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DESCRIPTION

BACKGROUND

1. Field of the Invention

[0001] The invention relates to the production of high molecular weight heparosan polymers.

2. Description of the Related Art

[0002] Biomaterials (loosely defined as compounds or assemblies that are used to augment or substitute for components of natural tissues or body parts) are and will continue to be integral components of tissue engineering and regenerative medicine approaches. Complex procedures including transplants and stem cell therapies promise to enhance human health, but limited supplies of donor organs/tissues and the steep learning curves (as well as ethical debates) for pioneering approaches are obstacles. There is a growing demand for more routine applications of biomaterials, such as in reconstructive surgery, cosmetics, and medical devices. Therefore, there is a need in the art for new and improved biomaterials that may be used, for example but not by way of limitation, for dermal filler applications and for surface coatings for implanted devices.

[0003] Hyaluronan (HA), poly-L-lactic acid (poly[lactide]), calcium hydroxyapatite and collagen based products dominate the current market for biomaterials utilized in reconstructive surgery and cosmetic procedures. However, these products have a number of undesirable properties for which manufacturers and healthcare professionals are seeking improvements. These disadvantages include, but are not limited to, limited lifetime, potential for immunogenicity and/or allergenicity, and non-natural appearance in aesthetic procedures. For enhancing biocompatibility and durability of an implanted device, HA, heparin, bovine serum albumin, pyrolytic carbon, or lipid coatings are employed to enhance biocompatibility of stents, catheters, and other implanted material devices. However, these products often cause fouling, clogging, or thrombus formation due to reactivity with the human body. Therefore, there is a need in the art for new and improved biomaterial compositions that overcome the disadvantages and defects of the prior art.

[0004] There are numerous medical applications of HA. For example, HA has been widely used as a viscoelastic replacement for the vitreous humor of the eye in ophthalmic surgery during implantation of intraocular lenses in cataract patients. HA injection directly into joints is also used to alleviate pain associated with arthritis. Chemically cross-linked gels and films are also utilized to prevent deleterious adhesions after abdominal surgery. Other researchers using other methods have demonstrated that adsorbed HA coatings also improve the biocompatibility of medical devices such as catheters and sensors by reducing fouling and tissue abrasion.

[0005] The present disclosure overcomes the disadvantages and defects of the prior art. It relates to a biomaterial comprising heparosan, the natural biosynthetic precursor of heparin and heparan sulfate. This composition has numerous characteristics that provide improvements and advantages over existing products. While heparosan is very similar to HA and heparin, the molecule has greater stability within the body since it is not the natural final form of this sugar and therefore the body has no degradation enzymes or binding proteins that lead to loss of functionality. This property also reduces biofouling, infiltration, scarring and/or clotting. Heparosan is also more hydrophilic than synthetic coatings such as plastics or carbon. Finally, aside from bacterial HA, most other current filler biomaterials are typically animal-derived, which causes concern for side effects such as allergic reactions or stimulating granulation, and such side effects will not be a concern with heparosan. Also, most naturally occuring heparosan polymers are known to have certain size ranges of molecular weight, depending on origin of the heparosan biopolymer such as the biosynthesis pathways utilized, including types of catalysts, hosts, and supporting appratus. As is known in the art, the size distribution of the heparosan biopolymer affects its physical properties, such as viscosity, chain entanglement, and solubility. We have developed a means to produce extremely high molecular weight (MW) heparosan polymers that have higher viscosity and can be used at lower concentrations (either with or without chemical crosslinking) than the naturally occurring heparosan preparations.

[0006] The structure and function of *Pastruella multocida* heparosan synthases was shown by Otto et al (2012, J. Biol. Chem. 287 (10) 7203-7212). Sismey-Ragatz *et al* disclose that the chemoenzymatic synthesis of two distinct *Pastruella* heparosan synthases involves potential different active sites. The production of high molecular weight N,O-sulfated heparosans was disclosed by EP544592 and the production and use of heparosan-based biomaterials and coatings is discussed in US2008226690-A1.

SUMMARY OF INVENTION

[0007] The invention relates to a method to recombinatly produce high molecular weight heparosan polymer, the method comprising the steps of culturing a recombinant host cell containing a nucleotide sequence encoding a polypeptide having heparosan synthase activity under conditions appropriate for the expression of the heparosan synthase, wherein at least one of; (a) the polypeptide having heparosan synthase activity is at least 90% identical to at least one of SEQ ID NOS:2, 4, and 6-8, and the nucleotide sequence encoding the polypeptide has been gene-optimized for expression in the recombinant host cell; (b) the polypeptide having heparosan synthase activity has 1-20 amino acid additions, deletions, and/or substitutions when compared to at least one of SEQ ID NOS:2, 4, and 6-8, and the nucleotide sequence encoding the polypeptide has been gene-optimized for expression in the recombinant host cell; (c) the polypeptide is encoded by the nucleotide sequence of at least one of SEQ ID NOS:9-11; (d) the polypeptide is encoded by a nucleotide sequence that is at least 90% identical to at least one of SEQ ID NOS:9-11; and (e) the nucleotide sequence encodes a Pasteurella heparosan synthase; and isolating heparosan polymer produced by the heparosan synthase, wherein the isolated heparosan polymer is biocompatible with a mammalian patient and biologically inert within extracellular compartments of a mammalian patient, and wherein the isolated heparosan polymer is represented by the structure (-GlcUA-beta1,4- GlcNAc-alpha-1,4-)n, wherein n is a positive integer greater than or equal to 2,000.

[0008] The invention also relates to a biomaterial composition, the composition comprising an isolated heparosan polymer, wherein the isolated heparosan polymer is biocompatible with a mammalian patient and biologically inert in extracellular compartments of a mammalian patient, the isolated heparosan polymer being represented by the structure (-GlcUA-betal,4-GlcNAc-alpha-1,4-)n, wherein n is a positive integer greater than or equal to 2,000.

[0009] Another aspect of the invention relates to an isolated nucleotide sequence encoding a polypeptide having heparosan synthase activity, wherein at least one of; (a) the polypeptide having heparosan synthase activity is at least 90% identical to at least one of SEQ ID NOS:2, 4, and 6-8, and the nucleotide sequence encoding the polypeptide has been gene-optimized for expression in the recombinant host cell; and (b) the polypeptide having heparosan synthase activity has 1-20 amino acid additions, deletions, and/or substitutions when compared to at least one of SEQ ID NOS:2, 4, and 6-8, and the nucleotide sequence encoding the polypeptide has been gene-optimized for expression in the recombinant host cell; (c) the polypeptide is encoded by the nucleotide sequence of at least one of SEQ ID NOS:9-11; and (d) the polypeptide is encoded by a nucleotide sequence that is at least 90% identical to at least one of SEQ ID NOS:9-11.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010]

Figure 1A contains an alignment of two *E. coli* gene-optimized sequences (SEQ ID NOS:9 and 10) with a native *Pasteurella multocida* heparosan synthase gene (SEQ ID NO:1).

Figure 1B contains an alignment of the two *E. coli* gene-optimized sequences (SEQ ID NOS:9 and 10).

Figure 1C contains an alignment of a Bacillus gene-optimized sequence (SEQ ID NO:11) with a native *Pasteurella multocida* heparosan synthase gene (SEQ ID NO:12).

Figure 2 depicts a gel analysis demonstrating the production of ultra-high molecular weight heparosan polymer in *E. coli* K5 with plasmid-borne recombinant *PmHS1* gene from *P. multocida* Type D.

Figure 3 depicts a gel analysis demonstrating the production of ultra-high molecular weight heparosan polymer in *E. coli* BL21 (DE3) with either plasmid-borne recombinant *PmHS1* gene or an expression plasmid that produces a maltose-binding protein (MBP) PmHS1 fusion protein.

Figure 4 depicts a gel analysis demonstrating the production of ultra-high molecular weight heparosan polymer in *E. coli* BL21Express I^q transformed with the expression plasmid that produces the maltose-binding protein (MBP) PmHS1 fusion protein.

Figure 5 depicts a gel analysis demonstrating the production of ultra-high molecular weight heparosan polymer in *E. coli* $K5^-$ (in which the *kfiA, kfiB,* and *kfiC* genes have been deleted) with either plasmid-borne recombinant *PmHS1* gene or the expression plasmid that produces the maltose-binding protein (MBP) PmHS1 fusion protein.

DETAILED DESCRIPTION

[0011] Before explaining the invention in detail by way of exemplary drawings, experimentation, results, and laboratory procedures, it is to be understood that the disclosure is not limited in its application to the details of construction and the arrangement of the components illustrated in the drawings, experimentation and/or results. The invention is capable of other embodiments or of being practiced or carried out in various ways. As such, the language used herein is intended to be given the broadest possible meaning; and the embodiments are meant to be exemplary - not exhaustive.

[0012] Unless otherwise defined herein, scientific and technical terms used herein shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. Generally, nomenclatures utilized in connection with, and techniques of, cell and tissue culture, molecular biology, and protein and oligo- or polynucleotide chemistry and hybridization described herein are those well-known and commonly used in the art. Standard techniques are used for recombinant DNA, oligonucleotide synthesis, and tissue culture and transformation (e.g., electroporation, lipofection). Enzymatic reactions and purification techniques are performed according to manufacturer's specifications or as commonly accomplished in the art or as described herein. The foregoing techniques and procedures are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification. See e.g., Sambrook et al. Molecular Cloning: A Laboratory Manual (2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989) and Coligan et al. Current Protocols in Immunology (Current Protocols, Wiley Interscience (1994)). The nomenclatures utilized in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art. Standard techniques are used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, and delivery, and treatment of patients.

[0013] All patents, published patent applications, and non-patent publications mentioned in the specification are indicative of the level of skill of those skilled in the art.

[0014] All of the compositions and/or methods disclosed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein. As utilized in accordance with the present disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

[0015] The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one." The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or." Throughout this application, the term "about" is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects. The use of the term "at least one" will be understood to include one as well as any quantity more than one, including but not limited to, 2, 3, 4, 5, 10, 15, 20, 30, 40, 50, 100, etc. The term "at least one" may extend up to 100 or 1000 or more, depending on the term to which it is attached; in addition, the quantities of 100/1000 are not to be considered limiting, as higher limits may also produce satisfactory results. In addition, the use of the term "at least one of X, Y and Z" will be understood to include X alone, Y alone, and Z alone, as well as any combination of X, Y and Z.

[0016] Throughout the specification and claims, unless the context requires otherwise, the terms "substantially" and "about" will be understood to not be limited to the specific terms qualified by these adjectives/adverbs, but will be understood to indicate a value includes the inherent variation of error for the device, the method being employed to determine the value and/or the variation that exists among study subjects. Thus, said terms allow for minor variations and/or deviations that do not result in a significant impact thereto. For example, in certain instances the term "about" is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value and/or the variation that exists among study subjects. Similarly, the term "substantially" may also relate to 80% or higher, such as 85% or higher, or 90% or higher, or 95% or higher, or 99% or higher, and the like.

[0017] As used in this specification and claim(s), the words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

[0018] The term "or combinations thereof" as used herein refers to all permutations and combinations of the listed items preceding the term. For example, "A, B, C, or combinations thereof" is intended to include at least one of: A, B, C, AB, AC, BC, or ABC, and if order is important in a particular context, also BA, CA, CB, CBA, BCA, ACB, BAC, or CAB. Continuing with this example, expressly included are combinations that contain repeats of one or more item or term, such as BB, AAA, MB, BBC, AAABCCCC, CBBAAA, CABABB, and so forth. The skilled artisan will understand that typically there is no limit on the number of items or terms in any combination, unless otherwise apparent from the context.

[0019] The term "naturally-occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory or otherwise is naturally-occurring. Similarly, a sugar polymer or polysaccharide with intrinsic structural features (such as but not limited to, composition, molecular weight (MW) distribution, etc.) found in native organisms (i.e., unmodified by the hand of man) is termed "naturally occurring".

[0020] The term "patient" as used herein includes human and veterinary subjects. "Mammal" for purposes of treatment refers to any animal classified as a mammal, including human, domestic and farm animals, nonhuman primates, and any other animal that has mammary tissue.

[0021] The terms "administration" and "administering", as used herein will be understood to include all routes of administration known in the art, including but not limited to, oral, topical, transdermal, parenteral, subcutaneous, intranasal, mucosal, intramuscular, intraperitoneal, intravitreal and intravenous routes, including both local and systemic applications. In addition, the compositions disclosed herein (and/or the methods of administration of same) may be designed to provide delayed, controlled or sustained release using formulation techniques which are well known in the art.

[0022] The term "dermal augmentation" refers to any change of the natural state of a mammal's skin and related areas due to external acts. The areas that may be changed by dermal augmentation include, but not limited to, epidermis, dermis, subcutaneous layer, fat, arrector pill muscle, hair shaft, sweat pore, and sebaceous gland.

[0023] As used herein, the term "heparosan" will be understood to refer to the natural biosynthetic precursor of heparin and heparin sulfate. The sugar polymer heparosan is an unsulfated, unepimerized heparin molecule, and may also be referred to as "N-acetyl heparosan".

[0024] The term "tissue" as used herein will be understood to refer to a grouping of cells within an organism that are similarly characterised by their structure and function.

[0025] The term "biomaterial" as used herein will be understood to refer to any nondrug material that can be used to treat, enhance, protect, or replace any tissue, organ, or function in an organism. The term "biomaterial" also refers to biologically derived material that is used for its structural rather than its biological properties, for example but not by way of limitation, to the use of collagen, the protein found in bone and connective tissues, as a cosmetic ingredient, or to the use of carbohydrates modified with biotechnological processes as lubricants for biomedical applications or as bulking agents in food manufacture. A "biomaterial" is any material, natural or man-made, that comprises whole or part of a living structure or biomedical device that performs, auguments, protects, or replaces a natural function and that is compatible with the body.

[0026] As used herein, when the term "isolated" is used in reference to a molecule, the term means that the molecule has been removed from its native environment. For example, a polynucleotide or a polypeptide naturally present in a living organism is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated." Further, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present disclosure. Isolated RNA molecules include in vivo or in vitro RNA replication products of DNA and RNA molecules. Isolated nucleic acid molecules further include synthetically produced molecules. Additionally, vector molecules contained in recombinant host cells are also isolated. Overall, this also applies to carbohydrates in general. Thus, not all "isolated" molecules need be "purified."

[0027] As used herein, when the term "purified" is used in reference to a molecule, it means that the concentration of the molecule being purified has been increased relative to molecules associated with it in its natural environment. Naturally associated molecules include proteins, nucleic acids, lipids and sugars but generally do not include water, buffers, and reagents added to maintain the integrity or facilitate the purification of the molecule being purified.

[0028] As used herein, the term "substantially purified" refers to a compound that is removed from its natural environment and is at least 60% free, preferably 75% free, and most preferably 90% free from other components with which it is naturally associated.

[0029] As used herein, "substantially pure" means an object species is the predominant species present (i.e., on a molar basis it is more abundant than any other individual species in the composition), and preferably a substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition will comprise more than about 80 percent of all macromolecular species present in the composition, such as more than about 85%, 90%, 95%, and 99%. In one embodiment, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.

[0030] As used herein, the term "substrate" will be understood to refer to any surface of which a coating may be disposed. Examples of substrates that may be utilized include, but are not limited to, silica, silicon, glass, polymers, nanotubes, nanoparticles, organic compounds, inorganic compounds, metals and combinations thereof. When the substrate is a metal, the metal may include, but is not limited to, gold, copper, stainless steel, nickel, aluminum, titanium, thermosensitive alloys and combinations thereof.

[0031] The terms "gel" and "semi-solid" are used interchangeably herein and will be understood to include a colloidal system, with the semblance of a solid, in which a solid is dispersed in a liquid; the compound may have a finite yield stress. The term "gel" also refers to a jelly like material formed by the coagulation of a colloidal liquid. Many gels have a fibrous matrix and fluid filled interstices: gels are viscoelastic rather than simply viscous and can resist some mechanical stress without deformation. When pressue is applied to gels or semi-solids, they conform to the shape at which the pressure is applied.

