(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 July 2017 (06.07.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/117229 A1

(51) International Patent Classification:
A61F 2/08 (2006.01) *C12N 5/07* (2010.01)
A61L 27/38 (2006.01) *C12N 5/0797* (2010.01)

(21) International Application Number:
PCT/US2016/068891

(22) International Filing Date:
28 December 2016 (28.12.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/273,161 30 December 2015 (30.12.2015) US

(71) Applicant: **WAKE FOREST UNIVERSITY HEALTH SCIENCES** [US/US]; Medical Center Boulevard, Winston-Salem, North Carolina 27157 (US).

(72) Inventor: **BITAR, Khalil**; 631 Gramercy Street, Winston-Salem, North Carolina 27104 (US).

(74) Agent: **ENGELLENNER, Thomas, J.**; Pepper Hamilton LLP, 19th Floor, High Street Tower, 125 High Street, Boston, Massachusetts 02110 (US).

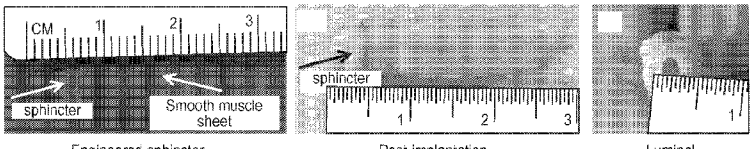
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

(54) Title: TISSUE-ENGINEERED GUT-SPHINCTER COMPLEXES AND METHODS OF MAKING THE SAME



Engineered sphincter-rectal cuff pre-implantation

FIG. 1A

Post implantation - sphincter marked with sutures

FIG. 1B

Luminal patency

FIG. 1C

(57) Abstract: Methods are disclosed for forming tissue engineered, tubular gut- sphincter complexes from intestinal circular smooth muscle cells, sphincteric smooth muscle cells and enteric neural progenitor cells. The intestinal smooth muscle cells and neural progenitor cells can be seeded on a mold with a surface texture that induces longitudinal alignment of the intestinal smooth muscle cells and co-cultured until an innervated aligned smooth muscle sheet is obtained. The innervated smooth muscle sheet can then be wrapped around a tubular scaffold to form an intestinal tissue construct. Additionally, the sphincteric smooth muscle cells and additional enteric neural progenitor cells can be mixed in a biocompatiable gel solution, and the gel and admixed cells applied to a mold having a central post such that the sphinteric smooth muscle and neural progenitor cells can be cultured to form an innerv - ated sphincter construct around the mold post.

WO 2017/117229 A1

TISSUE-ENGINEERED GUT-SPHINCTER COMPLEXES AND METHODS OF MAKING THE SAME

GOVERNMENT RIGHTS

[0001] This invention was made with government support from National Institutes of Diabetes, Digestive and Kidney Diseases under NIH/NIDDK Grant No. RO1DK071614 The United States Government has certain rights in the invention.

FIELD OF THE INVENTION

[0002] The present invention concerns bioengineering of tubular tissue structures, such as gastrointestinal tissues and, in particular, tissue-engineered gut-sphincter complexes.

BACKGROUND

[0003] The gastrointestinal (GI) tract or "gut" is a structurally complex hollow organ that displays diverse motility patterns to perform a variety of functions that aid in ingestion, digestion, absorption of nutritive elements, and excretion of waste. Gastrointestinal motility is a result of chemical and electrical interactions between smooth muscle, intramural innervation, interstitial cells, and mucosal epithelial layers.

[0004] Phasic neuromuscular structures of the GI tract contain orthogonal layers of smooth muscle, interlaced with enteric neuronal plexuses. They are also associated with interstitial cells and specialized mucosal layers. The propagating peristaltic wave defines the phasic nature of this neuromusculature. It encompasses contraction and relaxation of both the circular and longitudinal smooth muscle layers. The neuronal components as well as the interstitial cells generate electrical activity for the coordination of peristalsis. This activity is coupled in a highly coordinated manner to intracellular biochemical events in the smooth muscle layers to result in gut motility.

[0005] The principal components of the GI tract are the small intestine, the colon and the anal sphincter. The small intestine is the primary nutrient absorptive structure of the GI tract. Peristalsis and segmental contractions of the small intestine increase the surface area to facilitate greater absorption by the villi of the intestinal epithelium. The colon is contiguous with the small intestine, facilitating water absorption, and excretion of stool. The internal anal sphincter (IAS) contributes to 70% of the anal canal closure pressure, maintaining

continence. Weakened mechanical efficiency of the IAS due to idiopathic sphincteric degeneration, surgical, or obstetric trauma all lead to passive and active incontinence.

[0006] In addition to the anal sphincter, the gastrointestinal system has several other sphincters that control the transit of fluids through the gut, including the lower esophageal sphincter (LES) and the pyloric sphincter at the exit of the stomach.

[0007] In the basal state, the smooth muscle of the sphincters remains in a state of tonic contraction and closure to serve as a one-way valve to regulate flow through the opening controlled by the sphincter. Skeletal muscle sphincters are under voluntary control while smooth muscle sphincters are controlled by complex interactions between extrinsic nerves from the central nervous system (CNS) and intrinsic control by the enteric nervous system and the myogenic properties of specialized smooth muscle cells.

[0008] Sphincteric smooth muscles represent tonic muscles that remain contracted at rest and have small amplitude, slow contraction and slow relaxation response, while non-sphincteric smooth muscle represent phasic muscle that shows a wide range of contractile activity varying from a fully relaxed basal state to a large-amplitude rapid contraction and rapid relaxation response (Goyal et al., The Gastrointestinal System, Motility and Circulation, in Handbook of Physiology, J. D. Wood and S. G. Schultz, Editors. 1989, The American Physiological Society: Bethesda. p. 865-908).

[0009] There exists a need for functional tissue-engineered sphincteric constructs for repair and/or reconstruction of damaged sphincters as well as tissue engineered gut-sphincter complexes for gut-lengthening and other therapeutic interventions. Alternatively, such constructs could provide functional *in vitro* models of sphincters and gut segments for development of therapies and/or drug testing.

SUMMARY

[0010] Methods and constructs are disclosed for bioengineering of gastrointestinal tissue. In particular, three-dimensional, bioengineered, tubular gut-sphincter complexes are disclosed, together with methods of forming such constructs.

[0011] In one aspect, methods of forming tissue engineered, tubular gut-sphincter complexes are disclosed that start by obtaining intestinal circular smooth muscle cells from an intestinal donor source, obtaining sphincteric smooth muscle cells from a sphincteric donor source, and obtaining enteric neural progenitor cells from at least one neural progenitor

donor source. The intestinal smooth muscle cells can be seeded on a mold with a surface texture that induces longitudinal alignment of the intestinal smooth muscle cells with the neural progenitor cells added to the intestinal smooth muscle cells on the mold, such that the combination of intestinal smooth muscle cells and the neural progenitor cells can be cultured until an innervated aligned smooth muscle sheet is obtained. The innervated smooth muscle sheet can then be wrapped around a tubular scaffold to form an intestinal tissue construct. Additionally, the sphincteric smooth muscle cells and additional enteric neural progenitor cells can be mixed in a biocompatiable gel solution, and the gel and admixed cells can be applied to a mold having a central post; the sphinteric smooth muscle and neural progenitor cells can be cultured to form an innervated sphincter construct around the mold post. Once formed, the innervated sphincter construct can also be transferred to the tubular scaffold such that the intestinal tissue construct and sphincter construct contact each other, and the resulting combined sphincter and intestinal tissue constructs can be further cultured about the scaffold until a unified tubular gut-sphincter complex is obtained.

[0012] In other aspects, tissue engineered, tubular gut-sphincter complexes are disclosed for therapeutic inventions or for use as screening or testing platforms, e.g., for organ on a chip purposes, to test the effects of drugs or other therapies on functional gut tissue.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] Figures 1A-1C are photographs of an engineered sphincter-rectal cuff complex construction. Figure 1A shows the complex pre-implantation while Figure 1B shows the complex construction post-implantation and Figure 1C is an end view of the construction, showing maintenance of luminal patency.

[0014] Figure 2A is a graph of changes in basal tone over time during an induced contraction.

[0015] Figure 2B is a photograph of the sphincter-rectal cuff complex with the sphincter component delineated.

[0016] Figures 3A-3D are further photographs of a sphincter-rectal cuff complex preimplantation (Figure 3A), during implantation (Figure 3B), immediately following implantation (Figure 3C) and at harvest after 14 days in the animals abdominal cavity (Figure 3D).

[0017] Figure 4 is another photograph of sphincter-rectal cuff complex following implantation.

[0018] Figures 5A-5C are photographs of a gut-sphincter complex according to the invention. Figure 5A shows the complex pre-implantation, having a gut part 3 cm in length and a sphincter part (identified by a white arrow). Figure 5B shows the complex after 4 weeks of subcutaneous implantation in the abdominal wall and shows vascularization of the complex. Figure 5C shows that the complex maintained its luminal patency (0.3 cm internal diameter).

[0019] Figure 6A is a photograph of a gut-sphincter complex measured for intraluminal pressures. Figure 6B is a schematic of the intraluminal pressure measurement device and system. Figure 6C is a graph of *in vivo* intraluminal pressure measurements. The rats were anesthetized and the complex was accessed. A catheter with 4 circumferential sensors was inserted into the complex to measure luminal pressure. The catheter was connected to Sandhill equipment that records the pressures (as shown in the diagram for each sensor). Mean gut luminal pressure was 21 ± 2 mmHg while the sphincteric pressure was 52 ± 3 mmHg.

[0020] Figures 7A-7C are graphs showing the mechanical properties of the implanted complex. The tensile properties of the complex were lower than those of the native rat intestine; however they were not significantly different. Figure 7A shows that the tensile strength of the complex was 0.043 ± 0.007 MPa compared to 0.067 ± 0.006 MPa for the native rat intestine. Figure 7B shows that the elongation at break of the implant was 171 ± 30 % compared to 230 ± 13% for the native rat intestine. Figure 7C shows that Young's modulus of the implanted complex was 0.1 ± 0.01 MPa compared to 0.12 ± 0.01 MPa for the native rat intestine.

[0021] Figures 8A and 8B provide histological evaluations of the implants. Representative images of H&E for the sphincter(Figure 8A) and gut parts (Figure 8B)of the complex show maintenance of the circular alignment of the smooth muscle around the lumen of the tubular graft.

