过2次或更多次时，将羊膜在开始施行 制备的步骤IV之前洗涤至少1次。

在一个特殊的实施方案中，去细胞化的羊膜在无菌条件下贮存， 30 并且不施行进一步的处理，即没有干燥。在开始施行步骤IV之前，根 本的是评估所有的细菌学和血清学测试以确保所有的测试是阴性的。

步骤IV。本发明的方法的最后一个步骤包括干燥本发明的去细胞 化的羊膜，从而生产胶原生物织物。可以使用任何可干燥羊膜从而产 生平且干的胶原片的方法。可是，羊膜优选地在真空中干燥。

在一个特殊的实施方案中，对于干燥本发明的去细胞化的羊膜的 范例性方法包括下列步骤： 5 装配去细胞化的羊膜以便干燥。从无菌溶液中取出去细胞化的羊 膜，并轻轻地挤压出过量的液体。然后例如在托盘上将去细胞化的羊 膜轻轻地进行伸展直至其变平，在这过程中胎儿侧朝下。然后将去细 胞化的羊膜翻转，从而胎儿侧朝上，并置于干燥架上，优选为塑料网 状干燥架（例如 Quick Count® Plastic Canvas, Uniek, Inc., 10 Waunakee, WI)。在其他实施方案中，干燥架可以为耐高压加热的不 锈钢网。在一个最优选的实施方案中，大约0.5 cm的羊膜与干燥架的 边缘重查。在某些实施方案中，将伸展至干燥架之外的重叠的羊膜包 裹住架的顶部，例如用夹子或止血钳。一旦将羊膜置于干燥架上，就将无菌纱布置于热干燥器（凝胶干燥器）（例如583型，Bio-Rad 15 Laboratories, 200 Alfred Nobel Drive, Hercules, CA 94547) 的干燥平台上，从而覆盖住比置于塑料网状干燥架上的羊膜稍大的区 域。优选地，纱布层的总厚度不超过一个折叠成4M的纱布的厚度。

可以使用任何适合于干燥片样材料的热干燥装置。将干燥架置于干燥 平台上的纱布之上，从而塑料架的边缘伸出纱布边缘之外，优选地伸 20 出0.1-1.0 cm,更优选地伸出0.5-1.0 cm。在一个最优选的实施方 案中，将具有羊膜的干燥架置于无菌纱布之上，并且羊膜的胎儿侧朝 上。在一些实施方案中，将另一个塑料架网置于羊膜之上。网架和在 其中干燥的膜的图像显示在图4中。在另一些实施方案中，将薄塑料 片（例如 SW 182、透明的 PVC、AEP Industries Inc., South 25 Hackensack, NJ 07606) 或生物相容的娃氧坑置于膜覆盖的网上，从 而片正好延伸至所有边缘之外。在该实施方案中，第二个网架是不需 要的。

在一个可选择的实施方案中，将羊膜置于一个或多个无菌Tyvek 材料片（例如用于医学包装的Tyvek 片，Dupont Tyvek®, P.O. Box 30 80705，

may be used internally, e.g., as an implant for a permanent or semi-permanent structure in vivo. In some embodiments, the hydrogel composition 5 was formulated in a non-biodegradable. In other embodiments, the hydrogel composition is formulated to be biodegradable. In one particular embodiment, the hydrogel composition formulated as biodegradable within a few days. In another particular embodiment, the hydrogel composition is made with biodegradable within a few months.

In some embodiments, the fabric of the present invention, the biological collagen transplanted cells, so that in 10 of these cells are uniform and confluent. Can be used on a biological fabric of the present invention, the transplanted cells including stem cells (preferably human stem cells), who differentiated mature cells, totipotent stem cells, pluripotent stem cells, pluripotent stem cells, tissue-specific stem cells, embryonic-like stem cells, progenitors, type fibroblasts but not limited thereto. In other embodiments, the present invention comprises a fabric of the present invention on a biological graft special kind of progenitor cells, comprising 15 chondrocytes, hepatocytes, hematopoietic cells, pancreatic parenchymal cells, neuroblasts and progenitor muscle cells, but is not limited to thereto.

5.2 collagen bio-fabric production method of the present invention provides a method for preparing collagen biological fabric of the present invention. In particular, the present invention includes a method for the preparation of collagen bio-fabric, which comprises: providing 20 containing placenta and amnion chorion; amnion and chorion separation; and the decellularized amniotic membrane. In a particular embodiment, the method also requires washing and drying the decellularized amniotic membrane.

Preferably, the placenta from human placenta in a human subject to use. In some embodiments, the present invention comprises the placenta from the animal species for use in human subjects. In other embodiments, the present invention comprises the placenta from an animal species for 25 veterinary medicine in animal subjects.

In a preferred embodiment, the placenta is in the process of the present invention is used to obtain as quickly as possible after childbirth the newborn. In another preferred embodiment, the placenta in a cesarean birth of a healthy baby made

RegenX Science Exhibit 1004
Page 930

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0930

Wilmington, DE 19880-0705)上，可选择地用一个 Tyvek 片置于膜上（在放置塑料膜之前）。该可选择的方法将会产生更光滑 形式的生物织物（即没有顺着和垂直于材料的轴的不同纤维密集区域 的图案），这种生物织物可能对于某些应用是有利的，例如如此处所 述用作用于细胞扩展的基质。

羊膜的干燥。在一个优选的实施方案中，本发明包括在真空中加 热干燥本发明的羊膜。虽然真空中的干燥可以在大约01C-大约601C的 5 任何温度来完成，但是羊膜优选地于大约35TC-大约501C进行干燥， 最优选为大约501。应当注意，将会预期到胶原在50X：以上的温度有 一些降解。干燥温度优选地进行设置，并通过使用采用扩展性探针的 经校准的数字温度计来进行校验。优选地，将真空压力设置为大约-22 英寸Hg。继续干燥步骤，直至例如用水分分析仪测得羊膜的胶原基质 10 含有低于3-12%的水。为了达到这一点，羊膜可以进行加热-真空干燥 例如大约60分钟，从而获得脱水的羊膜。在一些实施方案中，将羊膜 干燥大约30分钟-2小时，优选为大约60分钟。虽然不想被任何作用 机制所束缚，但是据信低热环境联合真空压力使得羊膜能够达到脱水 的状态而不会让胶原变性。

15 在根据本发明完成干燥过程之后，在真空泵运转的情况下将羊膜 冷却大约2分钟。

羊膜的包装和贮存。一旦如所述将羊膜根据本发明的方法进行干 燥，就将膜轻轻地提离干燥架。“提起”膜可以包括下列步骤：当泵 仍然在运转时，将塑料膜从边角开始轻轻地从羊膜上去除，同时压住 20羊膜；将带有羊膜的框架提离干燥平台，然后置于切割板上，并让羊 膜一侧朝上；进行切割，沿着离框架边缘1-2 mm的边缘进行切割；然 后将羊膜剥离框架；和切开成由其随后的用途所决定的合适大小。优 选地，在这一阶段的羊膜的操作通过使用无菌手套来进行。

将羊膜置于无菌容器如揭片袋中，并密封。如上所述，根据本发 25 明的方法生产出的生物织物可以于室温贮存延长的一段时间。

在可选择的实施方案中，本发明提供了制备含有绒毛膜的胶原生 物织物的方法。期望上述方法将可应用于制备含有绒毛膜的生物织物 的方法。在一个特殊的实施方案中，本发明包括制备胶原生物织物的方法，其包括：提供含有羊膜和绒毛膜的胎盘；分离羊膜和绒毛膜； 30 和将绒毛膜进行去细胞化。在一个特殊的实施方案中，该方法此外还 需要洗涤和干燥去细胞化的绒毛膜。

immediately after the placenta. If the placenta collected under sterile conditions. In some embodiments, the time, the placenta prior to any further processing from the delivery reservoir 30 from 48 hours. In other embodiments, when the placenta prior to any further processing from birth onwards stored up to five days.

Preferably, the placenta, and umbilical cord blood is transferred from childbirth or maternity room to another locations such as the laboratory for further processing. Placenta is preferably transferred to a sterile transport device, e.g., a sterile bag or container, which optionally is insulated. In some embodiments, the placenta is stored at room temperature until further processing. In other embodiments, the placenta into the row refrigerated until further processing, ie at about 2-8X: temperature storage. In other embodiments, the 5 cases, the placenta prior to further processing stored up to five days under sterile conditions. In a most preferred embodiment, as is known to those skilled in the art, the operation and handling the placenta under sterile conditions. HEPA filter is preferably equipped laboratory system (clean room rating defined, with 1,000 or more). In a preferred embodiment, prior to the HEPA filtration system using a laboratory open at least one hour for the purposes of the present invention is a method.

10 In certain embodiments, the removal of placenta blood that remains after birth umbilical cord blood is completely drained. In some embodiments, the placenta blood removal rate of 70 percent, 80 percent, 90 percent, 95 percent, 99 percent.

The present invention comprises, prior to birth by those skilled in the art standard techniques known to screening for pregnant women to check for infectious diseases, including HIV, HBV, HCV, HTLV, syphilis, 15 CMV and other known pollution placenta organized viral pathogens, but not limited thereto. Preferably, the method for screening for infectious diseases to follow the Federal Drug Administration regulations proposed. Pregnant women can be screened within a month of birth (ie, obtain a blood sample for diagnostic purposes), preferably within two weeks of birth, and even more preferably within a week of birth, and most preferably birth time. Use only from such a donor group collected by 20 organizations to produce bio-fabric of the present invention, that his

5. 2. 1 制备三维支架和层压制品的方法 本发明提供了制备含有本发明胶原生物织物的三维支架、三维构 造和层压制品的方法。

在一些实施方案中，本发明提供了制备羊膜层压制品的方法，其 包括：提供含有羊膜和绒毛膜的胎盘，优选为人胎盘；用此处公开的 5 方法分离羊膜和绒毛膜；用此处公开的方法将羊膜进行去细胞化；用 此处公开的方法洗涤去细胞化的羊膜至少一次；将至少两层去细胞化 的羊膜以相互接触的方式层叠在一起，从而形成羊膜层压制品；和用 此处公开的方法干燥羊膜层压制品。

可选择地，在另一个实施方案中，制备羊膜层压制品的方法包括， 10 干燥至少两层根据本发明的方法制备得到的羊膜，和将至少两层羊膜 以相互接触的方式层叠在一起，从而形成羊膜层压制品。

在一些实施方案中，根据本发明的方法生产出的羊膜层可以在存 在粘合剂的条件下以相互接触的方式放置在一起，从而形成羊膜层压 制品。根据本发明的方法和组合物所使用的粘合剂可以为任何本领域 15 熟练技术人员已知的生物胶合剂，优选为生物相容的胶合剂，其包括 天然胶合剂如纤连蛋白、血纤蛋白以及合成的胶合剂，但并不局限于 此。在其他实施方案中，将根据本发明的方法制备得到的羊膜层相互 进行交联以形成羊膜层压制品。任何本领域熟练技术人员已知的交联 试剂和方法在本发明的范围之内，包括化学交联、肽交联、UV交联、 20 放射交联、纤连蛋白交联、血纤蛋白原交联、水凝胶交联，但并不局 限于此。在其他实施方案中，根据本发明的方法生产出的羊膜层压制 品不含有粘合剂。

5. 3胶原生物织物的贮存和揉作 本发明包括将本发明的胶原生物织物作为脱水的片状物于室温 25 (例如251C)进行贮存。在某些实施方案中，本发明的胶原生物织物 可以于至少10X：、至少151、至少20X：、至少25X：或至少291C的温 度进行贮存。优选地，本发明的胶原生物织物不进行冷藏。在一些实 施方案中，本发明的胶原生物织物可以于大约2-81C的温度进行冷藏。

在其他实施方案中，本发明的胶原生物织物可以于任何上面确定的温 30 度贮存延长的一段时间。在一个最优选的实施方案中，本发明的生物 织物在无菌和非氧化条件下进行贮存。根据本发明的方法生产出的生 物织物可以于任何指定的温度贮存12个月或更长，而生物化学或结构 完整性没有变化（例如没有降解），并且胶原生物织物的生物化学或 生物物理学特性没有任何改变。根据本发明的方法生产出的生物织物 可以贮存几年，而生物化学或结构完

mother has been tested for the above mentioned pathogens are negative or non-reactive. Preferably, the father as well as detailed information on medical and social history of the donor placenta membranes, including for example a detailed family history. use it is known to a person skilled in the art of standard serological and bacteriological tests to screen donors. Assay or diagnostic tests to identify any pathogens within the scope of the method of the present invention 25, it is preferred for the joint determination of those measured with high accuracy and high throughput capacity.

In one particular embodiment, the present invention includes those skilled in the art standard techniques known to return to check selected antigens and / or antibodies to the donor. Non-limiting examples of antigens and antibodies include: Antibody screening (ATY); alanine aminotransferase Screening (ALT); hepatitis core antibody (nucleic acids and ELISA); hepatitis B surface antigen; HCV antibodies; 30 HIV-I and HIV-2; HTLV-I and HTLV-2; syphilis test (RPR); CMV antibody testing; as well as hepatitis C and HIV testing. It may be used in the determination of nucleic acid-based assay or ELISA-based assay, which for the skilled artisan are known.

The present invention further comprises known to a person skilled in the art of standard techniques to detect blood from the umbilical cord of the newborn (see, for example, Cotorruelo et al., 2002, ClinLab 48 (5-6):. 271-81;. Maine et al., 2001, Expert Rev. Mol Diagn, 1 (1): 19-29; Nielsen et al., 1987, J. Clin Microbiol 25 (8):... 5 1406-10; all of which are incorporated herein impose its entirety by reference). In one embodiment, the person skilled in the art standard techniques known to detect blood from the umbilical cord of the newborn to check bacterial pathogens (including Gram-positive and Gram-negative bacteria, but not limited to) and fungi. In a particular embodiment, the umbilical cord blood to determine blood type and Rh factor skilled in the art standard techniques known.