[0032] The term "hydrogel" is utilized herein to describe a network of polymer chains that are water-insoluble, sometimes found as a colloidal gel in which water is the dispersion medium. Hydrogels are very absorbent natural or synthetic polymers, and may contain over 99% water. Hydrogels also possess a degree of flexibility very similar to natural tissue, due to their significant water content. In addition, peptides and/or larger biologically active substances can be enclosed in hydrogels, thereby forming a sustained release composition.

[0033] As used herein, the term "effective amount" refers to an amount of a biomaterial composition or conjugate or derivative thereof sufficient to exhibit a detectable therapeutic or prophylactic effect without undue adverse side effects (such as toxicity, irritation and allergic response) commensurate with a reasonable benefit/risk ratio. The effective amount for a subject will depend upon the type of subject, the subject's size and health, the nature and severity of the condition to be treated, the method of administration, the duration of treatment, the nature of concurrent therapy (if any), the specific formulations employed, and the like. Thus, it is not possible to specify an exact effective amount in advance. However, the effective amount for a given situation can be determined by one of ordinary skill in the art using routine experimentation based on the information provided herein.

[0034] As used herein, the term "nucleic acid segment" and "DNA segment" are used interchangeably and refer to a DNA molecule which has been isolated free of total genomic DNA of a particular species. Therefore, a "purified" DNA or nucleic acid segment as used herein, refers to a DNA segment which contains a Heparosan Synthase (HS) coding sequence yet is isolated away from, or purified free from, unrelated genomic DNA, for example, total *Pasteurella multocida*. Included within the term "DNA segment", are DNA segments and smaller fragments of such segments, and also recombinant vectors, including, for example, plasmids, cosmids, phage, viruses, and the like.

[0035] The term "expression" as used herein may include any step involved in the production of heparosan synthases, including but not limited to, transcription and translation.

[0036] The terms "gene-optimized" and "gene optimization" as used herein refers to changes in the nucleotide sequence encoding a protein to those preferentially used in a particular host cell such that the encoded protein is more efficiently expressed in the host cell when compared to the native nucleotide sequence. Gene-optimization involves various aspects of improving codon usage and messenger RNA structure to improve protein production. It is well known in the art that genes from one organism, the source, do not always perform well in a recipient organism. For example, some amino acids (AAs) are encoded by multiple tRNAs (the degenerate code), and each organism has a preferred codon(s) that is used more frequently. If a rare codon is used in a gene, then the ribosome must stall and wait for the rare tRNA to be found before the protein translation can move onto the next amino acid to be added; if the stalling occurs too long, then the ribosome can fall off, and the protein is not made. Similarly, if the mRNA has a secondary structure that interferes with ribosome movement and thus translation, then the ribosome can fall off the messenger RNA, again resulting in less protein production. By studying the DNA sequence of naturally highly produced proteins in the desired host or recipient organism, certain codons for AAs are noted. Therefore, the source gene can be converted to a more highly functional producer if the rare codons are removed, and the more used codons (with respect to the recipient) are used. The protein sequence is the same, but the DNA sequence can differ due to the degenerate tRNA code. As there are many aspects to the translation process, there are multiple important optimization issues that need to be addressed, including but not limited to, codon usage bias, GC content, CpG dinucleotides content, mRNA secondary structure, cryptic splicing sites, premature PolyA sites, internal chi sites and ribosomal binding site, negative CpG islands, RNA instability motifs (ARE), repeat sequences (direct repeat, reverse repeat, and Dyad repeat), addition of Kozak sequences and/or Shine-Dalgarno sequences to increase the efficiency of translational initiation, addition of stop codons to increase the efficiency of translational termination, and the like. Therefore, gene optimization, as used herein, refers to any changes in a nucleotide sequence made to address one or more of the optimization issues mentioned above.

[0037] A non-limiting example of a type of gene optimization is codon optimization. The terms "codon-optimized" and "codon optimization" refers to changes in the codons of the polynucleotide encoding a protein to those preferentially used in a particular organism such that the encoded protein is efficiently expressed in the organism of interest. Although the genetic code is degenerate in that most amino acids are represented by several codons, called "synonyms" or "synonymous" codons, it is well known that codon usage by particular organisms is nonrandom and biased towards particular codon triplets. This codon usage bias may be higher in reference to a given gene, genes of common function or ancestral origin, highly expressed proteins versus low copy number proteins, and the aggregate protein coding regions of an organism's genome. In some embodiments, the polynucleotides encoding enzymes may be codon-optimized for optimal production from the host organism selected for expression.

[0038] "Preferred, optimal, high codon usage bias codons" refers interchangeably to codons that are used at higher frequency in the protein coding regions than other codons that code for the same amino acid. The preferred codons may be determined in relation to codon usage in a single gene, a set of genes of common function or origin, highly expressed genes, the codon frequency in the aggregate protein coding regions of the whole organism, codon frequency in the aggregate protein coding regions of related organisms, or combinations thereof. Codons whose frequency increases with the level of gene expression are typically optimal codons for expression. A variety of methods are known for determining the codon frequency (e.g., codon usage, relative synonymous codon usage) and codon preference in specific organisms, including multivariate analysis, for example, using cluster analysis or correspondence analysis, and the effective number of codons used in a gene (See GCG Codon Preference, Genetics Computer Group Wisconsin Package; CodonW, John Peden, University of Nottingham; McInerney, J. O, 1998, Bioinformatics 14:372-73; Stenico et al., 1994, Nucleic Acids Res. 222437-46; Wright, F., 1990, Gene 87:23-29). Codon usage tables are available for a growing list of organisms (see for example, Wada et al., 1992, Nucleic Acids Res. 20:2111-2118; Nakamura et al., 2000, Nucl. Acids Res. 28:292; Duret, et al., supra; Henaut and Danchin, "Escherichia coli and Salmonella," 1996, Neidhardt, et al. Eds., ASM Press, Washington D.C., p. 2047-2066). The data source for obtaining codon usage may rely on any available nucleotide sequence capable of coding for a protein. These data sets include nucleic acid sequences actually known to encode expressed proteins (e.g., complete protein coding sequences-CDS), expressed sequence tags (ESTs), or predicted coding regions of genomic sequences (see for example, Mount, D., Bioinformatics: Sequence and Genome Analysis, Chapter 8, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2001; Uberbacher, E. C., 1996, Methods Enzymol. 266:259-281; Tiwari et al., 1997, Comput. Appl. Biosci. 13:263-270).

[0039] The Dalton (Da) is the international unit of molecular mass based on 1/12 of the mass of carbon 12. A kiloDalton (kDa) is 1,000 Da. A mega-Dalton (MDa) is 1,000 kDa.

[0040] Compositions that include an isolated high molecular weight (HMW) heparosan polymer are included and described in detail herein, along with methods of producing and using same. In certain embodiments, the composition is a biomaterial composition. In particular embodiments, the isolated heparosan polymer is biocompatible with a mammalian patient and biologically inert in extracellular compartments of the mammalian patient. The heparosan polymer is substantially not susceptible to vertebrate (such as but not limited to, mammalian) hyaluronidases or vertebrate (such as but not limited to, mammalian) heparanses and thereby is not substantially degraded *in vivo* in extracellular compartments of the mammalian patient. In addition, the heparosan polymer may be recombinantly produced as described in detail herein utilizing a combination of host cell and synthase biosynthesis, where features of both of these factors influence the MW made by the live cell.

[0041] The isolated heparosan polymer of the invention is represented by the structure (-GlcUA-betal,4-GlcNAc-alpha-1,4-)*n*, wherein *n* is a positive integer greater than or equal to about 2,000. Polymers of this size are hitherto unreported in the scientific literature and prior art. Each single n unit is approximately 400 Da, and therefore the isolated heparosan polymer has a molecular weight (MW) of greater than or equal to about 800 kDa. The n can be a positive integer in a range of from about 2,000 to about 17,000, and therefore the isolated heparosan polymer has a MW in a range of from about 0.8 MDa to about 6.8 MDa. In addition, n may be a positive integer such as but not limited to, 2,250; 2,500; 2,750; 3,000; 3,250; 3,500; 3,750; 4,000; 4,250; 4,500; 4,750; 5,000; 5,250; 5,500; 5,750; 6,000; 6,250; 6,500; 6,750; 7,000; 7,250; 7,500; 7,750; 8,000; 8,250; 8,500; 8,750; 9,000; 9,250; 9,500; 9,750; 10,000; 10,250; 10,500; 10,750; 11,000; 11,250; 11,500; 11,750; 12,000; 12,250; 12,500; 12,750; 13,000; 13,250; 13,500; 13,750; 14,000; 14,250; 14,500; 14,750; 15,000; 15,250; 15,500; 15,750; 16,000; 16,250; 16,500; 16,750; and 17,000; as well as within a range of any of the above.

[0042] The heparosan polymer may be linear or cross-linked. The compositions disclosed herein may be administered to a patient by any means known in the art; for example, the compositions may be injectable and/or implantable. In addition, the compositions may be in a gel or semi-solid state, a suspension of particles, or the compositions may be in a liquid form.

[0043] Alternatively, the heparosan polymer may be attached to a substrate. When attached to a substrate, the isolated heparosan polymer may be covalently (via a chemical bond) or non-covalently (via weak bonds) attached to the substrate. Any substrate known in the art or otherwise contemplated herein may be utilized, so long as the substrate is capable of being attached to the heparosan polymer and functioning. Examples of substrates that may be utilized include, but are not limited to, silica, silicon, semiconductors, glass, polymers, nanotubes, nanoparticles, organic compounds, inorganic compounds, metals, and combinations thereof. Non-limiting examples of metals that may be utilized include gold, copper, stainless steel, nickel, aluminum, titanium, thermosensitive alloys, and combinations thereof.

[0044] The present disclosure also comprises biomaterial compositions comprising a cross-linked gel that includes an isolated heparosan polymer and at least one cross-linking agent. The cross-linking agent may be any cross-linking agent known or otherwise contemplated in the art; specific non-limiting examples of cross-linking agents that may be utilized include aldehydes, epoxides, polyaziridyl compounds, glycidyl ethers, divinyl sulfones, and combinations and derivatives thereof. An advantage of the currently described invention is that lower concentrations of this high MW (greater than 1 MDa or 1,000 kDa) polymer may be used to produce useful gels than if a lower MW polymer was employed.

[0045] Any of the biomaterial compositions of the present disclosure may be a moisturizing biomaterial that protects from dehydration; alternatively, the disclosed biomaterial compositions may be a lubricating biomaterial.

[0046] Another aspect of the present disclosureis related to kits for *in vivo* administration of any of the compositions described herein above or otherwise contemplated herein to a mammalian patient. The kit may also include instructions for administering the composition to the mammalian patient. The kit may optionally also contain one or more other compositions for use in accordance with the methods described herein.

[0047] The invention is further directed to a method of recombinantly producing a high MW heparosan polymer. In the method, a recombinant host cell containing a nucleotide sequence encoding a heparosan synthase, the enzyme that polymerizes the monosaccharides from UDP-sugar precursors into heparosan polysaccharide or sugar polymer, is cultured under conditions appropriate for the expression of the heparosan synthase. The heparosan synthase produces the high MW heparosan polymer, which is then isolated.

[0048] The isolated high MW heparosan polymer may possess any or all of the characteristics described herein above, and may subsequently be utilized as a biomaterial composition. Thus, the method may further comprise one or more steps to this end, such as but not limited to, crosslinking the isolated heparosan polymer or attaching (either covalently or non-covalently) the isolated heparosan polymer to any of the substrates described or otherwise contemplated herein.

[0049] In certain non-limiting embodiments, the host cell is an *E. coli* host cell, and the heparosan synthase is a *Pasteurella* heparosan synthase.

[0050] Non-limiting examples of heparosan synthases that may be utilized in accordance with the invention are described in greater detail herein below.

[0051] Any host cell known in the art or otherwise contemplated herein may be utilized in accordance with the invention , so long as the host cell is capable of being made recombinant with a heparosan synthase gene and producing a high MW heparosan polymer upon expression of the heparosan synthase gene under the appropriate culture conditions. Non-limiting examples of host cells that may be utilized in accordance with the invention are described in greater detail below.

[0052] The present disclosure shows that *Pasteurella* heparosan synthases will perform the ultra-high MW heparosan biosynthesis operation in an *E. coli* host cell with the proper UDP-sugar and transport infrastructure. Most available *E. coli* strains employed in laboratories as well as most wild-type isolates are therefore not useful without further manipulation. The present disclosuredemonstrates that an *E. coli* K5 host (or strains that contain similar infrastructure) is amenable to high MW heparosan polymer production.

[0053] In theory, at least simple two models for controlling the size of a polymer are possible: (A) host cell-controlled biosynthesis or (B) synthase-controlled biosynthesis. In the former model, the nature of the supporting apparatus (*e.g.*, UDP-sugar precursors, transporters) defines the final size distribution made by the live cell. In the latter model, the intrinsic properties of the polymerizing catalyst (*e.g.*, elongation rate, processivity) control the polymer size distribution made by the live cell. A third model (C), combinatorial host cell/synthase biosynthesis, is possible where features of both factors influence the MW made by a live cell; this model is also the most complex, unpredictable, and non-obvious to decipher. Models A & B are inconsistent with the observed data; neither the *Escherichia coli* K5 host cell's product size

(~50-80 kDa) nor the *Pasteurella* heparosan synthase product size (~100-300 kDa) is similar to the heparosan made in this disclosure (>800 kDa) and should be considered a non-predictable outcome that has not been reported in the patent or scientific literature to date.

[0054] This disclosure also includes the use of alternative hosts with the potential for glycosaminoglycan production, including bacteria from both Gram-negative (e.g., *Pseudomonas,* etc.) and Gram-positive classes (e.g., *Bacilli, Lactoctococci,* etc.), as well as other microbes (fungi, archae, etc). The basic requirements of a recombinant host for use in heparosan production) include: (a) the glycosyltransferase(s) that produce heparosan, and (b) the UDP-sugar precursors UDP-GlcNAc and UDP-GlcUA. It should be noted that the latter requirement can be met by either native genes or introduced recombinant genes. The required genes can be either episomally and/or chromosomally located.

[0055] In certain embodiments, the host cell further comprises at least one gene encoding an enzyme for synthesis of a heparosan sugar precursor (i.e., UDP-GlcNAc or UDP-GlcUA). Non-limiting examples of genes encoding an enzyme for synthesis of a heparosan sugar precursor that may be utilized include pyrophosphorylases, transferases, mutases, dehydrogenases, and epimerases.

[0056] The ultra-high MW (= or > 1MDa) heparosan polymer is not known in nature and not been shown or reported by others. As well known in the polymer field, the size distribution affects its physical properties (e.g., viscosity, chain entanglement, solubility). The >1 MDa heparosan described is preferred over the naturally occurring heparosan with respect to performance in production of certain biomaterials, such as but not limited to, viscoelastics and hydrogels.

[0057] The present disclosure is also related to methods of augmenting tissue in a mammalian patient. In such methods, an effective amount of any of the biomaterial compositions described herein above or otherwise contemplated herein is administered to the mammalian patient. The biomaterial composition may be administered to the patient by any method known in the art, including, but not limited to, injection and/or implantation. When injected, the biomaterial composition may be in a liquid state or a suspension of particles, whereas when implanted, the biomaterial composition may be in a gel or semi-solid state, or may be attached to a substrate.

[0058] The present disclosure also relates to methods of repairing voids in tissues of mammals. In the method, any of the biomaterial compositions described herein above or otherwise contemplated herein is administered into the voids. The biomaterial composition may be injected and/or implanted into the voids.

[0059] The present disclosurealso relates to methods of creating voids or viscus in tissues of mammals. In the method, any of the biomaterial compositions described herein above or otherwise contemplated herein are disposed into a tissue or a tissue engineering construct to create the voids or viscus. The biomaterial composition may be injected and/or implanted into the tissue/tissue engineering construct to create the voids or viscus.

[0060] The present disclosure also relates to methods of reparative surgery or plastic surgery. In the method, any of the biomaterial compositions described herein above or otherwise contemplated herein is administered to a patient and serves as a filling material at the site to which it is administered. The biomaterial composition may be injected and/or implanted into the patient.

