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(54) Title: NANOFIBER STRUCTURES ON ASPERITIES FOR SEQUESTERING, CARRYING AND TRANSFERRING SUBSTANCES

(57) Abstract: A device which comprises a substrate which includes a plurality of asperities, such as microneedles, extending from the surface of the substrate, and an electrospun material in contact with the substrate. The electrospun material may comprise a plurality of nanofibers which carry at least one biologically active agent. Such devices are useful as transdermal delivery devices for delivering pharmaceuticals and other biologically active agents.

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NANOFIBER STRUCTURES ON ASPERITIES FOR SEQUESTERING, CARRYING AND TRANSFERRING SUBSTANCES

This application claims priority based on provisional application Serial No. 60/798,928, filed May 9, 2006, provisional application Serial No. 60/848,506, filed September 29, 2006, provisional application Serial No. 60/848,507, filed September 29, 2006, provisional application Serial No. 60/848,504, filed September 29, 2006, provisional application Serial No. 60/848,505, filed September 29, 2006, and provisional application Serial No. 60/848,213, filed September 29, 2006, the contents of which are incorporated herein by reference in their entireties.

FIELD OF THE INVENTION

The present invention relates to structures that contain one or more fiber and/or nanofiber structures on asperities, including, but not limited to, pins, microprojections, and microneedles. In one embodiment, the present invention relates to structures that contain one or more fiber and/or nanofiber structures on asperities including pins, microprojections, and microneedles where the nanofiber and/or fiber structures are designed to sequester, carry and/or encapsulate one or more substances. In still another embodiment, the present invention relates to a process for forming one or more fibers, nanofibers or structures made therefrom on asperities, such as pins, microprojections, and microneedles where such fibers and/or structures are designed to sequester, carry, and/or encapsulate one or more substances and to deliver such substances, such as therapeutic drugs and preventive medicines, through the skin.

BACKGROUND OF THE INVENTION

There is an interest in methods to sequester, entrap, encapsulate and/or deposit various compounds or substances on the surfaces of and/or within various structures (e.g., polymer, metal, or ceramic structures). One such method that holds promise is the use of fibers and/or nanofibers that are designed to carry one or more compounds or substances, where such fibers, nanofibers, or

structures made therefrom are placed, deposited or formed on one or more surfaces of a material (e.g.. a polymer, metal, ceramic or other material).

Such fibers could be used as a vehicle for the topical delivery of drugs. Topical delivery of drugs is a useful method for achieving systemic or localized pharmacological effects. An example is disclosed in U.S. Pat. No. 3,964,482 (by Gerstel), in which an array of either solid or hollow microneedles is used to penetrate through the stratum corneum, into the epidermal layer, but not to the dermal layer; however, hollow microneedles create engineering issues and require a reservoir or other source of fluid. Solid needles do not offer sufficient loading of the substance, let alone sufficient preservation and stabilization of the substance..

Thus, there is a need in the art for products that can be designed to incorporate various fibers and/or nanofibers, where the fibers and/or nanofibers are designed to carry, sequester and/or encapsulate one or more compounds or substances, thereby providing for efficient loading of the substance, sufficient preservation and stabilization of the substance, and desired release characteristics.

SUMMARY OF THE INVENTION

In accordance with an aspect of the present invention, there is provided a device which includes a substrate comprising a surface and a plurality of asperities extending from said surface, and an electrospun material in contact with said substrate. The electrospun material may be any of a variety of forms, including, but not limited to, fibers, such as nanofibers and microfibers, including fiber mats, three-dimensional films, spheres, tubes, or any other geometrical shape, and the like. In one non-limiting embodiment, the electrospun material is in the form of nanofibers. In a non-limiting embodiment the electrospun material may be formed from a polymer, including but not limited to those described hereinbelow.

The electrospun material, in one embodiment, comprises at least one carrier substance, such as a polymer, capable of carrying a biologically active agent, and at least one biologically active

agent.

The present invention also is directed to administering at least one biologically active agent, such as a drug, or a prophylactic or therapeutic agent, to a human or non-human animal by providing a device including a substrate, and an electrospun material, which includes the biologically active agent, in contact with the substrate as hereinabove described. The human or non-human animal is contacted with the device such that the at least one biologically active agent is released into the human or non-human animal.

In accordance with yet another aspect of the present invention, there is provided a device which comprises an ionically cross-linked macromolecular assembly and at least one biologically active agent, such as those described herein, contained in said assembly. In a non-limiting embodiment, the ionically cross-linked macromolecular assembly comprises an ionically cross-linked water soluble polymer.

In another non-limiting embodiment, the device further comprises a substrate which comprises a surface and a plurality of asperities, such as microprojections and microneedles, extending from the surface. The macromolecular assembly containing the at least one biologically active agent is in contact with the substrate.

In a non-limiting embodiment, the ionically cross-linked macromolecular assembly containing the at least one biologically active agent may be in the form of an electrospun material which may be any of a variety of forms, including, but not limited to, fibers, such as nanofibers and microfibers, including fiber mats, three-dimensional films, spheres, tubes, or any other geometrical shape, and the like.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention now will be described with respect to the drawings, wherein:

Figure 1 is a low magnification optical image of grounded carbon nanofibers coated with polyethylene oxide nanofibers;

Figure 2 shows a carbon fiber tip coated with nanofibers, and spanning nanofibers near the tip;

Figure 3 shows the tip of a carbon fiber coated with a ball of nanofibers at the tip;

Figure 4 shows a drawing of an asperity which has an increased surface area near the tip, and has corners that protect the nanofibers from shearing forces;

Figure 5 shows a drawing of an embodiment of an asperity having barbs and circumferential grooves;

Figure 6 shows a drawing of another embodiment of an asperity having barbs;

Figure 7A shows one embodiment of a two-polymer device, wherein the microneedle array comprises a soluble first layer which dissolves upon administration (Figure 7B) thereby releasing a relatively less soluble second layer into the skin;

Figure 8 shows one embodiment of a flexible microneedle array(s);

Figures 9A and 9B show one embodiment of a device comprising an elastomeric element which, upon pressure, changes form to hold the array(s) on the skin;

Figure 10 is a scanning electron microscope picture showing an overview of one embodiment of a microneedle array with a particular density;

Figure 11 is a scanning electron microscope picture showing a close up, side view of one embodiment of a single microneedle (e.g. within an array) with a particular shape and height;

Figure 12 is a scanning electron microscope picture showing a close up, top view of one embodiment of a single microneedle (e.g. within an array) with eight sides (i.e. eight surfaces) coming together at the top to form a point or tip;

Figure 13 shows a prospective microneedle array with a higher density;

Figure 14 shows an embodiment wherein a hydrogel is fabricated on the surface of an embodiment of the device of the present invention (e.g., a microneedle array) using aqueous based methods;

Figure 15 shows an alternative embodiment wherein physiologically active substances can be dissolved or dispersed in a solution of a cross-linker (e.g., a solution having multivalent or polyvalent ions);

Figure 16 shows a C. botulinum type A toxin amino acid sequence;

Figure 17 shows another C. botulinum type A toxin amino acid sequence;

Figure 18 shows the amino acid sequence of wild-type anthrax protective antigen;

Figure 19 shows the nucleotide sequence encoding wild-type anthrax protective antigen;

Figure 20 shows the amino acid sequences of human 1 - 34 PTH and bovine 1 - 34 PTH. (The present invention is not limited only to administering human hormones. The hormone homolog from other species (e.g., salmon, porcine, etc.) may have equal or better results);

Figure 21 shows the amino acid sequences for GLP-1, Exendin-4, Exendin-4 (3 - 39), and Exendin-4 (9 - 39);

Figure 22 shows the nucleotide sequence of the coding region for the C. botulinum type A neurotoxin;

Figure 23 shows C. botulinum type A toxin expression constructs; constructs used to provide C. botulinum or C. difficile sequences also are shown;

Figure 24 shows the nucleotide sequence of the C. Botulinum C fragment gene sequences contained within pAlterBot;

Figure 25 shows the C. botulinum type A toxin expression constructs; constructs used to provide C. botulinum sequences also are shown;

Figure 26 shows the nucleotide sequence present in the pETHisa vector which encodes the pHisBot fusion protein;

Figure 27 shows the amino acid sequence of the pHisBot protein; and

Figure 28 shows the expected results in a test of electrospun PA functionality.

DETAILED DESCRIPTION OF THE INVENTION

As used herein the word "asperities" means the microscopic surface elevations present on the surface of a material due to surface roughness of a material, such as pins, microprojections, and microneedles. The asperities, microprojections, and microneedles preferably are in the form of piercing elements which are dimensioned to penetrate into or through a desired body part such as a tissue, or organ, or which may deliver a biological material transdermally, intradermally, intraepidermally, or transmucosally. In a non-limiting embodiment, the asperity, microprojection, or microneedle is dimensioned such that it penetrates through the stratum corneum into the underlying epidermis layer, and in some embodiments, the dermal layer of the skin.

The term "transdermal" are used herein means the delivery of an agent into and/or through at least the top layer of the skin. The term "intradermal" means the delivery or release within the skin. The term "intra-epidermal" means the delivery or release specifically within the epidermal layer of the skin.

While the microneedle embodiment (described below) may be employed, other systems and apparatus that employ tiny skin piercing elements to enhance transdermal agent delivery are also contemplated, as disclosed in U.S. Pat. Nos. 5,879,326, 3,814,097, 5,250,023, 3,964,482, Reissue U.S. Pat. No. 25,637, and PCT Publication Nos. WO 96/37155, WO 96/37256, WO 96/17648, WO 97/03718, WO 98/11937, WO 98/00193, WO 97/48440, WO 97/48441, WO 97/48442, WO 98/00193, WO 99/64580, WO 98/28037, WO 98/29298, and WO 98/29365; all incorporated herein by reference in their entireties.

It is not intended that the present invention be limited to a precise geometry or topology of the microneedles. In one embodiment, the microneedles are defined by a plurality of surfaces sloping upwards from a relatively broad base to a tip (e.g. a pyramidal shape). In another embodiment, the microneedles have a generally conical-shaped body (e.g. a single curved surface).

Furthermore, within the scope of the present invention, the asperities, such as microprojections and microneedles, in one embodiment, may include one or more indentations

and/or barbs, which aid in retaining the nanofibers on the asperities.

It is not intended that the present invention be limited by the precise dimensions of the microneedles. In one embodiment, the microneedles described herein have a microneedle height to width (measured at the base) ratio of between 1.2 and 2.0 (and in one embodiment between 1.4 and 1.7), and the structure is predominantly solid, rather than hollow. These factors contribute to the force required to penetrate human skin being smaller than that required to break the penetrating elements. This applies to both single microneedles and various microneedle arrays, for which penetration forces are different depending on the number of penetrating elements, their height and spacing between adjacent microneedles.

In one embodiment, a prototypical microneedle has a diameter of between 200 and 500 microns (and in another embodiment, between 300 and 400 microns) at its broad end (e.g., at the base), and tapers to a sharp tip or chisel edges with a somewhat smaller diameter at its other end. The diameter of the tip may, for example, be in the range from about 50 microns to about 1 microns.

In one embodiment, the microprojections or microneedles have a length (or height) or less than 1000 microns, and in one embodiment less than 700 microns, but more than 250 microns. In another embodiment, the microneedles have a height of between 550 and 650 microns (such as, for example, between 580 and 620 microns) with a height to width ratio of between 1.5 and 1.7. The microprojections may be formed in different shapes, such as needles, blades, pins, punches, and combinations thereof. In one embodiment, the microneedles are pyramidal in shape (e.g., having between 6 and 12 sides, and in one embodiment, eight sides).

The term "fiber" includes not only structures that are cylindrical, but also includes structures which vary from a cylindrical shape, such as, for example, structures which are spherical, acicular, droplet shaped, or flattened or ribbon shaped. A "fiber-forming" material is capable of being fabricated into fibers. The fiber-forming materials that may be used in this invention include polymers, which can be in a liquid state, such as melted, in solution, or in suspension.

Water, organic solvents, or mixtures thereof or heat can be used to convert solid polymer into a liquid "fiber-forming" material. In one embodiment, electrospinning creates "pearls on a string" or encapsulated balls on very thin fibers. This technique may be useful when compounds do not mix well, or when one needs to meet extended release requirements, or when one wants to deliver balls and the fibers are just a simple carrier.

Additionally, as used herein, nanofibers are fibers having an average diameter in the range of about 1 nanometer to about 25,000 nanometers (25 microns). In another embodiment, the nanofibers of the present invention are fibers having an average diameter in the range of about 1 nanometer to about 10,000 nanometers, or about 1 nanometer to about 5,000 nanometers, or about 3 nanometers to about 3,000 nanometers, or about 7 nanometers to about 1,000 nanometers, or even about 10 nanometers to about 500 nanometers, In another embodiment, the nanofibers of the present invention are fibers having an average diameter of less than 25,000 nanometers, or less than 10,000 nanometers, or even less than 5,000 nanometers. In still another embodiment, the nanofibers of the present invention are fibers having an average diameter of less than 3,000 nanometers, or less than about 1,000 nanometers, or even less than about 500 nanometers.

In another embodiment, the nanofibers may have a diameter as small as 0.3 nanometers. In yet another embodiment the nanometers have a diameter between 3 nanometers and about 25 microns. In a further embodiment, the nanofibers have a diameter of from about 100 nanometers to about 25 microns. In still another embodiment the nanofibers have a diameter of from about 100 nanometers to about a micron. Such small diameters provide a high surface area to mass ratio, such as, for example, about $300m^2/g$. Within the scope of the present invention, a fiber may be any length. The term "fiber" also encompasses particles that are drop-shaped, flat, or that may otherwise form a cylindrical shape. Additionally, it should be noted that here, as well as elsewhere in the text, ranges may be combined.

The at least one fiber-forming material used in this invention may be selected from any

fiber-forming material which can be dissolved and is otherwise compatible with the biological material to be preserved. Fiber-forming materials which may be used in the practice of the method of the present invention include water soluble polymers, for example. In particular, acceptable fiber-forming materials, by way of example and not of limitation, include polyvinyl pyrrolidone) (PVP), polyethyl oxazoline (PEOZ), polyethylenlmine (PEI), polyethylene oxide (PEO) and mixtures thereof. Other polymers of interest include polyvinylalcohol, poly(ethylene glycol), polyoxymethylene, poly(hydroxyethyl methacrylate), carboxymethyl cellulose, hydroxypropylcellulose, alginic acid, chitosan, poly(glutamic acid), poly(isobutylacrylamide), poly(butyl methacrylate), poly(ethyl methacrylate), poly(vinylidene fluoride) poly(hydroxyvalerate), poly(L-lactic acid), polycaprolactone, poly(lactide-co-glycolide), poly(hydroxybutyrate), poly(hydroxybutyrate-co-valerate), polydioxanone, polyorthoester, polyanhydride, poly(glycolic acid), poly(vinyl methyl ether), polyvinylidene chloride, polyacrylonitrile, poly(trimethylene carbonate), poly(iminocarbonate), and copolymers thereof. Other illustrative polymers are set forth in Table 1 below.

TABLE 1

A. Suitable Hydrophilic Polymers

Polyacrylamide
Poly(Acrylic Acid-Co-Hypophosphite)
Polyacrylic Acid, Sodium Salt
Poly(Alkyl(C 16-22)Acrylate)
Poly(Ethylene Glycol) or Poly(Propylene Glycol)
Poly(Vinyl Alcohol)
Polypyrrolidone or Polyvinylpyrrolidone
Polysaccharides Such As Chitosan, Alginate, Amylose
Water Soluble Fractions Of Proteins Such As Collagen, Gelatin

B. Suitable Biostable, Hydrophobic Polymers

Poly(Divinylbenzene-Co-Ethylstyrene)
Polyisobutylene
Polylimonene
Polymaleic Acid Polyoxyethylene Dioleate
Poly(Vinyl Acetate) or Poly(Vinyl Chloride)

Polystyrene, Polyurethane, Polyurethane-Poly(Ethylene Oxide) Graft Copolymer
Poly(Ether Urethane) or Poly(Ether Urethane Urea)
Polyethylene
Polycarbonate
Poly(Ester Amide)
Polyacrylonitrile
Poly(Aryl Ether Keton)
Poly(Dimethyl Siloxane) And Other Polysiloxanes
Poly(Ethylene Terephthalate) And Other Polyesters
Poly(2-Hydroxyethylmethacrylate) or Polymethylmethacrylate

C. Suitable Biodegradable, Hydrophobic Polymers
Poly(Glycolic Acid), Poly(Lactic Acid) And Co-Polymers Thereof
Polyhydroxybutyrate (Phb)
Polyhydroxyvalerate (Phv), And Co-Polymers Thereof
Polycaprolactone
Polydioxanone And Other Synthetic Degradable Polyesters, Blends Thereof, And Copolymers Thereof
Polyanhydrides
Poly(Amino Acids) Such As Poly(Benzyl Glutamate)
"Pseudo"-Poly(Amino Acids) Such As Tyrosine-Derived Polycarbonates And Polyarylates And Copolymers Thereof With Poly(Ethylene Glycol)
Poly(Ortho Ester)
Polyphosphazenes Poly(Propylene Fumarate)

Although not specifically limited to any one production method, the fibers and/or nanofibers of the present invention are, in one embodiment, formed via an electrospinning process. The electrospinning of liquids and/or solutions capable of forming fibers, also known within the fiber forming industry as electrostatic spinning, is well known and has been described in a number of patents as well as in the general literature. The process of electrospinning generally involves the creation of an electrical field at the surface of a liquid. The resulting electrical forces create a jet of liquid that carries electrical charge. Thus, the liquid jets may be attracted to other electrically charged objects at a suitable electrical potential. As the jet of liquid elongates and travels, it will harden and dry. The hardening and drying of the elongated jet of liquid may be caused by cooling of the liquid, i.e., where the liquid is normally a solid at room temperature: evaporation of a solvent, e.g., by dehydration, (physically induced hardening), or by a curing mechanism (chemically induced hardening) or by a combination of such methods. The produced fibers are collected on a suitably

located, oppositely charged receiver and subsequently removed from it as needed, or directly applied to an oppositely charged or grounded generalized target area. In a non-limiting embodiment, the fibers are collected on a receiver such as, for example, a polystyrene or polyester net or a foil slide.

Fibers produced by this process have been used in a wide variety of applications, and are known, from United States Patent Nos. 4,043,331: 4,878,908; and 6,753,454, all of which are incorporated herein by reference in their entireties. One of the major advantages of electrospun fibers is that very thin fibers can be produced having diameters, usually on the order of about 50 nanometers to about 25,000 nanometers (25 microns), or even on the order of about 50 nanometers to about 5,000 nanometers (5 microns), or even on the order of about 50 nanometers to about 1,000 nanometers.

It will be appreciated that, because of the very small diameter of the fibers, the fibers have a high surface area per unit of mass. This high surface area to mass ratio permits fiber-forming material solutions to be transformed from solvated fiber-forming materials to solid nanofibers in fractions of a second. When biological materials are dissolved or suspended in a fiber-forming material solution which then is formed into fibers, the samples experience a rapid loss of excess solvent. This invention thereby also provides a fiber containing a mixture of at least one fiber-forming material and at least one preserved biological material and optionally other materials (e.g. carriers, fillers, etc.). The mixture may, in some embodiments, be substantially homogeneous; however, the present invention also contemplates non-homogeneous mixtures.

As one skilled in the art will recognize, the fibers may be spun using a wide variety of conditions such as potential difference, flow rate, and gap distance. These parameters will vary with conditions such as humidity or other environmental conditions, the size of the biological material or other additive, the solution viscosity, the collection surface, and the polymer conductivity, among others. The at least one fiber-forming material is in a liquid state when it is electrospun with the biological material to form a fiber containing a mixture of the at least one fiber-forming material and

the at least one biological material. In one embodiment, the present invention contemplates mixtures of the at least one fiber-forming material and at least one biological material, including mixtures where the biological material is soluble in the at least one fiber-forming material in its liquid state (homogeneous or single phase systems) and those mixtures in which the at least one biological material is insoluble in the at least one fiber-forming material in its liquid state (heterogeneous or multi-phase systems). When the biological material is insoluble in the at least one fiber-forming material in its liquid state, the biological material may take the form of a suspension or emulsion in the fiber-forming material. Whether the biological material is soluble or insoluble in the fiber-forming material, the biological material and the fiber-forming material may be mixed by any method which forms a substantially uniform mixture, including, for example, mechanical shaking or stirring, although other methods (such as sonication) may be used. As one skilled in the art will recognize, solubility of the biological material in the fiber-forming material solution will depend on the characteristics of the material itself, as well as factors such as, for example, the requirements of the material for a specific pH range, osmolarity, or the presence of co-factors for the material.

By way of a non-limiting example, polyethylene oxide (PEO) (Mw 400,000), and polyvinylpyrrolidone (PVP) (Mw 360,000) can be used as polymer substrates for the electrospinning of fibers. In case of PEO, about 2.5 mg/ml of bioactive agent(s) in approximately 6% w/v polymer solution can be used in electrospinning the sample. A cone cap is typically used to do the spinning at room temperature, using a voltage of 14 KV, a current of 80 nA, and positive polarity. The gap distance can be 28.5 cm, and the sample can be collected on polyester net or other suitable substrate.

By way of a non-limiting example, PVP fibers can be electrospun from a solution containing about 2.5 mg/ml of bioactive agent(s) in 22% w/v polymer. A cone cap is again typically used to do the spinning at room temperature, using a voltage of 22 KV, a current of 360 nA, and positive polarity. The gap distance can be 23 cm, and the sample collected on a polyester net or other suitable substrate.

Although not limited solely hereto, suitable arrays of asperities can made by the growth of elongated cylindrical crystals by a vapor-liquid-solid process; growth of polycyanoacrylate fibers from small deposits of catalyst material; MEMs technology of the sort utilized in the semiconductor electronics industry; removing, by dissolution, fracture, or decomposition, the matrix from a composite that contains acicular particles; and in many other ways known in the art.

In one non-limiting embodiment, the asperities are microneedles which are anisotropically etched MEMs microneedles in silicon.

In one embodiment, the solid microneedles are fabricated in a crystal silicon material suitable for use in the administration of the various preparations discussed herein. Without limiting the invention in any manner to any particular mechanism, it is believed that the biologically active materials or drug(s) is delivered by microporation of the stratum corneum, and polymer-drug deposition within the patient's skin and subsequent dissolution or erosion of the polymer. The drug becomes thereby bioavailable; it can dissolve and diffuse to the biological target, or alternatively, it can remain at the site of administration. Micropores are made into the stratum corneum by means of a microneedle array penetration, which optionally can be enhanced further by applying energy in the form of ultrasonic, heat and/or electric signals across or through the skin.

As is discussed above, it is possible to incorporate, encapsulate, entrap and/or deposit one or more compounds or substances on, in or around fibers and/or nanofibers. One manner by which this can be accomplished is by dissolving and/or dispersing the desired substance(s) or compound(s) in a polymer solution that is to be electrospun before it is electrospun. In one embodiment, the material inside the nanofiber is sequestered and preserved by the polymer matrix provided by the nanofiber.

Pharmaceutically active or bioactive substances which may be included in the electrospun material are listed in the Physicians' Desk Reference, 57th Edition (2003), and include allergens, amebicides and trichomonacides, analeptic agents, analgesics, anorexics, antacids, antihelmintics, antialcohol preparations, antiarthritics, antiasthma agents, antibacterials and antiseptics, antibiotics,

antiviral antibiotics, anticancer preparations, anticholinergic drug inhibitors, anticoagulants, anticonvulsants, antidepressants, antidiabetic agents, antidiarrheals, antidiuretics, antienuresis agents, antifibrinolytic agents, antifibrotics (systemic), antiflatulents, antifungal agents, antigonadotropin, antihistamines, antihyperammonia agents, anti-inflammatory agents, antimalarials, antimetabolites, anti-migraine preparations, antinauseants, antineoplastics, anti-obesity preparations, antiparasitics, anti-parkinsonism drugs, antipruritics, antipyretics, antispasmodics and antichloinergics, antitoxoplasmosis agents, antitussives, antivertigo agents, antiviral agents, bone metabolism regulators, bowel evacuants, bronchial dilators, calcium preparations, cardiovascular preparations, central nervous system stimulants, cerumenolytics, chelating agents, choleretics, cholesterol reducers and anti-hyperlipemics, colonic content acidifiers, cough and cold preparations, decongestants, expectorants and combinations, diuretics, emetics, enzymes and digestants, fertility agents, fluorine preparations, galactokinetic agents, geriatrics, germicides, hematinics, hemorrhoidal preparations, histamine H. receptor antagonists, hormones, hydrocholeretics, hyperglycemic agents, hypnotics, immunosuppressives, laxatives, mucolytics, muscle relaxants, narcotic antagonists, narcotic detoxification agents, ophthalmological osmotic dehydrating agents, otic preparations, oxytocics, parashypatholytics, parathyroid preparations, pediculicides, premenstrual therapeutics, psychostimulants, quinidines, radiopharmaceuticals, respiratory stimulants, salt substitutes, scabicides, sclerosing agents, sedatives, sympatholytics, sympathomimetics, thrombolytics, thyroid preparations, toxins for therapeutic vaccine use, tranquilizers, tuberculosis preparations, uricosuric agents, urinary acidifiers, urinary alkalinizing agents, urinary tract analgesic, urological irrigants, uterine contractants, vaginal therapeutics and vitamins and each specific compound or composition listed under each of the foregoing categories in the Physicians' Desk Reference.

They include, but not limited to water-soluble molecules possessing pharmacological activity, such as a peptide, protein, enzyme, enzyme inhibitor, antigen, cytostatic agent, anti-

inflammatory agent, antibiotic, DNA-construct, RNA-construct, or growth factor. Examples of therapeutic proteins are interleukins, albumins, growth hormones, aspariginase, superoxide dismutase, monoclonal antibodies. Biological agents include also water-insoluble drugs, such as camptothecin and related topoisomerase I inhibitors, gemcitabine, taxanes and paclitaxel derivatives. Other compounds include, for example, peptides, including peptidoglycans, as well as anti-tumor agents, cardiovascular agents such as forskolin; anti-neoplastics such as combretastatin, vinbiastine, doxorubicin, maytansine; anti-infectives such as vancomycin, erythromycin; anti-fungals such as nystatin, amphotericin B, triazoles, papulocandins, pneumocandins, echinocandins, polyoxins, nikkomycins, pradimicins, benanomicins; anti-anxiety agents, gastrointestinal agents, central nervous system-activating agents, analgesics, fertility agents, anti-inflammatory agents, steroidal agents, anti-urecemic agents, cardiovascular agents, vasodilating agents, vasoconstricting agents and the like.

Other biologically active agents which may be included in the electrospun material include vaccine antigens. The vaccine antigens of the invention can be derived from a cell, a bacteria or virus particle or a portion thereof. The antigen can be a protein, peptide, polysaccharide, glycoprotein, glycolipid, or combination thereof which elicits an immunogenic response in a human; or in an animal, for example, a mammal, bird, or fish. The immunogenic response can be humoral, mucosal, or cell mediated. Examples are viral proteins, such as influenza proteins, human immunodeficiency virus (HIV) proteins, Herpes virus proteins, and hepatitus A and B proteins. Additional examples include antigens derived from rotavirus, measles, mumps, rubella, and polio; or from bacterial proteins and lipopolysaccharides such as Gram-negative bacterial cell walls. Further antigens may also be those derived from organisms such as Haemophilus influenza, Clostridium antigens, including but not limited to, Clostridium tetani, Corynebacterium diphtheria, and Nesisseria gonhorrhoae, as well as anthrax antigens.

In another embodiment, the bioactive agent in the electrospun formulation comprises a

cytokine. Cytokines are hormone-like substances secreted by a wide variety of cells, including (but not limited to) lymphocytes (e.g., T cells), macrophages, fibroblasts, and endothelial cells. It is now known that cytokines consist of a broad class of glycoproteins that have the ability to regulate intercellular communication (e.g., cell-cell interaction) in both normal and pathologic situations. Cytokines generally contain from approximately 60 to 200 amino acid residues, with a relative molecular weight of between 15 and 25 kd. At least 35 distinct cytokines have been elucidated. It is not intended that the present invention be limited by the particular cytokine. Table 2 provides illustrative examples.

TABLE 2

Name	Abbr.	Type	Specific Name
Interferons	IFN	alpha	Leukocyte Interferon
		beta	Fibroblast Interferon
		gamma	Macrophage Activation Factor
Interleukins	IL-1	1 alpha	Endogenous Pyrogen
		1 beta	Lymphocyte-Activating Factor
		1 ra	IL-1 Receptor Antagonist
	IL-2		T-cell Growth Factor
	IL-3		Mast Cell Growth Factor
	IL-4		B-cell Growth Factor
	IL-5		Eosinophil Differentiation Factor
	IL-6		Hybridoma Growth Factor
	IL-7		Lymphopoietin
	IL-8		Granulocyte Chemotactic Protein
	IL-9		Megakaryoblast Growth Factor
	IL-10		Cytokine Synthesis Inhibitor Factor
	IL-11		Stromal Cell-Derived Cytokine
	IL-12		Natural Killer Cell Stimulatory Factor
Tumor Necrosis Factors	TNF	alpha	Cachectin
		beta	Lymphotoxin
Colony Stimulating Factors	CSF	GM-CSF	Granulocyte-macrophage Colony-Stimulating Factor
		Mp-CSF	Macrophage Growth Factor
		G-CSF	Granulocyte Colony-stimulating Factor
		EPO	Erythropoietin

Transforming Growth Factor	TGF	beta 1	Cartilage-inducing Factor
		beta 2	Epstein-Barr Virus-inducing Factor
		beta 3	Tissue-derived Growth Factor
Other Growth Factors	LIF		Leukemia Inhibitory Factor
	MW		Macrophage Migration-inhibiting Factor
	MCP		Monocyte Chemoattractant Protein
	EGF		Epidermal Growth Factor
	PDGF		Platelet-derived Growth Factor
	FGF	alpha	Acidic Fibroblast Growth Factor
		beta	Basic Fibroblast Growth Factor
	ILGF		Insulin-like Growth Factor
	NGF		Nerve Growth Factor
	BCGF		B-cell growth factor

Thus, the present invention provides a method of administering one or more drugs to a patient comprising the steps of penetrating at least an outer layer of the skin of the patient with one or more asperities, such as pins, microprojections, or microneedles extending from a surface of a substrate, wherein at least one of the one or more asperities, such as pins, microprojections, or microneedles (or portion thereof), is contacted or coated (uniformly or non-uniformly) with an electrospun polymer/drug(s) preparation, and releasing at least one of the one or more drugs into the patient.

The relative amounts of fiber-forming material and biological material that may be present in the fiber of the present invention may vary. In one embodiment, the biological material comprises between about 1 and about 50 percent (more typically 30 percent) by weight to volume (w/v) of the mixture from which the fiber is electrospun. In another example, the biological material comprises 10 percent of the mixture or less. In still another example, the

biological material may be 25 percent, 5 percent, 0.5 percent of the mixture by weight to volume. It is envisioned that larger or smaller concentrations of biological material may also be utilized.

It is advantageous to coat the asperities (including microprojections and microneedles) with nanofibers which contain useful substances inside or on the nanofiber. Different substances can be concentrated near the tips of the asperities. The high surface area per unit volume of a fiber, in particular a nanofiber, makes it possible to carry a high mass of particles containing bioactive material per unit volume on the outside surface of the fiber and/or nanofiber. In one embodiment, it is even more useful to coat the tips of the asperities with nanofibers carrying one or more useful substances, and to have nanofibers that sequester a second set of useful substances span the regions between the asperities.

If, in one embodiment, the surfaces with asperities are mechanically pressed into contact with a second surface, the nanofibers on top of the asperities will be transferred onto, or impressed into the second surface. If, in one embodiment, the second surface is a sheet of material thinner than the height of the asperities, the nanofibers may be pushed through the sheet and available for chemical reactions or other purposes on the other side of the sheet while the nanofibers that span the spaces between the asperities are pressed onto the first surface of the sheet.

In one embodiment, the present invention permits a designer to specify the relative amounts and kinds of useful materials, in each of three regions: on the surface near the tip of the asperity, a ball on top of the asperity, (including microprojections and microneedles) and in the space between the asperities. Such designs can be manufactured by one or methods in accordance with the present invention.

In one embodiment, quantities of around 1 microgram of one or more bioactive substances per pin and/or asperities have been found to be useful. For a material with a specific gravity near one, a cube with edges that are 0.1 mm (100 microns) long, would weigh 1 microgram. For nanofibers, the specific gravity is only about 0.2, so the volume required for 1 microgram is

correspondingly larger The edge of a 1 microgram cube of nanofibers is about 170 microns long.

Coatings made of fibers and/or nanofibers for an array of asperities can be designed to produce a variety of useful arrangements of the nanofibers. Thus, in one embodiment, the present invention utilizes an attraction between electrically charged fibers and/or nanofibers and a sharp point. Further, because electrospun nanofibers are very long, the same nanofiber may span the space between adjacent tips and/or be attached to a large number of tips, resulting in the creation of a thin, non-woven sheet supported on the tips of the array of pins.

Other arrangements of the nanofibers, created by the methods of the present invention provide a number of advantages.

In one embodiment, the nanofibers are wrapped around the tip of each asperity, such as microprojections or microneedles, to form a relatively thick coating on the tip, with relatively few nanofibers spanning the spaces between the tips. This can be accomplished by controlling the electrospinning process so that the electrospinning jet is collected on the tips held near the location where the onset of the electrically driven bending instability occurs, and further, the jet is nearly dry, but is still soft and can readily bend. These conditions also favor mechanical buckling as the jet is caught and stopped on the tip. Via the present invention, the ability to create such nanofiber structures can be realized.

The combination of these effects, and the presence of the pin, causes the nanofibers to generate a cotton-swab-like ("Q-Tip"-like) collection of nanofibers near the tip of the pin.

Conglutination of the nanofibers can also be controlled, so that the fibers may range from fibers that are not attached to each other and slide past each other at crossing points, or fibers that are mechanically attached to each other at crossing points. The material on which the one or more asperities are formed must be a good enough electrical conductor that charge carried via or on the arriving jet(s) is conducted away. A sufficient level of ionic conductivity exists on the surface of ordinary glass at a normal ambient relative humidity. If a material with an excessively low

conductivity is utilized, the effective conductivity of the asperities can be increased sufficiently by coating them with a ten to 1000 nanometer thick layer of carbon or other conductor or conductive material. This can be accomplished by any suitable technique including, but not limited to, vapor deposition, or by evaporation of carbon or metal onto the surface bearing the one or more asperities.

It should be noted that cotton-swab-like shapes can be used as collectors for electrospun nanofibers where. in one embodiment, such collectors are placed on one or more rotating mandrels In another embodiment, the present invention does not require the use of a rotating pin Instead, in this embodiment. the arriving nanofiber rotates around the tip region of the pin. This is made to happen locally and separately at the tip of each asperity. When the arriving fiber moves from one asperity to an adjacent one, a nanofiber segment remains between the two asperities, but the total amount of material in the spanning nanofibers is much less than the total amount accumulated near the tips of all the asperities.

In another embodiment, the arriving nanofibers can be accumulated in a "ball" attached to the tip if the electrical conductivity of the collected fibers is kept at a relatively high value as the fiber accumulates. The conductivity of the solidifying nanofibers can be adjusted by addition of ions such as lithium or sodium, and, optionally. with a less volatile liquid substance added to the solvent used in the electrospinning process. The less volatile substance supports ionic conduction of the electrical charge on the arriving nanofiber to the asperity, which is maintained thereby at an attractive electrical potential. This condition allows the arriving nanofiber (which can, in one embodiment, be regarded as a jet solidified enough to retain its fiber-like shape, but still quite soft, sticky, and electrically conducting) to be collected on top of nanofibers that have already been collected. The less volatile solvent can be retained in the collected nanofibers, or in another embodiment this solvent can be evaporated in a time slightly longer than the time required to collect the desired mass of nanofibers.

In the absence of such ionic conduction through the collected fibers, charge retained on the

topmost layer will repel the arriving nanofiber, and cause it to move toward uncoated pins. The uncoated pins may be some distance away: so long lengths of spanning nanofiber can be generated, in form of a non-woven mat and utilized in some embodiments of the present invention.

Three modes of collection of nanofiber are available to the device designer, although the present invention is not limited thereto. Two cause the nanofiber to be collected on or in front of the tip of the asperity, where the probability of its being pressed mechanically through a thin layer of material is high. The third mode is the a "non-woven mat" supported on the tips of the asperities Only a relatively small fraction of the material in the "non-woven mat' will be carried through the surface as the as the asperities are pressed against the surface. The remaining matter is brought into intimate contact with the outer surface of the thin layer, where it can be used to protect the surface or in other ways.

In one embodiment, the present invention relates to a structure where the tips of one or more asperities are coated with a cotton-swab-like arrangement of nanofibers bearing one useful material. Another set of useful materials is collected in balls attached to the tip of each asperity. A third set of useful materials is collected in nanofibers that span the spaces between asperities.

In another embodiment, the present invention relates to multilayer structures where material is collected in the vicinity of the tip of the pin in the 'cotton-swab-like" part of the structure, the material collected in "balls" in front of the tip of the asperity, and the "spanning segments" that extend from the tip of one asperity to the tip of an adjacent asperity.

An electrospinning jet that wrapped polyethylene oxide nanofibers around or on the tip of each asperity in an array, was demonstrated by collecting nanofibers on closely spaced carbon fibers of the sort used in reinforced composites, which are electrically conducting, very stiff, and have diameters of about 7 microns. The jet used was, in one embodiment. less than a centimeter in length. Potential differences between the electrospinning tip and the electrically grounded carbon fibers were in the range between about one and about two kilovolts, although the present invention is not

limited to just these differences. The diameters of the collected nanofibers were about one micron.

The velocity of the jet as it arrived at the tips of the carbon fibers was low, perhaps less than about one meter per second, or perhaps even about one tenth of one meter per second. The specifications above were of a representative experiment and are not intended to act as limitations on the scope of the present invention.

The carbon nanofibers, separated by various distances, were mounted on adhesive tape at lateral distances ranging from about one millimeter to side by side contact. The carbon fibers were projected at different distances toward the jet. Cottonswab-like coatings were formed on fibers separated by less than one millimeter. Balls were grown on fibers that projected furthest in the direction from which the jet arrives. The balls and cotton-swab-like structures sometimes formed at the tips of two carbon fibers which were separated by less than a few tens of microns A relatively small number of spanning nanofibers extended between fibers that are about one millimeter apart.

Figure 1 is a low magnification optical image of 7 micron diameter carbon fibers supported on transparent tape. The divisions in the scale shown in the image are one millimeter apart. The tips of the fibers were coated with polyethylene oxide nanofibers by the electrospinning methods described in this invention. The carbon nanofibers were grounded, and then held about 1 cm below the tip at which the electrospinning jet was created. The orifice from which the jet issued was at the end of a slender glass tube which had an internal diameter of about 60 microns. The potential difference between the electrospinning tip and the carbon nanofibers was about 1.5 kV. The position at which the electrically driven bending instability occurred is noted, and the tips of the carbon fibers were held. by hand and moving slightly, near this position. The collection of the nanofibers required about 10 or 20 seconds, and was terminated when white spots are visible at the ends of the carbon fibers. The nanofiber coating extended all the way around the tip of each of the carbon fibers.

Figure 2 shows a coated carbon fiber tip at higher magnification. The diameter of the coated

fiber was about 70 microns, so the radial thickness of the coating was about 30 microns. Several spanning nanofibers can be seen near the tip. showing that the number of spanning nanofibers was smaller than the number of nanofibers on the tip. The end of the coated fiber is rounded. The diameter varied slightly at different places along the axis. The upper part of the figure is out of focus, and is seen, by focusing on this region, to be smaller in diameter than the region near the tip.

