventive composition comprising a pharmacological agent, the composition maintains between about 5% and 95% of the pharmacological agent after soaking in an infinite sink in phosphate buffer saline for 24 hours, for 4 days, for 7 days, or for 14 days.

[0070] In further embodiments of the inventive composition comprising a pharmacological agent, the composition maintains between about 50% and 95% of the pharmacological agent after soaking in an infinite sink in phosphate buffer saline for 24 hours, for 4 days, for 7 days, or for 14 days.

[0071] In other embodiments of the inventive composition comprising a pharmacological agent, the composition maintains at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, or at least about 90% of the pharmacological agent after soaking in an infinite sink in phosphate buffer saline for 24 hours, for 4 days, for 7 days, or for 14 days.

[0072] In further embodiments of the inventive composition comprising a pharmacological agent, the amount of pharmacological agent is maintained after soaking in an infinite sink in phosphate buffer saline for 4 days, for 7 days, or for 14 days is within about 10%, within about 15%, within about 20%, or within about 25% of the amount of pharmacological agent present in the composition at 24 hours.

[0073] The modified biological materials disclosed herein can be used in reconstructive surgery including but not limited to hernia repair, breast reconstruction, abdominal wall repair, chest wall repair, urological repair, bone and cartilage implantation, gynecological repair, plastic surgery, tendon repair, burn and wound treatment and vein/artery repair.

[0074] The modified biological materials disclosed herein are optionally packaged in a sterile container for transport and storage.

[0075] The following examples are set forth to assist in understanding the invention and should not be construed as specifically limiting the invention described and claimed herein. Such variations of the invention, including the substitution of all equivalents now known or later developed, which would be within the purview of those skilled in the art, and changes in formulation or minor changes in experimental design, are to be considered to fall within the scope of the invention incorporated herein.

EXAMPLES

Example 1: Solvent exchange

[0076] FlexHD® samples were immersed in absolute ethanol for 15 minutes with gentle shaking. This step was repeated until a total of 3 cycles had been completed. The samples were then immersed in dichloromethane for 15 minutes with gentle shaking. This step was repeated once more so that a total of 2 cycles were completed. The samples were then placed in a vacuum oven at 30°C for 1 hour to remove residual solvent.

Example 2: Plasma Activation

[0077] Lyophilized acellular biological material, or a sample dehydrated according to Example 1, was placed inside a plasma chamber using air or oxygen as the process gas. When the pressure inside the chamber was below 2 Torr, the plasma process was started. The samples were treated by plasma for 0.5 to 5 minutes depending on sample size, with the radio frequency level set to "HIGH" (30W).

Example 3: Attachment of Coupler to Activated Sample

[0078] Immediately after processing according to Example 2, samples were immersed in 50 mL of tetrahydrofuran (THF) containing 175 mg of 1,1'-carbonyldiimidazole (CDI) for 30 minutes in a 50 mL Falcon tube at room temperature to yield samples to which CDI was chemically bound to the samples. For lyophilized samples, this example was carried out in a desiccator connected to a vacuum pump, vacuumed to a pressure of about 30-60 Torr for about 5-15 minutes to remove air bubbles inside the sample.

Example 4: Attachment of Antibiotic

[0079] The CDI-bound samples of Example 3 were rinsed with dry THF and immersed in 50 mL of an aqueous gentamicin solution (10 mg/mL) at room temperature for 2 hours to bind the gentamicin to the sample. Following the gentamicin conjugation, the tissue samples were washed with deionized water and then soaked in 1X PBS for at least 16 hours to remove any unbound gentamicin.

Example 5: Gentamicin Retention vs. Untreated Control

[0080] Samples prepared according to Example 4 were soaked in an infinite sink in PBS for 28 days. Untreated controls were also soaked under the same conditions. Gentamicin content (in nanograms per sample) was measured *in vitro* at 0, 24, 48, 168, 336, and 672 hours (Day 0, Day 1, Day 2, Day 7, Day 14, and Day 28, respectively). Figure 2 depicts the retention of gentamicin in samples taken at these points in time. As shown, the gentamicin content per sample dropped drastically between Day 0 and Day 1, which reflects the initial elution of unbound gentamicin from the sample. The amount of gentamicin per sample remained steadily between about 1500 ng and 500 ng from Day 1 to Day 28.

Example 6: Gentamicin Retention vs. Soaked Control

[0081] Samples prepared according to Example 4 were compared with: (i) samples that were soaked in gentamicin but not treated with oxygen plasma or CDI, (ii) samples that were dehydrated and exposed to air plasma (but not treated with CDI) and then soaked in gentamicin, and (iii) samples that were not treated at all. All samples were soaked in an infinite sink in PBS for 14 days. Gentamicin content (in nanograms per sample) was measured *in vitro* at 0, 24, 96, 168, and 336 hours (Day 0, Day 1, Day 4, Day 7, and Day 14, respectively). Figure 3 depicts the retention of gentamicin in samples taken at these points in time. As shown, the gentamicin content of the treated sample dropped drastically between Day 0 and Day 1, which reflects the initial elution of unbound gentamicin from the sample. The amount of gentamicin in the treated sample remained steadily between about 70 ng and 85 ng from Day 1 to Day 14. After Day 0, the control samples drastically dropped to no more than about 10 ng of gentamicin from Day 1 to Day 14. This data demonstrates that preparing a sample according to the direct plasma process described in Examples 1-4 enables it to retain significantly more gentamicin than just soaking the sample in gentamicin in the absence of CDI and oxygen plasma.

Example 7: Bacterial Outgrowth with *E. Coli*

[0082] Samples prepared according to Example 4, as well as untreated controls, were implanted into non-inoculated subcutaneous pockets of rats for a set amount of time and then explanted. The treated and untreated samples were then evaluated in an outgrowth assay in which bacteria were seeded onto the samples and then placed in bacterial growth media. Figure 4 depicts bacterial growth in each of the treated and untreated samples at Day 0, Day 4, Day 7, and Day 14. As shown, the explanted treated tissue retains antimicrobial activity after almost 7 days of implantation time.

[0083] It is noted that although the *in vitro* data from Examples 5 and 6 demonstrate that gentamicin was retained in the sample for at least 28 days, the *in vivo* data of this Example 7 shows cessation of efficacy after 7 days. This can be explained by the natural

remodeling process going on in the organism's body. As soon as the sample is implanted, it becomes coated with serum proteins as the body begins to incorporate and remodel it. Over time, the foreign material will become coated with new connective tissue and nascent vasculature. In the case of the explanted tissue, by Day 7, the sample has already been coated with connected tissue and is well on its way to being remodeled. In other words, although the gentamicin in the matrix of the sample is likely still bound, it has been sequestered by the newly formed connective tissue and is not exposed to the bacterial colonies in the outgrowth assay.

Example 8: Attachment of Antibiotic Using a Vacuum

[0084] Plasma-activated samples were placed in solution containing CDI. For lyophilized samples, the solution was placed in a desiccator connected to a vacuum pump, and the desiccator was vacuumed to a pressure of about 30 to 60 Torr for 5 to 15 minutes to remove air bubbles inside the sample to achieve better solution penetration. After 30 minutes to 2 hours of activation under agitation, samples were immersed in another solution containing a target bioactive molecule for 2 to 8 hours under agitation in order to attach the molecule to the surface of the sample. The samples were then washed with deionized water 5 to 10 times to remove any unattached molecules.

[0085] Figure 5 shows that gentamicin retention is significantly improved after introducing a short vacuum during the CDI activation step. The antimicrobial efficacy is also enhanced dramatically in the bacteria outgrowth assay, as shown in Figure 6.

Example 9: Bacterial Outgrowth Assay

[0086] An overnight culture of *E. coli* was diluted down to OD_{600nm}=0.03 (approximately 10^6 CFU/mL) in LB (Lennox) broth (Fisher) and 20 µL of this bacterial dilution was pipetted onto 6 mm biopsy punches of untreated human dermis as a control or plasma gentamicin-treated dermis. The samples incubated for 10 minutes at room temperature before each one was placed into a sterile 50 mL Erlenmeyer flask containing

10mL LB media. The flasks were shaken at 37°C at 225 RPM in an orbital shaker (Lab-Line), and optical density measurements were taken every hour to monitor bacterial growth until turbidity was achieved or no growth was observed.

[0087] As depicted in Figure 7, at 2, 3, and 4 hours of outgrowth assay, there were elevated numbers of *E. coli* in the untreated tissue compared with the treated samples. The control samples showed an exponential growth pattern while the treated samples showed no growth, indicative of complete bacterial eradication during the soaking period.

Example 10: Fibroblast Biocompatibility

[0088] Human dermal fibroblasts derived from neonatal foreskin were grown to confluency at 37°C in a CO_2 incubation chamber, in essential media supplemented with 10% FBS and pen/strep and split in a 1:10 dilution. All studies were performed with passage four cells. Plasma treated and controls samples were prepared using 6 mm skin biopsy cores to create test samples. The samples were soaked in 70% EtOH for 30 minutes and then placed into essential media for an overnight soak for at least 16 hours. The soak media was aspirated and the samples were placed individually into wells of a 24-well plate and seeded with 10,000 fibroblasts per sample delivered in 50 µL of complete media. After 30 minutes, an additional 500 µL of complete media was added to each well. Cells were assayed for metabolic activity at 1 hour and 24 hours after post-seeding using the TACS MTT assay (Trevigen, MD, USA) according to the manufacturer's instructions. Standard curves were seeded 24 hours prior to performing the MTT assay for the 24 hour samples. For the 1 hour samples, the standard curve was prepared at the time of test article preparation, which may not have allowed for metabolic activity to resume. For each time point, n=4 treated and control samples were tested. In addition four treated and control samples were measured for MTT activity without being cell seeded.

[0089] Readings from these samples were not different between treated and untreated test samples. Therefore, the dermis background to be subtracted from the cell seeding

values was determined by averaging these 8 samples together. The MTT activity for the 1 hour samples was determined by assaying the dermis and media samples together, however, it was observed that significant portion of the formazin product was sequestered in the dermis. Therefore, for the 24 hour study, the media and dermis were assayed separately and background form control dermis was also assayed separately.

[0090] The results of the fibroblast assay are shown in Figure 8. There was no significant different between the treated and control samples at either time point.

Example 11: Cell Viability

[0091] Cell viability was assessed at 1 and 4 days of exposure to the dermal matrices. For each timepoint, eight samples from each test group were placed with the epidermal surface facing up into the wells of a 96 well TCPS plate. The samples were washed with 100 µL of complete media for 5 minutes followed by aspiration of the media and placement of either cells or fresh media without cells onto the samples. For the cell-seeded samples, 10,000 human dermal fibroblasts were seeded onto 4 of the 8 human dermis test samples in 100 µL of complete media.

[0092] At the time of initial test sample cell-seeding for the 1 day cell viability samples, a standard curve was prepared in duplicate using the human dermal fibroblasts. To produce the standard curve, 25,000, 12,500, and 6,250 cells were seeded directly onto the TCPS wells. For the 4 day study, the standard curve was prepared three days post cell seeding. The media was exchanged every 24 hours during the study and just prior to the termination of the assay.

[0093] The CellTiter 96 Aqueous One Solution Assay (Promega, USA) was used to assess cell viability at 1 and 4 days post cell seeding. The kit was used according to the manufacturer's instruction with a few minor modifications made to accommodate use with human dermis tissue. To each well of the standard curve and the test samples, 20 µL of MTS solution was added. After 90 minutes, the dermis was gently transferred to

clean wells leaving the media behind. The colorimetric intensity of each well was measured at 490 nm in both the dermis and media containing wells. The non-cell seeded blanks were averaged together for both the media and dermis and subtracted from the cell-seeded samples to obtain normalized readings. It was necessary to use separate blanks for each of the treatment because it was observed that modifications made to the tissue could change the background color levels of both the media and dermis. The total normalized color observed in the media and dermis was added together and the cell number was determined using the standard curve.

[0094] Figure 9 depicts the results using the MTS assay. Relative cell number was extrapolated from a standard curve produced from dermal fibroblasts grown on TCPS. There was no significant difference between control and plasma treated samples and controls samples at either point in time.

Example 12: Gentamicin Quantification

[0095] The amount of gentamicin loaded into the human dermis was measured in samples prepared as described in Example 11. Three separate dermis samples from the control and plasma groups were weighed just prior to gentamicin determination. The samples were placed into 5mL of 0.25M HCl in borosilicate test tubes covered with aluminum foil. The samples were autoclaved at 121°C with a 1 hour cycle in a Steris autoclave (SG-120 Scientific Gravity Sterilizer) to degrade and solubilize the collagen. In earlier studies, the stability of gentamicin in 0.25M HCl and under autoclave conditions was assessed. No significant loss of gentamicin detection was observed.