10 In another embodiment, the present standard methods known to those skilled in the art to obtain the difference from the CBC with blood from the umbilical cord of newborns. In another embodiment, the present standard methods known to those skilled in the art to obtain aerobic bacterial culture from blood from the umbilical cord

整性没有变化（例如没有降解）， 并且胶原生物织物的生物化学或生物物理学特性没有任何改变。期望 5 根据本发明的方法制备得到的本发明的生物织物会无限期地保持着。

生物织物可以贮存在任何适合于长期贮存的容器中。优选地，本发明 的胶原生物织物贮存在无菌的双揭片袋包装中。

本发明包括在其干燥状态操作本发明的胶原生物织物。在一个特 殊的实施方案中，将胶原生物织物在使用之前进行修整，例如在用作 10 外科移植物之前。本发明包括任何与其用途相适应的本发明生物织物 的维数，这是由本领域的熟练技术人员来确定的。在一些实施方案中， 本发明包括为lx2 cm、2x3cm、4M cm、5x5 cm或6x8 cm的胶原生 物织物。可以将本发明的生物织物切割成任何所需的大小，该大小位 于羊膜的大小的限制之内。

15 本发明的胶原生物织物的表面方向可以通过肉眼来鉴定。本发明 的胶原生物织物具有"网格"图案，这使得本领域熟练技术人员能够 用肉眼来鉴定母体和胎儿侧的表面。在一个特殊的实施方案中，胶原生物织物的表面方向在放大的条件下进行鉴定。本领域熟练技术人员 将会意识到，胶原生物织物的胎儿侧可以通过其凹陷的即下凹的网格 图案来进行鉴定。相反地，母体侧可以通过其凸起的即升高的网格图 案来进行鉴定。

本发明的胶原生物织物需要最少的使用之前的准备时间。在一个 优选的实施方案中，本发明的胶原生物织物可在5分钟或更少时间之 内、在10分钟或更少时间之内、在15分钟或更少时间之内准备好进 行使用。本发明的胶原生物织物在使用例如用作外科移植物之前的准 备时间包括通过再水化来活化脱水的胶原生物织物。在一些实施方案 中，本发明的胶原生物织物当在外科部位上时进行水化。在其他实施 方案中，本发明的胶原生物织物在皿中于无菌条件下进行水化。本发 明包括用无菌生理缓冲液来水化本发明的胶原生物织物。在一个特殊 的实施方案中，本发明包括用无菌盐溶液如0.9%无菌NaCl溶液来水 化本发明的胶原生物织物。在一些实施方案中，无菌盐溶液是缓冲液。

在某些实施方案中，本发明的胶原生物织物的水化需要至少2分钟、 至少5分钟、至少10分钟、至少15分钟或至少20分钟。在一个优选 的实施方案中，本发明的胶原生物织物的水化在5分钟之内完成。在 另一个优选的实施方案中，本发明的胶原生物织物的水化在10分钟之 内完成。在另一个实施方案中，本发明的胶原生物织物的水化不需要 5 超过10分钟。

of newborns. Use only from the tissue collected by this donor to produce the present invention biological fabric, that it has within normal limits CBC (for example, no significant departure 15 levels were normal exception or deviation), the detection of serological and bacteriology is negative, and tested for infectious diseases and contamination of negative or non-reactive.

Collagen exemplary methods for the preparation of biological fabric of the present invention comprises the following steps.

Step I. The present invention includes a processing placenta membrane, in order to (optionally) separating the umbilical cord from the placenta, and amniotic and chorionic separation. In a preferred embodiment, the tire 20 before cutting the amniotic and chorionic membrane separation. Isolated amnion and chorionic preferably from the edge of the placental membrane starts. In another preferred embodiment, use blunt dissection to separate amnion and chorion, for example, with gloved fingers. After the amnion and chorionic and placental separation, for example, under the stump cut with scissors, and away from the placenta. In certain embodiments, when the organization can not be separated without tearing the amnion and chorion, the package 25 of the present invention comprises the amnion and chorion as a cut from the placenta down, and then they peeled apart.

The amniotic membrane is then preferably stored in sterile saline. In some embodiments, the sterile saline solution is a buffer solution. In a particular preferred embodiment, amnion for storing sterile 0.9 percent saline solution was sterile NaCl solution. Preferably, the cold storage at a temperature of at least 41C to store the amnion. In certain embodiments, the amniotic membrane at least 21C, at least 61C 30 or until 8TC temperature refrigeration. At this point, if the amniotic membrane kept refrigerated and covered with a sterile saline, the amnion can be stored for up to 5 days. Preferably, before the next step in the program of the amnion separated from childbirth onwards refrigerated up to 72 hours.

Step II. Once the amniotic and chorionic separation, the present invention comprises decellularized amniotic membrane. The method of the present invention include any known to those

RegenX Science Exhibit 1004
Page 933

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0933

一旦将本发明的胶原生物织物进行水化以便使用例如用作外科移 植物，根据本领域熟练技术人员的测定，其相对于本领域中的羊膜具 有增强的可缝合性。本发明的胶原生物织物不像本领域中的羊膜一样 容易撕裂，其也不像本领域中的羊膜一样易碎。本发明包括能够有效 10 地缝合的胶原生物织物。

5. 3. 1 本发明的生物织物的灭菌优选地使用本领域熟练技术人员已知的 方法通过电子束辐射来进行，例如Gorham, D. Byrom (编者），1991, Biomaterials, Stockton Press, New York, 55-122。任何足够杀 15 死至少99. 9%的细菌或其他潜在污染生物的辐射剂量在本发明的范围 之内。在一个优选的实施方案中，使用至少18-25 kGy的剂量来完成 本发明的生物织物的最终灭菌。可是，本发明的生物织物的灭菌不包 括在抗生素或甘油中的贮存。

5.4使用本发明的胶原生物织物的方法 20 本发明的胶原生物织物在内科和外科领域有着许多的应用，这部 分地是由于其与本领域中所使用的传统膜相比的物理特性，例如生物 机械强度、屈曲性、可缝合性和低免疫原性。例如期望本发明的胶原 生物织物对于在现有技术的膜上的引导性组织再生具有增强的治疗效 用，这些膜例如为合成的不可再吸收性PTFE膜如Goretex?;合成的 25 可再吸收性膜，其由乙交酯和丙交酯共聚物所形成；WO-88/08305、DE-2631909、U. S.专利号5，837，278中所公开的膜。

制备本发明的胶原生物织物的方法确保保留了生物织物的三级和 四级结构，因此使得该生物织物对于其在内科和外科领域中的想要的 用途来说是理想的。正如下面详细描述的，本发明提供了这样的胶原 30 生物织物，即该胶原生物织物的物理特性使得其适合于在多种内科和 牙科应用中使用，包括血管修复、子宫修复、腱的替换、角膜的替换、 人造皮肤、牙周病的治疗和伤口愈合，但并不局限于此。依赖于其想 要的用途，本发明包括使用胶原生物织物作为二维的膜，例如可以制 作形成管状容器的膜；作为三维的支架，例如植入物；或者作为一维 的纤维。

5.4.1 使用本发明的胶原生物织物进行治疗的方法 5 5.4.1.1 处理皮肤状况的方法 人皮肤是表皮和真皮的复合材料。表皮的上面部分是角质层，其 是皮肤最硬的一层，以及是受周围环境影响最大的一层。在角质层的 下面是表皮的内部。在表皮的下面是乳突状真皮，其由相对疏松的结 締组织组成，这些结締组织决定了皮肤的微起伏。位于乳突状真皮之 10 下的网状真皮是有间隙地组织在一起的紧密结締组织。网状真皮还与 粗糙的皱纹有关。在真皮之下是皮下层。

skilled in the decellularized method, but for the provisions of the present invention is a method amniotic decellularized does not include any steps frozen amniotic membrane. When used herein, refers to cells of all cellular material and 5 to remove cell debris (for example, all visible cellular material and cellular debris) from the amniotic membrane of the present invention. amnion cells to ensure that substantially all of the collagen matrix and is usually linked to the amniotic cells are removed. The decellularized amniotic membrane of the present invention removes "substantially all" collagen matrix associated with the cells, which is preferably at least 90 percent of the cells were removed, more preferably at least 95 percent removal of the cells, and most preferably at least 99 removed percent of the cells. For 10 decellularized amniotic membrane according to the method of the present invention is to uniformly thin thickness that is 10 to 40 microns, is smooth (measured by touch) and clear in appearance.

In a preferred embodiment, the decellularized amniotic membrane of the present invention comprises removing substantially all cellular material and cellular debris from the mother's side of the amniotic membrane, followed by removal of all cellular material and cellular debris from the fetal side of the amniotic membrane. In a particular embodiment, the amniotic membrane 15 of the present invention to include a physical scraping of cells, and joint use of a sterile solution rinse. In another particular embodiment, amnion, including the use of physical scraper scraping sterile cell scraper. In another particular embodiment, the sterile solution during the decellularization of the amniotic aqueous rinse, i.e., physiological buffer or salt solution containing a solution, such as 0. 9 percent NaCl solution.

20 decellularized amniotic membrane comprises removing naturally occurring cells and other antigens and cellular debris from the amniotic membrane, and optionally processed to produce new immune suppression sites. During the decellularized amniotic membrane, the removal of native viable cells as well as other possible cause of an adverse immune response or a cellular and non-cellular structural components. According to the cell technology used in the present invention should not cause significant damage to the amniotic anatomy or alter the biomechanical properties of its structural component 25, namely

皮肤的主要功能包括保护、排泄、分泌、吸收、温度调节、色素 形成、蓄积、感官知觉和免疫过程的调节。这些功能不利地受到由于 老化和过度阳光暴露所引起的皮肤结构变化的影响。与皮肤老化相关 15 的生理性变化包括屏障功能的削弱和降低的表皮细胞的更新率，例如 见 Cerimele, D.等人，1990, Br. J. Dermatol., 122 Suppl. 35， 13-2 0页。现有技术中的方法和组合物对于改善皮肤状况例如改善皮肤 的弹性和柔软性或者去除皱纹取得了有限的成功。

本发明的胶原生物织物具有医疗以及美容的应用。本发明的胶原 20 生物织物在处理皮肤状况中具有临床和治疗效用，包括皮肤损伤、老 化的皮肤、皱纹、细纹、变薄、降低的皮肤弹性、粗糙的皮肤、先天 的和变性的皮肤状况、VII型胶原缺乏以及阳光损害的皮肤。在某些实 施方案中，本发明的胶原生物织物可以用作用于皮肤状况如痤疮瘢 痕、眉间沟、切除性瘢痕或者任何其他本领域中已知的软组织缺损的 25 皮下植入物。本发明的胶原生物织物在处理与皮肤老化相关的变化中 具有临床和治疗效用。在某些实施方案中，本发明的胶原生物织物对于改善皮肤皱纹和/或其他状况如皮肤弹性和柔软性具有效用。本发明 的胶原生物织物可以用作用于处理皮肤状况的植入物、层压制品或三 维卷缩的形式。期望本发明的胶原生物织物相对于本领域中已知用于 30 处理这种皮肤状况的方法例如U. S.专利号5，972，999、5，418, 875、 、5，198, 465具有增强的临床效用，这部分地是由于其稳定 性，这可能提供了更长的持续效应。

在优选的实施方案中，本发明的胶原生物织物进一步用一种或多 种本领域中已知用于处理皮肤状况的试剂来加以补充。这种试剂的例 子包括维生素、矿物质、基于儿茶素的制剂、N-乙酰葡糖胺和葡糖胺(见例如，Neldner, 1993，Amer. Acad. Derm. Annl. Mtg. Wash. 5 DC.； Lubell 1996, Cosmetic Dermatol, 9(7): 58-60; Swaine 等 人，1995, J. Am. Board of Family Practice, 8 (3): 206-16; Shan 等人，1994, Kidney International, 46 (2): 388-95; 所有这些文 献在此处以其整体进行引用作为参考），但并不局限于此。

本发明的胶原生物织物可以用任何可用于处理皮肤状况的生物分 10 子进行浸渍，包括抗生素（例如克林霉素、二甲胺四环素、强力霉素、 庆大霉素）、激素、生长因子、抗肿瘤剂、抗真菌剂、抗病毒剂、疼 痛药物、抗组胺剂、抗炎剂、抗感染剂（包括银（例如银盐包括硝酸 银和磺胺嘧啶银，但并不局限于此）、元素银、抗生素、杀菌的酶（例如溶菌酶），但并不局限于此）、伤口愈合剂（例如细胞因子包括PDGF、 15 TGF,但并不局限于

collagen, elastin and fibronectin also possible the structure and biochemistry integrity is not decellularized influence. Specifically is the strong chemical treatment and protease treatment within the scope of the amniotic decellularized technique is not of the present invention.

Preferably, the decellularized amniotic membrane include the use of detergents containing solution. The method according to the present invention can be used in detergents include non-ionic detergent, Triton X-100, 30 sub anionic detergent or sodium lauryl sulfate, but is not limited thereto. In the method of the present invention, the detergent may be used alone or in combination. You can use any mild anionic detergent method according to the present invention, a non-corrosive detergent, which has a 6-8 PH and low foaming.

In one particular embodiment, the use of 0.01-1 percent of a hydrated sodium deoxycholate in the decellularized amniotic membrane. While not wishing to be bound by a special mode of action, but the method according to the present invention, the decellularized amniotic membrane may be lysed cell membranes and help to remove cell debris from the amniotic membrane. However, measures should be taken to eliminate any residual detergent amniotic level, 5 for example, to avoid interference and later again in the amniotic membrane transplantation of living cells. it is the fundamental limitation of protease activity in the preparation of the present invention, the biological fabric. Additives such as metal ion chelating agent may be formed for many proteases for hostile environment, e.g., 1,10-phenanthroline and ethylenediaminetetraacetic acid (EDTA). Provide for proteases such as collagenase is not optimum conditions during the cleavage step can help protect the amniotic components such as collagen not drop 10 solution. Protease is not suitable for most conditions can be obtained through such that the formulation of low permeability lysis solution to eliminate or limit the number of calcium and zinc ions in solution available. Many protein enzyme in the presence of calcium and zinc ions are active, and lose a lot of their activity in the absence of calcium and zinc ions environment. Preferably, the hypotonic lysis solution will be prepared by selecting the following conditions, namely pH, reduce the availability of calcium and zinc ions, the presence of metal ion chelators and the use of 15 specific protease inhibitor for collagenase, so that the best solution would be cleaved naturally

RegenX Science Exhibit 1004
Page 935

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0935

此；胸腺素）、作为伤口愈合剂的透明质酸、伤口封闭剂（例如有或没有凝血酶的血纤蛋白）、细胞吸引剂和支架试剂 (例如纤连蛋白）等等，但并不局限于此。在一个特殊的实施方案中， 胶原生物织物可以用至少一种生长因子进行浸渍，例如成纤维细胞生 长因子、上皮细胞生长因子等等。生物织物还可以用小分子进行浸渍， 20 例如小有机分子如特殊生物化学过程的特异性抑制剂，如膜受体抑制剂、激酶抑制剂、生长抑制剂、抗癌药物、抗生素等等 创伤和烧伤 与本领域中已知的其他生物材料例如U.S.专利号 、 4' 320，201、3' 800，792、4，837，285、5，116' 620 中所描述的那些相 25 比，期望本发明的胶原生物织物作为用于促进硬组织和/或软组织修复 的创伤敷料具有增强的临床效用，这部分地是由于其物理特性。因为 本发明的胶原生物织物保持了胶原的天然四级结构，所以其造成增强 了组织通过细胞迁移而向内生长进胶原基质的间隙中。本发明的生物 织物使得细胞能够粘附在胶原基质上，并生长进胶原基质中，和使得 30 细胞能够合成它们的大分子。细胞由此产生出能够让新组织生长的新 的基质。这种细胞生长没有在其他已知的胶原形式例如纤维、毛状物 和可溶性胶原上观察到。

在一些实施方案中，本发明包括通过将本发明的胶原生物织物直 接置于受试者的创伤部位的皮肤上即角质层上来治疗创伤，因此例如 用粘合带覆盖伤口。在其他实施方案中，本发明包括使用本发明的胶 原生物织物作为植入物例如作为皮下植入物来治疗创伤。

5 本发明包括通过添加能够促进组织向内生长进本发明的胶原生物 织物中的大分子来增加伤口愈合率。这种大分子包括透明质酸、纤连 蛋白、层粘连蛋白和蛋白聚糖（见例如，Doillon等人（1987) Biomaterials 8: 195-200 ； 以及 Doillon 和 Silver (1986) Biomaterials 7: 3-8)，但并不局限于此。

10 本发明此外还包括在此处描述的本发明的胶原生物织物中掺入药 理活性试剂以便如在5.4.2.7节中用于将药物递送给皮肤，和掺入任 何上述的生物分子，药理活性试剂包括血小板衍生生长因子、胰岛素 样生长因子、表皮生长因子、转化生长因子-P、血管生成因子、抗生 素、抗真菌剂、杀精剂、激素、酶、酶抑制剂，但并不局限于此。药 15 理活性试剂优选地以生理有效剂量进行提供。

在一些实施方案中，进一步于应用于创伤部位之前在胶原生物织 物上移植活细胞，包括异源干细胞、干细胞和自身成熟细胞，但并不 局限于此。

occurring cells, while protecting the amnion does not adversely following degradation by the proteasome. E.g., hypotonic lysis solution may include water, buffer solution, the PH of 5. 5-8, preferably 7-8, free of calcium and zinc ions, and containing a metal ion chelator such as EDTA. Further, during the processing with a hypotonic lysis solution amnion, controlling the temperature and time parameters it may also be used to limit the activity of proteases 20.