Figure 3 shows the tip of a carbon fiber, of the same sort as in Figures 1 and 2, coated with a ball of nanofibers at the tip. This carbon fiber is extended beyond its neighbors, and is in a position to collect more, less dry fibers, which is consistent with the mechanisms described in this disclosure, for the creation of balls on the tips by enhanced electrical conductivity of the collected fibers.

If the jet were wet when collected, balls or droplets also form. The fluid jet carries liquid into the droplet until the charge accumulates to a high enough value to cause the jet to move to a new location, usually leaving a spanning fiber to mark its path. These droplets do not dry as rapidly as nanofibers, but probably still provide significant sequestration and protection to bioactive substances in the fluid. The distinctions between balls of nanofibers and fluid droplets are noted but not emphasized or critical to the present invention.

In one embodiment, the devices of the present invention are employed for the injection of bioactive substances through the skin. Bioactive substances, (for therapeutic, diagnostic, vaccination, immunization, behavior modification, health monitoring, and other such purposes) need to be injected through the skin. This can be accomplished by coating the bioactive substance on a tapered pin which pierces the skin and deposits the bioactive material inside, either as a particle or by dissolving it in blood or other body fluids.

In practice it is desirable to use arrays of many pins, or microprojections, or microneedles to achieve efficacious concentrations of the bioactive material inside the body. In most instances below, references to a pin or microprojection or microneedle are intended to describe both a single pin, microprojection, or microneedle, and an array of a large number of similar pins,

microprojections, or microneedles. A prototypical pin, microprojection, or microneedle for this purpose, in one embodiment, has a diameter of about 100 microns at its broad end and tapers to a sharp tip of chisel edges with somewhat smaller diameter at its other end. The diameter of the tip may for example, be in the range from about 50 microns to about 5 microns. Such a pin, microprojection, or microneedle can be inserted through the skin and withdrawn with minimal damage to the skin tissue.

It is advantageous to coat the tip with nanofibers which contain a useful substance inside or on the nanofiber. When the coated pin, microprojection, or microneedle is inserted through the skin, the coating material is distributed between the outer surface of the skin, the surface of the hole created in the skin, and the interior of the body.

In another embodiment, the present invention relates to nanofibers that are carried on a pin, microprojection, or microneedle that will and/or are designed to absorb substances from inside the body and retain a sample of these substances when the pin is withdrawn. The retained substances can then be analyzed for diagnosis, control, or other purposes. Glucose concentration monitoring provides an example.

Nanofibers adhere tightly to the tips and to each other during insertion. When wet with bodily fluids the fibers may become slippery, dissolve, degrade, release bioactive substances by leaching or chemical reaction or produce other useful effects. The material used for the nanofiber and the known possibilities for forming nanofibers from complex mixtures of substances provide many options, which are included in this invention.

The bioactive materials are to be injected through the skin, as described herein. The spanning nanofibers are applied in a thin layer. For example, the spanning fibers could be a nitric oxide (or chlorine dioxide) releasing structure of the sort described in other co-owned University of Akron patents. This sort of non-woven mat applied over the top of pins, microprojections, or microneedles, already coated with the bioactive substances for injection through the skin, could be used both to

keep the device sterile between manufacture and use, and to sterilize the skin during and after use. The non-injected material in the spanning nanofibers contributes to an advantageous design that utilizes both injected and non-injected components of the nanofibers.

Because the pins used to carry the nanofibers through the skin are much thicker than the carbon fibers, and in ordered arrays, it is clear from the results obtained with the thin carbon fibers, that microgram quantities of nanofibers can be placed at desired locations, described above, on the arrays of pins created by MEMs fabrication methods.

The cotton-swab like shape is a desirable arrangement of nanofibers, since the majority of the useful material is attached directly to the part of the pin that will be inside the skin when the device is used, Reactions between reagents in dry fibers that span the spaces between pins can release a material to disinfect the skin, or to adhere to the skin after the pins are removed and manage fluids escaping from the puncture holes, or for other purposes.

Because, in one embodiment, the nanofibers of the present invention are much smaller than the asperities needed to penetrate the skin, the shape of the tips of the pins can be modified to carry more nanofibers through the skin.

Figure 4 shows radial steps which both increase the surface area near the tip and provide corners that protect some of the nanofibers from the shearing forces that would otherwise wipe the nanofibers off the tip as it is forced through the skin.

Figure 5 provides even more protection of against wiping off of material to be injected, obtained by the creation of forward facing barbs and also illustrates the use of circumferential grooves to protect the fibers from wiping.

Figure 6 has backward facing barbs that protect some of the nanofibers from wiping as the point is inserted, and also tends to carry some of the nanofibers as the tip is withdrawn. The withdrawn material would then be available for analysis. Only the region close to the tip of the pin is shown in these three figures. The patterns shown are not limiting. These patterns may continue in

the same way or with variations along the entire surface of the pin.

In another embodiment, two drugs are employed in an electrospun formulation. In some embodiments, the first drug and the second drug are different, but selected from antidepressant compounds, analgesic compounds, anti-inflammatory steroidal compounds (corticosteroids), non-steroidal anti-inflammatory compounds (NSAIDs), antibiotic compounds, anti-fungal compounds, antiviral compounds, antiproliferative compounds, antiglaucoma compounds, immunomodulatory compounds, cell transport/mobility impeding agents, cytokines and peptides/proteins skin-treating compounds sunscreens skin protectants, leukotrienes such as LTB4, antimetabolite compounds, antipsoriatic compounds, keratolytic compounds, anxiolytic compounds, and antipsychotic compounds. In a preferred embodiment, the polymer/two drug electrospun preparation is delivered transdermally, intradermally, intra-epidermally, transmucosally, or subcutaneously. Alternatively, the electrospun material including the two drugs is delivered IP, IV, or IM.

The present invention further contemplates, in one embodiment, a drug delivery system comprising a) a substrate comprising asperities such as microprojections or microneedles, and b) an embodiment of the various electrospun preparations (mentioned above). The term "substrate", as used herein, includes materials out of which the asperities are made including, but not limited to, silicon. When microprojections or microneedles are used, they may be, in one embodiment, solid. In another embodiment, they may be hollow. It is not intended that the present invention be limited to the manner in which the microneedles are contacted with the electrospun preparation. In one embodiment, the microneedles are contacted only at the microneedle tips. In one embodiment, the preparation contacts the entire substrate (or substantially the entire substrate) such that the preparation contacts even the spaces between an array of microneedles. It is not intended that the present invention be limited to uniform coatings of the preparation. In one embodiment, the preparation is present on the substrate in a non-uniform manner. In one embodiment, an electrospun polymer/drug(s) preparation is sprayed on said microneedles. In one embodiment, said

microneedles are dipped into an electrospun preparation.

In one embodiment of the multidrug microneedle array, the drugs are separated on the array into different regions or zones in the manner of a mosaic. Conceptually, the mosaic approach provides a means of packaging, storing and delivering to the patient drug combinations without encountering conventional formulation issues. Incompatible drugs need not be chemically "compatibilized" if they are deposited and stored at different addresses on a microneedle array or other type of skin patch.

One particularly useful application of the mosaic design comprises a multidrug microneedle embodiment where a non-polar and a polar drug are to be co-administered. While such drugs are not easily mixed into a single vehicle, the mosaic design permits both drugs to be on the same delivery platform, albeit separated. Another particularly useful application of the mosaic design comprises a multidrug microneedle embodiment wherein it is desired to carry drugs that cancel one another's side effects or that have synergistic effects. Such uses would be particularly appropriate where traditional formulation happens to be problematic.

It is not intended that the present invention be limited by the nature of the substrate comprising said microneedles. In one embodiment, the microneedles are formulated out of polymer. In another embodiment, the microneedles are made with a mold. In a preferred embodiment, the microneedles are etched out of a silicon substrate. In a preferred embodiment, said silicon microneedles are solid and the electrospun polymer/drug(s) formulation is deposited on said microneedles.

It is not intended that the present invention be limited to a single polymer or a single layer on said microneedles. In one embodiment, a first layer comprises a first polymer and a second layer a second polymer, where said second polymer is less soluble (e.g. in water) than said first polymer (Figure 7A). When the microneedles are administered, the microenvironment of the skin causes the first layer to dissolve (or substantially dissolve) thereby releasing the second (or top) layer from the

microneedles (Figure 7B). The bioactive agent (e.g. drug, antigen or other substance to be delivered) can be in the first layer, second layer, or both. Preferably, it is in the second layer. For example, in one embodiment, the present invention contemplates a substance delivery device comprising: a substrate having a back surface and a front surface; a plurality of solid microneedles extending upwards from the front surface of the substrate; a first polymer layer in contact with said microneedles; and a second polymer layer in contact with said first polymer layer, said second polymer layer comprising at least one bioactive substance.

It is also not intended that the present invention be limited to inflexible microneedle arrays. Indeed, embodiments of flexible microneedle arrays are contemplated. In one embodiment of a flexible microneedle, the present invention contemplates separating microneedles into individual "islands" by cutting into (and even through) the substrate so as to define such islands or regions separated by channels or streets (which can be, in one embodiment, filled or partially filled with polymer or drug). In one embodiment, the present invention contemplates mounting the substrate onto an adhesive material (e.g. adhesive tape) and dicing or cutting through the substrate to generate flexible arrays (Figure 8). In this manner, the risk of breakage when pushing against the back of the silicon substrates, when applying the patch to the skin, is reduced.

Various features can be added to the microneedle arrays to assure proper delivery. In one embodiment, the present invention contemplates the use of a plastic or otherwise elastomeric device positioned above the array relative to the skin (or attached or incorporated into the substrate or upper layer) that snaps into place once pressure is applied against the patch to push and keep the array of microneedles in the skin while the patch is on (to make sure needles are inside the skin and to avoid the need for an applicator in the final product, which is fully disposable in this embodiment). In one embodiment, the elastomeric element takes a first form prior to administration (Figure 9A) and then takes a second form after application of pressure (Figure 9B). In other words, the elastomeric element (which can be arched, curved or generally U-shaped) undergoes a shape change or

deformation upon receiving the pressure from pushing the array into contact with the skin (e.g. from concave to convex).

Thus, the present invention contemplates formulations and devices, including but not limited to delivery devices, as well as methods of making formulations and devices. In one embodiment, the present invention contemplates a method of creating a substance delivery device, comprising: providing i) an electrospun polymer formulation comprising at least one substance, and ii) a substrate comprising a plurality of microprojections; and depositing at least a portion of said preparation onto at least a portion of said substrate, so as to create a substance delivery device. The present invention also contemplates, as a device, the treated substrate prepared according to the above-described method. In one embodiment, said electrospun formulation comprises nanofibers.

It is not intended that the present invention be limited to a particular water-soluble polymer. In one embodiment, said water-soluble polymer is selected from the group consisting of PVP, hydroxyethylcellulose, carboxymethyl cellulose, alginic acid, chitosan, and poly(glutamic acid).

It is not intended that the present invention be limited by the nature of the substance. In one embodiment, said substance is a therapeutic protein. In another embodiment, said substance is a vaccine antigen. In one embodiment, first and second substances are used (e.g., a vaccine antigen as a first substance, and adjuvant as the second substance).

The present invention, as mentioned above, also contemplates methods of administering substances (including but not limited to pharmaceuticals). In one embodiment, the present invention contemplates a method of administering a substance, comprising: providing a subject and the delivery device described above; and contacting said subject with said delivery device under conditions such at least a portion of said substance is released from said device. The term "subject" includes human and non-human animals. In the case of humans, the term includes more than patients. The term also includes healthy, asymptomatic recipients. In one embodiment, said contacting comprises piercing the subject's skin with said asperities such as microprojections or

microneedles.

The present invention also contemplates, in one embodiment, a substance delivery device comprising: a substrate having a back surface and a front surface; and a plurality of solid microneedles extending upwards from the front surface of the substrate, the microneedles comprising an electrospun polymer formulation, said formulation comprising at least one substance. In one embodiment, said formulation provides for a sustained release of substance. In another embodiment, said formulation provides for various release rates of said substance.

When in an array, the density of the microprojections is, in one embodiment, at least 10 microprojections/cm^2, in another embodiment, at least 200 microprojections/cm^2, and, in some embodiments, at least 1000 microprojections/cm^2. In one embodiment, each microneedle is spaced (when measured center to center with another microneedle) between 300 microns and 2.7 mm apart. In one embodiment, the spacing is approximately three times the height of the microneedle, i.e. for a microneedle that is 600 microns (plus or minus 200 microns) in height, the spacing may be 1.8 mm, while for a microneedle that is 900 microns in height, the spacing may be 2.7 mm, while for a microneedle that is 300 microns in height, the spacing may be 900 microns.

The figures herein provide illustrative examples of microneedles and corresponding arrays. Figure 10 is a scanning electron microscope picture showing an overview of one embodiment of a microneedle array (each microneedle is approximately 620 microns, plus or minus 20 microns, in height) with a particular density (the distance between microneedles, when measured center to center, is approximately 1.8 mm, plus or minus 200 microns. Figure 11 is a scanning electron microscope close up, side view of one embodiment of a single microneedle (e.g. within the array) of approximately 620 microns (plus or minus 20 microns) in height showing the pyramidal shape. Figure 12 is a scanning electron microscope close up, top view of one embodiment of a single microneedle (e.g. within the array) with eight sides (i.e. eight surfaces) coming together at the top to form a point or tip, wherein the width is approximately 385 microns, plus or minus 5 microns, and

the height is approximately 620 microns, plus or minus 20 microns.

It is not intended that the present invention be limited to a particular density. Figure 13 shows a prospective microneedle array with a higher density of microneedles.

In another non-limiting embodiment, the present invention comprises a microfabricated device for transdermal, intra-epidermal, intradermal or transmucosal drug delivery comprising ionically cross-linked macromolecular assemblies, which provides sufficient mechanical strength, sufficient capacity of loading various amounts of drug, stability, and release characteristics. After the device is brought in contact with a subject's skin, the substance is released.

Ionically cross-linked macromolecular assemblies are polymer networks that are formed through the establishment of reversible links between the macromolecular chains as described in *"J. Berger, M Reist, J.M. Mayer, 0. Felt, N.A. Peppas, R. Gurny, Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications, European Journal of Pharmaceutics and Biopharmaceutics 57 (2004) 19-34."* They can include polyelectrolyte complexes; multilayer polyelectrolyte materials, complexes of polyelectrolytes with low molecular weight ions; assemblies of dendrimers; ionically cross-linked hydrogels, which can be in the form of film; micro-and nanospheres; micro- and nanocapsules; micro- and nanofibers. The properties of cross-linked hydrogels depend mainly on their cross-linking density, namely the ratio of moles of cross-linking units to the moles of polymer repeating units, polymer characteristics, and microstructure of the material. Ionically cross-linked assemblies include those in which gradient exists in the density of the cross-linking agent.

Ionically cross-linked macromolecular assemblies of the present invention comprise a substance to be delivered. The preferred substances of the present invention are biologically active agents such as those described herein, including but not limited to, pharmaceutically active substances, such as therapeutic drugs, which can include small molecule drugs, proteins, peptides, nucleic acids, hormones, or vaccines, which can include antigens and adjuvants.

The formulation containing the pharmaceutically active substance can be in the form of an ionically cross-linked three dimensional film, sphere, tube, fiber, such as a nanofiber or microfiber, or in any other geometrical shape, and is attached the surface of the device by chemical or physical means.

The polymers of this aspect of the present invention may be water-soluble polymers. In one embodiment they are polyelectrolytes - polymers containing ionic or ionizable groups. In another embodiment they are polymers capable of establishing ionic complexes, such as sodium – poly(ethylene oxide), or sodium -poly[di(methoxyethoxyethoxy)phosphazene complex. In one embodiment the polymers are biodegradable polymers. Typical examples of such polymers are poly(ethylene glycol), polyvinylpyrrolidone, polyvinylalcohol, poly(ethylene oxide), polyoxymethylene, poly(hydroxyethyl methacrylate), carboxymethylcellulose, hydroxyethylcellulose, hydroxypropylcellulose, alginic acid, chitosan, poly(glutamic acid), poly(isobutylacrylamide), poly(ethylenimine), and copolymers thereof. The polymers can be linear, branched, brush- or comb-like in their macromolecular architecture. Copolymers can be random or block copolymers, or biomolecules (such as fibrin, fibrinogen, cellulose, starch, collagen and hyaluronic acid). In yet another embodiment, the polymers can be hydrophobic, but contain hydrophilic groups or hydrophilic segments capable of forming ionic cross-links or complexes. Examples of hydrophobic polymers are poly(butyl methacrylate), poly(ethyl methacrylate), poly(vinylidene fluoride) poly(hydroxyvalerate), poly(L-lactic acid), polycaprolactone, poly(lactide-co-glycolide), poly(hydroxybutyrate), poly(hydroxybutyrate-co-valerate), polydioxanone, polyorthoester, polyanhydride, poly(glycolic acid), poly(vinyl methyl ether), polyvinylidene chloride, polyacrylonitrile, poly(trimethylene carbonate), poly(iminocarbonate), and other derivitized polyurethanes, and poly(organosiloxanes).

It is contemplated that the ionically cross-linked macromolecular assembly/drug(s) preparation can be deposited on a surface (in a uniform or non-uniform manner). In one

embodiment, the preparation is deposited (e.g. by dipping, coating, spin coating, spraying, or by suitable applicator) on an array of asperities such as those described herein, such as microprojections or microneedles for transdermal, intradermal, or transmucosal delivery of the bioactive substance.

While the microneedle embodiment (described herein) is preferred, other systems and apparatus that employ tiny skin piercing elements to enhance transdermal agent delivery are also contemplated, as disclosed in U.S. Pat. Nos. 5,879,326, 3,814,097, 5,250,023, 3,964,482, Reissue U.S. Pat. No. 25,637, and PCT Publication Nos. WO 96/37155, WO 96/37256, WO 96/17648, WO 97/03718, WO 98/11937, WO 98/00193, WO 97/48440, WO 97/48441, WO 97/48442, WO 98/00193, WO 99/64580, WO 98/28037, WO 98/29298, and WO 98/29365; all incorporated herein by reference in their entirety.

In one non-limiting embodiment, the macromolecular assembly can be prepared by ionic cross-linking or complexation of nanofibers. Polymeric materials can be fabricated in fibers, nano- and microfibers. The term "fiber" includes not only structures that are cylindrical, but also includes structures which vary from a cylindrical shape, such as for example, structures which are flattened or ribbon shaped. When fine fibers are randomly distributed they can form an interlocking net (non-woven fine fiber).

A "fiber-forming" material is capable of being fabricated into fibers. The fiber-forming materials used in this invention include polymers, which can be in a liquid state, such as melted, in solution, or in suspension. Water, organic solvents, or mixtures thereof or heat can be used to convert solid polymer into a liquid "fiber-forming" material. Electrostatic solution spinning is one method of making nanofibers and microfiber. Such electrospun material is then treated with a solution of an ion-complexing agent for cross-linking.

In one embodiment, the macromolecular assembly can be fabricated on the surface of the device using an aqueous based method (Figure 14). The advantages of such method for making ionically cross-linked hydrogels are that it avoids the use of organic solvents, heat, and, complicated

manufacturing equipment. In one embodiment, the method involves the following steps. First, a solution of containing polymer and pharmaceutically active substance can be applied to a device using methods, such as dipcoating, spin coating, electrospinning, spraying, or with a brush or other suitable applicator. The preferred solution is a solution in water or in aqueous buffer; however organic solutions can be used or added if beneficial. The surface of a device can be dried after the application if desired. Secondly, a solution containing multivalent or polyvalent ions (cross-linker), is applied to the surface using the previously described methods.

Multivalent ions can be selected from the group if inorganic ions, such as calcium, zinc bismuth, barium, magnesium, aluminum, copper, cobalt, nickel, or cadmium, or organic ions, such as spermine, spermidine, or polylysine. In one embodiment, the salt of the monovalent ion is a calcium salt, such as calcium chloride, or $CaCl_2$. The salt of the multivalent ion may be present in the solution at any concentration and pH, preferably from about 1% to about 25% and a pH from 4 to 9. The Ca^{2+} ions in the solution serve as a cross-linker, whereby the film is stabilized when they contact the solution containing the calcium salt.

The device can then be dried by freeze-drying, treating it with ethanol or other water miscible organic solvent, or under vacuum.

Alternatively, a physiologically active substance can be dissolved or dispersed in a solution of the cross-linker (multivalent or polyvalent ions) (Figure 15). In one embodiment of such a method, the physiologically active substance is introduced in the film at the same stage as the film is stabilized.

In one embodiment, the ionic cross-linking process can be applied to the total volume of the polymer film. In another embodiment the cross-linking process is limited to portion of the film, such as a portion localized on the elevated part of the device. In yet another embodiment, the cross-linking process is only applied to a portion of the film, which is localized on the tip of the elevated part of the device.

It is realized that the nature of the cross-linking agent used in the process can influence the stability of the film and thus the disintegration time and the release kinetics upon the administration. It is generally known that the disintegration and release time increases as the stability of the ionic links in hydrogel matrices increases in the following way: two valent ionic cross-linker, such as calcium ion, three valent ionic cross-linker, such as aluminum ion, multivalent organic ions, such as spermine or spermidine, polyvalent organic ions, such as poly(L-lysine). It is also understood that an increase in the cross-linking density of the hydrogel matrix can also lead to the increase in the disintegration of the hydrogel formulation and release time of the pharmaceutically active substance.

In one embodiment multiple ions are used to cross-link hydrogel matrix. In another embodiment the gradient of ions is achieved through the depth of the film. In yet another embodiment, the cross-linking density is also varied through the volume of the film. This can be achieved via varying the cross-linker and its concentration with time in the cross-linking solution. Alternatively, the polymer hydrogel is composed of interpenetrating networks of ionically cross-linked polyelectrolytes.

In a preferred embodiment, only one type of a cross-linker is used and no additional cross-linking agent is added in the course of the process.

In yet another embodiment, the type of cross-linker and the concentration of the cross-linkers varies in the various zones of the device. Thus, various modes of release kinetics can be achieved using the same device.

The present invention contemplates administering one or more of the various embodiments of the formulations (described herein) comprising a drug, such as a prophylactic or therapeutic to humans and animals. In one embodiment, the present invention contemplates a method comprising: a) providing i) a preparation comprising at least one drug in a ionically-cross-linked macromolecular assembly and ii) a subject, wherein said subject is a human or non-human animal; and b) contacting said subject with said preparation (e.g. under conditions such that at least a portion of said drug is

released into said subject). In one embodiment, the present invention contemplates a method comprising: a) providing i) a preparation comprising one or more drugs in a macromolecular assembly formulation and ii) a subject, wherein said subject is a human or non-human animal; and b) contacting said subject with said preparation (e.g. under conditions such that at least a portion of said drug is released into said subject).

In one embodiment, two drugs are employed in a macromolecular assembly formulation. In some embodiments, first drug and second drug are different, but selected from antidepressant compounds, analgesic compounds, anti-inflammatory steroidal compounds (corticosteroids), non-steroidal antiinflammatory compounds (NSAIDs), antibiotic compounds, anti-fungal compounds, antiviral compounds, antiproliferative compounds, antiglaucoma compounds, immunomodulatory compounds, cell transport/mobility impeding agents, cytokines and peptides/proteins skin-treating compounds sunscreens skin protectants, leukotrienes such as LTB_4, antimetabolite compounds, antipsoriatic compounds, keratolyiic compounds, anxiolytic compounds, and antipsychotic compounds. In a preferred embodiment, the hydrogel/two drug preparation is delivered transdermally, intradermally, intra-epidermally, transmucosally, subcutaneously, IP, IV or IM.

The present invention further contemplates, in one embodiment, a drug delivery system comprising a) a substrate comprising asperities such as those described herein, such as microprojections or microneedles, and an embodiment of the various ionically cross-linked preparations (mentioned above). When microneedles are used, they may be hollow, but preferably they are solid. It is not intended that the present invention be limited to the manner in which the microneedles are contacted with the macromolecular assembly preparation. In one embodiment, the microneedles are contacted only at the microneedle tips. In a preferred embodiment, the preparation contacts the entire substrate (or substantially the entire substrate) such that the preparation contacts even the spaces between an array of microneedles. It is not intended that the present invention be

limited to uniform coatings of the preparation. In one embodiment, the preparation is present on the substrate in a non-uniform manner. In one embodiment, a ionically cross-linked macromolecular assembly/drug(s) preparation is sprayed on said microneedles. In one embodiment, said microneedles are dipped into a preparation.

Thus, the present invention contemplates formulations and devices, including but not limited to delivery devices, as well as methods of making formulations and devices. In one embodiment, the present invention contemplates a method of creating a substance delivery device, comprising: providing i) an ionically cross-linked macromolecular assembly which includes at least one substance, such as a biologically active agent as described herein and ii) a substrate comprising a plurality of asperities such as those described herein, such as microprojections and microneedles; and depositing at least a portion of said preparation onto at least a portion of said substrate, so as to create a substance delivery device. The present invention also contemplates, as a device, the treated substrate prepared according to the above-described method. In one embodiment, said ionically cross-linked macro-molecular assembly comprises an ionically cross-linked water-soluble polymer. In a preferred embodiment, said ionically cross-linked macromolecular assembly has a multilayer structure. In another embodiment, said ionically cross-linked macromolecular assembly comprises ionically cross-linked nanofibers.

It is not intended that the present invention be limited to a particular water-soluble polymer. In one embodiment, said water-soluble polymer is selected from the group consisting of carboxymethyl cellulose, alginic acid, chitosan, poly(glutamic acid).

It is not intended that the present invention be limited by the nature of the nanofibers. In one embodiment, wherein said ionically cross-linked nanofibers comprise ionically cross-linked chitosan nanofibers.

It is not intended that the present invention be limited by the nature of the substance. In one embodiment, said substance is a therapeutic protein. In another embodiment, said substance is

vaccine antigen. In one embodiment, first and second substances are used (e.g. a vaccine antigen as a first substance, and adjuvant as the second substance).

The present invention, as mentioned above, also contemplates methods of administering substances (including but not limited to pharmaceuticals). In one embodiment, the present invention contemplates a method of administering a substance, comprising: providing: a subject and the delivery device described above; and contacting said subject with said delivery device under conditions such at least a portion of said substance is released from said device. In one embodiment, said contacting comprises piercing the subject's skin with said microprojections.

The present invention also contemplates, in one embodiment, a substance delivery device comprising: a substrate having a back surface and a front surface; and a plurality of solid microneedles extending upwards from the front surface of the substrate, the microneedles comprising a ionically cross-linked macromolecular assembly formulation, said formulation comprising at least one substance. In one embodiment, said ionically cross-linked macromolecular assemblies provide for a sustained release of substance. In another embodiment,said ionically cross-linked macromolecular assemblies provide for various release rates of said substance.

"Polyelectrolytes" are defined here as polymers that contain ionic (ionized or ionizable) groups, which groups impart to the polymer anionic, cationic, or amphiphilic character. The ionic groups can be in the form of a salt, or, alternatively, an acid or base that is, or can be, at least partially dissociated. Polyelectrolytes can also contain non-ionic side groups. Polyelectrolyte can be biodegradable (e.g. to prevent eventual deposition and accumulation of polymer molecules in the body) or non-biodegradable under the conditions of use. A preferred polyelectrolyte is a polyanion and contains ionic groups that include carboxylic acid, sulfonic acid, hydroxyl, or phosphate moieties.

Pharmaceutically active substances which may be included in the resulting preparation which includes the macromolecular assembly hereinabove described may be those described herein.

In one non-limiting embodiment, a hydrogel/drug(s) preparation is delivered transdermally, intra-epidermally, intradermally, or transmucosally using anisotropically etched MEMS microneedles in silicon. In one embodiment, the invention contemplates solid asperities such as those herein described, such as microprojections or microneedles which are fabricated in a crystal silicon material suitable for use in the administration of the various preparations discussed above. Without limiting the invention in any manner to any particular mechanism, it is believed that the drug(s) is delivered by microporation of the stratum corneum, and polymer-drug deposition within the patient's skin and subsequent dissolution or erosion of the polymer. The drug becomes thereby bioavailable; it can dissolve and diffuse to the biological target, or alternatively, it can remain at the site of administration. Micropores are made into the stratum corneum by means of a microneedle array penetration, which can optionally be further enhanced by applying energy in the form of ultrasonic, heat and/or electric signals across or through the skin.

In one embodiment, the present invention contemplates a method of administering one or more drugs to a patient comprising the steps of penetrating at least an outer layer of the skin of the subject with one or more microneedles extending from a front surface of a substrate, at least one of the one or more microneedles (or portion thereof) contacted or coated (uniformly or non-uniformly) by a macromolecular assembly/drug(s) preparation (discussed above), and releasing at least one of the one or more drugs into the subject.

In another, non-limiting embodiment, the at least one biologically active agent is a toxin.

For example, several species of Clostridium produce toxins of significance to human and animal health. [C.L. Hatheway, Clin. Microbiol. Rev. 3:66-98 (1990).] The effects of these toxins range from diarrheal diseases that can cause destruction of the colon, to paralytic effects that can cause death (see Table 3). Particularly at risk for developing clostridial diseases are neonates and humans and animals in poor health (e.g., those suffering from diseases associated with old age or immunodeficiency diseases).

Clostridium botulinum produces the most poisonous biological toxins known. The lethal human dose is a mere 10^{-9} mg/kg bodyweight for toxin in the bloodstream. THe closely related isoforms of botulinal toxin blocks nerve transmission to the muscles, resulting in flaccid paralysis. When the toxin reaches airway and respiratory muscles, the consequence is respiratory failure that can cause death. [S. Arnon, J. Infect. Dis.154:201-206 (1986).]

C. botulinum spores are carried by dust and are found on vegetables taken from the soil, on fresh fruits, and on agricultural products such as honey. Under conditions favorable to the organism, the spores germinate to vegetative cells which produce toxin. [S. Arnon, Ann. Rev. Med. 31:541 (1980).] Botulism disease may be grouped into four types, based on the method of introduction of the toxin isoform into the bloodstream. Food-borne botulism results from ingesting improperly preserved and inadequately heated food that contains botulinal toxin. [K.L. MacDonald et al., Am. J. Epidemiol. 124:794 (1986).] The death rate due to botulinal toxin is 12% and can be higher in particular risk groups. [C.O. Tacket et al., Am. J. Med. 76:794 (1984).] Wound-induced botulism results from C. botulinum penetrating traumatized tissue and producing toxin that is absorbed into the bloodstream.

TABLE 3

Clostridium Species of Medical and Veterinary Importance*

Species	Disease
C. aminovalericum	Bacteriuria (pregnant women)
C. argentinense	Infected wounds; Bacteremia; Botulism; Infections of amniotic fluid
C. baratii	Infected war wounds; Peritonitis; Infectious processes of the eye, ear and prostate
C. beijerinckikii	Infected wounds
C. bifermentans	Infected wounds; Abscesses; Gas Gangrene; Bacteremia
C. botulinum	Food poisoning; Botulism (wound, food, infant)
C. butyricum	Urinary tract, lower respiratory tract, pleural cavity, and abdominal infections; Infected wounds; Abscesses; Bacteremia
C. cadaveris	Abscesses; Infected wounds

C. carnis	Soft tissue infections; Bacteremia
C. chauvoei	Blackleg
C. clostridioforme	Abdominal, cervical, scrotal, pleural, and other infections; Septicemia; Peritonitis; Appendicitis
C. cochlearium	Isolated from human disease processes, but role in disease unknown.
C. difficile	Antimicrobial-associated diarrhea; Pseudomembranous enterocolitis; Bacteremia; Pyogenic infections
C. fallax	Soft tissue infections
C. ghnoii	Soft tissue infections
C. glycolicum	Wound infections; Abscesses; Peritonitis
C. hastiforme	Infected war wounds; Bacteremia; Abscesses
C. histolyticum	Infected war wounds; Gas gangrene; Gingival plaque isolate
C. indolis	Gastrointestinal tract infections
C. innocuum	Gastrointestinal tract infections; Empyema
C. irregulare	Penile lesions
C. leptum	Isolated from human disease processes, but role in disease unknown

TABLE 3

Clostridium Species of Medical and Veterinary Importance*

Species.	Disease
C. limosum	Bacteremia; Peritonitis; Pulmonary infections
C. malenominatum	Various infectious processes
C. novyi	Infected wounds; Gas gangrene; Blackleg, Big head (ovine); Redwater disease (bovine)
C. oroticum	Urinary tract infections; Rectal abscesses
C. paraputrificum	Bacteremia; Peritonitis; Infected wounds; Appendicitis
C. perfringens	Gas gangrene; Anaerobic cellulitis; Intra-abdominal abscesses; Soft tissue infections; Food poisoning; Necrotizing pneumonia; Empyema; Meningitis; Bacteremia; Uterine Infections; Enteritis necrotans; Lamb dysentery Struck; Ovine Enterotoxemia
C. putrefaciens	Bacteriuria (Pregnant women with bacteremia)
C. putrificum	Abscesses; Infected wounds; Bacteremia
C. ramosum	Infections of the abdominal cavity, genital tract, lung, and biliary tract; Bacteremia
C. sartagoforme	Isolated from human disease processes, but role in disease unknown.
C. septicum	Gas gangrene; Bacteremia; Suppurative infections; Necrotizing enterocolitis; Braxy
C. sordellii	Gas gangrene; Wound infections; Penile lesions; Bacteremia; Abscesses; Abdominal and vaginal infections
C. sphenoides	Appendicitis; Bacteremia; Bone and soft tissue infections; Intraperitoneal infections; Infected war wounds; Visceral gas gangrene; Renal abscesses
C. sporogenes	Gas gangrene; Bacteremia; Endocarditis; central nervous system and europulmonary infections; Penile lesions; Infected war wounds; Other pyogenic infections
C. subterminale	Bacteremia; Empyema; Biliary tract, soft tissue and bone infections

C. symbiosum	Liver abscesses; Bacteremia; Infections resulting due to bowel flora
C. tertium	Gas gangrene; Appendicitis; Brain abscesses; Intestinal tract and soft tissue infections; infected war wounds; penodonitis; Bacteremia

TABLE 3

Clostridium Species of Medical and Veterinary Importance*

Species	Disease
C. tetani	Tetanus; Infected gums and teeth; Corneal ulcerations; Mastoid and middle ear infections; Intraperitoneal infections; Tetanus neonatorum; Postpartum uterine infections; Soft tissue infections, especially related to trauma (including abrasions and lacerations); Infections related to use of contaminated needles
C. thermosaccharolyticum	Isolated from human disease processes, but role in disease unknown.

* Compiled from P.G. Engelkirk et al. "Classification", Principles and Practice of Clinical Anaerobic Bacteriology, pp. 22-23, Star Publishing Co., Belmont, CA (1992); J. Stephen and R.A. Petrowski, "Toxins Which Traverse Membranes and Deregulate Cells," in Bacterial Toxins, 2d ed., pp. 66-67, American Society for Microbiology (1986); R. Berkow and A.J. Fletcher (eds.), "Bacterial Diseases," Merck Manual of Diagnosis and Therapy, 16th ed., pp. 116-126, Merck Research Laboratories, Rahway, N.J. (1992); and O.H. Sigmund and C.M. Fraser (eds.), "Clostridial Infections, "Merck Veterinary Manual, 5th ed., pp. 396-409, Merck & Co., Rahway, N.J. (1979).

[M.N. Swartz, "Anaerobic Spore-Forming Bacilli: The Clostridia," pp. 633-646, in B.D. Davis et al.,(eds.), Microbiology, 4th edition, J.B. Lippincott Co. (1990).] Inhalation botulism has been reported as the result of accidental exposure in the laboratory [E. Holzer, Med. Klin. 41:1735 (1962)] and could arise if the toxin is used as an agent of biological warfare [D.R. Franz et al., in Botulinum and Tetanus Neurotoxins, B.R. DasGupta, ed., Plenum Press, New York (1993), pp. 473-

476]. Infectious infant botulism results from C. botulinum colonization of the infant intestine with production of toxin and its absorption into the bloodstream. It is likely that the bacterium gains entry when spores are ingested and subsequently germinate. [S. Arnon, J. Infect. Dis. 154:201 (1986).]

Different strains of Clostridium botulinum each produce one of seven structurally similar but antigenically distinct toxin isoforms designated by the letters A-G. Serotype A toxin has been implicated in 26% of the cases of food botulism; types B, E and F have also been implicated in a smaller percentage of the food botulism cases [H. Sugiyama, Microbiol. Rev. 44:419 (1980)]. Wound botulism has been reportedly caused by only types A or B toxins [H. Sugiyama, supra]. Nearly all cases of infant botulism have been caused by bacteria producing either type A or type B toxin. (Exceptionally, one New Mexico case was caused by Clostridium botulinum producing type F toxin and another by Clostridium botulinum producing a type B-type F hybrid.) [S. Arnon, Epidemiol. Rev. 3:45 (1981).] Type C toxin affects waterfowl, cattle, horses and mink. Type D toxin affects cattle, and type E toxin affects both humans and birds.

A trivalent antitoxin derived from horse plasma is commercially available from Connaught Industries Ltd. as a therapy for toxin types A, B, and E. However, the antitoxin has several disadvantages. First, extremely large dosages must be injected intravenously and/or intramuscularly. Second, multiple administrations of antitoxin can trigger serious side effects, such as acute anaphylaxis which can lead to death, and serum sickness. Finally, the efficacy of the antitoxin is uncertain and the treatment is costly.

[C.O. Tacket et al., Am. J. Med. 76:794 (1984).]

A heptavalent equine botulinal antitoxin which uses only the F(ab')2 portion of the antibody molecule has been tested by the United States Military. [M. Balady, USAMRDC Newsletter, p. 6 (1991).] This was raised against impure toxoids in those large animals and is not a high titer preparation.

A pentavalent human antitoxin has been collected from immunized human subjects for use

as a treatment for infant botulism. The supply of this antitoxin is limited and cannot be expected to meet the needs of all individuals stricken with botulism disease. In addition, collection of human sera must involve screening out HIV and other potentially serious human pathogens. [P.J. Schwarz and S.S.on, Western J. Med. 156:197 (1992).]

Immunization of subjects with toxin preparations has been done in an attempt to induce immunity against botulinal toxins. A C. botulinum vaccine comprising chemically inactivated (i.e., formaldehyde-treated) type A, B,C, D and E toxin is commercially available for human usage. However, this vaccine preparation has several disadvantages. First, the efficacy of this vaccine is variable (in particular, only 78% of recipients produce protective levels of anti-type B antibodies following administration of the primary series). Second, immunization is painful (deep subcutaneous inoculation is required for administration), with adverse reactions being common (moderate to severe local reactions occur in approximately 6% of recipients upon initial injection; this number rises to approximately 11% of individuals who receive booster injections) [Informational Brochure for the Pentavalent (ABCDE) Botulinum Toxoid, Centers for Disease Control]. Third, preparation of the vaccine is dangerous as active toxin must be handled by laboratory workers.

What are needed are safe and effective vaccine preparations for administration to those at risk of exposure to C. botulinum toxins.