[0096] Following the autoclave step, the gentamicin was measured using a commercially available ELISA kit (BioO, USA) according to the instruction included in the kit. Human dermis samples were handled according to the protocol as if they were milk samples. Using anticipated drug loads determined from earlier zone of inhibition studies, the autoclaved samples were diluted into 1X sample extraction buffer 1:40. Standards provided with the kit were used to create a standard curve and subsequently to

calculate total gentamicin in each sample. The gentamicin load was expressed as parts per million (ppm) by dividing the weight of gentamicin by the weight of the dermis sample.

[0097] Figure 10 depicts the results of the assay. Plasma treated samples contained an average of 24 ppm of residual gentamcin. The control tissue, which did not contain any gentamicin, showed an ELISA value of 0.1 ppm, which represents the baseline background level inherent to the assay.

Example 13: Antimicrobial Activity

[0098] The experimental procedure described in the quantitative method of ASTM 2180 was modified and used to evaluate the antimicrobial effectiveness of gentamicin bound to treated and untreated human dermis. The surface area of the test samples was reduced from 3 x 3 cm squares to 6 mm diameter round dermal biopsy cores. The slurry inoculum volume was reduced from 0.5-1.0 to 6 µL, which provided the required 1mm depth of slurry across the sample. The neutralizing broth volume was therefore reduced to 600 µL. Finally, the duration of the experiment was increased from 24 hours to 48 hours of exposure of the inoculum to the test surfaces. Consequently, the moisture level of the dermis samples had to be controlled by placing the samples on hydrated squares of sterile filter paper moistened with 200 µL sterile PBS in order to recover viable cells from the untreated samples at 2 days post-slurry inoculation. The filter paper squares were checked form moisture level daily and approximately 200 µL of PBS was added each day.

[0099] Briefly, 18 hour bacterial cultures of *S. aureus* (ATCC 25923 and 10390), *E. coli* (ATCC 25404), or *P. aeruginosa* (ATCC 27853) were grown and diluted to OD_{600}=1.0, 0.5, 0.5, or 0.5, respectively, in an agar slurry containing 0.85% NaCl and 0.3% agar which was sterilized and equilibrated in a water bath to 45°C. Treated and untreated (control) dermal biopsy cores (in triplicate for each time point) were placed into sterile 35 x 10 mm petri dishes containing 2.4 cm^2 sterile filter papers, and 6 µL of

inoculated slurry was pipetted onto each sample. The samples were allowed to gel for 10 minutes at room temperature before 200 µL sterile PBS was pipetted onto the filter paper squares to keep the cores hydrated. The petri dishes were placed into the 37°C incubator for a specified contact time (1 hour, 1 day, or 2 days). Following the specified contact time, the untreated and treated samples were collected in 600 µl neutralizing broth (TSB for *S. aureus* and *P. aeruginosa;* LB for *E. coli*) to form a 1:100 dilution of the initial inoculum. The samples were sonicated for 1 min in a non-cavitating sonic bath, followed by 1 min of vigorous mechanical vortexing to release the agar slurry from the samples. Serial dilutions were performed with the neutralizing broth, plated (TSA for *S. aureus* and *P. aeruginosa;* LB for *E. coli*), and incubated overnight at 37°C. Percent reduction was calculated by comparing the CFU recovered from the untreated versus treated samples.

[00100] *K. pneumoniae, C. perfringens* and *P. mirabilis* were tested using almost identical conditions with a 24 hour contact time at Gibraltar Labs in Fairfield, NJ. For their studies, all bacteria were cultured for 18 hours and used at OD 0.5 in the agar slurry. The only other difference was that 1 ml was used as the volume for the neutralizing broth in the Gibraltar studies.

[00101] The internal ASTM 2180 experiments examined the antimicrobial effects of plasma treated tissue at three different exposure times (1 hour, 1 day, and 2 days) against *E. coli*, *P. aeruginosa*, and *S. aureus* 25923 and 10390. The percent killing and log bacterial reduction for the plasma treated samples are shown below in Table 1, while the percent killing and standard deviation for the plasma treated sample data set are shown in Figure 11.

Table 1. Percent killing and log bacterial reduction for plasma treated samples

Bacterial Species	Percent Killing			Log Reduction		
	1 Hour	1 Day	2 Days	1 Hour	1 Day	2 Days
E. coli 25404	99.94	100	100	3.19	4.73	5.34
P. aeruginosa 27853	100	100	100	4.75	6.13	6.64
S. aureus 25923	96	99.99	99.99	1.36	4.67	4.64
S. aureus 10390	81.3	99.7	99.9	0.72	2.49	2.84

[00102] The external ASTM 2180 experiments performed at Gibraltar Labs in Fairfield, NJ utilized a single 24 hour (1 day) exposure time of the bacterial slurry on the tissue and three bacterial strains: *Klebsiellapenumoniae*, *Proteus mirabilis*, and *Clostridium perfringens* (vegetative cells). They tested the antimicrobial activity of plasma treated dermis. The percent killing and log bacterial reduction for these treated samples can be found in Table 2 below. Both of the treated samples resulted in 99.999% killing with a 5-log reduction of *K. pneumonia*, 99.99% killing with a 4.34-log reduction of *Proteus mirabilis*, and 99.999% killing with a 5.61-log reduction of *C. perfringens*. Both treated tissue samples were effective surface antimicrobial against all three of these bacterial species with a 24 hour contact time.

Table 2. Percent killing and log bacterial reduction in plasma treated dermis against three bacterial strains through external ASTM 2180 testing.

Bacterial Species	Percent Killing	Log Reduction
Klebsiella pneumoniae	99.999	5
Proteus mirabilis	99.99	4.34
Clostridium perfringens (vegetative cells)	99.999	5.61

Example 14: Bone Tissue Treatment Process

[00103] Bone samples were soaked in PBS or 0.9% saline solution at 37°C for three days. Bone samples were then immersed in 100% ethanol with gentle shaking for 15 minutes. This immersion step was repeated until a total of three cycles had been completed. Finally the bone samples were vacuum dried at room temperature for 2 hours.

[00104] The dried bone samples were them treated with oxygen plasma for 30 seconds. Immediately thereafter, the bones were immersed in 1,1'-carbonyldiimidazole solution for 30 minutes, and then immersed in a 10 mg/mL gentamicin aqueous solution for 2 hours followed by washing with deionized water. Finally, the bone samples were soaked in PBS for three days to remove any unbound gentamicin.

[00105] The present invention is not to be limited in scope by the specific embodiments disclosed in the examples which are intended as illustrations of a few aspects of the invention and any embodiments that are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art and are intended to fall within the scope of the appended claims.

We Claim:

1. A composition comprising:
a biological material activated by oxygen plasma and
a ligand bound to a surface of the biological material.

2. The composition of claim 1, wherein the biological material is collagen.

3. The composition of claim 1, wherein the biological material is tissue.

4. The composition of claim 1, wherein the biological material is bone.

5. The composition of claim 3, wherein the tissue is dermal tissue.

6. The composition of claim 5, wherein the dermal tissue is acellular.

7. The composition of claim 5, wherein the dermal tissue is derived from a bacterial source.

8. The composition of claim 5, wherein the dermal tissue is derived from a mammalian source.

9. The composition of claim 8, wherein the mammalian source is selected from the group consisting of humans, non-human primates, pigs, cows, horses, goats, sheep, dogs, cats, rabbits, guinea pigs, gerbils, hamsters, rats, mice and transgenic variants thereof.

10. The composition of claim 8, wherein the dermal tissue is derived from a human cadaver.

11. The composition of claim 1, wherein the biological material is dehydrated.

12. The composition of claim 11, wherein the biological material is dehydrated by lyophilization.

13. The composition of claim 11, wherein the biological material is dehydrated by a solvent exchange process.

14. The composition of claim 13, wherein the solvent exchange process comprises soaking the biological material in a solvent.

15. The composition of claim 13, wherein the solvent exchange process comprises soaking the biological material in a hydrophilic solvent followed by soaking the biological material in an organic solvent.

16. The composition of claim 13, wherein the solvent exchange process comprises soaking the biological material in a hydrophilic solvent followed by soaking the biological material in an organic solvent followed by placing the biological material under vacuum.

17. The composition of claim 14, wherein the solvent is selected from the group consisting of ethanol, n-propanol, iso-propanol, n-butanol, sec-butanol, iso-butanol,, tert-butanol, allyl alcohol, benzyl alcohol, furfuryl alcohol, chclohexanol, benzyl alcohol, tetrahydrofuran, chloroform, methyl ethyl ketone, benzene, and one of the following: ethyl acedtate, cyclohexane, benzene, chloroform, carbon tetrachloride, ethylene chloride, acetonitrile, toluene, methyl ethyl ketone, n-hexane, n-heptane, carbon disulfide, diethyl ketone, n-propyl acetate, toluene, carbon tetrachloride, methanol, acetone, aqueous mixtures thereof and combinations thereof.

18. The composition of claim 15, wherein the organic solvent is selected from the group consisting of dichloromethane, tetrahydrofuran ethyl ether, methyl t-butyl ether, pentane, hexane and a mixture thereof.

19. The composition of claim 13, wherein the biological material is placed under vacuum at room temperature or with heat.

20. The composition of claim 1, wherein the ligand is a coupling agent.

21. The composition of claim 20, wherein the coupling agent is selected from the group consisting of 1,1'-carbonyldiimidazole, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride, N,N'-disuccinimidyl carbonate, N-hydroxysuccimimidyl chloroformate, isocyanate, salts thereof, and a combination thereof.

22. The composition of claim 1, further comprising a second ligand bonded to the composition.

23. The composition of claim 22, wherein the second ligand is a pharmacological agent.

24. The composition of claim 23, wherein the ligand creates a covalent bond between the pharmacological agent and the biological material.

25. The composition of claim 1, wherein the ligand is a biologically active molecule.

26. The composition of claim 23, wherein the pharmacological agent is an antimicrobial agent.

27. The composition of claim 26, wherein the antimicrobial agent is an antibiotic.

28. The composition of claim 27, wherein the antibiotic has an available nucleophilic group.

29. The composition of claim 26, wherein the antimicrobial agent is selected from the group consisting of amikacin, gentamicin, kanamycin, neomycin, netilmicin, tobramycin, paromomycin, geldanamycin, herbimycin, loracarbef, ertapenem, doripenem , imipenem/cilastatin, meropenem, cefadroxil, cefazolin, cefalotin, cefalexin, cefaclor, cefamandole, cefoxitin, cefprozil, cefuroxime, cefditoren, cefoperazone, cefotaxime, cefpodoxime, ceftazidime, ceftibuten, ceftizoxime, ceftriaxone, cefepime, ceftaroline fosamil, ceftobiprole, teicoplanin, vancomycin, telavancin, clindamycin, lincomycin, daptomycin, azithromycin, clarithromycin, dirithromycin, erythromycin, roxithromycin, troleandomycin, telithromycin, spectinomycin, spiramycin, aztreonam, furazolidone, nitrofurantoin, amoxicillin, ampicillin, azlocillin, carbenicillin, cloxacillin, dicloxacillin, flucloxacillin, mezlocillin, methicillin, nafcillin, oxacillin, penicillin G, penicillin V, piperacillin, temocillin, ticarcillin, amoxicillin/clavulanate, ampicillin/sulbactam, piperacillin/tazobactam, ticarcillin/clavulanate, bacitracin, colistin, polymyxin b, ciprofloxacin, enoxacin, gatifloxacin, levofloxacin, lomefloxacin, moxifloxacin, nalidixic acid, norfloxacin, ofloxacin, trovafloxacin, grepafloxacin, sparfloxacin, temafloxacin, mafenide , sulfonamidochrysoidine, sulfacetamide, sulfadiazine, silver sulfadiazine, sulfamethizole, sulfamethoxazole, sulfanilimide, sulfasalazine, sulfisoxazole, trimethoprim, trimethoprim-sulfamethoxazole, demeclocycline, doxycycline, minocycline, oxytetracycline, tetracycline, clofazimine, dapsone, capreomycin, cycloserine, ethambutol, ethionamide, isoniazid, pyrazinamide, rifampicin, rifabutin, rifapentine, streptomycin, arsphenamine, chloramphenicol, fosfomycin, fusidic acid, linezolid, metronidazole, mupirocin, platensimycin, quinupristin/dalfopristin, rifaximin, thiamphenicol, tigecycline, tinidazole, pharmaceutically acceptable salts thereof, and a combination thereof.

30. The composition of claim 26, wherein the antimicrobial agent is selected from the group consisting of the list of chlorhexidine, biguanides, quaternary ammonium compounds, pharmaceutically acceptable salts thereof, and a combination thereof.