Preferably, the decellularization treatment of the amniotic membrane also limits the formation of new immunological sites. Because it is believed that enzymatic degradation of collagen results in increased immunogenicity, the present invention comprises an enzyme nuclease treatment e.g. amnion, inhibition of these enzymes in cellular metabolism, protein production and cell division are effective, they amniotic protease component Solutions to a minimum, thus saving the 25 underlying structures amnion. Examples of the method according to the present invention can be used are those effective nuclease digestion in naturally occurring DNA and RNA in the cells of nucleases including exonucleases and endonucleases. Including cytostatic activity exonuclease method according to Non-limiting examples of the present invention can be used as a nuclease nuclease DNAase I (SIGMA Chemical Company, St. Louis, Mo.) and RNAase A (SIGMA Chemical Company, St. Louis, 30 Mo.), and the inhibition of cell activity such as endonuclease EcoR I (SIGMA Chemical Company, St. Louis, Mo.) and Hind III (SIGMA Chemical Company, St. Louis, Mo.). More preferably, the selected nucleases such as magnesium, calcium physiological buffer solution containing ions applied, these ions on the activity of nuclease be the most appropriate.

Preferably, the farmers of the ion buffer solution, the treatment temperature and treatment duration by one skilled skilled in the art by routine experimentation to choose, in order to ensure the desired level of nuclease activity. Buffer is preferably hypotonic to promote access to the interior cellular nucleases.

5 In one particular embodiment, the amniotic membrane of the present invention will be decellularized comprises the following steps. First, the amnion was transferred to a clean sterile container, and optionally rinsed with sterile water,

本发明的胶原生物织物对于治疗伤口感染是特别有用的，例如随 20 后出现外科或外伤性伤口的贯穿的伤口感染。在一个特别的实施方案 中，胶原生物织物用治疗有效剂量的用于治疗伤口感染的试剂进行浸 渍，包括抗生素、抗微生物剂和抗菌剂，但并不局限于此。本发明的 胶原生物织物在治疗由任何本领域中已知的微生物所引起的伤口感染 之中具有临床和治疗效用，例如源自人体内的感染伤口的微生物，这 25 是一个已知的病原生物库，或者源自环境来源的感染伤口的微生物。

其在伤口中的生长可以被本发明的方法和组合物所减少或防止的微生 物的非限制性例子为金黄色葡萄球菌（s. aureus)、表皮葡萄球菌（St. epidermis)、p-溶血性链球菌(Streptococcus)、大肠杆菌(E. coli)、克雷伯氏菌属（Klebsiella)物种和假单胞菌属 30 (Pseudomonas)物种，和在厌氧细菌中为韦氏梭菌（Clostridium welchii)或（tartium),它们是造成气性坏疽的原因，主要是在深 的外伤性伤口中。

在其他实施方案中，本发明的胶原生物织物用于创伤治疗，包括 表皮伤口、皮肤伤口、慢性伤口、急性伤口、外部伤口、内部伤口（例 如胶原生物织物可以在外科手术期间包裹在吻合部位的周围，以便防 止血从缝合线中渗漏出来，和防止身体与缝合材料形成粘连）、先天 5 性伤口（例如营养不良性大疱性表皮松解症），但并不局限于此。特 别地，胶原生物织物在治疗压力性溃疡（例如褥疮性溃疡）中具有增 强的效用。压力性溃疡常常出现在长期卧床休息的患者中，例如四肢 瘫痪患者和截瘫患者，他们由于局部压力的影响而遭受皮肤损失。结 果造成的压力性溃疡表现出真皮糜烂以及表皮和皮肤附属物的损失。

10 本发明的胶原生物织物还可以用于治疗烧伤，包括一度烧伤、二 度烧伤（部分厚度烧伤）、三度烧伤（完全厚度烧伤）、烧伤伤口的 感染、切除的和未切除的烧伤伤口的感染、移植伤口的感染、供体部 位的感染、先前移植的或愈合的烧伤伤口或者皮肤移植供体部位的上 皮损失以及烧伤伤口脓疱病，但并不局限于此。

15 5.4.1.3 组织工程 本发明包括使用本发明的胶原生物织物作为用于运送培养皮肤细 胞的载体，例如作为对于上皮细胞生长和分化的支持物。在某些实施 方案中，为了在生物织物上移植细胞以形成组织和/或类器官（即在表 面外观或结构上与身体的任何器官或腺体类似），在生物织物上再次 20 移植这种新细胞的过程期间，生物织物可以用细胞粘着因子进行处理 以增强细胞与生物织物的附着。在某些实施方案中，细胞附着程度是 通过用血清处理羊

and then dried with sterile gauze. Then placed on a sterile tray amniotic, and maternal and side facing up. With a sterile cell scraper (for example, 32 cm, PE blades, PS to hand, NalgeNunc International) and from the mother side by physically visible to all except 10 of cellular material to the amnion amniotic portion decellularized. If necessary, use sterile water to help remove cells and cell debris. After the completion of part of the amniotic cells to the parent side of the amniotic flip side up so that the fetus. Use a cell scraper to gently remove all visible on the side of the fetal cell debris, amniotic membrane in this process for minimum pressure to prevent tearing. You can use sterile water to help remove cells and debris.

15 In one embodiment, the decellularized amniotic membrane before further processing into a sterile container, the container is filled with a sterile physiological solution such as sterile 0.9 percent NaCl solution. The method according to the present invention, amniotic next processing step should be placed no later than amnion after 2-3 hours in sterile physiological solution into effect. In a particular embodiment, when the step II Step III immediately implemented, without amniotic placed in a container containing a sterile solution of 20.

During the cleaning process, for example, sterile scalpel amniotic membrane can be cut, thus its shape trimmed in order to be easier to clean, can not be removed or cleaned areas.

Step III. After decellularization, amniotic washed to ensure removal of cell debris may include cell proteins, lipids and cells, including cellular nucleic acid, and removal of any foreign cells such as broken 25 extracellular soluble proteins, lipids and proteoglycans. While not wishing to be bound by any action mechanism, but removed to reduce the immunogenicity of such cells and amniotic debris outside the cell.

Once the amniotic membrane of the present invention have been decellularized, further washed amniotic so effectively to achieve complete removal of all visible cellular material and cellular debris from both sides of the amnion.

The solution is preferably formulated to be effective in cracking naturally occurring tissue cells from 30 children hypotonic or low strength aqueous solution. This solution may be hypotonic deionized water or low water seepage buffer

RegenX Science Exhibit 1004
Page 937

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0937

膜来增加的，例如人血清或胎牛血清。在其他实施方案中，细胞附着程度是通过用纤连蛋白处理羊膜来增加的。

本发明的胶原生物织物可以用于引导性组织再生技术，例如再生 25 或替换有病的或受损的组织。本发明包括本发明的生物织物的使用， 即将生物织物直接植入于治疗部位，或者形成假体装置。本发明的胶 原生物织物在任何情况下都是特别有用的，例如在外科手术尤其是口 部或牙科外科手术之后（在5.4.2.2节中有更详细的描述），其中有 望获得提高的伤口愈合和/或真皮替换。本发明的胶原生物织物在引导 30 性组织再生中的效用部分地是由于其能够提供这样的条件，即这些条 件防止了其他组织向内生长进需要再生的区域中。

例如，在由于腐烂或疾病而去除了牙根的实质部分的地方，希望 出现健康的骨的再生以替换去除的骨组织。可是，已经发现由于去除 了骨而留下的空腔很快被结締组织充满，而且这种结締组织的向内生长有效地防止了骨再生。为了防止这种向内生长，可以将本发明的胶 原生物织物通过外科手术插入从而环绕在伤口空腔的边缘。生物织物 5 防止或阻止伤口空腔被不想要的细胞类型侵入，因此能够让首选的细 胞生长入空腔中，由此愈合了伤口。

在一些实施方案中，胶原生物织物用作用于眼表面重建的移植 物，例如在患有Stevens Johnson综合征、化学灼伤之后的继发性达 缘干细胞缺乏的受试者中，或者在具有化学灼伤和/或热灼伤的受试者 10 中。期望本发明的生物织物例如通过促进上皮形成而具有增强的临床 效用。期望本发明的生物织物相对于在本领域中用于组织工程目的的 羊膜具有增强的临床效用，见例如，Gomes等人，2003, Opthalmology, 119: 166-73; Ti 等人' 2001，Opthalmology, 108: 1209-1217; Meller 等人，2000, Opthalmology, 107: 980-9; Gris 等人，2002, 15 Opthalmology, 109: 508-12; Koizumi 等人，2000，Invest. Opthal. and Visual Science, 41: 2506-13 ; Pires 等人，1999，Arch. Opthalmol. 117: 1291-7; Tseng 等人•，1998, Arch. Opthalmol. 116: 431-441; Heiligenhaus 等人，2001，Invest. Opthal. and Visual Science 42: 1969-74 ; Anderson 等人，2001，Br. J. 20 Opthalmol. 85: 567-75。

本发明包括在胶原生物织物上移植活细胞，包括成熟组织细胞、 自身细胞和干细胞，但并不局限于此。在本发明的方法中使用的干细 胞可以为全能细胞、多能细胞或分化的组织特异性细胞。在本发明的 方法中使用的干细胞可以通过本领域熟练技术人员已知的标准方法来 25 获得。优选地，根据于

solution. Moreover, the low water seepage buffer solution preferably contains additives that are provided for in the activity of the protease is not the most suitable conditions, e.g., it may be due to cell lysis and release of collagenase.

Preferably, the amnion and gently shaking in detergents, for example, on a rocking platform, and help from decellularized. In certain embodiments, the amniotic shaken for at least 15 minutes, at least 20 minutes, at least 30 minutes or until 120 minutes. After detergent decellularized, sheep 5 film as described above can be physical again decellularized; when needed can be repeated physical and detergent decellularization step, just keep the integrity of the amnion, until there is no remaining under visible cellular material and cellular debris.

In a particular embodiment, the amniotic membrane comprises the steps of washing: The decellularized amniotic membrane placed in a sterile container and then spent the cells of the solution sufficient to cover the full amount of amniotic number 10 container; then and to the container containing the amniotic membrane of the cell solution is placed on a shaker platform (such as 100, VWR Scientific Products Corp., PO Box 640169, Pittsburgh, PA 15264-0169). Then decellularized amniotic membrane solution shaken in 15-120 minutes shaking platform. After the rockers move step, the amniotic removed from the container, and placed in a sterile solution, such as full 0.9 percent NaCl solution clean sterile tray. Use new sterile cell scraper to remove 15 of the remaining solution to the cells and removing any remaining cellular material from both sides of the amnion. This step can be repeated as many times as necessary to remove all visible residual cellular material from both sides of the amnion.

In certain embodiments, the decellularized amniotic membrane after step immediately (ie within 30 minutes) and dried. Alternatively, when not immediately further processed, amniotic membrane 20 may be refrigerated up to 28 days prior to drying, such as stored in 2-8X: temperature. When the decellularized amniotic membrane storage time over three days but less than 28 days, covering the amnion preferably sterile solutions to be replaced regularly, for example, changed every 1-3 days.