The present invention contemplates, in a non-limiting embodiment, a preparation comprising at least one polymer and at least one Clostridium antigen or toxin (or fragment thereof) such as those selected from a species set forth in Table 3. The present invention contemplates, in one embodiment, such an antigen or toxin (or fragment thereof) in a preparation suitable for transdermal, intradermal, or transmuscosal delivery. In a preferred embodiment, the species is C. botulinum. In one embodiment, the antigen or toxin (or fragment thereof) is in a hydrogel formulation. In one embodiment, the antigen or toxin (or fragment thereof) is attached or linked to a carrier. In one embodiment, the antigen or toxin (or fragment thereof) is part of a fusion protein. In one

embodiment, the antigen or toxin (or fragment thereof) is in a micelle formulation. In one embodiment, the antigen or toxin (or fragment thereof) is in or on nanofibers, nanotubes, nanospheres, or nanocapsules. In one embodiment, the antigen or toxin (or fragment thereof) is complexed with one or more dendrimers. In one embodiment, the antigen or toxin (or fragment thereof) is in a liposome preparation (e.g. for liposome delivery). In a preferred embodiment, the antigen or toxin (or fragment thereof) is on the surface of a substrate, e.g. on a skin patch, microneedle array, or the like. Combinations of the above are specifically contemplated, including but not limited to, substrates comprising the Clostridium antigen(s) or toxin(s) (or fragment(s) thereof) in a polymer formulation, or in an assembly of nanofibers. In a particularly preferred embodiment, the toxin is functionally impaired (e.g. it will not cause toxicity).

In one embodiment, the Clostridium antigen or toxin (or fragment thereof) is in a hydrogel formulation. It is not intended that the present invention be limited by the nature of the hydrogel since a variety of types can be used, including but not limited to ionically cross-linked hydrogels or hydrogel films. It is contemplated that the hydrogel/antigen(s) or hydrogel/toxin(s) preparation can be deposited on a surface (in a uniform or non-uniform manner). In one embodiment, the preparation is deposited (e.g. by dipping, coating, spin coating, spraying, or by suitable applicator) on an may of microprojections or microneedles for transdermal, intradermal or transmucosal delivery of the antigen(s). In one embodiment, the present invention contemplates a method comprising: a) providing i) a preparation comprising one or more Clostridium antigens or toxins (or fragments thereof) in a hydrogel formulation and ii) a subject, wherein said subject is a human or non-human animal; and b) contacting said subject with said preparation (e.g. under conditions such that said subject generates an immune response to said one or more antigens or toxins).

When polymers are used, it is not intended that the present invention be limited by the nature of the polymer since a variety of types can be used (see Table 1). In one embodiment, the polymer comprises polyvinylpyrrolidone or hydroxy-propylcellulose. In one embodiment, the polymer

comprises hydroxyethylcellulose.

It is not intended that the present invention be limited to the manner in which the preparation of polymer and antigen (or toxin) is prepared. However, in a preferred embodiment, the preparation is electrospun to create fibers or fiber mats. It is preferred that at least a portion of the antigen (or toxin) in the electrospun fibers or mats be capable of release or escape from the polymer (e.g. upon contact with an aqueous or semi-aqueous environment). In certain embodiments (e.g. cosmetic), it is desired that the toxin be functional. In other embodiments (e.g. vaccines), it is not necessary that the toxin be functional (e.g. mutants and chemically treated toxins can be used).

In a preferred embodiment, the toxin polypeptides comprise one or more Clostridium botulinum neurotoxins. The invention contemplates the use of polypeptides derived from C. botulinum toxin as immunogens for the production of vaccines and antitoxins, as well as for cosmetic purposes. The C. botulinum vaccines and antitoxins find use in humans and other animals. In one embodiment, the present invention contemplates a fusion protein comprising a non-toxin protein sequence and a portion of the Clostridium botulinum type A toxin. In one embodiment, the C. botulinum type A toxin sequences comprise a portion of the sequence shown in Figure 16. In another embodiment, the C. botulinum type A toxin sequences comprise a portion of the sequence shown in Figure 17. It is not intended that the present invention be limited by the nature of the fusion protein. For example, the fusion protein may comprise the Clostridium botulinum type A toxin sequence as shown in Figure 17 along with a poly-histidine tract. The toxins are conveniently made in host cells containing a recombinant expression vector, wherein the vector encodes a fusion protein comprising a non-toxin protein sequence and a portion of the Clostridium botulinum type A toxin sequence shown in Figure 16. In this embodiment, the host cell is capable of expressing the encoded Clostridium botulinum type A toxin protein as a soluble protein at a level greater than or equal to 0.25% to 10% of the total cellular protein and preferably at a level greater than or equal to 0.75% of the total cellular protein. It is not intended that the present invention be limited by the

nature of the fusion protein expressed by the recombinant vector in the host cell. For example, the fusion protein may comprise the Clostridium botulinum type A toxin sequence as set forth shown in Figure 17, along with a poly-histidine tract.

Regardless of whether a wild-type, mutant, or toxin fusion proteins are used, the present invention contemplates administering one or more of the various embodiments of the preparations described above as a vaccine to humans and animals, or as a cosmetic. In one embodiment, the present invention contemplates a vaccine-related method comprising: a) providing i) a preparation comprising at least one polymer and one or more C. botulinum toxins and ii) a subject, wherein said subject is a human or non-human animal; and b) contacting said subject with said preparation (e.g. under conditions such that said subject generates an immune response to said one or more antigens). In a preferred embodiment, the toxin is electrospun with one or more polymers such as those described herein so as to create an electrospun preparation. As discussed below, in a preferred embodiment, the electrospun preparation is part of a vaccine delivery system or a cosmetic.

The present invention further contemplates, in one embodiment, a vaccine delivery system comprising a) a substrate such as described herein comprising asperities such as those described herein, such as microprojections or microneedles, and an embodiment of the various preparations (mentioned above) comprising polymer and toxin. In a preferred embodiment, the asperities, such as microprojections or microneedles have dimensions such that they penetrate into the stratum corneum, the epidermal layer, and, in some embodiments, the dermal layer. In one embodiment, the microprojections or microneedles have a length (or height) or less than 1000 microns, more preferably less than 500 microns, and still more preferably less than 250 microns. The microprojections may be formed in different shapes, such as needles, blades, pins, punches, and combinations thereof. When in an array, the density of the microprojections is at least 10 microprojections/cm^2, more preferably, at least 200 microprojections/cm^2, and, in some embodiments, at least 1000 microprojections/cm^2. The microneedles may be hollow or solid.

It is not intended that the present invention be limited by the nature of the substrate comprising said microneedles. In one embodiment, the microneedles are formulated out of polymer. In one embodiment, the microneedles are formulated (at least in part) out of a polymer/antigen(s) or polymer/toxin(s) preparation. In another embodiment, the microneedles are made with a mold. In a preferred embodiment, the microneedles are etched out of a silicon substrate. In a preferred embodiment, said silicon microneedles are solid and an electrospun polymer/antigen(s) preparation is deposited on said microneedles.

Thus, the present invention contemplates novel conjugates, formulations, delivery devices, methods of conjugation, methods of formulation, and methods of delivery. In one embodiment, the present invention contemplates a method of creating a formulation: providing i) at least one fiber-forming material, and ii) a preparation comprising at least one Clostridium toxin; mixing said fiber-forming material with said preparation so as to create a mixture; and electrospinning said mixture to create an electrospun formulation. In one embodiment, the method further comprises: depositing at least a portion of said electrospun material onto at least a portion of a substrate to create a treated substrate (e.g. microneedles, including but not limited to etched solid microneedles). The present invention also contemplates, as a device, the treated substrate prepared according to the above-described method. In one embodiment, said fiber-forming material comprises hydroxypropylcellulose, while in another embodiment said fiber-forming material comprises PVP. In one embodiment, said preparation comprises first and second Clostridium toxins. In one embodiment, said first toxin comprises at least a portion of a C. difficile toxin and said second toxin comprises at least a portion of C. botulinum toxin. In one embodiment, said first and second toxin are part of a fusion protein.

In one embodiment, the present invention contemplates a method of creating a toxin delivery device: providing i) at least one fiber-forming material, ii) a preparation comprising at least one Clostridium toxin, and iii) a substrate comprising a plurality microprojections; mixing said fiber-

forming material with said preparation so as to create a mixture; electrospinning said mixture to create an electrospun material; and depositing at least a portion of said electrospun material onto at least a portion of said substrate, so as to create a toxin delivery device. In one embodiment, said Clostridium toxin is a C. botulinum toxin. The present invention also contemplates, as a device, the treated substrate prepared according to the above-described method. In one embodiment, said fiber-forming material comprises hydroxypropylcellulose, while in another embodiment said fiber-forming material comprises PVP.

The present invention, as mentioned above, also contemplates methods of administering antigens and vaccines. In one embodiment, the present invention contemplates a method of administering antigen, comprising: providing: a subject and the toxin delivery device described above; and contacting said subject with said antigen delivery device under conditions such at least a portion of said antigen is released from said device. In one embodiment, said contacting of step b) comprises piercing the subject's skin with said microprojections.

The present invention also contemplates, in one embodiment, a toxin delivery device comprising: a substrate having a back surface and a front surface; a plurality of solid microneedles extending upwards from the front surface of the substrate, the microneedles comprising a formulation, said formulation comprising at least one polymer and at least one Clostridium toxin (e.g. a C. botulinum toxin).

As used herein, the term "fusion protein" refers to a chimeric protein containing the protein of interest (i.e., C. botulinum toxin A or B and fragments thereof) joined to an exogenous protein fragment (the fusion partner which consists of a non-toxin protein). The fusion partner may enhance solubility of the C. botulinum protein as expressed in a host cell, may provide an affinity tag to allow purification of the recombinant fusion protein from the host cell or culture supernatant, or both. If desired, the fusion protein may be removed from the protein of interest (i.e., toxin protein or fragments thereof) prior to immunization by a variety of enzymatic or chemical means known to the

art.

As used herein, the term "poly-histidine tract" when used in reference to a fusion protein refers to the presence of two to ten histidine residues at either the amino- or carboxy-terminus of a protein of interest. A poly-histidine tract of six to ten residues is preferred. The poly-histidine tract is also defined functionally as being a number of consecutive histidine residues added to the protein of interest which allows the affinity purification of the resulting fusion protein on a nickel-chelate column.

The botulinal neurotoxin is synthesized as a single polypeptide chain which is processed into a heavy (H) and a light (L) chain; these two chains are held together via disulfide bonds in the active toxin [B.R. DasGupta and H. Sugiyama, Biochem. Biophys. Res. Commun. 48:108 (1972); reviewed in B.R. DasGupta, J. Physiol. 84:220 (1990) and H. Sugiyama, Microbiol. Rev. 44:419 (1980)]. Antisera raised against purified preparations of isolated H and L chains have been shown to protect mice against the lethal effects of the toxin; however, the effectiveness of the two antisera differ with the anti-H sera being more potent (H. Sugiyama, supra).

While the different botulinal toxins show structural similarity to one another, the different serotypes are reported to be immunologically distinct (i.e., sera raised against one toxin type does not cross-react to a significant degree with other types). Thus, the generation of multivalent vaccines may require the use of more than one type of toxin. Purification methods have been reported for native toxin types A, B, C, D, E, and F [reviewed in G. Sakaguchi, Pharmac. Ther. 19:165 (1983)].

As the different botulinal toxins are structurally related, the invention contemplates polymer/toxin formulations with any of the botulinal toxins (e.g., types A-30 F) as soluble recombinant fusion proteins, and in particular electrospun formulations. In one preferred embodiment, toxins A and B of C. difficile are contemplated as immunogens (either separately or together). In one embodiment, recombinant C. botulinum toxin proteins are used as antigens in mono- and multivalent vaccine preparations. For example, soluble, substantially endotoxin-free

recombinant C. botulinum type A toxin proteins may be used alone or in conjunction with either recombinant or native toxins or toxoids from C. botulinum, C. difficile and C. tetani as antigens for the preparation of these mono- and multivalent vaccines. It is contemplated that, due to the structural similarity of C. botulinum and C. tetani toxin proteins, a vaccine comprising C. difficile and botulinum toxin proteins (native or recombinant or a mixture thereof) be used to stimulate an immune response against C. botulinum, C. tetani and C. difficile.

The adverse consequences of exposure to botulinal toxin would be avoided by immunization of subjects at risk of exposure to the toxin with nontoxic preparations which confer immunity such as chemically or genetically detoxified toxin. In general, chemical detoxification of bacterial toxins using agents such as formaldehyde, glutaraldehyde or hydrogen peroxide can be used for the generation of vaccines or antitoxins. A delicate balance must be struck between too much and too little chemical modification. If the treatment is insufficient, the vaccine may retain residual toxicity. If the treatment is too excessive, the vaccine may lose potency due to destruction of native immunogenic determinants.

Vaccines which confer immunity against one or more of the toxin types A, B, E and F would be useful as a means of protecting humans from the deleterious effects of those C. botulinum toxins known to affect man. Vaccines which confer immunity against one or more of the toxin types C, D and E would be useful for veterinary applications.

The C fragment of the C. botulinum type A neurotoxin heavy chain has been evaluated as a vaccine candidate. The C. botulinum type A neurotoxin gene has been cloned and sequenced [D.E. Thompson et al., Eur. J. Biochem. 189:73 (1990)]. The C fragment of the type A toxin was expressed as either a fusion protein comprising the botulinal C fragment fused with the maltose binding protein (MBP) or as a native protein [H.F. LaPenotiere et al., supra]. The plasmid construct encoding the native protein was reported to be unstable, while the fusion protein was expressed in inclusion bodies as insoluble protein. Immunization of mice with crudely purified MBP fusion

protein resulted in protection against IP challenge with 3 LD_{50} doses of toxin [LaPenotiere et al., supra]. However, this recombinant C. botulinum type A toxin C fragment/MBP fusion protein is not a suitable immunogen for the production of vaccines as it is expressed as an insoluble protein in E. coli. Furthermore, this recombinant C. botulinum type A toxin C fragment/MBP fusion protein was not shown to be substantially free of endotoxin contamination. Experience with recombinant C. botulinum type A toxin C fragment/MBP fusion proteins shows that the presence of the MBP on the fusion protein greatly complicates the removal of endotoxin from preparations of the recombinant fusion protein.

Inclusion body protein must be solubilized prior to purification and/or administration to a host. The harsh treatment of inclusion body protein needed to accomplish this solubilization may reduce the immunogenicity of the purified protein. Ideally, recombinant proteins to be used as vaccines are expressed as soluble proteins at high levels (i.e., greater than or equal to about 0.75% of total cellular protein) in E. coli or other host cells. This facilitates the production and isolation of sufficient quantities of the immunogen in a highly purified form (i.e., substantially free of endotoxin or other pyrogen contamination). The ability to express recombinant toxin proteins as soluble proteins in E. coli is advantageous due to the low cost of growth compared to insect or mammalian tissue culture cells.

In one embodiment, the vaccine comprises the C fragment of the C. botulinum type A toxin and a poly-histidine tract (also called a histidine tag). In a particularly preferred embodiment, a fusion protein comprising the histidine tagged C fragment (expressed using the pET series of expression vectors (Novagen)) is employed in one of the polymer/toxin formulations of the present invention, and in particular an electrospun formulation. The pET expression system utilizes a vector containing the T7 promoter which encodes the fusion protein and a host cell which can be induced to express the T7 DNA polymerase (i.e., a DE3 host strain). The production of C fragment fusion proteins containing a histidine tract is not limited to the use of a particular expression vector and

host strain. Several commercially available expression vectors and host strains can be used to express the C fragment protein sequences as a fusion protein containing a histidine tract (For example, the pQE series (pQE-8, 12, 16, 17, 18, 30, 31, 32, 40, 41, 42, 50, 51, 52, 60 and 70) of expression vectors (Qiagen) which are used with the host strains M15[pREP4] (Qiagen) and SG13009[pREP4] (Qiagen) can be used to express fusion proteins containing six histidine residues at the amino-terminus of the fusion protein).

When recombinant clostridial toxin proteins produced in gram-negative bacteria (e.g., E. coli) are used as vaccines, they are purified to remove endotoxin prior to administration to a host animal. In order to vaccinate a host, an immunogenicallyeffective amount of purified substantially endotoxin-free recombinant clostridial toxin protein is administered in any of a number of physiologically acceptable carriers known to the art. When administered for the purpose of vaccination, the purified substantially endotoxin-free recombinant clostridial toxin protein may be used alone or in conjunction with known adjutants, including potassium alum, aluminum phosphate, aluminum hydroxide, Gerbu adjuvant (GmDP; C.C. Biotech Corp.), RMI adjuvant (MPL; RJBI Immunochemical research, Inc.), QS21 (Cambridge Biotech). The alum and aluminum-based adjutants are particularly preferred when vaccines are to be administered to humans.

A variety of routes of immunization may be used, such as nasal, oral, intramuscular, intra-peritoneal, etc. However, it is preferred that immunization be through the skin (e.g. subcutaneous, or more preferably transdermally, intradermally or transmucosally using asperities such as microprojections or microneedles, as discussed herein).

In one embodiment, the present invention contemplates using Clostridium antigen(s) and/or toxin(s) in polymer formulations for vaccines and cosmetics. In a preferred embodiment, such antigens and toxins are electrospun with one or more polymers or hydrogels, such as those discussed herein.

In a preferred embodiment, the preparation comprising Clostridium antigen(s) or toxin(s) is

capable, after electrospinning, of acting as an antigen by eliciting an immune response in a human or animal. It is envisioned that in such an embodiment, a medically acceptable fiber-forming material may be used to preserve the antigen for later rehydration and use as a vaccine. In general, rehydration of the fiber of the present invention may be accomplished by mixing the fiber with a solvent for the fiber-forming material. When the fiber is used to preserve an antigen for use in a vaccine, the solvent will optimally be a medically acceptable compound.

It is not intended that the present invention be limited to the method by which the electrospun antigen(s) or toxin(s) is administered. The resulting vaccine may be injected or an ingested vaccine. However, in a preferred embodiment, the vaccine is administered at or in the skin. In a particularly preferred embodiment, the electrospun polymer/ antigen(s) material is delivered on microneedles or on a patch such that antigen(s) is released into the skin.

In one non-limiting embodiment, the toxin may be contained in an ionically cross-linked macromolecular assembly, such as those hereinabove described, including but not limited to hydrogels, such as those hereinabove described.

In one embodiment, the present invention contemplates a method of administering one or more antigens such as the toxins hereinabove described, to a patient comprising the steps of penetrating at least an outer layer of the skin of the patient with one or more microneedles extending from a front surface of a substrate, at least one of the one or more microneedles (or portion thereof) contacted or coated (uniformly or non-uniformly) by an antigen preparation (discussed above), and releasing at least one of the one or more antigens into the patient.

One use of microneedle vehicle for delivery of the polymer/toxin(s) preparations described above involves cosmetic applications, and in particular, facial cosmetic applications. The administration of an appropriate dose of, for example, botulinum toxin, to attenuate tone of muscles about the eyes and forehead can in many cases remove wrinkles characteric of aging in skin overlying the muscle while inducing only mild, often acceptable muscle weakness. Such

chemodenervating agents also may be used to induce cosmetic improvement in hemifacial paralysis by intentionally inducing partial paralysis in the contralateral side of the face thereby to improve bilateral facial symmetry.

Administration of low doses of the agents into muscles activating the jaw can retard tooth wear caused by involuntary or unconscious clenching of the teeth.

Examples of direct effects are the treatment of unwanted involuntary pathologic muscle stimulation, i.e., spasm, rigidity, or hyperstimulation, by direct administration throughout, or in the area of innervation of, the affected muscle or muscles. Thus, diseases involving muscle spasticity in general can be treated, typically without regard for its cause. The drug may be used to alleviate overstimulation, rigidity, or spasticity in muscle or muscle groups caused by stroke, cerebral palsey, multiple sclerosis, unilateral or bilateral parkinsonism, and other diseases characterized by spasmodic or continuous muscle hyperstimulation.

Accordingly, it is an object of the invention to provide a novel method of standardizing chemodenervating neurotoxin-derived pharmaceuticals such as botulinumderived pharmaceuticals by using microneedle administration. Another object is to standardize botulinum toxin preparations with respect to their zone of denervation when administered in vivo. Another object is to provide novel (lower) dosage forms of such agents. Yet another object is to provide novel therapies for muscle spasticity andlor hyperactivation heretofore untreatable or treatable only imperfectly with systemic drugs or surgery.

While many such neurotoxins are known, the currently most promising reagents of this type are the family of toxins derived from Clostridium botulinum, and the most preferred is pharmaceutical grade botulinum toxin Type A available commercially from Allergan Pharmaceuticals, Inc. under the tradename OCULINUM. The therapetic effects are achieved at dosage levels in the range between a few I.U. to 500 to 1000 I.U., preferably no more than 500 I.U., and most preferably no more than 300 I.U., and still most preferably between 50 I.U. and 200 I.U.,

administered on a microneedle array about a muscle or muscle group. The dimensions of the microneedle array control the zone of destruction of a subset of the neuromuscular junctions innervating the muscle, leaving others in a functional state.

Facial expression lines such as the transverse forehead lines or the nasolabial fold are created by attachments of projections of facial muscles into the dermis. Contraction of facial muscles generally is well known to produce the various characteristic forms of facial expressions such as smiling, grimacing, etc. In addition, exaggeration of facial lines also is associated with the aging process. The general principle of the application of the toxin is to limit the tonic contractile state of facial muscles so as to reduce muscle tone and to improve or change the quality and characteristics of facial expression.

The transverse forehead lines may be reduced in intensity by administering a quantity of toxin with a diffusion field of approximately 5 to 10 mms at the superior border of the forehead and at a point approximately 15 mms superior to the brow. This can be done symmetrically on both sides of the forehead. The glabellar lines (the frowning lines in the mid position of the forehead) may be targeted by treated the glabellar muscles with a toxin quantity producing a field of denervation of 5 to 10 mms The toxin is administered 15 mms. above the brow line in the mid position of the forehead. The nasal labial fold lines can be diminished in their intensity by treating the zygomatic major and minor muscles which emanate from the zygomatic arch and extend diagonally to the position of the nasal labial fold.

Thus, in one embodiment, the present invention contemplates a method of cosmetically decreasing facial wrinkling in a subject, the method comprising: providing i) a subject, and ii) a microneedle array comprising a chemodenervating pharmaceutical in a polymer formulation; and placing said microneedle array on the skin of said subject under conditions whereby said wrinkling is reduced. In a preferred embodiment said denervating pharmaceutical comprises a neurotoxin derived from Clostridium botulinum. In a particularly preferred embodiment, said neurotoxin is in

the form of a fusion protein. In one embodiment, said chemodenervating agent is Acetyl hexapeptide-3 (trade name Argireline). Argireline is manufactured by a Spanish company Lipotec and is a hexapeptide (a chain of 6 amino acids) attached to the acetic acid residue. It is believed to work by inhibiting the release of neurotransmitters. When applied to the skin, Argireline relaxes facial tension leading to the reduction in facial lines and wrinkles with regular use.

In a further embodiment, the present invention contemplates a method of decreasing sweating (e.g. excessive sweating of the underarms, palms, feet, etc.) of a subject, the method comprising: providing i) a subject, and ii) a microneedle array comprising a chemodenervating pharmaceutical in a polymer formulation; and placing said microneedle array on the skin of said subject under conditions whereby said sweating is reduced. In a preferred embodiment said denervating pharmaceutical comprises a neurotoxin derived from Clostridium botulinum. In a particularly preferred embodiment, said neurotoxin is in the form of a fusion protein.

Alternatively, the present invention contemplates, in one embodiment, that alpha-Bungarotoxin molecules can be used in the manner of botulinum toxin A. In one embodiment, native alpha-Bungarotoxin is employed. As used herein, "native" alphabungarotoxin molecules are those found in the venom of Bungarus multicinctus. The complete amino acid sequence of native alpha-bungarotoxin molecules and the DNA sequences that encode them have been published (see, e.g., GenBank accession numbers X91990, AF056400-AF056417, AJ131356, Y17057, Y17058, Y17693, Y17694). In one embodiment, the native sequence is IVCHTTATSPISAVTCPPGENLCYRKMWCD-AFCSSRGKVVELGCAATCPSKKPYEEVTCCSTDKCNPHPKQRPG.

In a preferred embodiment, modified alpha-bungarotoxin molecules that have altered binding specificities are employed. In particular, modification of residues 38 (Lys) and 42 (Leu) to Pro and Gln, respectively, altered the binding specificity of alphabungarotoxin from alpha7-containing nicotinic acetylcholine receptors to alpha3beta.2-containing nicotinic acetylcholine receptors.

Additional modifications to these amino acid residues and other amino acid residues are possible.

In another non-limiting embodiment, the at least one biologically active agent is an anthrax antigen. Anthrax is an acute infectious disease caused by the bacterium B. anthracis. It occurs in wild and domestic lower vertebrates (cattle, sheep, goats, etc.), but can also occur in humans (e.g. do to exposure to infected animals or because of bioterrorism). The virulence of B. anthracis is dependent on Anthrax Toxin and the capsule, a polymer of gamma-D-glutamic acid. Anthrax Toxin (AT) consists of two enzymatic moieties, edema factor (EF) and lethal factor (LF), and a single receptor-binding moiety, protective antigen (PA).

The capsule is a poor immunogen. Wild-type PA (the amino acid sequence of which is shown in Figure 18) is also a poor immunogen. Indeed, the currently licensed human anthrax vaccine (AVA, BioPort Corporation, Lansing Mich.) requires six vaccinations over eighteen months followed by yearly boosters to induce and maintain protective anti-PA titers (Pittman et al., Vaccine 20:1412-20, 2002; Pittman et al., Vaccine 20:972-78, 2001). In some vaccines, this regimen is associated with undesirable side effects (Pittman et al., Vaccine 20:972-78, 2001). Thus, there is a need for an effective and safe vaccine that would require fewer doses to confer immunity to anthrax.

The present invention, in a non-limiting embodiment, an anthrax antigen (or fragment thereof) in a preparation suitable for transdermal, intra-epidermal, intradermal or intramucosal delivery. In another embodiment, antigen from Y. pestis or F. tularensis is contemplated in a preparation suitable for transdermal delivery. In a preferred embodiment, the present invention contemplates mutant anthrax protective antigen (e.g. the mutant antigens set forth in Table 4 below, or fragment thereof) in a preparation suitable for transdermal delivery.

Table 4. amino acid residues at which Cys substitutions inhibited PA acrivity by at least 100-fold.

Residues

Domain	
1	I210, K225, T240, K245
2	S337, G342, W346, T357, I364, P379, T380, S382, T390, T393, K397, N399, Y411, I419, A420, N422, F427, S248, D451, D453, V455, N458
3	E515
4	I656, N657, I665, D683, L687

In one embodiment, the mutant protective antigen (or fragment thereof) is attached or linked to a carrier. In one embodiment, the mutant protective antigen (or fragment thereof) is part of a fusion protein. In one embodiment, the mutant protective antigen (or fragment thereof) is in a micelle formulation. In one embodiment, the mutant protective antigen (or fragment thereof) is in or on nanofibers, nanotubes, nanospheres, or nanocapsules. In one embodiment, the mutant protective antigen (or fragment thereof) is complexed with one or more dendrimers. In one embodiment, the mutant protective antigen (or fragment thereof) is in a liposome preparation (e.g. for liposome delivery). In a preferred embodiment, the mutant protective antigen (or fragment thereof) is on the surface of a substrate, e.g. on a skin patch, such as an array, of asperities such as those described herein, such as microprojections or microneedles or the like. Combinations of the above are contemplated specifically, including but not limited to substrates comprising the mutant protective antigen (or fragment thereof) in a polymer formulation, or in an assembly of nanofibers. In a particularly preferred embodiment, the mutant is functionally impaired (e.g. it will not form pores or channels, or will otherwise not cause toxicity).

The present invention contemplates, in one embodiment, a preparation suitable for transdermal, intradermal, intra-epidermal, transmucosal, subcutaneous, intravenous, or intramuscular delivery comprising a first anthrax antigen (or fragment thereof) in a mixture with a second anthrax antigen (or fragment thereof). The present invention contemplates, in one

embodiment, a preparation suitable for transdermal, transmucosal, subcutaneous, intravenous, or intramuscular delivery comprising a first anthrax antigen (or fragment thereof) conjugated to a second anthrax antigen (or fragment thereof). These first and second antigens (or fragments thereof) can be prepared simply as a mixture or, alternatively, they can be linked together in a conjugate. In one embodiment, the first and second antigens (or fragments thereof) are in a micelle formulation. In one embodiment, the first and second antigens (or fragments thereof) are in or on nanofibers, nanotubes, nanospheres, or nanocapsules. In one embodiment, the first and second antigens (or fragments thereof) are complexed with one or more dendrimers. In one embodiment, the first and second antigens (or fragments thereof) are in a liposome preparation (e.g. for liposome delivery). In a preferred embodiment, the first and second antigens (or fragments thereof) are on the surface of a substrate, e.g. on a skin patch, microneedle array, or the like. Combinations of the above are specifically contemplated, including but not limited to substrates comprising the first and second antigens (or fragments thereof) in a polymer formulation, or in an assembly of nanofibers. While edema factor and/or lethal factor (or fragments thereof) can be used as first and second antigens, in a preferred embodiment, said first and second antigens comprises (respectively) poly-glutamic acid and protective antigen (or fragment thereof). The present invention contemplates, in one embodiment, a preparation suitable for transdermal delivery comprising wild-type protective antigen or mutant protective antigen (e.g. the mutant antigens set forth in Table 4) conjugated to poly-glutamic acid. In a preferred embodiment, the present invention contemplates an array of microprojections or microneedles comprising anthrax antigen(s) selected from the group consisting of wild-type protective antigen (or fragment thereof), mutant protective antigen (or fragment thereof), a conjugate of wild-type protective antigen (or fragment thereof) and poly-glutamic acid, and a conjugate of mutant protective antigen (or fragment thereof) and poly-glutamic acid. In a particularly preferred embodiment, the mutant is functionally impaired (e.g. it will not form pores or channels, or will otherwise not cause toxicity). In a preferred embodiment, the microprojections or

microneedles have dimensions such that they penetrate into the stratum comeum, the epidermal layer, and, in some embodiments, the dermal layer. In one embodiment, the microprojections or microneedles have a length (or height) or less than 1000 microns, more preferably less than 500 microns, and still more preferably less than 250 microns. The microprojections may be formed in different shapes, such as needles, blades, pins, punches, and combinations thereof. When in an array, the density of the microprojections is at least microprojections/cm^2, more preferably, at least 200 microprojections/cm^2, and, in some embodiments, at least 1000 microprojections/cm^2.

The present invention further contemplates, in one embodiment, a preparation comprising at least one polymer and at least one anthrax antigen (or fragment thereof). In another embodiment, antigen from Y. pestis or F. tularensis is contemplated in such a polymer preparation. It is not intended that the present invention be limited by the nature of the polymer since a variety of types can be used (see Table 1). In one embodiment, cross-linked polymers are employed. However, in a preferred embodiment, the polymer comprises polyvinylpyrrolidone or hydroxypropylcellulose. It is not intended that the present invention be limited by the nature of the antigen(s) in the polymer preparation. While anthrax edema factor and/or lethal factor (or fragments thereof) can be use, in one embodiment, the antigen(s) is/are selected from the group consisting of wild-type anthrax protective antigen (or fragment thereof), mutant protective antigen (or fragment thereof), a conjugate of wild-type protective antigen (or fragment thereof) and poly-glutamic acid, and a conjugate of mutant protective antigen (or fragment thereof) and poly-glutamic acid. In a particularly preferred embodiment, the mutant is functionally impaired (e.g. it will not form pores or channels, or will otherwise not cause toxicity). It is contemplated that the polymer/antigen(s) preparation can be administered by a variety of routes (e.g. IP, IM, IV, inhalation, transdermal, intradermal, intra-epidermal, transmucosal, subcutaneous, etc.)

It is not intended that the present invention be limited to the manner in which the preparation of polymer and antigen(s) is prepared. However, in one embodiment, the preparation is electrospun

to create fibers or fiber mats. It is preferred that at least a portion of the antigen in the electrospun fibers or mats be capable of release or escape from the polymer (e.g. upon contact with an aqueous or semi-aqueous environment).

While edema factor and/or lethal factor can be used as antigens, in one embodiment, said antigen in said polymer preparation comprises poly-glutamic acid and protective antigen (or fragment thereof). These antigens can be prepared simply as a mixture. However, in a preferred embodiment, the antigen comprises a conjugate of poly-glutamic acid and protective antigen (or fragment thereof). In one embodiment, the conjugate is prepared by covalently linking poly-glutamic acid to protective antigen (or fragment thereof). In one embodiment, the protective antigen (or fragment thereof) is wild-type. In a preferred embodiment, the protective antigen (or fragment thereof) is a mutant. In a particularly preferred embodiment, the mutant is functionally impaired (e.g. it will not form pores or channels, or will otherwise not cause toxicity).

It is not intended that the present invention be limited to a particular mutant. A variety of mutants have been described that are functionally impaired. For example, U.S. Patent No. 7,037,503 (hereby incorporated by reference) to Collier et al. describes a group of dominant negative mutants having point mutations at positions 397 and/or 425 of the PA protein (see Figure 18 for the wild-type sequence of the PA protein). In addition, Mourez et al. (PNAS 100:13803 2003) describe 568 mutants, thirty-three of which demonstrate reduced ability to mediate toxicity (see Table 4). The present invention contemplates various embodiments wherein one or more of such mutants are employed (whether conjugated or unconjugated).

It is not intended that the present invention be limited to the nature of the conjugates (when conjugates are used). U.S. Patent Application 20060134143 to Schneerson et al. (hereby incorporated by reference) describes immunogenic conjugates of poly-gamma-glutamic acid (PGA) made using a variety of different PGA types (including but not limited to PGA comprising D-glutamic acid and PGA comprising L-glutamic acid) extracted from culture supernatants of B.

anthracis. In one example, recombinant wild-type PA was derivatized with adipic acid dihydrazide and reacted. On the other hand, Aulinger et al. (Infection & Immunity 73:3408 2005) describes conjugating PA (or a PA mutant) to purified PGA via a carbodiimide reaction. Poly-L-glutamic acid and poly-D-glutamic acid sodium salt are commercially available from Sigma-Aldrich (St. Louis). Protective antigen and fragments thereof (i.e. the 63kDa and 20kDa fragments) are commercially available from List Biological Laboratories, Inc. When a fragment is used, it is preferred that the 63kDa fragment is used (or a mutant thereof).

The present invention further contemplates, in one embodiment, a preparation comprising at least one anthrax antigen (or fragment thereof) in a hydrogel formulation. In another embodiment, antigen from Y. pestis or F. tularensis is contemplated in such a preparation. It is not intended that the present invention be limited by the nature of the hydrogel since a variety of types can be used, including but not limited to ionically cross-linked hydrogels or hydrogel films. It is not intended that the present invention be limited by the nature of the antigen(s) in the hydrogel preparation. While edema factor and/or lethal factor (or fragments thereof) can be use, in one embodiment, the antigen(s) is/are selected from the group consisting of wild-type protective antigen (or fragment thereof), mutant protective antigen (or fragment thereof), a conjugate of wild-type protective antigen (or fragment thereof) and poly-glutamic acid, and a conjugate of mutant protective antigen (or fragment thereof) and poly-glutamic acid. In a particularly preferred embodiment, the mutant is functionally impaired (e.g. it will not form pores or channels, or will otherwise not cause toxicity). It is contemplated that the hydrogel/antigen(s) preparation can be deposited on a surface (in a uniform or non-uniform manner). In one embodiment, the preparation is deposited (e.g. by dipping, coating, spin coating, spraying, or by suitable applicator) on an array of microprojections or microneedles for transdermal or transmucosal delivery of the antigen(s).

Regardless of whether a mutant or wild-type is used, the present invention contemplates administering one or more of the various embodiments of the preparations described above as a

vaccine to humans and animals. In one embodiment, the present invention contemplates a method comprising: a) providing i) a preparation comprising at least one polymer and one or more anthrax antigens (or fragments thereof) and ii) a subject, wherein said subject is a human or non-human animal; and b) contacting said subject with said preparation (e.g. under conditions such that said subject generates an immune response to said one or more antigens). In one embodiment, the present invention contemplates a method comprising: a) providing i) a preparation comprising one or more anthrax antigens (or fragments thereof) in a hydrogel formulation and ii) a subject, wherein said subject is a human or non-human animal; and b) contacting said subject with said preparation (e.g. under conditions such that said subject generates an immune response to said one or more antigens). In a preferred embodiment, a conjugate of two anthrax antigens is employed (as discussed above). In a preferred embodiment, the conjugate is electrospun with one or more polymers so as to create an electrospun preparation. As discussed below, in a preferred embodiment, the electrospun preparation is part of a vaccine delivery system. In a preferred embodiment, the electrospun preparation is delivered transdermally, intradermally, intra-epidermally, transmucosally, subcutaneously, IP, IV or IM.

The present invention further contemplates, in one embodiment, a vaccine delivery system comprising a) a substrate comprising microprojections or microneedles,and an embodiment of the various preparations (mentioned above) comprising either i) antigen(s) alone (or fragments thereof), ii) antigen(s) (or fragments thereof) and carrier alone, iii) polymer (or hydrogel) and antigen(s) (or fragments thereof), iv) conjugates of poly-glutamic acid and wild-type or mutant protective antigen (or fragment thereof), or v) iv) polymer (or hydrogel) and conjugates of poly-glutamic acid and wild-type or mutant protective antigen (or fragment thereof). When microneedles are used, they may be solid or hollow. It is not intended that the present invention be limited to the manner in which the microneedles are contacted with the preparation. In one embodiment, the microneedles are contacted only at the microneedle tips. In a preferred embodiment, the preparation contacts the

entire substrate (or substantially the entire substrate) such that the preparation contacts even the spaces between an may of microneedles. It is not intended that the present invention be limited to uniform coatings of the preparation. In one embodiment, the preparation is present on the substrate in a non-uniform manner.

In one embodiment, a polymer/antigen(s) preparation is sprayed on said microneedles. In one embodiment, said microneedles are dipped into a preparation comprising at least one polymer and at least one anthrax antigen (or fragment thereof). In a preferred embodiment, an electrospun polymer/antigen(s) preparation is deposited on said microneedles. In another embodiment, antigen from Y. pestis or F. tularensis is contemplated in such an electrospun polymer/antigen(s) preparation and this preparation is deposited on said microneedles.

In one embodiment, the present invention contemplates using at least two different anthrax protective antigens (or fragments thereof). For example, in order to generate effective immunity against more virulent strains of Bacillus anthracis, such as Vollum 1B, the Ames strain, two or more different sources of protective antigens (or fragments thereof) are contemplated in one embodiment, and mutants in another embodiment. Protective antigen from variants of such strains are also contemplated.

Thus, the present invention contemplates novel conjugates, formulations, delivery devices, methods of conjugation, methods of formulation, and methods of delivery. In one embodiment, the present invention contemplates a method of creating a formulation: providing i) at least one fiber-forming material, and ii) a preparation comprising at least one anthrax antigen; mixing said fiber-forming material with said preparation so as to create a mixture; and electrospinning said mixture to create electrospun formulation. In one embodiment, the method further comprises: depositing at least a portion of said electrospun material onto at least a portion of a substrate to create a treated substrate (e.g. a substrate comprising microneedles). The present invention also contemplates, as a

device, the treated substrate prepared according to the above-described method. In one embodiment, said fiber-forming material comprises hydroxypropylcellulose, while in another embodiment said fiber-forming material comprises PVP. In one embodiment, said preparation comprises first and second anthrax antigens (e.g. said first anthrax antigen comprises poly-gamma-glutamic acid and said second anthrax antigen comprises protective antigen). In one embodiment, said protective antigen is wild-type, while in another embodiment, said protective antigen is mutant. In a preferred embodiment, said first and second antigens are linked to form a conjugate.