31. The composition of claim 1, wherein the activated biological material has surface hydroxyl groups, surface carboxyl groups, or a combination thereof.

32. The composition of claim 1, wherein the source of oxygen plasma is O_2.

33. The composition of claim 1, wherein the source of oxygen plasma is air.

34. The composition of claim 1, wherein the ligand is covalently bound to one or more chemical moieties on the surface of the biological material.

35. The composition of claim 23, wherein from about 5% to about 95% of the pharmacological agent is maintained in the composition after soaking in an infinite sink in phosphate buffer saline for 24 hours.

36. The composition of claim 23, wherein from about 50% to about 95% of the pharmacological agent is maintained in the composition after soaking in an infinite sink in phosphate buffer saline for 24 hours.

37. The composition of claim 23, wherein from about 5% to about 95% of the pharmacological agent is maintained in the composition after soaking in an infinite sink in phosphate buffer saline for 4 days.

38. The composition of claim 23, wherein from about 50% to about 95% of the pharmacological agent is maintained in the composition after soaking in an infinite sink in phosphate buffer saline for 4 days.

39. The composition of claim 23, wherein from about 5% to about 95% of the pharmacological agent is maintained in the composition after soaking in an infinite sink in phosphate buffer saline for 7 days.

40. The composition of claim 23, wherein from about 50% to about 95% of the pharmacological agent is maintained in the composition after soaking in an infinite sink in phosphate buffer saline for 7 days hours.

41. The composition of claim 23, wherein from about 5% to about 95% of the pharmacological agent is maintained in the composition after soaking in an infinite sink in phosphate buffer saline for 14 days hours.

42. The composition of claim 23, wherein from about 50% to about 95% of the pharmacological agent is maintained in the composition after soaking in an infinite sink in phosphate buffer saline for 14 hours.

43. The composition of claim 31 or 32, wherein the amount of pharmacological agent maintained after soaking in an infinite sink in phosphate buffer saline for 4 days is within 25% of the amount of pharmacological agent at 24 hours.

44. The composition of claim 35 or 36, wherein the amount of pharmacological agent maintained after soaking in an infinite sink in phosphate buffer saline for 4 days is within 10% of the amount of pharmacological agent at 24 hours.

45. The composition of claim 35 or 36, wherein the amount of pharmacological agent maintained after soaking in an infinite sink in phosphate buffer saline for 7 days is within 25% of the amount of pharmacological agent at 24 hours.

46. The composition of claim 35 or 36, wherein the amount of pharmacological agent maintained after soaking in an infinite sink in phosphate buffer saline for 7 days is within 10% of the amount of pharmacological agent at 24 hours.

47. The composition of claim 35 or 36, wherein the amount of pharmacological agent maintained after soaking in an infinite sink in phosphate buffer saline for 14 days is within 25% of the amount of pharmacological agent at 24 hours.

48. The composition of claim 35 or 36, wherein the amount of pharmacological agent maintained after soaking in an infinite sink in phosphate buffer saline for 14 days is within 10% of the amount of pharmacological agent at 24 hours.

49. A composition comprising a biological material activated by oxygen plasma.

50. A composition comprising:
acellular tissue;
a coupling agent bound to the acellular tissue; and
a pharmacological agent bound to the coupling agent.

51. A method of treating biological material comprising: contacting biological material with oxygen plasma to form activated biological material.

52. The method of claim 51, further comprising bonding a ligand to the activated biological material.

53. The method of claim 51, wherein the biological material is collagen.

54. The method of claim 51, wherein the biological material is tissue.

55. The method of claim 51, wherein the biological material is bone.

56. The method of claim 54, wherein the tissue is dermal tissue.

57. The method of claim 56, wherein the dermal tissue is acellular.

58. The method of claim 56, wherein the dermal tissue is derived from a bacterial source.

59. The method of claim 56, wherein the dermal tissue is derived from a mammalian source.

60. The method of claim 59, wherein the mammalian source is selected from the group consisting of humans, non-human primates, pigs, cows, horses, goats, sheep, dogs, cats, rabbits, guinea pigs, gerbils, hamsters, rats, mice and transgenic variants thereof.

61. The method of claim 56, wherein the dermal tissue is derived from a human cadaver.

62. The method of claim 51, wherein the biological material is dehydrated.

63. The method of claim 62, wherein the biological material is dehydrated by lyophilization.

64. The method of claim 62, wherein the biological material is dehydrated by a solvent exchange process.

65. The method of claim 64, wherein the solvent exchange process comprises soaking the biological material in a solvent.

66. The method of claim 64, wherein the solvent exchange process comprises soaking the biological material in a hydrophilic solvent followed by soaking the biological material in an organic solvent.

67. The method of claim 64, wherein the solvent exchange process comprises soaking the biological material in a hydrophilic solvent followed by soaking the biological material in an organic solvent followed by placing the biological material under vacuum.

68. The method of claim 65, wherein the solvent is selected from the group consisting of ethanol, n-propanol, iso-propanol, n-butanol, sec-butanol, iso-butanol,, tert-butanol, allyl alcohol, benzyl alcohol, furfuryl alcohol, chlorohexanol, benzyl alcohol, tetrahydrofuran, chloroform, methyl ethyl ketone, benzene, and one of the following: ethyl acetate, cyclohexane, benzene, chloroform, carbon tetrachloride, ethylene chloride, acetonitrile, toluene, methyl ethyl ketone, n-hexane, n-heptane, carbon disulfide, diethyl ketone, n-propyl acetate, toluene, carbon tetrachloride, methanol, acetone, aqueous mixtures thereof and combinations thereof.

69. The method of claim 66, wherein the organic solvent is selected from the group consisting of dichloromethane, tetrahydrofuran ethyl ether, methyl t-butyl ether, pentane, hexane and a mixture thereof.

70. The method of claim 67, wherein the biological material is placed under vacuum at room temperature or with heat.

71. The method of claim 5, wherein the ligand is a coupling agent.

72. The method of claim 71, wherein the coupling agent is selected from the group consisting of 1,1'-carbonyldiimidazole, 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride, N,N'-Disuccinimidyl carbonate, N-hydroxysuccimimidyl chloroformate, isocyanate, salts thereof, and a combination thereof.

73. The method of claim 51, further comprising bonding a second ligand to the ligand bonded material.

74. The method of claim 73, wherein the second ligand is a pharmacological agent.

75. The method of claim 74, wherein the ligand creates a covalent bond between the pharmacological agent and the biological material.

76. The method of claim 51, wherein the ligand is a biologically active molecule.

77. The method of claim 74, wherein the pharmacological agent is an antimicrobial agent.

78. The method of claim 77, wherein the antimicrobial agent is an antibiotic.

79. The method of claim 78, wherein the antibiotic has an available nucleophilic group.

80. The method of claim 77, wherein the antimicrobial agent is selected from the group consisting of amikacin, gentamicin, kanamycin, neomycin, netilmicin, tobramycin, paromomycin, geldanamycin, herbimycin, loracarbef, ertapenem, doripenem , imipenem/cilastatin, meropenem, cefadroxil, cefazolin, cefalotin, cefalexin, cefaclor, cefamandole, cefoxitin, cefprozil, cefuroxime, cefditoren, cefoperazone, cefotaxime, cefpodoxime, ceftazidime, ceftibuten, ceftizoxime, ceftriaxone, cefepime, ceftaroline fosamil, ceftobiprole, teicoplanin, vancomycin, telavancin, clindamycin, lincomycin, daptomycin, azithromycin, clarithromycin, dirithromycin, erythromycin, roxithromycin, troleandomycin, telithromycin, spectinomycin, spiramycin, aztreonam, furazolidone, nitrofurantoin, amoxicillin, ampicillin, azlocillin, carbenicillin, cloxacillin, dicloxacillin, flucloxacillin, mezlocillin, methicillin, nafcillin, oxacillin, penicillin g, penicillin v, piperacillin, penicillin g, temocillin, ticarcillin, amoxicillin/clavulanate, ampicillin/sulbactam,

piperacillin/tazobactam, ticarcillin/clavulanate, bacitracin, colistin, polymyxin b, ciprofloxacin, enoxacin, gatifloxacin, levofloxacin, lomefloxacin, moxifloxacin, nalidixic acid, norfloxacin, ofloxacin, trovafloxacin, grepafloxacin, sparfloxacin, temafloxacin, mafenide , sulfonamidochrysoidine, sulfacetamide, sulfadiazine, silver sulfadiazine, sulfamethizole, sulfamethoxazole, sulfanilimide, sulfasalazine, sulfisoxazole, trimethoprim, trimethoprim-sulfamethoxazole, demeclocycline, doxycycline, minocycline, oxytetracycline, tetracycline, clofazimine, dapsone, capreomycin, cycloserine, ethambutol, ethionamide, isoniazid, pyrazinamide, rifampicin, rifabutin, rifapentine, streptomycin, arsphenamine, chloramphenicol, fosfomycin, fusidic acid, linezolid, metronidazole, mupirocin, platensimycin, quinupristin/dalfopristin, rifaximin, thiamphenicol, tigecycline, tinidazole, pharmaceutically acceptable salts thereof, and a combination thereof.

81. The method of claim 77, wherein the antimicrobial agent is selected from the group consisting of the list of chlorhexidine, biguanides, quaternary ammonium compounds, pharmaceutically acceptable salts thereof, and/or mixtures thereof.

82. The method of claim 51, wherein the source of oxygen plasma is O_2.

83. The method of claim 51, wherein the source of oxygen plasma is air.

84. The method of claim 51, wherein the biological material is contacted with oxygen plasma at high radio frequency level.

85. The method of claim 51, wherein the biological material is contacted with oxygen plasma for a time period from about 10 seconds to about 10 minutes.

86. The method of claim 51, wherein the biological material is contacted with oxygen plasma for a time period from about 30 seconds to about 5 minutes.

87. The method of claim 51, wherein the biological material is contacted with oxygen plasma at a pressure below about 1 Torr.

88. The method of claim 51, wherein the biological material is contacted with oxygen plasma at a pressure below about 0.5 Torr.

89. The method of claim 71, wherein the biological material is contacted with the coupling agent under pressure of about 20 to about 80 Torr.

90. The method of claim 71, wherein the biological material is contacted with the coupling agent under pressure of about 30 to about 60 Torr.

91. The method of claim 71, wherein the bonding comprises contacting the biological material with coupling agent for a time period from about 1 minute to about 24 hours.

92. The method of claim 71, wherein the bonding comprises contacting the biological material with the coupling agent under agitation.

93. The method of claim 74, wherein the pharmacological agent bonding comprises contacting the pharmacological agent with the ligand bonded material for a time period from about 1 minute to about 24 hours.

94. The method of claim 74, wherein the pharmacological agent bonding comprises contacting the ligand bonded material with the pharmacological agent under agitation.

95. The method of claim 74, further comprising washing the pharmacological agent bonded material.

96. The method of claim 74, further comprising sterilizing the pharmacological agent bonded biological material.

97. The method of claim 74, further comprising disinfecting the pharmacological agent bonded biological material.

98. A product prepared according to any one of the methods of claim 51-97.

99. A method of performing reconstructive surgery in a patient in need thereof comprising implanting or grafting a composition of any of claims 1-49 in a patient in need thereof.

100. A method of administering a drug to a patient in need thereof comprising implanting or grafting a composition of any of claims 23-30 and 35-48 in a patient in need thereof.

101. A method of dehydrating a biological material comprising soaking the biological material in a hydrophilic solvent followed by soaking the biological material in an organic solvent.

102. The method of claim 101, wherein further comprising placing the biological material under vacuum.

103. The method of claim 102, wherein the biological material is placed under vacuum at room temperature or with heat.

104. A method of dehydrating a biological material comprising soaking the biological material in a solvent miscible with or capable of forming an azeotrope with water.