[0061] The presently disclosure further relates to methods of dermal augmentation and/or treatment of skin deficiency in a patient. In the method, any of the biomaterial compositions described herein above or otherwise contemplated herein is administered to the patient. The biomaterial composition may be injected and/or implanted into the patient. The biomaterial composition is biocompatible, swellable, hydrophilic, and substantially non-toxic, and the biomaterial composition swells upon contact with physiological fluids at the administration/injection/implantation site.

[0062] The dermal augmentation method of the present disclosure is especially suitable for the treatment of skin contour deficiencies, which are often caused by various conditions/exposures, including but not limited to, aging, environmental exposure, weight loss, child bearing, injury, surgery, in addition to diseases such as acne and cancer. Non-limiting examples of contour deficiencies include frown lines, worry lines, wrinkles, crow's feet, marionette lines, stretch marks, and internal and external scars resulted from injury, wound, bite, surgery, or accident.

[0063] In addition, the present disclosurealso relates to methods of medical or prophylactic treatment of a mammalian patient. In the method, any of the compositions described herein above or otherwise contemplated herein is administered to the mammalian patient in need of such a treatment. The composition may be injected and/or implanted into the mammalian patient.

[0064] Further, the present disclosurealso relates to methods of treatment or prophylaxis of tissue augmentation in a mammalian patient. In the method, a medical or prophylactic composition comprising a polysaccharide gel composition that includes any of the biomaterial compositions described herein above or otherwise contemplated herein is administered to the mammalian patient.

[0065] The present disclosure is further related to a delivery system for a substance having biological or pharmacological activity. The system comprising a molecular cage formed of a cross-linked gel of heparosan or a mixed cross-linked gel of heparosan and at least one other hydrophilic polymer co-polymerizable therewith. The system further includes a substance having biological or pharmacological activity dispersed therein, wherein the substance is capable of being diffused therefrom in a controlled manner.

[0066] The biomaterials disclosed herein may be utilized in any methods of utilizing biomaterials known or otherwise contemplated in the art. For example but not by way of limitation, the biomaterial compositions may be utilized in any of the methods of utilizing other known biomaterials that are described in US Patent Nos. 4,582,865, issued to Balazs et al. on April 15, 1986; 4,636,524, issued to Balazs et al. on January 13, 1987; 4,713,448, issued to Balazs et al. on December 15, 1987; 5,137,875, issued to Tsununaga et al. on August 11, 1992; 5,827,937, issued to Ang on October 27, 1998; 6,436,424, issued to Vogel et al. on August 20, 2002; 6,685,963, issued to Taupin et al. on February 3, 2004; and 7,060,287, issued to Hubbard et al. on June 13, 2006. Other specific examples of uses for the biomaterial compositions presently disclosed include, but are not limited to: (a) a persistent lubricating coating on a surface, such as, but not limited to, surgical devices; (b) a long lasting moisturizer; (c) a viscoelastic supplement for joint maladies; and (d) a non-thrombotic, non-occluding blood conduit (such as, but not limited to, a stent or artificial vessel, etc.). In addition, any of the biomaterial compositions disclosed herein may be utilized in tissue engineering to form a viscus or vessel duct or lumen by using the biomaterial compositions of the presently disclosed inventive concept(s) as a three-dimensional space maker; in this instance, the surrounding cells will not bind to the biomaterial compositions), thereby making such biomaterial compositions well suited for this technology.

[0067] Compositions disclosed herein may be produced using recombinant heparosan synthases as described or otherwise known in the art, including but not limited to, the heparosan synthases disclosed in the inventor's prior patents U.S. Patent Nos. 7,307,159, issued December 11, 2007; 7,771,981, issued May 8, 2002; and 8,088,604, issued January 3, 2012; as well as the heparosan synthases disclosed in the inventor's published patent applications US 2008/0226690, published September 18, 2008; US 2010/0036001, published February 11, 2010; and US 2012/0108802, published May 3, 2012..

[0068] Heparosan synthases that may be utilized in accordance with the invention include: a recombinant heparosan synthase having an amino acid sequence as set forth in at least one of SEQ ID NOS: 2, 4, and 6-8, and a recombinant heparosan synthase encoded by the nucleotide sequence of at least one of SEQ ID NOS: 9-11. Further embodiments describe a recombinant heparosan synthase that is at least 90% identical to at least one of SEQ ID NOS: 2, 4, and 6-8; a recombinant heparosan synthase that is at least 95% identical to at least one of SEQ ID NOS: 2, 4, and 6-8; a recombinant heparosan synthase encoded by a nucleotide sequence that is at least 90% identical to at least one of SEQ ID NOS: 9-11; and a recombinant heparosan synthase encoded by a nucleotide sequence that is at least 95% identical to at least one of SEQ ID NOS: 9-11.

[0069] The use of truncated heparosan synthase genes to produce any of the compositions described or otherwise contemplated herein also falls within the scope of the present disclosure . For instance, the removal of the last 50 residues or the first 77 residues of PmHS1 (SEQ ID NOS: 7 and 8, respectively) does not inactivate its catalytic function (Kane et al., 2006). Those of ordinary skill in the art would appreciate that simple amino acid removal from either end of the heparosan synthase sequence can be accomplished. The truncated versions of the sequence simply have to be checked for activity in order to determine if such a truncated sequence is still capable of producing heparosan.

[0070] Similarly, the use of fusion proteins that add other polypeptide segments (to either termini or internally) to the heparosan synthase sequence is also disclosed. The fusion protein partner (such as but not limited to, maltose-binding protein, thioredoxin, etc.) can increase stability, increase expression levels in the cell, and/or facilitate the purification process, but the catalytic activity for making the heparosan polymer remains the same.

[0071] One of ordinary skill in the art, given a nucleic acid sequence or an amino acid sequence, could make substitutions and changes to the nucleic acid/amino acid sequence without changing its functionality (specific examples of such changes are given hereinafter and are generally set forth in SEQ ID NOS:7-8).

TABLE 1

Amino Acid Group	Conservative and Semi-Conservative Substitutions
NonPolar R Groups	Alanine, Valine, Leucine, Isoleucine, Proline, Methionine, Phenylalanine, Tryptophan
Polar, but uncharged, R Groups	Glycine, Serine, Threonine, Cysteine, Asparagine, Glutamine
Negatively Charged R Groups	Aspartic Acid, Glutamic Acid
Positively Charged R Groups	Lysine, Arginine, Histidine

[0072] Therefore, the present disclosure also includes the use of heparosan synthases that have amino acid sequences that differ from at least one of SEQ ID NOS:2, 4, and 6-8 by at least one of the following: the presence of 1-20 amino acid additions, deletions, or substitutions when compared to at least one of SEQ ID NOS:2, 4, and 6-8; the presence of 1-15 amino acid additions, deletions, or substitutions when compared to at least one of SEQ ID NOS:2, 4, and 6-8; the presence of 1-10 amino acid additions, deletions, or substitutions when compared to at least one of SEQ ID NOS:2, 4, and 6-8; and the presence of 1-5 amino acid additions, deletions, or substitutions when compared to at least one of SEQ ID NOS:2, 4, and 6-8.

[0073] Allowing for the degeneracy of the genetic code as well as conserved and semi-conserved substitutions, sequences which have between about 90% and about 99% *identity to* the nucleotides of at least one of SEQ ID NO: 9-11 will be sequences which are "essentially as set forth in at least one of SEQ ID NO: 9-11."

[0074] The present disclosure also include the use of nucleotide sequences encoding any of the heparosan synthases described herein, wherein the nucleotide sequences are synthetic sequences that have been gene-optimized for expression in a particular host cell. Specific, non-limiting examples of gene-optimized heparosan synthase encoding nucleotide sequences are provided in SEQ ID NOS:9-11. SEQ ID NOS:9-10 include nucleotide sequences encoding the heparosan synthase of SEQ ID NO:2 and which have been gene-optimized for expression in *E. coli.* SEQ ID NO:11 includes a nucleotide sequence encoding the heparosan synthase of SEQ ID NO:2 and which have been gene-optimized for expression in *Bacillus.*

[0075] The use of gene-optimized sequences is known and used in the art to increase expression of the gene sequence within the heterologous host. However, a novel product was unexpectedly produced from the heparosan synthase expressed in *E. coli* when the *Pasteurella* gene sequence was gene-optimized and expressed in *E. coli.* The invention discloses the production of mega-Dalton molecular weight heparosan polymers, and this novel species has never before been reported in any known microbes. One of ordinary skill in the art would assume that optimization of a gene sequence encoding an enzyme would result in increased expression of that enzyme in the heterologous host, thereby resulting in increased production of the same enzyme-derived product (i.e., higher amounts of the heparosan polymer of the typical size found in the native microbes) produced in the native host. Unexpectedly, the expression of gene-optimized *Pasteurella multocida* heparosan synthase in *E. coli* resulted in a new species of product - an ultra-high molecular weight heparosan polymer. Production of heparosan polymers of this size have not been reported for any other microbe. In addition, the heparosan polymers produced exhibit superior and advantageous properties compared to the lower molecular weight products currently known in the art. These properties provide enhanced utility for the heparosan polymer in the biomaterials field. For example, but not by way of limitation, the ultra-high molecular weight (MW) heparosan polymers produced in accordance with the invention exhibit enhanced solution viscosity and can be used at lower concentrations (either with or without chemical crosslinking) than the naturally occurring heparosan preparations.

[0076] The present disclosure further includes isolated nucleotide sequences, along with recombinant host cells, that contain any of the gene-optimized heparosan synthase sequences of the invention.

[0077] Heparosan, a sugar polymer that is the natural biosynthetic precursor of heparin and heparan sulfate, has numerous characteristics that indicate that this material exhibits enhanced performance in a variety of medical applications or medical devices. In comparison to HA and heparin, two very structurally similar polymers used in many current applications in several large markets, heparosan is more stable in the body, as no naturally occurring enzymes degrade heparosan, and therefore the biomaterial compositions of the invention should have longer lifetimes compared to presently used biomaterials. In addition, heparosan interacts with fewer proteins (thus less fouling) and cells (thus less infiltration, scarring, or clotting) when compared to existing biomaterials.

[0078] The heparosan chain does not contain sulfate groups; thus, the degrading enzyme heparanase, the anticoagulation system proteins of blood, the cell surface binding receptors, and growth factors and cytokines will not specifically bind the polymer. This characteristic leads to an inert character in the body, thereby providing long half-life in the extracellular space in addition to not stimulating or inducing cellular behaviors (e.g., growth, migration, binding, activation, etc). However, once in the cell, the heparosan chain can be degraded by normal metabolic systems such as the exoglycosidases in the lysososme.

[0079] In comparison to synthetic plastics or carbon, the natural hydrophilicity (aka water-loving) characteristics of heparosan also enhance tissue compatibility. Animal-derived proteins (*e.g.*, collagen, bovine serum albumin) and calcium hydroxyapatite often have side effects, including but not limited to, eliciting an allergic response and/or stimulating granulation (5). On the other hand, even certain pathogenic bacteria use heparosan to hide in the body since this polymer is non-immunogenic (8-10). The biomaterial compositions of the invention produced from a non-animal source also promise to be free of adventitious agents (*e.g.*, vertebrate viruses, prions) that could potentially contaminate animal- or human-derived sources.

[0080] Certain carbohydrates play roles in forming and maintaining the structures of multicellular organisms in addition to more familiar roles as nutrients for energy. Glycosaminoglycans [GAGs], long linear polysaccharides consisting of disaccharide repeats that contain an amino sugar, are well known to be essential in vertebrates (9, 11-15). The GAG structures possess many negative groups and are replete with hydroxyl groups, therefore these sugars have a high capacity to adsorb water and ions. Heparin/heparan (backbone [β4GlcUA-α4GlcNAc]$_n$), chondroitin (backbone [β4GlcUA-β3GalNAc]$_n$), and hyaluronan (HA; backbone [β4GlcUA-β3GlcNAc]$_n$) are the three most prevalent GAGs in humans. Depending on the tissue and cell type, the GAGs are structural, adhesion, and/or signaling elements. A few clever microbes also produce extracellular polysaccharide coatings, called capsules, composed of GAG chains that serve as virulence factors (9, 10). The capsule is thought to assist in the evasion of host defenses such as phagocytosis and complement. As the microbial polysaccharide is identical or very similar to the host GAG, the antibody response is either very limited or non-existent.

[0081] In humans, heparosan only exists transiently, serving as a precursor to the more highly modified final products of heparan sulfate and heparin. In contrast, the bacterial strains set forth herein produce heparosan as their final product (16). Due to the less complex makeup of bacterial cells and to the relative ease with which their growth and expression can be modulated, harvesting a polymer from microbes is much easier, more scalable, and less expensive than extracting from animal tissues. In addition, the polymer in the currently described invention, namely the ultra high MW (1 to 6.8 MDa) heparosan derived from our recombinant system has not previously existed or been reported in nature.

[0082] Dermal fillers serve as soft tissue replacements or augmentation agents (5, 6). The need for a dermal filler may arise from aging (loss of HA and elastin), trauma (loss of tissue), acne (severe pitting), and/or atrophy (certain wasting diseases including lipoatrophy). Three important characteristics that dermal fillers must possess include a) space-filling ability, *b*) maintenance of hydration, and c) biocompatibility (5). Currently, polysaccharides, proteins, plastics, and ceramics have been used as biomaterials in dermal fillers. With respect to aesthetic appearance and ease of implantation, softer injectable gels have better attributes; thus, polysaccharides and proteins are widely used. In addition to therapeutic uses, cosmetic applications are becoming more widespread. Alternatives to dermal filler treatment are the use of (*i*) plastic surgery (tightening the skin), (*ii*) nerve killing agents such as BOTOX® (relax muscles), and (*iii*) the use of autologous fat. Compared to dermal fillers, these alternatives are more invasive and/or leave the patient with an unnatural appearance (5, 6). For victims of trauma, scarring, or severe disease, an aim of the therapy is to instill more self-confidence and better disposition; this effect should not be discounted, as a patient's state of mind is important for overall healing.

[0083] A major goal of bioengineering is the design of implanted artificial devices to repair or to monitor the human body. High-strength polymers, durable alloys, and versatile semiconductors have many properties that make these materials desirable for bioengineering tasks. However, the human body has a wide range of defenses and responses that evolved to prevent infections and to remove foreign matter that hinders the utilization of modern man-made substances (17, 18). Improving the biocompatibility of these materials will remove a significant bottleneck in the advancement of bioengineering.

[0084] A leading example of a medical need for improved surface coatings lies in cardiovascular disease. Damage from this disease is a very prevalent and expensive problem; the patient's system is oxygen- and nutrient-starved due to poor blood flow. The availability of blood vessel grafts from transplants (either autologous or donor) is limited as well as expensive. Therefore, the ability to craft new artificial vessels is a goal, but will take more time to perfect due to the complex engineering and biological requirements. Another current, more approachable therapeutic intervention employs stents, artificial devices that prop open the inner cavity of a patient's blood vessel. As summated by Jordan & Chaikof, "The development of a clinically durable small-diameter vascular graft as well as permanently implantable biosensors and artificial organ systems that interface with blood, including the artificial heart, kidney, liver, and lung, remain limited by surface-induced thrombotic responses" (7). Thus, to advance this technology further, thromboresistant surface coatings are needed that inhibit: (*i*) protein and cell adsorption, (*ii*) thrombin and fibrin formation, and (*iii*) platelet activation and aggregation.

[0085] Artificial plastics (poly[lactide] in SCULPTRA® (Sanofi-Aventis) or poly[methylmethacrylate] in ARTECOLL® (Artes Medical, Inc., San Diego, CA), ceramics (calcium hydroxyapatite in RADIESSE® (Bioform Medical, Inc., San Mateo, CA)) or pure carbon have utility for many therapeutic applications (1,5,7,18), but in many respects, their chemical and physical properties are not as optimal as polysaccharides for the targeted goals of dermal fillers or surface coatings. The most critical issues are lack of good wettability (due to poor interaction with water) and/or hardness (leading to an unnatural feel or brittleness). The present disclosure is related to the use of heparosan to replace and supplant useful sugar polymers that are hydrophilic (water loving) and may be prepared in a soft form.