In one embodiment, the present invention contemplates a method of creating an antigen delivery device, comprising: providing i) at least one fiber-forming material, ii) a preparation comprising at least two anthrax antigens, and iii) a substrate comprising a plurality of microprojections; mixing said fiber-forming material with said preparation so as to create a mixture; electrospinning said mixture to create electrospun material; and depositing at least a portion of said electrospun material onto at least a portion of said substrate, so as to create an antigen delivery device. In one embodiment, said two anthrax antigens comprises poly-gamma-glutamic acid and protective antigen (e.g. linked to form a conjugate). In one embodiment, said protective antigen is wild-type, while in another embodiment, said protective antigen is mutant. In one embodiment, said fiber-forming material comprises hydroxypropylcellulose, while in another embodiment said fiber-forming material comprises PVP. The present invention also contemplates, as a device, the antigen delivery device prepared according to the above-described method.

The present invention also contemplates, in one embodiment, a drug delivery device comprising: a substrate having a back surface and a front surface; a plurality of solid microneedles extending upwards from the front surface of the substrate, the microneedles comprising a formulation, said formulation comprising at least one polymer and at least one anthrax antigen (e.g. a two antigen conjugate as described above).

Anthrax toxin is a member of the binary bacterial toxins, a subset of intracellularly acting toxins in which the enzymatic and receptor-binding moieties are secreted by the bacteria as discrete monomeric proteins. While an understanding of the mechanism of action of these toxins is not needed for the successful practice of the various embodiments of the present invention, it is believed these proteins assemble at the surface of receptor-bearing eukaryotic cells to form toxic hetero-oligomeric complexes. The complexes are internalized and delivered to an acidic compartment, where the receptor-binding moiety inserts into the membrane and mediates translocation of the enzymatic moiety to the cytosol. Within the cytosol, the enzymatic moiety modifies a molecular target, disrupting cell physiology and causing cytopathic effects.

Anthrax toxin consists of two enzymatic moieties, edema factor (EF; 89 kDa) and lethal factor (LF; 90 kDa), and a single receptor-binding moiety, protective antigen (PA; 83 kDa), named for its ability to elicit protective immunity. The amino acid sequence for wild-type PA is shown in Figure 18. The nucleic acid sequence encoding wild-type PA is shown in Figure 19. EF is a Ca 2+ and calmodulin-dependent adenylate cyclase, and LF is a Zn^{2+} -dependent protease that cleaves mitogen-activated protein kinase kinases.

The crystallographic structure of intact PA has been solved and shows that the protein is organized into four domains. Domain 1, the N-terminal domain, contains the furin cleavage site and therefore encompasses PA_{20}. That portion of domain 1 within PA_{63}, termed domain F, contains two calcium atoms and participates in EF/LF binding and oligomerization. Domain 2 contains a flexible loop (213_2-213_3) that is believed to form the transmembrane region of the pore. While not limiting the invention in any manner by reference to proposed mechanisms, it is believed (based on ion conductance experiments on Cys-substitution mutants) that the 213_2-213_3 loops from the seven subunits of the prepore combine to form a transmembrane 14-strand 13 -barrel. Domain 3 is believed to be involved in oligomerization, and domain 4, the C-terminal domain, contains the receptor-binding site. In addition to monomeric PA, the structure of a soluble form of the PA_{63}

heptamer has been solved; it is believed to represent the conformation of the heptamer before pore formation and has been termed the prepore.

PA mutants have been described that block the intoxication process. There has been some interest in mutations that make the protein dominantly negative (DN), thereby converting it into a potent antitoxin. Without limiting the invention in any manner by reference to proposed mechanisms, it is believed that DN-PA co-oligomerizes with wild-type PA_{63} and inhibits its ability to form pores and mediate translocation. Point mutations at D425 and F427, a deletion or substitution of the 213_2-213_3 loop, or combinations of these mutations, have been shown to confer varying degrees of DN activity on PA. Interestingly, the conjugation of such a•DN mutant to PGA has been reported to generate a much improved immune response (as compared to wild-type PA alone or even wild-type PA conjugated to PGA). Aulinger et al. (Infection & Immunity 73:3408 2005). In one embodiment, the present invention contemplates transdermal delivery of such conjugates, wherein said mutant comprises a substitution at K397, D425 or both. In a preferred embodiment, said mutant is selected from the group consisting of K397A, K397D, K397C, and K397Q. In another embodiment, said mutant is selected from the group consisting of D425A, D425N, D425E, and D425K. In another embodiment, said mutant is defined by a substitution at position F427 (including but not limited to F427A, F427D, and F427K). In one embodiment, the present invention contemplates transdermal delivery of such conjugates, wherein said mutant comprises a substitution at K397, D425 and F427. In one embodiment, the present invention contemplates transdermal delivery of such conjugates, wherein said mutant comprises a deletion or substitution of the 213_2-2 133 loop.

The analysis of large arrays of PA mutants has recently become feasible, following identification of ways to obtain small-scale preparations of PA mutants and screen them rapidly and reliably for defects in PA functions. To search PA for additional DN sites, researchers at the University of Oklahoma developed a protocol to mutate each of the 568 amino acids of PA_{63} to Cys

and characterize the resulting mutants. They restricted their study to PA_{63} because PA_{20} does not appear to play a role in intoxication besides preventing toxin assembly in solution. Cys-replacements were chosen because this amino acid is absent from PA and because the thiol-containing side chain is amenable to specific derivatization, facilitating structure-function studies. These researchers first screened for mutations that a caused large (>100-fold) reduction in PA's ability to mediate toxicity (these mutations are listed in Table 4). Such mutations were identified in all four domains, but a majority were in domain 2. Mutations conferring the DN phenotype were found exclusively within domain 2, in the 213_6 strand, the 213_7 strand, or the 21310-21311 loop. Because DN mutants prevent the conformational transition of PA_{63} from the prepore to the pore state, they concluded that these structural elements play major roles in this change.

As noted above, in another embodiment, antigen from Y. pestis or F. tularensis is contemplated in a preparation suitable for transdermal delivery. It is not intended that the present invention be limited by the particular antigens from these organisms. However, in one embodiment, an F1 capsule antigen from Y. pestis is employed in a polymer formulation (e.g. electrospun formulation) deposited on a microneedle array. In another embodiment, the LcrV protein (or fragment thereof) from Y. pestis is similarly employed. In a particular embodiment, these two antigens are used together (as a mixture, conjugate or fusion protein). In still another embodiment, one or more virulence-associated factors are employed, including but not limited to catalase-peroxidase (KatY), murine toxin (Ymt), plasminogen activator (Pla), and F1 capsule antigen (Can) from Y. pestis. In one embodiment, a specific sequence of the outer membrane protein encoded by the fopA gene of F. tularensis is employed.

In one embodiment, the present invention contemplates using new and previously identified PA mutants (or fragments thereof) in vaccines. In a preferred embodiment, such mutants are conjugated to PGA and electrospun with one or more polymers.

In a preferred embodiment, the preparation comprising anthrax antigen(s) is capable, after electrospinning, of acting as an antigen by eliciting an immune response in a human or animal. It is envisioned that in such an embodiment, a medically acceptable fiber-forming material may be used to preserve the antigen for later rehydration and use as a vaccine. In general, rehydration of the fiber of the present invention may be accomplished by mixing the fiber with a solvent for the fiber-forming material. When the fiber is used to preserve an antigen for use in a vaccine, the solvent will optimally be a medically acceptable compound.

It is not intended that the present invention be limited to the method by which the electrospun anthrax antigen(s) is administered. The resulting vaccine may be injected or an ingested vaccine. However, in a preferred embodiment, the vaccine is administered at or in the skin. In a particularly preferred embodiment, the electrospun polymer/anthrax antigen(s) material is delivered on microneedles or on a patch such that antigen(s) is released into the skin.

In one embodiment, the anthrax antiget(s) may be contained in an ionically cross-linked macromolecular assembly such as those hereinabove described, including a hydrogel, as described hereinabove.

In one embodiment, the present invention contemplates a method of administering one or more anthrax antigens to a patient comprising the steps of penetrating at least an outer layer of the skin of the patient with one or more microneedles extending from a front surface of a substrate, at least one of the one or more microneedles (or portion thereof) contacted or coated (uniformly or non-uniformly) by an anthrax antigen preparation (discussed above), and releasing at least one of the one or more anthrax antigens into the patient.

In another non-limiting embodiment, the at least one biologically active agent is a hormone.

Osteoporosis is a bone disorder characterized by progressive bone loss that predisposes an individual to an increased risk of fracture, typically in the hip, spine and wrist. The progressive bone

loss, which typically begins between the ages of 40 and 50, is mainly asymptomatic until a bone fracture occurs, leading to a high degree of patient morbidity and mortality. Eighty percent of those affected by osteoporosis are women and, based on recent studies, during the six years following the onset of menopause, women lose one third of their bone mass.

Parathyroid hormone (PTH) is a hormone secreted by the parathyroid gland that regulates the metabolism of calcium and phosphate in the body. PTH has stirred great interest in the treatment of osteoporosis for its ability to promote bone formation and, hence, dramatically reduced incidence of fractures. Large-scale clinical trials have shown that PTH effectively and safely reduces the percentage of vertebral and non-vertebral fractures in women with osteoporosis. PTH-based agents have also stirred interest in the treatment of bone fractures (in both men and women) by virtue of their ability to accelerate bone healing.

To this end, various stabilized formulations of PTH-based agents have been developed that can be reconstituted for subcutaneous injection, which, as discussed below, is the conventional means of delivery. Illustrative are the formulations disclosed in U.S. Pat. No. 5,563,122 ("Stabilized Parathyroid Hormone Composition") and U.S. patent application Pub. No. 2002/0107200 ("Stabilized Teriparatide Solutions"), which are incorporated by reference herein in their entirety.

A currently approved injectable PTH-based agent is FORTEO (an rDNA derived teriparatide injection), which contains recombinant human parathyroid hormone (1-34), (rhPTH (1-34)). FORTEO™ is typically prescribed for women with a history of osteoporotic fracture, who have multiple risk factors for fracture, or who have failed or are intolerant of previous osteoporosis therapy, based on a physician's assessment. In postmenopausal women with Osteoporosis, FORTEO™ has been found to increase bone mineral density (BMD) and reduce the risk of vertebral and non-vertebral fractures.

FORTEO™ has also been found to increase bone mass in men with primary or hypogonadal osteoporosis who are at high risk for fracture. These include men with a history of osteoporotic

fracture, or who have multiple risk factors for fracture, or who have failed or are intolerant to previous osteoporosis therapy. In men with primary or hypogonadal osteoporosis, FORTEO™ has similarly been found to increase BMD.

Despite the efficacy of PTH in treating disorders such as osteoporosis, there are several drawbacks and disadvantages associated with the disclosed prior art methods of delivering PTH, particularly, via subcutaneous injection. A major drawback is that subcutaneous injection is a difficult and uncomfortable procedure, which often results in poor patient compliance.

What is needed is a more comfortable hormone delivery approach with good patient compliance.

The present invention contemplates, in one embodiment, a hormone, including but not limited to PTH (or a fragment thereof, such as the 1-34 fragment), in a preparation suitable for transdermal, intradermal, intra-epidermal or intramucosal delivery. In one embodiment, the hormone (or fragment thereof) is attached or linked to a carrier. In one embodiment, the hormone (or fragment thereof) is part of a fusion protein. In one embodiment, the hormone (or fragment thereof) is in a micelle formulation. In one embodiment, the hormone (or fragment thereof) is in or on nanofibers, nanotubes, nanospheres, or nanocapsules. In one embodiment, the hormone (or fragment thereof) is complexed with one or more dendrimers. In one embodiment, the hormone (or fragment thereof) is in a liposome preparation (e.g. for liposome delivery). In a preferred embodiment, the hormone (or fragment thereof) is on the surface of a substrate, e.g. on a skin patch, an array, of asperities such as those herein described, such as microprojections or microneedles, or the like. Combinations of the above are contemplated specifically, including but not limited to substrates comprising the hormone (or fragment thereof) in a polymer formulation, or in an assembly of nanofibers. In a particularly preferred embodiment, the hormone is functional and not functionally impaired.

The present invention contemplates, in one embodiment, a preparation suitable for

transdermal, intradermal, intra-epidermal, transmucosal, subcutaneous, intravenous, or intramuscular delivery comprising a first hormone (or fragment thereof) in a mixture with a second hormone (or fragment thereof). These first and second hormones (or fragments thereof) can be prepared simply as a mixture or, alternatively, they can be linked together in a conjugate. In one embodiment, the first and second hormones (or fragments thereof) are in a micelle formulation. In one embodiment, the first and second hormones (or fragments thereof) are in or on nanofibers, nanotubes, nanospheres, or nanocapsules. In one embodiment, the first and second hormones (or fragments thereof) are complexed with one or more dendrimers. In one embodiment, the first and second hormones (or fragments thereof) are in a liposome preparation (e.g. for liposome delivery). In a preferred embodiment, the first and second hormones (or fragments thereof) are on the surface of a substrate, e.g. on a skin patch, microneedle array, or the like. Combinations of the above are specifically contemplated, including but not limited to substrates comprising the first and second hormones (or fragments thereof) in a polymer formulation, or in an assembly of nanofibers. In a preferred embodiment, said first hormone is PTH (or the 1-34 fragment thereof) and the second hormone is calcitonin (e.g. human or salmon). In a preferred embodiment, the hormone(s) preparation is placed on microprojections or microneedles having dimensions such that they penetrate into the stratum corneum, the epidermal layer, and, in some embodiments, the dermal layer. In one embodiment, the microprojections or microneedles have a length (or height) or less than 1000 microns, more preferably less than 500 microns, and still more preferably less than 250 microns. The microprojections may be formed in different shapes, such as needles, blades, pins, punches, and combinations thereof. When in an array, the density of the microprojections is at least 10 microprojections/cm^2, more preferably, at least 200 microprojections/cm^2, and, in some embodiments, at least 1000 microprojections/cm^2.

The present invention further contemplates, in one embodiment, a preparation comprising at least one polymer and at least one hormone (or fragment thereof). It is not intended that the present

invention be limited by the nature of the polymer since a variety of types can be used (see Table 1). In one embodiment, cross-linked polymers are employed. However, in a preferred embodiment, the polymer comprises polyvinylpyrrolidone or hydroxypropylcellulose. In another embodiment, said polymer is hydroxyethylcellulose. It is not intended that the present invention be limited by the nature of the hormone(s) in the polymer preparation. However, in a particularly preferred embodiment, a functional PTH (or the 1-34 PTH fragment) is contemplated for use in the polymer formulation. Human and bovine sequences of 1-34 PTH are shown in Figure 20. It is contemplated that the polymer/hormone(s) preparation can be administered by a variety of routes (e.g. IP, IM, IV, inhalation, transdermal, intradermal, 'infra-epidermal, transmucosal, subcutaneous, etc.).

It is not intended that the present invention be limited to the manner in which the preparation of polymer and hormone(s) is prepared. However, in a preferred embodiment, the preparation is electrospun to create fibers or fiber mats. It is preferred that at least a portion of the hormone in the electrospun fibers or mats be capable of release or escape from the polymer (e.g. upon contact with an aqueous or semi-aqueous environment). In one embodiment, the electrospun polymer/hormone(s) fiber mat is placed directly on the skin. In another embodiment, the polymer/hormone(s) fiber mat is part of a delivery vehicle (e.g. skin patch, microneedle array, etc.).

The present invention further contemplates, in one embodiment, a preparation comprising at least one hormone (or fragment thereof) in a hydrogel formulation. It is not intended that the present invention be limited by the nature of the hydrogel since a variety of types can be used, including but not limited to ionically cross-linked hydrogels or hydrogel films. It is not intended that the present invention be limited by the nature of the hormone(s) in the hydrogel preparation. In a particularly preferred embodiment, the hormone comprises a functional PTH (or 1-34 fragment thereof). It is contemplated that the hydrogel/hormone(s) preparation can be deposited on a surface (in a uniform or non-uniform manner). In one embodiment, the preparation is deposited (e.g. by dipping, coating, spin coating, spraying, or by suitable applicator) on an array of asperities such as microprojections

or microneedles as described herein for transdermal, intradermal, intra-epidermal, or transmucosal delivery of the hormone(s).

Other systems and apparatus that employ tiny skin piercing elements to enhance transdermal agent delivery are disclosed in U.S. Pat. Nos. 5,879,326, 3,814,097, 5,250,023, 3,964,482, Reissue U.S. Pat. No. 25,637, and PCT Publication Nos. WO 96/37155, WO 96/37256, WO 96/17648, WO 97/03718, WO 98/11937, WO 98/00193, WO 97/48440, WO 97/48441, WO 97/48442, WO 98/00193, WO 99/64580, WO 98/28037, WO 98/29298, and WO 98/29365; all incorporated herein by reference in their entirety.

The present invention contemplates administering one or more of the various embodiments of the preparations described above as a prophylactic or therapeutic to humans and animals. In one embodiment, the present invention contemplates a method comprising: a) providing i) a preparation comprising at least one polymer and one or more hormones (or fragments thereof) and ii) a subject, wherein said subject is a human or non-human animal; and b) contacting said subject with said preparation (e.g. under conditions such that at least a portion of said hormone is released into said subject). In one embodiment, the present invention contemplates a method comprising: a) providing i) a preparation comprising one or more hormones (or fragments thereof) in a hydrogel formulation and ii) a subject, wherein said subject is a human or non-human animal; and b) contacting said subject with said preparation (e.g. under conditions such that at least a portion of said hormone is released into said subject). In a preferred embodiment, two hormones are employed (as discussed above). In a preferred embodiment, the hormones are electrospun with one or more polymers so as to create an electrospun preparation. As discussed below, in a preferred embodiment, the electrospun preparation is part of a hormone delivery system. In a preferred embodiment, the electrospun preparation is delivered transdermally, intradermally, intra-epidermal, transmucosally, subcutaneously, IP, IV or IM.

The present invention further contemplates, in one embodiment, a hormone delivery system

comprising a) a substrate comprising microprojections or microneedles, and an embodiment of the various preparations (mentioned above) comprising either i) hormone(s) alone (or fragments thereof), ii) hormone(s) (or fragments thereof) and carrier alone, or iii) polymer (or hydrogel) and hormone(s) (or fragments thereof). When microneedles are used, they may be solid or hollow. It is not intended that the present invention be limited to the manner in which the microneedles are contacted with the preparation. In one embodiment, the microneedles are contacted only at the microneedle tips. In a preferred embodiment, the preparation contacts the entire substrate (or substantially the entire substrate) such that the preparation contacts even the spaces between an array of microneedles. It is not intended that the present invention be limited to uniform coatings of the preparation. In one embodiment, the preparation is present on the substrate in a non-uniform manner. In one embodiment, a polymer/hormone(s) preparation is sprayed on said microneedles. In one embodiment, said microneedles are dipped into a preparation comprising at least one polymer and at least one hormone (or fragment thereof). In a preferred embodiment, an electrospun polymer/hormone(s) preparation is deposited on said microneedles.

It is not intended that the present invention be limited by the nature of the substrate comprising said microneedles. In one embodiment, the microneedles are formulated out of polymer. In one embodiment, the microneedles are formulated (at least in part) out of a polymer/hormone(s) preparation. In another embodiment, the microneedles are made with a mold. In a preferred embodiment, the microneedles are etched out of a silicon substrate. In a preferred embodiment, said silicon microneedles are solid and an electrospun polymer/hormone(s) preparation is deposited on said microneedles.

Thus, the present invention contemplates novel formulations, delivery devices, methods of formulation, and methods of delivery. In one embodiment, the present invention contemplates a method of creating a formulation: providing i) at least one fiber-forming material, and ii) a preparation comprising at least one hormone (or fragment thereof); mixing said fiber-forming

material with said preparation so as to create a mixture; and electrospinning said mixture to create electrospun formulation. In one embodiment, the method further comprises: depositing at least a portion of said electrospun material onto at least a portion of a substrate (e.g. a substrate comprising microneedles, including but not limited to etched solid microneedles) to create a treated substrate.. The present invention also contemplates, as a device, the treated substrate prepared according to the above-described method. In one embodiment, said fiber-forming material comprises hydroxypropyl-cellulose, while in another embodiment said fiber-forming material comprises PVP. In one embodiment, said preparation comprises first and second hormones. In a preferred embodiment, said first hormone comprises PTH (1-34).

In one embodiment, the present invention contemplates a method of creating a hormone delivery device, comprising: providing i) at least one fiber-forming material, ii) a preparation comprising providing i) at least one fiber-forming material, ii) a preparation comprising at least one hormone (or fragment thereof), and iii) a substrate comprising a plurality microprojections; mixing said fiber-forming material with said preparation so as to create a mixture; electrospinning said mixture to create electrospun material; and depositing at least a portion of said electrospun material onto at least a portion of said substrate, so as to create a hormone delivery device. In a preferred embodiment, said hormone comprises PTH (1-34). In one embodiment, said fiber-forming material comprises hydroxypropyl-cellulose, while in another embodiment said fiber-forming material comprises PVP. The present invention also contemplates, as a device, the hormone delivery device prepared according to the above-described method.

The present invention, as mentioned above, also contemplates methods of administering hormones. On one embodiment, the method of administering hormone comprises: providing: a subject and the hormone delivery device described above; and contacting said subject with said delivery device under conditions such at least a portion of said hormone is released from said device. In one embodiment, said contacting of step b) comprises piercing the subject's skin with said

microprojections.

The present invention also contemplates, in one embodiment, a hormone delivery device comprising: a substrate having a back surface and a front surface; a plurality of solid microneedles extending upwards from the front surface of the substrate, the microneedles comprising a formulation, said formulation comprising at least one polymer and at least one hormone (or fragment thereof). In a preferred embodiment, said hormone comprises PTH (1-34).

Importantly, the present invention contemplates utilizing, in some embodiments, peptide hormones that are protease resistant. In one embodiment, such protease-resistant peptides are peptides comprising protecting groups. In some embodiments, the peptide hormone (such as 1-34 PTH) has the amino terminus blocked by standard methods to prevent digestion by exopeptidases, for example by acetylation. In another embodiment, the carboxyl terminus is blocked by standard methods to prevent digestion by exopeptidases, for example, by amidation. In some embodiments, both the amino terminus and the carboxyl terminus are blocked, e.g. the amino terminus is blocked with an acetal group, and said peptide's carboxyl terminus is blocked with an acetate group. In another embodiment, endoprotease-resistance is achieved using peptides which comprise at least one D-amino acid.

The present invention contemplates a variety of hormones and hormone combinations in the above-indicated formulations and delivery vehicles. Vertebrate hormones fall into three chemical classes: Amine derived hormones (examples are catecholamines and thyroxine, as well as melatonin and serotonin); Peptide hormones (examples are TRH and vasopression, as well as leuteinizing hormone, follicle-stimulating hormone, EPO, angiotensin, gastrin, growth hormone and thyroid-stimulating hormone; preferred embodiments of peptide hormones include glucagon, glucagon-like peptide GLP-1, and Exendin-4); Lipid and phospholipids (examples are steroid hormones such as testosterone and cortisol, sterol hormones such as calcitriol, and eicosanoids such as prostaglandins). The most commonly-prescribed hormones are estrogens and progestagens, thyroxine (such as

levothyrroxin) and steroids. Of course, insulin is used by diabetics.

A "pharmacologic dose" of a hormone is a medical usage referring to an amount of a hormone far greater than naturally occurs in a healthy body. The effects of pharmacologic doses of hormones may be different from responses to naturally-occurring amounts and may be therapeutically useful. An example is the ability of pharmacologic doses of glucocorticoid to suppress inflammation.

In a preferred embodiment, the present invention contemplates formulations of a PTH-based agent, such as PTH-based agent is selected from the group consisting of hPTH(1-34), hPTH salts and analogs, teriparatide and related peptides. Throughout this application, the terms "PTH-based agent" and "PTH(1-34)" include, without limitation, recombinant hPTH(l-34), synthetic hpTH(1-34), PTH(1-34), teriparatide, hPTH(I-34) salts, simple derivatives of hPTH(1- 34), such as hPTH(1-34) amide, and closely related molecules, such as hPTH(1-33) or hPTH(1-31) amide, or any other closely related osteogenic peptide. Synthetic hPTH(1-34) is the most preferred PTH agent (however, 1-34 PTH from other species is also contemplated).

Examples of pharmaceutically acceptable hPTH salts include, without limitation, acetate, propionate, butyrate, pentanoate, hexanoate, heptanoate, levulinate, chloride, bromide, citrate, succinate, maleate, glycolate, gluconate, glucuronate, 3-hydroxyisobutyrate, tricarballylicate, malonate, adipate, citraconate, glutarate, itaconate, mesaconate, citramalate, dimethylolpropinate, tiglicate, glycerate, methacrylate, isocrotonate,l3-hydroxibutyrate, crotonate, angelate, hydracrylate, ascorbate, aspartate, glutamate, 2-hydroxyisobutyrate, lactate, malate, pyruvate, fumarate, tartarate, nitrate, phosphate, benzene, sulfonate, methane sulfonate, sulfate and sulfonate.

Preferably, the PTH-based agent is present in the coating formulation at a concentration in the range of approximately 1-30 wt. %.

More preferably, the amount of PTH-based agent contained in the solid biocompatible coating (i.e., microprojection member or product) is in the range of approximately 1μg-1000 μg,

even more preferably, in the range of approximately 10-100 μg.

In a further embodiment of the invention, the coating formulation includes at least one polymeric material or polymer that has amphiphilic properties, which can comprise, without limitation, cellulose derivatives, such as hydroxyethylcellulose (HEC), hydroxypropylmethylcellulose (HPMC), hydroxypropycellulose (HPC), methylcellulose (MC), hydroxyethylmethylcellulose (HEMC), or ethylhydroxy-ethylcellulose (EHEC), as well as pluronics.

In one embodiment of the invention, the concentration of the polymer presenting amphiphilic properties in the coating formulation is in the range of approximately 0.01-20 wt. %, and in one embodiment in the range of approximately 0.03-10 wt.% of the coating formulation.

In another embodiment, the coating formulation includes a hydrophilic polymer selected from the following group: hydroxyethyl starch, carboxymethyl cellulose and salts of, dextran, poly(vinyl alcohol), poly(ethylene oxide), poly(2-hydroxyethylmethacrylate), poly(n-vinyl pyrolidone), polyethylene glycol and mixtures thereof, and like polymers.

In one embodiment, the concentration of the hydrophilic polymer in the coating formulation is in the range of approximately 1-30 wt. %, and in another embodiment, in the range of approximately 1-20 wt. % of the coating formulation.

In one embodiment, the present invention contemplates utilizing an electrospun formulation (e.g. alone or on a delivery vehicle described herein) comprising at least one substance to improve the look and/or feel of skin, wherein said substance is selected from the group consisting of vitamins (e.g. A, C, D and/or E), polysaccharides, botanicals, proteins/peptides (e.g. collagen, keratin, elastin, and peptide fragments thereof, including but not limited to pentapeptides such as KTTKS), fatty acids (omega-3, omega-6, etc.), and enzymes/coenzymes. In one embodiment, bioactive factors from whey (e.g. lactalbumin, lactoferrin, lactose, etc.) are contemplated for such formulations.

With respect to botanicals, both plant extracts and purified bioactive agents from plants (e.g.

in electrospun polymer formulations) are contemplated in some embodiments. It is not intended that the present invention be limited to a particular plant or group of plants. Illustrative plants (from which extracts can be made or agents can be purified) include but are not limited to aloe vera, St. John's Wort (hypericum crean), Noni (Morinda citrifolia), coffee plant, white and green tea (camellia sinensis), avocado (persea Americana), jojoba (simmondsia chinensis), almond (prunus dulcis), olive oils (olea europea), Sea Kulp, Yacon (smallanthus sonchifolius), soy (glycine max), comfrey plant (symphytum uplandicum), gotu cola (centella asiatica), and African Baobab Tree (adansonia digitata). In one embodiment, the present invention contemplates extracts or purified agents useful for treating cellulite, wherein said plants are selected from the group consisting of meadowsweet (spiraea ulmaria), coffee plant (caffeine), ginkgo (ginkgo biloba), birch (betula), common ivy (hedera helix), and lemon (citrus lemon). In one embodiment, the present invention contemplates extracts or purified agents useful for treating acne, wherein said plants are selected from the group consisting of saw palmetto (sereonnoa repens), green tea (camellia sinensis), soy (glycyne soja), burdock (arctium lappa), tea tree (melaleuca alternifolia), wild pansy (viola tricolor), kiwi (actinidia chinensis), kokum (garcinia indica), and licorice (glycyrrhiza glabra). In one embodiment, the present invention contemplates extracts or purified agents useful as skin whiteners, wherein said plant is selected from the group consisting of bearbeny (arctostaphylos uva-ursi), aloe vera, lemon citrus, white mulberry (moms alba), Chinese sage (salvia miltrionhiza), watercress (nasturtium officinale), and amla (embelica officinalis). In one embodiment, the present invention contemplates extracts or purified agents useful for specific skin conditions, wherein said plant is selected from the group consisting of yarrow flowers (achillea millefolium), oats (avena sativa), pot marigold (calendula officinalis), chesnut bark (castaneva sativa), hawthorn fruits (crataegus monogina), cucumber seeds (cucumis sativus), semitake mushrooms (cordyceps sabolifera), wormwood (artemesia carvifolia), oriental horsetail (equisetum arvense), and tamanu (calophyllum inophyllum).

In one embodiment, the present invention contemplates utilizing a delivery vehicle described herein comprising at least one hormone (e.g. electrospun hormone) to treat (or prevent) a skin condition. In one embodiment, the hormone (e.g., estradiol, or a vitamin) is prescribed to treat a condition, including but not limited to a skin condition that is the result of photodamage or photoaging. In one embodiment, the present invention contemplates treating a skin condition associated with inflammation with such a delivery device described herein comprising one or more retinoids (the vitamin A family). In yet another embodiment, the present invention contemplates utilizing a delivery vehicle described herein (e.g. microneedle array) comprising Vitamin D (and/or analogs) for the treatment of psoriasis. In one embodiment of the present invention, the delivery device comprising Vitamin E is utilized for the prevention and treatment of skin scars and/or keloids. In another embodiment, the present invention contemplates utilizing one of the delivery devices described herein comprising selenium in order to a) protect keratinocytes against damage from ultraviolet radiation, b) promote the healing of wounds and burns, and c) reduce the chance of skin cancer (i.e. an anti-oncogenic treatment). In one embodiment, the present invention contemplates such a delivery device comprising combinations of the above-named vitamins and minerals (e.g. vitamin D in combination with selenium) in a formulation (e.g. an electrospun formulation or hydrogel formulation).

In one embodiment, the present invention contemplates utilizing a delivery vehicle described herein (e.g. microneedle array) for delivery of Vitamin C (e.g. in an electrospun polymer formulation) in subjects (e.g. subjects who cannot take Vitamin C orally) through the skin. In another embodiment, the present invention contemplates the delivery of Vitamin C to the skin without utilizing a microneedle may (e.g. in an electrospun polymer formulation or on a skin patch). In still another embodiment, Vitamin C is administered using both of the above approaches are utilized so as to achieve both local and systemic administration.

In one embodiment, the present invention contemplates utilizing a delivery vehicle described

herein comprising at least one hormone (e.g. electrospun hormone) to treat (or prevent) type II diabetes. In one embodiment, the hormone is GLP-1. In another embodiment, the hormone is Exendin-4 or fragments thereof such as Exendin-4 (3-39) and Exendin-4 (9-39). As noted above, in some embodiments, protease resistance versions of such peptide hormones are contemplated, including but not limited to N-acetal GLP-1 (commercially available from Phoenix Pharmaceuticals, Inc.). The amino acid sequences for such peptide hormones are shown in Figure 21.

In one embodiment, the present invention contemplates electrospun hormones, including but not limited to PTH(1-34). In a preferred embodiment, such hormones are electrospun with one or more polymers such as those described herein.

In a preferred embodiment, the preparation comprising hormone(s) is capable, after electrospinning, of releasing some functional hormone into the human or animal. It is envisioned that in such an embodiment, a medically acceptable fiber-forming material may be used to preserve the hormone(s) for later rehydration and use. In general, rehydration of the fiber of the present invention may be accomplished by mixing the fiber with a solvent for the fiber-forming material. When the fiber is used to preserve a hormone, the solvent will optimally be a medically acceptable compound.

It is not intended that the present invention be limited to the method by which the electrospun hormone(s) is administered. However, in a preferred embodiment, the hormone(s) is administered at or in the skin. In a particularly preferred embodiment, the electrospun polymer/hormone(s) material is delivered on microneedles or on a patch such that hormone(s) is released into the skin.

In a non-limiting embodiment, the hormones(s) is (are) contained in an ionically cross-linked macromolecular assembly such as those hereinabove described, including but not limited to a hydrogel, such as those hereinabove described.

Ionically cross-linked macromolecular assemblies of the present invention comprise a

substance to be delivered such as one or more hormones. The formulation containing the pharmaceutically active substance can be in the form of an ionically cross-linked three dimensional film, sphere, tube, fiber, or in any other, geometrical shape and is attached the surface of the device by chemical or physical means.

It is contemplated that the ionically cross-linked macromolecular assembly/drug(s) preparation can be deposited on a surface (in a uniform or non-uniform manner). In one embodiment, the preparation is deposited (e.g. by dipping, coating, spin coating, spraying, or by suitable applicator) on an array of microprojections or microneedles for transdermal, intradermal or transmucosal delivery of the hormone(s).

In one embodiment, the present invention contemplates a method of administering one or more hormones to a patient comprising the steps of penetrating at least an outer layer of the skin of the patient with one or more asperities such as those hereinabove described, such as microprojections or microneedles extending from a front surface of a substrate, at least one of the one or more microneedles (or portion thereof) contacted or coated (uniformly or non-uniformly) by an hormone preparation (discussed above), and releasing at least one of the one or more hormones into the patient.

Other applications for spatially controlled deposition of electrospun fibers: Appropriate nanofibers could be applied to the edge of a blade, such as a razor blade, or on or between multiple blades or to the piercing edges of a syringe needle. The fibers could aid in the lubrication, prevent corrosion, or minimize bleeding for example.

The needle could be coated with the fibers. These fibers may be adjuvant polymers, such as PVP. Upon injection of the vaccine, the adjuvant During injection, the coating provides lubrication during the insertion. The same or another kind of fiber can also serve as an adjuvant.

Multiple layers of fibers or composites that have varied chemistries, like the NO delivering patches, with Vitamin C spun out on one pin and nitrite spun out on another, or fibers containing

each are spun onto the same needle The fibers deliver the NO when moisture from the skin, or from a puncture of the skin activates the reagents. For some applications the reagents may be placed on the separate pins which are inserted at the same time.

The spinning methods utilized in the present invention can also be used to coat only the surface of a stent, without forming a web between the struts of a stent. Only the metal is coated; thus there is not made a stent graft but a fibrous coating that allows fluids to flow through the open interstices of the stent during insertion, and fluids can have direct access to the interior surface of the blood vessel through these interstices after the stent is inserted

The steerable (directable) nature of the jets used in this invention can improve application of fibers to skin. Fine details of the wound can be coated with less "bridging" than when a typical bending and coiling jet is used. Before when it was attempted to spin out fibers in attempts to control bleeding wounds, such as, for example, when fibers were spun onto a liver wound on a dog, the fibers tented over the wound surface. Now the fibers may be delivered directly to a specific surface, such as a grounded surface on a wound, where one would want only to deliver a polymer containing a small quantity of growth factor to enhance healing of the wound. This may be useful in plastic surgery, where only a small incision is treated in this way. A small wire may be laid on top of the incision, and function as the target or ground, that provides even greater control of the place at which the fiber is attached to the wound.

Controlled jets of the sort described in this invention can be used in laparoscopic surgery to cover, protect. medicate, mechanically stabilize or treat tissues to which a surgeon has only very limited access.

In another embodiment, the asperities coated with nanofibers in accordance with the present invention may be employed as ion and fluid transport membranes for utilization of hierarchical structures of multiwall nanotubes on carbon nanofibers.

The hierarchical structure described includes, in its simplest concept, carbon nanofibers with

radial branches which may or may not have metal particles at the end of each radial branch. Each radial branch is connected electrically through a path that is a part of the carbon hierarchical structure, to an external electrical circuit. The methods described in this invention can be used to coat the end of each branch and metal particle with a porous network of polymer nanofibers. If these pores are filled with an ionized network this network can function as a selective ion transport membrane, which is an essential component of a fuel cell. Thus, in one embodiment, the present invention makes it possible to make radical new designs for fuel cells, batteries, and bioelectric contacts. It could also lead to new designs for electrochemical sensors which depend on the motion of ions in the vicinity of an electrode, which is a large class of sensors.

The ability to deposit one kind of nanofiber at the tips of the carbon nanotube branches and other kinds of nanofibers to span the interstices between the tips of the branches makes it feasible to consider new ways of creating essential paths for the flow of gases and liquids through the hierarchical structures of carbon and other materials, including the electrically conducting variations of titanium oxide known as Magnèli phases, and other such conducting or semiconducting materials, some of which are listed in the following reference by J. R. Smith, et al.

The use of electrospun nanofibers to form durable ion transport membranes is also an enabling technology for the manufacture of light emitting diodes and photoelectric diodes that depend on the motion of ions in the vicinity of asperities on a surface. ("Electrodes Based on Magnelipahse Titanium Oxides", J. R. Smith, F. C. Walsh, and R L Clarke, Journal of Applied Electrochemistry, Vol. 28 (1988), pages 1021-1033. Reviews in Applied Electrochemistry, Number 50).

EXAMPLES

The invention now will be described with respect to the following examples, however, the scope of the present invention is not intended to be limited thereby.

EXAMPLE 1

Construction And Expression Of C. botulinum C Fragment Fusion Proteins

The C. botulinum type A neurotoxin gene has been cloned and sequenced [Thompson, et al., Eur. J. Biochem. 189:73 (1990)]. The nucleotide sequence of the toxin gene is available from the EMBL/GenBank sequence data banks under the accession number X52066; the nucleotide sequence of the coding region is set forth in Figure 22. The amino acid sequence of the C. botulinum type A neurotoxin is shown in Figure 16. The type A neurotoxin gene is synthesized as a single polypeptide chain which is processed to form a dimer composed of a light and a heavy chain linked via disulfide bonds. The 50 kD carboxy-terminal portion of the heavy chain is referred to as the C fragment or the Hc domain.

A number of attempts by others to express polypeptides comprising the C fragment of C. botulinum type A toxin as a native polypeptide (e.g., not as a fusion protein) in E. coli have been unsuccessful [H.F. LaPenotiere, et al. in Botulinum and Tetanus Neurotoxins, DasGupta, Ed., Plenum Press, New York (1993), pp. 463-466]. Expression of the C fragment as a fusion with the E. coli MBP was reported to result in the production of insoluble protein (H.F. LaPenotiere, et al., supra).

In order to produce soluble recombinant C fragment proteins in E. coli, Dr. Williams has shown that it is desirable to construct fusion proteins comprising a synthetic C fragment gene derived from the C. botulinum type A toxin and either a portion of the C. difficile toxin protein or the MBP. See Williams et al., U.S. Patent 5,919,665 (hereby incorporated by reference).

a) Construction Of Plasmids Encoding C Fragment Fusion Proteins

Fusion proteins comprising a fusion between the MBP, portions of the C. difficile toxin A repeat domain (shown to be expressed as a soluble fusion protein) and the C fragment of the C. botulinum type A toxin have been described. See Williams et al., U.S. Patent 5,919,665 (hereby incorporated by reference). A fusion protein comprising the C fragment of the C. botulinum type A toxin and the MBP was also constructed by Dr. Williams.

Figure 23 provides a schematic representation of the botulinal fusion proteins along with the donor constructs containing the C. difficile toxin A sequences or C. botulinum C fragment sequences which were used to generate the botulinal fusion proteins. In Figure 23, the solid boxes represent C. difficile toxin A gene sequences, the open boxes represent C. botulinum C fragment sequences and the solid black ovals represent the E. coil MBP. When the name for a restriction enzyme appears inside parenthesis, this indicates that the restriction site was destroyed during construction. An asterisk appearing with the name for a restriction enzyme indicates that this restriction site was recreated at the cloning junction.