105. The method of claim 104, further comprising soaking the biological material in a volatile organic solvent.

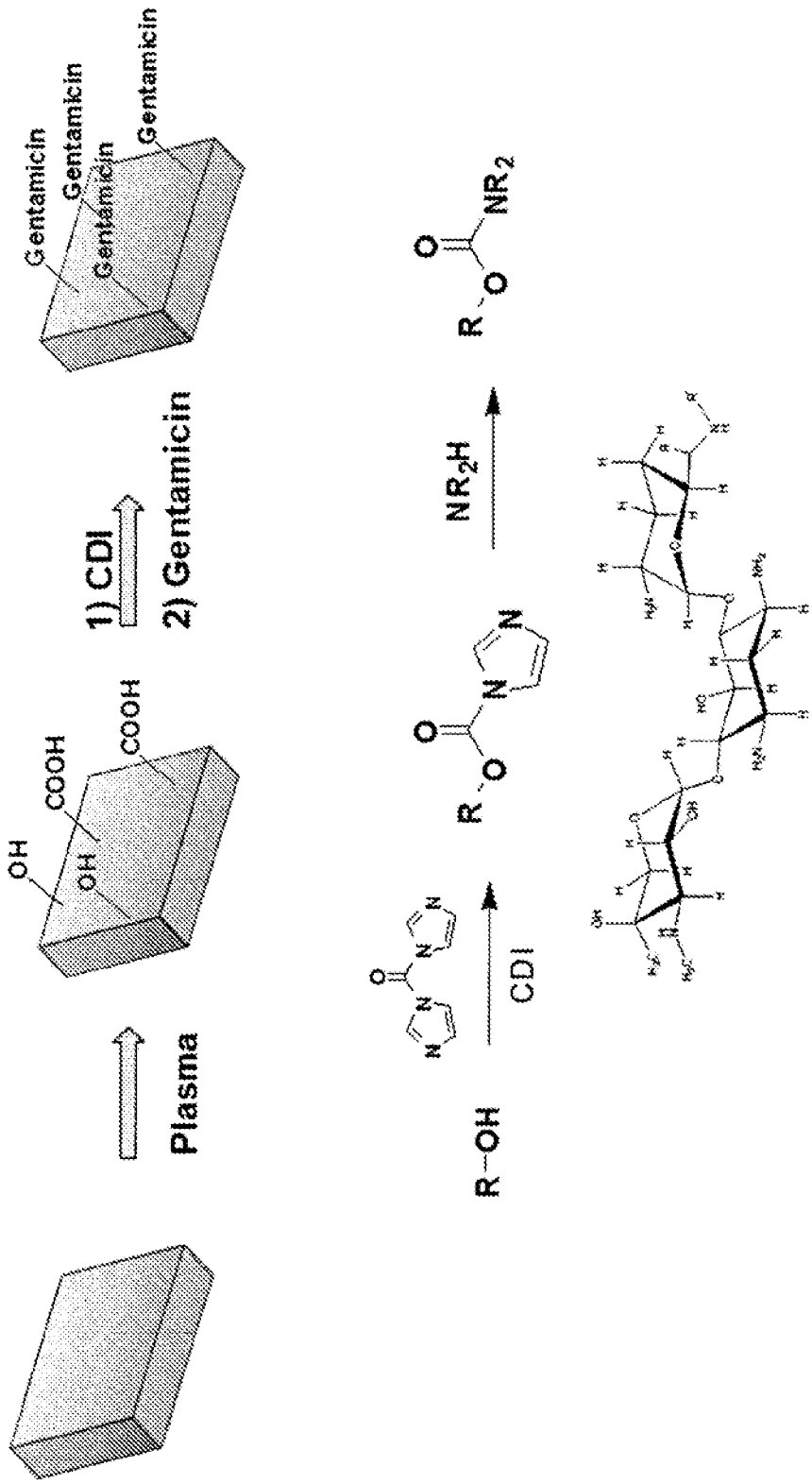
Gentamicin
Gentamicin
Gentamicin
Gentamicin
1) CDI
2) Gentamicin
COOH
COOH
COOH
OH
OH
Plasma
CDI
R–OH
NR_2H
NR_2

Figure 1

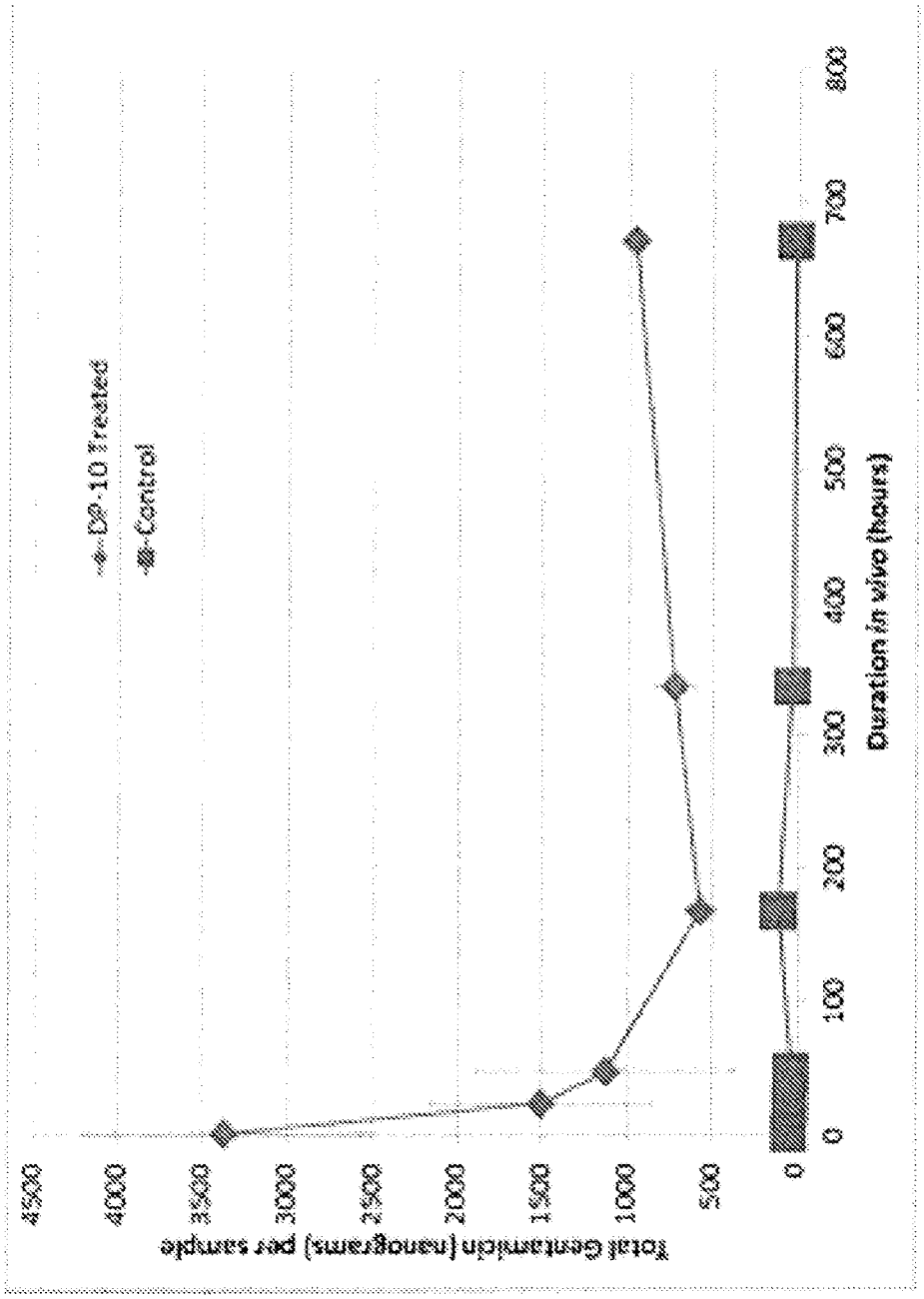
DP-10 Treated
Control
Total Gentamicin (nanograms) per sample
4500
4000
3500
3000
2500
2000
1500
1000
500
0
100
200
300
400
500
600
700
800
Duration in vivo (hours)

Figure 2

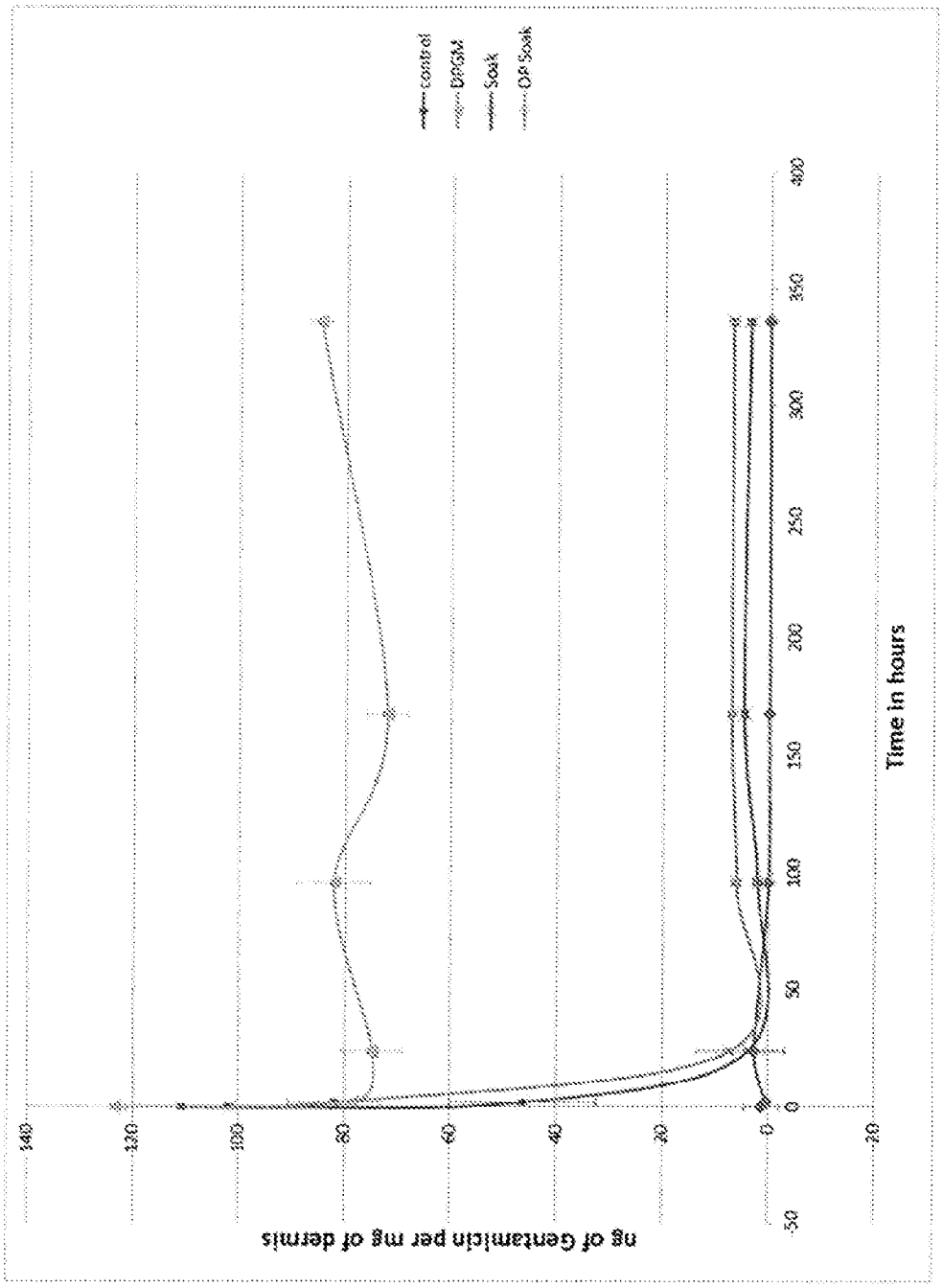
Control
DPSM
Soak
OS Soak
ng of Gentamicin per mg of dermis
Time in hours
140
120
100
80
60
40
20
0
-20
-50
0
50
100
150
200
250
300
350
400