Step

2002年2月13日提交的U. S.申请号10/74，976 中所公开的方法来收集干细胞，在此处引用该文献作为参考。

本发明包括将本发明的胶原生物织物（例如三维支架）用于形成 生物工程改造的组织和类器官，包括血管、心脏瓣膜、肝、胰腺和韧 带，但并不局限于此。虽然不想被任何作用机制所束缚，但是本发明 30 的生物织物作为生物工程改造的组织的效用部分地是由于其在促进血 液凝固中的止血特性。本发明的生物织物对于血管假体和作为血管外 科中的移植物是特别有用的。本发明的生物织物可以例如用作在血管 外科程序期间环绕在血管（或者血管与移植物）吻合部位上的覆盖物， 以防止血从缝合线中渗漏出来，和防止身体与缝合材料形成粘连。

5.4.2 在外科程序中使用胶原生物织物的方法 本发明包括使用本发明的胶原生物织物作为外科移植物。本发明 5 包括含有本发明的胶原生物织物或其层压制品的外科移植物。本发明 此外还包括制备和使用外科移植物的方法。

在一些实施方案中，本发明包括在外科程序中使用外科移植物的 方法，从而将外科移植物直接应用于受试者的外科部位，例如内部部 位或外部部位。在一些实施方案中，在外科程序期间使用胶原生物织 10 物作为外科移植物，这在下面会更详细地进行公开，以便例如防止血 从缝合线中渗漏出来，和防止身体与缝合材料形成粘连。在其他实施 方案中，外科移植物用作吻合部位上的覆盖物，例如在GI外科手术期 间GI管道的吻合部位，以便防止肠液和胆汁从缝合线中渗漏出来，和 防止身体与缝合材料形成粘连。

15 本发明包括使用生物织物作为覆盖烧伤或经过外科手术的皮肤伤 口的移植物或敷料；防止在所有腹膜内外科手术中或者在其他于覆盖 腹部、胸腔和心包的浆膜面上的重建中出现粘连；重建衬在口腔和鼻 腔、呼吸道、胃肠道和泌尿生殖道内的所有粘膜面；作为支持脑外科 中的硬脑膜修复的基质；作为促进在中枢和外周神经系统中的神经再 20 生的基质；和重建软组织以防止在关节或腱修复中的粘连。

本发明包括用一种或多种生物分子浸渍本发明的外科移植物，优 选为治疗剂，这取决于特殊的想要的外科用途。这种生物分子包括抗 生素（例如克林霉素、二甲胺四环素、强力霉素、庆大霉素）、激素、 生长因子、抗肿瘤剂、抗真菌剂、抗病毒剂、疼痛药物、抗组胺剂、 25 抗炎剂、抗感染剂（包括银（例如银盐包括硝酸银和磺胺嘧啶银，但 并不局限于此）、元素银、抗生素、杀菌的酶（例如溶

In certain embodiments, when the amniotic membrane is not refrigerated after washing, the amniotic membrane prepared at the beginning of the implementation of IV washed at least three times before. In other embodiments, the membrane 25 when the sheep have been frozen and sterile solutions have replaced 1 times, washed at least twice prior to the step at the start of the implementation of the amniotic membrane preparation IV. In other embodiments, when the amniotic membrane has been refrigerated and carried the sterile solution has been replaced 2 times or more, prior to the step at the start of the implementation of the amniotic membrane prepared IV washed at least once.

In a particular embodiment, the decellularized amniotic membrane stored under sterile conditions, and 30 are not in operation for further processing, ie no drying. Before starting the implementation of step IV, the root of all this is to assess the bacteriological and serological tests to ensure that all tests are negative.

Step IV. The final step of the method of the present invention comprises drying the decellularized amniotic membrane of the present invention to produce collagen bio-fabric. You can use any dried amniotic membrane, and thus the life of the production method of dry collagen sheet. However, the amnion preferably dried in vacuo.

In a particular embodiment, the decellularized drying of the present invention exemplary method comprises the steps of amniotic: 5 assembly decellularized amniotic membrane to dry. Removed from the sterile solution to the cell membrane of the sheep, and gently squeeze out excess liquid. For example, in the tray and then will gently be decellularized amniotic stretch until it flattened in the process of fetal side down. Then to the cells of the amniotic inverted, whereby the fetal side up, and placed drying rack, preferably a plastic mesh drying frame (e.g., Quick Count(R) Plastic Canvas, Uniek, Inc., 10 Waunakee, WI). In other embodiments, the drying rack can be autoclavable stainless steel mesh. In a most preferred embodiment, the edge of about 0.5 cm and drying rack amniotic weight check. In certain embodiments, it will extend to the top of the amniotic bag wrapped rack drying rack outside the overlap, for example, with a clip or hemostat. Once the amniotic membrane is placed on the drying rack, sterile gauze will be placed in a

菌酶），但并 不局限于此）、伤口愈合剂（例如细胞因子包括PDGF、TGF,但并不 局限于此；胸腺素）、作为伤口愈合剂的透明质酸、伤口封闭剂（例 如有或没有凝血酶的血纤蛋白）、细胞吸引剂和支架试剂（例如纤连 30 蛋白）等等，但并不局限于此。在一个特殊的实施方案中，胶原生物 织物可以用至少一种生长因子进行浸渍，例如成纤维细胞生长因子、 上皮细胞生长因子等等。生物织物还可以用小有机分子进行浸渍，例 如特殊生物化学过程的特异性抑制剂，如膜受体抑制剂、激酶抑制剂、 生长抑制剂、抗癌药物、抗生素等等。

本发明此外还包括在本发明的外科移植物上移植活细胞，包括干 细胞、全能干细胞、多能干细胞、多潜能干细胞、组织特异性干细胞、 5 胚胎样干细胞、定向祖细胞、类成纤维细胞，但并不局限于此。在其 他实施方案中，本发明包括在本发明的外科移植物上移植特殊种类的 祖细胞，包括软骨细胞、肝细胞、造血细胞、胰实质细胞、成神经细 胞和肌肉祖细胞，但并不局限于此 眼科学 10 本发明的胶原生物织物在治疗眼相关疾病或病症中具有临床和治 疗效用。本发明的胶原生物织物对于治疗和/或预防眼表面疾病是特别 有用的，包括角膜溃疡/穿孔、大泡性角膜病变、眼部皮样囊肿/肿瘤、原发性翼状胬肉、持续性角膜上皮缺损、急性和慢性碱灼伤、热灼伤、 无虹膜、特应性角膜炎、特发性边缘干细胞缺乏、角膜翳、新血管生 15 成、类风湿性角膜融化、眼瘢痕性类天疱疮、渗漏性滤过性水泡、暴 露的Ahmed瓣膜管、具有随后的睑球粘连的沙雷氏菌（Serratia)蜂 窝组织炎、急性和慢性Stevenson Johnson综合征，但并不局限于此。

本发明的胶原生物织物在下列应用中是特别有效的，即促进具有溃疡 的持续性角膜上皮缺损的愈合；促进上皮形成；促进上皮细胞和干细 20 胞的生长；减轻炎症和疼痛、抑制血管生成和瘢疤形成；上皮表型的 恢复；和作为在翼状胬肉的"裸露巩膜"消除期间的结膜自体移植物 的可选择替代的基质。

本发明包括用本发明的胶原生物织物进行移植以治疗症状性大泡 性角膜病变，优选是在人中，最优选是在具有差的视力的人中。期望 25 本发明的生物织物相对于本领域中用于治疗症状性大泡性角膜病变 (明确地说就是结膜瓣构建）的其他标准程序来说具有增强的治疗和 临床效用。相对于本领域中用于治疗症状性大泡性角膜病变（明确地 说就是结膜瓣构建）的其他标准程序来说，用本发明的生物织物进行 移植的优点包括减轻疼痛、容易实施、美容上更加能够接受的外观以 30 及减少并发症如上睑下垂和边缘干细胞缺乏，但并不局限于此。本发 明的胶原生物织物提供了改良的结膜瓣的可选择替代

thermal drier (drier gel) (e.g., model 583, Bio-Rad 15 Laboratories, 200 Alfred Nobel Drive, Hercules, CA 94547) the drying platform, thereby covering the area than the plastic mesh drying rack placed amniotic slightly larger. Preferably, the total thickness of the gauze layer does not exceed the thickness of a fold into a gauze of 4M.

You can use any heat-drying device suitable for drying sheet-like material. The drying rack on top of the gauze on the drying platform, thereby extending edges of the plastic frame beyond the edges of gauze, preferably extending 20 0.1-1.0 cm, more preferably extend 0.5-1.0 cm. In a most preferred embodiment, the case, having amnion drying rack placed on sterile gauze, and fetal amniotic membrane on the side toward. In some embodiments, the web was placed on top of the other plastic frame amnion. In which the image grid and the dried film are shown in Figure 4. In other embodiments, the thin plastic sheet (e.g., SW 182, transparent PVC, AEP Industries Inc., South 25 Hackensack, NJ 07606) or oxygen hole Wa biocompatible film covering the medium is placed so that sheet just extend beyond all edges. In this embodiment, the second grid is not required for.

In an alternative embodiment, the amniotic membrane placed in a sterile Tyvek or more sheet material (e.g., Tyvek sheet for medical packaging, Dupont Tyvek(R), PO Box 30 80705, Wilmington, DE 19880-0705), and optionally with a Tyvek sheet placed on the film (before placing a plastic film). The alternative method will produce a smoother fabric forms of life (i.e., there is no pattern of dense areas of different fibers and along an axis perpendicular to the material), this biological fabric may be advantageous for certain applications, e.g., as herein the matrix used for cell expansion. amnion dry. In one preferred embodiment, the present invention comprises a vacuum heating drying amniotic present invention. Although the vacuum drying may be any temperature from about 5 01C- about 601C to complete, but amnion preferably about 501C to about 35TC- dried, most preferably about 501. It should be noted, will be expected to collagen 50X: temperatures above some degradation. The drying temperature is preferably set, and to verify the use of scalability by using a probe calibrated digital thermometer. Preferably, the vacuum pressure is set at about -22 inches Hg. Continue drying step, for example until the moisture analyzer was

物以促进具有溃 疡的角膜上皮缺损的愈合；改进的用于对于睑球粘连消散的结膜表面 重建的方法；改进的用于从结膜或角膜表面外科去除肿瘤、损伤或瘢 痕组织的方法；改进的用于通过矫正疱疹的渗漏而进行青光眼外科手 术的方法；在翼状胬肉的"裸露巩膜"消除期间的结膜自体移植物的 可选择替代的改良基质；和用于防止带状角膜病变的复发。

5 本发明还提供了生物织物用作眼外科移植物的用途。本发明包括 移植物的制备，这些移植物含有进一步含有一种或多种治疗剂的本发 明的生物织物，当给予受体时可以将这些治疗剂递送给受体。治疗剂 的例子包括匹鲁卡品、红霉素、庆大霉素、万古霉素、妥布拉霉素、 奈替米星、硫酸多粘菌素B、三甲氧苄二氨嘧啶、两性霉素B、抗癌剂、 10 抗生素、环孢菌素等等，但并不局限于此。

本发明的胶原生物织物还在减少由于受激准分子激光器光折射/ 治疗角膜切除术所引起的角膜模糊中具有效用。

用于眼科程序的本发明的胶原生物织物可以多种构造进行提供， 包括插入物、防护物、颗粒、凝胶、含水注射剂、海棉状物、薄膜， 15 但并不局限于此。

本领域熟练技术人员已知的用于眼科移植物的常规外科技术包括 在本发明的范围内。

使用本发明的胶原生物织物作为眼外科移植物的范例性方案可以 包括下列步骤：去除有病的角膜和/或结膜组织；在无菌条件下提供根 20 据本发明的方法制备得到的外科移植物，并通过自由的技术进行修整 以覆盖角膜上皮或裸露的巩膜。通过缝合将移植物锚定于裸露的巩膜 与结膜的连接处；进行主要的缝合；然后使用中断的缝合来形成移植 物表面上的张力平均分布；以致于避免在移植物与受体巩膜或角膜之 间出现任何空隙。在移植物和宿主的连接处，移植物的游离边缘必须 25 保持在受体结膜之下，以允许宿主上皮于与供体组织的连接点处在基 底层上滑动，否则移植物会被受体挤压出来 ^tlt 本发明的胶原生物织物在牙科学中具有特别的效用，例如牙周外 科、用于牙周组织再生的引导性组织再生、引导性骨再生和牙根覆盖。

30 本发明包括使用本发明的胶原生物织物来促进牙周骨内缺损的再生， 包括匹配性两侧牙周缺损、齿间骨内缺损、3层壁深的骨内缺损、2层 壁深的骨内缺损以及骨内缺损2和3,但并不局限于此。期望本发明的 胶原生物织物相对于本领域中已知的其他技术来说在治疗牙周骨内缺 损中具有增强的治疗效用和提高的临床参数，本领域中已知的其他技 术例如为

amniotic collagen matrix 10 contains less than 3-12 percent water. To achieve this, amniotic membrane can be heated - for example, about 60 minutes was dried in vacuo to obtain a dehydrated amniotic membrane. In some embodiments, the amniotic membrane was dried for about 30 minutes to 2 hours, preferably about 60 minutes. While not wishing to be bound by any mechanism of action, it is believed that the low thermal environment combined with vacuum pressure causes the amnion to reach a state of dehydration rather than make collagen degeneration.

15 after completion of the drying process according to the present invention, in the case of the vacuum pump running amniotic cool for about 2 minutes. amniotic packaging and storage. Once described as the amniotic membrane was dried according to the method of the present invention, the film will be gently lifted off the drying rack. "Lift" film may include the steps of: when the pump is still in operation, will begin to gently remove the plastic film from the corner from the amniotic membrane, amniotic Squeeze 20; the frame with the amniotic liftoff drying platform, then set cutting board and let the amniotic membrane side up; cutting along the edge of the frame 1-2 mm from the edge of the cut; then peeled amnion framework; and cut into appropriate size by the subsequent use of the decision. Preferably, at this stage of the operation carried out by amniotic use of sterile gloves. will be placed in a sterile container, such as exposing amniotic sheet bag and seal. As described above, produced according to the present invention a biological fabric 25 can be stored at room temperature for an extended period of time.

In an alternative embodiment, the present invention provides a method for preparing a collagen containing human chorionic biological fabric. We expect the method described above can be applied to a method for preparing a biological fabric containing human chorionic. In one particular embodiment, the present invention includes a method for the preparation of collagen bio-fabric, which comprises: providing a placenta comprising amnion and chorion; the separation of amnion and chorion; 30 and will be decellularized chorionic. In one particular embodiment, the method also requires washing and drying decellularized chorionic.

使用交联的胶原膜，例如在下列文献中公开的那些，即 Quteish 等人，1992, J. Clin. Periodontol. 19 (7): 476-84; Chung 5 等人，1990，J. Periodontol. 61 (12): 732-6; Mattson 等人，1995， J. Periodontol. 66 (7): 635-45; Benque 等人，1997，J. Clin. Periodontol. 24 (8): 544-9; Mattson 等人，1999，J. Periodontol. 70(5): 510-7。通过使用本发明的胶原生物织物而得到改善的参数的 例子包括斑块和齿龈指数评分、探测袋深度、探测附着深度以及分叉 10 牵涉和骨缺损的分类，这些对于本领域熟练技术人员来说是已知的，但并不局限于此。

本发明还包括在治疗II类分叉缺损中使用本发明的生物织物，包 括两侧缺损、成对的口腔II类下颌臼齿分叉缺损和两侧下领分叉缺 损，但并不局限于此。本发明的胶原生物织物在治疗II类分叉缺损中 15 的效用可以部分地用其能够再生出在分叉缺损中丧失的牙周组织的能 力来解释。期望本发明的生物织物相对于本领域中用于治疗II类分叉 缺损的胶原膜来说具有增强的治疗和临床效用，本领域中用于治疗II 类分叉缺损的胶原膜例如为在下列文献中公开的那些，即Paul等人， 1992，Int. J. Periodontics Restorative Dent. 12: 123-31; Wang 20 等人，1994，J. Periodontol. 65: 1029-36; Blumenthal, 1993, J. Periodontol. 64: 925-33; Black 等人，1994，J. Periodontol. 54: 598-604; Yukna 等人，1995，J. Periodontol. 67: 650-7。

本发明此外还包括在牙根覆盖程序中使用本发明的生物织物。本 发明的生物织物在牙根覆盖中的效用可以解释为这是部分地由于其能 25 够基于引导性组织再生的原理而替换丧失的、损伤的或有病的牙龈组 织。由于上面说到的原因，期望本发明的生物织物与本领域中传统用 于牙根覆盖的胶原膜相比具有增强的临床效用，本领域中传统用于牙 根覆盖的胶原膜例如为在下列文献中公开的那些，即Shieh 等人，1997, L Periodontol. 68: 770-8; Zahedi 等人，1998，J. Periodontol. 30 69: 975-81; Ozcan 等人，1997, J. Marmara Univ. Dent. Fa. 2: 588-98; Wang 等人，1997，J. Dent. Res. 78 (Spec Issue): 119 (Abstr. 106)。

本发明此外还包括在患有牙周病的受试者中使用胶原生物织物， 包括牙周炎和牙龈炎，但并不局限于此。本发明的生物织物还具有附 加于刮治和根面平整程序的临床效用。本发明包括用本发明的胶原生物织物治疗患有牙周病的受试者。使用本发明的胶原生物织物在受试 5 者中治疗牙周病的范例性方法包括将优选地用抗生素如葡萄糖酸氯己 定浸渍的胶原生物织物插入受试者中的一个或多个牙周袋中，

The method of preparation of scaffolds and laminate 5. 2.1 The present invention provides a three-dimensional collagen scaffold was prepared containing biological fabric of the present invention, the three-dimensional structure made of laminate and method.