In Figure 23, a restriction map of the pMA1870-2680 and pPA1100-2680 constructs which contain sequences derived from the C. difficile toxin A repeat domain are shown; these constructs can be used as the source of C. difficile toxin A gene sequences for the construction of plasmids encoding fusions between the C. botulinum C fragment gene and the C. difficile toxin A gene. Dr. Williams demonstrated that the pMA1870-2680 expression construct expresses high levels of soluble, intact fusion protein (20 mg/liter culture) which can be affinity purified on an amylose column. See Williams et al., U.S. Patent 5,919,665 (hereby incorporated by reference). The pAlterBot construct was used by Dr. Williams as the source of C. botulinum C fragment gene sequences for the botulinal fusion proteins (obtained from J. Middlebrook and R. Lemley at the U.S. Department of Defense). pAlterBot contains a synthetic C. botulinum C fragment inserted in to the pALTER-1 vector (Promega). This synthetic C fragment gene encodes the same amino acids as does the naturally occurring C fragment gene. The naturally occurring C fragment sequences, like most clostridial genes, are extremely A/T rich (Thompson et al., supra). This high A/T content creates expression difficulties in E. coli and yeast due to altered codon usage frequency and fortuitous polyadenylation sites, respectively. In order to improve the expression of C fragment proteins in E. coli, a synthetic version of the gene was created by Dr. Williams in which the non-preferred codons were replaced with preferred codons. See Williams et al., U.S. Patent 5,919,665 (hereby

incorporated by reference).

The nucleotide sequence of the C. botulinum C fragment gene sequences contained within pAlterBot is shown in Figure 24. The first six nucleotides (ATGGCT) encode methionine and alanine residues, respectively. These two amino acids result from the insertion of the C. botulinum C fragment sequences into the pALTER vector and provide the initiator methionine residue. The amino acid sequence of the C. botulinum C fragment encoded by the sequences contained within pAlterBot is shown in Figure 17. The first two amino acids (Met Ala) are encoded by vector-derived sequences. From the third amino acid residue onward (Arg), the amino acid sequence is identical to that found in the C. botulinum type A toxin gene.

The pMA1870-2680, pPA1100-2680 and pAlterBot constructs were used by Dr. Williams as progenitor plasmids to make expression constructs in which fragments of the C. difficile toxin A repeat domain were expressed as genetic fusions with the C. botulinum C fragment gene using the pMAL-c expression vector (New England BioLabs). See Williams et al., U.S. Patent 5,919,665 (hereby incorporated by reference). The pMAL-c expression vector generates fusion proteins which contain the MBP at the amino-terminal end of the protein. A construct, pMBot, in which the C. botulinum C fragment gene was expressed as a fusion with only the MBP was constructed by Dr. Williams (Figure 23). Fusion protein expression can be induced from E. coli strains harboring the above plasmids, and induced protein was affinity purified on an amylose resin column.

i) Construction Of pBlueBot

In order to facilitate the cloning of the C. botulinum C fragment gene sequences into a number of desired constructs, the botulinal gene sequences can be removed from pAlterBot and were inserted into the pBluescript plasmid (Stratagene) to generate pBlueBot (Figure 23). pBlueBot was constructed by Dr. Williams as follows. Bacteria containing the pAlterBot plasmid were grown in medium containing tetracycline and plasmid DNA was isolated using the QlAprep-spin Plasmid Kit (Qiagen). One microgram of pAlterBot DNA was digested with Ncol and the resulting 3'

recessed sticky end was made blunt using the Klenow fragment of DNA polymerase I (here after the Klenow fragment). The pAlterBot DNA was then digested with HindIII to release the botulinal gene sequences (the Bot insert) as a blunt (filled NcoI site)-HindIII fragment. pBluescript vector DNA was prepared by digesting 200 ng of pBluescript DNA with SmaI and HindIII. The digestion products from both plasmids were resolved on an agarose gel. The appropriate fragments were removed from the gel, mixed and purified utilizing the Prep-a-Gene kit (BioRad). The eluted DNA was then ligated using T4 DNA ligase and used to transform competent DH5a cells (Gibco-BRL). Host cells were made competent for transformation using the calcium chloride protocol of Sambrook et al., supra at 1.82-1.83. Recombinant clones were isolated and confirmed by restriction digestion using standard recombinant molecular biology techniques (Sambrook et al, supra). The resultant clone, pBlueBot, contains several useful unique restriction sites flanking the Bot insert (i.e., the C. botulinum C fragment sequences derived from pAlterBot) as shown in Figure 23. See Williams et al., U.S. Patent 5,919,665 (hereby incorporated by reference).

ii) Construction Of C. difficile/C. botulinum/MBP Fusion Proteins Constructs encoding fusions between the C. difficile toxin A gene and the C. botulinum C fragment gene and the MBP were made by Dr. Williams utilizing the same recombinant DNA methodology outlined above; these fusion proteins contained varying amounts of the C. difficile toxin A repeat domain. See Williams et al., U.S. Patent 5,919,665 (hereby incorporated by reference).

The pMABot clone contains a 2.4 kb insert derived from the C. difficile toxin A gene fused to the Bot insert (i.e, the C. botulinum C fragment sequences derived from pAlterBot). pMABot (Figure 23) was constructed by mixing gel-purified DNA from NotUHindIII digested pBlueBot (the 1.2 kb Bot fragment), SpeI/NotI digested pPA1100-2680 (the 2.4 kb C. difficile toxin A repeat fragment) and XbaUHindIII digested pMAL-c vector. Recombinant clones were isolated by Dr. Williams, confirmed by restriction digestion and purified using the QIAprep-spin Plasmid Kit (Qiagen). This clone expresses the toxin A repeats and the botulinal C fragment protein sequences

as an in-frame fusion with the MBP.

The pMCABot construct contains a 1.0 kb insert derived from the C. difficile toxin A gene fused to the Bot insert (i.e, the C. botulinum C fragment sequences derived from pAlterBot). pMCABot was constructed by digesting the pMABot clone with EcoRl to remove the 5' end of the C. difficile toxin A repeat (see Figure 23, the pMAL-c vector contains a EcoRI site 5' to the C. difficile insert in the pMABot clone). The restriction sites were filled and religated together after gel purification. The resultant clone (pMCABot, Figure 23) generated an in-frame fusion between the MBP and the remaining 3' portion of the C. difficile toxin A repeat domain fused to the Bot gene. See Williams et al., U.S. Patent 5,919,665 (hereby incorporated by reference).

The pMNABot clone contains the 1 kb SpeUEcoRl (filled) fragment from the C. difficile toxin A repeat domain (derived from clone pPA1100-2680) and the 1.2 kb C. botulinum C fragment gene as a Ncol (filled)/HindIII fragment (derived from pAlterBot). These two fragments were inserted by Dr. Williams into the pMAL-c vector digested with XbaUHindIII. The two insert fragments were generated by digestion of the appropriate plasmid with EcoRI (pPA1100-2680) or Ncol (pAlterBot) followed by treatment with the Klenow fragment. After treatment with the Klenow fragment, the plasmids were digested with the second enzyme (either Spel or HindIII). All three fragments were gel purified, mixed and Prep-a-Gene purified prior to ligation. Following ligation and transformation, putative recombinants were analyzed by restriction analysis; the EcoRl site was found to be regenerated at the fusion junction, as was predicted for a fusion between the filled EcoRI and Ncol sites. See Williams et al., U.S. Patent 5,919,665 (hereby incorporated by reference).

A construct encoding a fusion protein between the botulinal C fragment gene and the MBP gene was constructed (i.e., this fusion lacks any C. difficile toxin A gene sequences) and termed pMBot. The pMBot construct was made by Dr. Williams by removal of the C. difficile toxin A sequences from the pMABot construct and fusing the C fragment gene sequences to the MBP. This

was accomplished by digestion of pMABot DNA with StuI (located in the pMALc polylinker 5' to the XbaI site) and XbaI (located 3' to the NotI site at the toxA-Bot fusion junction), filling in the XbaI site using the Klenow fragment, gel purifying the desired restriction fragment, and ligating the blunt ends to circularize the plasmid. Following ligation and transformation, putative recombinants were analyzed by restriction mapping of the Bot insert (i.e., the C. botulinum C fragment sequences). See Williams et al., U.S. Patent 5,919,665 (hereby incorporated by reference).

b) Expression Of C. botulinum C Fragment Fusion Proteins In E. coli

Large scale (1 liter) cultures of the pMAL-c vector, and each recombinant construct described above in (a) can be grown, induced, and soluble protein fractions isolated as described in Williams et al., U.S. Patent 5,919,665 (hereby incorporated by reference), who reported yields in excess of 20 mg fusion protein per liter culture.

EXAMPLE 2

Production Of Soluble C. botulinum C Fragment

Protein Substantially Free Of Endotoxin Contamination

In order to determine if the solubility of the botulinal C fragment was enhanced by expressing the C fragment protein as a native protein, an N-terminal His-tagged protein or as a fusion with glutathione-S-transferase (GST), alternative expression plasmids have been constructed. See Williams et al., U.S. Patent 5,919,665 (hereby incorporated by reference). Figure 25 provides a schematic representation of the vectors described below.

In Figure 25, the following abbreviations are used. pP refers to the pET23 vector. pHIS refers to the pETHisa vector. pBlue refers to the pBluescript vector. pM refers to the pMAL-c vector and pG refers to the pGEX3T vector. The solid black lines represent C. botulinum C fragment gene sequences; the solid black ovals represent the MBP; the hatched ovals represent GST; "HHHHH" represents the poly-histidine tag. In Figure 25, when the name for a restriction enzyme appears inside parenthesis, this indicates that the restriction site was destroyed during construction. An

asterisk appearing with the name for a restriction enzyme indicates that this restriction site was recreated at a cloning junction.

i) Construction Of pPBot

In order to express the C. botulinum C fragment as a native (i.e., non-fused) protein, the pPBot plasmid (shown schematically in Figure 25) was constructed by Dr. Williams as follows. The C fragment sequences present in pAlterBot were removed by digestion of pAlterBot with NcoI and HindIII. The NcoUHindI11 C fragment insert was ligated to pETHisa vector which was digested with NcoI and HindIII. This ligation creates an expression construct in which the NcoI-encoded methionine of the botulinal C fragment is the initiator codon and directs expression of the native botulinal C fragment. The ligation products were used to transform competent BL21(DE3)pLysS cells (Novagen). Recombinant clones were identified by restriction mapping. See Williams et al., U.S. Patent 5,919,665 (hereby incorporated by reference).

ii) Construction Of pHisBot

In order to express the C. botulinum C fragment containing a poly-histidine tag at the amino-terminus of the recombinant protein, the pHisBot plasmid (shown schematically in Figure 8) was constructed by Dr. Williams as follows. The NcoUHindIII botulinal C fragment insert from pAlterbot was ligated into the pETHisa vector which was digested with Nhel and HindiII. The Ncol (on the C fragment insert) and Nhel (on the pETHisa vector) sites were filled in using the Klenow fragment prior to ligation; these sites were then blunt end ligated (the Ndel site was regenerated at the clone junction as predicted). The ligation products were used to transform competent BL21(DE3)pLysS cells and recombinant clones were identified by restriction mapping. See Williams et at., U.S. Patent 5,919,665 (hereby incorporated by reference).

The resulting pHisBot clone expresses the botulinal C fragment protein with a histidine-tagged N-terminal extension having the following sequence: MetGlyHisHisHisHisHisHisHisHisHisSerSerGlyHisIleGluGlyArgHisMetAla; the amino acids

encoded by the botulinal C fragment gene are underlined and the vector encoded amino acids are presented in plain type. The nucleotide sequence present in the pETHisa vector which encodes the pHisBot fusion protein is set forth in Figure 26. The amino acid sequence of the pHisBot protein is set forth in Figure 27.

iii) Construction Of pGBot

The botulinal C fragment protein was expressed as a fusion with the glutathione-S-transferase protein by constructing the pGBot plasmid (shown schematically in Figure 24). This expression construct was created by Dr. Williams by cloning the NotUSall C fragment insert present in pBlueBot into the pGEX3T vector which was digested with SmaI and XhoI. The NotI site (present on the botulinal fragment) was made blunt prior to ligation using the Klenow fragment. The ligation products were used to transform competent BL21 cells. See Williams et al., U.S. Patent 5,919,665 (hereby incorporated by reference).

iv) solubility and purification

The pGBot protein was almost entirely insoluble under the utilized conditions, while the pHisBot protein was soluble. See Williams et al., U.S. Patent 5,919,665 (hereby incorporated by reference). For improved affinity purification of the soluble pHisBot protein (in terms of both yield and purity), an alternative poly-histidine binding affinity resin (Ni-NTA resin; Qiagen) is desirable. The Ni-NTA resin has a superior binding affinity (K_d= 1 x 10^{-13} at pH 8.0; Qiagen user manual) relative to the His-bind resin.

Based upon the foregoing disclosure, it should now be apparent that Clostridium toxins, and in particular, C. botulinum toxins, together with polymer (e.g. in an electrospun formulation) can be used as vaccines. A particularly desirable administration approach is transdermal, via microprojections (e.g. an array of microneedles). A variety of polymers, as well as delivery vehicles, can be used without departing from the spirit of the invention. In one embodiment, the electrospun polymer/toxin(s) fiber mat is placed directly on the skin. In another embodiment, the

polymer/toxin(s) fiber mat is part of a delivery vehicle (e.g. skin patch, microneedle array, etc.)

EXAMPLE 3

Mutagenesis. To generate a complete library of Cys mutants of PA_{63} one can use the QuikChange (Stratagene) method to generate point mutations, in conjunction with automated systems currently used for high-throughput DNA sequencing. The pag gene encoding PA is AT rich (34% GC) and does not present significant technical hurdles. Automated systems can be used to synthesize the mutagenic oligonucleotide pairs, and the quality of mutagenic oligonucleotides can be confirmed by automated capillary electrophoresis. In this manner, a full plate can be processed in a day, and the oligonucleotides needed for complete mutagenesis of PA_{63} can be synthesized and checked in 12 days. After manually performing the PCR mutagenesis in 96-well plates and transforming the resulting mutated plasmids into E. coli, automation can again be used for preparing DNA from the colonies and sequencing of the region of interest. Clones bearing the desired mutation can be used for functional screens.

As noted above, researchers at the University of Oklahoma used this protocol to generate 568 mutants, corresponding to the replacement of every amino acid of PA_{63} with Cys. Thirty three mutants (6% of the total number of mutants tested) were both strongly defective and well expressed. As shown in Table 3, the 33 mutations were distributed among the four domains of PA, with a majority in domain 2.

Some Inactive Mutants Are Dominant. The 33 inactive mutants were tested by the researchers at the University of Oklahoma for ability to inhibit the toxicity of a mixture of PA and LF_NDTA toward CHO cells at ratios of mutated to wild-type PA up to 8:1. Those that showed measurable inhibitory activity under these conditions were defined, for the purpose of this study, as "dominant negative" (DN). Nine preparations of mutant PA were inhibitory: I364, T380, S382, T393, K397, N399, Y411, N422, and F427. The present invention contemplates using such mutant protective antigens both alone and in conjugates (as described above). Like the DN mutants

characterized earlier, the purified DN Cys-mutants showed defects specifically in pore formation and translocation. PA bearing T393C, T380C, or S382C showed strong DN activity, only slightly weaker than that of the K397D + D425K double mutant characterized earlier. N399C and N422C showed moderate DN activity, and 1364C and Y411 C showed weak inhibitory activity.

One of the strongest of the five DN mutants, T393C, was tested by researchers at the University of Oklahoma for ability to protect Fisher 344 rats from a lethal challenge with a mixture of PA and LF. Like the other DN mutants tested, T393C prevented symptoms of intoxication until the time of death, whereas rats challenged with native toxin alone became moribund 75 rain after challenge (data not shown). In the end, 33 well-expressed mutants with defects in toxicity were described by the Oklahoma group.

In contrast to the Cys substitutions, Ala substitutions created by the Oklahoma group at selected sites gave different results: Y411A showed strong DN activity; T380A and N399A showed weak DN activity; and N422 and S382A showed no DN activity. At position 393, Cys or Lys gave strong DN activity; Asp or Ala gave moderate DN activity; and Ser gave no DN activity. The present invention contemplates employing these Ala substituted mutant protective antigens alone or as conjugates (as described above).

Conjugating wild-type and mutant PA to PGA. Conjugates are readily synthesized as follows. To 1 mg of PA in 0.5 ml phosphate-buffered saline (PBS; pH 7.0), 0.5 mg of degraded PGA can be added. After PGA has dissolved, 5 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride should be added, and the solution mixed on a rotary shaker at 25C for 4 hours. One can monitor the conjugation process by SDS-PAGE. Moreover, the conjugates can be purified on a PD10 column (Amersham Biosciences), verified by SDS-PAGE, and stored at -20C until use.

Alternatively, gamma-PGA polypeptides can be synthesized (with various lengths, e.g. 5, 10, 15 or 20 residues) and can be bound to carrier proteins at either the C-or N-termini (for further

reactions). The various types of these polypeptides are described in U.S. Patent Application 20060134143 to Schneerson et al. (hereby incorporated by reference) including but not limited to PGA comprising D-glutamic acid and PGA comprising L-glutamic acid:

Type I: NBrAcGly$_3$-γDPGA$_n$- COOH(Br-Gly$_3$-γDPGA$_n$-C)

NBrAc-Gly$_3$-γLPGA$_n$-COOH(Br-Gly$_3$-γLPGA$_n$-C)

Type II: NAc-L-Cys-Gly$_3$-γDPGA$_n$,-COOH(Cys-Gly$_3$-γDPGA$_n$-C)

NAc-L-Cys-Gly$_3$-γLPGA$_n$,-COOH(Cys-Gly$_3$-γLPGA$_n$-C)

Type III: NAc-γDPGA$_n$-Gly$_3$-L-Cys-CONH$_2$(N- γDPGA$_n$-Gly$_3$-Cys)

NAc-γLPGA$_n$-Gly$_3$-L-Cys-CONH$_2$(N-γLPGA$_n$-Gly$_3$-Cys)

Type IV: CHO-Gly$_3$- γDPGA$_n$-COOH

Type V: NAc-γDPGA$_n$-Gly$_3$-CO-AH

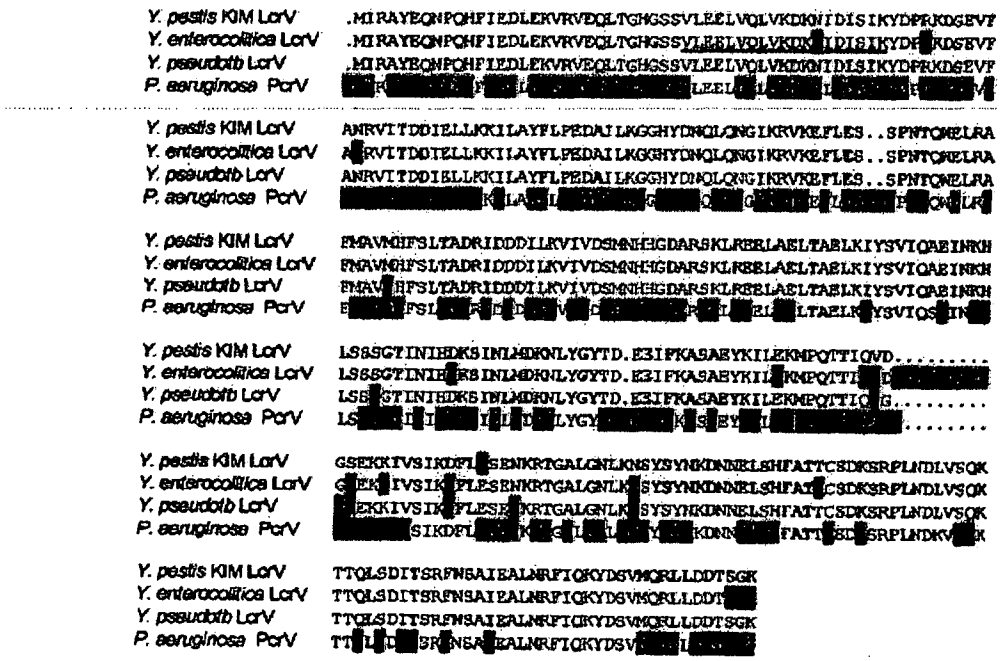
NAc-γDPGA$_n$-CO-AH

Type VI: NAc-γDPGA$_n$-Cys-CONH$_2$

In one example, recombinant wild-type PA is derivatized with adipic acid dihydrazide and reacted with one or more of these polypeptides.

Using Y. pestis antigens. As noted above, in another embodiment, antigen from Y. pestis is contemplated in a preparation suitable for transdermal delivery. It is not intended that the present invention be limited by the particular antigens from these organisms. However, in one embodiment, an F1 capsule antigen from Y. pestis is employed in a polymer formulation (e.g. electrospun formulation) deposited on a microneedle array. In another embodiment, a protein (or fragment) from the injectisome from Y. pestis is employed. In a preferred embodiment, the LcrV protein (or fragment thereof) from the Y. pestis injectisome is similarly employed. With regard to the latter, Table 5 shows the amino acid sequence for LcrV of Y. pestis, as well as from some other sources.

TABLE 5



Because of the similarity between three of the organisms (but not P. aeruginosa), the present invention contemplates, in one embodiment, using the LcrV protein (or fragment thereof) of any of these three organisms in order to generate protection against Y. pestis. If a fragment is used, typically the fragment will be at least twenty (20) amino acids and selected in a region of high conservation (see Table 5).

Where F1 capsule antigen and LcrV protein are made as a fusion protein, the fusion protein may be made by selecting appropriate coding sequences from GenBank accession No. AF074611. Using F. tularensis antigens. As noted above, in another embodiment, antigen from F. tularensis is contemplated in a preparation suitable for transdermal delivery. It is not intended that the present invention be limited by the particular antigens from these organisms. However, in one embodiment, a specific sequence of the outer membrane protein (or fragment thereof) encoded by the fopA gene of F. tularensis is employed.

Alternatively, a protein (or fragment thereof) encoded by the tul4 gene is employed. The PCR primers that will conveniently amplify these genes are given below:

*tul4 primers (forward, ATTACAATGGCAGGCTCCAGA; reverse, GCCCAAGTTTTATCGTTCTTCTCA) will specifically amplify an 89-bp fragment of the tul4 gene (GenBank accession no. M32059) encoding a 17-kDa lipoprotein.

* fopA primers (forward, AACAATGGCACCTAGTAATATTTCTGG; reverse, CCACCAAAGAACCATGTTAAACC) will amplify an 86 bp fragment of the fopA gene (GenBank accession no. AF097542) which encodes a 43-kDa outer membrane protein.

The proteins can be expressed in vitro or in vivo and then formulated (e.g. in an electrospun formulation) for application to the microneedle array.

Electrospinning Anthrax Protective Antigen. In this example, the protocol for electrospinning anthrax antigen is described. Pure native PA (List Biological Laboratories) will be reconstituted in 1 ml of distilled water containing 0.1 % Bovine Serum Albumin (BSA) at a concentration of 1 mg/ml. 400 Al of the solution will be further diluted with equal volume of aqueous 0.1% BSA to prepare the PA solution which will be mixed appropriately with PVP as described below. The "unspun" 500 µg/ml PA solution will be used as the control for the evaluation of retention of functionality (although this is not required for a vaccine). Two electrospinning solutions will be prepared utilizing the PA solution. The first will consist of 400 µl of PA solution, and 1600 Al of 0.075 mM Polyvinylpyrrolidone (PVP) dissolved in absolute ethanol, and the second will have distilled water as the solvent. Both solutions will be utilized in preparation of electrospun mats, and tested in the assay of functionality to confirm that protein activity has been retained upon introduction of polymer solution.

Because an approximate concentration of 100 µg/ml of PA is required for the functionality test, and the volume needed for the cell-based assay is 1ml, 100 mg of the material is deposited onto the collector. It is estimated that the mixture of PA and PVP in ethanol will be electrospun at 20

uUmin and 16 kV, while the completely aqueous mixture will be electrospun at 5-10 uUmin and 20 kV. From previous experiments it can be estimated that, regardless of which solution is electrospun, only 50% to 75% of the total material will be deposited on the collector. 1500 µl of each solution is electrospun onto aluminum foil, coated with Teflon to ease the removal of the electrospun mat from the foil. The entire material deposited on the sheet will be peeled off and dissolved in 1 ml of MatTek tissue culture medium to provide samples for the test of functionality. This stock solution will be further diluted with culture medium to a final estimated concentration of 10 µg/ml; this concentration is 50-100 times the required amount for the test of functionality.

The cells used in this test are MonoMac 6 cells (DSMZ, Braunschweig, Germany)) maintained in RPMI 1640 (Hyclone) medium supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 1% non-essential amino acids, 1 mM oxaloacetate, 0.45 mM pyruvate, 0.2 U/ml insulin (OPI media supplement, Sigma, St.Louis, MO), and 1% (v/v) penicillin streptomycin solution (final concentrations 100 IU penicilin G and 100 gg streptomycin per ml, Sigma). Cells are washed with Hank's Balanced Salt Solution (HBSS; Hyclone) at 37°C once prior to commencing experiments, which are all carried in serum free media without antibiotics.

The functionality experiments are based on examination of Lethal Factor (LF) proteolytic activity in the cytosol of viable cells. We have shown in previous experiments that LF added to the surrounding medium cannot enter MonoMac 6 cells unless functionally active PA is present to facilitate its transport from the medium into the cytosol. MonoMac 6 cells are washed with warm DPBS and are resuspended in Macrophage Serum Free Medium (Gibco) for plating at a density of 1 x 10^6/ml/well in 24 well microplates. After addition of each of the PA solutions to be tested (two different dissolved electrospun preparations and an aliquot of the "unspun" solution) and a 100 ng/ml solution of LF (List Biolabs) to separate wells of cells, the cells are incubated for different periods of time, and at each designated time point a 200 µl aliquot of cell suspension is removed. Cells are pelleted by centrifugation and are then lysed in a buffer containing 0.1% Nonidet P-40

(NP40), 150 mM NaCl, 40 mM Tris @H 7.2), 10% 5 glycerol, 5 mM NaF, 1 mM Na pyrophosphate, 1mM Na o-vanadate, 10 mM ophenanthroline, and 100 ng/ml phenylmethyl sulfonyl fluoride (PMSF) at 70°C for 10 min .

The lysates are separated by SDS-polyacrylamide gel electrophoresis and the presence of intact or cleaved MEK-2 is quantitated by immunoblotting. Aliquots of 7.5-10 ug total cell lysate protein per well are loaded onto 10% SDS gels (Novex) and electrophoresed followed by transfer to nitrocellulose membranes in an electrotransfer apparatus (Novex) for 1 h at 30 V. The membranes are blocked with 5% low-fat milk in This-buffered saline buffer, pH 7.6, containing 0.1% Tween 20 for 1 h at room temperature and then probed with anti-MEK-2 rabbit polyclonal antibody (Santa Cruz) at a dilution of 1:2000. Goat anti-rabbit IgG is used at a dilution of 1:2000 as the secondary antibody.

Blots are washed after probing and processed with the Enhanced Chemiluminescence detection kit according to the manufacturer's directions.

The predicted results for a successful delivery of LF are illustrated in Figure 28.

As can be seen in Figure 28, it is expected that after 4 hours of incubation of MonoMac 6 cells with LF alone or with PA alone, there is no degradation of the MAP Kinase MEK-2 detected in an immunoblot, or "Western blot," of the cell lysates, whereas when LF and PA are both present, MEK-2 is destroyed within the cells in 4 hours. If the electrospun PA behaves in a manner similar to unspun PA (as is to be expected), one can anticipate seeing MEK-2 cleavage as shown in Figure 28.

Delivering Electrospun PA To A Tissue Model. In this example, the protocol for delivering electrospun PA through MatTek EpiDerm 200 epidermal equivalent tissue, using 600 um microspike-bearing silicon chips and collecting the surrounding medium from the base of the MatTek units for challenge with LF and MonoMac 6 cells, is described.

Testing Antigenicity of Electrospun PA. In this example, the protocol for testing for preservation of antigenicity of the electrospun PA using a commercially available polyclonal goat

anti-PA antibody from List Biological Laboratories is described. This antibody recognizes PA even after it has been subjected to SDS-polyacrylamide gel electrophoresis, indicating that, at least in goats, the PA can be prepared as an immunogen to generate a robust immune response. Aliquots of the dissolved samples of electrospun PA generated from the two solvent systems will be separated by SDS polyacrylamide gel electrophoresis along with aliquots of the "unspun" PA control and transferred to nitrocellulose membranes as described above. The membranes will be probed with an appropriate dilution of the goat anti-PA antibody, while a rabbit anti-goat polyclonal antibody will be used as the secondary antibody. The membranes will then be examined by enhanced chemiluminescence as described above. This test is less stringent than the functionality assay (and all that is needed for a vaccine) because even PA which has been denatured by the electrospinning protocol (which is not expected) will retain its capacity to be recognized by the goat antibody. However, we can still quantitate the delivery of immunoreactive PA after electrospinning and even after delivery across an epidermal equivalent model by using a semiquantitative immunoblotting procedure known as a "slot blot." The slot-blot apparatus allows direct application of samples collected after electrospinning, or after delivery through a skin equivalent, onto a nitrocelulose membrane. The apparatus can accommodate 48 samples at a time, including a series of sequential dilutions of the "unspun" PA control, the PA concentrations of which are all precisely known. After all the samples are applied, the membrane is removed and incubated with goat anti-PA primary antibody and rabbit-antigoat secondary antibody, and examined by enhanced chemiluminescence as described above.

Based upon the foregoing disclosure, it should now be apparent that anthrax antigens, and in particular, certain conjugates of two anthrax antigens (one of which being a mutant), taken alone or together with polymer (e.g. in an electrospun formulation) can be used as vaccines. A particularly desirable administration approach is transdermal, via microprojections (e.g. an array of microneedles). A variety of polymers and mutants, as well as delivery vehicles, can be used without

departing from the spirit of the invention. In one embodiment, the electrospun polymer/antigen(s) fiber mat is placed directly on the skin. In another embodiment, the polymer/antigen(s) fiber mat is part of a delivery vehicle (e.g. skin patch, microneedle array, etc.)

EXAMPLE 4

Parathyroid Hormone (PTH 1-34) Polyvinylpyrrolidone (PVP) molecular weight of ~ 1,300,000 from Sigma-Aldrich was dissolved at a concentration of 0.075mM in 0.4% Human Serum Albumin (HSA) in water and magnetically stirred for 3 hours. A 550 pg/ml peptide solution supplied by Immunotopics as part of its PTH 1-34 Elisa kit was used as the hormone source. The electrospinning PTH 1-34 solution was prepared by mixing the PVP and PTH solutions at an 80% / 20% (v/v) PVP/PTH ratio. The electrospinning method was utilized to deposit a nanocomposite of the solution onto the substrates. A total of 1.0 ml was electrospun onto the target. The coating was created at the flow rate of 80min, voltage of 20 kV, and a distance between the collector and needle of 10 cm. The coated devices were attached to microcentrifuge tubes with crazy glue to add stability, and ease the handling process. The transdermal delivery experiments were carried out using EpiDerm Full Thickness (EFT-300) tissue models from MatTek Corporation. The samples were placed on top of the tissue model units with sufficient pressure to ensure that the spikes had entered the tissue, thereby beginning the time release study. At each time point the tissue model its with the devices in place were moved into pre-warmed media, and supernatant was collected. After the 24 hour harvest, the devices were removed, the tissue was cut out of the holders and homogenized into 500 µl of tissue culture media in a microfuge tube using a small motor-driven pestle. The ground tissue was centrifuged, and analysis of the supernatant medium was performed. To test tissue viability after the 24 hour time point, mitochondrial dehydrogenase activity was assayed using Alamar Blue reduction. Due to this assay's interaction with the HRP assay only two tissue model units from each set were examined for viability.

Based upon the foregoing disclosure, it should now be apparent that hormones, and in

particular, PTH(1-34), together with polymer (e.g. in an electrospun formulation) can be used prophylatically (to prevent) or therapeutically (to treat) bone loss. A particularly desirable administration approach is transdermal, via microprojections (e.g. an array of microneedles). A variety of polymers and hormones (or hormone combinations), as well as delivery vehicles, can be used without departing from the spirit of the invention. For example, iIn one embodiment, the electrospun polymer/hormone(s) fiber mat is placed directly on the skin. In another embodiment, the polymer/hormone(s) fiber mat is part of a delivery vehicle (e.g. skin patch, microneedle array, etc.).

The disclosures of all patents and publications (including published patent applications) are hereby incorporated by reference to the same extent as if each patent and publication were individually and specifically incorporated by reference.

Although the invention has been described in detail with particular reference to certain embodiments detailed herein, other embodiments can achieve the same results. Variations and modifications of the present invention will be obvious to those skilled in the art and the present invention is intended to cover in the appended claims all such modifications and equivalents.

WHAT IS CLAIMED IS:

1. A device, comprising:

a substrate comprising a surface and a plurality of asperities extending from said surface; and

an electrospun material in contact with said substrate.

2. The device of Claim 1 wherein said asperties are microneedles.

3. The device of Claim 1 wherein said electrospun material is in the form of nanofibers.

4. The device of Claim 1 wherein said electrospun material comprises at least one carrier substance capable of carrying a biologically active agent, and at least one biologically active agent.

5. The device of Claim 4 wherein said carrier material is a water-soluble polymer.

6. The device of Claim 5 wherein said polymer is selected from the group consisiting of PVP, carboxymethyl cellulose,0 hydroxyethylcellulose, hydroxypropylcellulose, alginic acid, and chitosan.

7. The device of Claim 4 wherein said at least one biologically active agent is a therapeutic protein.

8. The device of Claim 4 wherein said biologically active agent is a cytokine.

9. The device of Claim 4 wherein said biologically active agent is a Clostridium toxin.

10. The device of claim 4 wherein said biologically active agent is an anthrax antigen.

11. The device of Claim 4 wherein said biologically active agent is a hormone.

12. A method of administering a biologically active agent, comprising:

contacting a subject with the device of Claim 4 under conditions such that at least a portion of said agent is released from said device.