Figure 3

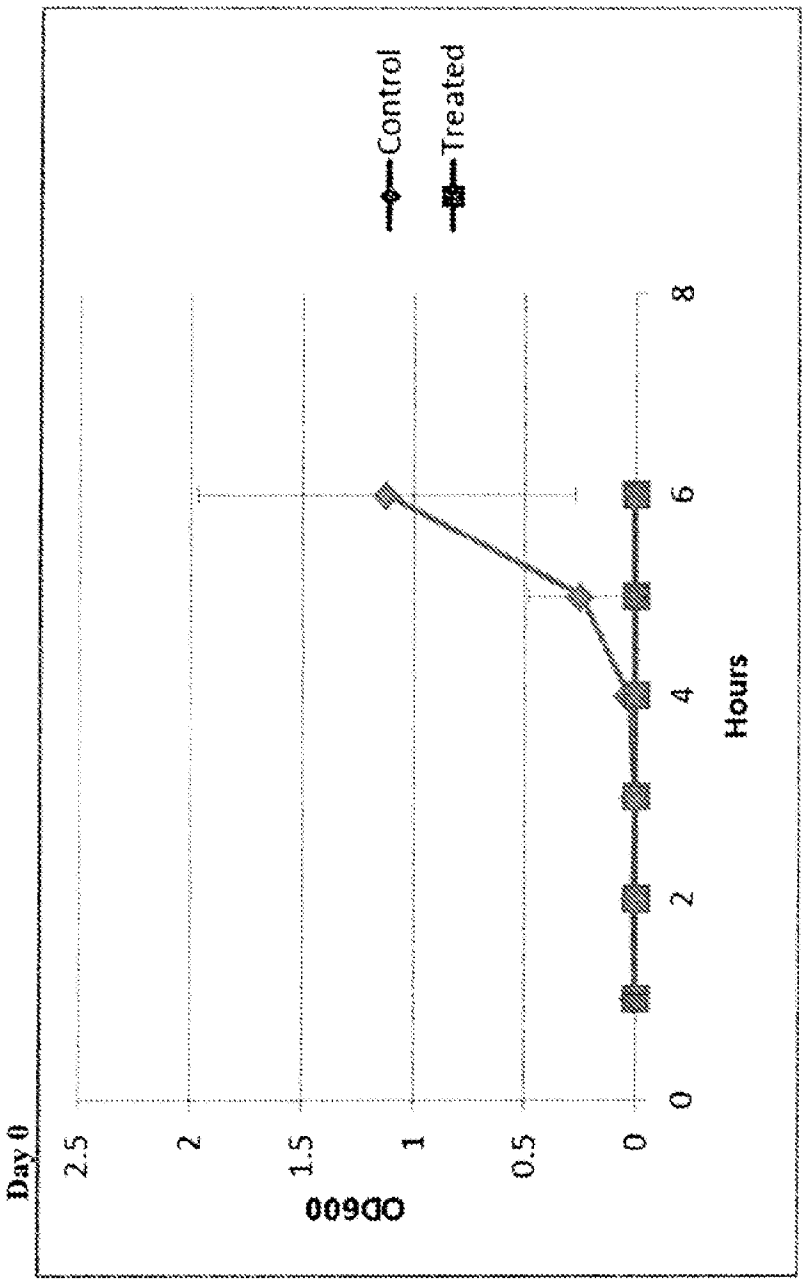

Figure 4A

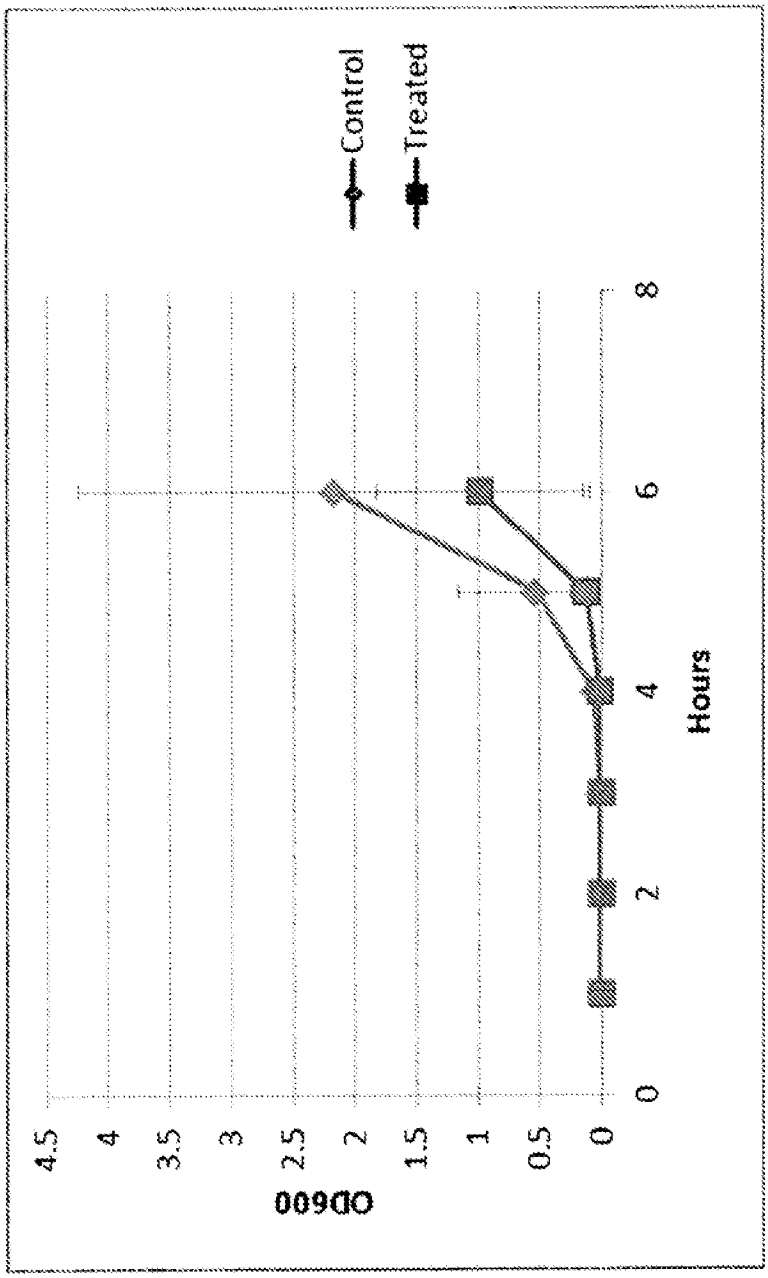

Figure 4B

Day 4

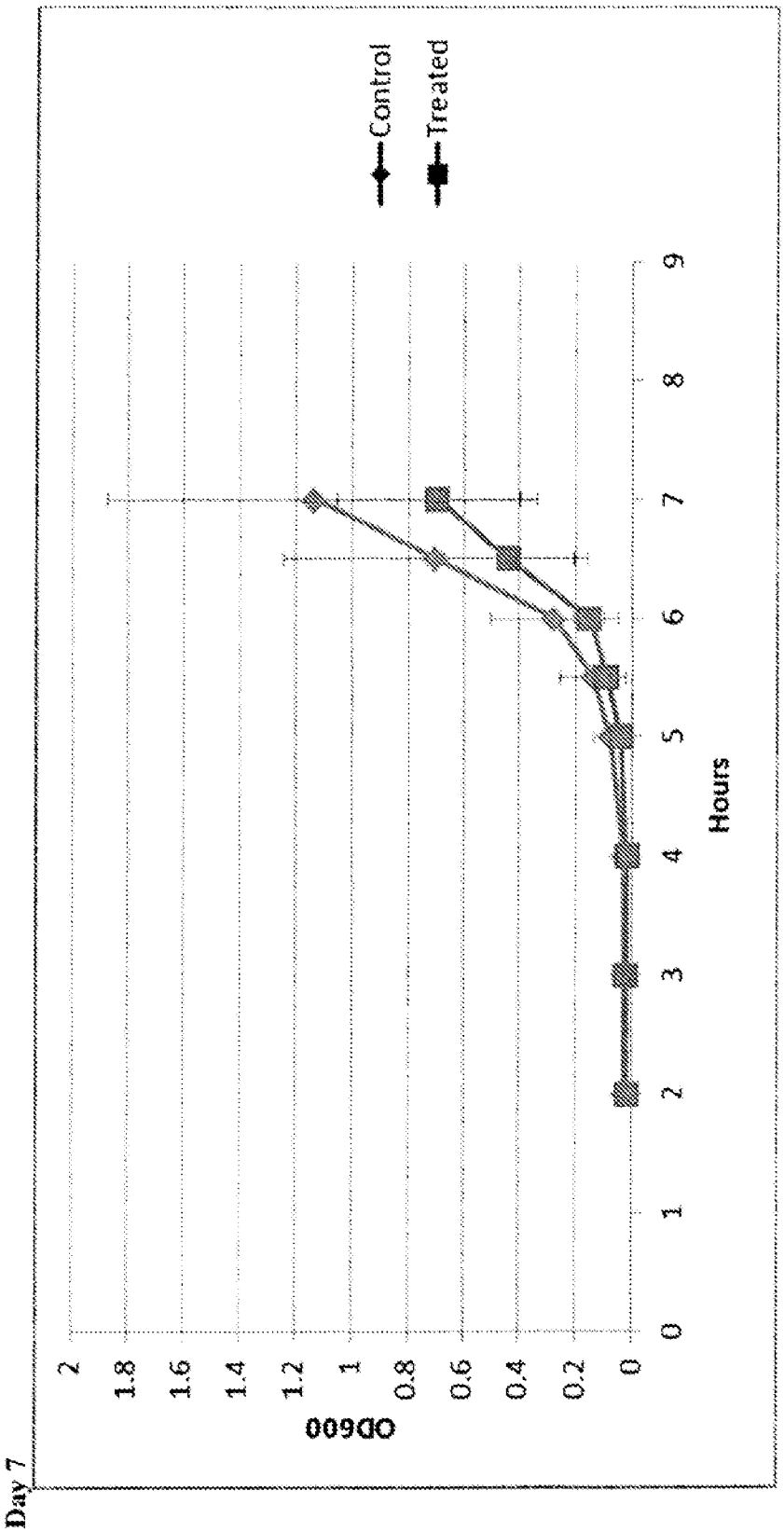
Day 7
Control
Treated
OD600
Hours
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
0
1
2
3
4
5
6
7
8
9