[0022] Figures 9A-9F are photographs showing the immunofluorescence of the implants. Smooth muscle of the sphincter (Figure 9A) and the gut parts (Figure 9B) of the complex maintained their contractile phenotype as shown by positive stain for smoothelin after 4 weeks of implantation. Figure 9C demonstrates the innervation of the complex as

demonstrated by positive stain with βIII tubulin, indicating that the neural progenitor cells differentiated into neurons. Figure 9D shows the presence of excitatory motor neurons, demonstrated by positive stain with ChAT. Figure 9E shows inhibitory motor neurons stained positive with nNOS. Figure 9F demonstrates vascularization by positive stain with von Willebrand factor.

[0023] Figure 10A-10C are graphs showing *in vitro* physiological functionality of the implants. Figure 10A shows that the implanted sphincter maintained its capacity to generate a spontaneous basal tone of 382 ± 79 μN. Figure 10B shows that the addition of potassium chloride (KCl) resulted in a contraction of 427 ± 42 μN above the basal tone in the sphincter. Figure 10C shows that KCl resulted in a robust and sustained contraction (434 ± 17 μN) in the gut part of the complex.

[0024] Figures 11A-11D are further graphs showing *in vitro* physiological functionality of the implants. Electrical field stimulation (EFS) of both the sphincter (Figure 11A & Figure 11B) and the gut parts (Figure 11C) of the complex caused relaxation of the smooth muscle (black trace). Pre-incubation of the implants with nNOS inhibitor LNAME significantly reduced the magnitude of relaxation (Figure 11D), indicating the presence of functional nitrergic neurons (Figures 11B & 11C,grey traces).

DETAILED DESCRIPTION

[0025] As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural references unless the context clearly dictates otherwise. Thus, for example, references to "the method" includes one or more methods, and/or steps of the type described herein which will become apparent to those persons skilled in the art upon reading this disclosure and so forth. The steps of any method can be practiced in feasible order and are restricted to a sequential order merely because they are so recited in a claim.

[0026] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods and materials are now described.

[0027] "Differentiation" refers to a change that occurs in cells to cause those cells to assume certain specialized functions and to lose the ability to change into certain other specialized functional units. Cells capable of differentiation may be any of totipotent,

pluripotent or multipotent cells. Differentiation may be partial or complete with respect to mature adult cells.

[0028] Stem cells are undifferentiated cells defined by the ability of a single cell both to self-renew, and to differentiate to produce progeny cells, including self-renewing progenitors, non-renewing progenitors, and terminally differentiated cells. Stem cells are also characterized by their ability to differentiate *in vitro* into functional cells of various cell lineages from multiple germ layers (endoderm, mesoderm and ectoderm), as well as to give rise to tissues of multiple germ layers following transplantation, and to contribute substantially to most, if not all, tissues following injection into blastocysts. Neural stem cells can be isolated from embryonic and adult central nervous system (CNS) tissue, neural tube tissue or enteric nervous system (ENS) tissue.

[0029] Stem cells can be further classified according to their developmental potential as: (1) totipotent; (2) pluripotent; (3) multipotent; (4) oligopotent; and (5) unipotent. Totipotent cells are able to give rise to all embryonic and extra-embryonic cell types. Pluripotent cells are able to give rise to all embryonic cell types. Multipotent cells include those able to give rise to a subset of cell lineages, but all within a particular tissue, organ, or physiological system (for example, hematopoietic stem cells (HSC) can produce progeny that include HSC (self-renewal), blood cell-restricted oligopotent progenitors, and all cell types and elements (e.g., platelets) that are normal components of the blood). Cells that are oligopotent can give rise to a more restricted subset of cell lineages than multipotent stem cells; and cells that are unipotent typically are only able to give rise to a single cell lineage.

[0030] In a broader sense, a progenitor cell is a cell that has the capacity to create progeny that are more differentiated than itself, and yet retains the capacity to replenish the pool of progenitors. By that definition, stem cells themselves are also progenitor cells, as are the more immediate precursors to terminally differentiated cells. When referring to the cells of the present invention, as described in greater detail below, this broad definition of progenitor cell may be used. In a narrower sense, a progenitor cell is often defined as a cell that is intermediate in the differentiation pathway, i.e., it arises from a stem cell and is intermediate in the production of a mature cell type or subset of cell types. This type of progenitor cell is generally not able to self-renew. Accordingly, if this type of cell is referred to herein, it will be referred to as a non-renewing progenitor cell or as an intermediate progenitor or precursor cell.

[0031] As used herein, the phrase "differentiates into a neural lineage or phenotype" refers to a cell that becomes partially or fully committed to a specific neural phenotype of the CNS or PNS, i.e., a neuron or a glial cell, the latter category including without limitation astrocytes, oligodendrocytes, Schwann cells and microglia. The term "neural" as used herein is intended to encompass all electrical active cells, e.g., cells that can process or transmit information through electrical or chemical signals, including the aforementioned neurons, glial cells, astrocytes, oligodendrocytes, Schwann cells and microglia.

[0032] For the purposes of this disclosure, the terms "neural progenitor cell" or "neural precursor cell" mean a cell that can generate progeny that are either neuronal cells (such as neuronal precursors or mature neurons) or glial cells (such as glial precursors, mature astrocytes, or mature oligodendrocytes). Typically, the cells express some of the phenotypic markers that are characteristic of the neural lineage. Typically, they do not produce progeny of other embryonic germ layers when cultured by themselves *in vitro*, unless dedifferentiated or reprogrammed in some fashion.

[0033] A "neuronal progenitor cell" or "neuronal precursor cell" is a cell that can generate progeny that are mature neurons. These cells may or may not also have the capability to generate glial cells. A "glial progenitor cell" or "glial precursor cell" is a cell that can generate progeny that are mature astrocytes or mature oligodendrocytes. These cells may or may not also have the capability to generate neuronal cells.

[0034] The phrase "biocompatible substance" and the terms "biomaterial" and "substrate" are used interchangeably and refer to a material that is suitable for implantation or injection into a subject. A biocompatible substance does not cause toxic or injurious effects once implanted in the subject. In one embodiment, the biocompatible substrate includes at least one component of extracellular matrix. In other embodiments, the substrate can also include a polymer with a surface that can be shaped into the desired structure that requires repairing or replacing. The polymer can also be shaped into a part of a body structure that requires repairing or replacing. In another embodiment, the biocompatible substrate can be injected into a subject at a target site.

[0035] In one embodiment, the substrate is an injectable or implantable biomaterial that can be composed of crosslinked polymer networks which are typically insoluble or poorly soluble in water, but can swell to an equilibrium size in the presence of excess water. For example, a hydrogel can be injected into desired locations within the organ. In one

embodiment, the collagen can be injected alone. In another embodiment, the collagen can be injected with other hydrogels. The hydrogel compositions can include, without limitation, for example, poly(esters), poly(hydroxy acids), poly(lactones), poly(amides), poly(ester-amides), poly(amino acids), poly(anhydrides), poly(ortho-esters), poly(carbonates), poly(phosphazines), poly(thioesters), polysaccharides and mixtures thereof. Furthermore, the compositions can also include, for example, a poly(hydroxy) acid including poly(alpha-hydroxy) acids and poly(beta-hydroxy) acids. Such poly(hydroxy) acids include, for example, polylactic acid, polyglycolic acid, polycaproic acid, polybutyric acid, polyvaleric acid, and copolymers and mixtures thereof.

[0036] Hydrogels with effective pore sizes in the 10-100 nm range and in the 100 nm-10 micrometer range are termed "microporous" and "macroporous" hydrogels, respectively. Microporous and macroporous hydrogels are often called polymer "sponges." When a monomer, e.g., hydroxyethyl methacrylate (HEMA), is polymerized at an initial monomer concentration of 45 (w/w) % or higher in water, a hydrogel is produced with a porosity higher than the homogeneous hydrogels. Hydrogels can also expand in the presence of diluent (usually water). The matrix materials of present invention encompass both conventional foam or sponge materials and the so-called "hydrogel sponges." For a further description of hydrogels, see U.S. Pat. No. 5,451,613 (issued to Smith et al.) herein incorporated by reference.

[0037] The term "extracellular matrix" or "ECM" is used herein to denote compositions comprising one or more of the following: collagen I, collagen IV, laminin, heparan sulfate, or fragments of one or more of such proteins.

[0038] "Collagen I" refers to collagen I or collagen I compositions derived from cell culture, animal tissue, or recombinant means, and may be derived from human, murine, porcine, or bovine sources. "Collagen I" also refers to substances or polypeptide(s) at least substantially homologous to collagen I or collagen I compositions. Additionally, "collagen I" refers to collagen I or collagen I compositions that do not include a collagen I fragment, e.g., including essentially only a complete collagen I protein.

[0039] "Collagen IV" refers to collagen IV or collagen IV compositions derived from cell culture, animal tissue, or recombinant means, and may be derived from human, murine, porcine, or bovine sources. "Collagen IV" also refers to substances or polypeptide(s) at least substantially homologous to collagen IV or collagen IV compositions. Additionally,

"collagen IV" refers to collagen IV or collagen IV compositions that do not include a collagen IV fragment, e.g., including essentially only a complete collagen I protein.

[0040] "Laminin" refers to laminin, laminin fragments, laminin derivatives, laminin analogs, or laminin compositions derived from cell culture, recombinant means, or animal tissue. "Laminin" can be derived from human, murine, porcine, or bovine sources. "Laminin" refers to laminin or laminin compositions comprising laminin-1, laminin-2, laminin-4, or combinations thereof. "Laminin" also refers to substances or polypeptide(s) at least substantially homologous to laminin-1, laminin-2, or laminin-4. Additionally, "laminin" refers to laminin or laminin compositions that do not include a laminin fragment, e.g., including essentially only a complete laminin protein.

[0041] The term "subject" as used herein refers to any living organism, including, but not limited to, humans, nonhuman primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats, rabbits and guinea pigs, and the like. The term does not denote a particular age or sex. In a specific embodiment, the subject is human.

[0042] The terms "treating," "treatment" or "intervention" refer to the administration of one or more therapeutic agents or procedures to a subject who has a condition or disorder or a predisposition toward a condition or disorder, with the purpose to prevent, alleviate, relieve, alter, remedy, ameliorate, improve, affect, slow or stop the progression, slow or stop the worsening of the disease, at least one symptom of condition or disorder, or the predisposition toward the condition or disorder.