In some embodiments, the present invention provides a process for the preparation of amniotic membrane laminate, comprising: providing a placenta comprising amnion and chorion, preferably a human placenta; 5 method disclosed herein isolated amnion and chorion; the method disclosed herein will be performed using amniotic decellularized; method disclosed herein decellularized amniotic membrane was washed at least once; at least two layers of decellularized amniotic membrane in contact with each other stacked together to form a layer of amniotic pressed product; and methods disclosed herein dried amniotic membrane laminate.

Alternatively, in another embodiment, a method for preparing a laminate comprising amnion, dried for at least 10 layers laminated in contact with each other according to the method of the present invention is obtained by preparing amniotic membrane, amniotic membrane and the at least two together to form a laminate amnion.

In some embodiments, produced according to the method of the present invention, amnion layer may exist under the conditions of the adhesive in mutual contact placed together to form a laminate amnion. The adhesive compositions and methods of the present invention may be used in any of the 15 known to those skilled in the field of bio-adhesives, preferably biocompatible glues, natural gums including agents such as fibronectin, Blood and synthetic fibrin glue, but not limited thereto. In other embodiments, each other according to the method of the present invention amniotic membrane prepared amniotic membrane to form a crosslinked laminate. Any cross-linking reagents and methods known to a person skilled in the art within the scope of the present invention, including chemical cross-linking, cross-linking peptide, UV crosslinking, radiation crosslinking 20, crosslinked fibronectin, fibrinogen crosslinked hydrogel crosslinked, but not limited thereto. In other embodiments, the methods noted in the present invention to produce the amniotic laminate product does not contain a binder.

RegenX Science Exhibit 1004
Page 942

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0942

例如插 入大于或等于5 mm。胶原生物织物优选地是可生物降解的。

在牙科学中使用的本发明的胶原生物织物取决于待治疗的牙科病 症的类型而可以用一种或多种生物分子进行浸渍。本领域中已知用于 10 治疗牙科病症的任何生物分子包括在本发明的方法和组合物之内。在 一个特殊的实施方案中，在治疗伴随有感染的牙科病症中使用的胶原 生物织物可以用一种或多种抗生素进行浸渍，包括强力霉素、四环素、 葡萄糖酸氯己定和二甲胺四环素，但并不局限于此 神经学 15 本发明的胶原生物织物还用于修复受损伤的神经特别是修复切断 的外周神经，以及用于神经外科程序。本发明包括例如在硬脑膜替换 和在外周神经修复中使用胶原生物织物。由于上面提及的原因，本发 明的胶原生物织物与本领域中使用的其他方法相比在用作硬脑膜替代 物时或者在神经修复中具有增强的临床效用，本领域中使用的其他方 20 法例如为 Berger 等人，1970，Acta. Neurochir. 23: 141; Ducker 等人，1968, Mil. Med. 133: 298 ； U. S•专利号 4，778，467 ； ; 3，961, 805; 5, 354，305。

在一些实施方案中，本发明的胶原生物织物可以用作环绕神经吻 合的假体，例如其可以用于包裹外周神经吻合。本发明的胶原生物织 25 物在神经修复中有特别的效用，这例如是通过这样的过程来完成的， 即促进穿过修复区域的纵向结締组织框架的形成，因此引起鞘细胞和 轴突再生的纵向式样。

外周神经的修复在称为nerorraphy (Jennings等人，1955， Surgery: 206)的程序中通常通过使用缝合来进行。可是，该方法只 30 获得了有限的成功，因为缝合切断的神经的方法是困难的。因此，本 发明的胶原生物织物为神经修复的无缝合方法提供了另一种选择，由 此例如将神经末端装入含有本发明的生物织物的管状假体装置中，因 此将切断的末端靠得很近以便再生 泌尿学 本发明的胶原生物织物在矫正尿失禁中具有特别的效用。尿失禁 是由于尿道不能在储尿期间保持关闭而造成的。该原因可以是外在 5 的，例如尿道和膀胱颈的差的解剖学支持导致失禁，和对于骨盆底重 悬作出响应。尿道失效可选择地也可以是内在的，即弱的尿道功能。

在传统上，尿失禁通过使用膨胀剂如胶原、脂肪、硅氧烷来矫正（见 综述 Lightner, 2002，Current Opinion in Urology, 12 (4)： 333-8)。本发明的胶原生物织物相对于本领域中所述膨胀剂来说具有 10 增强的效用，即增强了自控能力。此外，本发明的胶原生物织物还具 有增强的治疗效用，例如减少局部的并发症、减少尿道感染、无宿主 反应、可靠的耐久性和更高的安全性。

Storage 5.3 collagen bio-textiles and shoving as the present invention includes collagen biological fabric of the present invention as the dehydrated flakes were stored at room temperature for 25 (for example, 251C). In certain embodiments, the collagen biological fabric of the invention may be at least 10X:, at least 151, at least 20X:, at least 25X: or at least 291C temperature for storage. Preferably, the collagen biological fabric of the present invention is not refrigerated. In some embodiments, the collagen biological fabric of the present invention can be refrigerated at a temperature of about 2-81C.

In other embodiments, the collagen biological fabric of the present invention can be determined at any temperature above 30 degrees stored for an extended period of time. In a most preferred embodiment, the biological fabrics of the present invention under non-oxidizing conditions and aseptically stored. The method according to the present invention may be stored in the fabric produced in any given biological temperature for 12 months or longer, but no change in biochemical or structural integrity (e.g., no degradation), and collagen fabric biological biochemical or biophysical characteristics without any change. Produced according to the method of the present invention can be stored for several years biological fabric, while no change in biochemical or structural integrity (e.g., no degradation), biochemical or biophysical and biological properties of the collagen fabric without any change. 5 expected indefinitely to maintain the biological fabric according to the method of the present invention is prepared according to the present invention. bio-fabric can be stored in any container suitable for long-term storage in. Preferably, the collagen biological fabric of the present invention is stored in a sterile bag in double exposing sheet.

The present invention includes an operation of the present invention in its dry state collagen bio-fabric. In a special embodiment, the biological collagen fabric trimmed prior to use, for example, used as a surgical graft before 10. The present invention includes any number of dimensions to its intended use of the present invention, the biological fabric, which is by the person skilled in the art to determine. In some embodiments, the present invention includes lx2 cm, 2x3cm, 4M cm, 5x5 cm or 6x8 cm collagen raw material fabric. Biological fabric of the present invention can be cut to any desired size, which is located within the

RegenX Science Exhibit 1004
Page 943

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0943

本发明的胶原生物织物在矫正尿失禁中的效用部分地是由于此处 描述的胶原生物织物独特的物理特性，特别是其无免疫原性。在一些 15 实施方案，将胶原生物织物用作植入物，例如尿道植入物。虽然不想 被任何特殊的理论所束缚，但是本发明的胶原生物织物可能通过增加 尿道闭合压力和对于尿液被动流出的抗性而具有增强的治疗效用 矫形学 本发明的胶原生物织物可以在矫形外科程序中使用。在某些实施 20 方案中，胶原生物织物可以用于矫形缺陷，例如后天或先天缺陷。由 于外伤或者骨肿瘤的外科恢复引起的局部缺陷的重建是矫形外科或领 面外科中的主要问题。通常地，已经使用了合成的骨替代物或胶原膜， 这部分是由于它们的诱导骨生成的活性（见例如，Rao等人，1995，J. Biomater. Sci. Polymer Edn. 7 (7): 623-45)。可是，常见的问 25 题是由于植入的材料造成的全身性感染。可是，本发明的胶原生物织 物比本领域中所使用的材料是有利的，这特别是由于其作为用于矫形 缺陷的骨替代物的低免疫原性。在一些实施方案中，胶原生物织物可 以用作用于重建腱、韧带和软骨的假体。在其他实施方案中，本发明 的胶原生物织物可以用作作为骨替代物的假体。

30 心血管外科 本发明的胶原生物织物可以在心血管外科程序中使用，例如用作 假体以用于构建大和小血管；用于修复先天性血管畸形和有病的瓣 本发明的生物织物特别可用作血管外科中的移植物，例如静脉或 动脉移植物。生物织物在血管外科中的效用部分地是由于其无毒性； 无免疫原性；稳定性；容易操作；抗凝血酶原的特性；最小的血管壁 5 移植孔隙度；在多种大小的可用性；材料的再生产性。在一些实施方 案中，本发明的生物织物可以用作瓣膜替代物 药物递送 本发明的胶原生物织物可以用作用于药物例如治疗剂的可控递送 的药物递送载体。在一些实施方案中，胶原生物织物递送一种或多种 10 治疗剂给受试者，优选为人。包括在本发明的范围内的治疗剂为蛋白 质、肽、多糖、多糖缀合物、基于遗传的疫苗、减毒活疫苗、完整的 细胞。在本发明的方法中使用的药物的非限制性例子为抗生素、抗癌 剂、抗菌剂、抗病毒剂、疫苗、麻醉剂、镇痛药、抗哮喘剂、抗炎剂、 抗抑郁剂、抗关节炎剂、抗糖尿病剂、抗精神病药、中枢神经系统刺 15 激剂、激素、免疫抑制剂、肌肉松弛剂、前列腺素。

胶原生物织物可以用作用于向受试者优选为人可控递送一种或多 种小分子的递送载体。在一些实施方案中，胶原生物织物递送一种或 多种小分子给受试者，优选为人。在此处使用时，术语"小分子"和 类似的术语包括肽、肽模拟物、氨基酸、氨基酸类似物、多核苷酸、 多核苷酸类似物、核苷酸、核苷酸

limits of the size of the amniotic membrane. surface direction 15 of the invention collagen bio-fabric can be identified by the naked eye. Collagen biological fabric of the present invention has a "grid" pattern, which makes the person skilled in the art to be able to visually identify surface side of the mother and fetus. In one particular embodiment, the surface of the fabric in the direction of collagen biological conditions identified under magnification. Those skilled in the art will recognize that the fetal side of the fabric may be biological collagen identified by its concave i.e. recessed grid pattern. Conversely, maternal side can be identified by its projection that is elevated grid pattern.

Collagen biological fabric of the present invention requires minimal preparation time prior to use. In a preferred embodiment, the collagen biological fabric of the present invention may be in the time of 5 minutes or less, in 10 minutes or less, for the period of time of 15 minutes or less ready to carry out use. Biological collagen fabric of the present invention is used for example in preparation time as the surgical graft comprising rehydration prior to activation of the dehydrated biological collagen fabric. In some embodiments, the present invention collagen bio-fabric on the surgical site when hydrated. In other embodiments, the collagen biological fabric of the present invention dish hydration under sterile conditions. The present invention comprises a sterile physiological buffer to collagen hydrated biological fabric of the present invention. In one particular embodiment, the present invention comprises a sterile saline solution, such as sterile 0.9 percent NaCl solution runoff collagen biological fabric of the present invention. In some embodiments, the sterile saline solution is a buffer solution.

In certain embodiments, the fabric of the present invention, the biological collagen Hydration requires at least 2 minutes, at least 5 minutes, at least 10 minutes, at least 15 minutes or at least 20 minutes. In a preferred embodiment, the fabric of the present invention, the biological collagen hydration completed within 5 minutes. In another preferred embodiment, the fabric of the present invention, the biological collagen hydration completed within 10 minutes. In another embodiment, the present invention collagen bio-fabric does not require hydration 5 over 10 minutes.

RegenX Science Exhibit 1004
Page 944

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0944

类似物、具有低于大约10，000克/ 摩尔的分子量的有机或无机化合物（即包括杂有机化合物和有机金属 化合物）、具有低于大约5, 000克/摩尔的分子量的有机或无机化合 物、具有低于大约1,000克/摩尔的分子量的有机或无机化合物、具 有低于大约500克/摩尔的分子量的有机或无机化合物、具有低于大约 100克/摩尔的分子量的有机或无机化合物，以及这样的化合物的盐、 酯和其他药物上可接受的形式，但并不局限于此。还包括了这样的化 合物的盐、酯和其他药物上可接受的形式。

在某些实施方案中，相对于本领域中已知的其他药物递送系统来 说，本发明的胶原生物织物用作用于药物递送的载体导致药物的增强 的吸收；改善的药物动力学特性；和药物的全身性分布。改善的药物动力学表示，当例如用标准药物动力学参数进行测量时获得了药物动 力学特性的增强，例如达到最大血浆浓度的时间（T...）；最大血浆浓 度的数量（Cm）；引起可检测的血液或血浆浓度的时间（Tlag)。增强 的吸收表示，当用这样的参数进行测量时，药物的吸收得到了改善。

药物动力学参数的测量按照本领域的常规方法来进行。

6. 实施例 5 6.1生产胶原生物织物的方法 材料 下面的材料用于制备胶原生物织物。

材料/设备 • 分娩记录的复本 • 材料/家族健康史/知情同意的复本 • 来源条形码标记（供体ID号） • 收集# (将连续的号分配给收入的材料） • 组织处理记录（文件ID #ANT-19F)；保留每个批号的处理 的详细i已录 • 人胎盘（在处理开始时小于48小时的存放时间） • 无菌的外科夹子/止血钳 • 无菌的剪刀 • 无菌的解剖刀 • 无菌的细胞刮刀（Nalgene NUNC Int. R0896) • 无菌的纱布（非无菌的PSS 4416,灭菌） • 无菌的漂洗不锈钢托盘 • 消毒的处理不锈钢托盘 • 消毒的塑料箱 • 无菌 0. 9% NaCl 溶液（Baxter 2F7124) • 无菌水（Milli Q plus 09195 或 Baxter 2F7113) • 无菌的样品容器（VWR 15704-014) • 个人防护设备（包括无菌的或非无菌的手套） • 具有证明书的洁净室 • 事先制备的去细胞化溶液（D-细胞）；0. 01-1%—水合脱氧胆 酸钠 • 消毒的箱 • 摇动平台（VWR 100型） • 记时器(VWR) • 消毒的塑料架网 • PVC包裹膜 • 真空泵（Schuco-Vac 5711-130) 5 • 凝胶干燥器（即热干燥器；BioRad 583型） • 消毒的不锈钢切割板 • 用于包装的袋 • 无菌的不诱钢尺（General Tools MFG. Co 1201) • 可追踪的数字温度计（ 61161-364型，Control公司） 10 • Accu-Seal 自动密封器（Accu-Seal, 630-1B6 型） 在生育时对孕妇进行筛选以检查传染病，例如HIV、HBV、HCV、 HTLV、梅

Once the collagen biological fabric of the present invention will be hydrated in order to be used as a surgical graft using, for example, as determined by the skilled person, with respect to those skilled in the amniotic membrane with an enhanced resistance to suture. Collagen biological fabric of the present invention, unlike in the art as easily torn amnion, which is also not in the art of amnion as easily. The present invention includes effective biological collagen 10 stitched fabric.