[0086] In addition to HA and heparin, other polysaccharides such as dextran ($[\alpha 6\mathrm{Glc}]_n$), cellulose ($[\beta 4\mathrm{Glc}]_n$), or chitosan ($[\beta 4\mathrm{GlcN}]_n$) have many useful properties, but since they are not naturally anionic (negatively charged), these polymers do not mimic the natural extracellular matrix or blood vessel surfaces. Cellulose and dextran can be chemically transformed into charged polymers that help increase their biocompatibility and improve their general physicochemical properties, but harsh conditions are required leading to batch-to-batch variability and quality issues. On the other hand, GAGs, the natural polymers, have intrinsic negative charges.

[0087] HA and heparin have been employed as biomaterial coatings for vascular prosthesis and stents (artificial blood vessels and supports), as well as coatings on intraocular lenses and soft-tissue prostheses (7, 22). The rationale is to prevent blood clotting, enhance fouling resistance, and prevent post-surgery adhesion (when organs stick together in an undesirable fashion). The biomaterial compositions presently disclosed should also be suitable as a coating, as described in greater detail herein after.

[0088] A key advantage with heparosan is that it has increased biostability in the extracellular matrix when compared to other GAGs. As with most compounds synthesized in the body, new molecules are made, and after serving their purpose, are broken down into smaller constituents for recycling. Heparin and heparan sulfate are eventually degraded and turned over by a single enzyme known as heparanase (23, 24). Experimental challenge of heparosan and N-sulfo-heparosan with heparanase, however, shows that these polymers lacking O-sulfation are <u>not</u> sensitive to enzyme action *in vitro* (25, 26). These findings demonstrate that heparosan is not fragmented enzymatically in the body. Overall, this indicates that heparosan is a very stable biomaterial.

EXAMPLES

[0089] Examples are provided hereinbelow. However, the present invention is to be understood to not be limited in its application to the specific experimentation, results and laboratory procedures. Rather, the Examples are simply provided as one of various embodiments and are meant to be exemplary, not exhaustive.

EXAMPLE 1

[0090] Gene-optimized pmHS1 sequences for expression in *E. coli* and *Bacillus.* Three gene-optimized sequences encoding the *Pasteurella multocida* heparosan synthase of SEQ ID NO:2 were obtained. Two of the sequences (SEQ ID NOS:9 and 10) were gene-optimized for expression in *E. coli,* while the third sequence (SEQ ID NO:11) was gene-optimized for expression in *Bacillus.*

[0091] Figure 1A contains an alignment of the two *E. coli* gene-optimized sequences, SEQ ID NOS:9 and 10, with a native *Pasteurella multocida* heparosan synthase gene (SEQ ID NO:1). Figure 1B contains an alignment of only the two *E. coli* gene-optimized sequences, SEQ ID

NOS:9 and 10. Figure 1C contains an alignment of the *Bacillus* gene-optimized sequence (SEQ ID NO:11) with a native *Pasteurella multocida* heparosan synthase gene (SEQ ID NO:12).

[0092] Table 2 illustrates the percent identity between the two gene-optimized sequences of SEQ ID NOS:9-10 and the native *Pasteurella multocida* gene sequence (SEQ ID NO:1). Note that all three sequences encode amino acid sequences that are 100% identical to the amino acid sequence of SEQ ID NO:2. As can be seen, the two gene-optimized sequences are approximately 74% identical to the native *Pasteurella* gene sequence. It is also noted that the two gene-optimized sequences are only 95% identical to each other, so there is some variation obtained from the algorithm that is being used to generate the optimized sequence.

TABLE 2: Percent Identities of Gene-optimized and Native Heparosan Synthase Gene Sequences

	pmHS1 (SEQ ID NO:1)	pmHS1-opt1 (SEQ ID NO:9)	pmHS1-opt2 (SEQ ID NO:10)
pmHS1 (SEQ ID NO:1)		74.3%	73.7%
pmHS1-opt1 (SEQ ID NO:9)	74.3%		94.5%
pmHS1-opt2 (SEQ ID NO:10)	73.7%	94.5%	

EXAMPLE 2

[0093] Production of High MW Heparosan Polysaccharide. There are two types of naturally occurring micobes, (a) certain *Pasteurella multocida* bacteria (Type D) and their related brethren such as certain *Avibacteria,* and (b) *Escherichia coli* K5 and their related brethren that make an extracellular coating composed of unsulfated heparosan polymer that is readily harvested from the culture media. An unexpected and advantageous characteristic has been discovered for the recombinant (gene-optimized *Pasteurella* gene in an *E. coli* host) heparosan over both natural bacterial heparosan and mammalian heparin; the heparosan produced herein has a higher molecular weight of approximately 1 to 6.8 MDa (1,000 to 6,800 kDa); therefore, gels or liquid viscoelastics formed of this recombinant heparosan should be easier to produce.

[0094] *Transformation of gene-optimized pmHS1 into E. coli:* Synthetic *pmHS1* gene-optimized nucleotide sequence (SEQ ID NO:9) was obtained from GenScript USA Inc. (Piscataway, NJ) and ligated into a pKK223-3 plasmid. The plasmid containing the pmHS1 gene was then transformed into chemically competent *E. coli* K5 cells.

[0095] *Heparosan Production and Testing:* *E. coli* K5 cells expressing the gene-optimized *pmHS1* gene were grown in synthetic media at 30°C in a 14 L fermentor for approximately 40 hours. Spent culture medium (the liquid part of culture after microbial cells are removed) was harvested (by centrifugation at 10,000 x *g* for 60 minutes), and aliquots thereof were analyzed by agarose gel electrophorsis (1X TAE buffer, 0.8-1.5% agarose) followed by visualization with Stains-All (Lee & Cowman, Anal. Biochem., 1994). The heparosan polymer size was determined by comparison to monodisperse HA size standards (HiLadder, Hyalose, LLC).

[0096] The yield of the heparosan in the spent media was checked by carbazole assays for uronic acid. The carbazole assay is a spectrophotometric chemical assay that measures the amount of uronic acid in the sample via production of a pink color; every other sugar in the heparosan chain is a glucuronic acid. The detection limit of the carbazole assay is approximately 5 micrograms of polymer.

[0097] The identity of the polymer as heparosan was tested by heparin lyase III (*Pedobacter*) digestion; any heparin-like polysaccharide will be cleaved into small fragments (oligosaccharides) that run at the dye front on an agarose gel and do not stain well with Stains-All.

[0098] Various advantages of the presently disclosed heparosan are outlined in Tables 3 and 4.

Table 3:

Comparison of Heparosan and Existing Surgical Biomaterials for Coating Applications

Key Variable	Project Target	Current Practice	Associated Barrier of Current Procedure	Innovative Approaches of Inventive Concept(s)
Coating Stability	Long lasting (weeks-months).	HA, heparin, Bovine serum albumin (BSA)	Degraded by body's natural enzymes	Use heparosan, a polymer that is not enzymatically digested in human body.
		Carbon (C)	---	
		Lipids (L)	Shed from surface	
Wettability	Freely interacts with water.	BSA, HA, heparin, L	---	Use water-loving heparosan polymer.
		C	Hydrophobic	
Fouling, Clotting	Surface does not bind proteins or cells.	HA, heparin	Blood cells & clotting factors bind	Use relatively biologically inert heparosan polymer.
		BSA, C,L	---	
Disease Transmission	Zero risk of animal virus or prions.	HA [chicken], CG	Potential risk	Use non-animal, bacterially derived heparosan.
		HA [bacterial],	---	

Comparison of Heparosan and Existing Surgical Biomaterials for Coating Applications				
Key Variable	Project Target	Current Practice	Associated Barrier of Current Procedure	Innovative Approaches of Inventive Concept(s)
		PP, CHP		

Table 4:

Comparison of Heparosan and Existing Biomaterials for Surface Coating Applications				
Key Variable	**Project Target**	**Current Practice**	**Associated Barrier of Current Procedure**	**Innovative Approaches of Inventive Concept(s)**
Semi-stable Gel Formation	Injectable, Soft, long-lasting (>12-24 months), but not permanent gel.	Hyaluronan Gel (HA) Collagen Gel (CG)	Too short lifetime	Use heparosan, a polymer that is not enzymatically digested in human body, and is not a coarse, hard material.
		Plastic Particles (PP)	Grainy appearance & too long lifetime	
		Ca Hydroxyapatite Particles (CHP)	Grainy appearance too long lifetime, & cannot inject easily	
Immunogenicity, Allergenicity	No antibody generation.	HA [bacterial], PP, CHP	---	Use heparosan polymer that looks 'human' and does not trigger immune system.
		HA [chicken], CG [bovine>human]	Immune or allergic response	
Infiltration	Reduce cell adhesion and/or signaling.	HA	Proteins & cells bind	Use heparosan polymer that lacks known adhesion domains or chemotactic signals.
		PP, CHP	---	
		CG	Cells bind	
Disease Transmission	Zero risk of human or animal virus and/or prions.	HA [chicken], CG	Potential risk	Use non-animal, bacterially derived heparosan.
		HA [bacterial], PP, CHP	---	
X-ray Imaging Compatible	No opaque or marked areas.	HA, CG	---	Use X-ray-transparent heparosan.
		PP, CHP	Obscures images	

<table>
<tr><th colspan="5">Comparison of Heparosan and Existing Biomaterials for Surface Coating Applications</th></tr>
<tr><th>Key Variable</th><th>Project Target</th><th>Current Practice</th><th>Associated Barrier of Current Procedure</th><th>Innovative Approaches of Inventive Concept(s)</th></tr>
<tr><td rowspan="2">Abundant Resource</td><td rowspan="2">Renewable & not overly expensive to produce.</td><td>CG [human]</td><td>Limited tissue bank supply or cell culture derived (costly)</td><td rowspan="2">Use heparosan made via bacterial fermentation.</td></tr>
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EXAMPLE 3

[0099] ***<u>Production of mega-Dalton molecular weight heparosan.</u>*** Agarose gel analysis of ultra-high molecular weight heparosan polymer produced according to the method of Example 2 was performed. The agarose gel analysis (1X TAE, Stains-All detection) shown in Figure 2 demonstrated that the construct of the plasmid-borne recombinant *PmHS1* gene from *P. multocida* Type D in *E. coli* K5 **(Ec K5 + pmHS1)** produced a very high MW heparosan polymer (~1 to ~4.5 MDa; band marked with a *bracket*). As a negative control, the same *E. coli* host with vector alone **(Ec K5 + vector)** only produced a low MW polymer (~50 kDa to ~100 kDa; marked with an *arrow*). **Std** = SelectHA MegaLadder/SelectHA HiLadder/Select HA LoLadder (Hyalose LLC) with bands from top to bottom: 6100, 4570, 3050, 1510, 1090, 966, 572, 495, 310, 214, 110, 27 kDa (kDa = 1,000 Da; MDa = 1,000 kDa). Plasmids: **Vector** = (pKK223-3); **PmHS1** = (pKK223-3/PmHS1).

EXAMPLE 4

[0100] ***<u>Production of mega-Dalton molecular weight heparosan in E. coli BL21(DE3).</u>*** *E. coli* BL21(DE3) [NEB], an *E. coli* strain with distinct genetics from K5 and K12 strains, was transformed with either pKK223-3/gene-optimized PmHS1 (**P**) or pMAL-C4e/gene-optimized PmHS1 (**M**), an expression plasmid producing a maltose-binding protein (MBP)-PmHS1 fusion protein. Cultures of the transformants were induced with IPTG and then grown overnight in either LB (**LB**) or a synthetic media (**Syn**). The culture media was then clarified by centrifugation and the heparosan polymer concentrated by ethanol precipitation. The identity of the heparosan polymer was confirmed by digestion with heparin lyase III **(+LYASE).** The agarose gel analysis (1X TAE, Stains-All detection) shown in Figure 3 demonstrated that the construct of the plasmid-borne recombinant *PmHS1* gene from *P. multocida* Type D in *E. coli* BL21(DE3) produced a very high MW heparosan polymer (~2 to 6.8 MDa; extent of the high MW band marked with a *bracket)*. **Mega** = SelectHA MegaLadder (Hyalose LLC) with bands from top to bottom: 6100, 4570, 3050, 1510 kDa, **Std** = SelectHA HiLadder/SelectHA LoLadder (Hyalose LLC) with bands from top to bottom: 1510, 1090, 966, 572, 495, 310, 214, 110, 27 kDa (kDa = 1,000 Da; MDa = 1,000 kDa).

EXAMPLE 5

[0101] ***Production of mega-Dalton molecular weight heparosan in E. coli BL21 Express I^q.*** *E. coli* BL21Express I^q (NEB) was transformed with pMAL-C4e/gene-optimized PmHS1, an expression plasmid producing an MBP-PmHS1 fusion protein. Cultures of the transformants were induced with IPTG and then grown overnight in synthetic media. The culture media was then clarified by centrifugation, and the heparosan polymer concentrated by ethanol precipitation. The identity of the heparosan polymer was confirmed by digestion of the polymer (**START**) with heparin lyase III (**+LYASE**). The agarose gel analysis (1X TAE, Stains-All detection) shown in Figure 4 demonstrated that the construct of the plasmid-borne recombinant, gene-optimized *PmHS1* gene (encoding PmHS from *P. multocida* Type D) in *E. coli* BL21 Express I^q produced a very high MW heparosan polymer (~2 to 6.8 MDa; band marked with a *bracket*). **Mega** = SelectHA MegaLadder (Hyalose LLC) with bands from top to bottom: 6100, 4570, 3050, 1510 kDa.

EXAMPLE 6

[0102] ***Effect of deletion of heparosan production in E. coli K5 on production of mega-Dalton molecular weight heparosan.*** The *kfiA, kfiB,* and *kfiC* genes in *E. coli* K5 were deleted, and the resulting strain (**K5**-) no longer produces the 50-80 kDa heparosan usually produced by K5. The K5- strain was transformed with either pKK223-3/gene-optimized PmHS1 (**P**) or pMAL-C4e/gene-optimized PmHS1 (**M**). Cultures of the transformants were induced with IPTG and then grown overnight in either LB. The culture media was then clarified by centrifugation, and the heparosan polymer concentrated by ethanol precipitation. The identity of the heparosan polymer was confirmed by digestion with heparin lyase III (**+LYASE**). The agarose gel analysis (1X TAE, Stains-All detection) shown in Figure 5 demonstrated that the construct of the plasmid-borne recombinant gene-optimized *PmHS1* gene from *P. multocida* Type D, expressed in *E. coli* K5 with no *kfiA, kfiB,* or *kfiC* genes, produced a very high MW heparosan polymer (~2 MDa; band marked with a *bracket*). **Std** = SelectHA MegaLadder/SelectHA HiLadder/SelectHA LoLadder (Hyalose LLC) with bands from top to bottom: 6100, 4570, 3050, 1510, 1090, 966, 572, 495, 310, 214, 110, 27 kDa (kDa = 1,000 Da; MDa = 1,000 kDa).

[0103] Thus, the *kfiA, kfiB,* and *kfiC* genes are not involved in the production of ultra-high MW heparosan in *E. coli* K5.