[0043] The gut is responsible for ingestion of food, propulsion of luminal content and excretion of waste. These functions are conducted by the basic unit of the musculature of the gut, which is the smooth muscle. Function of the smooth muscle of the gut is highly regulated, mainly by the enteric nervous system (ENS) and the interstitial cells of Cajal (ICCs). The smooth muscle receives regulatory inputs which are critical to produce a coordinated response. While the gut is considered a continuous tubular muscular organ (except for the stomach), several sphincters exist as checkpoints along the length of the gut. Those sphincters possess high pressure zone that regulate the propulsion of luminal content. Neuro-muscular diseases of the gut alter the normal motility patterns. Even though surgical

intervention remains the standard treatment, preservation of the sphincter attached to the rest of the gut is challenging.

[0044] Motility disorders result when neuro-muscular functions of the gut are disturbed. In certain cases of neuro-muscular diseases, sphincter integrity and function is also impaired. In esophageal achalasia, impairment of the enteric neurons at the level of the smooth muscle of the lower esophagus causes loss of relaxation of the lower esophageal sphincter (LES). Treatments aim to reduce the contractility of the LES. This can be done using different drugs/blockers, balloon dilatation, injection of botulinum toxin or myotomy of the LES. On the other hand, gastroparesis is characterized by delayed gastric emptying. This is partially attributed to the inability of the pyloric sphincter to relax. Common treatments include botulinum toxin injection, drugs/diet change, gastric electric stimulation or pyloroplasty. In patients with colorectal cancer, preservation of the sphincter following surgical resection of the tumor is challenging. The formation of a stoma is a common treatment; however patients suffer from low quality of life (social and physical problems). In other cases, congenital anomalies such as anorectal malformation involve the rectum along with the anus. Children with anorectal malformation require surgical intervention which consists of either surgical repair or a colostomy. Fecal incontinence is a major complication among other physical and social morbidities resulting from anorectal malformation. All of the listed treatments for neuro-muscular disorders are either associated with complications or provide a short-term relief for the patients. New long term therapeutic strategies are needed.

[0045] Methods and constructs are disclosed for bioengineering of gastrointestinal tissue. In particular, three-dimensional, bioengineered, tubular gut- sphincter complexes (TGSC) are disclosed, together with methods of forming such constructs. Specifically, engineered innervated human smooth muscle sheets and innervated human sphincters with a pre-defined alignment are disclosed for placement around tubular scaffolds to create a gut-sphincter complex.

[0046] In certain embodiments, a method of forming a tissue engineered, tubular gut-sphincter complex comprises: isolating intestinal circular smooth muscle cells from an intestinal donor source, isolating sphincteric smooth muscle cells from a sphincteric donor source, isolating enteric neural progenitor cells from at least one neural progenitor donor source, seeding the isolated intestinal circular smooth muscle cells on a mold with a surface texture that induces longitudinal alignment of the intestinal circular smooth muscle cells, adding the isolated enteric neural progenitor cells to the intestinal circular smooth muscle

cells on the mold, co-culturing the intestinal circular smooth muscle cells and the enteric neural progenitor cells until an innervated aligned smooth muscle sheet is obtained, disposing the innervated aligned smooth muscle sheet around a tubular scaffold to form an innervated intestinal tissue construct, admixing the sphincteric smooth muscle cells and additional enteric neural progenitor cells in a biocompatiable gel solution, applying the gel and admixed cells to a mold having a central post; co-culturing the sphinteric smooth muscle cells and neural progenitor cells to form an innervated sphincter construct around the mold post, transferring and applying the innervated sphincter construct to the tubular scaffold such that the innervated intestinal tissue construct and the innervated sphincter construct contact each other, and further culturing the combined sphincter and intestinal tissue constructs about the scaffold until a unified tubular gut-sphincter complex is obtained.

[0047] In embodiments described herein, the steps of isolating the intestinal circular smooth muscle cells, sphincteric smooth muscle cells and enteric neural progenitor cells may further comprise obtaining each type of cell from a single subject. In some embodiments, the intestinal circular smooth muscle cells, sphincteric smooth muscle cells and enteric neural progenitor cells are obtained from a subject who is between the ages of about 1 year to about 80 years.

[0048] In embodiments described herein, at least one co-culturing step may comprise culturing cells in a collagen suspension.

[0049] In embodiments described herein, the step of disposing the innervated aligned smooth muscle sheet around a tubular scaffold may comprise wrapping the innervated aligned smooth muscle sheet around a chitosan scaffold.

[0050] In embodiments described herein, the method of forming a tissue engineered, tubular gut-sphincter complex may further comprise connecting two or more innervated aligned smooth muscle sheets together to form a composite structure.

[0051] In some embodiments, the unified tubular gut-sphincter complex has a length of about 1 cm to about 100 cm. In certain embodiments, the unified tubular gut-sphincter complex has a width of about 0.1 cm to about 10 cm.

[0052] In certain embodiments, a tubular gut-sphincter complex is formed by isolating intestinal circular smooth muscle cells from an intestinal donor source, isolating sphincteric smooth muscle cells from a sphincteric donor source, isolating enteric neural progenitor cells from at least one neural progenitor donor source, seeding the isolated intestinal circular

smooth muscle cells on a mold with a surface texture that induces longitudinal alignment of the intestinal circular smooth muscle cells, adding the isolated enteric neural progenitor cells to the intestinal circular smooth muscle cells on the mold, co-culturing the intestinal circular smooth muscle cells and the enteric neural progenitor cells until an innervated aligned smooth muscle sheet is obtained, disposing the innervated aligned smooth muscle sheet around a tubular scaffold to form an innervated intestinal tissue construct, admixing the sphincteric smooth muscle cells and additional enteric neural progenitor cells in a biocompatiable gel solution, applying the gel and admixed cells to a mold having a central post; co-culturing the sphinteric smooth muscle cells and neural progenitor cells to form an innervated sphincter construct around the mold post, transferring and applying the innervated sphincter construct to the tubular scaffold such that the innervated intestinal tissue construct and the innervated sphincter construct contact each other, and further culturing the combined sphincter and intestinal tissue constructs about the scaffold until a unified tubular gut-sphincter complex is obtained.

[0053] In embodiments described herein, steps of isolating the intestinal circular smooth muscle cells, sphincteric smooth muscle cells and enteric neural progenitor cells in the formation of the tubular gut-sphincter complex may further comprise obtaining each type of cell from a single subject. In some embodiments, the intestinal circular smooth muscle cells, sphincteric smooth muscle cells and enteric neural progenitor cells are obtained from a subject who is between the ages of about 1 year to about 80 years.

[0054] In embodiments described herein, in the formation of the tubular gut-sphincter complex at least one co-culturing step may comprise culturing cells in a collagen suspension.

[0055] In embodiments described herein, in the formation of the tubular gut-sphincter complex the step of disposing the innervated aligned smooth muscle sheet around a tubular scaffold may comprise wrapping the innervated aligned smooth muscle sheet around a chitosan scaffold.

[0056] In embodiments described herein, the formation of a tissue engineered, tubular gut-sphincter complex may further comprise connecting two or more innervated aligned smooth muscle sheets together to form a composite structure.

[0057] In some embodiments, the tubular gut-sphincter complex has a length of about 1 cm to about 100 cm. In certain embodiments, the unified tubular gut-sphincter complex has a width of about 0.1 cm to about 10 cm.

[0058] In certain embodiments, a tubular gut-sphincter complex comprises: a tubular scaffold; an innervated intestinal tissue construct disposed about the tubular scaffold; and an innervated sphincteric tissue construct also disposed about the tubular scaffold joined to the intestinal tissue construct at an end of the intestinal tissue construct; and the complex exhibits directionally oriented smooth muscle cells, basal tone and choleric contractions in response to a contractile stimulus.

[0059] In embodiments described herein, the tubular scaffold may further comprise a chitosan scaffold.

[0060] In some embodiments, the tubular gut-sphincter complex has a length of about 1 cm to about 100 cm. In certain embodiments, the unified tubular gut-sphincter complex has a width of about 0.1 cm to about 10 cm.

[0061] To demonstrate the utility of the present invention, engineered complexes were subcutaneously implanted in the abdomen of the rats for 4 weeks. The implanted tissues exhibited vascularization and *in vivo* manometry revealed luminal pressure at the gut and the sphincter zone. Tensile strength, elongation at break and Young's modulus of the engineered complexes were similar to those of native rat intestine. Histological and immunofluorescence assays showed maintenance of smooth muscle circular alignment in the engineered tissue, maintenance of smooth muscle contractile phenotype and innervation of the smooth muscle. Electrical field stimulation induced a relaxation of the smooth muscle of both the sphincter and the gut parts. Relaxation was partially inhibited by nitric oxide inhibitor indicating nitrergic contribution to the relaxation. The sphincteric part of TGSC maintained the basal tone characteristic of a native sphincter. The gut part also maintained its specific neuro-muscular characteristics. The results of this study provide a promising therapeutic approach to restore gut continuity and motility.

Example 1 – Spincter-Rectal Cuff Complex

[0062] Innervated aligned smooth muscle sheets were engineered using the co-culture technique of isolated human smooth muscle cells and human enteric neural progenitor cells derived from the small intestine, as described above. The sheets were wrapped around tubular chitosan scaffolds. Innervated pyloric sphincters were engineered using human pyloric smooth muscle and enteric neural progenitor cells, again as described above. The sphincters were placed on one end around the tubular scaffold. The combination of the

tissues around the tubular scaffold is referred to as sphincter-rectal cuff. The sphincter-rectal cuff was 3 cm in length and 3mm internal diameter.

[0063] Athymic rats were used for this study to avoid implant rejection since the implant was made of human cells. Following sedation of the rats, a 5 cm midline skin incision was made. The engineered tubular tissue was implanted subcutaneously and fixed in place using non-resorbable sutures. Figure. 5A is a photograph of bioengineered sphincter-rectal cuff pre-implantation.