5. 3. 1 of the present biosensor, the fabric of the present invention is preferably sterilized using those skilled in the art by methods known to electron beam radiation, e.g., Gorham, D. Byrom (Editor), 1991, Biomaterials, Stockton Press, New York, 55-122. Any enough to kill at least 15 dead 99.9 percent of bacteria or other biological contamination potential radiation dose within the scope of the present invention. In a preferred embodiment, at least 18-25 kGy dose to complete the final sterilization of biological fabric of the present invention. However, sterilization of biological fabric of the present invention does not include antibiotics or glycerol storage.

5.4 collagen collagen bio-textiles biological fabric of the present invention 20. The method of the present invention in the medical and surgical field has many applications, this is partly due to the physical characteristics of its traditional film and used in the art as compared to such as bio-mechanical strength, buckling can be sewn and low immunogenicity. E.g., a desired biological collagen fabric of the present invention in the prior art for guided tissue regeneration membranes with enhanced therapeutic utility, for example, these films can not be synthesized resorbable PTFE membranes, such as Goretex?; synthetic bioresorbable 25 film, and lactide ester copolymer formed its post acetic; WO-88/08305, DE-2631909, US Patent No. 5,837,278 disclosed a film.

The method for preparing collagen biological fabric of the present invention ensure the preservation of the tertiary and quaternary structure of biological fabric, thus making the creature fabric is ideal for its use in the medical and surgical field is desired. As described in detail below, the present invention provides such a biological collagen fabric 30, i.e., the physical properties of the fabric such that the biological collagen suitable for use in a variety of medical and dental applications, including repair,

RegenX Science Exhibit 1004
Page 945

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0945

毒、CMV和其他病毒，以及其他会污染待收集的胎盘组织的 病原体。只使用从这样的供体中收集得到的组织来生产本发明的胶原 生物织物，即其母亲经检测对于上面提及的病原体呈阴性或者无反应 15 性。

在正常生育之后，胎盘、脐带和脐带血自然地从收缩的子宫中排 出。在生育之后收集胎盘、脐带和脐带血。将这些材料转移至实验室， 在那儿它们在具有HEPA过滤系统的洁净室中于无菌条件下进行处理， HEPA过滤系统在处理之前开启至少1小时。在操作产品时总是戴上手 20 套（无菌的或非无菌的，在适当的时候）。一旦可以实施的时候就处 置掉所有的羊膜/绒毛膜的未使用（多余）部分和在组织处理期间产生 的污染液体。

步骤I 用无菌的Steri-Wrap片设置出一个无菌场地，并将下列用于处理 25 的器械和附件放置在该场地上。

• 无菌的托盘包 • 无菌的细胞刮刀 • 无菌的解剖刀 • 消毒的处理托盘 30 在处理记录中记录下无菌包ID #0 从转移容器中取出胎盘，并置于消毒的不锈钢托盘上。用外科夹 子和剪刀从胎盘上剪下大约2英寸的脐带。将脐带置于单独的无菌容 器中以用于进一步的处理。容器用组织ID条形码进行标记；和鉴定材 料和贮存溶液的存在（例如介质的类型）。在一些情况下，如果不需 要用于其他计划，则去弃脐带。

从胎盘膜的边缘开始，通过使用手指的钝器解剖来分离羊膜和绒 5 毛膜。这一操作在切割膜之前进行。

在将羊膜与绒毛膜和胎盘的整个表面分离之后，用剪刀环绕脐带 残端切割羊膜，并将羊膜与胎盘分开。在一些情况下，如果不撕裂组 织就不可能分离羊膜和绒毛膜，将羊膜和绒毛膜作为一块从胎盘上切 割下来，然后将它们剥离开。

10 将绒毛膜置于单独的样品容器中，以便用于其他计划。容器用组 织ID条形码进行标记，鉴定材料和贮存溶液的存在（例如介质的类 型），标上首字母缩写和标上日期。

如果羊膜片仍然粘附在胎盘上，将其从胎盘上剥离，并用剪刀环 绕脐带切割下来。将胎盘放回转移容器中以用于其他计划。

15 在组织处理记录中记录下适当的数据。

羊膜用无菌0.9% NaCl溶液保持在托盘中。优选地，在处理的下 一个步骤之前，将羊膜从分娩的时

uterus repair, tendon replacement vessel, Alternatively cornea, artificial skin, wound healing and treatment of periodontal disease, but is not limited thereto. Want to be dependent on its use, the present invention includes the use of collagen as a two-dimensional fabric of biological membranes, e.g., tubular container can be produced as a film is formed; a three-dimensional scaffold, e.g., implants; or as a one-dimensional fiber.

5.4.1 Use of biological collagen fabric of the present invention is a method for treating skin conditions treated 5 5.4.1.1 way human skin is the epidermis and dermis of composite materials. The upper part of the epidermis is the stratum corneum, which is the hardest layer of the skin, and is most affected by the surrounding environment layer. Here in the stratum corneum is the internal skin. Below the epidermis is the papillary dermis, which consists of relatively loose connective tissue composed of connective tissue which determines the skin microrelief. Located in the papillary dermis of the reticular dermis 10 under is organized together with clearance close connective tissue. Reticular dermis is also related with coarse wrinkles. Under the dermis is the subcutaneous layer.

The main function of the skin include protection, excretion, secretion, absorption, temperature regulation, pigment formation, accumulation, sensory perception and regulation of the immune process. These features adversely affected by changes in the structure of the skin due to aging and excessive sun exposure caused. Skin aging-related physiological changes, including weakened 15 barrier function and reduced epidermal cells update rate, see for example Cerimele, D. et al., 1990, Br. J. Dermatol., 122 Suppl. 35, 13-2 0 page. The prior art methods and compositions for improving skin conditions such as improving skin elasticity and softness or remove wrinkles achieved limited success.

Collagen biological fabric of the present invention has a medical and cosmetic applications. Collagen fabric 20 of the present invention has a biological treatment of the skin condition in clinical and therapeutic utility, including skin damage, skin aging, wrinkles, fine lines, thinning, reduced skin elasticity, rough skin, and the inherent variability of the skin condition, VII collagen and the lack of sun damaged skin. In certain embodiments, the

RegenX Science Exhibit 1004
Page 946

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0946

候起通过冷藏贮存最多72小时。

步骤II 从样品容器中一次取出一片羊膜，并置于消毒的不锈钢托盘上。

20 将其他片置于单独的注满无菌水的无菌不锈钢托盘中，直至准备对它 们进行清洁。从处理托盘上移去多余的羊膜片，并置于单独的注满无 菌水的漂洗不锈钢托盘中。

如果被血液、母体或胎儿液体/材料严重污染，则用无菌水漂洗羊 膜，并在需要时更换无菌水。

25 将羊膜置于处理托盘上，并保持母体侧朝上。用无菌的细胞刮刀 从羊膜的母体侧小心地去除尽可能多的可见污染物和细胞材料。（注 意：对于该步骤应当使用最小的压力以防止撕裂膜）。使用无菌水来 帮助去除细胞和细胞碎片。羊膜进一步在单独的无菌不锈钢漂洗托盘 中用无菌水漂洗。

30 将羊膜翻转从而将胎儿侧朝上，并将其放回处理托盘上，然后用 无菌水漂洗。用细胞刮刀轻轻地去除可见的细胞材料和碎片（注意： 对于该步骤应当使用最小的压力以防止撕裂膜）。使用无菌水来帮助 去除细胞和细胞碎片。

在单独的无菌漂洗托盘中于两个清洁循环之间用无菌水漂洗羊 膜。以按需要一样多的次数（清洁循环数）来清洁组织，以便从膜的 两侧去除大部分（如果不是全部）可见的细胞材料和碎片。在两次漂 5 洗之间更换漂洗托盘中的无菌水。

在每次清洁循环之后用无菌水漂洗处理托盘。

所有其他的羊膜片以同样的方式进行处理，并置于同一个容器 中。贴上组织ID条形码，鉴定材料和贮存溶液的存在（例如介质的类 型），加上首字母缩写和日期。

10 在组织处理记录中记录下适当的信息和日期。

步骤III 从漂洗托盘中（或者从贮存容器中）取出羊膜，用手指轻轻地挤 压出过量的液体，并将膜置于无菌样品容器中。容器用D-细胞溶液装 满至150 ml 标线，并保证覆盖住所有的羊膜和关闭了容器。

15 将容器置于摇动平台上的箱中。开启摇动平台，并让膜于设置#6 在D-细胞溶液中摇动至少15分钟和至多120分钟。

以步骤I中同样的方式用新的无菌设备和消毒的托盘设置出一个 新的无菌场地。在处理记录中记录下无菌包ID #. 在完成摇动之后，关闭摇动平台，并从容

collagen biological fabric of the present invention can be used for skin conditions such as acne scarring, eyebrows ditch, scar removal, or any other known in the art of soft tissue defects 25 subcutaneous implants. Collagen biological fabric of the present invention has clinical and therapeutic utility in dealing with aging-related changes in the skin. In certain embodiments, the collagen biological fabric of the present invention to improve skin wrinkles and / or other conditions such as skin elasticity and softness have utility. Collagen biological fabric of the present invention can be used as a form of treatment of the skin condition of the implant, laminate or three-dimensional crimp. Desired biological collagen fabric of the invention with respect to methods known in the art for this skin condition 30 processes, for example, US Patent Nos. 5,972,999,5,418, 875, 5,198, 465 with enhanced clinical utility, which is partly due to its stability, it is possible to provide a longer duration effect.

In a preferred embodiment, the collagen biological fabric of the invention further with one or more reagents known in the art for treatment of the skin condition to be supplemented. Examples of such agents include vitamins, minerals, tea-based child pigment preparations, N- acetylglucosamine and glucosamine (see, for example, Neldner, 1993, Amer. Acad. Derm. Annl. Mtg. Wash. 5 DC.; Lubell 1996, Cosmetic Dermatol, 9 (7): 58-60; Swaine et al., 1995, J. Am Board of Family Practice, 8 (3):. 206-16; Shan et al., 1994, Kidney International, 46 (2): 388-95; all incorporated herein by reference in its entirety), but is not limited thereto.

Collagen biological fabric of the present invention can be used for any biological treatment of the skin condition in 10 sub-impregnated, including antibiotics (such as clindamycin, minocycline, doxycycline, gentamicin), hormones, growth factors, anti-tumor agents, antifungal agents, antiviral agents, pain medications, antihistamines, anti-inflammatory agents, anti-infective agents (including silver (including silver salts such as silver nitrate and silver sulfadiazine, but not limited to thereto), elemental silver, antibiotics, bactericidal enzymes (e.g., lysozyme), but not limited to), wound healing agents (e.g. cytokines include PDGF, 15 TGF, but is not limited thereto; thymosin), as a wound healing agent, hyaluronic acid, a wound sealant (e.g., with or without fibrin thrombin), cellular attractant and bracket reagent (e.g. fibronectin) and the like, but is not limited

器中取出膜。将膜置于 20 新的不锈钢处理托盘中，加入无菌0. 9% NaCl溶液以覆盖托盘的底部。

用新的无菌细胞刮刀从组织的两侧去除残留的D-细胞和细胞材料 (如果有的话）。该步骤以按需要一样多的次数进行重复以便从两侧 的整个表面上去除尽可能多的可见的残留细胞材料。于两个清洁循环之间在单独的漂洗托盘中用无菌0.9% NaCl溶液漂洗膜。在两次漂洗 25 之间更换漂洗托盘中的无菌0. 9% NaCl溶液。

在最后的清洁循环完成之后，膜用无菌0. 9% NaCl溶液进行漂洗， 并置于新的注满无菌0. 9% NaCl溶液的无菌样品容器中。

所有余下的羊膜片以完全一样的方式进行处理。

当所有羊膜片都进行了处理并置于含有无菌0.9% NaCl溶液的容 30 器中时，将容器置于摇动平台上的箱中，以便于设置#6摇动至少5分 钟。在摇动完成之后,从样品容器中取出膜，更换容器中的无菌0. 9% NaCl溶液，和将膜放回样品容器中。

样品容器用组织ID条形码和检疫标签进行标记。鉴定材料和贮存 溶液的存在（例如介质的类型），标上首字母缩写和标上日期。将样 品容器置于洁净的拉链锁袋中，并置于冰箱（2-81C)中。

在组织处理记录中记录下所有适当的数据。

5 当血清学结果显示可用时，在检疫标签之上放上适当的标签（血 清学阴性或仅供研究使用），并将那些容器与经检验的分开。

步骤IV 在用步骤IV进行处理之前，检查组织状态检验以确保所有可应用 的检测结果都是阴性的。

10 以步骤II和III中同样的方式用无菌的Steri-Wrap片设置出一 个无菌场地，并放置好所有无菌和消毒的器械和附件。

从冰箱中取出膜，并置于新的无菌不锈钢处理托盘中。加入无菌 0.9% NaCl溶液以覆盖托盘的底部。

用新的无菌细胞刮刀轻轻地去除所有可见的细胞材料和碎片（如 15 果有的话）（注意：对于该步骤应当使用最小的压力以防止撕裂膜）。

使用无菌0. 9% NaCl溶液来帮助去除细胞和碎片。

在单独的注满无菌0.9% NaCl溶液的无菌不锈钢漂洗托盘中漂洗 膜。在两次清洁循环之间更换0.9% NaCl溶液。将膜置于新的无菌样 品容器中，容器

thereto. In a particular embodiment, the collagen may be at least one biological fabric impregnated growth factors, e.g., fibroblast growth factor, epidermal growth factor, and so on. Biological fabric may be impregnated with a small molecule, e.g., small organic molecules 20 as specific inhibitors of particular biochemical processes, such as a membrane receptor inhibitors, kinase inhibitors, growth inhibitors, anticancer drugs, antibiotics, trauma and the like burns and others known in the art of biological materials such as US Patent No. 4 '320,201,3' 800,792,4,837,285,5,116 '620 to those described in the 25 than expected this collagen biological fabric of the invention as a wound dressing for promoting hard and / or soft tissue repair with enhanced clinical utility, in part because of their physical properties. Because the collagen fabric of the present invention maintains the biological native quaternary structure of the collagen, so that it causes enhanced tissue ingrowth and cell migration through the collagen matrix into the gap. Biological fabric of the present invention enables the cells can adhere collagen matrix, and grow into the collagen matrix, and so 30 cells are able to synthesize their macromolecules. Thereby allowing the cells to produce new tissue growth of new matrix. For example, it not observed on fibers, hairs and other soluble collagen in the form of collagen known that cell growth.