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SEQUENCE LISTING

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gatgatagag tgtttcgctg tctctattat cttccgttag ccagtttgct ggtcttgaaa 180

tacaaatctg aagaatatta tttttcttac acaagagaga gaaatagata tcagccatgc 240

ctgaatgggt aaagtcagaa agagaaaatt gattaaagag actgactcta aagctaacag 300

ttcctgtacc taatacattg accgctttgt ctttttccag aggtttatag aagctatata 360

ccagtctatc cgccgaaaaa tatttggtca ttctacttgg aaagagaatg ccgtgtaaac 420

caataaccgc tttatcatcg tattcattca gcttcttgat catcgtattg atgtaatcgc 480

ttggatagat aatgtcatca tcacaggtta tataatatcc atcttgattt ttttcaatca 540

actcttccag taaaatgaat ttgccattat ctctaatgga gttatcttta tctttgcaat 600

gaacaacggt tgctttatta cctaaatttt ttatgaagtc agggatttct acatagccat 660

caagataaat atgaaaatga tcacattgat tttttagtat gccgataata cgtcgtaatt 720

gcgctattct tgagggaata gaacaaatat tgatataaac aggaatctta ggattggaca 780

acttactcat ttcttgtggt actggtaagg catcgtaaat acgagggaat tgaaaaagat 840

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tatcatcatc tacaataata atttctatgt ttttatatgt ttgtaacaat aatgaattaa 1500

tagaagcttc gataaattgc gctgtattgt gagatgtcat gataatactg actaatggat 1560

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tatatatttt ttcaaattct tgcagaacca attggagttg ttttaataaa agtttatttt 1740

cgttttcaag ggatgcggat agcggatgtt tactgtcctg ttttgccaat aaagtttgtt 1800

gagaaataat gtctttgttt aaagttgttt ttagactatc aattttattt tgaaaggtgt 1860

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<210> 4

<211> 651

<212> PRT

<213> Pasteurella multocida

<400> 4

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Asp Ser Leu Lys Thr Thr Leu Asn Lys Asp Ile Ile Ser Gln Gln Thr
35 40 45

Leu Leu Ala Lys Gln Asp Ser Lys His Pro Leu Ser Ala Ser Leu Glu
50 55 60

Asn Glu Asn Lys Leu Leu Leu Lys Gln Leu Gln Leu Val Leu Gln Glu
65 70 75 80

Phe Glu Lys Ile Tyr Thr Tyr Asn Gln Ala Leu Glu Ala Lys Leu Glu
85 90 95

Lys Asp Lys Gln Thr Thr Ser Ile Thr Asp Leu Tyr Asn Glu Val Ala
100 105 110

Lys Ser Asp Leu Gly Leu Val Lys Glu Thr Asn Ser Val Asn Pro Leu
115 120 125

Val Ser Ile Ile Met Thr Ser His Asn Thr Ala Gln Phe Ile Glu Ala
130 135 140

Ser Ile Asn Ser Leu Leu Leu Gln Thr Tyr Lys Asn Ile Glu Ile Ile
145 150 155 160

Ile Val Asp Asp Asp Ser Ser Asp Asn Thr Phe Glu Ile Ala Ser Arg
165 170 175

Ile Ala Asn Thr Thr Ser Lys Val Arg Val Phe Arg Leu Asn Ser Asn
180 185 190

Leu Gly Thr Tyr Phe Ala Lys Asn Thr Gly Ile Leu Lys Ser Lys Gly
195 200 205

Asp Ile Ile Phe Phe Gln Asp Ser Asp Asp Val Cys His His Glu Arg
210 215 220

Ile Glu Arg Cys Val Asn Ile Leu Leu Ala Asn Lys Glu Thr Ile Ala
225 230 235 240

Val Arg Cys Ala Tyr Ser Arg Leu Ala Pro Glu Thr Gln His Ile Ile
245 250 255

Lys Val Asn Asn Met Asp Tyr Arg Leu Gly Phe Ile Thr Leu Gly Met
260 265 270

His Arg Lys Val Phe Gln Glu Ile Gly Phe Phe Asn Cys Thr Thr Lys
275 280 285

Gly Ser Asp Asp Glu Phe Phe His Arg Ile Ala Lys Tyr Tyr Gly Lys
290 295 300

Glu Lys Ile Lys Asn Leu Leu Leu Pro Leu Tyr Tyr Asn Thr Met Arg
305 310 315 320

Glu Asn Ser Leu Phe Thr Asp Met Val Glu Trp Ile Asp Asn His Asn
325 330 335

Ile Ile Gln Lys Met Ser Asp Thr Arg Gln His Tyr Ala Thr Leu Phe
340 345 350

Gln Ala Met His Asn Glu Thr Ala Ser His Asp Phe Lys Asn Leu Phe
355 360 365

Gln Phe Pro Arg Ile Tyr Asp Ala Leu Pro Val Pro Gln Glu Met Ser
370 375 380

Lys Leu Ser Asn Pro Lys Ile Pro Val Tyr Ile Asn Ile Cys Ser Ile
385 390 395 400

Pro Ser Arg Ile Ala Gln Leu Arg Arg Ile Ile Gly Ile Leu Lys Asn
405 410 415

Gln Cys Asp His Phe His Ile Tyr Leu Asp Gly Tyr Val Glu Ile Pro
420 425 430

Asp Phe Ile Lys Asn Leu Gly Asn Lys Ala Thr Val Val His Cys Lys
435 440 445

Asp Lys Asp Asn Ser Ile Arg Asp Asn Gly Lys Phe Ile Leu Leu Glu
450 455 460

Glu Leu Ile Glu Lys Asn Gln Asp Gly Tyr Tyr Ile Thr Cys Asp Asp
465 470 475 480

Asp Ile Ile Tyr Pro Ser Asp Tyr Ile Asn Thr Met Ile Lys Lys Leu
485 490 495

Asn Glu Tyr Asp Asp Lys Ala Val Ile Gly Leu His Gly Ile Leu Phe
500 505 510

Pro Ser Arg Met Thr Lys Tyr Phe Ser Ala Asp Arg Leu Val Tyr Ser
515 520 525

Phe Tyr Lys Pro Leu Glu Lys Asp Lys Ala Val Asn Val Leu Gly Thr
530 535 540

Gly Thr Val Ser Phe Arg Val Ser Leu Phe Asn Gln Phe Ser Leu Ser
545 550 555 560

Asp Phe Thr His Ser Gly Met Ala Asp Ile Tyr Phe Ser Leu Leu Cys
565 570 575

Lys Lys Asn Asn Ile Leu Gln Ile Cys Ile Ser Arg Pro Ala Asn Trp
580 585 590

Leu Thr Glu Asp Asn Arg Asp Ser Glu Thr Leu Tyr His Gln Tyr Arg
595 600 605

Asp Asn Asp Glu Gln Gln Thr Gln Leu Ile Met Glu Asn Gly Pro Trp
610 615 620

Gly Tyr Ser Ser Ile Tyr Pro Leu Val Lys Asn His Pro Lys Phe Thr
625 630 635 640

Asp Leu Ile Pro Cys Leu Pro Phe Tyr Phe Leu
645 650

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<211> 1854
<212> DNA
<213> Pasteurella multocida

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gatatatgta aaaaaaatat aacacaatca aaaagtaata aaatagaaga agataatatt 180

tctggagaaa acaaattttc agtatcaata aaagatctat ataacgaaat aagcaatagt 240

gaattaggga ttacaaaaga aagactagga gcccccccctc tagtcagtat tataatgact 300

tctcataata cagaaaaatt cattgaagcc tcaattaatt cactattatt gcaaacatac 360

aataacttag aagttatcgt tgtagatgat tatagcacag ataaaacatt tcagatcgca 420

tccagaatag caaactctac aagtaaagta aaaacattcc gattaaactc aaatctaggg 480

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gatagcgatg atgtatgtca ccatgaaaga atcgaaagat gtgttaatgc attattatcg      600

aataaagata atatagctgt tagatgtgca tattctagaa taaatctaga aacacaaaat      660

ataataaaag ttaatgataa taaatacaaa ttaggattaa taactttagg cgtttataga      720

aaagtattta atgaaattgg tttttttaac tgcacaacca aagcatcgga tgatgaattt      780

tatcatagaa taattaaata ctatggtaaa aataggataa ataacttatt tctaccactg      840

tattataaca caatgcgtga agattcatta ttttctgata tggttgagtg ggtagatgaa      900

aataatataa agcaaaaaac ctctgatgct agacaaaatt atctccatga attccaaaaa      960

atacacaatg aaaggaaatt aaatgaatta aaagagattt ttagctttcc tagaattcat     1020

gacgccttac ctatatcaaa agaaatgagt aagctcagca accctaaaat tcctgtttat     1080

ataaatatat gctcaatacc ttcaagaata aaacaacttc aatacactat tggagtacta     1140

aaaaaccaat gcgatcattt tcatatttat cttgatggat atccagaagt acctgatttt     1200

ataaaaaaac tagggaataa agcgaccgtt attaattgtc aaaacaaaaa tgagtctatt     1260

agagataatg gaaagtttat tctattagaa aaacttataa aggaaaataa agatggatat     1320

tatataactt gtgatgatga tatccggtat cctgctgact acataaacac tatgataaaa     1380

aaaattaata aatacaatga taaagcagca attggattac atggtgttat attcccaagt     1440

agagtcaaca agtatttttc atcagacaga attgtctata attttcaaaa acctttagaa     1500

aatgatactg ctgtaaatat attaggaact ggaactgttg cctttagagt atctattttt     1560

aataaatttt ctctatctga ttttgagcat cctggcatgg tagatatcta tttttctata     1620

ctatgtaaga aaaacaatat actccaagtt tgtatatcac gaccatcgaa ttggctaaca     1680

gaagataaca aaaacactga gaccttattt catgaattcc aaaatagaga tgaaatacaa     1740

agtaaactca ttatttcaaa caacccttgg ggatactcaa gtatatatcc attattaaat     1800

aataatgcta attattctga acttattccg tgtttatctt tttataacga gtaa           1854
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<210> 6
<211> 617
<212> PRT
<213> Pasteurella multocida

<400> 6

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Glu Ser Leu Val Lys Tyr Asn Ile Asp Ile Cys Lys Lys Asn Ile Thr
        35                  40                  45

Gln Ser Lys Ser Asn Lys Ile Glu Glu Asp Asn Ile Ser Gly Glu Asn
    50                  55                  60

Lys Phe Ser Val Ser Ile Lys Asp Leu Tyr Asn Glu Ile Ser Asn Ser
65                  70                  75                  80
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65 70 75 80

Glu Leu Gly Ile Thr Lys Glu Arg Leu Gly Ala Pro Pro Leu Val Ser
85 90 95

Ile Ile Met Thr Ser His Asn Thr Glu Lys Phe Ile Glu Ala Ser Ile
100 105 110

Asn Ser Leu Leu Leu Gln Thr Tyr Asn Asn Leu Glu Val Ile Val Val
115 120 125

Asp Asp Tyr Ser Thr Asp Lys Thr Phe Gln Ile Ala Ser Arg Ile Ala
130 135 140

Asn Ser Thr Ser Lys Val Lys Thr Phe Arg Leu Asn Ser Asn Leu Gly
145 150 155 160

Thr Tyr Phe Ala Lys Asn Thr Gly Ile Leu Lys Ser Lys Gly Asp Ile
165 170 175

Ile Phe Phe Gln Asp Ser Asp Asp Val Cys His His Glu Arg Ile Glu
180 185 190

Arg Cys Val Asn Ala Leu Leu Ser Asn Lys Asp Asn Ile Ala Val Arg
195 200 205

Cys Ala Tyr Ser Arg Ile Asn Leu Glu Thr Gln Asn Ile Ile Lys Val
210 215 220

Asn Asp Asn Lys Tyr Lys Leu Gly Leu Ile Thr Leu Gly Val Tyr Arg
225 230 235 240

Lys Val Phe Asn Glu Ile Gly Phe Phe Asn Cys Thr Thr Lys Ala Ser
245 250 255

Asp Asp Glu Phe Tyr His Arg Ile Ile Lys Tyr Tyr Gly Lys Asn Arg
260 265 270

Ile Asn Asn Leu Phe Leu Pro Leu Tyr Tyr Asn Thr Met Arg Glu Asp
275 280 285

Ser Leu Phe Ser Asp Met Val Glu Trp Val Asp Glu Asn Asn Ile Lys
290 295 300

Gln Lys Thr Ser Asp Ala Arg Gln Asn Tyr Leu His Glu Phe Gln Lys
305 310 315 320

Ile His Asn Glu Arg Lys Leu Asn Glu Leu Lys Glu Ile Phe Ser Phe
325 330 335

Pro Arg Ile His Asp Ala Leu Pro Ile Ser Lys Glu Met Ser Lys Leu
340 345 350

Ser Asn Pro Lys Ile Pro Val Tyr Ile Asn Ile Cys Ser Ile Pro Ser
355 360 365

Arg Ile Lys Gln Leu Gln Tyr Thr Ile Gly Val Leu Lys Asn Gln Cys

370 375 380

Asp His Phe His Ile Tyr Leu Asp Gly Tyr Pro Glu Val Pro Asp Phe
385 390 395 400

Ile Lys Lys Leu Gly Asn Lys Ala Thr Val Ile Asn Cys Gln Asn Lys
405 410 415

Asn Glu Ser Ile Arg Asp Asn Gly Lys Phe Ile Leu Leu Glu Lys Leu
420 425 430

Ile Lys Glu Asn Lys Asp Gly Tyr Tyr Ile Thr Cys Asp Asp Asp Ile
435 440 445

Arg Tyr Pro Ala Asp Tyr Ile Asn Thr Met Ile Lys Lys Ile Asn Lys
450 455 460

Tyr Asn Asp Lys Ala Ala Ile Gly Leu His Gly Val Ile Phe Pro Ser
465 470 475 480

Arg Val Asn Lys Tyr Phe Ser Ser Asp Arg Ile Val Tyr Asn Phe Gln
485 490 495

Lys Pro Leu Glu Asn Asp Thr Ala Val Asn Ile Leu Gly Thr Gly Thr
500 505 510

Val Ala Phe Arg Val Ser Ile Phe Asn Lys Phe Ser Leu Ser Asp Phe
515 520 525

Glu His Pro Gly Met Val Asp Ile Tyr Phe Ser Ile Leu Cys Lys Lys
530 535 540

Asn Asn Ile Leu Gln Val Cys Ile Ser Arg Pro Ser Asn Trp Leu Thr
545 550 555 560

Glu Asp Asn Lys Asn Thr Glu Thr Leu Phe His Glu Phe Gln Asn Arg
565 570 575

Asp Glu Ile Gln Ser Lys Leu Ile Ile Ser Asn Asn Pro Trp Gly Tyr
580 585 590

Ser Ser Ile Tyr Pro Leu Leu Asn Asn Asn Ala Asn Tyr Ser Glu Leu
595 600 605

Ile Pro Cys Leu Ser Phe Tyr Asn Glu
610 615

<210> 7
<211> 565
<212> PRT
<213> Pasteurella multocida

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Lys Asp Ala Leu Thr Leu Tyr Glu Asn Ile Ala Lys Ile Tyr Gly Ser
20 25 30