Figure 4C

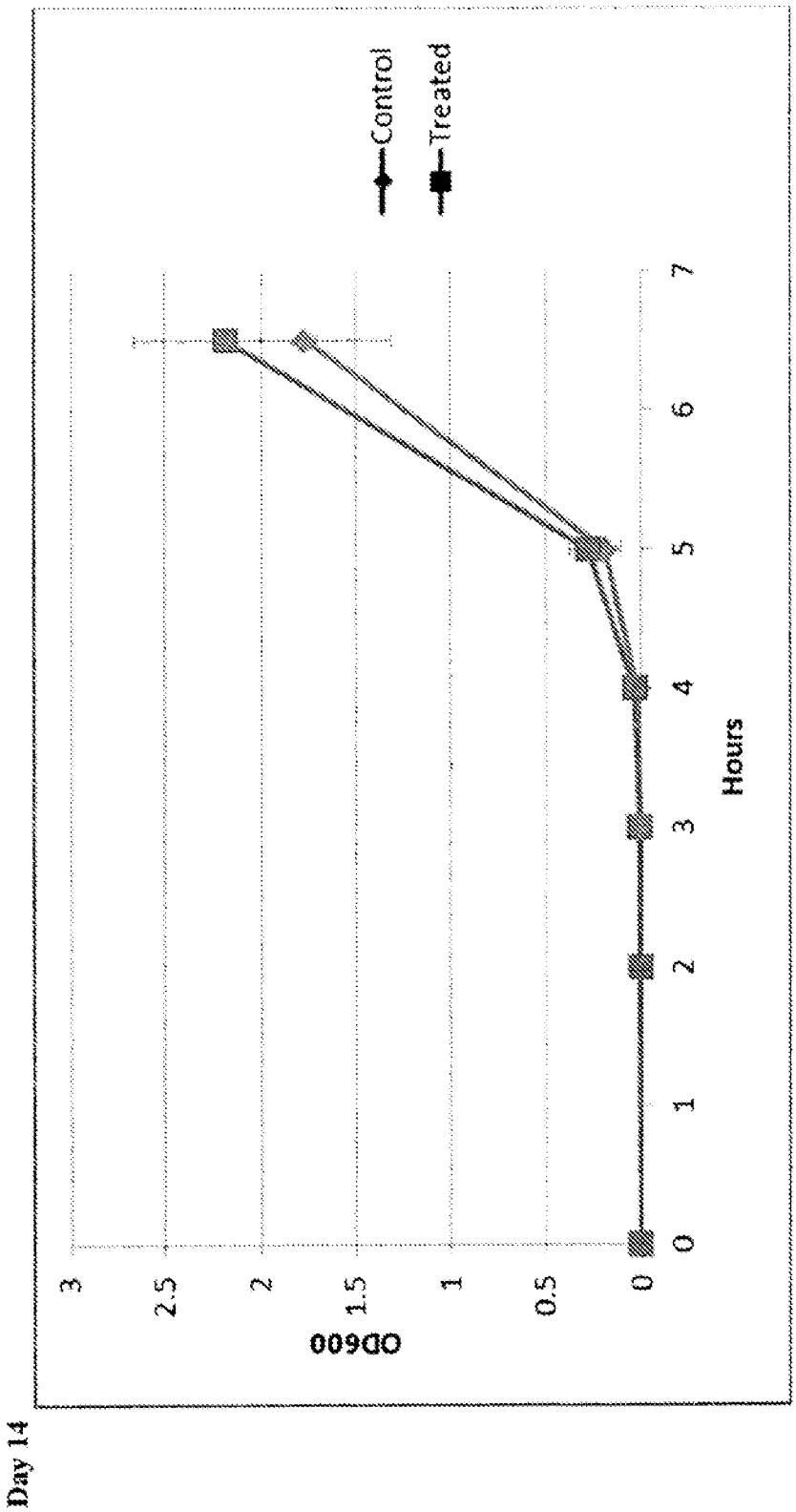

Figure 4D

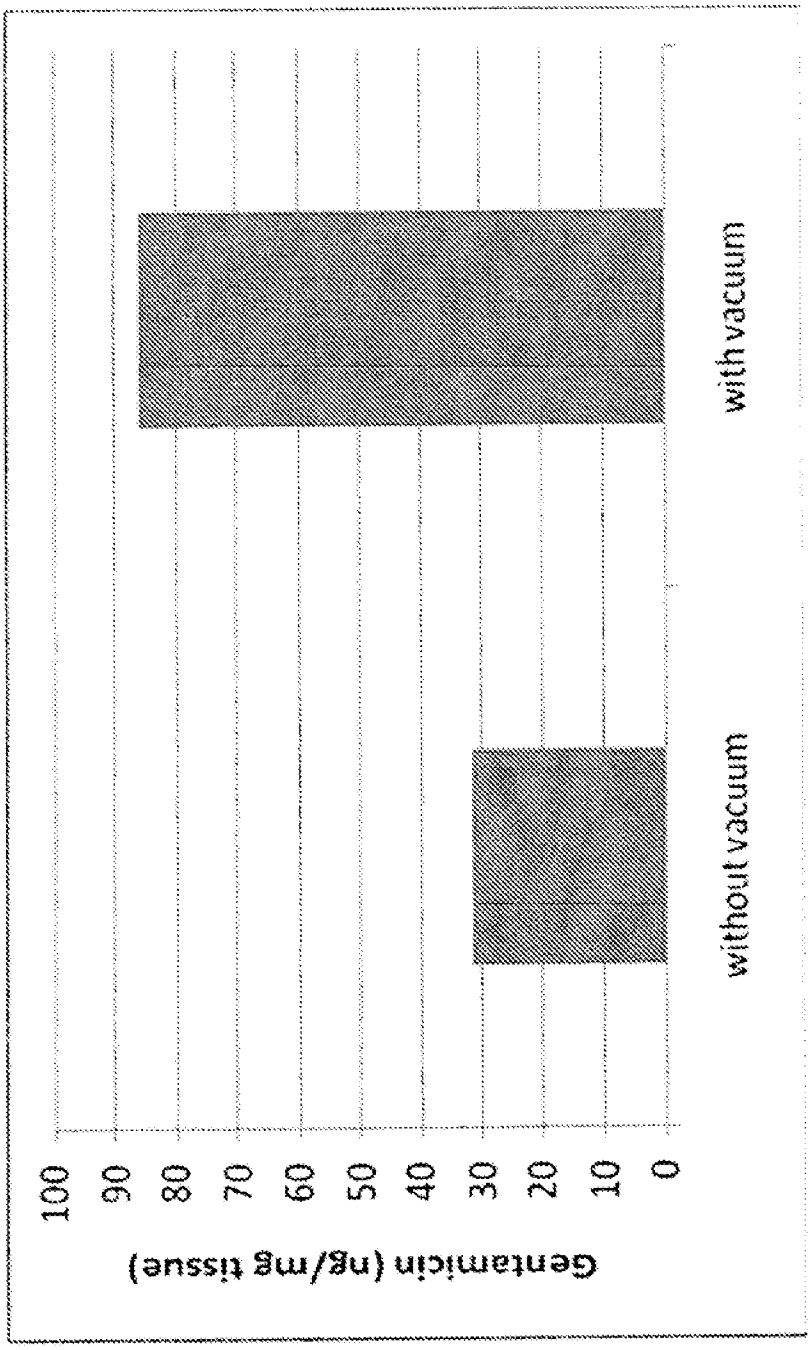
Gentamicin (ng/mg tissue)
100
90
80
70
60
50
40
30
20
10
0
without vacuum
with vacuum

Figure 5

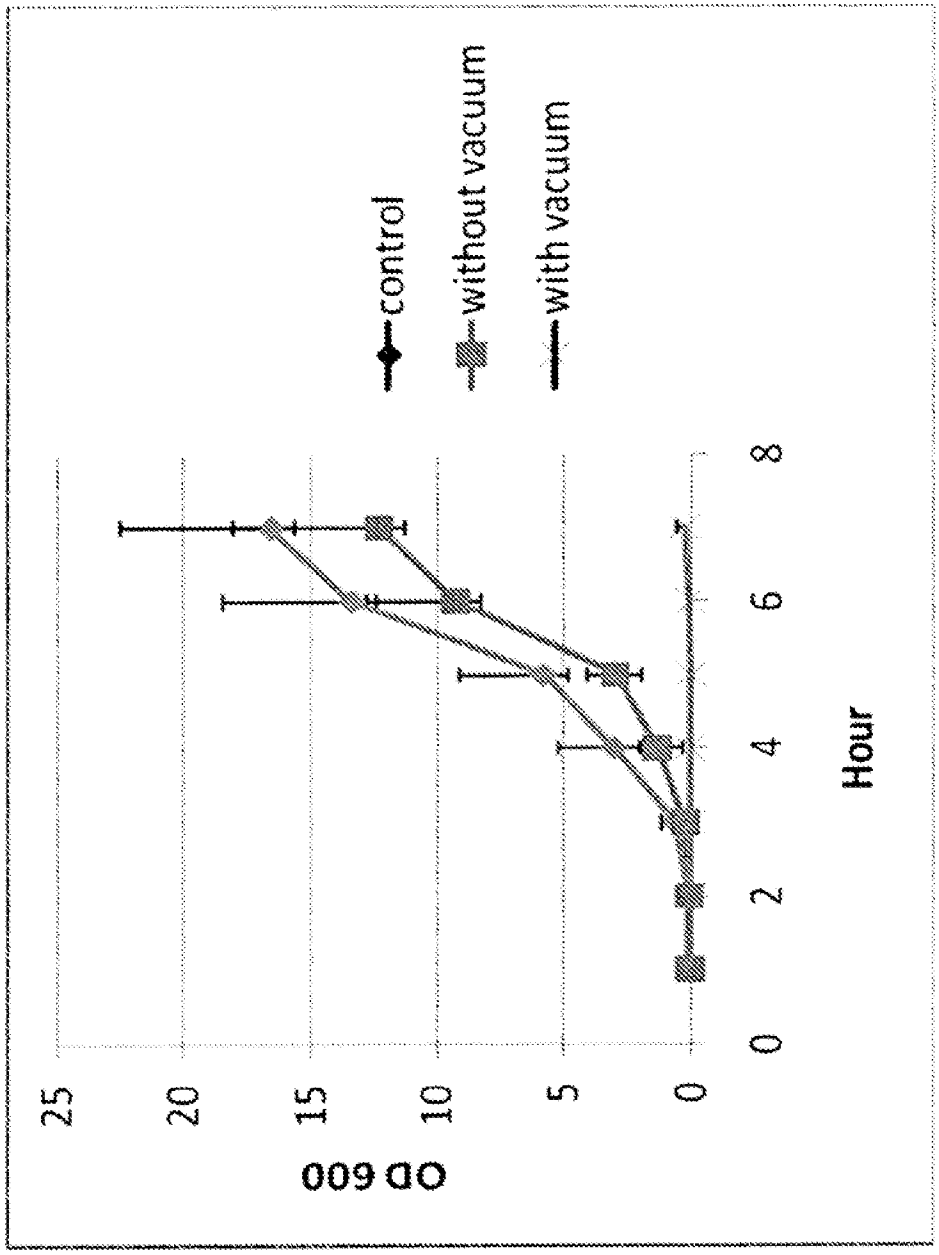
control
without vacuum
with vacuum
OD 600
25
20
15
10
5
0
0
2
4
6
8
Hour