On

In some embodiments, the present invention includes collagen by biological fabric of the present invention is directly placed in the subject wound up parts of the skin stratum corneum, the treatment of wounds, so for example, cover the wound with adhesive tape. In other embodiments, the present invention includes the use of biological collagen fabric of the present invention as an implant, for example as a subcutaneous implant for the treatment of wounds.

5 of the present invention can include the addition of collagen to promote tissue ingrowth into the fabric of the present invention in biological macromolecules to increase the rate of wound healing. Such macromolecules include hyaluronic acid, fibronectin, laminin, and proteoglycans (see, for example, Doillon et al (1987) Biomaterials 8: 195-200; and Doillon and Silver (1986) Biomaterials 7: 3-8), but it is not limited thereto.

RegenX Science Exhibit 1004
Page 948

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0948

用新鲜的无菌0.9% NaCl溶液注满，并置于摇动平台 20 上以便于设置#6摇动至少5分钟。

前述步骤重复3次，并在每次摇动之间更换无菌0. 9% NaCl溶液。

在组织处理记录中记录下适当的数据。

从样品容器中一次取出一片膜，用手指轻轻地挤压出过量的液 体，并将膜置于无菌处理托盘上。轻轻地将膜进行伸展直至其变平； 25 并保证其胎儿侧朝下。

框架通过用无菌剪刀切割消毒的塑料片来制备。框架的大小应当 在每个方向上比膜部分小大约0. 5 cm。在注满无菌0. 9% NaCl溶液的 漂洗托盘中漂洗框架。

将框架置于稍微伸展的膜表面上，并轻轻地在上面进行按压。必 30 要的是将塑料框架的光滑的一侧朝向组织。

用解剖刀环绕框架切割膜，并保留大约0. 5 cm伸展至框架边缘之 夕卜。多余的膜放回样品容器中。

用夹子或镊子将伸展至框架之外的膜边缘包衮住框架的边缘，并 暂时放在同一个托盘中备用。

下一片膜以同样的方式进行处理。重要的是，将要进行干燥的总 区域不要超过300 cm2/热干燥器。在"架开"这一片膜的同时，未上 5 架的片应当保持在容器中的无茵0.9% NaCl溶液中。

设置干燥器的干燥温度，并使用采用扩展性探针的经校准的数字 温度计来进行校验。将干燥温度设置于5 0IC。在组织处理记录中记录 下数椐。

开启真空泵。

10 将无菌纱布置于热干燥器的干燥平台上，覆盖住比架开的膜的区 域稍大的区域。重要的是确保纱布层的总厚度不超过一个折叠成4M 的纱布的厚度。

将一片塑料架网置于纱布之上。塑料网的边缘应当伸展出纱布边 缘大约 0. 5-1. 0 cm。

15 将架开的膜轻轻地提起，并置于位于塑料网之上的热干燥器平台 上，同时保持膜一侧朝上。重复该步骤直至将最大数量的膜（不超过 300 cm2)置于热干燥平台上。（注意：羊膜的胎儿侧朝上）。

将一片PVC包裹膜切割成足够的大小以覆盖热干燥器的整个干燥 平台还加上额外的脚。

10 The present invention also includes collagen biological fabric of the present invention are described herein for incorporation of pharmacologically active agents such as for drug delivery to the skin in section 5.4.2.7, and the incorporation of any of the above biomolecules pharmacologically active agents include platelet-derived growth factor, insulin-like growth factor, epidermal growth factor, transforming growth factor -P, angiogenic factors, antibiotics, antifungal agents, spermicides, hormones, enzymes, enzyme inhibitors, but not limited thereto. 15 drug physiologically active agent, preferably in a physiologically effective dose available.

Before

In some embodiments, it is further applied to the wound site in transplantation of living cells on collagen bio-fabric comprising a heterologous stem cells, stem cells and mature cells themselves, but not limited thereto.

Collagen biological fabric of the present invention are useful for the treatment of infected wounds are particularly useful, such as the emergence of 20 after a surgical wound infection or trauma wounds penetrated. In a particular embodiment, the collagen bio-fabric with a therapeutically effective dose for the treatment of infected wounds impregnated with a reagent, including antibiotics, antimicrobials and antimicrobial agents, but is not limited thereto. Collagen biological fabric of the present invention has clinical and therapeutic utility in the treatment of wound infection by any of the art known among microbes caused, for example, derived from the human body microbe infected wounds, the 25 is a known pathogen library, or infected wounds from environmental sources of microbes. growth in the wound can be reduced or prevented by the methods and compositions of the present invention, non-limiting examples of microorganisms Staphylococcus aureus (s. Aureus), Staphylococcus epidermidis (St. epidermis), p - hemolytic streptococcus (Streptococcus), Escherichia coli (E. coli), Klebsiella (Klebsiella) species and Pseudomonas 30 (Pseudomonas) species, and anaerobic bacteria is Clostridium Webster (Clostridium welchii) or (tartium), which is the cause of gas gangrene, mainly in deep traumatic wounds.

In other embodiments, the collagen fabric of the invention for the biological treatment of wounds,

20 在真空泵运转的同时，用塑料膜轻轻地覆盖热干燥器的整个干燥 平台，并在两侧让1/2的脚伸展出干燥平台边缘之外。要注意的是， 膜紧紧地拉在膜和框架片之上（即其被真空"吸进去了"），而且没有漏气和在组织区域上没有起皱纹。随后关闭盖子。

将真空泵设置为大约-22英寸Hg的真空。在2-3分钟的干燥循环 25 之后记录泵表的数值。将膜进行热-真空干燥大约60分钟。干燥过程 中大约15-30分钟，在热干燥器中放置上一层新的无菌纱布。纱布层 的总厚度一定不能超过一个折叠成4x4的纱布的厚度。

在改变之后，要注意的是，塑料膜紧紧地拉在膜和框架片之上， 而且没有漏气和在膜区域上没有起皱纹。

30 真空密封的完整性通过检查泵压单计量表而定时进行检查。在完 成干燥处理之后，打开热干燥器，在泵运转的同时让膜冷却大约2分 钟。

用无菌的Steri-Wrap和位于其下的消毒的不锈钢切割板设置出 一个新的无菌场地。在这一点上使用无菌的手套。在泵仍在运转的同 时，从边角上开始轻轻地从膜片上去除塑料膜，并用戴着手套的手压 住膜片。将框架与膜一起轻轻地提离干燥平台，并置于位于消毒的不 5 锈钢切割板之上的无菌场地上，同时让膜一侧朝上。用解剖刀通过沿 着离框架边缘1-2 mm的边缘形成切口而对膜片进行切割。用戴着手套 (无菌手套）的手将膜保持在原处。通过将膜片缓慢地剥离而将其轻 轻地提离框架，然后置于切割板之上的无菌场地上。

用解剖刀或锋利的剪刀将膜片切割成指定尺寸的片段。在包装之 10 前，在无菌的场地上切割所有的片并进行妥善保管。用一只手（无菌 的）将单片的膜置于内部的揭片袋包装中，同时用另一只手（非无菌 的）拿住袋子。要注意的是不要用"无菌的"手接触袋子。在将所有 的片置于内袋中之后，将它们密封。在袋子外部的指定区域中贴上具 有适当的信息（例如部分#、批#等等）的标签。所有的膜片以同样的 15 方式进行处理。将经标记和密封的揭片袋包放置于防水的拉链锁袋中 进行贮存，直至准备将它们运送到灭菌设备或分配器中。在组织处理 记录中记录下所有适当的数据。

6. 2胶原生物织物的水化/可缝合性的评价 为了获得关于本发明生物织物的外科操作、水化期和可缝合性的 20 定性和定量的反馈，将根据本发明的方法制备得到的羊膜样品提供给 四位富有经验的、非常知名的眼表面外科医生以进行评价。样品由外 科医

including skin wounds, skin wounds, chronic wounds, acute wounds, external wounds, internal wounds (e.g., collagen fabrics may biological package during surgery ?around the anastomotic site in order to prevent leakage of blood from suture, suture material and prevent the body and the formation of adhesions), congenital 5 wounds (such as dystrophic epidermolysis bullosa disease), but is not limited to this. In particular, biological collagen fabric has enhanced utility in the treatment of pressure ulcers (such as decubitus ulcers) in. Pressure Ulcer often occurs in patients with long-term bed rest, such as quadriplegics and paraplegics patients, due to their partial pressures suffered skin damage. Results caused by pressure ulcers showed erosion and the loss of the epidermis and dermis skin appendages.

Collagen biological fabric 10 of the present invention may also be used to treat burns, including first degree burns, second degree burns (partial thickness burns), third-degree burns (full thickness burns), burn wound infections, removal and uncut burn wound infection, wound infection transplantation, donor site of the infection, previous transplant or healing of burn wounds or skin graft body portion for epithelial damage and burn wounds impetigo, but is not limited thereto.

15 5.4.1.3 tissue engineering of the present invention includes the use of collagen as a biological fabric of the present invention for conveying plant body cultured skin cells, such as the epithelial cell growth and differentiation of support. In certain embodiments, the fabric for the transplanted cells to form a biological tissue and / or organ class (that is, like any organ or gland of the body on the surface appearance or structure), again on a biological fabric 20 such transplants during the process of new cells, bio-fabric can be treated with a cell adhesion factor to enhance cell attachment and biological fabric. In certain embodiments, the extent of cell adhesion by increasing serum treated amniotic membrane, such as human serum or fetal calf serum. In other embodiments, the degree of cell adhesion by treatment with amniotic fibronectin to increase.

Collagen biological fabric of the present invention can be used for guided tissue regeneration techniques, such as damaged or diseased tissue regeneration or replacement 25. The present invention includes the use of biological fabric of

RegenX Science Exhibit 1004
Page 950

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0950

生通过下列方法进行评价，即在猪眼试样上施行组织移植以确定 本发明生物织物的外科操作特性和可缝合性。

每个外科医生可能使用下列的方法： 25 (1)将生物织物在干燥的状况下进行切割以适合猪眼的单个象限； (2)将切割出的生物织物置于猪眼的表面上；(3)在猪眼上用无菌的盐溶液将生物织物进行水化而让移植物进 行活化即再水化，其水化期时间为2、5、10和20分钟； 30 (4)将生物织物通过几个9-0 Vicryl缝合咬合而缝合到猪眼的 上皮上；和 (5)外科医生作出关于经水化的羊膜的组织品质、一致性和可缝 合性的定性评注。

等同方案： 不是要用此处描述的特殊实施方案来限制本发明的范围。事实 上，从前面的描述和附图中，除了所述的那些之外的本发明的多种修 5 饰对于本领域熟练技术人员来说将会是显而易见的。这种修饰将会落 在附加的权利要求的范围之内。

此处引用了多种出版物、专利和专利申请，它们的公开内容以其 整体进行引用作为参考。

僻知灘伊 一：續 滅麵 图 2B 图3 说明书附图

the present invention, the fabric is about to be implanted directly in biological treatment site, or to form prosthetic device. Biological collagen fabric of the present invention in any case is particularly useful, for example, after surgery especially oral or dental surgery unit (described in more detail in Section 5.4.2.2), which is expected to enhance wound healing and / or dermal replacement. Biological collagen fabric of the present invention in tissue regeneration in the guide 30 is partly due to the effectiveness of its ability to provide such conditions that these conditions prevent tissue ingrowth into the other area in need of regeneration.

For example, due to disease or decay away in addition to a substantial portion of the root where you want to appear healthy bone tissue regeneration to replace bone removed. However, it has been found that because of the removal of the bone cavity and left quickly filled by connective tissue, and this inward growth of connective tissue effectively prevents bone regeneration. To prevent this ingrowth, can be biologically collagen fabric of the invention is surgically inserted around the edges of the wound so that the cavity. Biological fabric 5 wound cavity is to prevent or stop an unwanted cell type invasion, it is possible to make the preferred cell growth into the cavity, thereby healing the wound.

In some embodiments, the collagen bio-fabric is used for ocular surface reconstruction grafts, for example, with Stevens Johnson syndrome, chemical burns after the secondary of limbal stem cell deficiency in a subject, or having chemical burns and / or thermal burns in 10 subjects. The fabric of the present invention the desired biological e.g., by promoting epithelialization and an enhanced clinical utility. Desired biological fabric of the present invention with respect to the art for the purpose of amniotic membrane tissue engineering has enhanced clinical utility, see, for example, Gomes et al., 2003, Opthalmology, 119: 166-73; Ti et al. '2001, Opthalmology, 108: 1209-1217; Meller et al., 2000, Opthalmology, 107: 980-9; Gris et al., 2002, 15 Opthalmology, 109: 508-12; Koizumi et al., 2000, Invest Opthal and Visual Science, 41...:. 2506-13; Pires et al., 1999, Arch Opthalmol 117: 1291-7; Tseng et al. , 1998, Arch Opthalmol 116:... 431-441; Heiligenhaus et al., 2001, Invest Opthal and Visual Science 42:.. 1969-74; Anderson et al., 2001, Br J. 20 Opthalmol 85: 567-75.

The present invention comprises a bio-fabric on collagen transplantation of living cells, including mature tissue cells, and stem cells themselves, but not limited thereto. Stem cells in the method of the present invention may be used in totipotent cells, pluripotent cells or differentiated tissue-specific cells. In the method of the present invention can be used in stem cell known to those skilled in the art by standard methods to get 25. Preferably, the method of U. S. Application No. 13 February 2002, submitted 10 / 74,976 disclosed to collect stem cells, in the literature cited herein by reference.