[0064] Four weeks following implantation, the rats were brought to the procedure room and anesthetized using isoflurane. A midline incision was made and the surgical site was re-accessed. The implanted sphincter-rectal cuff was healthy in color and highly vascularized

[0065] The implant maintained its luminal patency for 4 weeks. A manometry catheter was used to measure the luminal pressure in the sphincter-rectal cuff. The sensors were linearly aligned along the catheter and were 2 mm apart. The catheter was inserted into the lumen of the sphincter-rectal cuff while still attached under the skin. Luminal pressure was recorded in all 3 channels and averaged 40 mmHg. Spontaneous small amplitude contractions were seen in all 3 channels. The rat was then euthanized and the implant was harvested.

[0066] Figures 1A-1C are photographs of an engineered sphincter-rectal cuff complex construction. Figure 1A shows the complex pre-implantation while Figure 1B shows the complex construction post-implantation and Figure 1C is an end view the construction, showing maintenance of luminal patency. Figure 2A is a graph of changes in basal tone over time during an induced contraction. Figure 2B is a photograph of the sphincter-rectal cuff complex with the sphincter component delineated. Figures 3A-3D are further photographs of a sphincter-rectal cuff complex preimplantation (Figure 3A), during implantation (Figure 3B), immediately following implantation (Figure 3C) and at harvest after 14 days in the animals abdominal cavity (Figure 3D). Figure 4 is another photograph of sphincter-rectal cuff complex following implantation.

[0067] Following measurement of luminal pressure, one end of the sphincter-rectal cuff was tightly clamped while the other end was left open. A volume of 1 ml of liquid was pipetted through the open end. The tissue expanded in the center as it was filled with liquid. The tissue then returned to its original shape after the liquid solution was cleared. There was no sign of leakage or disruption.

[0068] Cross sections of the sphincter and the rectal cuff were used for physiological studies. The sphincter maintained its ability to generate a spontaneous basal tone similar to an engineered sphincter prior to its implantation. This indicates that the implanted sphincter maintains its tonic characteristic *in vivo*. The electromechanical coupling integrity of the smooth muscle was also evaluated in the presence of potassium chloride. Following depolarization of the smooth muscle membrane in both the sphincter and the rectal cuff, a fast and robust contraction was observed. This indicates that the voltage-dependent calcium channels were maintained in the sphincter-rectal cuff after 4 weeks of implantation. Next, we evaluated the response of the sphincter-rectal cuff to exogenous neurotransmitters. The addition of acetylcholine (Ach) caused a rapid contraction, which was significantly attenuated when the tissue was pre-treated with the neurotoxin tetrodotoxin (TTX). This indicates that Ach acted on both the smooth muscle and the neurons and caused muscle contraction. After blocking the neurons with TTX, Ach acted on smooth muscle receptors only and caused a lower response. Relaxation was evaluated in the presence of VIP (vasoactive intestinal polypeptide). In the presence of TTX, the observed VIP-induced relaxation was significantly diminished. Again, this indicates that VIP relaxation was mediated through receptors on the smooth muscle and neurons. To further confirm the functionality of the neurons, electrical field stimulation (EFS) was applied on the implant. EFS caused a rapid relaxation which was completely inhibited by TTX. This indicated that the response was purely neuronally mediated.

[0069] Additional cross sections of the sphincter and the rectal cuff were fixed in formalin and processed for histological analysis. H&E staining showed maintenance of alignment of smooth muscle around the lumen of the tubular scaffold. Maintenance of smooth muscle phenotype was confirmed by positive immunostaining for smooth muscle specific markers. Innervation was also confirmed by positive satining for neural marker βIII tubulin. Vascularization was further confirmed by staining for von Willebrand factor.

[0070] Cross sections of the implanted tissue were obtained and were mechanically evaluated using a mechanical testing machine (Instron). The tissues were subjected to tensile stress and strain testing. Young's modulus of the implant was higher than the scaffold only, indicating that the innervated smooth muscle sheet remodeled around the scaffold following implantation resulting in higher Young's modulus. This is an indication that the implant was able to handle higher stretch without breaking when compared to scaffolds only.

Example 2 – Sphincter-Intestine Complex

[0071] *Materials and methods:*

[0072] Cell culture reagents were purchased from Life Technologies (Grand Island, NY, US) unless otherwise specified. Smooth muscle growth medium consisted of Dulbecco's modified Eagle medium, 10% fetal bovine serum, 1.5% antibiotic-antimycotic, and 0.6% L-glutamine. Neural growth medium consisted of neurobasal, 1X N2 supplement, recombinant human Epidermal Growth Factor (EGF 20 ng/mL, Stemgent, San Diego, CA, US), recombinant basic Fibroblast Growth Factor (bFGF 20 ng/mL, Stemgent, CA, US), and 1X antibiotic–antimycotic. Neural differentiation media consisted of neurobasal medium-A supplemented with 2% fetal bovine serum, 1X B27 supplement and 1X antibiotic–antimycotic. Medium molecular weight chitosan (75–85% deacetylation), tetrodotoxin (TTX), and neuronal nitric oxide synthase (nNOS)-blocker N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) were purchased from Sigma (St. Louis, MO). Sylgard [poly(dimethylsiloxane); PDMS] was purchased from World Precision Instruments (Sarasota, FL). Type I rat tail collagen was purchased from BD Biosciences.

[0073] Human intestinal and pyloric tissues were ethically obtained from organ donors through Carolina Donor Services and Wake Forest Baptist Medical Center (IRB No. 00007586). Tissues were obtained from three donors aged 2, 18, and 67 years.

[0074] Human intestinal circular smooth muscle cells: Smooth muscle cells were isolated from human duodenum. The duodena (10 cm below the pyloric sphincter) were obtained consistently for cell isolation. Human duodena were cleaned of any luminal content and were washed extensively in ice-cold Hank's balanced salt solution (HBSS). The tissues were cut into smaller pieces and the circular smooth muscle was obtained by stripping off the mucosa and the longitudinal muscle. The circular smooth muscle tissue was then minced, washed extensively in HBSS and incubated in a digestion mix containing type II collagenase (Worthington, Lakewood, NJ) and DNAse (Roche, Indianapolis, IN) for one hour at 37°C with agitation. The digested tissue was then extensively washed in HBSS and subjected to a second digest. Digested cells were washed, resuspended in warm smooth muscle growth media and expanded in tissue culture flasks at 37°C with 5% CO_2.

[0075] Human pyloric smooth muscle cells: Human pylori were dissected off for smooth muscle isolation. Pylorus tissues were cleaned of any fat and mucosa and extensively washed with HBSS. Tissues were then minced and washed again with sterile HBSS. Minced tissues

were digested twice at 37°C with agitation in type II collagenase (Worthington Biochemical, Lakewood, NJ) and DNAse (Roche, Indianapolis, IN) for one hour each. Cells were pelleted down with centrifugation, resuspended in smooth muscle growth media and expanded in tissue culture flasks at 37°C with 5% CO_2.

[0076] Human enteric neural progenitor cells: Human enteric neural progenitor cells were isolated from the small intestine. Human duodenal tissues were finely minced followed by extensive washing in HBSS. Tissues were then digested in a mixture of type II collagenase, dispase, and DNAse. The cells were passed through 70 µm cell strainer followed by extensive washing. The cells were then passed through 40 µm cell strainers. Following centrifugation, cells were resuspended in neural growth media and cultured in non-tissue culture treated plates at 37°C and 7% CO_2. The cultured cells formed free-floating clusters referred to as neurospheres which have been shown to stain positive for neural crest-derived cell marker p75.

[0077] Preparation of tubular gut-sphincter segment: Tubular chitosan/collagen scaffolds were engineered as described by Zakhem et al. in "Chitosan-based scaffolds for the support of smooth muscle constructs in intestinal tissue engineering," *Biomaterial,*. 2012;33:4810-7 and Zakhem et al. in "Development of Chitosan Scaffolds with Enhanced Mechanical Properties for Intestinal Tissue Engineering Applications," *Journal of Functional Biomaterials*, 2015;6:999-1011. A 2% w/v chitosan solution was prepared in 0.2 M acetic acid. The chitosan solution was then mixed with type I collagen in a 1:1 ratio. The mix was then poured into a custom-made 3-cm long tubular mold with a diameter of 0.7 cm. The lumen of the scaffold was created by inserting an inner tubing of 0.3 cm diameter in the center of the main tubular mold. This created a scaffold with length of 3 cm and internal diameter of 0.3 cm. The scaffolds were frozen at -80°C for 3 hours followed by lyophilization overnight. The scaffolds were then neutralized in 0.2 NaOH and washed extensively with PBS and distilled water. The scaffolds were sterilized in 70% ethanol and then washed extensively with sterile 1X PBS before cell seeding.

[0078] Innervated aligned smooth muscle sheets were engineered as previously described by Zakhem et al. in "Successful implantation of an engineered tubular neuromuscular tissue composed of human cells and chitosan scaffold," *Surgery*, 2015;158:1598-608. and Zakhem et al. in "Development of Chitosan Scaffolds with Enhanced Mechanical Properties for Intestinal Tissue Engineering Applications," *Journal of Functional Biomaterials*, 2015;6:999-1011. Briefly, smooth muscle cells were seeded onto wavy molds made of Sylgard with

longitudinal grooves and allowed to align. Five days after smooth muscle alignment, neural progenitor cells were collected and suspended in a mixture of 10% FBS, 1× DMEM, 1× antibiotic–antimycotic, 10 μg/ml mouse laminin and 0.4 mg/ml type I rat tail collagen. Neural progenitor cells were then mixed in collagen/laminin gel and laid on top of the smooth muscle. Engineered human innervated smooth muscle sheets were then circumferentially wrapped around the tubular scaffolds as described previously to mimic the circular muscle layer. The sheets around the scaffolds are referred to as the gut part of the complex. Human innervated pyloric smooth muscle sphincters were engineered as previously described by Rego et al. in "Bioengineered human pyloric sphincters using autologous smooth muscle and neural progenitor cells," *Tissue engineering*, 2015. A suspension of 200 000 enteric neural progenitor cells was suspended in a gel mix. The mixture was pipetted on a Sylgard-coated plate that had a central cylindrical post and allowed to gel for about 20 min at 37°C. Pyloric smooth muscle cells were trypsinized and 500 000 cells were obtained. The cells were resuspended in a similar gel mixture. The mixture was then pipetted on top of the first neural layer. Following gelation, differentiation media was supplemented every other day for 10 days. The sphincters were also placed, at one end, around the tubular scaffolds and were referred to as the sphincter part of the complex.