13. The method of Claim 12 wherein said contacting comprises piercing the skin of said subject with said asperities.

Figure 1

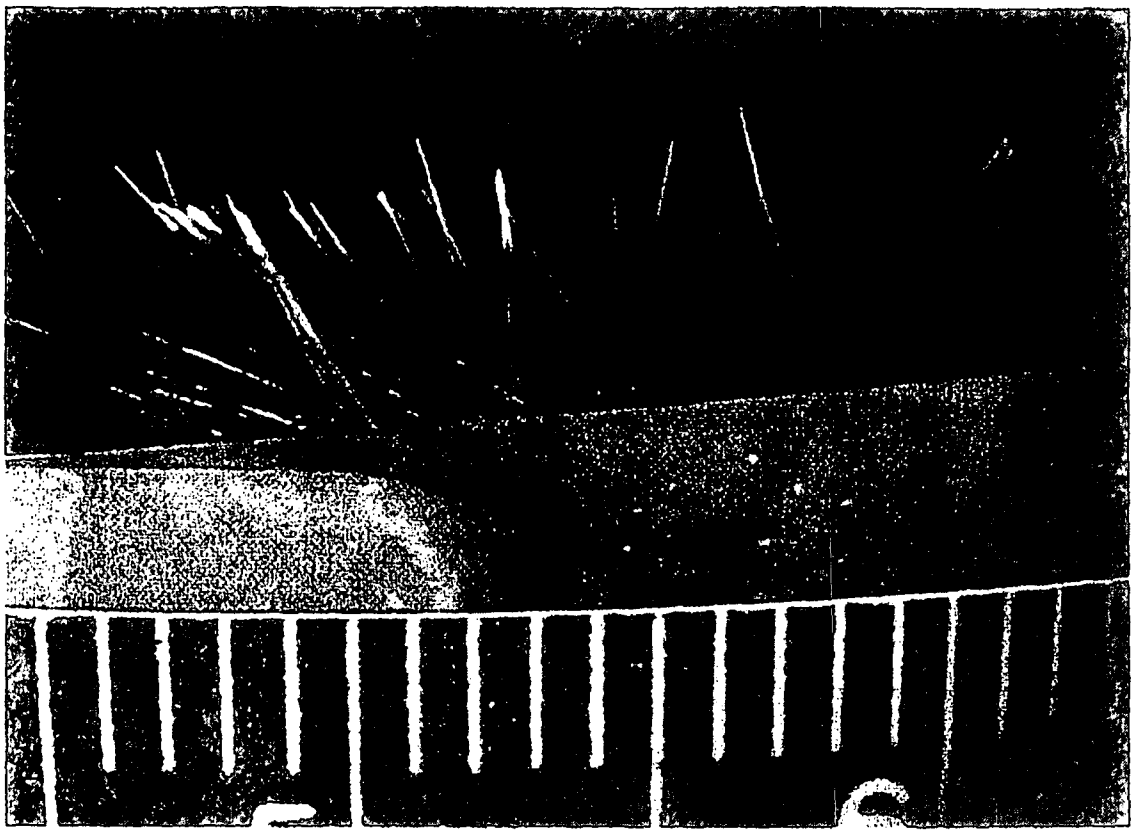


Figure 2

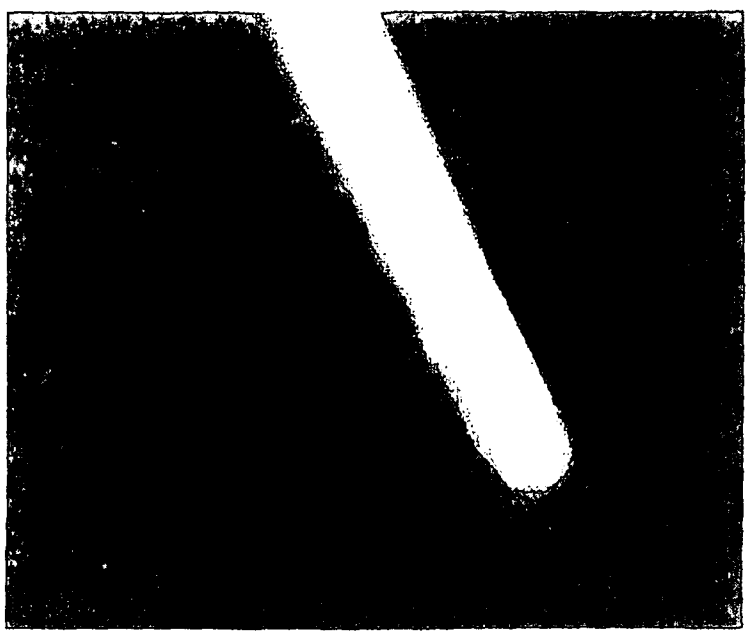
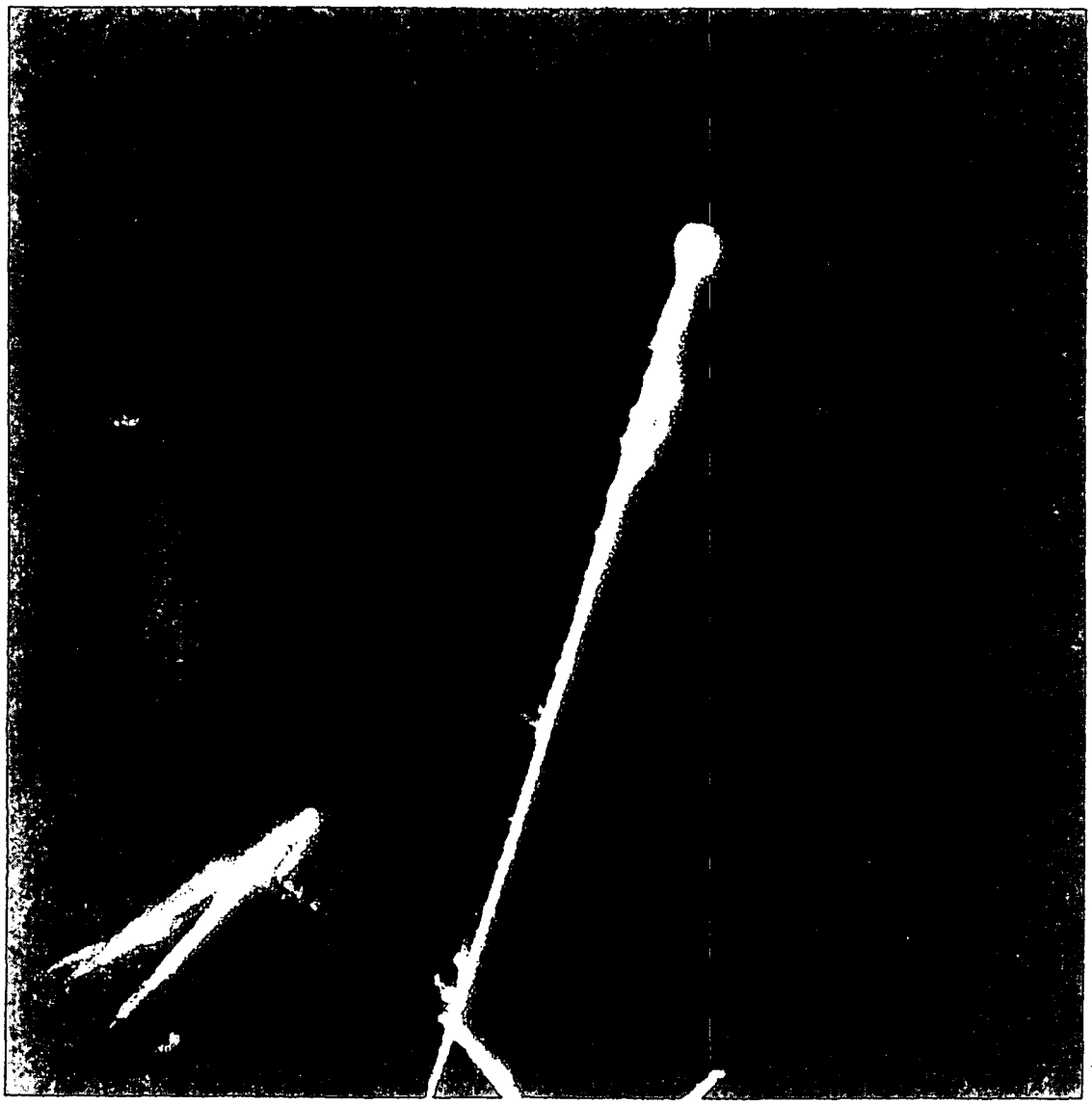


Figure 3



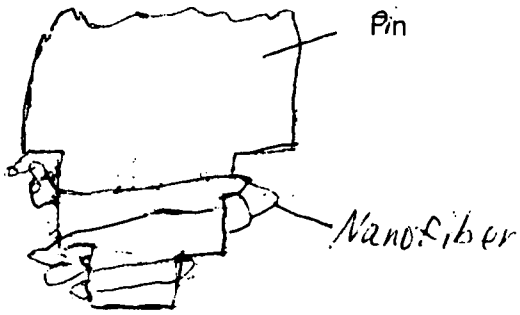


Figure 4

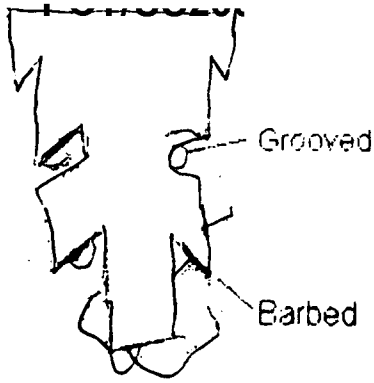


Figure 5

Figure 6

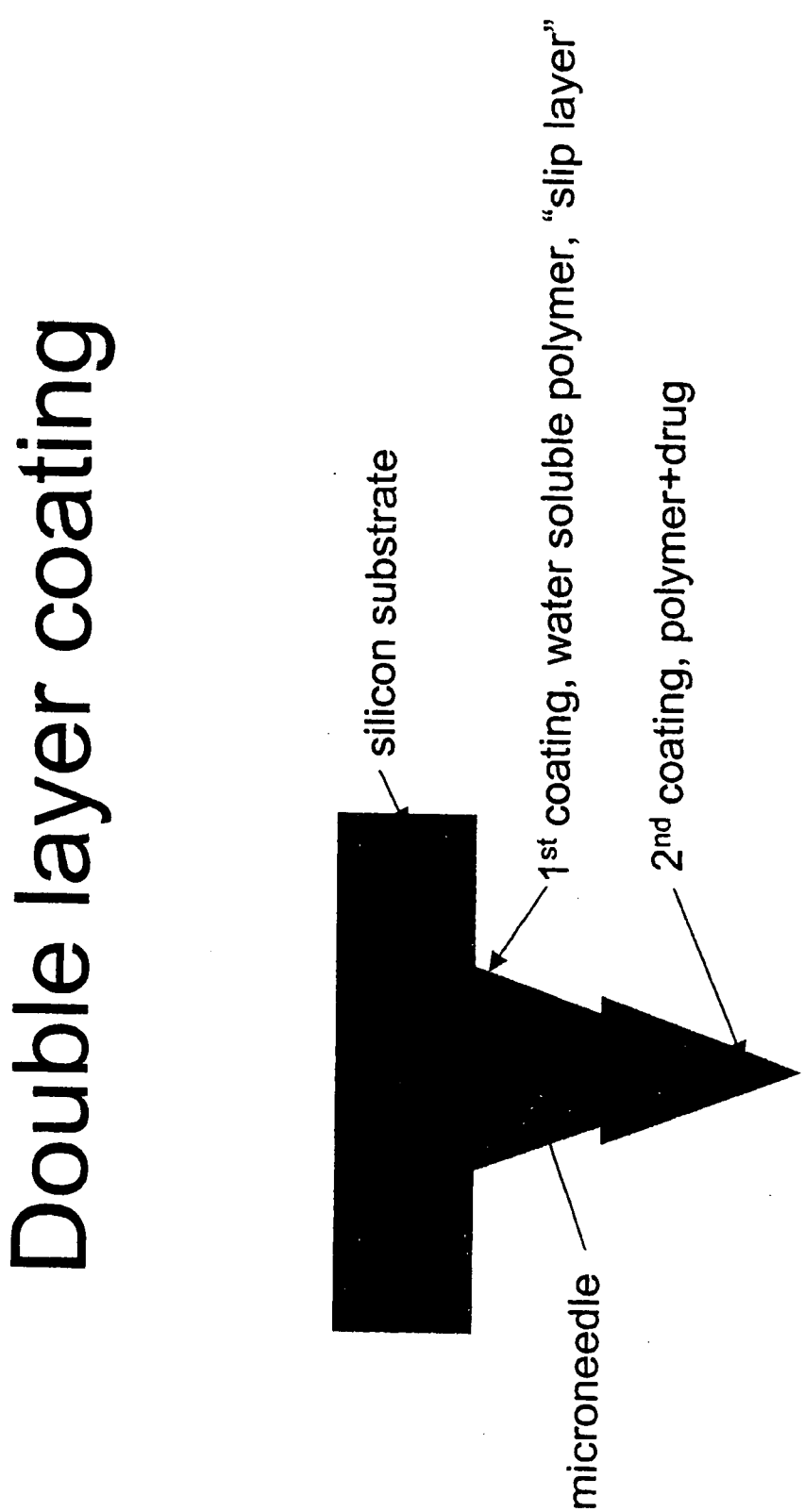

Double layer coating
silicon substrate
1st coating, water soluble polymer, "slip layer"
2nd coating, polymer+drug
microneedle

FIG 7A

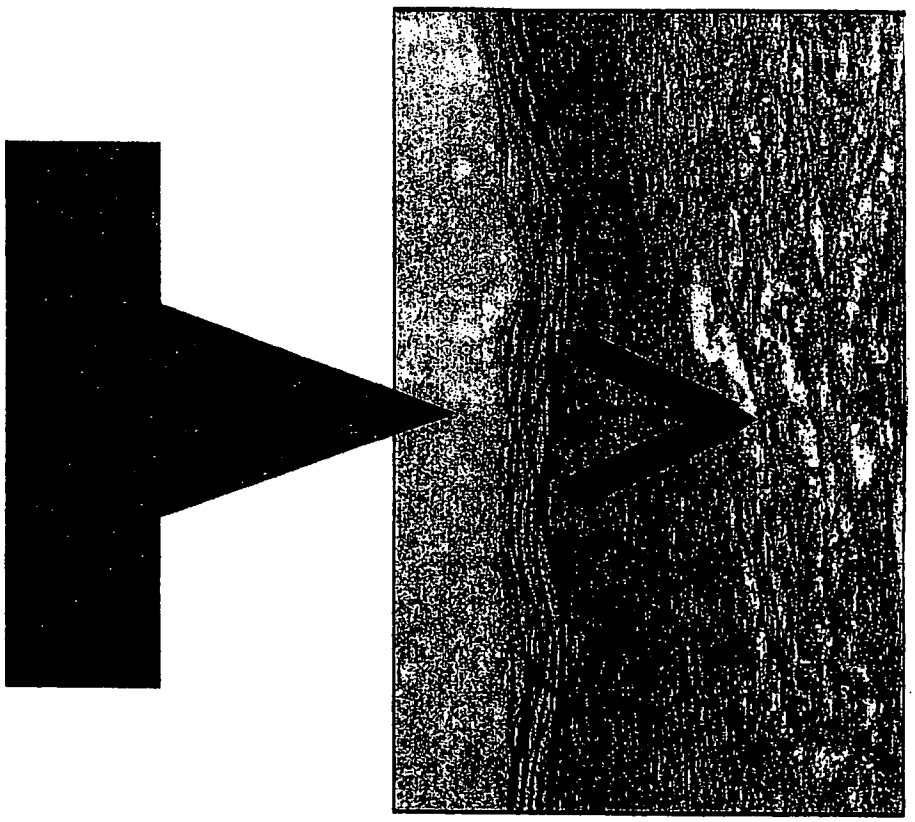


FIG 7B

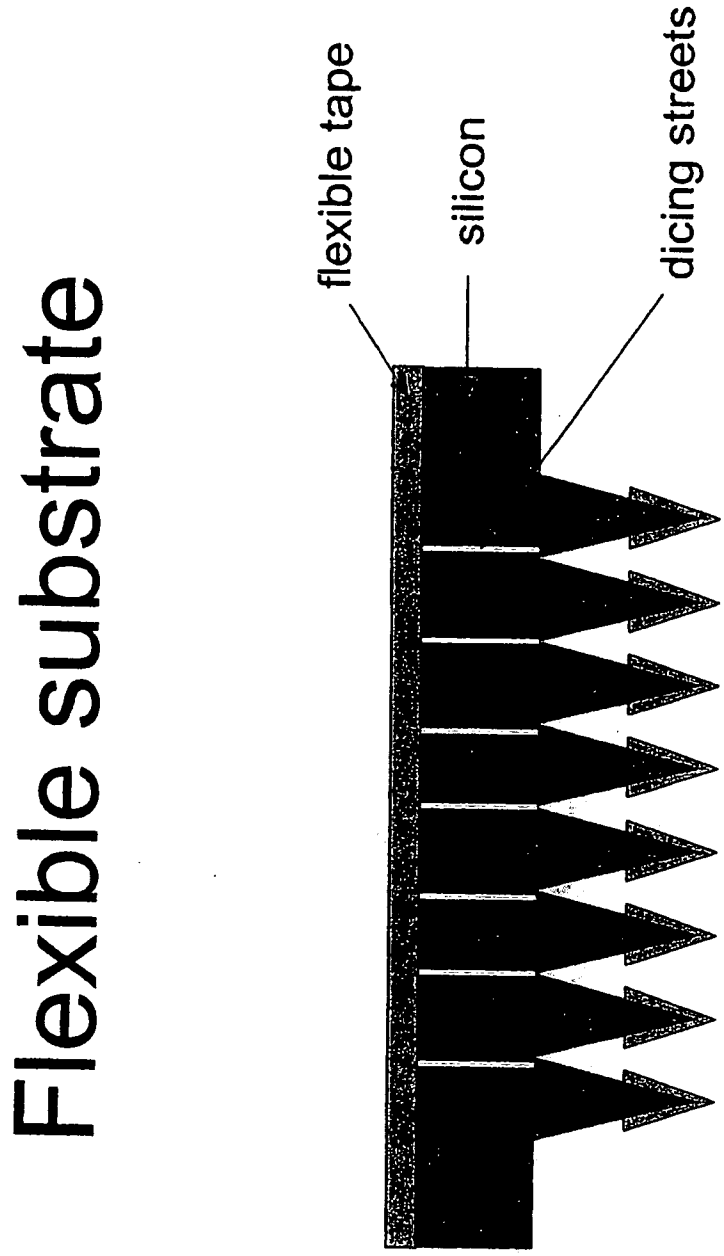


FIG 8

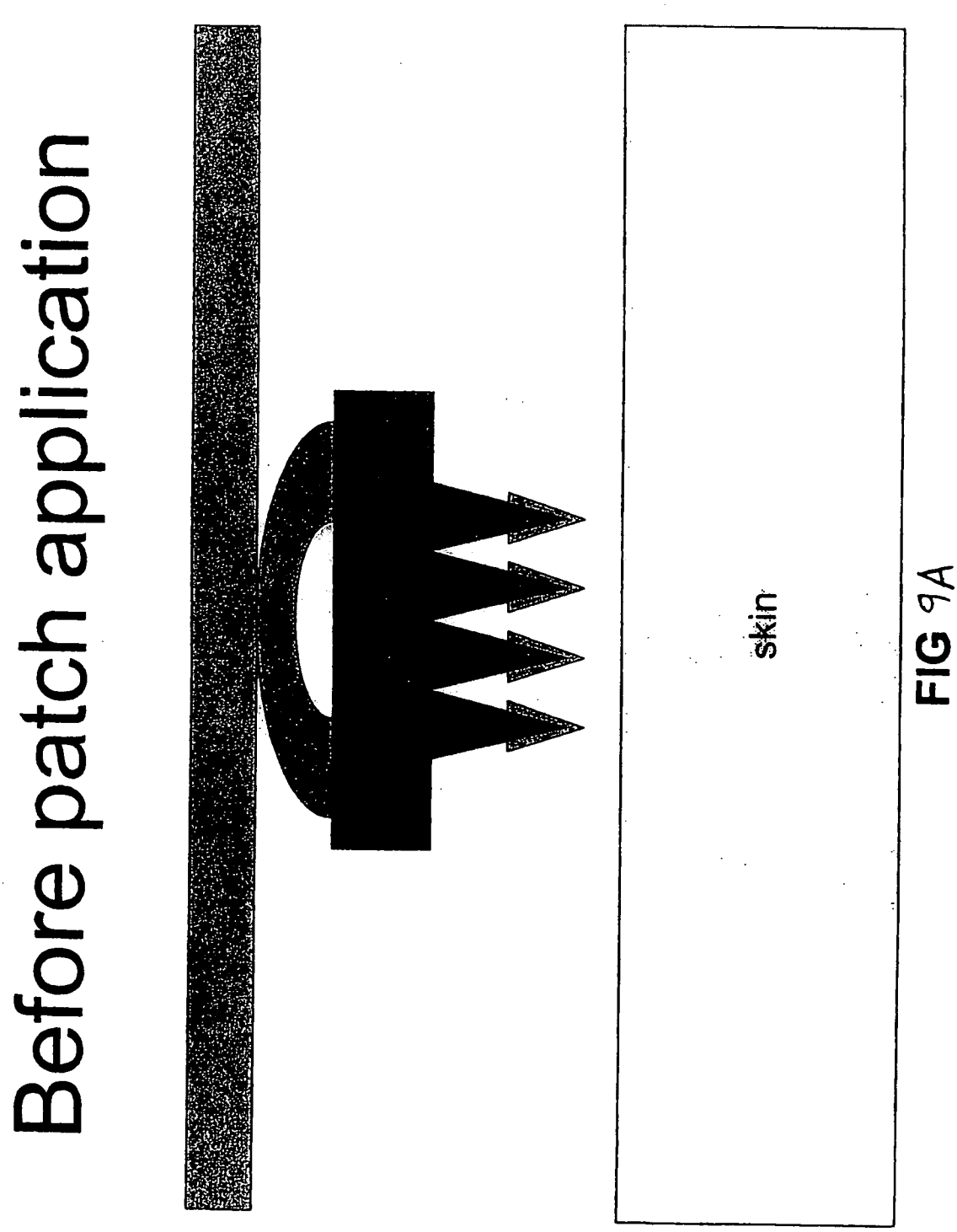


FIG 9A

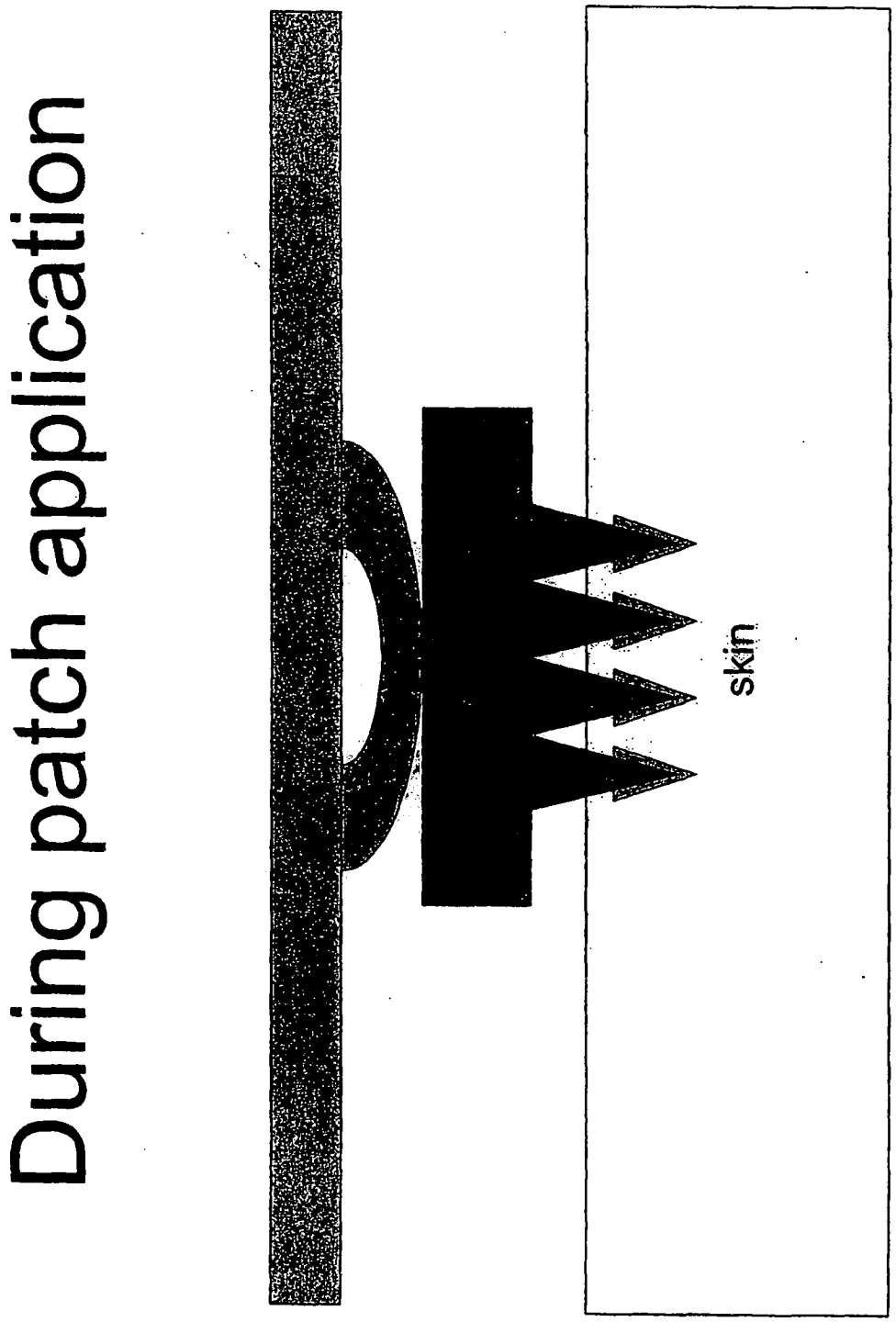


FIG 1B

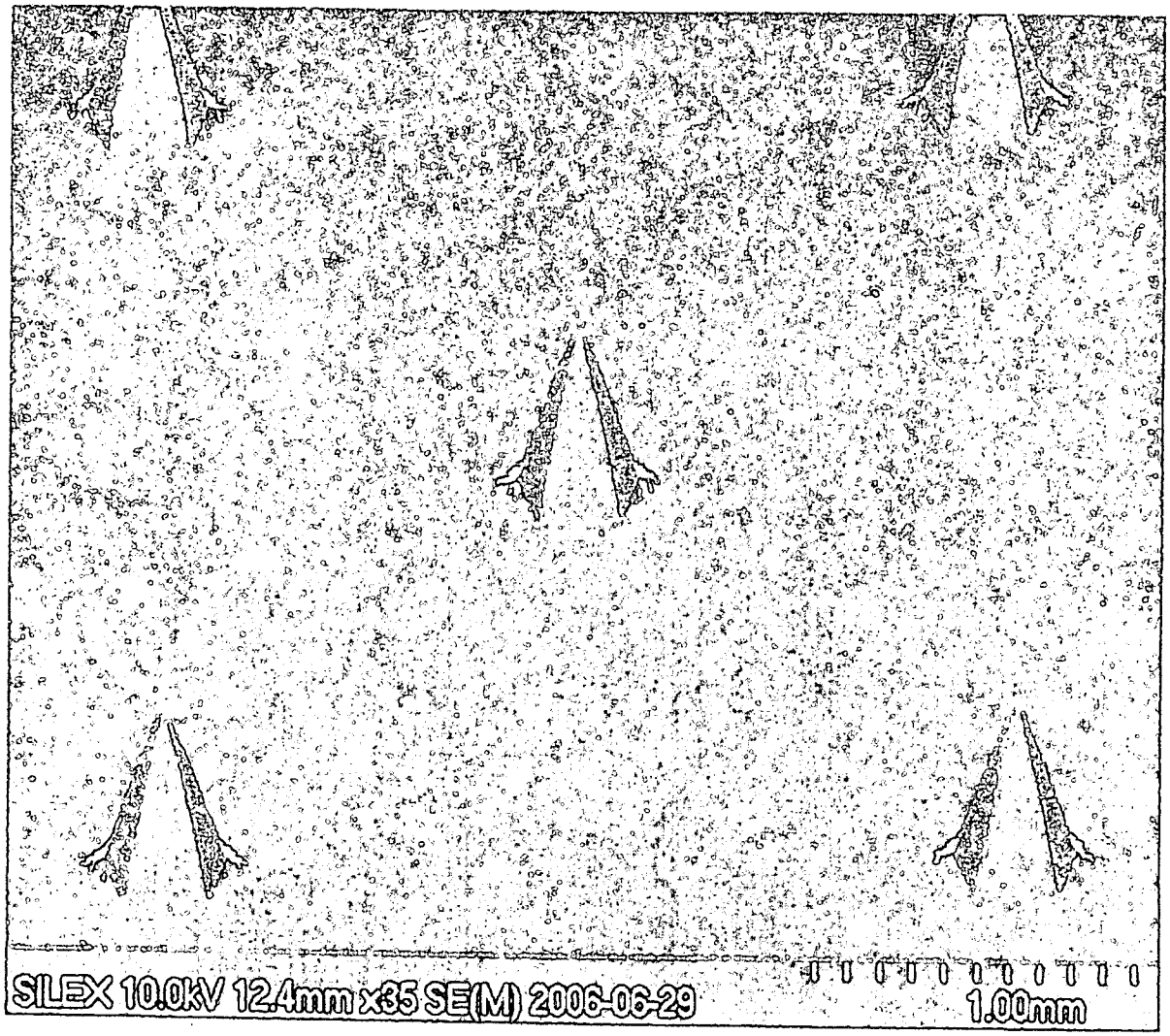


FIG 10

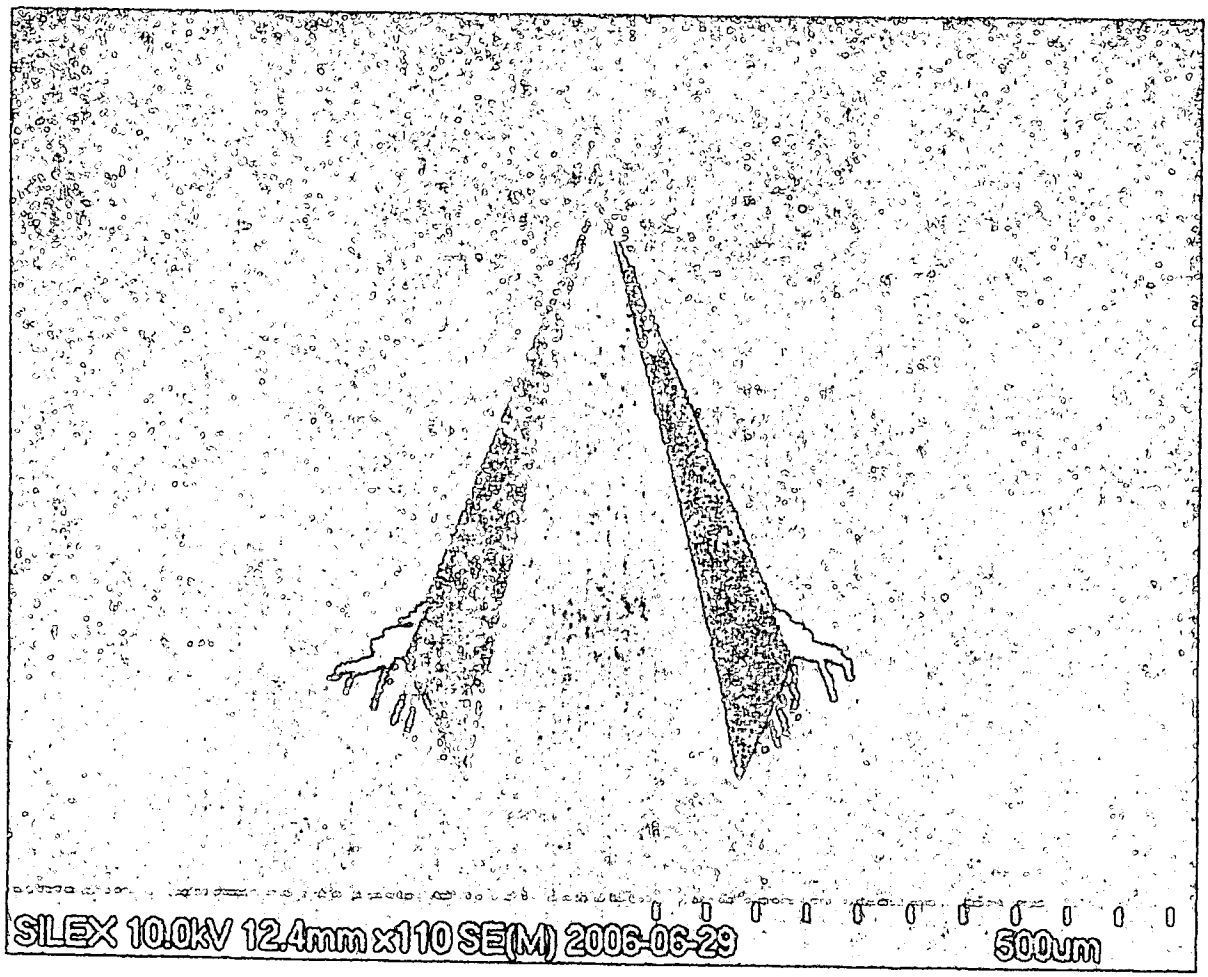


FIG 11

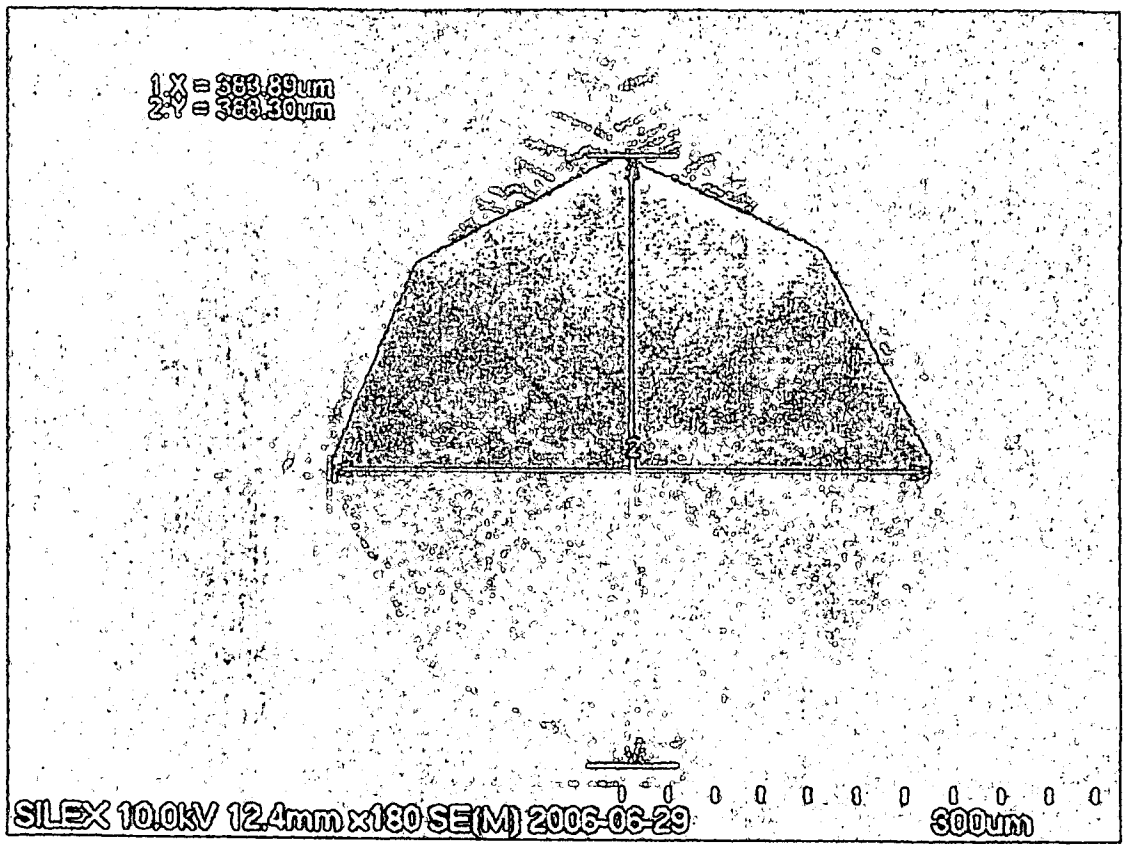

1.X = 383.89um
2.Y = 388.30um
SILEX 10.0kV 12.4mm x180 SE(M) 2006-06-29
300um

FIG 12

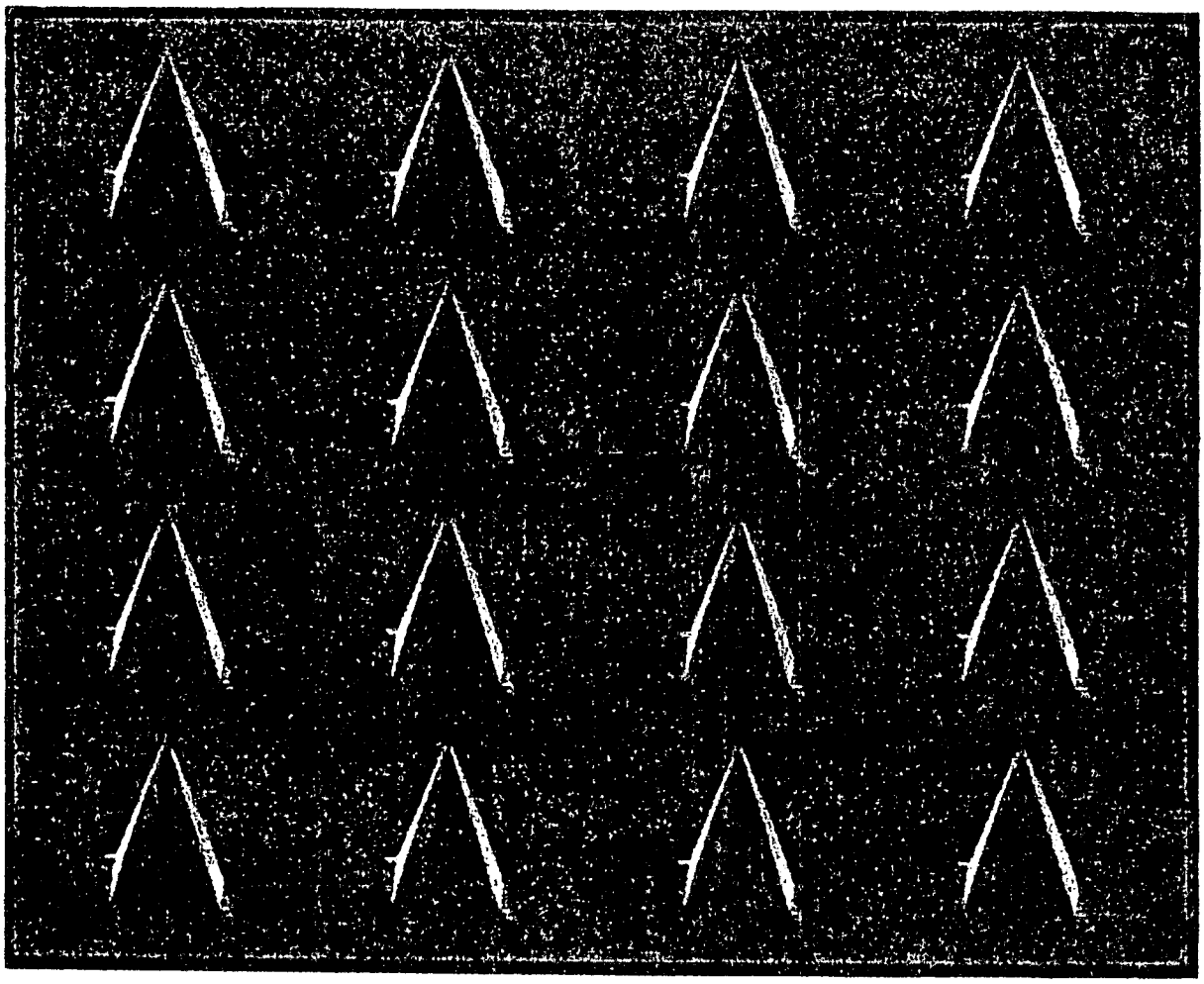


FIG 13

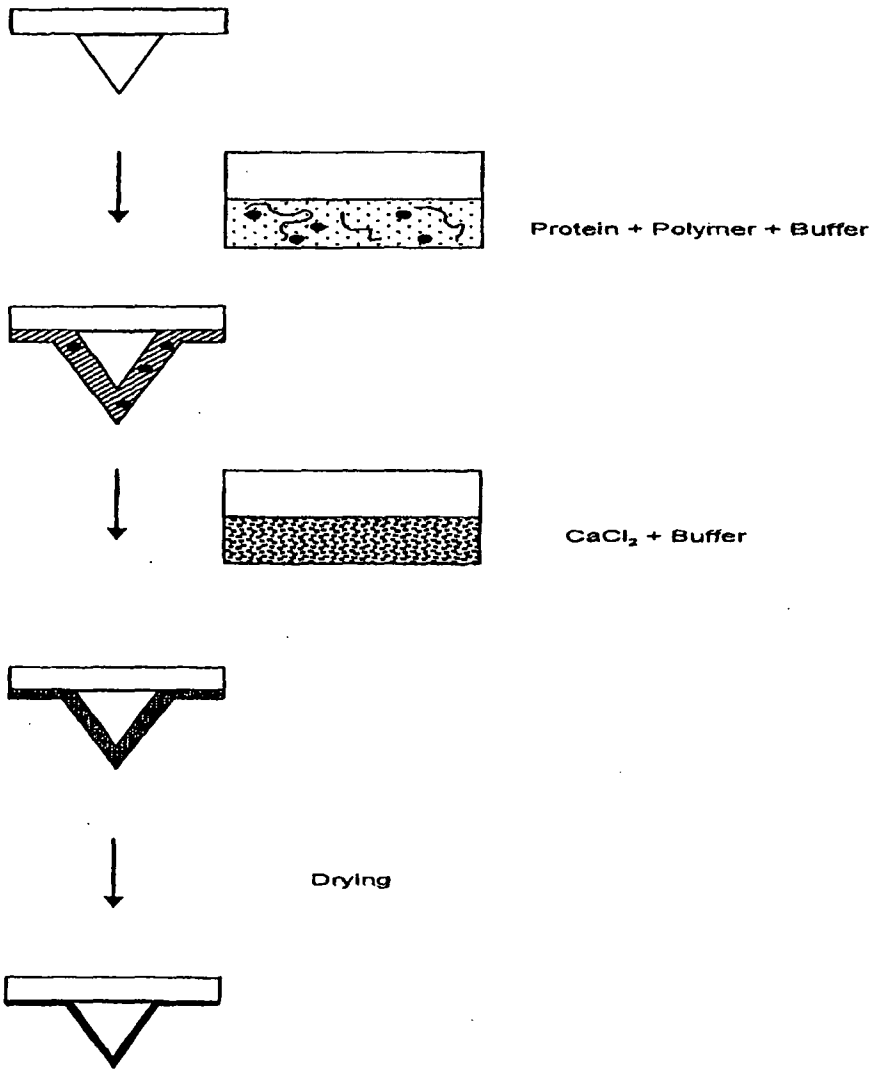


FIG 14

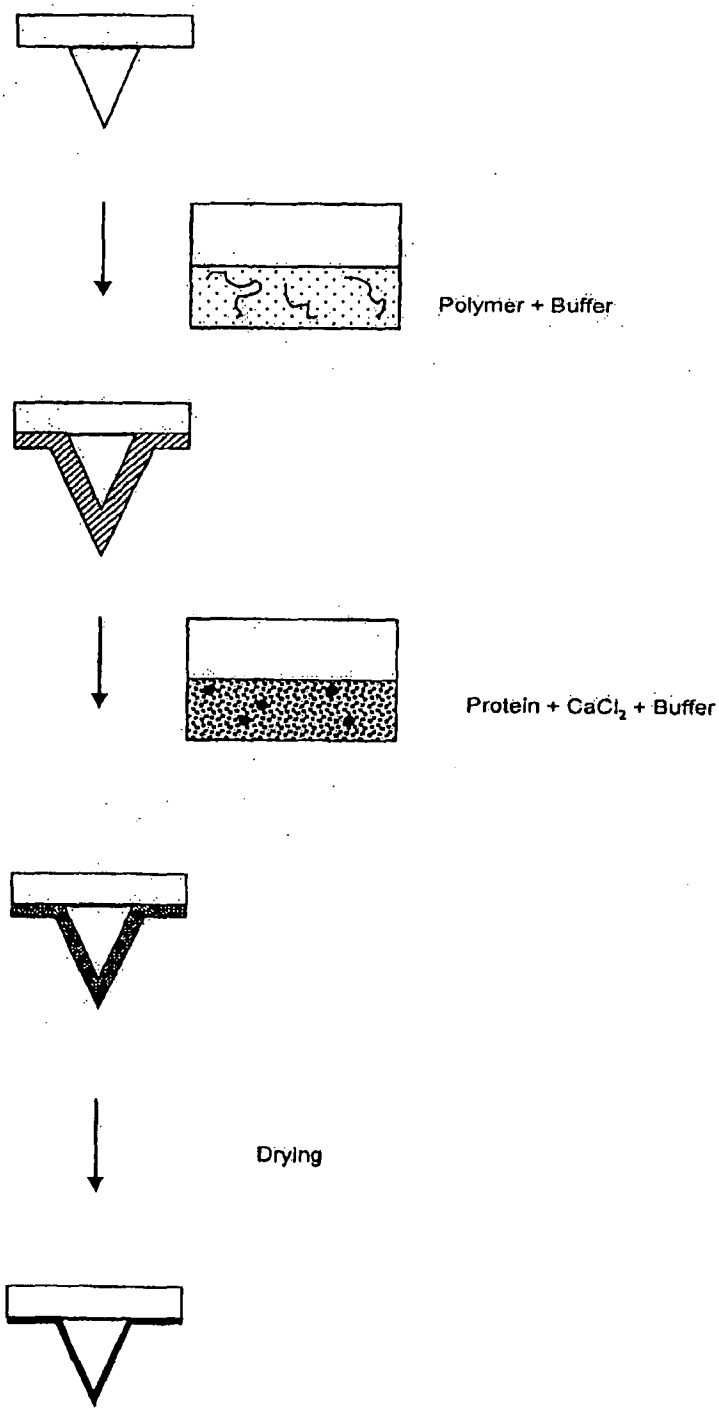


FIG 15

```
Met Gln Phe Val Asn Lys Gln Phe Asn Tyr Lys Asp Pro Val Asn Gly
 1               5                  10                  15

Val Asp Ile Ala Tyr Ile Lys Ile Pro Asn Val Gly Gln Met Gln Pro
            20                  25                  30

Val Lys Ala Phe Lys Ile His Asn Lys Ile Trp Val Ile Pro Glu Arg
        35                  40                  45

Asp Thr Phe Thr Asn Pro Glu Glu Gly Asp Leu Asn Pro Pro Pro Glu
    50                  55                  60

Ala Lys Gln Val Pro Val Ser Tyr Tyr Asp Ser Thr Tyr Leu Ser Thr
65                  70                  75                  80

Asp Asn Glu Lys Asp Asn Tyr Leu Lys Gly Val Thr Lys Leu Phe Glu
                85                  90                  95

Arg Ile Tyr Ser Thr Asp Leu Gly Arg Met Leu Leu Thr Ser Ile Val
            100                 105                 110

Arg Gly Ile Pro Phe Trp Gly Gly Ser Thr Ile Asp Thr Glu Leu Lys
        115                 120                 125

Val Ile Asp Thr Asn Cys Ile Asn Val Ile Gln Pro Asp Gly Ser Tyr
    130                 135                 140

Arg Ser Glu Glu Leu Asn Leu Val Ile Ile Gly Pro Ser Ala Asp Ile
145                 150                 155                 160

Ile Gln Phe Glu Cys Lys Ser Phe Gly His Glu Val Leu Asn Leu Thr
                165                 170                 175

Arg Asn Gly Tyr Gly Ser Thr Gln Tyr Ile Arg Phe Ser Pro Asp Phe
            180                 185                 190

Thr Phe Gly Phe Glu Glu Ser Leu Glu Val Asp Thr Asn Pro Leu Leu
        195                 200                 205

Gly Ala Gly Lys Phe Ala Thr Asp Pro Ala Val Thr Leu Ala His Glu
    210                 215                 220

Leu Ile His Ala Gly His Arg Leu Tyr Gly Ile Ala Ile Asn Pro Asn
225                 230                 235                 240

Arg Val Phe Lys Val Asn Thr Asn Ala Tyr Tyr Glu Met Ser Gly Leu
                245                 250                 255

Glu Val Ser Phe Glu Glu Leu Arg Thr Phe Gly Gly His Asp Ala Lys
            260                 265                 270

Phe Ile Asp Ser Leu Gln Glu Asn Glu Phe Arg Leu Tyr Tyr Tyr Asn
        275                 280                 285

Lys Phe Lys Asp Ile Ala Ser Thr Leu Asn Lys Ala Lys Ser Ile Val
    290                 295                 300

Gly Thr Thr Ala Ser Leu Gln Tyr Met Lys Asn Val Phe Lys Glu Lys
305                 310                 315                 320

Tyr Leu Leu Ser Glu Asp Thr Ser Gly Lys Phe Ser Val Asp Lys Leu
                325                 330                 335

Lys Phe Asp Lys Leu Tyr Lys Met Leu Thr Glu Ile Tyr Thr Glu Asp
            340                 345                 350
```