Glu Ser Leu Val Lys Tyr Asn Ile Asp Ile Cys Lys Lys Asn Ile Thr
35 40 45

Gln Ser Lys Ser Asn Lys Ile Glu Glu Asp Asn Ile Ser Gly Glu Asn
50 55 60

Lys Phe Ser Val Ser Ile Lys Asp Leu Tyr Asn Glu Ile Ser Asn Ser
65 70 75 80

Glu Leu Gly Ile Thr Lys Glu Arg Leu Gly Ala Pro Pro Leu Val Ser
85 90 95

Ile Ile Met Thr Ser His Asn Thr Glu Lys Phe Ile Glu Ala Ser Ile
100 105 110

Asn Ser Leu Leu Leu Gln Thr Tyr Asn Leu Glu Val Ile Val Val Asp
115 120 125

Asp Tyr Ser Thr Asp Lys Thr Phe Gln Ile Ala Ser Arg Ile Ala Asn
130 135 140

Ser Thr Ser Lys Val Lys Thr Phe Arg Leu Asn Ser Asn Leu Gly Thr
145 150 155 160

Tyr Phe Ala Lys Asn Thr Gly Ile Leu Lys Ser Lys Gly Asp Ile Ile
165 170 175

Phe Phe Gln Ser Asp Asp Val Cys His His Glu Arg Ile Glu Arg Cys
180 185 190

Val Asn Ala Leu Leu Ser Asn Lys Asp Asn Ile Ala Val Arg Cys Ala
195 200 205

Tyr Ser Arg Ile Asn Leu Glu Thr Gln Asn Ile Ile Lys Val Asn Asp
210 215 220

Asn Lys Tyr Lys Leu Gly Leu Ile Thr Leu Gly Val Tyr Arg Lys Val
225 230 235 240

Phe Asn Glu Ile Gly Phe Phe Asn Cys Thr Thr Lys Ala Ser Asp Asp
245 250 255

Glu Phe Tyr His Arg Ile Ile Lys Tyr Tyr Gly Lys Asn Arg Ile Asn
260 265 270

Asn Leu Phe Leu Pro Leu Tyr Tyr Asn Thr Met Arg Glu Asp Ser Leu
275 280 285

Phe Ser Asp Met Val Glu Trp Val Asp Glu Asn Asn Ile Lys Gln Lys
290 295 300

Thr Ser Asp Ala Arg Gln Asn Tyr Leu His Glu Phe Gln Lys Ile His
305 310 315 320

Asn Glu Arg Lys Leu Asn Glu Leu Lys Glu Ile Phe Ser Phe Pro Arg
325 330 335

Ile His Asp Ala Leu Pro Ile Ser Lys Glu Met Ser Lys Leu Ser Asn
340 345 350

Pro Lys Ile Pro Val Tyr Ile Asn Ile Cys Ser Ile Pro Ser Arg Ile
355 360 365

Lys Gln Leu Gln Tyr Thr Ile Gly Val Leu Lys Asn Gln Cys Asp His
370 375 380

Phe His Ile Tyr Leu Asp Gly Tyr Pro Glu Val Pro Asp Phe Ile Lys
385 390 395 400

Lys Leu Gly Asn Lys Ala Thr Val Ile Asn Cys Gln Asn Lys Asn Glu
405 410 415

Ser Ile Arg Asp Asn Gly Lys Phe Ile Leu Leu Glu Lys Leu Ile Lys
420 425 430

Glu Asn Lys Asp Gly Tyr Tyr Ile Thr Cys Asp Asp Asp Ile Arg Tyr
435 440 445

Pro Ala Asp Tyr Thr Asn Thr Met Ile Lys Lys Ile Asn Lys Tyr Asn
450 455 460

Asp Lys Ala Ala Ile Gly Leu His Gly Val Ile Phe Pro Ser Arg Val
465 470 475 480

Asn Lys Tyr Phe Ser Ser Asp Arg Ile Val Tyr Asn Phe Gln Lys Pro
485 490 495

Leu Glu Asn Asp Thr Ala Val Asn Ile Leu Gly Thr Gly Thr Val Ala
500 505 510

Phe Arg Val Ser Ile Phe Asn Lys Phe Ser Leu Ser Asp Phe Glu His
515 520 525

Pro Gly Met Val Asp Ile Tyr Phe Ser Ile Leu Cys Lys Lys Asn Asn
530 535 540

Ile Leu Gln Val Cys Ile Ser Arg Pro Ser Asn Trp Leu Thr Glu Asp
545 550 555 560

Asn Lys Asn Thr Glu
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<210> 8

<211> 538

<212> PRT

<213> Pasteurella multocida

<400> 8

Ser Asn Ser Glu Leu Gly Ile Thr Lys Glu Arg Leu Gly Ala Pro Pro
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            20                  25                  30

Ala Ser Ile Asn Ser Leu Leu Leu Gln Thr Tyr Asn Leu Glu Val Ile
        35                  40                  45

Val Val Asp Asp Tyr Ser Thr Asp Lys Thr Phe Gln Ile Ala Ser Arg
    50                  55                  60

Ile Ala Asn Ser Thr Ser Lys Val Lys Thr Phe Arg Leu Asn Ser Asn
65                  70                  75                  80

Leu Gly Thr Tyr Phe Ala Lys Asn Thr Gly Ile Leu Lys Ser Lys Gly
                85                  90                  95

Asp Ile Ile Phe Phe Gln Ser Asp Asp Val Cys His His Glu Arg Ile
            100                 105                 110

Glu Arg Cys Val Asn Ala Leu Leu Ser Asn Lys Asp Asn Ile Ala Val
        115                 120                 125

Arg Cys Ala Tyr Ser Arg Ile Asn Leu Glu Thr Gln Asn Ile Ile Lys
    130                 135                 140

Val Asn Asp Asn Lys Tyr Lys Leu Gly Leu Ile Thr Leu Gly Val Tyr
145                 150                 155                 160

Arg Lys Val Phe Asn Glu Ile Gly Phe Phe Asn Cys Thr Thr Lys Ala
                165                 170                 175

Ser Asp Asp Glu Phe Tyr His Arg Ile Ile Lys Tyr Tyr Gly Lys Asn
            180                 185                 190

Arg Ile Asn Asn Leu Phe Leu Pro Leu Tyr Tyr Asn Thr Met Arg Glu
        195                 200                 205

Asp Ser Leu Phe Ser Asp Met Val Glu Trp Val Asp Glu Asn Asn Ile
    210                 215                 220

Lys Gln Lys Thr Ser Asp Ala Arg Gln Asn Tyr Leu His Glu Phe Gln
225                 230                 235                 240

Lys Ile His Asn Glu Arg Lys Leu Asn Glu Leu Lys Glu Ile Phe Ser

                245                 250                 255

Phe Pro Arg Ile His Asp Ala Leu Pro Ile Ser Lys Glu Met Ser Lys
            260                 265                 270

Leu Ser Asn Pro Lys Ile Pro Val Tyr Ile Asn Ile Cys Ser Ile Pro
        275                 280                 285

Ser Arg Ile Lys Gln Leu Gln Tyr Thr Ile Gly Val Leu Lys Asn Gln
    290                 295                 300
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Cys Asp His Phe His Ile Tyr Leu Asp Gly Tyr Pro Glu Val Pro Asp
305                 310                 315                 320

Phe Ile Lys Lys Leu Gly Asn Lys Ala Thr Val Ile Asn Cys Gln Asn
                325                 330                 335

Lys Asn Glu Ser Ile Arg Asp Asn Gly Lys Phe Ile Leu Leu Glu Lys
            340                 345                 350

Leu Ile Lys Glu Asn Lys Asp Gly Tyr Tyr Ile Thr Cys Asp Asp Asp
        355                 360                 365

Ile Arg Tyr Pro Ala Asp Tyr Thr Asn Thr Met Ile Lys Lys Ile Asn
    370                 375                 380

Lys Tyr Asn Asp Lys Ala Ala Ile Gly Leu His Gly Val Ile Phe Pro
385                 390                 395                 400

Ser Arg Val Asn Lys Tyr Phe Ser Ser Asp Arg Ile Val Tyr Asn Phe
                405                 410                 415

Gln Lys Pro Leu Glu Asn Asp Thr Ala Val Asn Ile Leu Gly Thr Gly
            420                 425                 430

Thr Val Ala Phe Arg Val Ser Ile Phe Asn Lys Phe Ser Leu Ser Asp
        435                 440                 445

Phe Glu His Pro Gly Met Val Asp Ile Tyr Phe Ser Ile Leu Cys Lys
    450                 455                 460

Lys Asn Asn Ile Leu Gln Val Cys Ile Ser Arg Pro Ser Asn Trp Leu
465                 470                 475                 480

Thr Glu Asp Asn Lys Asn Thr Glu Thr Leu Phe His Glu Phe Gln Asn
                485                 490                 495

Arg Asp Glu Ile Gln Ser Lys Leu Ile Ile Ser Asn Asn Pro Trp Gly
            500                 505                 510

Tyr Ser Ser Ile Tyr Pro Leu Leu Asn Asn Asn Ala Asn Tyr Ser Glu
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Leu Ile Pro Cys Leu Ser Phe Tyr Asn Glu
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<210> 9
<211> 1864
<212> DNA
<213> Artificial Sequence

<220>
<223> Pasteurella multocida heparosan synthase sequence gene-optimized for expression in E. coli

<400> 9

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ccagagcaaa ctgattatta gcaacaatcc gtggggctat agcagcatct atccgctgct    1800
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atcc                                                                 1864
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<210> 10
<211> 1864
<212> DNA
<213> Artificial Sequence

<220>
<223> Pasteurella multocida heparosan synthase sequence gene-optimized for expression in E. coli

<400> 10
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Patentkrav

1. Fremgangsmåde til rekombinant fremstilling af en højmolekylvægt heparosanpolymer, hvilken fremgangsmåde omfatter trinnene:

at dyrke en rekombinant værtscelle indeholdende en nukleotidsekvens der koder for et polypeptid med heparosansyntaseaktivitet under betingelser der er passende til ekspressionen af heparosansyntasen, hvor mindst en af:

(a) polypeptidet med heparosansyntaseaktivitet er mindst 90% identisk med mindst en af SEQ ID NO:2, 4, og 6-8, og nukleotidsekvensen der koder for polypeptidet er blevet gen-optimeret til ekspression i den rekombinante værtscelle;

(b) polypeptidet med heparosansyntaseaktivitet har 1-20 aminosyreadditioner, deletioner, og/eller substitutioner ved sammenligning med mindst en af SEQ ID NO:2, 4, og 6-8, og nukleotidsekvensen der koder for polypeptidet er blevet gen-optimeret til ekspression i den rekombinante værtscelle;

(c) polypeptidet er kodet af nukleotidsekvensen af mindst en af SEQ ID NO:9-11;

(d) polypeptidet er kodet af en nukleotidsekvens der er mindst 90% identisk med mindst en af SEQ ID NO:9-11; og

(e) nukleotidsekvensen koder for en *Pasteurella* heparosansyntase; og

at isolere heparosanpolymer fremstillet ved heparosansyntasen, hvor den isolerede heparosanpolymer er biokompatibel med en pattedyrspatient og biologisk inert inden i ekstracellulære afsnit hos en pattedyrspatient, og hvor den isolerede heparosanpolymer er repræsenteret ved strukturen (-GlcUA-beta1,4-GlcNAc-alpha-1,4-)$_n$, hvor n er et positivt heltal større end eller lig med 2.000.

2. Fremgangsmåde ifølge krav 1, hvor den rekombinante værtscelle endvidere omfatter mindst et gen der koder for et enzym til syntese af en heparosansukkerprecursor, hvor det mindst ene gen, der koder for et enzym til syntese af en heparosansukkerprecursor er valgt fra gruppen bestående af en pyrophosphorylase, en transferase, en mutase, en dehydrogenase og en epimerase, der er i stand til at fremstille UDP-GlcNAc eller UDP-GlcUA.

3. Fremgangsmåde ifølge krav 1 eller 2, endvidere omfattende mindst et af trinnene:

(a) at tværbinde den isolerede heparosanpolymer; og/eller

(b) at kovalent og/eller ikke-kovalent forbinde den isolerede heparosanpolymer med mindst en del af en overflade af et substrat; eventuelt hvor substratet er valgt fra gruppen bestående af silica, silicium, halvledere, glas, polymerer, nanorør, nanopartikler, organiske forbindelser, uorganiske forbindelser, metaller og kombinationer deraf; eventuelt hvor mindst en del af substratet er et metal valgt fra gruppen bestående af guld, kobber, rustfrit stål, nikkel, aluminium, titanium, termosensitive legeringer og kombinationer deraf.

4. Fremgangsmåde ifølge et hvilket som helst af kravene 1-3, hvor den rekombinante værtscelle er en *E. coli* rekombinant værtscelle.

5. Fremgangsmåde ifølge et hvilket som helst af kravene 1-4, hvor den isolerede heparosanpolymer endvidere er defineret som havende en værdi for n i et område fra ca. 2.000 til ca. 17.000.

6. Fremgangsmåde ifølge et hvilket som helst af kravene 1-5, hvor den isolerede heparosanpolymer i det væsentlige ikke er modtagelig for pattedyrshyaluronidaser eller -heparanaser og derved i det væsentlige ikke nedbrydes *in vivo* i ekstracellulære afsnit hos en pattedyrspatient.

7. Biomaterialesammensætning, hvilken sammensætning omfatter:

en isoleret heparosanpolymer, hvor den isolerede heparosanpolymer er biokompatibel med en pattedyrspatient og biologisk inert i ekstracellulære afsnit hos en pattedyrspatient, idet den isolerede heparosanpolymer er repræsenteret med strukturen (-GlcUA-beta1,4- GlcNAc-alpha-1,4-)$_n$, hvor *n* er et positivt heltal, der er større end eller lig med 2.000.

8. Biomaterialesammensætning ifølge krav 7, hvor den isolerede heparosanpolymer endvidere er defineret som havende en værdi n i et område fra ca. 2.000 til ca. 17.000.

9. Biomaterialesammensætning ifølge krav 7 eller 8, hvor heparosanpolymeren er lineær.

10. Biomaterialesammensætning ifølge krav 7 eller 8, hvor heparosanpolymeren er tværbunden.

11. Biomaterialesammensætning ifølge et hvilket som helst af kravene 7-10, endvidere defineret som værende i en gel, halvfast, og/eller partikelformet tilstand, og hvor biomaterialesammensætningen endvidere er defineret som værende en implanterbar biomaterialesammensætning.

12. Biomaterialesammensætning ifølge et hvilket som helst af kravene 7-10, endvidere defineret som værende en flydende biomaterialesammensætning, og hvor biomaterialesammensætningen endvidere er defineret som værende en injicerbar biomaterialesammensætning.

13. Biomaterialesammensætning ifølge et hvilket som helst af kravene 7-12, endvidere omfattende et substrat som den isolerede heparosanpolymer er kovalent og/eller ikke-kovalent forbundet med, eventuelt hvor substratet er valgt fra gruppen bestående af silica, silicium, halvledere, glas, polymerer, nanorør, nanopartikler, organiske forbindelser, uorganiske forbindelser, metaller og kombinationer deraf; eventuelt hvor mindst en del af substratet er et metal valgt fra gruppen bestående af guld, kobber, rustfrit stål, nikkel, aluminium, titanium, termosensitive legeringer og kombinationer deraf.

14. Biomaterialesammensætning ifølge et hvilket som helst af kravene 7-13, hvor den isolerede heparosanpolymer i det væsentlige ikke er modtagelig for pattedyrshyaluronidaser eller -heparanaser og derved i det væsentlige ikke nedbrydes *in vivo* i ekstracellulære afsnit hos en pattedyrspatient.

15. Isoleret nukleotidsekvens der koder for et polypeptid med heparosansyntaseaktivitet, hvor mindst en af:

(a) polypeptidet med heparosansyntaseaktivitet er identisk med mindst en af SEQ ID NO:2, 4, og 6-8, og nukleotidsekvensen, der koder for polypeptidet, er blevet gen-optimeret til ekspression i den rekombinante værtscelle; og

(b) polypeptidet med heparosansyntaseaktivitet har 1-20 aminosyreadditioner, -deletioner og/eller -substitutioner ved sammenligning med mindst en af SEQ ID NO: 2, 4, og 6-8, og nukleotidsekvensen, der koder for polypeptidet, er blevet gen-optimeret til ekspression i den rekombinante værtscelle;

(c) polypeptidet er kodet af nukleotidsekvensen af mindst en af SEQ ID NO; 9-11; og

(d) polypeptidet er kodet af en nukleotidsekvens der er mindst 90% identisk med mindst en af SEQ ID NO:9-11.