[0079] Implantation of the engineered tubular gut-sphincter complex: Athymic rats (n=6) were used as recipients of the tubular gut-sphincter complexes. Surgical procedures described in this work were performed following the guidelines set forth by IACUC. Rats were anesthetized by continuous isoflurane masking throughout the surgery. The surgical area was shaved and aseptically prepared. A midline skin incision of up to 5 cm was made in the abdominal wall. The engineered tubular gut-sphincter complex was fixed using 5-0 prolene sutures to mark the tissue at the time of harvest. The rats were allowed to recover in their cages in standard fashion and were given the appropriate analgesics.

[0080] *In vivo* intraluminal pressure measurement: Four weeks following implantation, the rats were brought back to the procedure room. The rats were anesthetized by continuous isoflurane masking. The surgical site was re-accessed. The implants were located by the prolene sutures. An air-charged catheter with circumferential sensors (7 mm spacing between the sensors) was used to measure the luminal pressure of the tubular implants. The catheter was inserted into the tubular implant entering from the gut part until the first sensor of the catheter reached the sphincteric area. The remaining sensors measured the pressure at the gut part of the tubular implant. Luminal pressure was recorded using InSIGHT Acquisition

(version 5.2.4, Sandhill scientific Inc, Highland Ranch, CO, USA). The recorded pressures were analyzed using BioVIEW system (version 5.6.3.0 Sandhill scientific Inc, Highland Ranch, CO, USA). Following pressure readings, the rats were euthanized. The implants were dissected from the surrounding tissue. The harvested implants were further evaluated.

[0081] Immediately after harvest, the implants were tested for their tensile properties using a uniaxial load test machine (Instron model # 5544, Issaquah, WA, USA). Tubular specimens were obtained and hooked onto the machine equipped with a 2kN load cell. Tensile strength, elongation at break and Young's modulus were obtained. Rat native intestines served as control.

[0082] A pressure transducer catheter with an inflatable balloon was used to measure the burst strength pressure of the implants. The catheter was inserted into the lumen of the implants and the luminal pressure was increased until failure occurred. The pressure was slowly increased until failure occurred and the pressures were recorded.

[0083] Immediately after harvest, sections of the sphincter and the gut tissues were fixed in formaldehyde, processed and paraffin embedded. Sections were deparaffinized and stained with hematoxylin and eosin (H&E) for morphological analysis. Phenotype of smooth muscle and differentiated neurons was analyzed by incubating the sections in primary antibodies directed against smoothelin and β-III tubulin, respectively. Neuronal nitric oxide synthase (nNOS) and choline acetyltransferase (ChAT) antibodies were used to confirm the presence of inhibitory and excitatory motor neurons, respectively. Vascularization was confirmed by immunostaining of the sections with von Willebrand (vWF) factor. Appropriate fluorophore-conjugated secondary antibodies were used.

[0084] Circular strips of the harvested sphincters and gut tissues were also immediately obtained and evaluated for physiological functionality. A force transducer apparatus (Harvard Apparatus, Holliston, MA) was used to measure real time force generation. The tissues were hooked to a stationary fixed pin from one side and to the measuring arm of the force transducer from the other side. The tissues were kept in a warm tissue bath throughout the experiments. Establishment of basal tone by the sphincters, electromechanical coupling integrity of the smooth muscle of both the sphincters and the gut tissues, and the functionality of the differentiated neurons were evaluated.

[0085] Electromechanical coupling integrity was evaluated in the presence of 60mM potassium chloride (KCl). Functionality of neurons was evaluated using electrical field

stimulation (EFS) in the absence and presence of neurotoxin, tetrodotoxin (TTX) and nitric oxide synthase (nNOS) blocker N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME).

[0086] The difference in tensile strength, elongation at break and Young's modulus between the implants and the native rat intestines was evaluated by Student's *t*-test. Analysis of acquired force data was acquired using Powerlab and exported to GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego CA; www.graphpad.com). Second order Savitzky–Golay smoothing was applied to data. Student paired *t*-test was used to compare the means of forces in the absence and presence of inhibitors. All values were expressed as means ± SEM. A *p*-value less than 0.05 was considered significant.

[0087] *Results:*

[0088] Gut-sphincter complex were engineered by combining innervated smooth muscle sheets and engineered innervated pyloric sphincters around tubular chitosan scaffolds (Figure 5A). The engineered innervated smooth muscle sheets were wrapped circumferentially around the tubular scaffolds to form the circular muscle layer. The sheets constitute the gut part of the gut-sphincter complex. The engineered sphincters of the complex were placed at one end of the scaffolds. The bioengineered tissues were implanted subcutaneously in the abdomen of athymic rats for 4 weeks. At the end of 4 weeks implantation, the tissue engineered sphincter became integrated with the gut sphincter complex and formed a single continuous functional unit .The implants showed healthy pink color upon harvest (Figure 5B). The implants were 3 cm in length and 0.5 cm diameter (Figure 5C). The luminal patency of the implants was maintained for 4 weeks post-implantation. There were no signs of inflammation, infection or tissue necrosis. Neovascularization was visually demonstrated by the presence of blood vessels around the implants.

[0089] The rats were anesthetized by continuous isoflurane masking. The implants were re-accessed. The catheter with circumferential sensors was calibrated before any measurement. The pressure reading was set to zero prior to insertion of the catheter into the lumen of the implant. The catheter was inserted into the lumen of the implant by increment of 1 sensor at a time until all sensors were inserted (Figures 6A-6C). Pressures started increasing as the catheter was inserted into the tissue. Pressure reading from each sensor was reflected on a separate channel. The catheter was inserted from the gut side of the implant (opposite end of the sphincter). Pressure reading of the sphincter is shown on the top graph (Figure 6C). The other lower channels were measuring the gut part of the complex (Figure 6C, lower three

graphs). The mean luminal pressure (of all sensors) of the gut zone was (21 ± 2 mmHg). The mean pressure recorded at the sphincter zone was (52 ± 3 mmHg). The pressures were stable over time. The luminal patency was further confirmed by completely inserting the catheter through the length of the implant without obstruction.

[0090] Uniaxial tensile properties of the implants were compared to those of native rat intestine. The tensile strength was significantly different between the implants and the native rat intestine (Figure 7A). The tensile strength of the native rat intestine was 0.067 ± 0.006 MPa whereas the average tensile strength of the implants was 0.043 ± 0.007 MPa (n = 4, p = 0.02). Elongation at break (Figure 7B) and Young's modulus (Figure 7C) of the implants were lower than those of the native rat intestine, however, they were not significantly different (n = 4, p = 0.1 and p = 0.37 respectively). Elongation at break and Young's modulus were 230 ± 13 % and 0.12 ± 0.01 MPa, respectively for the native rat intestine. The implant's elongation at break and Young's modulus averaged 171 ± 30 % and 0.1 ± 0.01 MPa, respectively.

[0091] A pressure transducer catheter with an inflatable balloon was inserted inside the lumen of the tubular implants. Pressure was increased gradually until failure of the implant. The pressure at failure was recorded as the burst pressure strength. The mean burst pressure strength of the implants was 1396 ± 60 mmHg.

[0092] Paraffin cross sections of the harvested engineered sphincters and gut tissues of 6 μm thickness were prepared. Representative H&E staining of both the engineered sphincters and gut tissues after implantation is shown in Figures 8A and 8B, respectively. The engineered innervated smooth muscle sheets were wrapped circumferentially around the tubular scaffolds to form circular muscle layer. The smooth muscle of the engineered sphincters was also circumferentially aligned. H&E stains showed maintenance of smooth muscle alignment around the lumen of the tubular tissues for both the gut segment and the sphincter. H&E shows dense aligned smooth muscle.

[0093] Sections of both the engineered sphincters and the engineered gut segments stained positive for the smooth muscle specific marker smoothelin (Figure 9A & 9B, respectively). This indicated that smooth muscle contractile phenotype of both the engineered sphincter and the gut segment was maintained over a period of 4 weeks post-implantation. Engineered tissues also stained positive for pan-neuronal marker, βIII-tubulin, indicating the presence of differentiated neurons in the engineered complex 4 weeks post-implantation

(Figure 9C). Additional positive staining for neural nitric oxide synthase (nNOS) (Figure 9D) and choline acetyltransferase (ChAT) (Figure 9E) indicate the presence of inhibitory and excitatory motor neurons in the implanted complex. Positive stain for Von Willebrand Factor (vWF) confirmed the vascularization of the implants (Figure 9F).

[0094] Circular strips of implanted engineered sphincter and gut segments were hooked to a force transducer measuring arm and allowed to establish baseline. The engineered sphincters exhibited the spontaneous ability to generate basal tone that averaged 382 ± 79 μN (Figure 10A). Electromechanical coupling integrity of both the sphincters and the gut segments was demonstrated by the robust contraction of the tissues after the addition of KCl. Sphincters demonstrated a contraction of 427 ± 42 μN above the basal tone (Figure 10B) while the gut tissues exhibited a mean peak contraction of 434 ± 17 μN (Figure 10C).

[0095] Functionality of neurons in the segment was evaluated by electrical field stimulation (8 Hz and 0.5 ms). Smooth muscle of both the implanted engineered sphincters and the gut segments relaxed following excitation of the nerves (Figure 11). Relaxation of the implanted sphincters averaged -294 ± 26 μN below the basal tone (Figure 11A and 11B- black traces) and the maximal relaxation averaged -355 ± 8 μN in the gut segment (Figure 11C- black trace). The tissues were then washed with fresh warm buffer and pre-treated with TTX. Upon excitation of the nerves, relaxation was completely abolished in both the sphincters and the gut segments. This indicated that the relaxation of the smooth muscle observed following EFS without TTX was due to excitation of neurons only. This also indicates that the neural progenitor cells within the complexes differentiated into functional neurons. Further characterization of the relaxation response was studied in the presence of nNOS inhibitor LNAME. The tissues were pre-treated with LNAME followed by EFS. Relaxation was significantly reduced to -140 ± 6 μN in the sphincters (Figure 11B – grey trace) and -223 ± 29 in the gut segments (Figure 11C – grey trace). This inhibition indicates that EFS-induced relaxation of the smooth muscle was partially mediated by functional nitrergic neurons. The data from Figures 11B and 11C are quantified in the bar graph depicted in Figure 11D.