Figure 6

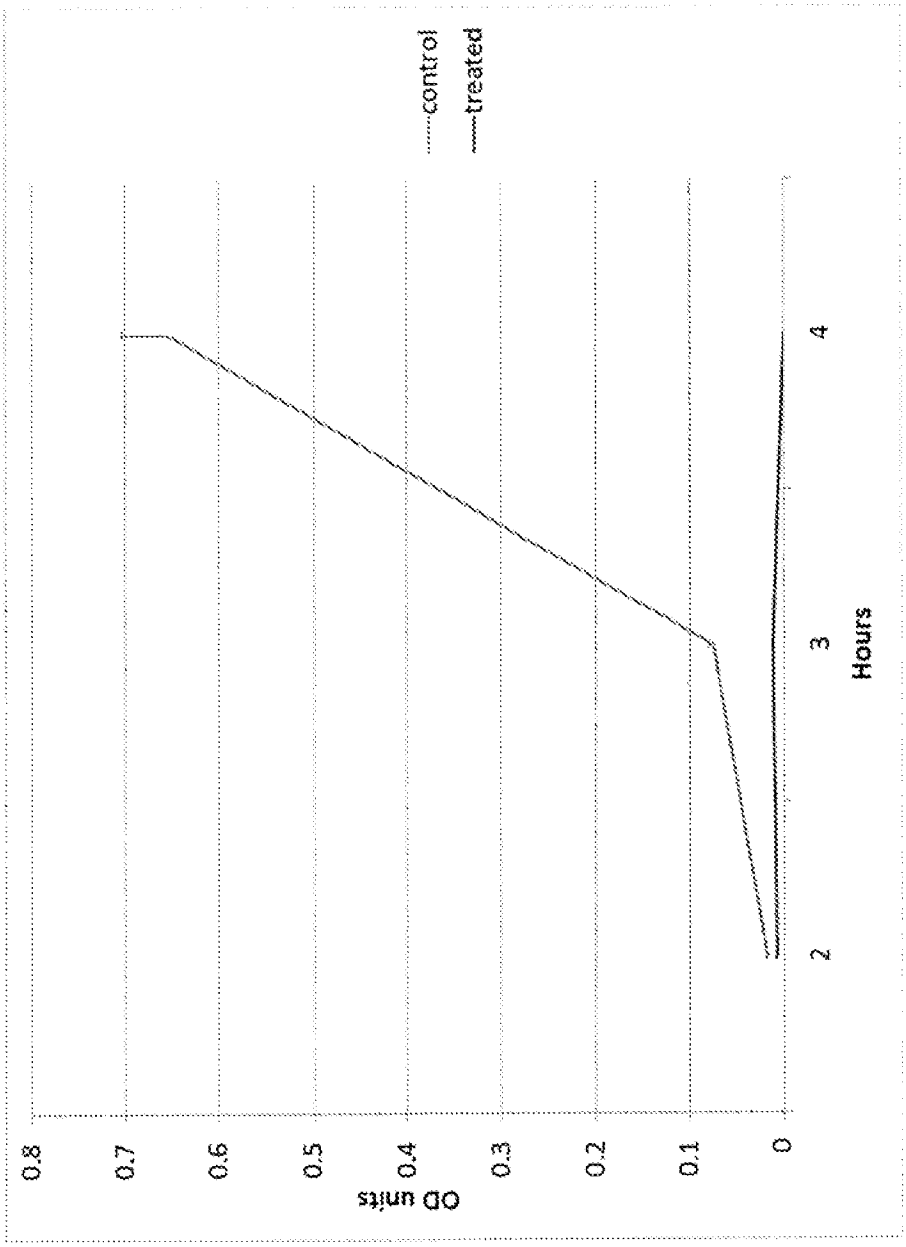

Figure 7

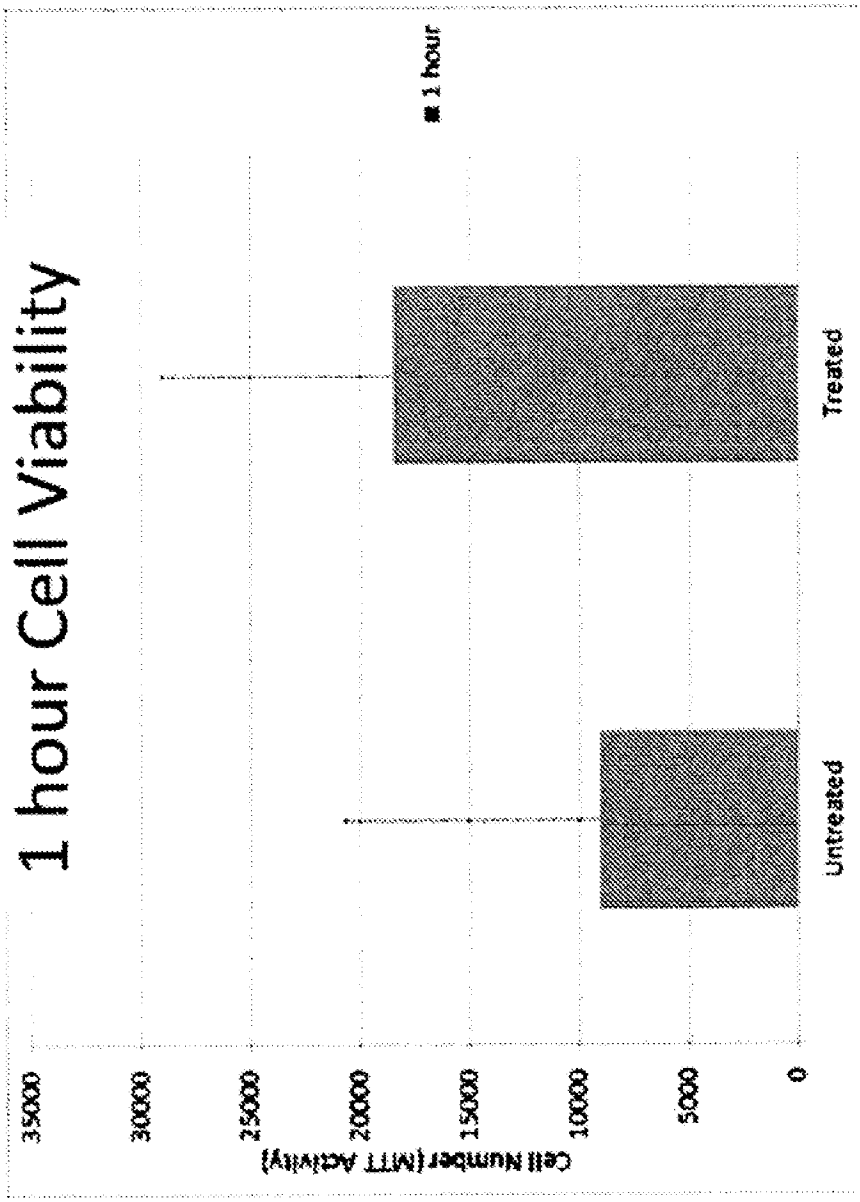
1 hour Cell Viability
1 hour
Cell Number (MTT Activity)
35000
30000
25000
20000
15000
10000
5000
0
Untreated
Treated

Figure 8A

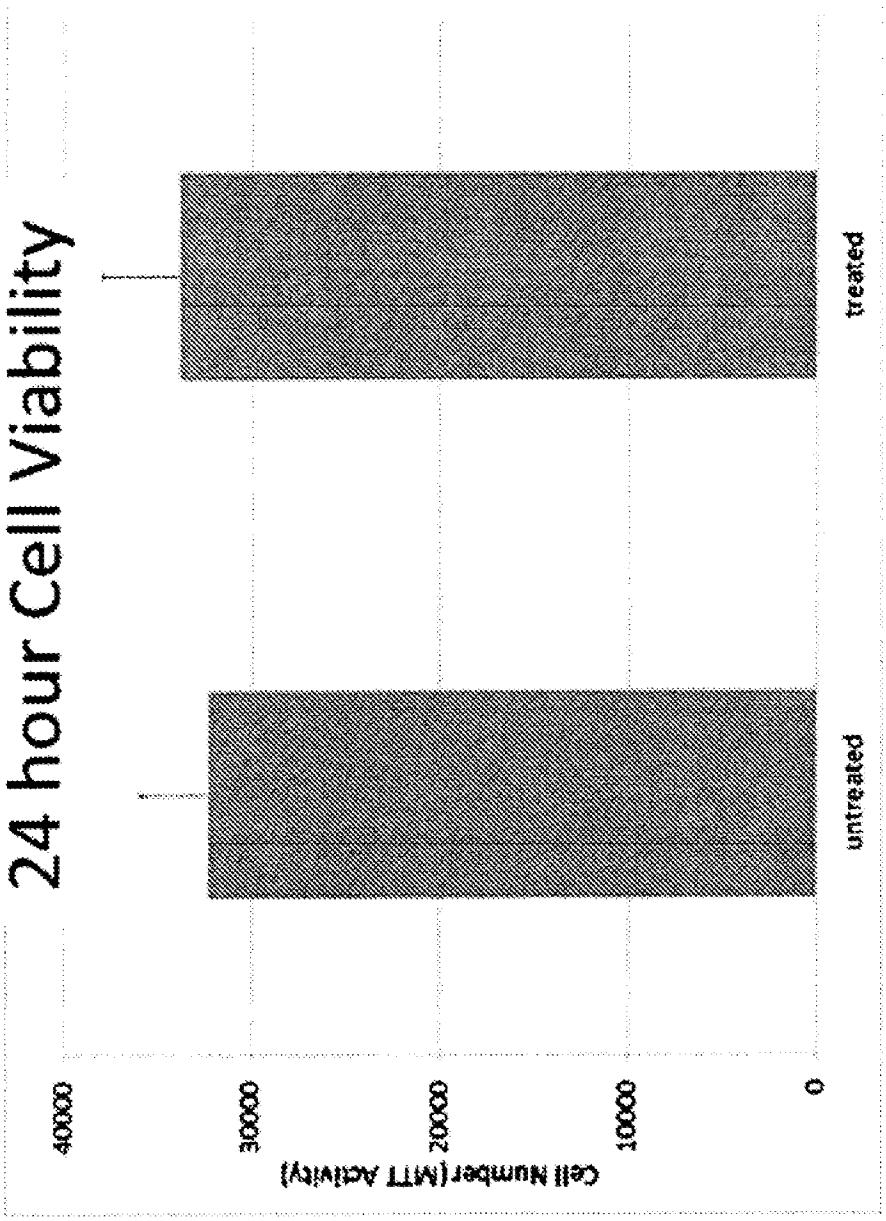
24 hour Cell Viability
Cell Number (MTT Activity)
40000
30000
20000
10000
0
untreated
treated

Figure 8B

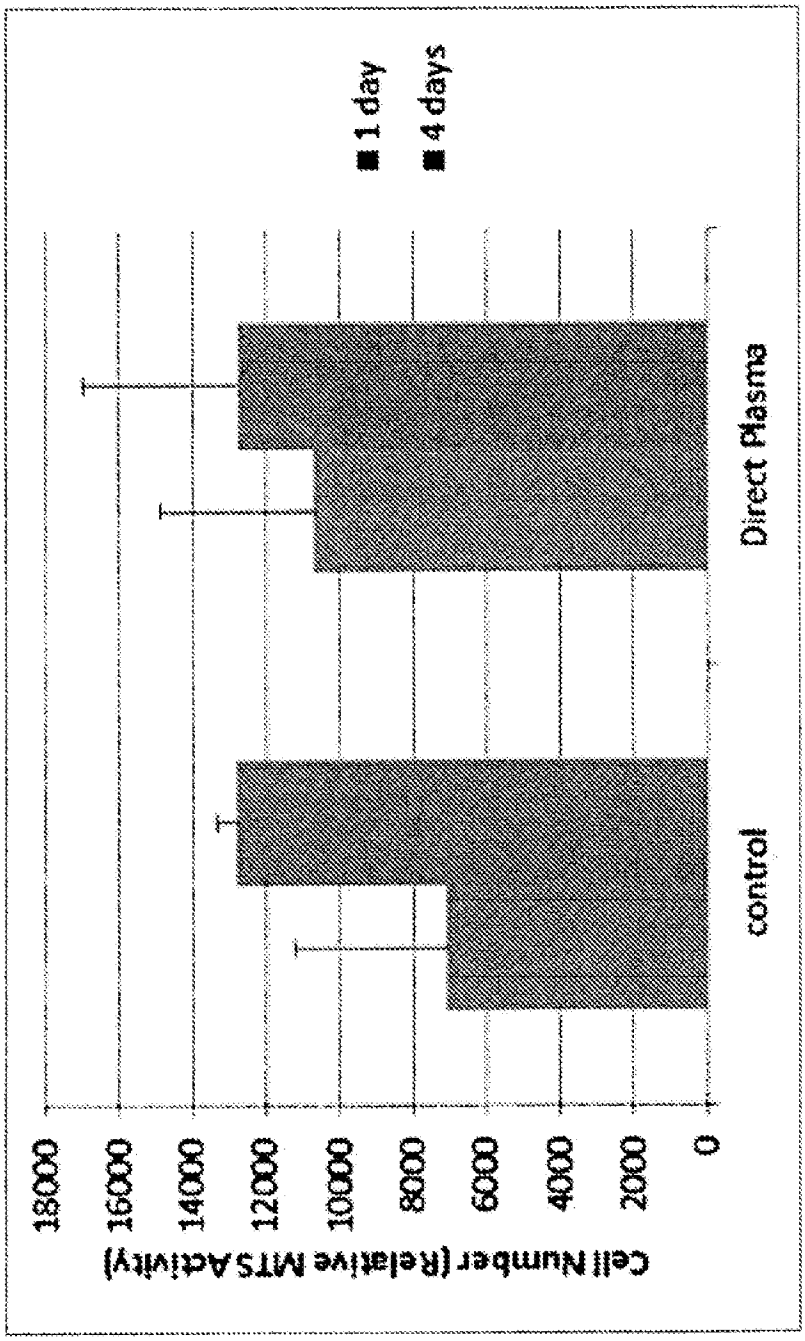
Cell Number (Relative MTS Activity)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
control
Direct Plasma
1 day
4 days

Figure 9

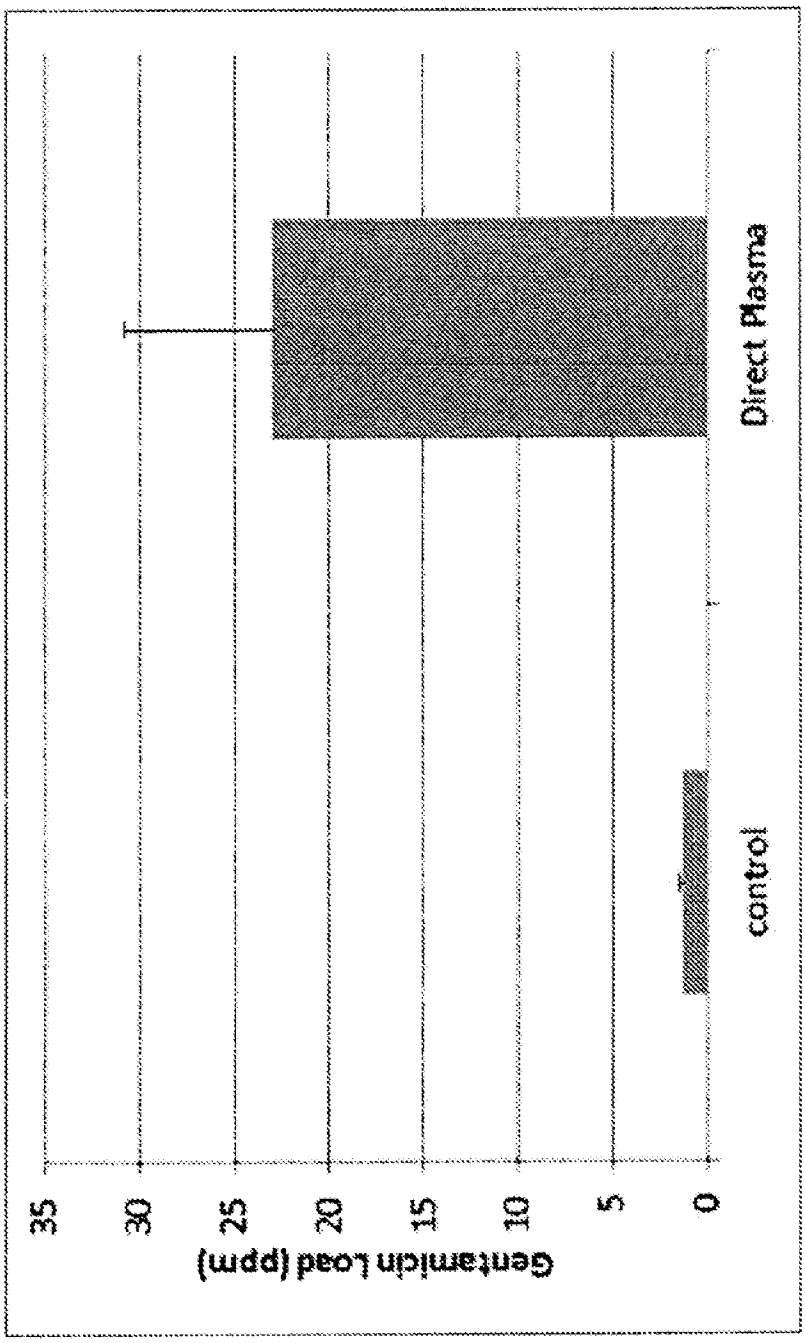
Gentamicin Load (ppm)
35
30
25
20
15
10
5
0
Direct Plasma
control