FIG 16

```
Asn Phe Val Lys Phe Phe Lys Val Leu Asn Arg Lys Thr Tyr Leu Asn
        355                 360                 365

Phe Asp Lys Ala Val Phe Lys Ile Asn Ile Val Pro Lys Val Asn Tyr
    370                 375                 380

Thr Ile Tyr Asp Gly Phe Asn Leu Arg Asn Thr Asn Leu Ala Ala Asn
385                 390                 395                 400

Phe Asn Gly Gln Asn Thr Glu Ile Asn Asn Met Asn Phe Thr Lys Leu
                405                 410                 415

Lys Asn Phe Thr Gly Leu Phe Glu Phe Tyr Lys Leu Leu Cys Val Arg
            420                 425                 430

Gly Ile Ile Thr Ser Lys Thr Lys Ser Leu Asp Lys Gly Tyr Asn Lys
        435                 440                 445

Ala Leu Asn Asp Leu Cys Ile Lys Val Asn Asn Trp Asp Leu Phe Phe
    450                 455                 460

Ser Pro Ser Glu Asp Asn Phe Thr Asn Asp Leu Asn Lys Gly Glu Glu
465                 470                 475                 480

Ile Thr Ser Asp Thr Asn Ile Glu Ala Ala Glu Glu Asn Ile Ser Leu
                485                 490                 495

Asp Leu Ile Gln Gln Tyr Tyr Leu Thr Phe Asn Phe Asp Asn Glu Pro
            500                 505                 510

Glu Asn Ile Ser Ile Glu Asn Leu Ser Ser Asp Ile Ile Gly Gln Leu
        515                 520                 525

Glu Leu Met Pro Asn Ile Glu Arg Phe Pro Asn Gly Lys Lys Tyr Glu
    530                 535                 540

Leu Asp Lys Tyr Thr Met Phe His Tyr Leu Arg Ala Gln Glu Phe Glu
545                 550                 555                 560

His Gly Lys Ser Arg Ile Ala Leu Thr Asn Ser Val Asn Glu Ala Leu
                565                 570                 575

Leu Asn Pro Ser Arg Val Tyr Thr Phe Phe Ser Ser Asp Tyr Val Lys
            580                 585                 590

Lys Val Asn Lys Ala Thr Glu Ala Ala Met Phe Leu Gly Trp Val Glu
        595                 600                 605

Gln Leu Val Tyr Asp Phe Thr Asp Glu Thr Ser Glu Val Ser Thr Thr
    610                 615                 620

Asp Lys Ile Ala Asp Ile Thr Ile Ile Ile Pro Tyr Ile Gly Pro Ala
625                 630                 635                 640

Leu Asn Ile Gly Asn Met Leu Tyr Lys Asp Asp Phe Val Gly Ala Leu
                645                 650                 655

Ile Phe Ser Gly Ala Val Ile Leu Leu Glu Phe Ile Pro Glu Ile Ala
            660                 665                 670

Ile Pro Val Leu Gly Thr Phe Ala Leu Val Ser Tyr Ile Ala Asn Lys
        675                 680                 685

Val Leu Thr Val Gln Thr Ile Asp Asn Ala Leu Ser Lys Arg Asn Glu
    690                 695                 700

Lys Trp Asp Glu Val Tyr Lys Tyr Ile Val Thr Asn Trp Leu Ala Lys
705                 710                 715                 720

Val Asn Thr Gln Ile Asp Leu Ile Arg Lys Lys Met Lys Glu Ala Leu
                725                 730                 735

Glu Asn Gln Ala Glu Ala Thr Lys Ala Ile Ile Asn Tyr Gln Tyr Asn
            740                 745                 750

Gln Tyr Thr Glu Glu Glu Lys Asn Asn Ile Asn Phe Asn Ile Asp Asp
        755                 760                 765

Leu Ser Ser Lys Leu Asn Glu Ser Ile Asn Lys Ala Met Ile Asn Ile
    770                 775                 780
```

FIG 16 CONT.

```
Asn Lys Phe Leu Asn Gln Cys Ser Val Ser Tyr Leu Met Asn Ser Met
785                 790                 795                 800

Ile Pro Tyr Gly Val Lys Arg Leu Glu Asp Phe Asp Ala Ser Leu Lys
                805                 810                 815

Asp Ala Leu Leu Lys Tyr Ile Tyr Asp Asn Arg Gly Thr Leu Ile Gly
            820                 825                 830

Gln Val Asp Arg Leu Lys Asp Lys Val Asn Asn Thr Leu Ser Thr Asp
        835                 840                 845

Ile Pro Phe Gln Leu Ser Lys Tyr Val Asp Asn Gln Arg Leu Leu Ser
    850                 855                 860

Thr Phe Thr Glu Tyr Ile Lys Asn Ile Ile Asn Thr Ser Ile Leu Asn
865                 870                 875                 880

Leu Arg Tyr Glu Ser Asn His Leu Ile Asp Leu Ser Arg Tyr Ala Ser
                885                 890                 895

Lys Ile Asn Ile Gly Ser Lys Val Asn Phe Asp Pro Ile Asp Lys Asn
            900                 905                 910

Gln Ile Gln Leu Phe Asn Leu Glu Ser Ser Lys Ile Glu Val Ile Leu
        915                 920                 925

Lys Asn Ala Ile Val Tyr Asn Ser Met Tyr Glu Asn Phe Ser Thr Ser
    930                 935                 940

Phe Trp Ile Arg Ile Pro Lys Tyr Phe Asn Ser Ile Ser Leu Asn Asn
945                 950                 955                 960

Glu Tyr Thr Ile Ile Asn Cys Met Glu Asn Asn Ser Gly Trp Lys Val
                965                 970                 975

Ser Leu Asn Tyr Gly Glu Ile Ile Trp Thr Leu Gln Asp Thr Gln Glu
            980                 985                 990

Ile Lys Gln Arg Val Val Phe Lys Tyr Ser Gln Met Ile Asn Ile Ser
        995                 1000                1005

Asp Tyr Ile Asn Arg Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu
    1010                1015                1020

Asn Asn Ser Lys Ile Tyr Ile Asn Gly Arg Leu Ile Asp Gln Lys Pro
1025                1030                1035                1040

Ile Ser Asn Leu Gly Asn Ile His Ala Ser Asn Asn Ile Met Phe Lys
                1045                1050                1055

Leu Asp Gly Cys Arg Asp Thr His Arg Tyr Ile Trp Ile Lys Tyr Phe
            1060                1065                1070

Asn Leu Phe Asp Lys Glu Leu Asn Glu Lys Glu Ile Lys Asp Leu Tyr
        1075                1080                1085

Asp Asn Gln Ser Asn Ser Gly Ile Leu Lys Asp Phe Trp Gly Asp Tyr
    1090                1095                1100

Leu Gln Tyr Asp Lys Pro Tyr Tyr Met Leu Asn Leu Tyr Asp Pro Asn
1105                1110                1115                1120

Lys Tyr Val Asp Val Asn Asn Val Gly Ile Arg Gly Tyr Met Tyr Leu
                1125                1130                1135

Lys Gly Pro Arg Gly Ser Val Met Thr Thr Asn Ile Tyr Leu Asn Ser
            1140                1145                1150

Ser Leu Tyr Arg Gly Thr Lys Phe Ile Ile Lys Lys Tyr Ala Ser Gly
        1155                1160                1165

Asn Lys Asp Asn Ile Val Arg Asn Asn Asp Arg Val Tyr Ile Asn Val
    1170                1175                1180

Val Val Lys Asn Lys Glu Tyr Arg Leu Ala Thr Asn Ala Ser Gln Ala
1185                1190                1195                1200
```

FIG 16C CONT.

```
Gly Val Glu Lys Ile Leu Ser Ala Leu Glu Ile Pro Asp Val Gly Asn
                1205                1210                1215

Leu Ser Gln Val Val Val Met Lys Ser Lys Asn Asp Gln Gly Ile Thr
            1220                1225                1230

Asn Lys Cys Lys Met Asn Leu Gln Asp Asn Asn Gly Asn Asp Ile Gly
        1235                1240                1245

Phe Ile Gly Phe His Gln Phe Asn Asn Ile Ala Lys Leu Val Ala Ser
    1250                1255                1260

Asn Trp Tyr Asn Arg Gln Ile Glu Arg Ser Ser Arg Thr Leu Gly Cys
1265                1270                1275                1280

Ser Trp Glu Phe Ile Pro Val Asp Asp Gly Trp Gly Glu Arg Pro Leu
                1285                1290                1295
```

FIG 16CONT.

```
Met Ala Arg Leu Leu Ser Thr Phe Thr Glu Tyr Ile Lys Asn Ile Ile
  1               5                  10                  15

Asn Thr Ser Ile Leu Asn Leu Arg Tyr Glu Ser Asn His Leu Ile Asp
            20                  25                  30

Leu Ser Arg Tyr Ala Ser Lys Ile Asn Ile Gly Ser Lys Val Asn Phe
        35                  40                  45

Asp Pro Ile Asp Lys Asn Gln Ile Gln Leu Phe Asn Leu Glu Ser Ser
    50                  55                  60

Lys Ile Glu Val Ile Leu Lys Asn Ala Ile Val Tyr Asn Ser Met Tyr
65                  70                  75                  80

Glu Asn Phe Ser Thr Ser Phe Trp Ile Arg Ile Pro Lys Tyr Phe Asn
                85                  90                  95

Ser Ile Ser Leu Asn Asn Glu Tyr Thr Ile Ile Asn Cys Met Glu Asn
            100                 105                 110

Asn Ser Gly Trp Lys Val Ser Leu Asn Tyr Gly Glu Ile Ile Trp Thr
        115                 120                 125

Leu Gln Asp Thr Gln Glu Ile Lys Gln Arg Val Val Phe Lys Tyr Ser
    130                 135                 140

Gln Met Ile Asn Ile Ser Asp Tyr Ile Asn Arg Trp Ile Phe Val Thr
145                 150                 155                 160

Ile Thr Asn Asn Arg Leu Asn Asn Ser Lys Ile Tyr Ile Asn Gly Arg
                165                 170                 175

Leu Ile Asp Gln Lys Pro Ile Ser Asn Leu Gly Asn Ile His Ala Ser
            180                 185                 190

Asn Asn Ile Met Phe Lys Leu Asp Gly Cys Arg Asp Thr His Arg Tyr
```

FIG-17

```
        195                 200                 205

Ile Trp Ile Lys Tyr Phe Asn Leu Phe Asp Lys Glu Leu Asn Glu Lys
    210                 215                 220

Glu Ile Lys Asp Leu Tyr Asp Asn Gln Ser Asn Ser Gly Ile Leu Lys
225                 230                 235                 240

Asp Phe Trp Gly Asp Tyr Leu Gln Tyr Asp Lys Pro Tyr Tyr Met Leu
                245                 250                 255

Asn Leu Tyr Asp Pro Asn Lys Tyr Val Asp Val Asn Asn Val Gly Ile
            260                 265                 270

Arg Gly Tyr Met Tyr Leu Lys Gly Pro Arg Gly Ser Val Met Thr Thr
        275                 280                 285

Asn Ile Tyr Leu Asn Ser Ser Leu Tyr Arg Gly Thr Lys Phe Ile Ile
    290                 295                 300

Lys Lys Tyr Ala Ser Gly Asn Lys Asp Asn Ile Val Arg Asn Asn Asp
305                 310                 315                 320

Arg Val Tyr Ile Asn Val Val Val Lys Asn Lys Glu Tyr Arg Leu Ala
                325                 330                 335

Thr Asn Ala Ser Gln Ala Gly Val Glu Lys Ile Leu Ser Ala Leu Glu
            340                 345                 350

Ile Pro Asp Val Gly Asn Leu Ser Gln Val Val Val Met Lys Ser Lys
        355                 360                 365

Asn Asp Gln Gly Ile Thr Asn Lys Cys Lys Met Asn Leu Gln Asp Asn
    370                 375                 380

Asn Gly Asn Asp Ile Gly Phe Ile Gly Phe His Gln Phe Asn Asn Ile
385                 390                 395                 400

Ala Lys Leu Val Ala Ser Asn Trp Tyr Asn Arg Gln Ile Glu Arg Ser
                405                 410                 415

Ser Arg Thr Leu Gly Cys Ser Trp Glu Phe Ile Pro Val Asp Asp Gly
            420                 425                 430

Trp Gly Glu Arg Pro Leu
        435
```

FIG 17C CONT.

```
Glu Val Lys Gln Glu Asn Arg Leu Leu Asn Glu Ser Glu Ser Ser Ser
 1               5                  10                  15
 Gln Gly Leu Leu Gly Tyr Tyr Phe Ser Asp Leu Asn Phe Gln Ala Pro
            20                  25                  30
 Met Val Val Thr Ser Ser Thr Thr Gly Asp Leu Ser Ile Pro Ser Ser
        35                  40                  45
 Glu Leu Glu Asn Ile Pro Ser Glu Asn Gln Tyr Phe Gln Ser Ala Ile
    50                  55                  60
 Trp Ser Gly Phe Ile Lys Val Lys Lys Ser Asp Glu Tyr Thr Phe Ala
65                  70                  75                  80
 Thr Ser Ala Asp Asn His Val Thr Met Trp Val Asp Asp Gln Glu Val
                85                  90                  95
 Ile Asn Lys Ala Ser Asn Ser Asn Lys Ile Arg Leu Glu Lys Gly Arg
            100                 105                 110
 Leu Tyr Gln Ile Lys Ile Gln Tyr Gln Arg Glu Asn Pro Thr Glu Lys
        115                 120                 125
 Gly Leu Asp Phe Lys Leu Tyr Trp Thr Asp Ser Gln Asn Lys Lys Glu
    130                 135                 140
 Val Ile Ser Ser Asp Asn Leu Gln Leu Pro Glu Leu Lys Gln Lys Ser
145                 150                 155                 160
 Ser Asn Ser Arg Lys Lys Arg Ser Thr Ser Ala Gly Pro Thr Val Pro
                165                 170                 175
 Asp Arg Asp Asn Asp Gly Ile Pro Asp Ser Leu Glu Val Glu Gly Tyr
            180                 185                 190
 Thr Val Asp Val Lys Asn Lys Arg Thr Phe Leu Ser Pro Trp Ile Ser
        195                 200                 205
 Asn Ile His Glu Lys Lys Gly Leu Thr Lys Tyr Lys Ser Ser Pro Glu
    210                 215                 220
 Lys Trp Ser Thr Ala Ser Asp Pro Tyr Ser Asp Phe Glu Lys Val Thr
225                 230                 235                 240
 Gly Arg Ile Asp Lys Asn Val Ser Pro Glu Ala Arg His Pro Leu Val
                245                 250                 255
 Ala Ala Tyr Pro Ile Val His Val Asp Met Glu Asn Ile Ile Leu Ser
            260                 265                 270
 Lys Asn Glu Asp Gln Ser Thr Gln Asn Thr Asp Ser Glu Thr Arg Thr
        275                 280                 285
 Ile Ser Lys Asn Thr Ser Thr Ser Arg Thr His Thr Ser Glu Val His
    290                 295                 300
 Gly Asn Ala Glu Val His Ala Ser Phe Phe Asp Ile Gly Gly Ser Val
305                 310                 315                 320
 Ser Ala Gly Phe Ser Asn Ser Asn Ser Ser Thr Val Ala Ile Asp His
                325                 330                 335
 Ser Leu Ser Leu Ala Gly Glu Arg Thr Trp Ala Glu Thr Met Gly Leu
            340                 345                 350
 Asn Thr Ala Asp Thr Ala Arg Leu Asn Ala Asn Ile Arg Tyr Val Asn
        355                 360                 365
 Thr Gly Thr Ala Pro Ile Tyr Asn Val Leu Pro Thr Thr Ser Leu Val
    370                 375                 380
 Leu Gly Lys Asn Gln Thr Leu Ala Thr Ile Lys Ala Lys Glu Asn Gln
385                 390                 395                 400
 Leu Ser Gln Ile Leu Ala Pro Asn Asn Tyr Tyr Pro Ser Lys Asn Leu
                405                 410                 415
 Ala Pro Ile Ala Leu Asn Ala Gln Asp Asp Phe Ser Ser Thr Pro Ile
            420                 425                 430
 Thr Met Asn Tyr Asn Gln Phe Leu Glu Leu Glu Lys Thr Lys Gln Leu
        435                 440                 445
 Arg Leu Asp Thr Asp Gln Val Tyr Gly Asn Ile Ala Thr Tyr Asn Phe
    450                 455                 460
 Glu Asn Gly Arg Val Arg Val Asp Thr Gly Ser Asn Trp Ser Glu Val
465                 470                 475                 480
```

FIG 18

```
Leu Pro Gln Ile Gln Glu Thr Thr Ala Arg Ile Ile Phe Asn Gly Lys
                485                 490                 495
Asp Leu Asn Leu Val Glu Arg Arg Ile Ala Ala Val Asn Pro Ser Asp
            500                 505                 510
Pro Leu Glu Thr Thr Lys Pro Asp Met Thr Leu Lys Glu Ala Leu Lys
        515                 520                 525
Ile Ala Phe Gly Phe Asn Glu Pro Asn Gly Asn Leu Gln Tyr Gln Gly
    530                 535                 540
Lys Asp Ile Thr Glu Phe Asp Phe Asn Phe Asp Gln Gln Thr Ser Gln
545                 550                 555                 560
Asn Ile Lys Asn Gln Leu Ala Glu Leu Asn Ala Thr Asn Ile Tyr Thr
                565                 570                 575
Val Leu Asp Lys Ile Lys Leu Asn Ala Lys Met Asn Ile Leu Ile Arg
            580                 585                 590
Asp Lys Arg Phe His Tyr Asp Arg Asn Asn Ile Ala Val Gly Ala Asp
        595                 600                 605
Glu Ser Val Val Lys Glu Ala His Arg Glu Val Ile Asn Ser Ser Thr
    610                 615                 620
Glu Gly Leu Leu Leu Asn Ile Asp Lys Asp Ile Arg Lys Ile Leu Ser
625                 630                 635                 640
Gly Tyr Ile Val Glu Ile Glu Asp Thr Glu Gly Leu Lys Glu Val Ile
                645                 650                 655
Asn Asp Arg Tyr Asp Met Leu Asn Ile Ser Ser Leu Arg Gln Asp Gly
            660                 665                 670
Lys Thr Phe Ile Asp Phe Lys Lys Tyr Asn Asp Lys Leu Pro Leu Tyr
        675                 680                 685
Ile Ser Asn Pro Asn Tyr Lys Val Asn Val Tyr Ala Val Thr Lys Glu
    690                 695                 700
Asn Thr Ile Ile Asn Pro Ser Glu Asn Gly Asp Thr Ser Thr Asn Gly
705                 710                 715                 720
Ile Lys Lys Ile Leu Ile Phe Ser Lys Lys Gly Tyr Glu Ile Gly Glx
                725                 730                 735
```

FIG 18 CONT.

```
gaagttaaac aggagaaccg gttattaaat gaatcagaat caagttccca ggggttacta 60
ggatactatt ttagtgattt gaattttcaa gcacccatgg tggttacctc ttctactaca 120
ggggatttat ctattcctag ttctgagtta gaaaatattc catcggaaaa ccaatatttt 180
caatctgcta tttggtcagg atttatcaaa gttaagaaga gtgatgaata tacatttgct 240
acttccgctg ataatcatgt aacaatgtgg gtagatgacc aagaagtgat taataaagct 300
tctaattcta acaaaatcag attagaaaaa ggaagattat atcaaataaa aattcaatat 360
caacgagaaa atcctactga aaaaggattg gatttcaagt tgtactggac cgattctcaa 420
aataaaaaag aagtgatttc tagtgataac ttacaattgc cagaattaaa acaaaaatct 480
tcgaactcaa gaaaaaagcg aagtacaagt gctggaccta cggttccaga ccgtgacaat 540
gatggaatcc ctgattcatt agaggtagaa ggatatacgg ttgatgtcaa aaataaaaga 600
acttttcttt caccatggat ttctaatatt catgaaaaga aaggattaac caaatataaa 660
tcatctcctg aaaaatggag cacggcttct gatccgtaca gtgatttcga aaaggttaca 720
ggacggattg ataagaatgt atcaccagag gcaagacacc cccttgtggc agcttatccg 780
attgtacatg tagatatgga gaatattatt ctctcaaaaa atgaggatca atccacacag 840
aatactgata gtgaaacgag aacaataagt aaaaatactt ctacaagtag gacacatact 900
agtgaagtac atggaaatgc agaagtgcat gcgtcgttct ttgatattgg tgggagtgta 960
tctgcaggat ttagtaattc gaattcaagt acggtcgcaa ttgatcattc actatctcta 1020
gcaggggaaa gaacttgggc tgaaacaatg ggtttaaata ccgctgatac agcaagatta 1080
aatgccaata ttagatatgt aaatactggg acggctccaa tctacaacgt gttaccaacg 1140
acttcgttag tgttaggaaa aaatcaaaca ctcgcgacaa ttaaagctaa ggaaaaccaa 1200
ttaagtcaaa tacttgcacc taataattat tatccttcta aaaacttggc gccaatcgca 1260
ttaaatgcac aagacgattt cagttctact ccaattacaa tgaattacaa tcaatttctt 1320
gagttagaaa aaacgaaaca attaagatta gatacggatc aagtatatgg gaatatagca 1380
acatacaatt ttgaaaatgg aagagtgagg gtggatacag gctcgaactg gagtgaagtg 1440
ttaccgcaaa ttcaagaaac aactgcacgt atcattttta atggaaaaga tttaaatctg 1500
gtagaaaggc ggatagcggc ggttaatcct agtgatccat tagaaacgac taaaccggat 1560
atgacattaa aagaagccct taaaatagca tttggattta acgaaccgaa tggaaactta 1620
caatatcaag ggaaagacat aaccgaattt gattttaatt tcgatcaaca aacatctcaa 1680
aatatcaaga atcagttagc ggaattaaac gcaactaaca tatatactgt attagataaa 1740
atcaaattaa atgcaaaaat gaatatttta ataagagata aacgttttca ttatgataga 1800
aataacatag cagttggggc ggatgagtca gtagttaagg aggctcatag agaagtaatt 1860
aattcgtcaa cagagggatt attgttaaat attgataagg atataagaaa aatattatca 1920
ggttatattg tagaaattga agatactgaa gggcttaaag aagttataaa tgacagatat 1980
gatatgttga atatttctag tttacggcaa gatggaaaaa catttataga ttttaaaaaa 2040
tataatgata aattaccgtt atatataagt aatcccaatt ataaggtaaa tgtatatgct 2100
gttactaaag aaaacactat tattaatcct agtgagaatg gggatactag taccaacggg 2160
atcaagaaaa ttttaatctt ttctaaaaaa ggctatgaga taggataa 2208
```

FIG 19

pTH (1-34) (bovine)
H-Ala- Val- Ser- Glu- Ile- Gln- Phe- Met- His- Asn- Leu - Gly- Lys -
His- Leu- Ser- Ser- Met- Glu- Arg- Val- Glu- Trp- Leu - Arg- Lys-
Lys- Leu- Gln-Asp-Val- His-Asn- Phe - OH

pTH (1-34) (human)
H- Ser- Val- Ser- Glu- Ile- Gln- Leu- Met- His- Asn- Leu- Gly- Lys -
His- Leu- Asn- Ser- Met- Glu- Arg- Val- Glu- Trp- Leu- Arg- Lys-
Lys- Leu- Gln- Asp- Val- His-Asn- Phe- OH

FIG 20

GLP-1 HADGTFTSDVSSYLEGQAAKEFIAWLVKGR-NH_2

Exendin-4 HGEGTFTSDLSKQMEEAVRLFIEWLKNGGPSSGAPPPS-NH_2

Exendin-4(3-39) EGTFTSDLSKQMEEAVRLFIEWLKNGGPSSGAPPPS-NH_2

Exendin-4(9-39) DLSKQMEEAVRLFIEWLKNGGPSSGAPPPS-NH_2

FIG 21

```
ATG CAA TTT GTT AAT AAA CAA TTT AAT TAT AAA GAT CCT GTA AAT GGT       48
Met Gln Phe Val Asn Lys Gln Phe Asn Tyr Lys Asp Pro Val Asn Gly
  1               5                  10                  15

GTT GAT ATT GCT TAT ATA AAA ATT CCA AAT GTA GGA CAA ATG CAA CCA       96
Val Asp Ile Ala Tyr Ile Lys Ile Pro Asn Val Gly Gln Met Gln Pro
            20                  25                  30

GTA AAA GCT TTT AAA ATT CAT AAT AAA ATA TGG GTT ATT CCA GAA AGA      144
Val Lys Ala Phe Lys Ile His Asn Lys Ile Trp Val Ile Pro Glu Arg
        35                  40                  45

GAT ACA TTT ACA AAT CCT GAA GAA GGA GAT TTA AAT CCA CCA CCA GAA      192
Asp Thr Phe Thr Asn Pro Glu Glu Gly Asp Leu Asn Pro Pro Pro Glu
    50                  55                  60

GCA AAA CAA GTT CCA GTT TCA TAT TAT GAT TCA ACA TAT TTA AGT ACA      240
Ala Lys Gln Val Pro Val Ser Tyr Tyr Asp Ser Thr Tyr Leu Ser Thr
65                  70                  75                  80

GAT AAT GAA AAA GAT AAT TAT TTA AAG GGA GTT ACA AAA TTA TTT GAG      288
Asp Asn Glu Lys Asp Asn Tyr Leu Lys Gly Val Thr Lys Leu Phe Glu
                85                  90                  95

AGA ATT TAT TCA ACT GAT CTT GGA AGA ATG TTG TTA ACA TCA ATA GTA      336
Arg Ile Tyr Ser Thr Asp Leu Gly Arg Met Leu Leu Thr Ser Ile Val
            100                 105                 110

AGG GGA ATA CCA TTT TGG GGT GGA AGT ACA ATA GAT ACA GAA TTA AAA      384
Arg Gly Ile Pro Phe Trp Gly Gly Ser Thr Ile Asp Thr Glu Leu Lys
        115                 120                 125

GTT ATT GAT ACT AAT TGT ATT AAT GTG ATA CAA CCA GAT GGT AGT TAT      432
Val Ile Asp Thr Asn Cys Ile Asn Val Ile Gln Pro Asp Gly Ser Tyr
    130                 135                 140

AGA TCA GAA GAA CTT AAT CTA GTA ATA ATA GGA CCC TCA GCT GAT ATT      480
Arg Ser Glu Glu Leu Asn Leu Val Ile Ile Gly Pro Ser Ala Asp Ile
145                 150                 155                 160

ATA CAG TTT GAA TGT AAA AGC TTT GGA CAT GAA GTT TTG AAT CTT ACG      528
Ile Gln Phe Glu Cys Lys Ser Phe Gly His Glu Val Leu Asn Leu Thr
                165                 170                 175

CGA AAT GGT TAT GGC TCT ACT CAA TAC ATT AGA TTT AGC CCA GAT TTT      576
Arg Asn Gly Tyr Gly Ser Thr Gln Tyr Ile Arg Phe Ser Pro Asp Phe
            180                 185                 190

ACA TTT GGT TTT GAG GAG TCA CTT GAA GTT GAT ACA AAT CCT CTT TTA      624
Thr Phe Gly Phe Glu Glu Ser Leu Glu Val Asp Thr Asn Pro Leu Leu
        195                 200                 205

GGT GCA GGC AAA TTT GCT ACA GAT CCA GCA GTA ACA TTA GCA CAT GAA      672
Gly Ala Gly Lys Phe Ala Thr Asp Pro Ala Val Thr Leu Ala His Glu
    210                 215                 220

CTT ATA CAT GCT GGA CAT AGA TTA TAT GGA ATA GCA ATT AAT CCA AAT      720
Leu Ile His Ala Gly His Arg Leu Tyr Gly Ile Ala Ile Asn Pro Asn
225                 230                 235                 240

AGG GTT TTT AAA GTA AAT ACT AAT GCC TAT TAT GAA ATG AGT GGG TTA      768
Arg Val Phe Lys Val Asn Thr Asn Ala Tyr Tyr Glu Met Ser Gly Leu
                245                 250                 255

GAA GTA AGC TTT GAG GAA CTT AGA ACA TTT GGG GGA CAT GAT GCA AAG      816
Glu Val Ser Phe Glu Glu Leu Arg Thr Phe Gly Gly His Asp Ala Lys
            260                 265                 270

TTT ATA GAT AGT TTA CAG GAA AAC GAA TTT CGT CTA TAT TAT TAT AAT      864
Phe Ile Asp Ser Leu Gln Glu Asn Glu Phe Arg Leu Tyr Tyr Tyr Asn
        275                 280                 285

AAG TTT AAA GAT ATA GCA AGT ACA CTT AAT AAA GCT AAA TCA ATA GTA      912
Lys Phe Lys Asp Ile Ala Ser Thr Leu Asn Lys Ala Lys Ser Ile Val
    290                 295                 300

GGT ACT ACT GCT TCA TTA CAG TAT ATG AAA AAT GTT TTT AAA GAG AAA      960
Gly Thr Thr Ala Ser Leu Gln Tyr Met Lys Asn Val Phe Lys Glu Lys
305                 310                 315                 320

TAT CTC CTA TCT GAA GAT ACA TCT GGA AAA TTT TCG GTA GAT AAA TTA     1008
Tyr Leu Leu Ser Glu Asp Thr Ser Gly Lys Phe Ser Val Asp Lys Leu
                325                 330                 335
```

FIG 22

```
AAA TTT GAT AAG TTA TAC AAA ATG TTA ACA GAG ATT TAC ACA GAG GAT     1056
Lys Phe Asp Lys Leu Tyr Lys Met Leu Thr Glu Ile Tyr Thr Glu Asp
            340                 345                 350

AAT TTT GTT AAG TTT TTT AAA GTA CTT AAC AGA AAA ACA TAT TTG AAT     1104
Asn Phe Val Lys Phe Phe Lys Val Leu Asn Arg Lys Thr Tyr Leu Asn
        355                 360                 365

TTT GAT AAA GCC GTA TTT AAG ATA AAT ATA GTA CCT AAG GTA AAT TAC     1152
Phe Asp Lys Ala Val Phe Lys Ile Asn Ile Val Pro Lys Val Asn Tyr
    370                 375                 380

ACA ATA TAT GAT GGA TTT AAT TTA AGA AAT ACA AAT TTA GCA GCA AAC     1200
Thr Ile Tyr Asp Gly Phe Asn Leu Arg Asn Thr Asn Leu Ala Ala Asn
385                 390                 395                 400

TTT AAT GGT CAA AAT ACA GAA ATT AAT AAT ATG AAT TTT ACT AAA CTA     1248
Phe Asn Gly Gln Asn Thr Glu Ile Asn Asn Met Asn Phe Thr Lys Leu
                405                 410                 415

AAA AAT TTT ACT GGA TTG TTT GAA TTT TAT AAG TTG CTA TGT GTA AGA     1296
Lys Asn Phe Thr Gly Leu Phe Glu Phe Tyr Lys Leu Leu Cys Val Arg
            420                 425                 430

GGG ATA ATA ACT TCT AAA ACT AAA TCA TTA GAT AAA GGA TAC AAT AAG     1344
Gly Ile Ile Thr Ser Lys Thr Lys Ser Leu Asp Lys Gly Tyr Asn Lys
        435                 440                 445

GCA TTA AAT GAT TTA TGT ATC AAA GTT AAT AAT TGG GAC TTG TTT TTT     1392
Ala Leu Asn Asp Leu Cys Ile Lys Val Asn Asn Trp Asp Leu Phe Phe
    450                 455                 460

AGT CCT TCA GAA GAT AAT TTT ACT AAT GAT CTA AAT AAA GGA GAA GAA     1440
Ser Pro Ser Glu Asp Asn Phe Thr Asn Asp Leu Asn Lys Gly Glu Glu
465                 470                 475                 480

ATT ACA TCT GAT ACT AAT ATA GAA GCA GCA GAA GAA AAT ATT AGT TTA     1488
Ile Thr Ser Asp Thr Asn Ile Glu Ala Ala Glu Glu Asn Ile Ser Leu
                485                 490                 495

GAT TTA ATA CAA CAA TAT TAT TTA ACC TTT AAT TTT GAT AAT GAA CCT     1536
Asp Leu Ile Gln Gln Tyr Tyr Leu Thr Phe Asn Phe Asp Asn Glu Pro
            500                 505                 510

GAA AAT ATT TCA ATA GAA AAT CTT TCA AGT GAC ATT ATA GGC CAA TTA     1584
Glu Asn Ile Ser Ile Glu Asn Leu Ser Ser Asp Ile Ile Gly Gln Leu
        515                 520                 525

GAA CTT ATG CCT AAT ATA GAA AGA TTT CCT AAT GGA AAA AAG TAT GAG     1632
Glu Leu Met Pro Asn Ile Glu Arg Phe Pro Asn Gly Lys Lys Tyr Glu
    530                 535                 540

TTA GAT AAA TAT ACT ATG TTC CAT TAT CTT CGT GCT CAA GAA TTT GAA     1680
Leu Asp Lys Tyr Thr Met Phe His Tyr Leu Arg Ala Gln Glu Phe Glu
545                 550                 555                 560

CAT GGT AAA TCT AGG ATT GCT TTA ACA AAT TCT GTT AAC GAA GCA TTA     1728
His Gly Lys Ser Arg Ile Ala Leu Thr Asn Ser Val Asn Glu Ala Leu
                565                 570                 575

TTA AAT CCT AGT CGT GTT TAT ACA TTT TTT TCT TCA GAC TAT GTA AAG     1776
Leu Asn Pro Ser Arg Val Tyr Thr Phe Phe Ser Ser Asp Tyr Val Lys
            580                 585                 590

AAA GTT AAT AAA GCT ACG GAG GCA GCT ATG TTT TTA GGC TGG GTA GAA     1824
Lys Val Asn Lys Ala Thr Glu Ala Ala Met Phe Leu Gly Trp Val Glu
        595                 600                 605

CAA TTA GTA TAT GAT TTT ACC GAT GAA ACT AGC GAA GTA AGT ACT ACG     1872
Gln Leu Val Tyr Asp Phe Thr Asp Glu Thr Ser Glu Val Ser Thr Thr
    610                 615                 620

GAT AAA ATT GCG GAT ATA ACT ATA ATT ATT CCA TAT ATA GGA CCT GCT     1920
Asp Lys Ile Ala Asp Ile Thr Ile Ile Ile Pro Tyr Ile Gly Pro Ala
625                 630                 635                 640

TTA AAT ATA GGT AAT ATG TTA TAT AAA GAT GAT TTT GTA GGT GCT TTA     1968
Leu Asn Ile Gly Asn Met Leu Tyr Lys Asp Asp Phe Val Gly Ala Leu
                645                 650                 655
```