[0010] The teachings of all patents, published applications and references cited herein are incorporated by reference in their entirety.

[0011] While the preferred embodiments of the invention have been illustrated and described, it will be clear that the invention is not so limited. Numerous modifications,

changes, variations, substitutions, and equivalents will occur to those skilled in the art without departing from the spirit and scope of the present invention as defined by the appended claims.

CLAIMS:

1. A method of forming a tissue engineered, tubular gut-sphincter complex comprising:
   isolating intestinal circular smooth muscle cells from an intestinal donor source,
   isolating sphincteric smooth muscle cells from a sphincteric donor source,
   isolating enteric neural progenitor cells from at least one neural progenitor donor source,
   seeding the isolated intestinal circular smooth muscle cells on a mold with a surface texture that induces longitudinal alignment of the intestinal circular smooth muscle cells,
   adding the isolated enteric neural progenitor cells to the intestinal circular smooth muscle cells on the mold,
   co-culturing the intestinal circular smooth muscle cells and the enteric neural progenitor cells until an innervated aligned smooth muscle sheet is obtained,
   disposing the innervated aligned smooth muscle sheet around a tubular scaffold to form an innervated intestinal tissue construct,
   admixing the sphincteric smooth muscle cells and additional enteric neural progenitor cells in a biocompatiable gel solution,
   applying the gel and admixed cells to a mold having a central post;
   co-culturing the sphinteric smooth muscle cells and neural progenitor cells to form an innervated sphincter construct around the mold post,
   transferring and applying the innervated sphincter construct to the tubular scaffold such that the innervated intestinal tissue construct and the innervated sphincter construct contact each other, and
   further culturing the combined sphincter and intestinal tissue constructs about the scaffold until a unified tubular gut-sphincter complex is obtained.

2. The method of claim 1, wherein steps of isolating the intestinal circular smooth muscle cells, sphincteric smooth muscle cells and enteric neural progenitor cells further comprise obtaining each type of cell from a single subject.

3. The method of claim 1, wherein at least one co-culturing step comprises culturing cells in a collagen suspension.

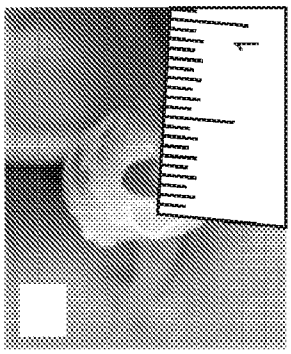
4. The method of claim 1, wherein the step of disposing the innervated aligned smooth muscle sheet around a tubular scaffold comprises wrapping the innervated aligned smooth muscle sheet around a chitosan scaffold.

5. The method of claim 1, wherein the method further comprises connecting two or more innervated aligned smooth muscle sheets together to form a composite structure.

6. A tubular gut-sphincter complex formed by the method of claim 1.

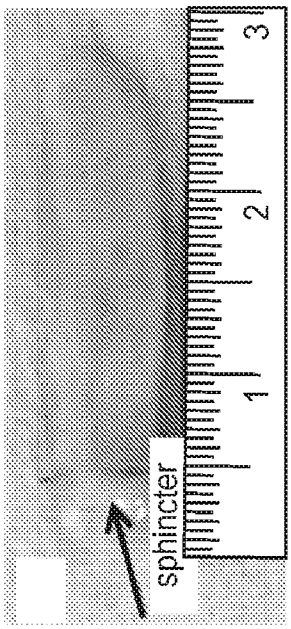
7. A tubular gut-sphincter complex comprising:
a tubular scaffold;
an innervated intestinal tissue construct disposed about the tubular scaffold; and
an innervated sphincteric tissue construct also disposed about the tubular scaffold joined to the intestinal tissue construct at an end of the intestinal tissue construct; and
the complex exhibiting directionally oriented smooth muscle cells, basal tone and choleric contractions in response to a contractile stimulus.

8. The structure of claim 7, wherein the tubular scaffold further comprises a chitosan scaffold.



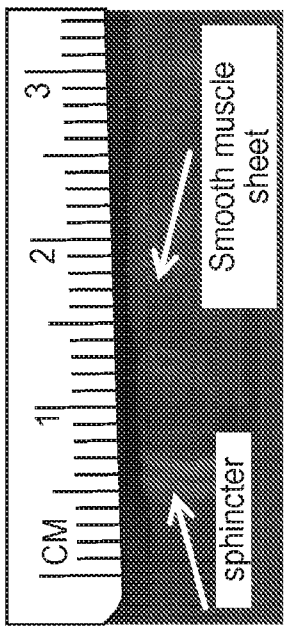
Luminal patency

FIG. 1C



Post-implantation - sphincter marked with sutures

FIG. 1B



Engineered sphincter-rectal cuff pre-implantation

FIG. 1A

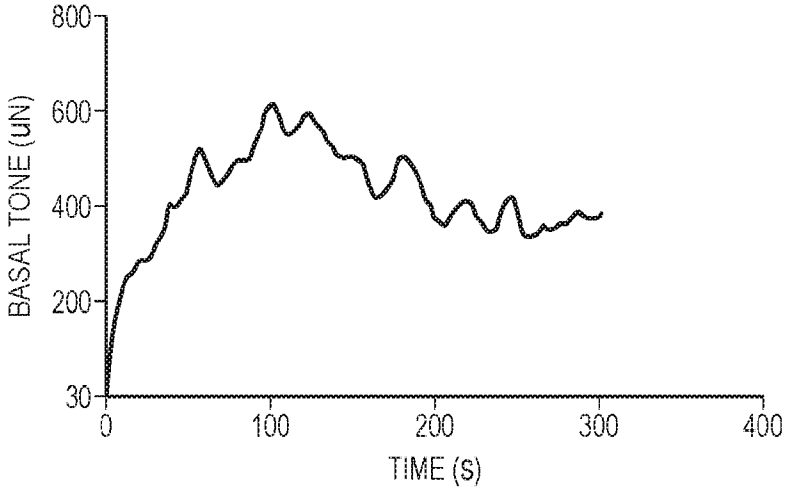

800
600
400
200
30
BASAL TONE (uN)
0
100
200
300
400
TIME (s)

FIG. 2A

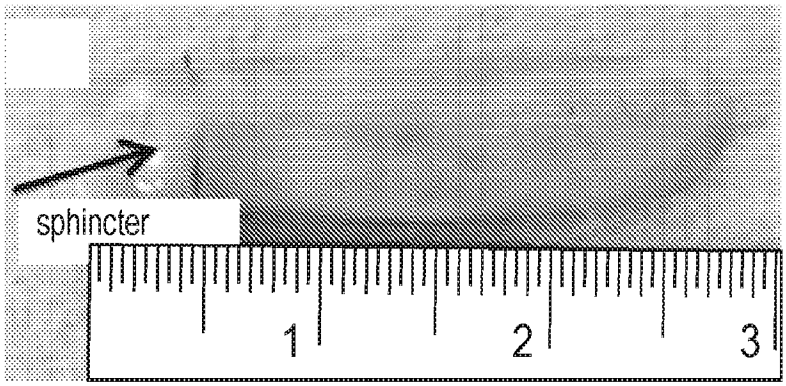
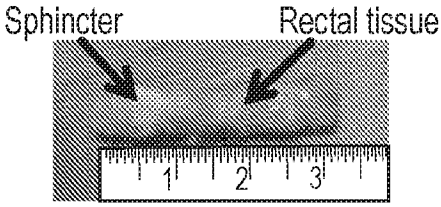

sphincter
1
2
3

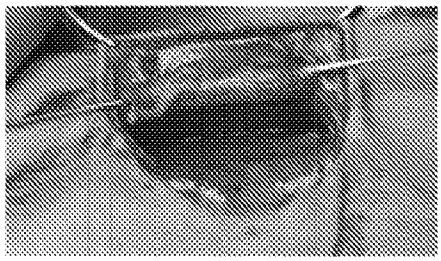
FIG. 2B

FIG. 3A



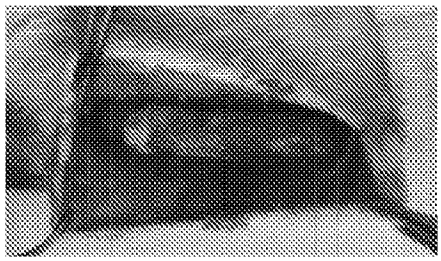
Sphincter-rectal cuff pre-implantation

FIG. 3B



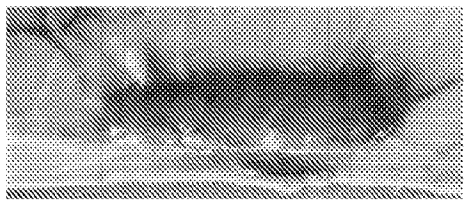
Implantation subcutaneously in the abdominal area