16. Isoleret nukleotidsekvens ifølge krav 15, hvor værtscellen, som nukleotidsekvensen er blevet kodon-optimeret for, er *E. coli.*

17. Rekombinant værtscelle, omfattende den isolerede nukleotidsekvens ifølge krav 15 eller 16.

DRAWINGS

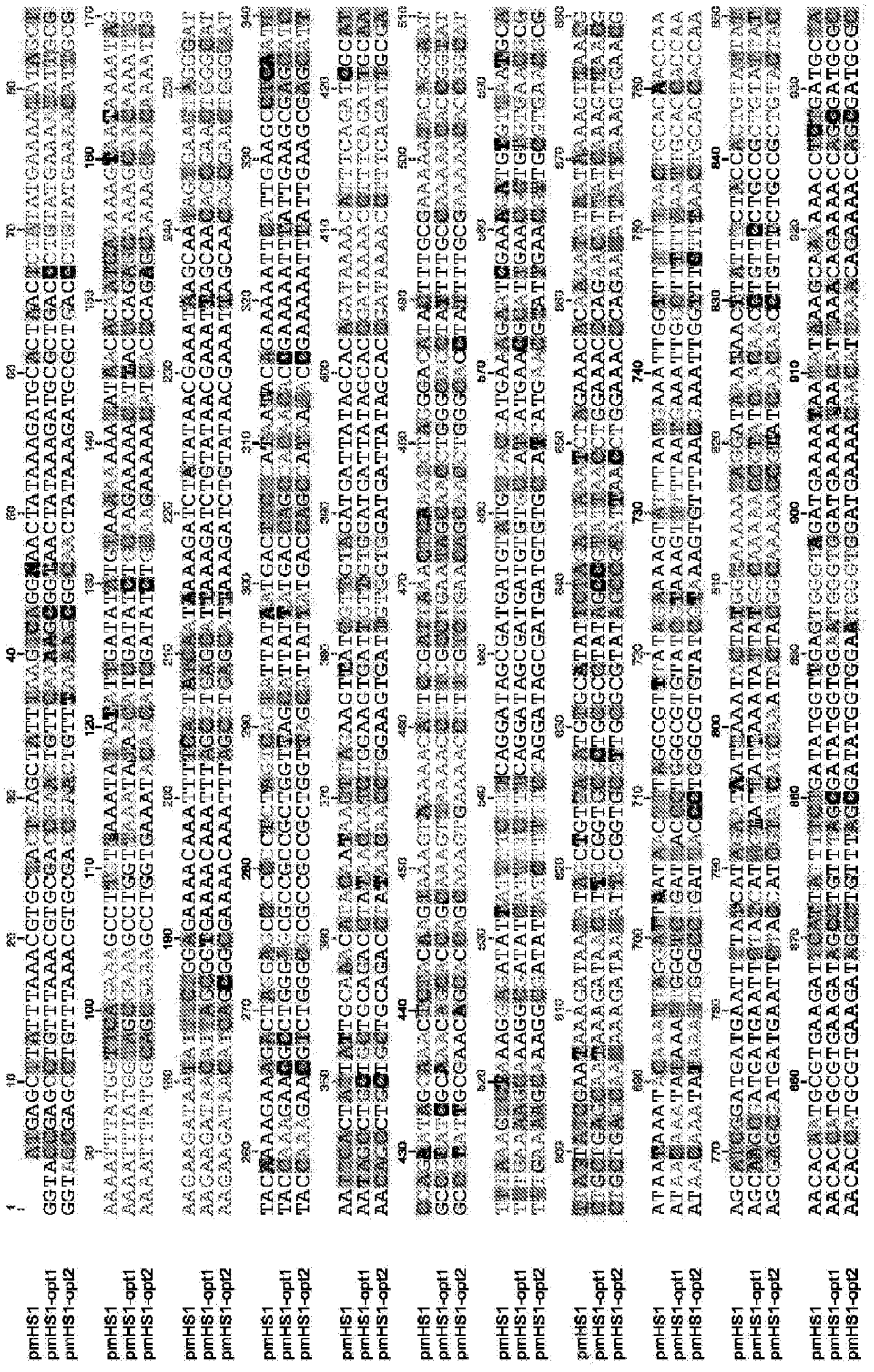

FIGURE 1A

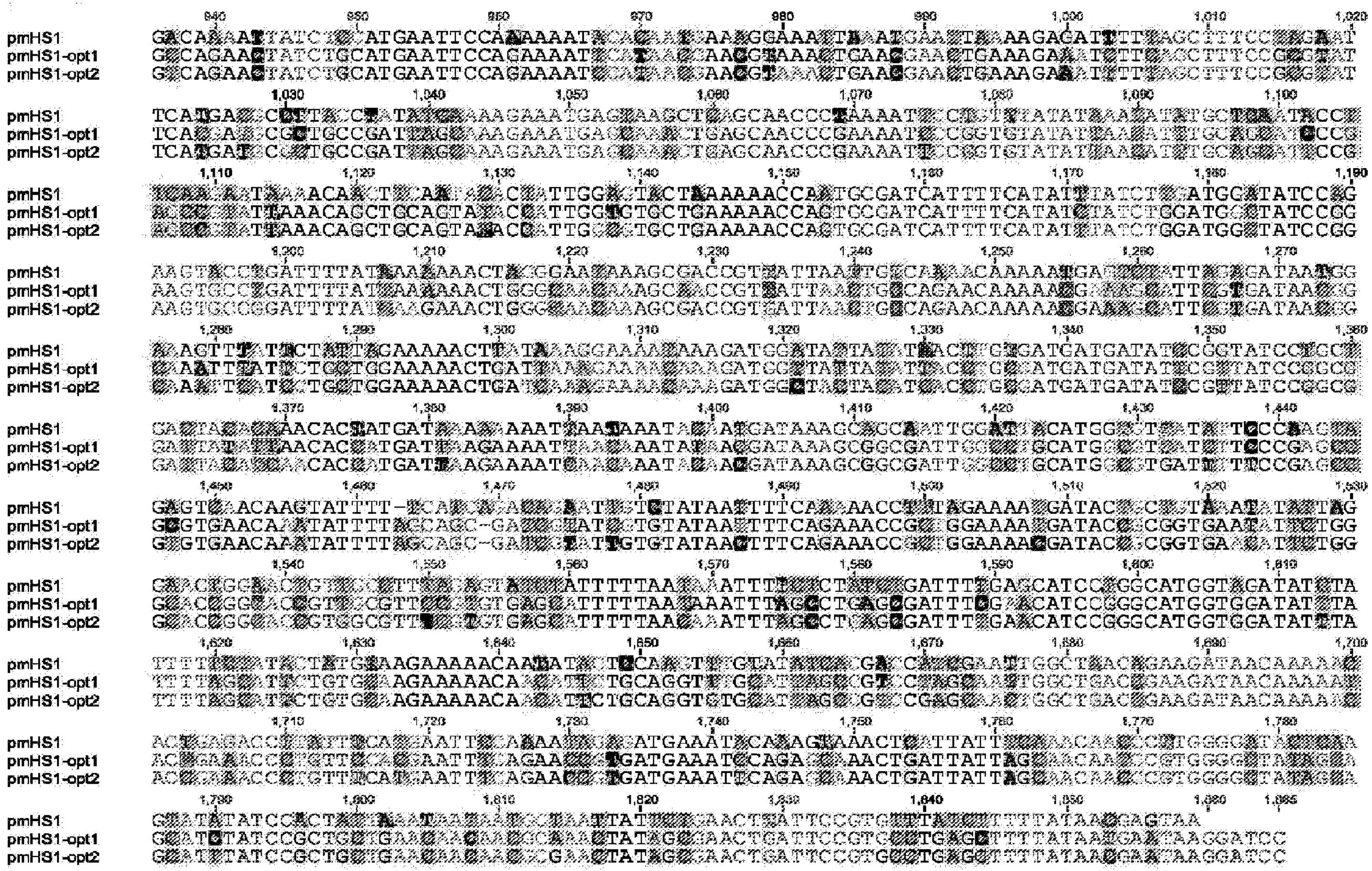

FIGURE 1A (Continued)

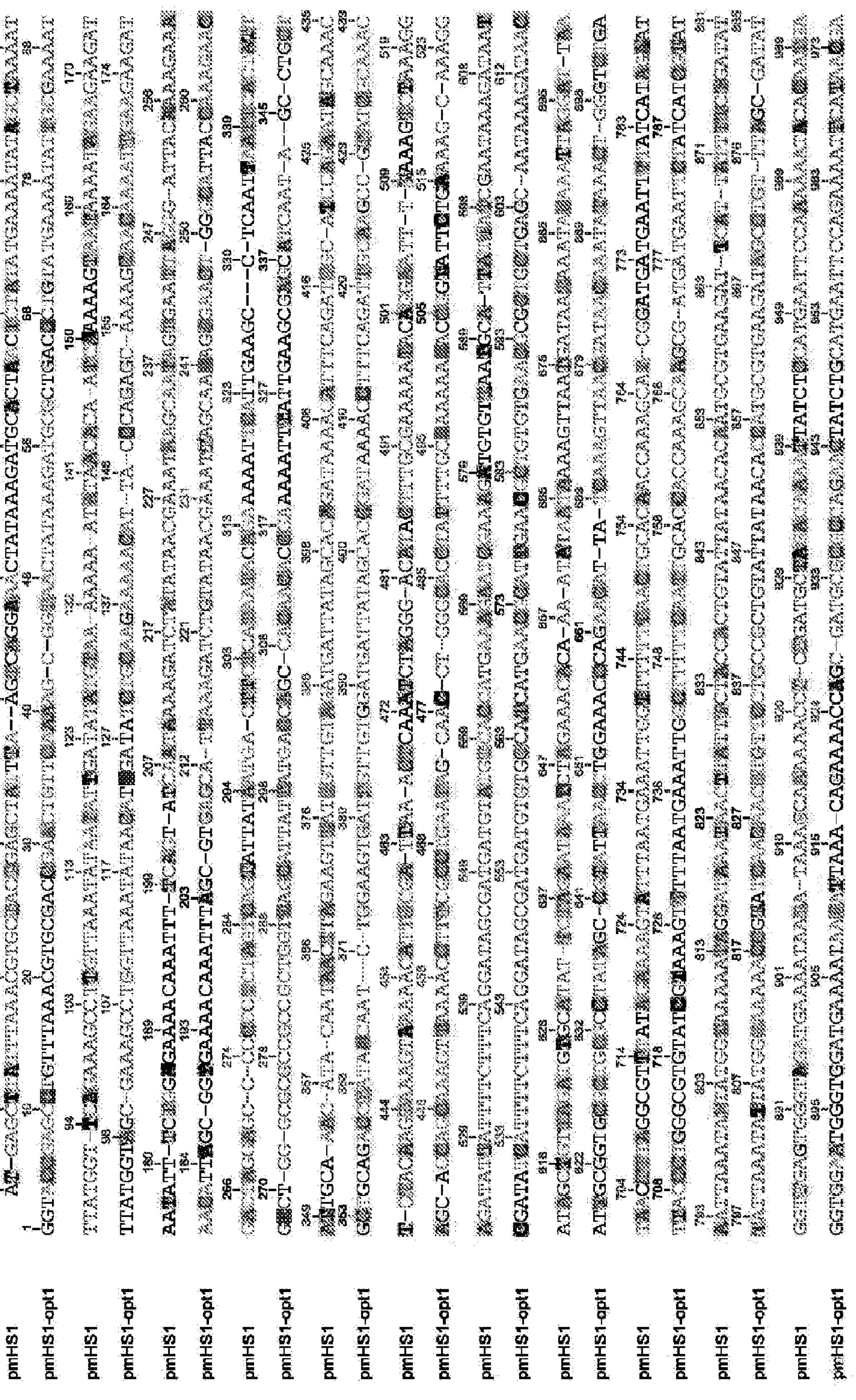

FIGURE 1B

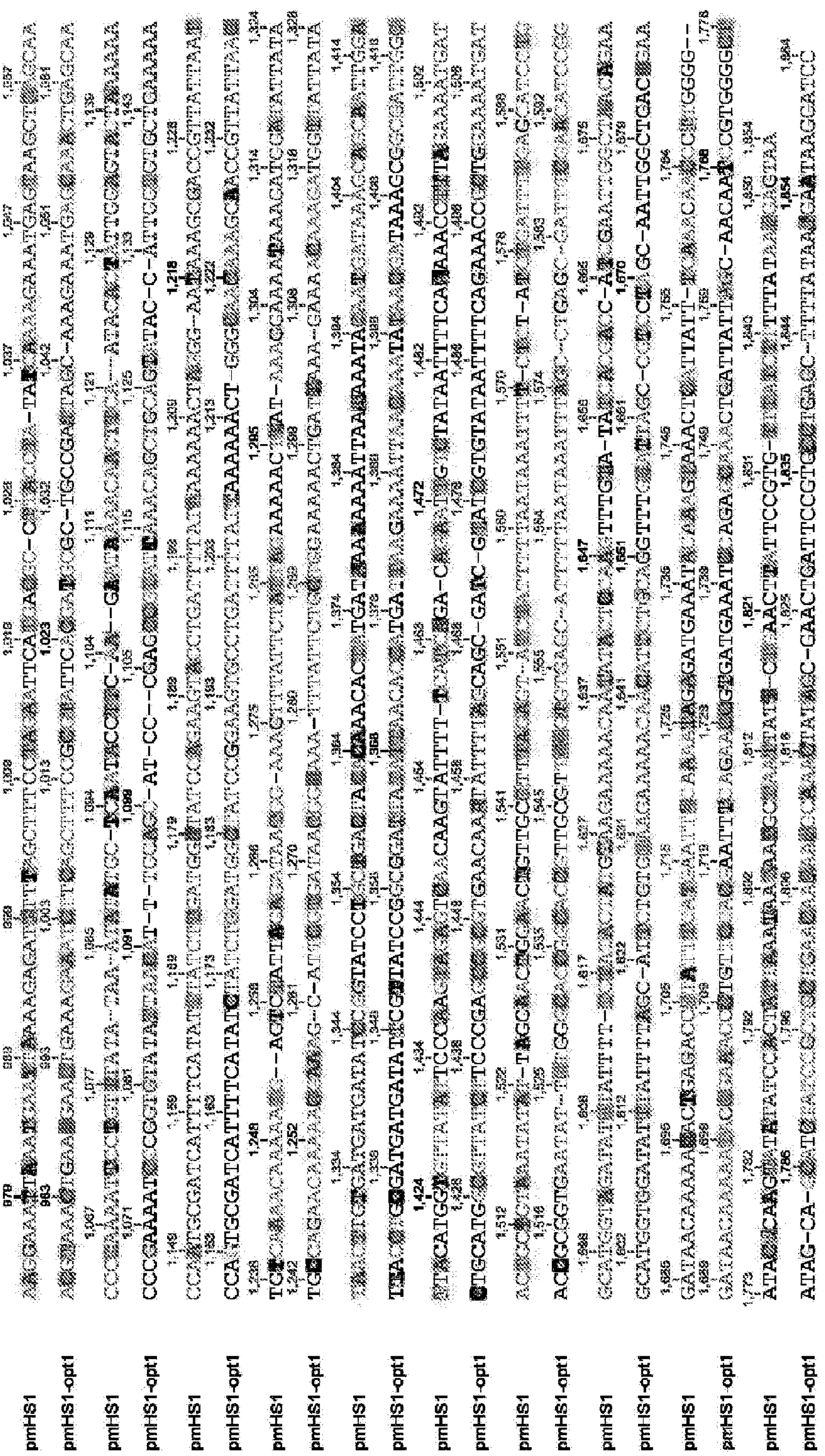

FIGURE 1B (Continued)

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PmHS-opt3    37 ATGGGTACCTCACTGTTTAAACGTGCTACGGAACTTTTTAAAAGCGGAAACTACAAAGAT
Native pmHS  37 ATGGGTACCTCACTGTTTAAACGTGCTACAGAACTTTTTAAAAGCGGCAACTACAAAGAT

PmHS-opt3    97 GCTCTTACATTGTACGAAAACATCGCCAAAATCTATGGCAGCGAATCTCTGGTTAAATAC
Native pmHS  97 GCTCTTACGTTGTACGAAAACATCGCCAAAATCTACGGAAGCGAATCTCTGGTTAAATAC

PmHS-opt3    157 AACATCGATATCTGCAAGAAAAATATCACGCAATCAAAAAGCAACAAAATCGAAGAAGAT
Native pmHS  157 AACATCGATATCTGCAAGAAAAATATTACACAATCAAAAAGCAACAAAATCGAAGAAGAT

PmHS-opt3    217 AACATCTCAGGAGAAAACAAATTTTCTGTTTCAATCAAAGATTTATATAACGAAATTAGC
Native pmHS  217 AACATCTCAGGCGAAAACAAATTTTCTGTTTCAATCAAAGATTTATATAACGAAATTAGC