Figure 10

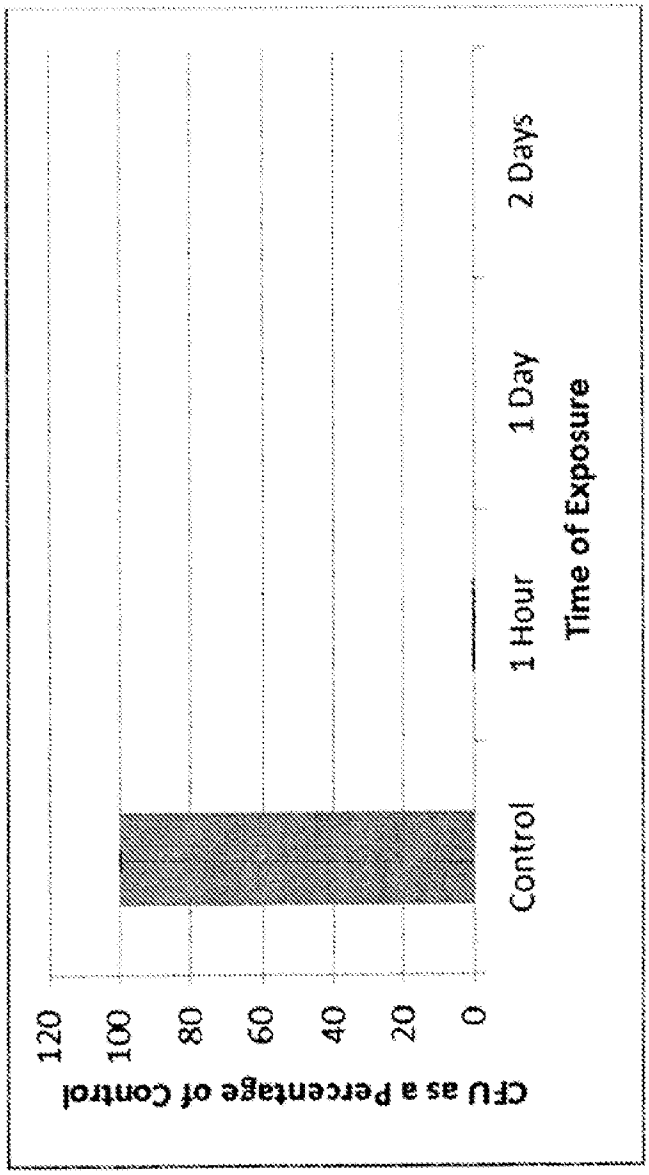

Figure 11A

E. Coli

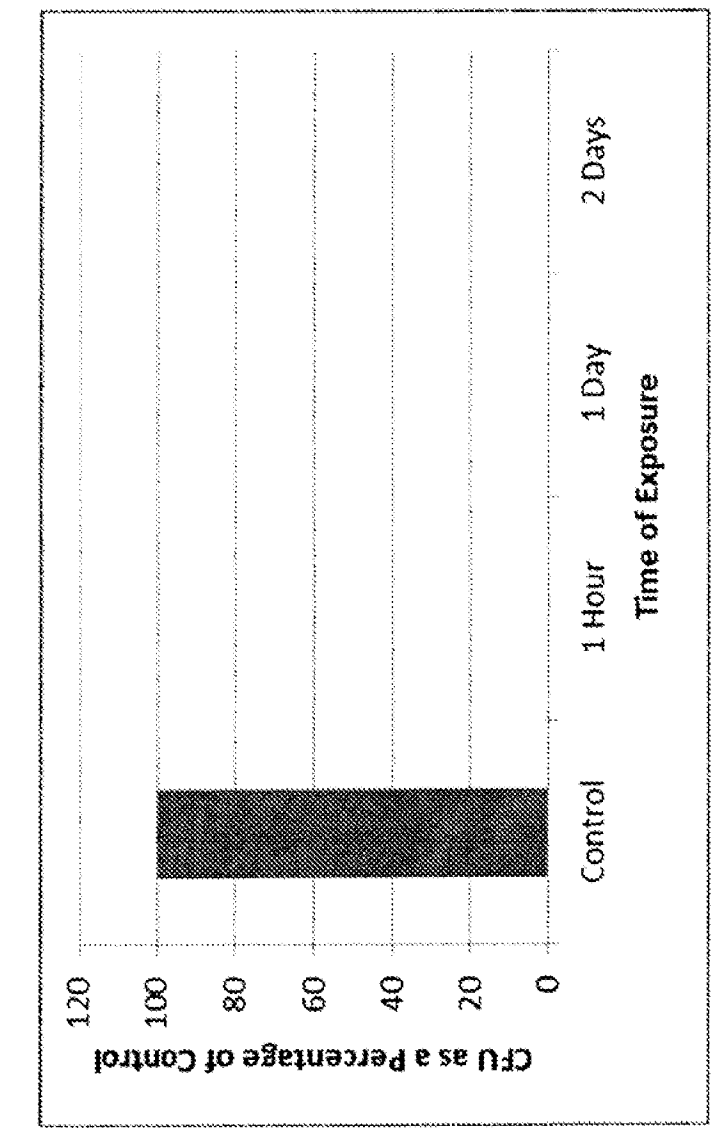
P. aeruginosa
CFU as a Percentage of Control
120
100
80
60
40
20
0
Control
1 Hour
1 Day
2 Days
Time of Exposure

Figure 11B

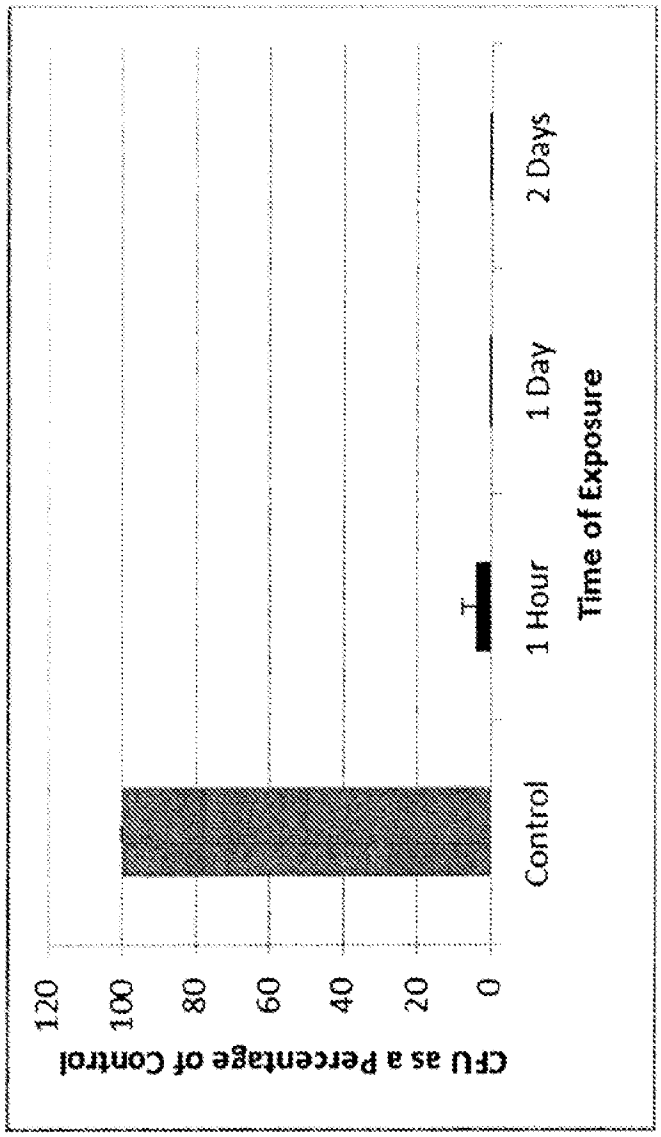
S. aureus 25923
CFU as a Percentage of Control
120
100
80
60
40
20
0
Control
1 Hour
1 Day
2 Days
Time of Exposure

Figure 11C

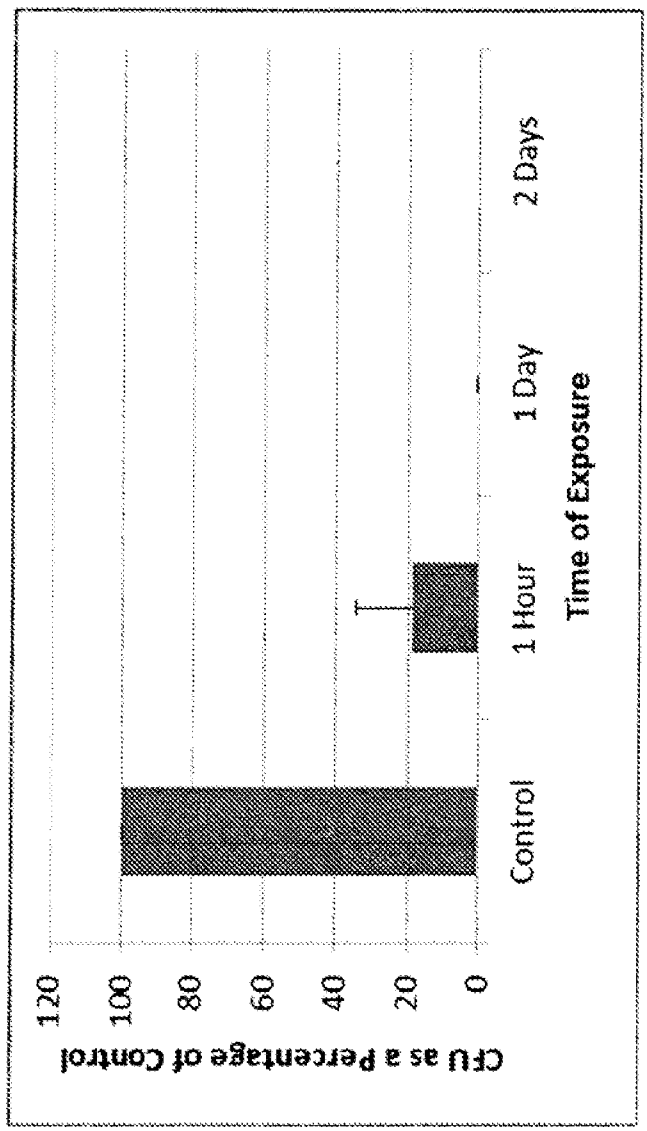
S. aureus 10390
CFU as a Percentage of Control
120
100
80
60
40
20
0
Control
1 Hour
1 Day
2 Days
Time of Exposure

Figure 11D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/35515

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C08F 2/46 (2012.01)
USPC - 427/488
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)
USPC - 427/488

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 427/488; 506/9, 506/40 (words only)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
*** Databases: WEST (PGPB, USPT, USOC, EPAB, JPAB); Google, Google Scholar *** Search Terms Used: Orthobond, Clevenger, Dong, Katz, activated, activating, functionalized, functionalizing, surface, material, carbonyldiimidazole, Disuccinimidyl, hydroxysuccimimidyl, ligand, coupling agent, antimicrobial, antibiotic, oxygen, plasma, collagen, tissue

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X – Y	US 2007/0005140 A1 (Kim et al.) 04 January 2007 (04.01.2007), especially abstract, para [0011], [0013], [0018], [0108], [0115],	1-2, 25, 31-34, 49, 51-53, 76, 82-83 and 98 --- 3-24, 26-30, 35-48, 54-75, 77-81 and 84-97
X	US 7,726,319 B1 (Boyce) 01 June 2010 (01.06.2010), especially col 5, ln 41-68; col 7, ln 7-39	101-105
Y	US 2008/0274184 A1 (Hunt) 06 November 2008 (06.11.2008), especially para [0015], [0034], [0043], [0066], [0115],	3-10, 50 and 54-61
Y	US 2008/0131522 A1 (Liu et al.) 05 June 2008 (05.06.2008), especially para [0037], [0069]-[0070],	11-19 and 62-70
Y	US 2007/0212386 A1 (Patravale et al.) 13 September 2007 (13.09.2007), especially para [0086], [0099]-[0100], [0104], [0106],	20-24, 26-30, 35-48, 50, 71-75, 77-81 and 89-97
Y	US 2011/0056882 A1 (Borenstein et al.) 10 March 2011 (10.03.2011), especially para [0011], [0024], [0026], [0029]-[0031], [0062]-[0064], [0088],	84-88

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"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)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"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
"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
"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
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 July 2012 (16.07.2012)	27 JUL 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/35515

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:

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.: 99-100
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:

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.