FIG 22 CONT.

```
ATA TTT TCA GGA GCT GTT ATT CTG TTA GAA TTT ATA CCA GAG ATT GCA      2016
Ile Phe Ser Gly Ala Val Ile Leu Leu Glu Phe Ile Pro Glu Ile Ala
            660                 665                 670

ATA CCT GTA TTA GGT ACT TTT GCA CTT GTA TCA TAT ATT GCG AAT AAG      2064
Ile Pro Val Leu Gly Thr Phe Ala Leu Val Ser Tyr Ile Ala Asn Lys
        675                 680                 685

GTT CTA ACC GTT CAA ACA ATA GAT AAT GCT TTA AGT AAA AGA AAT GAA      2112
Val Leu Thr Val Gln Thr Ile Asp Asn Ala Leu Ser Lys Arg Asn Glu
    690                 695                 700

AAA TGG GAT GAG GTC TAT AAA TAT ATA GTA ACA AAT TGG TTA GCA AAG      2160
Lys Trp Asp Glu Val Tyr Lys Tyr Ile Val Thr Asn Trp Leu Ala Lys
705                 710                 715                 720

GTT AAT ACA CAG ATT GAT CTA ATA AGA AAA AAA ATG AAA GAA GCT TTA      2208
Val Asn Thr Gln Ile Asp Leu Ile Arg Lys Lys Met Lys Glu Ala Leu
                725                 730                 735

GAA AAT CAA GCA GAA GCA ACA AAG GCT ATA ATA AAC TAT CAG TAT AAT      2256
Glu Asn Gln Ala Glu Ala Thr Lys Ala Ile Ile Asn Tyr Gln Tyr Asn
            740                 745                 750

CAA TAT ACT GAG GAA GAG AAA AAT AAT ATT AAT TTT AAT ATT GAT GAT      2304
Gln Tyr Thr Glu Glu Glu Lys Asn Asn Ile Asn Phe Asn Ile Asp Asp
        755                 760                 765

TTA AGT TCG AAA CTT AAT GAG TCT ATA AAT AAA GCT ATG ATT AAT ATA      2352
Leu Ser Ser Lys Leu Asn Glu Ser Ile Asn Lys Ala Met Ile Asn Ile
    770                 775                 780

AAT AAA TTT TTG AAT CAA TGC TCT GTT TCA TAT TTA ATG AAT TCT ATG      2400
Asn Lys Phe Leu Asn Gln Cys Ser Val Ser Tyr Leu Met Asn Ser Met
785                 790                 795                 800

ATC CCT TAT GGT GTT AAA CGG TTA GAA GAT TTT GAT GCT AGT CTT AAA      2448
Ile Pro Tyr Gly Val Lys Arg Leu Glu Asp Phe Asp Ala Ser Leu Lys
                805                 810                 815

GAT GCA TTA TTA AAG TAT ATA TAT GAT AAT AGA GGA ACT TTA ATT GGT      2496
Asp Ala Leu Leu Lys Tyr Ile Tyr Asp Asn Arg Gly Thr Leu Ile Gly
            820                 825                 830

CAA GTA GAT AGA TTA AAA GAT AAA GTT AAT AAT ACA CTT AGT ACA GAT      2544
Gln Val Asp Arg Leu Lys Asp Lys Val Asn Asn Thr Leu Ser Thr Asp
        835                 840                 845

ATA CCT TTT CAG CTT TCC AAA TAC GTA GAT AAT CAA AGA TTA TTA TCT      2592
Ile Pro Phe Gln Leu Ser Lys Tyr Val Asp Asn Gln Arg Leu Leu Ser
    850                 855                 860

ACA TTT ACT GAA TAT ATT AAG AAT ATT ATT AAT ACT TCT ATA TTG AAT      2640
Thr Phe Thr Glu Tyr Ile Lys Asn Ile Ile Asn Thr Ser Ile Leu Asn
865                 870                 875                 880

TTA AGA TAT GAA AGT AAT CAT TTA ATA GAC TTA TCT AGG TAT GCA TCA      2688
Leu Arg Tyr Glu Ser Asn His Leu Ile Asp Leu Ser Arg Tyr Ala Ser
                885                 890                 895

AAA ATA AAT ATT GGT AGT AAA GTA AAT TTT GAT CCA ATA GAT AAA AAT      2736
Lys Ile Asn Ile Gly Ser Lys Val Asn Phe Asp Pro Ile Asp Lys Asn
            900                 905                 910

CAA ATT CAA TTA TTT AAT TTA GAA AGT AGT AAA ATT GAG GTA ATT TTA      2784
Gln Ile Gln Leu Phe Asn Leu Glu Ser Ser Lys Ile Glu Val Ile Leu
        915                 920                 925

AAA AAT GCT ATT GTA TAT AAT AGT ATG TAT GAA AAT TTT AGT ACT AGC      2832
Lys Asn Ala Ile Val Tyr Asn Ser Met Tyr Glu Asn Phe Ser Thr Ser
    930                 935                 940

TTT TGG ATA AGA ATT CCT AAG TAT TTT AAC AGT ATA AGT CTA AAT AAT      2880
Phe Trp Ile Arg Ile Pro Lys Tyr Phe Asn Ser Ile Ser Leu Asn Asn
945                 950                 955                 960

GAA TAT ACA ATA ATA AAT TGT ATG GAA AAT AAT TCA GGA TGG AAA GTA      2928
Glu Tyr Thr Ile Ile Asn Cys Met Glu Asn Asn Ser Gly Trp Lys Val
                965                 970                 975
```

FIG 22 CONT.

```
TCA CTT AAT TAT GGT GAA ATA ATC TGG ACT TTA CAG GAT ACT CAG GAA     2976
Ser Leu Asn Tyr Gly Glu Ile Ile Trp Thr Leu Gln Asp Thr Gln Glu
            980                 985                 990

ATA AAA CAA AGA GTA GTT TTT AAA TAC AGT CAA ATG ATT AAT ATA TCA     3024
Ile Lys Gln Arg Val Val Phe Lys Tyr Ser Gln Met Ile Asn Ile Ser
        995                 1000                1005

GAT TAT ATA AAC AGA TGG ATT TTT GTA ACT ATC ACT AAT AAT AGA TTA     3072
Asp Tyr Ile Asn Arg Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu
    1010                1015                1020

AAT AAC TCT AAA ATT TAT ATA AAT GGA AGA TTA ATA GAT CAA AAA CCA     3120
Asn Asn Ser Lys Ile Tyr Ile Asn Gly Arg Leu Ile Asp Gln Lys Pro
1025                1030                1035                1040

ATT TCA AAT TTA GGT AAT ATT CAT GCT AGT AAT AAT ATA ATG TTT AAA     3168
Ile Ser Asn Leu Gly Asn Ile His Ala Ser Asn Asn Ile Met Phe Lys
                1045                1050                1055

TTA GAT GGT TGT AGA GAT ACA CAT AGA TAT ATT TGG ATA AAA TAT TTT     3216
Leu Asp Gly Cys Arg Asp Thr His Arg Tyr Ile Trp Ile Lys Tyr Phe
            1060                1065                1070

AAT CTT TTT GAT AAG GAA TTA AAT GAA AAA GAA ATC AAA GAT TTA TAT     3264
Asn Leu Phe Asp Lys Glu Leu Asn Glu Lys Glu Ile Lys Asp Leu Tyr
        1075                1080                1085

GAT AAT CAA TCA AAT TCA GGT ATT TTA AAA GAC TTT TGG GGT GAT TAT     3312
Asp Asn Gln Ser Asn Ser Gly Ile Leu Lys Asp Phe Trp Gly Asp Tyr
    1090                1095                1100

TTA CAA TAT GAT AAA CCA TAC TAT ATG TTA AAT TTA TAT GAT CCA AAT     3360
Leu Gln Tyr Asp Lys Pro Tyr Tyr Met Leu Asn Leu Tyr Asp Pro Asn
1105                1110                1115                1120

AAA TAT GTC GAT GTA AAT AAT GTA GGT ATT AGA GGT TAT ATG TAT CTT     3408
Lys Tyr Val Asp Val Asn Asn Val Gly Ile Arg Gly Tyr Met Tyr Leu
                1125                1130                1135

AAA GGG CCT AGA GGT AGC GTA ATG ACT ACA AAC ATT TAT TTA AAT TCA     3456
Lys Gly Pro Arg Gly Ser Val Met Thr Thr Asn Ile Tyr Leu Asn Ser
            1140                1145                1150

AGT TTG TAT AGG GGG ACA AAA TTT ATT ATA AAA AAA TAT GCT TCT GGA     3504
Ser Leu Tyr Arg Gly Thr Lys Phe Ile Ile Lys Lys Tyr Ala Ser Gly
        1155                1160                1165

AAT AAA GAT AAT ATT GTT AGA AAT AAT GAT CGT GTA TAT ATT AAT GTA     3552
Asn Lys Asp Asn Ile Val Arg Asn Asn Asp Arg Val Tyr Ile Asn Val
    1170                1175                1180

GTA GTT AAA AAT AAA GAA TAT AGG TTA GCT ACT AAT GCA TCA CAG GCA     3600
Val Val Lys Asn Lys Glu Tyr Arg Leu Ala Thr Asn Ala Ser Gln Ala
1185                1190                1195                1200

GGC GTA GAA AAA ATA CTA AGT GCA TTA GAA ATA CCT GAT GTA GGA AAT     3648
Gly Val Glu Lys Ile Leu Ser Ala Leu Glu Ile Pro Asp Val Gly Asn
                1205                1210                1215

CTA AGT CAA GTA GTA GTA ATG AAG TCA AAA AAT GAT CAA GGA ATA ACA     3696
Leu Ser Gln Val Val Val Met Lys Ser Lys Asn Asp Gln Gly Ile Thr
            1220                1225                1230

AAT AAA TGC AAA ATG AAT TTA CAA GAT AAT AAT GGG AAT GAT ATA GGC     3744
Asn Lys Cys Lys Met Asn Leu Gln Asp Asn Asn Gly Asn Asp Ile Gly
        1235                1240                1245

TTT ATA GGA TTT CAT CAG TTT AAT AAT ATA GCT AAA CTA GTA GCA AGT     3792
Phe Ile Gly Phe His Gln Phe Asn Asn Ile Ala Lys Leu Val Ala Ser
    1250                1255                1260

AAT TGG TAT AAT AGA CAA ATA GAA AGA TCT AGT AGG ACT TTG GGT TGC     3840
Asn Trp Tyr Asn Arg Gln Ile Glu Arg Ser Ser Arg Thr Leu Gly Cys
1265                1270                1275                1280

TCA TGG GAA TTT ATT CCT GTA GAT GAT GGA TGG GGA GAA AGG CCA CTG     3888
Ser Trp Glu Phe Ile Pro Val Asp Asp Gly Trp Gly Glu Arg Pro Leu
                1285                1290                1295
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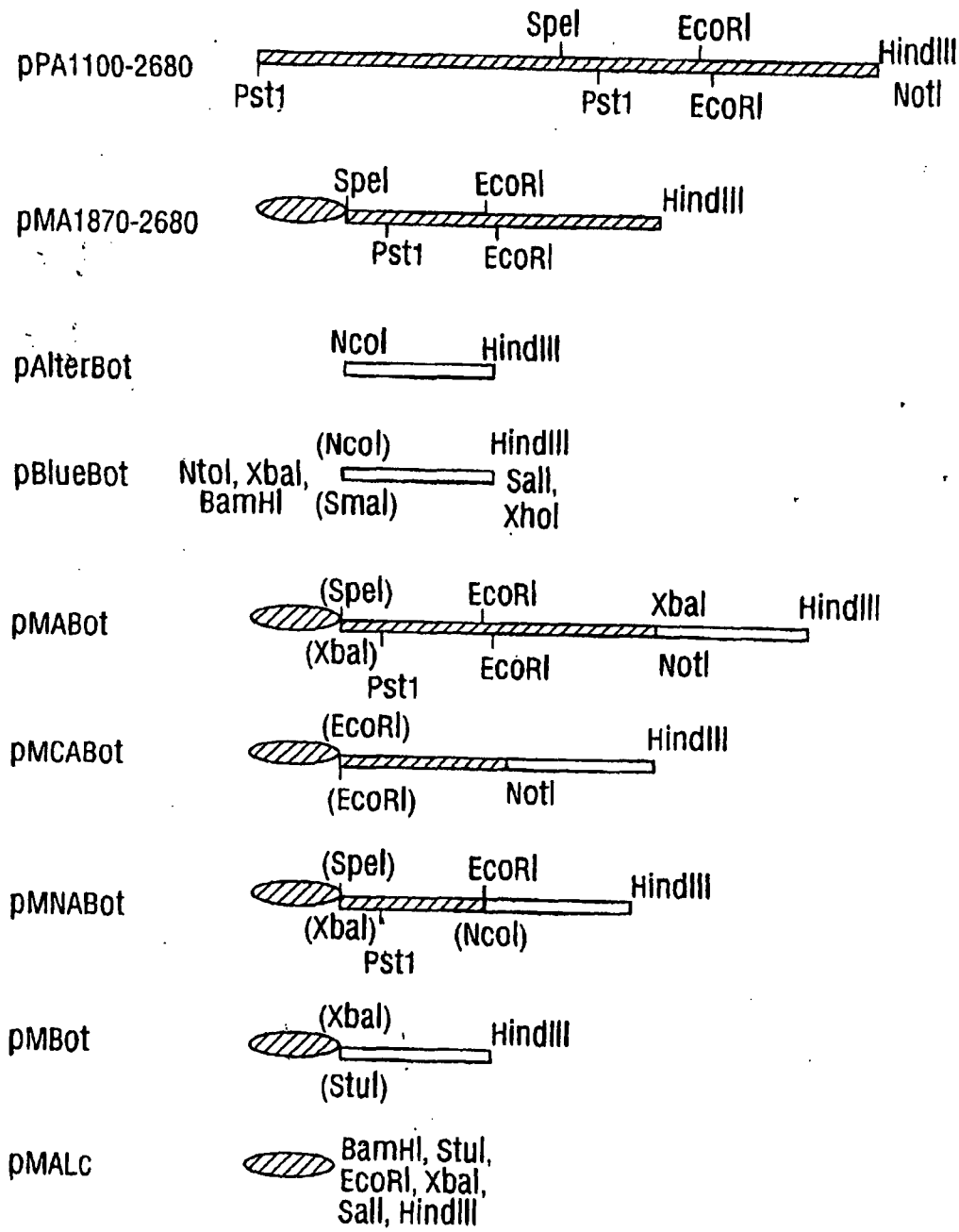
FIG 22 CONT.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EcoRI
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NcoI
HindIII
pBlueBot
(NcoI)
HindIII
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BamHI
(SmaI)
SalI,
XhoI
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(SpeI)
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HindIII
(XbaI)
Pst1
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NotI
pMCABot
(EcoRI)
HindIII
(EcoRI)
NotI
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(SpeI)
EcoRI
HindIII
(XbaI)
Pst1
(NcoI)
pMBot
(XbaI)
HindIII
(StuI)
pMALc
BamHI, StuI,
EcoRI, XbaI,
SalI, HindIII

FIG 23

```
ATG GCT CGT CTG CTG TCT ACC TTC ACT GAA TAC ATC AAG AAC ATC ATC        48

Met Ala Arg Leu Leu Ser Thr Phe Thr Glu Tyr Ile Lys Asn Ile Ile
 1               5                  10                  15

AAT ACC TCC ATC CTG AAC CTG CGC TAC GAA TCC AAT CAC CTG ATC GAC        96
Asn Thr Ser Ile Leu Asn Leu Arg Tyr Glu Ser Asn His Leu Ile Asp
            20                  25                  30

CTG TCT CGC TAC GCT TCC AAA ATC AAC ATC GGT TCT AAA GTT AAC TTC       144
Leu Ser Arg Tyr Ala Ser Lys Ile Asn Ile Gly Ser Lys Val Asn Phe
        35                  40                  45

GAT CCG ATC GAC AAG AAT CAG ATC CAG CTG TTC AAT CTG GAA TCT TCC       192
Asp Pro Ile Asp Lys Asn Gln Ile Gln Leu Phe Asn Leu Glu Ser Ser
    50                  55                  60

AAA ATC GAA GTT ATC CTG AAG AAT GCT ATC GTA TAC AAC TCT ATG TAC       240
Lys Ile Glu Val Ile Leu Lys Asn Ala Ile Val Tyr Asn Ser Met Tyr
65                  70                  75                  80

GAA AAC TTC TCC ACC TCC TTC TGG ATC CGT ATC CCG AAA TAC TTC AAC       288
Glu Asn Phe Ser Thr Ser Phe Trp Ile Arg Ile Pro Lys Tyr Phe Asn
                85                  90                  95

TCC ATC TCT CTG AAC AAT GAA TAC ACC ATC ATC AAC TGC ATG GAA AAC       336
Ser Ile Ser Leu Asn Asn Glu Tyr Thr Ile Ile Asn Cys Met Glu Asn
            100                 105                 110

AAT TCT GGT TGG AAA GTA TCT CTG AAC TAC GGT GAA ATC ATC TGG ACT       384
Asn Ser Gly Trp Lys Val Ser Leu Asn Tyr Gly Glu Ile Ile Trp Thr
        115                 120                 125

CTG CAG GAC ACT CAG GAA ATC AAA CAG CGT GTT GTA TTC AAA TAC TCT       432
Leu Gln Asp Thr Gln Glu Ile Lys Gln Arg Val Val Phe Lys Tyr Ser
    130                 135                 140

CAG ATG ATC AAC ATC TCT GAC TAC ATC AAT CGC TGG ATC TTC GTT ACC       480
Gln Met Ile Asn Ile Ser Asp Tyr Ile Asn Arg Trp Ile Phe Val Thr
145                 150                 155                 160

ATC ACC AAC AAT CGT CTG AAT AAC TCC AAA ATC TAC ATC AAC GGC CGT       528
Ile Thr Asn Asn Arg Leu Asn Asn Ser Lys Ile Tyr Ile Asn Gly Arg
                165                 170                 175

CTG ATC GAC CAG AAA CCG ATC TCC AAT CTG GGT AAC ATC CAC GCT TCT       576
Leu Ile Asp Gln Lys Pro Ile Ser Asn Leu Gly Asn Ile His Ala Ser
            180                 185                 190

AAT AAC ATC ATG TTC AAA CTG GAC GGT TGT CGT GAC ACT CAC CGC TAC       624
Asn Asn Ile Met Phe Lys Leu Asp Gly Cys Arg Asp Thr His Arg Tyr
        195                 200                 205

ATC TGG ATC AAA TAC TTC AAT CTG TTC GAC AAA GAA CTG AAC GAA AAA       672
Ile Trp Ile Lys Tyr Phe Asn Leu Phe Asp Lys Glu Leu Asn Glu Lys
    210                 215                 220

GAA ATC AAA GAC CTG TAC GAC AAC CAG TCC AAT TCT GGT ATC CTG AAA       720
Glu Ile Lys Asp Leu Tyr Asp Asn Gln Ser Asn Ser Gly Ile Leu Lys
225                 230                 235                 240

GAC TTC TGG GGT GAC TAC CTG CAG TAC GAC AAA CCG TAC TAC ATG CTG       768
Asp Phe Trp Gly Asp Tyr Leu Gln Tyr Asp Lys Pro Tyr Tyr Met Leu
                245                 250                 255

AAT CTG TAC GAT CCG AAC AAA TAC GTT GAC GTC AAC AAT GTA GGT ATC       816
Asn Leu Tyr Asp Pro Asn Lys Tyr Val Asp Val Asn Asn Val Gly Ile
            260                 265                 270

CGC GGT TAC ATG TAC CTG AAA GGT CCG CGT GGT TCT GTT ATG ACT ACC       864
Arg Gly Tyr Met Tyr Leu Lys Gly Pro Arg Gly Ser Val Met Thr Thr
        275                 280                 285

AAC ATC TAC CTG AAC TCT TCC CTG TAC CGT GGT ACC AAA TTC ATC ATC       912
Asn Ile Tyr Leu Asn Ser Ser Leu Tyr Arg Gly Thr Lys Phe Ile Ile
    290                 295                 300

AAG AAA TAC GCG TCT GGT AAC AAG GAC AAT ATC GTT CGC AAC AAT GAT       960
Lys Lys Tyr Ala Ser Gly Asn Lys Asp Asn Ile Val Arg Asn Asn Asp
305                 310                 315                 320

CGT GTA TAC ATC AAT GTT GTA GTT AAG AAC AAA GAA TAC CGT CTG GCT      1008
```

FIG 24

```
Arg Val Tyr Ile Asn Val Val Val Lys Asn Lys Glu Tyr Arg Leu Ala
                325                 330                 335

ACC AAT GCT TCT CAG GCT GGT GTA GAA AAG ATC TTG TCT GCT CTG GAA     1056
Thr Asn Ala Ser Gln Ala Gly Val Glu Lys Ile Leu Ser Ala Leu Glu
            340                 345                 350

ATC CCG GAC GTT GGT AAT CTG TCT CAG GTA GTT GTA ATG AAA TCC AAG     1104
Ile Pro Asp Val Gly Asn Leu Ser Gln Val Val Val Met Lys Ser Lys
        355                 360                 365

AAC GAC CAG GGT ATC ACT AAC AAA TGC AAA ATG AAT CTG CAG GAC AAC     1152
Asn Asp Gln Gly Ile Thr Asn Lys Cys Lys Met Asn Leu Gln Asp Asn
    370                 375                 380

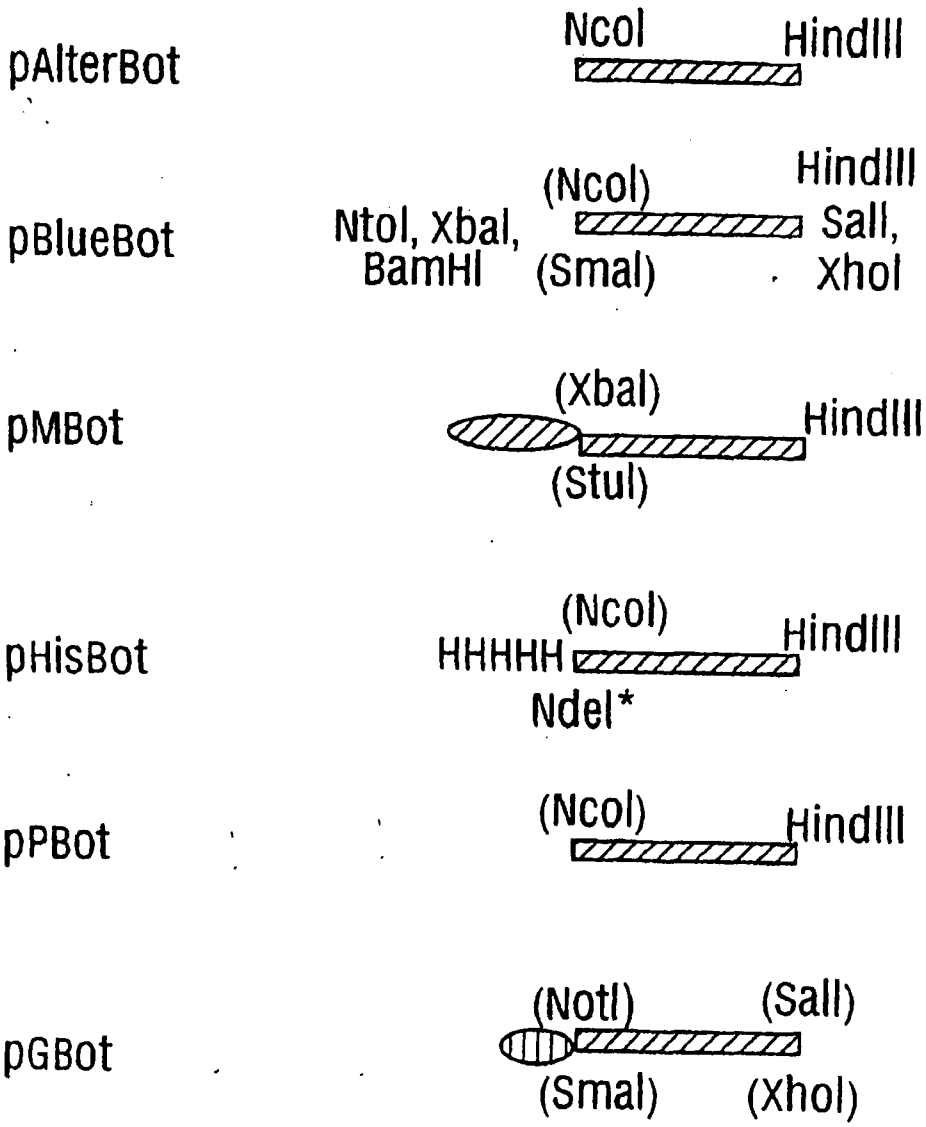
AAT GGT AAC GAT ATC GGT TTC ATC GGT TTC CAC CAG TTC AAC AAT ATC     1200
Asn Gly Asn Asp Ile Gly Phe Ile Gly Phe His Gln Phe Asn Asn Ile
385                 390                 395                 400

GCT AAA CTG GTT GCT TCC AAC TGG TAC AAT CGT CAG ATC GAA CGT TCC     1248
Ala Lys Leu Val Ala Ser Asn Trp Tyr Asn Arg Gln Ile Glu Arg Ser
                405                 410                 415

TCT CGC ACT CTG GGT TGC TCT TGG GAG TTC ATC CCG GTT GAT GAC GGT     1296
Ser Arg Thr Leu Gly Cys Ser Trp Glu Phe Ile Pro Val Asp Asp Gly
            420                 425                 430

TGG GGT GAA CGT CCG CTG TAACCCGGGA AAGCTT                           1330
Trp Gly Glu Arg Pro Leu
        435
```

FIG 24 CONT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(SmaI)
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XhoI
pMBot
(XbaI)
HindIII
(StuI)
pHisBot
(NcoI)
HHHHH
HindIII
NdeI*
pPBot
(NcoI)
HindIII
pGBot
(NotI)
(SalI)
(SmaI)
(XhoI)

FIG 25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```
ATG GGC CAT CAT CAT CAT CAT CAT CAT CAT CAT CAC AGC AGC GGC CAT      48
Met Gly His His His His His His His His His His Ser Ser Gly His
 1               5                  10                  15

ATC GAA GGT CGT CAT ATG GCT AGC ATG GCT CGT CTG CTG TCT ACC TTC      96
Ile Glu Gly Arg His Met Ala Ser Met Ala Arg Leu Leu Ser Thr Phe
            20                  25                  30

ACT GAA TAC ATC AAG AAC ATC ATC AAT ACC TCC ATC CTG AAC CTG CGC     144
Thr Glu Tyr Ile Lys Asn Ile Ile Asn Thr Ser Ile Leu Asn Leu Arg
        35                  40                  45

TAC GAA TCC AAT CAC CTG ATC GAC CTG TCT CGC TAC GCT TCC AAA ATC     192
Tyr Glu Ser Asn His Leu Ile Asp Leu Ser Arg Tyr Ala Ser Lys Ile
    50                  55                  60

AAC ATC GGT TCT AAA GTT AAC TTC GAT CCG ATC GAC AAG AAT CAG ATC     240
Asn Ile Gly Ser Lys Val Asn Phe Asp Pro Ile Asp Lys Asn Gln Ile
65                  70                  75                  80

CAG CTG TTC AAT CTG GAA TCT TCC AAA ATC GAA GTT ATC CTG AAG AAT     288
Gln Leu Phe Asn Leu Glu Ser Ser Lys Ile Glu Val Ile Leu Lys Asn
                85                  90                  95

GCT ATC GTA TAC AAC TCT ATG TAC GAA AAC TTC TCC ACC TCC TTC TGG     336
Ala Ile Val Tyr Asn Ser Met Tyr Glu Asn Phe Ser Thr Ser Phe Trp
            100                 105                 110

ATC CGT ATC CCG AAA TAC TTC AAC TCC ATC TCT CTG AAC AAT GAA TAC     384
Ile Arg Ile Pro Lys Tyr Phe Asn Ser Ile Ser Leu Asn Asn Glu Tyr
        115                 120                 125

ACC ATC ATC AAC TGC ATG GAA AAC AAT TCT GGT TGG AAA GTA TCT CTG     432
Thr Ile Ile Asn Cys Met Glu Asn Asn Ser Gly Trp Lys Val Ser Leu
    130                 135                 140

AAC TAC GGT GAA ATC ATC TGG ACT CTG CAG GAC ACT CAG GAA ATC AAA     480
Asn Tyr Gly Glu Ile Ile Trp Thr Leu Gln Asp Thr Gln Glu Ile Lys
145                 150                 155                 160

CAG CGT GTT GTA TTC AAA TAC TCT CAG ATG ATC AAC ATC TCT GAC TAC     528
Gln Arg Val Val Phe Lys Tyr Ser Gln Met Ile Asn Ile Ser Asp Tyr
                165                 170                 175

ATC AAT CGC TGG ATC TTC GTT ACC ATC ACC AAC AAT CGT CTG AAT AAC     576
Ile Asn Arg Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu Asn Asn
            180                 185                 190

TCC AAA ATC TAC ATC AAC GGC CGT CTG ATC GAC CAG AAA CCG ATC TCC     624
Ser Lys Ile Tyr Ile Asn Gly Arg Leu Ile Asp Gln Lys Pro Ile Ser
        195                 200                 205

AAT CTG GGT AAC ATC CAC GCT TCT AAT AAC ATC ATG TTC AAA CTG GAC     672
Asn Leu Gly Asn Ile His Ala Ser Asn Asn Ile Met Phe Lys Leu Asp
    210                 215                 220

GGT TGT CGT GAC ACT CAC CGC TAC ATC TGG ATC AAA TAC TTC AAT CTG     720
Gly Cys Arg Asp Thr His Arg Tyr Ile Trp Ile Lys Tyr Phe Asn Leu
225                 230                 235                 240

TTC GAC AAA GAA CTG AAC GAA AAA GAA ATC AAA GAC CTG TAC GAC AAC     768
Phe Asp Lys Glu Leu Asn Glu Lys Glu Ile Lys Asp Leu Tyr Asp Asn
                245                 250                 255

CAG TCC AAT TCT GGT ATC CTG AAA GAC TTC TGG GGT GAC TAC CTG CAG     816
Gln Ser Asn Ser Gly Ile Leu Lys Asp Phe Trp Gly Asp Tyr Leu Gln
            260                 265                 270

TAC GAC AAA CCG TAC TAC ATG CTG AAT CTG TAC GAT CCG AAC AAA TAC     864
Tyr Asp Lys Pro Tyr Tyr Met Leu Asn Leu Tyr Asp Pro Asn Lys Tyr
        275                 280                 285

GTT GAC GTC AAC AAT GTA GGT ATC CGC GGT TAC ATG TAC CTG AAA GGT     912
Val Asp Val Asn Asn Val Gly Ile Arg Gly Tyr Met Tyr Leu Lys Gly
    290                 295                 300

CCG CGT GGT TCT GTT ATG ACT ACC AAC ATC TAC CTG AAC TCT TCC CTG     960
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FIG 26

```
Pro Arg Gly Ser Val Met Thr Thr Asn Ile Tyr Leu Asn Ser Ser Leu
305                 310                 315                 320

TAC CGT GGT ACC AAA TTC ATC ATC AAG AAA TAC GCG TCT GGT AAC AAG     1008
Tyr Arg Gly Thr Lys Phe Ile Ile Lys Lys Tyr Ala Ser Gly Asn Lys
                325                 330                 335

GAC AAT ATC GTT CGC AAC AAT GAT CGT GTA TAC ATC AAT GTT GTA GTT     1056
Asp Asn Ile Val Arg Asn Asn Asp Arg Val Tyr Ile Asn Val Val Val
            340                 345                 350

AAG AAC AAA GAA TAC CGT CTG GCT ACC AAT GCT TCT CAG GCT GGT GTA     1104
Lys Asn Lys Glu Tyr Arg Leu Ala Thr Asn Ala Ser Gln Ala Gly Val
        355                 360                 365

GAA AAG ATC TTG TCT GCT CTG GAA ATC CCG GAC GTT GGT AAT CTG TCT     1152
Glu Lys Ile Leu Ser Ala Leu Glu Ile Pro Asp Val Gly Asn Leu Ser
    370                 375                 380

CAG GTA GTT GTA ATG AAA TCC AAG AAC GAC CAG GGT ATC ACT AAC AAA     1200
Gln Val Val Val Met Lys Ser Lys Asn Asp Gln Gly Ile Thr Asn Lys
385                 390                 395                 400

TGC AAA ATG AAT CTG CAG GAC AAC AAT GGT AAC GAT ATC GGT TTC ATC     1248
Cys Lys Met Asn Leu Gln Asp Asn Asn Gly Asn Asp Ile Gly Phe Ile
                405                 410                 415

GGT TTC CAC CAG TTC AAC AAT ATC GCT AAA CTG GTT GCT TCC AAC TGG     1296
Gly Phe His Gln Phe Asn Asn Ile Ala Lys Leu Val Ala Ser Asn Trp
            420                 425                 430

TAC AAT CGT CAG ATC GAA CGT TCC TCT CGC ACT CTG GGT TGC TCT TGG     1344
Tyr Asn Arg Gln Ile Glu Arg Ser Ser Arg Thr Leu Gly Cys Ser Trp
        435                 440                 445

GAG TTC ATC CCG GTT GAT GAC GGT TGG GGT GAA CGT CCG CTG             1386
Glu Phe Ile Pro Val Asp Asp Gly Trp Gly Glu Arg Pro Leu
    450                 455                 460

TAACCCGGGA AAGCTT                                                  1402
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FIG 26CONT

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Met Gly His His His His His His His His His Ser Ser Gly His
 1               5                  10                  15

Ile Glu Gly Arg His Met Ala Ser Met Ala Arg Leu Leu Ser Thr Phe
            20                  25                  30

Thr Glu Tyr Ile Lys Asn Ile Ile Asn Thr Ser Ile Leu Asn Leu Arg
        35                  40                  45

Tyr Glu Ser Asn His Leu Ile Asp Leu Ser Arg Tyr Ala Ser Lys Ile
    50                  55                  60

Asn Ile Gly Ser Lys Val Asn Phe Asp Pro Ile Asp Lys Asn Gln Ile
65                  70                  75                  80

Gln Leu Phe Asn Leu Glu Ser Ser Lys Ile Glu Val Ile Leu Lys Asn
                85                  90                  95

Ala Ile Val Tyr Asn Ser Met Tyr Glu Asn Phe Ser Thr Ser Phe Trp
            100                 105                 110

Ile Arg Ile Pro Lys Tyr Phe Asn Ser Ile Ser Leu Asn Asn Glu Tyr
        115                 120                 125

Thr Ile Ile Asn Cys Met Glu Asn Asn Ser Gly Trp Lys Val Ser Leu
    130                 135                 140

Asn Tyr Gly Glu Ile Ile Trp Thr Leu Gln Asp Thr Gln Glu Ile Lys
145                 150                 155                 160

Gln Arg Val Val Phe Lys Tyr Ser Gln Met Ile Asn Ile Ser Asp Tyr
                165                 170                 175

Ile Asn Arg Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu Asn Asn
            180                 185                 190

Ser Lys Ile Tyr Ile Asn Gly Arg Leu Ile Asp Gln Lys Pro Ile Ser
        195                 200                 205

Asn Leu Gly Asn Ile His Ala Ser Asn Asn Ile Met Phe Lys Leu Asp
    210                 215                 220

Gly Cys Arg Asp Thr His Arg Tyr Ile Trp Ile Lys Tyr Phe Asn Leu
225                 230                 235                 240

Phe Asp Lys Glu Leu Asn Glu Lys Glu Ile Lys Asp Leu Tyr Asp Asn
                245                 250                 255

Gln Ser Asn Ser Gly Ile Leu Lys Asp Phe Trp Gly Asp Tyr Leu Gln
            260                 265                 270

Tyr Asp Lys Pro Tyr Tyr Met Leu Asn Leu Tyr Asp Pro Asn Lys Tyr
        275                 280                 285

Val Asp Val Asn Asn Val Gly Ile Arg Gly Tyr Met Tyr Leu Lys Gly
    290                 295                 300

Pro Arg Gly Ser Val Met Thr Thr Asn Ile Tyr Leu Asn Ser Ser Leu
305                 310                 315                 320

Tyr Arg Gly Thr Lys Phe Ile Ile Lys Lys Tyr Ala Ser Gly Asn Lys
                325                 330                 335

Asp Asn Ile Val Arg Asn Asn Asp Arg Val Tyr Ile Asn Val Val Val
            340                 345                 350

Lys Asn Lys Glu Tyr Arg Leu Ala Thr Asn Ala Ser Gln Ala Gly Val
        355                 360                 365

Glu Lys Ile Leu Ser Ala Leu Glu Ile Pro Asp Val Gly Asn Leu Ser
    370                 375                 380

Gln Val Val Val Met Lys Ser Lys Asn Asp Gln Gly Ile Thr Asn Lys
385                 390                 395                 400

Cys Lys Met Asn Leu Gln Asp Asn Asn Gly Asn Asp Ile Gly Phe Ile
                405                 410                 415

Gly Phe His Gln Phe Asn Asn Ile Ala Lys Leu Val Ala Ser Asn Trp
            420                 425                 430

Tyr Asn Arg Gln Ile Glu Arg Ser Ser Arg Thr Leu Gly Cys Ser Trp
        435                 440                 445

Glu Phe Ile Pro Val Asp Asp Gly Trp Gly Glu Arg Pro Leu
    450                 455                 460
```

FIG 27

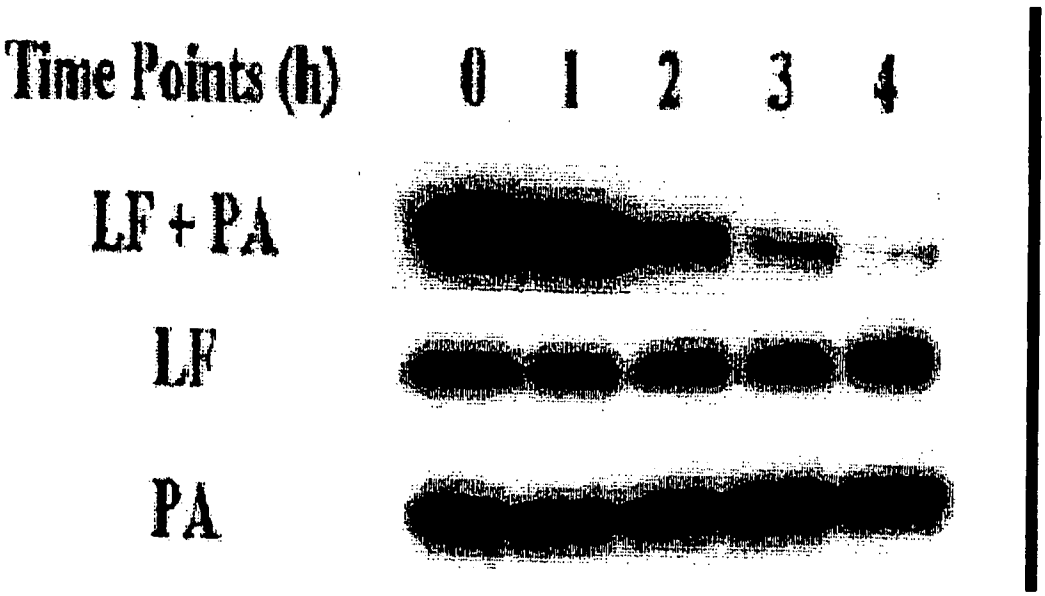

FIG 28