FIG. 3C



Implantation subcutaneously in the abdominal area

FIG. 3D



Harvest after 14 days

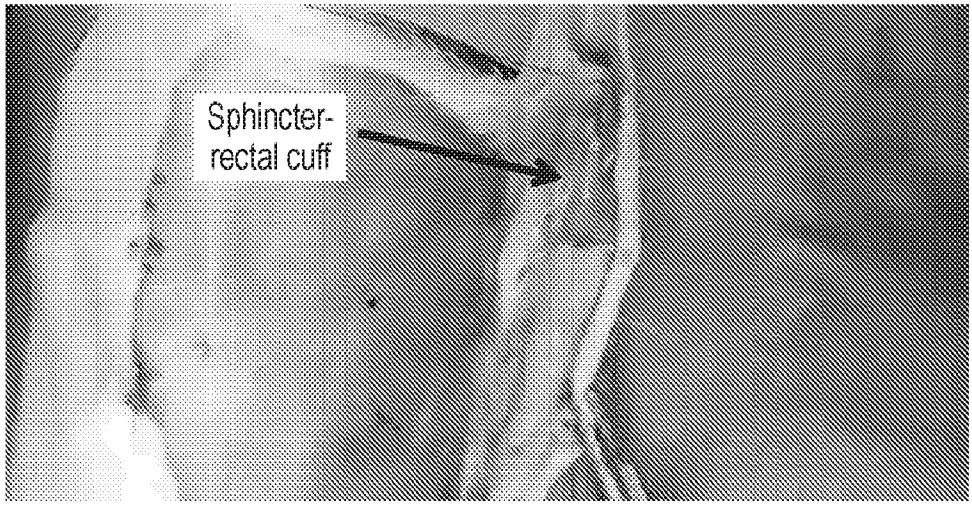


FIG. 4

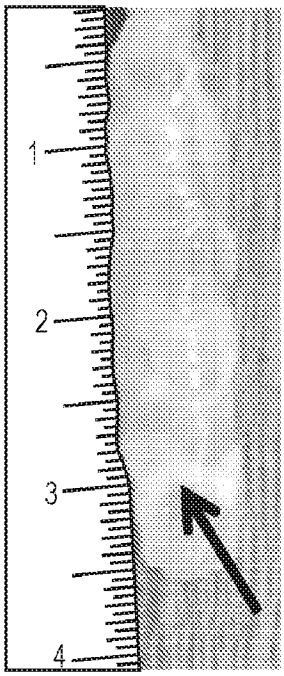


FIG. 5A

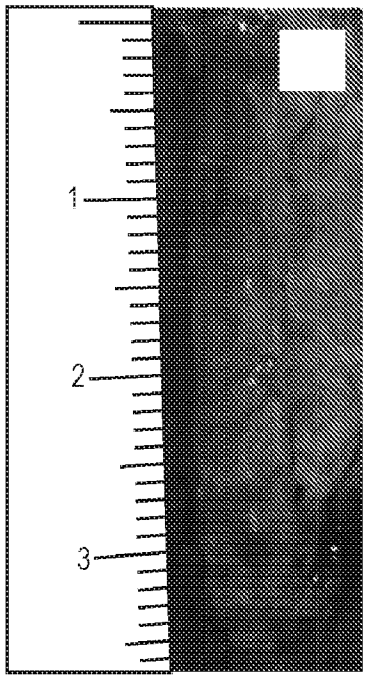


FIG. 5B

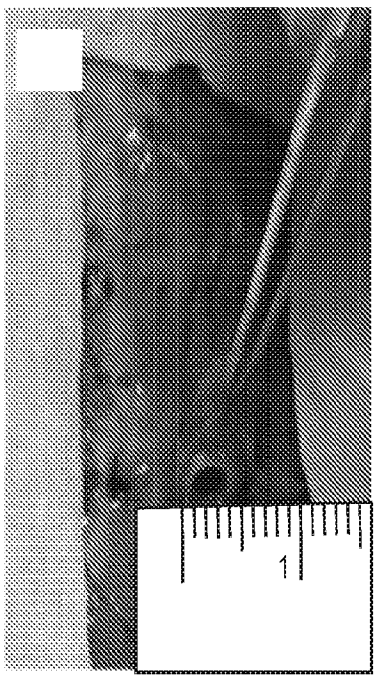


FIG. 5C

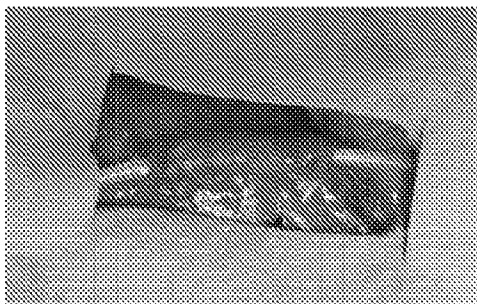


FIG. 6A

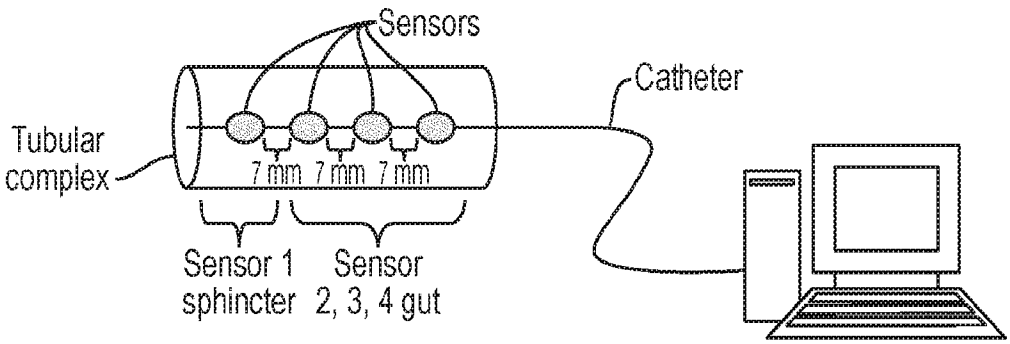


FIG. 6B

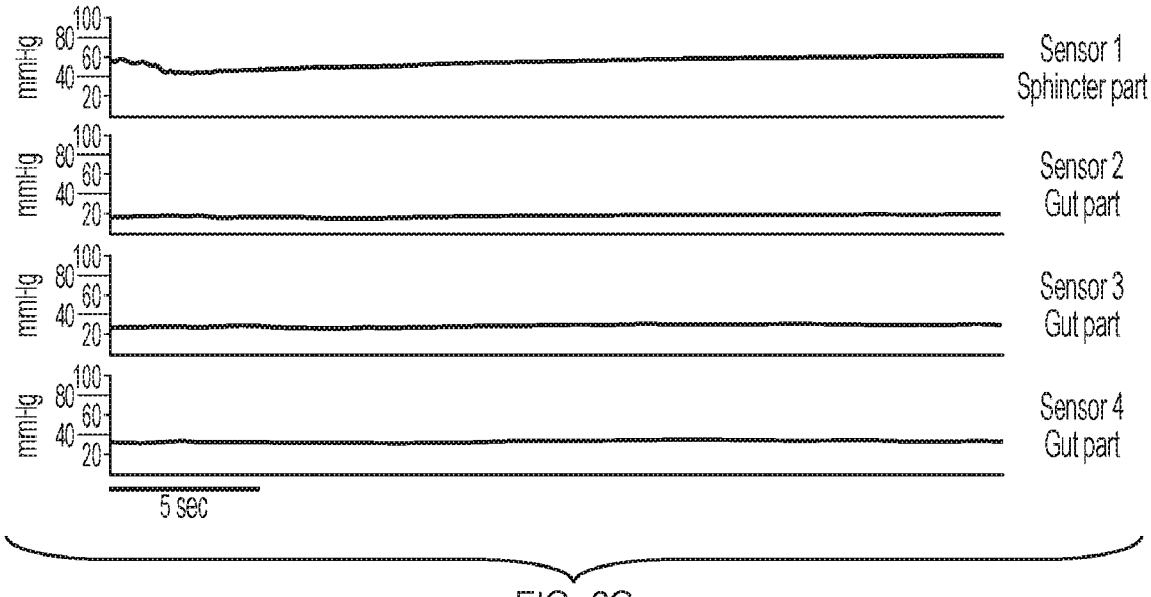


FIG. 6C

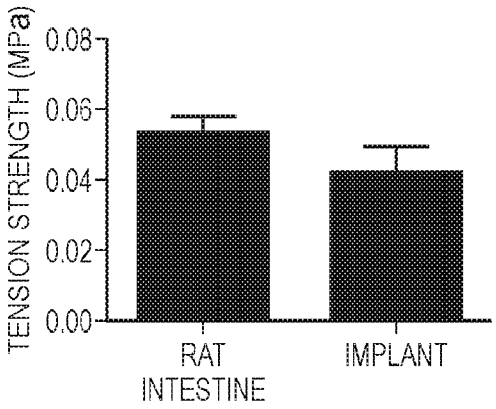


FIG. 7A

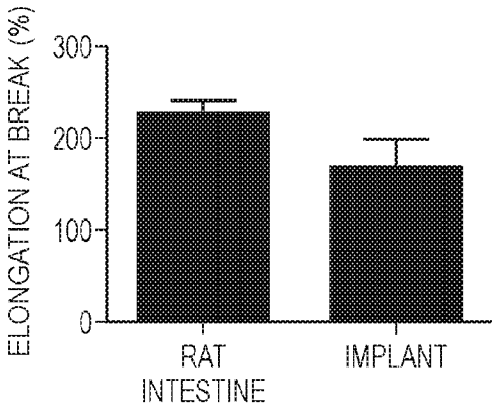


FIG. 7B

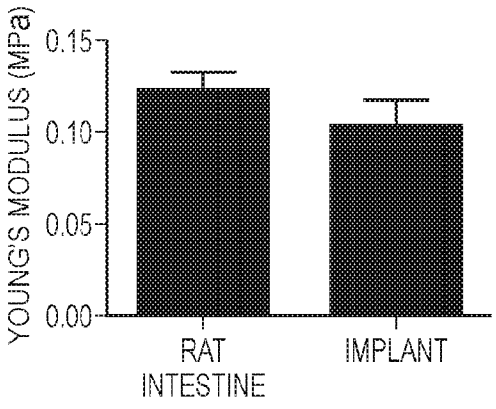


FIG. 7C

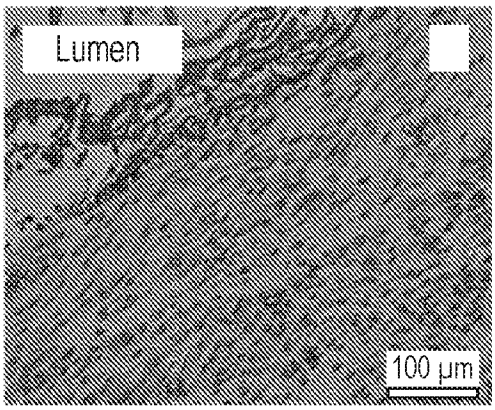


FIG. 8A

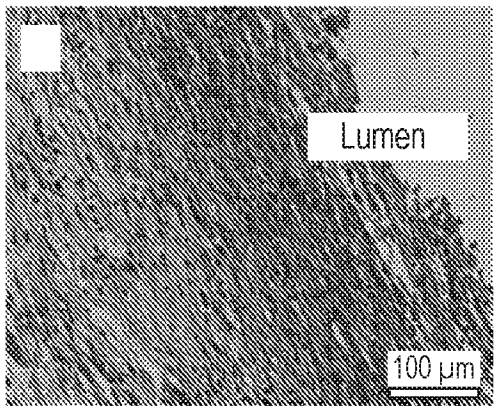


FIG. 8B

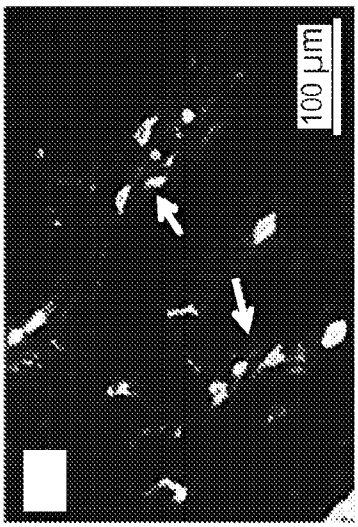


FIG. 9C

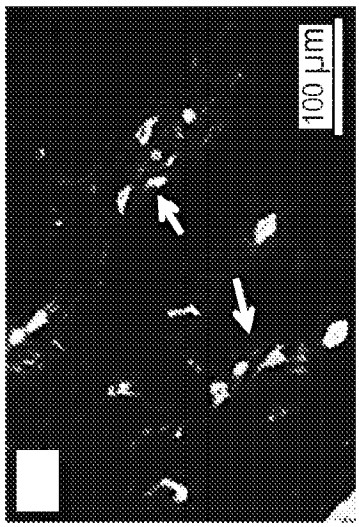


FIG. 9F

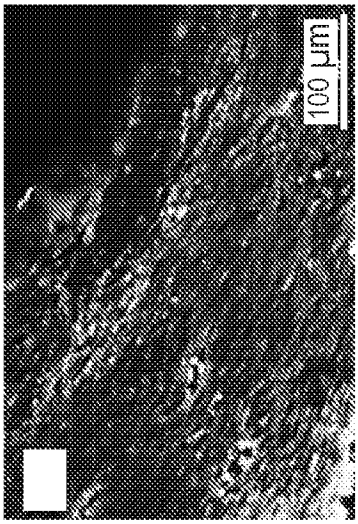


FIG. 9B

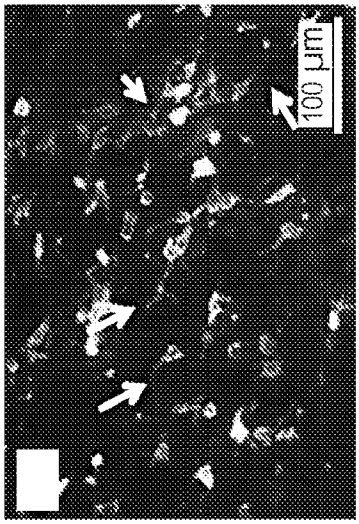


FIG. 9E

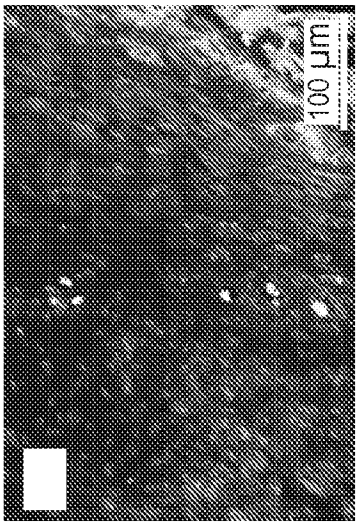


FIG. 9A

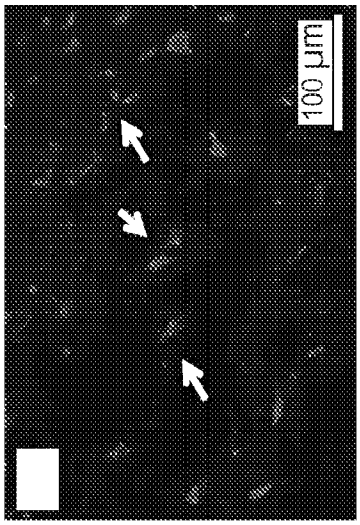


FIG. 9D

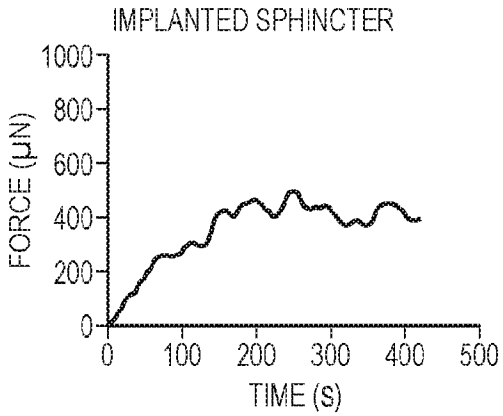


FIG. 10A

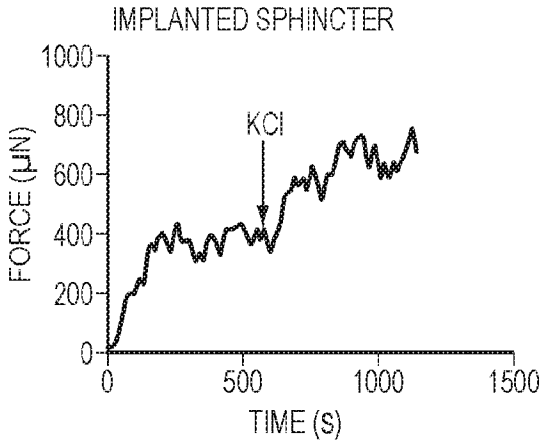


FIG. 10B

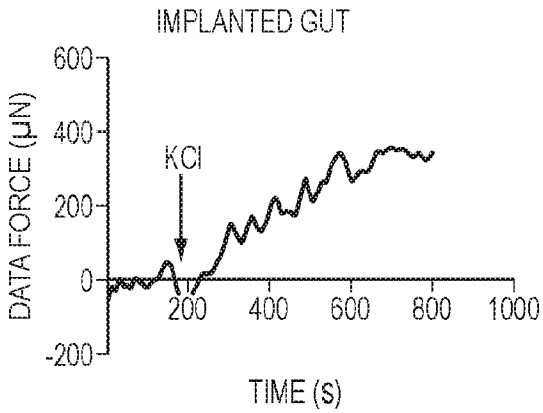


FIG. 10C

FIG. 11A