PmHS-opt3    277 AATTCTGAATTGGGCATCACAAAAGAACGGTTAGGCGCACCGCCTTTGGTGTCTATTATC
Native pmHS  277 AATTCTGAATTGGGCATCACAAAAGAACGGTTAGGAGCTCCGCCTTTGGTGTCTATTATC

PmHS-opt3    337 ATGACATCACATAACACGGAAAAATTTATCGAAGCCAGCATCAACTCTCTGCTTTTACAG
Native pmHS  337 ATGACATCACATAACACGGAAAAATTTATCGAAGCCAGCATCAACTCTCTGCTTTTGCAG

PmHS-opt3    397 ACATACAACAACCTTGAAGTCATCGTTGTGGATGATTACTCTACAGATAAAACGTTTCAA
Native pmHS  397 ACATACAACAACCTTGAAGTCATCGTTGTGGATGATTACTCTACAGATAAAACGTTTCAA

PmHS-opt3    457 ATCGCTTCAAGAATCGCCAATTCAACAAGCAAAGTAAAAACGTTTCGCTTAAACAGCAAT
Native pmHS  457 ATCGCTTCAAGAATCGCCAATTCAACAAGCAAAGTAAAAACGTTTCGCTTAAACAGCAAC

PmHS-opt3    517 TTGGGCACATACTTTGCTAAAAACACGGGCATCTTAAAAAGCAAAGGAGATATCATTTTC
Native pmHS  517 TTGGGAACATACTTTGCTAAAAACACGGGCATCTTGAAAAGCAAAGGAGATATCATTTTC

PmHS-opt3    577 TTTCAGGATTCTGATGATGTCTGCCATCATGAAAGAATTGAACGCTGTGTAAATGCCTTG
Native pmHS  577 TTTCAGGATTCTGATGATGTCTGCCATCATGAAAGAATTGAACGCTGTGTAAATGCCTTG

PmHS-opt3    637 CTGAGCAACAAAGATAATATTGCAGTCCGTTGCGCGTATTCTCGGATCAACCTGGAAACA
Native pmHS  637 CTGAGCAACAAAGATAATATTGCAGTCCGTTGCGCGTATTCTCGGATCAACCTGGAAACA

PmHS-opt3    697 CAAAACATCATCAAAGTAAACGATAACAAATACAAATTGGGCCTGATTACGCTTGGAGTT
Native pmHS  697 CAAAACATCATCAAAGTAAACGATAACAAATACAAATTGGGCCTGATTACGCTTGGAGTT

PmHS-opt3    757 TATCGTAAAGTGTTTAACGAAATCGGCTTTTTCAATTGTACAACGAAAGCCTCTGATGAT
Native pmHS  757 TATCGTAAAGTGTTTAACGAAATCGGCTTTTTCAATTGTACAACGAAAGCCTCTGATGAT

PmHS-opt3    817 GAATTTTACCATAGAATCATCAAATACTATGGAAAAAATCGCATTAATAACCTGTTTCTG
Native pmHS  817 GAATTTTACCATAGAATCATCAAATACTATGGAAAAAATCGCATTAATAACCTGTTTCTG

PmHS-opt3    877 CCGTTGTACTACAACACAATGCGTGAAGATTCATTATTTAGCGATATGGTCGAATGGGTA
Native pmHS  877 CCGTTGTACTACAACACAATGCGTGAAGATTCATTATTTAGCGATATGGTCGAATGGGTA

PmHS-opt3    937 GATGAAAACAACATCAAACAAAAAACGTCAGATGCACGGCAGAACTACTTGCATGAATTT
Native pmHS  937 GATGAAAACAACATCAAACAAAAAACGTCAGATGCACGGCAGAACTACTTGCATGAATTT
```

FIGURE 1C

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PmHS-opt3    997  CAAAAAATCCATAACGAACGTAAACTGAACGAACTTAAAGAAATTTTTAGCTTTCCGAGA
Native pmHS  997  CAAAAAATCCATAACGAACGTAAACTGAACGAACTTAAAGAAATTTTTAGCTTTCCGCGG

PmHS-opt3    1057 ATTCATGATGCGCTGCCTATCTCAAAAGAAATGTCTAAACTTTCAAACCCGAAAATCCCT
Native pmHS  1057 ATCCATGATGCGCTGCCTATCTCAAAAGAAATGTCTAAACTTTCAAACCCGAAAATCCCT

PmHS-opt3    1117 GTTTACATCAACATTTGCTCAATTCCGTCTCGCATCAAACAATTACAGTACACAATCGGA
Native pmHS  1117 GTTTACATCAACATCTGCAGCATTCCGTCTCGCATCAAACAATTACAGTATACAATTGGC

PmHS-opt3    1177 GTGTTGAAAAACCAGTGTGATCATTTTCATATCTACTTGGATGGCTATCCGGAAGTTCCT
Native pmHS  1177 GTGTTGAAAAACCAGTGTGATCATTTTCATATCTACTTGGATGGCTATCCGGAAGTTCCT

PmHS-opt3    1237 GATTTTATCAAAAAATTGGGAAACAAAGCAACGGTGATCAACTGCCAAAACAAAAACGAA
Native pmHS  1237 GATTTTATCAAAAAATTGGGAAACAAAGCAACGGTGATCAACTGCCAAAACAAAAACGAA

PmHS-opt3    1297 AGCATCAGAGATAACGGCAAATTTATCCTTTTAGAAAAATTGATCAAAGAAAACAAAGAT
Native pmHS  1297 AGCATCAGAGATAACGGCAAATTTATCCTTTTGGAAAAATTGATCAAAGAAAACAAAGAT

PmHS-opt3    1357 GGATACTACATCACATGTGATGATGATATTCGCTATCCTGCGGATTATATTAATACGATG
Native pmHS  1357 GGATACTACATCACATGTGATGATGATATTCGCTATCCTGCGGATTATATTAATACGATG

PmHS-opt3    1417 ATTAAGAAAATTAACAAATACAACGATAAAGCAGCGATCGGCCTGCATGGAGTTATCTTT
Native pmHS  1417 ATTAAGAAAATTAACAAATACAACGATAAAGCAGCGATCGGCCTGCATGGAGTTATCTTT

PmHS-opt3    1477 CCGTCTCGTGTGAACAAATACTTTTCAAGCGATCGGATCGTCTACAACTTTCAGAAACCT
Native pmHS  1477 CCGTCTCGTGTGAACAAATACTTTTCAAGCGATCGGATCGTCTACAACTTTCAGAAACCT

PmHS-opt3    1537 TTAGAAAACGATACAGCAGTAAACATCTTGGGCACAGGAACGGTCGCGTTTAGAGTATCA
Native pmHS  1537 TTGGAAAACGATACAGCAGTAAACATCTTGGGCACAGGAACGGTCGCGTTTAGAGTATCA

PmHS-opt3    1597 ATCTTTAACAAATTTTCTCTGTCAGATTTTGAACATCCGGGCATGGTTGATATCTACTTT
Native pmHS  1597 ATCTTTAACAAATTTTCTCTGTCAGATTTTGAACATCCGGGCATGGTTGATATCTACTTT

PmHS-opt3    1657 AGCATCCTGTGCAAGAAAAATAACATCCTTCAAGTGTGTATCTCAAGACCTAGCAATTGG
Native pmHS  1657 AGCATCCTGTGCAAGAAAAATAACATCCTTCAAGTGTGTATCTCAAGACCTAGCAATTGG

PmHS-opt3    1717 CTGACAGAAGATAACAAAAACACAGAAACGCTTTTTCATGAATTTCAAAACCGCGATGAA
Native pmHS  1717 CTGACAGAAGATAACAAAAACACAGAAACGCTTTTTCATGAATTTCAAAACCGCGATGAA

PmHS-opt3    1777 ATCCAGAGCAAACTTATCATCTCTAACAACCCGTGGGGATATTCTTCAATCTACCCTTTG
Native pmHS  1777 ATCCAGAGCAAACTTATCATCTCTAACAACCCGTGGGGATACTCTTCAATCTACCCTTTG

PmHS-opt3    1837 CTGAACAACAACGCAAACTACTCAGAACTGATCCCGTGTCTTAGCTTTTATAACGAATAA
Native pmHS  1837 CTGAACAACAACGCAAACTACTCAGAACTGATCCCGTGTCTTAGCTTTTATAACGAATAA
```

FIGURE 1C (continued)

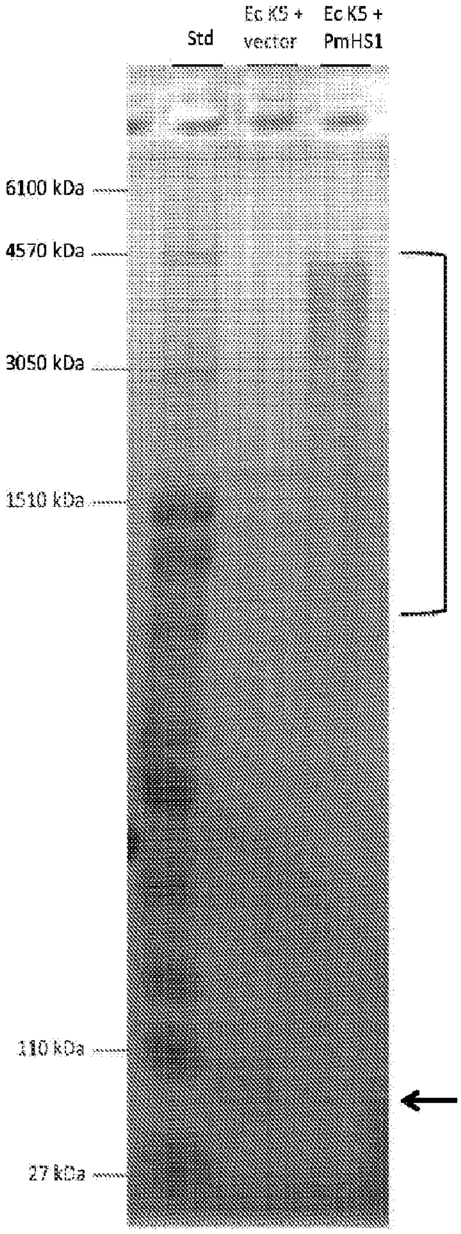
Std
Ec K5 + vector
Ec K5 + PmHS1
6100 kDa
4570 kDa
3050 kDa
1510 kDa
110 kDa
27 kDa

FIGURE 2

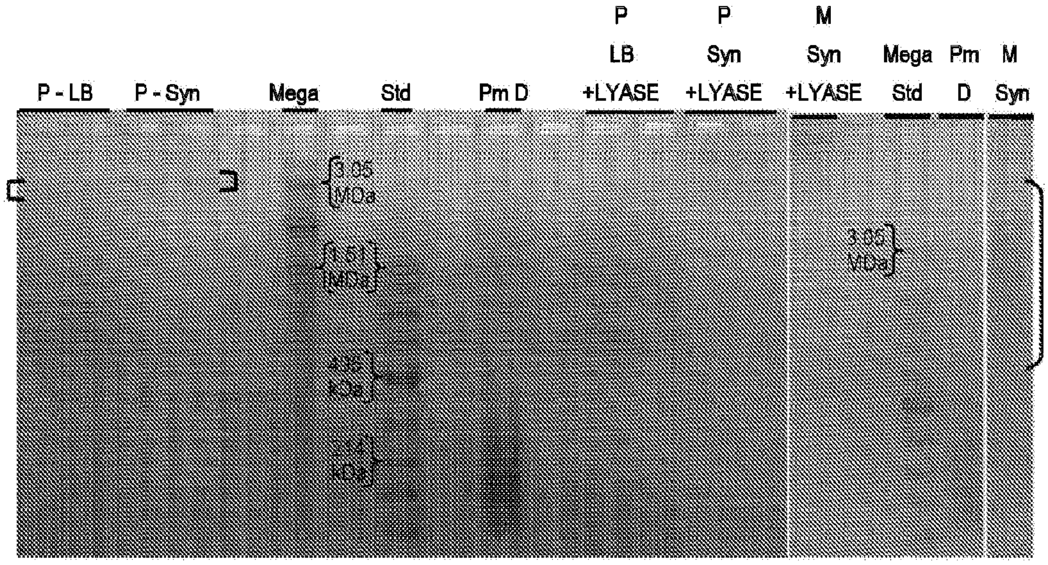
P - LB
P - Syn
Mega
Std
Pm D
P
LB
+LYASE
P
Syn
+LYASE
M
Syn
+LYASE
Mega
Std
Pm
D
M
Syn
3.05
MDa
1.51
MDa
436
kDa
214
kDa
3.05
MDa

FIGURE 3

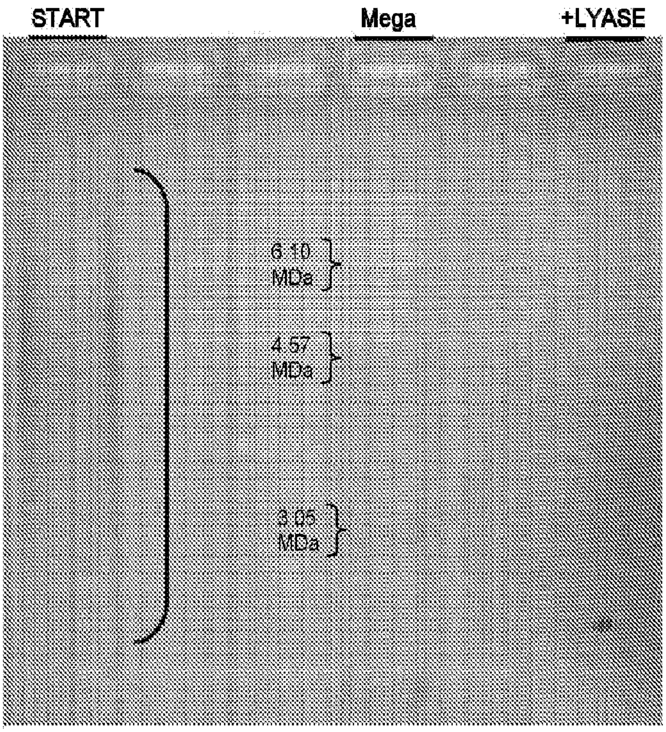

FIGURE 4

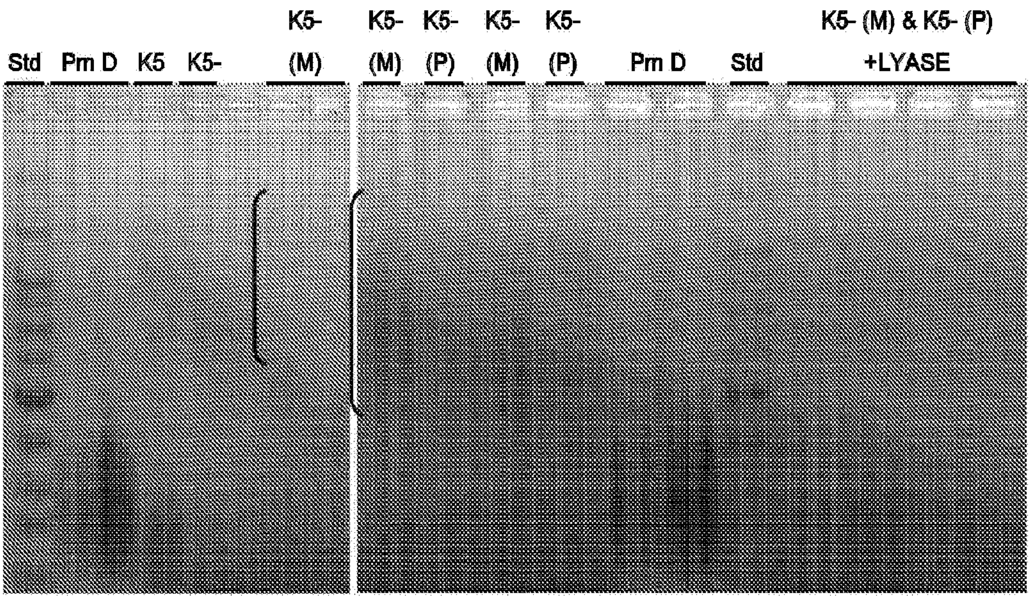

FIGURE 5