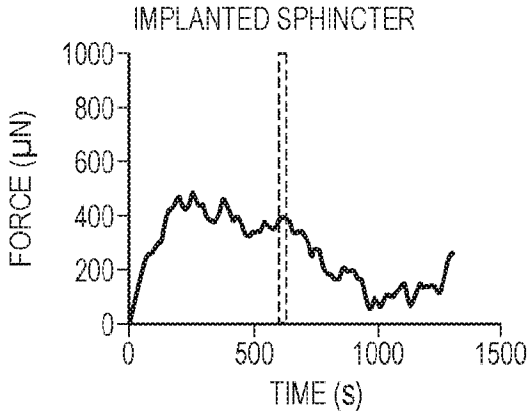


FIG. 11B

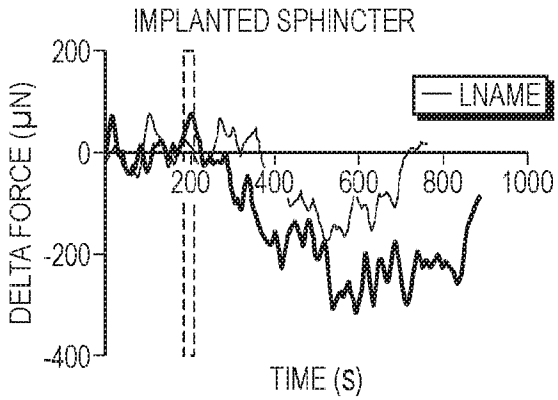


FIG. 11C

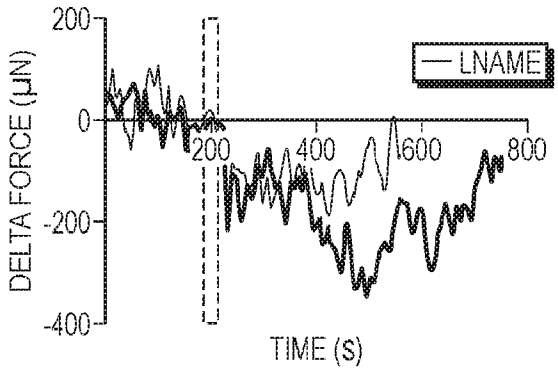
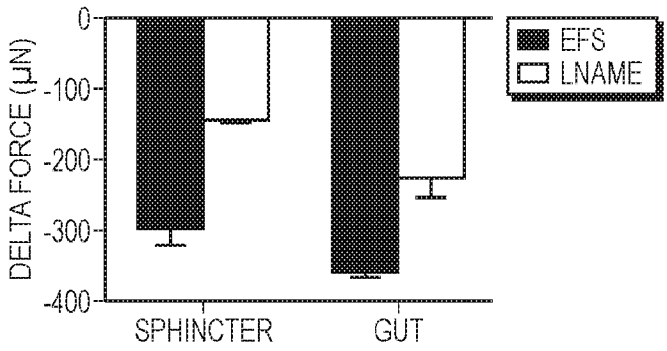


FIG. 11D



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US16/68891

A. CLASSIFICATION OF SUBJECT MATTER
IPC - A61F 2/08; A61L 27/38; C12N 5/07, 5/0797 (2017.01)
CPC - A61F 2/04, 2/08; A61K 35/30, 35/34, 35/34; C12N 5/0619, 5/0622

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2014/0377232 A1 (WAKE FOREST UNIVERSITY HEALTH SCIENCES) December 25, 2014; abstract; paragraphs [0016], [0040]-[0041], [0047], [0064]-[0065], [0079]-[0080], [0096], [0120]-[0121]; claims 1, 19-21	7-8
A	US 2006/0134076 A1 (BITAR, KN et al.) June 22, 2006; paragraphs [0009], [0018], [0025], [0103]-[0104]	1-6
A	US 2008/0102438 A1 (YANNAS, IV et al.) May 1, 2008; paragraphs [0028], [0030]	1-6
A	ZAKHEM, E et al. Successful Implantation of an Engineered Tubular Neuro-Muscular Tissue Composed of Human Cells and Chitosan Scaffold. June 19, 2015; Surgery 158(6); 1598-15608	1-6

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 February, 2017 (20.02.2017)	29 MAR 2017
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)