The present invention includes collagen biological fabric of the present invention (for example, three-dimensional scaffold) for the formation of tissues and organs bioengineered class, including blood vessels, heart valves, liver, pancreas and ligaments, but is not limited thereto. While not wishing to be bound by any mechanism of action, but the biological fabric 30 as bioengineered tissue of the present invention, in part because of its utility blood coagulation hemostasis promoting properties. Biological fabric of the present invention for a vascular prosthesis and the vascular surgical implants are particularly useful. Biological fabric of the present invention can be used for example during vascular surgery procedures around the blood vessel (or vessels and grafts) covering the anastomotic site to prevent leakage from the suture line bleeding out, and prevent the formation of adhesions body and suture material.

5.4.2 using collagen bio fabric in surgical procedures in the method of the present invention includes the use of collagen biological fabric of the present invention as a surgical graft. 5 of the invention of the present invention include those containing collagen biological fabrics or laminates surgical graft. The present invention also includes the use of surgical graft and preparation methods.

In some embodiments, the present invention includes a method for use in surgical procedures in surgical graft, which will be directly applied to surgical graft surgical site of a subject, such as internal or external parts of the unit bit. In some embodiments, during a surgical procedure using biological tissue collagen was used as a surgical graft 10, which will be disclosed in more detail below, for example, to prevent blood from leaking out of the suture and to prevent body and suture

material adhesions. In other embodiments, the surgical graft is used as mulch on the anastomotic site, for example in the anastomotic site GI GI pipes during the surgery in order to prevent intestinal fluid and bile leakage from the suture and to prevent body and suture material adhesions.

15 The present invention involves the use of bio-fabric is used as grafts or dressing after surgery or burns the skin wounds; to prevent other to cover the abdomen, chest and face serous pericardium in all peritoneal surgery or in the adhesions reconstruction appear; reconstruction lining in the mouth and nasal cavity, respiratory, gastrointestinal mucosal surface of all the urogenital tract and; as a support brain surgery to repair the dura matrix; as facilitators in the central and peripheral nervous system nerve regeneration 20 students substrate; and reconstruction of soft tissue in the joints to prevent sticking or tendon repair.

The present invention comprises one or more biological molecules impregnated surgical graft of the present invention, preferably therapeutic agents, depending upon the particular surgical purpose desired. Such biomolecules include antibiotics (such as clindamycin, two Yue amine tetracycline, doxycycline, gentamicin), hormones, growth factors, anti-tumor agents, antifungal agents, antiviral agents, pain medications, anti-group histamines, 25 anti-inflammatory agents, anti-infective agents (including silver (e.g., silver salts include silver nitrate and silver sulfadiazine, but not limited to), elemental silver, antibiotics, bactericidal enzymes (such as lysozyme), but is not limited to this), wound healing agents (e.g. cytokines include PDGF, TGF, but is not limited thereto; thymosin), hyaluronic acid as a wound healing agent, wound sealants (e.g., with or without thrombin blood fibrin), cellular attractant and bracket reagent (e.g. fibronectin protein 30), etc., but is not limited thereto. In a particular embodiment, the collagen may be at least one biological fabric impregnated growth factors, e.g., fibroblast growth factor, epidermal growth factor, and so on. Biological fabric may also be impregnated with small organic molecules, e.g., a specific inhibitor of particular biochemical processes, such as a membrane receptor inhibitors, kinase inhibitors, growth inhibitors, anticancer drugs, antibiotics, and so on.

The present invention also includes the surgical graft of the present invention transplantation of living cells, including stem cells, totipotent stem cells, pluripotent stem cells, pluripotent stem cells, tissue-specific stem cells, 5 embryonic-like stem cells, progenitor cells, the class into fibroblasts, but not limited thereto. In other embodiments, the present invention comprises a surgical graft of the present invention is a special kind of transplanted progenitor cells, including chondrocytes, hepatocytes, hematopoietic cells, pancreatic parenchymal cells, neuroblasts and progenitor muscle cells, but is not limited to biological collagen fabric 10 thereto ophthalmology invention has clinical and therapeutic utility in the treatment of eye related diseases or disorders. Collagen biological fabric of the invention for the treatment and / or prevention of ocular surface disease is particularly useful, including corneal ulcer / perforation, bullous keratopathy, ocular dermoid cysts / tumors, primary pterygium, persistent corneal epithelial defect, acute and chronic alkali burns, thermal burns, aniridia, atopic keratitis, idiopathic edge stem cell deficiency, pannus, neovascular 15 percent, phoenix class corneal melting moist eye scarring pemphigoid, leaky filtration blisters, Ahmed valve tube is exposed, with subsequent symblepharon marcescens (Serratia) cellulitis, acute and chronic Stevenson Johnson syndrome, but is not limited thereto.

Collagen biological fabric of the present invention in the following applications are particularly effective, namely the promotion of ulcer having persistent corneal epithelial defect healing; promote epithelial formation; promotion of epithelial cells and stem cell growth 20; reduce inflammation and pain, inhibition of angiogenesis and scarring; optional substitution matrix conjunctival autograft in pterygium and as "trees exposed sclera" Elimination period grafts; epithelial phenotype recovery.

The present invention includes collagen biological fabric of the present invention will be transplanted to treat symptomatic bullous keratopathy, preferably humans In humans, most preferably having poor eyesight. Desired biological fabric 25 of the invention with respect to the art for the treatment of symptomatic bullous keratopathy (specifically conjunctival flap is constructed) Other standard procedure for having enhanced therapeutic and clinical utility. With respect to the art for the treatment of symptomatic bullous

keratopathy (specifically conjunctival flap is constructed) Other standard procedures, the advantages of the biological fabric of the present invention will be transplanted include alleviating pain, easy to implement, on beauty more acceptable to 30 and reduce the appearance of complications, such as ptosis and edge stem cell deficiency, but not limited thereto. Collagen biological fabric of the present invention provides an improved conjunctival flap selectable alternatives to promote healing of ulcers have corneal epithelial defect; improved methods for symblepharon dissipate for conjunctival surface reconstruction; improved from the conjunctiva for surgical removal of a tumor or corneal surface, damage or scar tissue method; improved by correcting herpes leakage performed surgical method for glaucoma; in pterygium "trees exposed sclera" eliminate conjunctival autograft during the Band keratopathy and for preventing recurrence; optional alternative modified substrate.

5 The present invention also provides a biological fabric is used as ophthalmic surgical graft applications. The present invention includes the preparation of graft, these grafts contain further comprising one or more therapeutic agents of the present invention the biological fabric, these receptors when administered therapeutic agents can be delivered to the recipient. Examples of therapeutic agents include pilocarpine, erythromycin, gentamicin, vancomycin, tobramycin, netilmicin sulfate, polymyxin B, three Yue oxygen benzyl diamino pyrimidine, gender amphotericin B, anti-cancer agents, antibiotics 10, cyclosporin, etc., but is not limited thereto.

Collagen biological fabric of the present invention also reduce the excimer laser photorefractive / therapeutic keratectomy corneal blur caused by having utility.

Biological Collagen Fabrics

for ophthalmic procedures of the present invention can be configured to provide a variety of, including insert, protective, granules, gels, aqueous injection preparation, sponge-like, thin film, but not limited to 15. it is known to persons skilled in the art of conventional techniques for ophthalmic surgical implants within the scope of the present invention.

Use biological collagen fabric of the present invention as an ophthalmic surgical graft exemplary program may include the following

RegenX Science Exhibit 1004
Page 955

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0955

steps: removal of corneal and / or conjunctival tissue diseased; provide a root 20 under sterile conditions, according to the present invention has been prepared surgical implants, and trimmed by a free technique to cover the corneal epithelium or trees exposed sclera. By stitching the graft anchored to trees exposed junction of the sclera and conjunctiva; conduct major suture; then using interrupts tension on the suture to form a graft surface evenly distributed; so avoid graft with the receptor or sclera any gaps appear between the cornea. The free edge of the graft and the host connection, the graft must be kept below 25 receptor conjunctiva to allow the host to the point of attachment of epithelial tissue of the donor at the slide base layer, otherwise the graft recipient is extruded tlt collagen biological fabric of the present invention has particular utility in dentistry, such as periodontal surgery, periodontal tissue regeneration guided tissue regeneration, guided bone regeneration and root coverage.

30 of the present invention includes the use of collagen biological fabric of the present invention is to promote the regeneration of periodontal bone defects, including matching on both sides of periodontal defects, interdental bone defects, three layers deep within the walls of the bone defect, layer 2 defects and bone defects 2 and 3, but not limited to the inner wall of the bone-deep. Collagen desired biological fabric of the present invention known in the art with respect to other techniques for therapeutic utility in the treatment of periodontal bone defects with enhanced and improved clinical parameters, known in the art to use other techniques such as cross. linked collagen membrane, such as those disclosed in the following documents to those that Quteish et al., 1992, J. Clin Periodontol 19 (7): 476-84; Chung 5 et al., 1990, J Periodontol 61 (12...):. 732-6; Mattson et al., 1995, J. Periodontol 66 (7): 635-45; Benque et al., 1997, J Clin Periodontol 24 (8): 544-9; Mattson et al.,... 1999, J Periodontol 70 (5):. 510- 7. By using biological collagen fabric of the present invention obtained by the improved parameters Examples include patches and gum index score, probing pocket depth, probing depth and attachment fork 10 and involved bone defect classification for these skilled artisan It is known, but not limited thereto.

The present invention also includes the use of biological fabric of the present invention in the treatment of class II furcation defects, including

defects on both sides, a pair of oral mandibular molars class II furcation defects and lower sides of collar bifurcation defect, but does not limited thereto. Collagen biological fabric of the present invention in the treatment of class II furcation defect 15 utility may be able to part with its ability to regenerate periodontal tissue defects in bifurcated loss to explain. Desired biological fabric of the invention with respect to the collagen membrane in the art for the treatment of class II furcation defects and treatment for having enhanced clinical utility, type II collagen membrane treatment bifurcation defects in the art, for example, as in the following. those disclosed in the literature, that is, Paul et al., 1992, Int J. Periodontics Restorative Dent 12: 123-31; Wang 20 et al., 1994, J Periodontol 65:... 1029-36; Blumenthal, 1993, J.. Periodontol 64:.. 925-33; Black et al., 1994, J Periodontol 54: 598-604; Yukna et al., 1995, J Periodontol 67:.. 650-7.

The present invention also includes the use of biological fabric of the present invention in root coverage program. Biological fabric of the present invention in root coverage in utility can be interpreted as this is partly due to its principle can reach 25 guided tissue regeneration based on the replacement of lost, damaged or diseased gums group organizations. Due to the above mentioned reasons, the fabric of the present invention, the desired biological and conventional in the art for root coverage compared to collagen membrane having enhanced clinical utility, in the art of traditional collagen membrane for root coverage, for example in the following documents. those disclosed that Shieh et al., 1997, L Periodontol 68: 770-8; Zahedi et al., 1998, J Periodontol 30 69:.. 975-81; Ozcan et al., 1997, J. Marmara Univ Dent... Fa 2: 588-98; Wang et al., 1997, J Dent Res 78 (Spec Issue):.. 119 (Abstr 106.)..

The present invention also includes the use of collagen bio-fabric in a subject suffering from periodontal disease, including gingivitis and periodontitis, but is not limited thereto. Biological fabric of the present invention also have additional scaling and root planing on clinical utility program. The present invention includes collagen raw material fabric of the present invention is treating a subject suffering from periodontal disease. Use biological collagen fabric of the present invention in the treatment of periodontal disease in those subjects 5 examples of the preferred methods include use of antibiotics such as chlorhexidine

gluconate-impregnated collagen bio-fabric insert the subject of one or more of periodontal bag, e.g., greater than or equal inserted 5 mm. Biological collagen fabrics are preferably biodegradable.

Collagen biological fabric of the present invention

In dentistry used depends on the type of dental disorder to be treated and can be treated with one or more biological molecules impregnation. Any biological molecule known in the art for the treatment of dental disorders including 10 in the method of the present invention and compositions of. In one particular embodiment, the collagen used in the treatment of bio-fabric accompanied by dental infection disorders can be impregnated with one or more antibiotics, including doxycycline, tetracycline, chlorhexidine gluconate and two ? amine tetracycline, but not limited to neurological biological collagen fabric 15 of the invention it is also used to repair the damaged nerve in particular severed peripheral nerve repair, and a neurosurgical procedure. The present invention includes, for example the use of collagen in the dura mater replacement bio-textiles and peripheral nerve repair. When the above-mentioned reasons, the fabric of the present invention, the biological collagen and other methods in the art for use as a dural substitute compared or having enhanced clinical utility in nerve repair, the other parties in the art to use 20. France, for example, Berger et al., 1970, Acta Neurochir 23:.. 141; Ducker et al., 1968, Mil Med 133: 298; U. S ◆ Patent Nos. 4,778,467;; 3,961, 805;. 5, 354,305.

In some embodiments, the collagen biological fabric of the present invention may be used as surround nerve anastomosis of the prosthesis, which can be used to wrap such as peripheral nerve anastomosis. Collagen biological tissue 25 of the present invention has particular utility in nerve repair, which, for example, by such a process to be completed, namely, to promote the formation of longitudinal through repair of connective tissue framework of the region, thus causing sheath cells and axons regeneration longitudinal pattern. peripheral nerve repair called nerorraphy (Jennings et al., 1955, Surgery: 206) The program is usually carried out through the use of sutures. However, this method only 30 have met with limited success because of the method of suturing severed nerves is difficult. Therefore, no suture nerve repair provides an alternative to collagen biological fabric of the present invention

is, for example, thereby loading the nerve ends of the tubular prosthetic device containing biological fabric of the present invention, so will cut off the end of relying on close to regenerate collagen urology biological fabric of the present invention has particular utility in correcting urinary incontinence. Urinary incontinence is due to urethral not kept closed during urine storage caused. The causes can be external 5, for example, the difference between the anatomy of the urethra and bladder neck support lead to incontinence, and the pelvic floor resuspended to respond. Alternatively urethra failure may be intrinsic, ie weak urinary function.

Traditionally, urinary incontinence by using bulking agents such as collagen, fat, silicone to correct (see review Lightner, 2002, Current Opinion in Urology, 12 ?: 333-8). Collagen biological fabric of the invention with respect to the art of the bulking agent is enhanced with 10 utility that enhances self-control. In addition, collagen biological fabric of the present invention also have an enhanced therapeutic effect, such as reducing local complications, reduce urinary tract infection, host response, reliable durability and greater security.

Collagen biological fabric of the present invention to correct urinary incontinence utility here is partly due to the unique biological collagen fabric described physical properties, particularly its non-immunogenic. 15 In some embodiments, the collagen implants used as biological fabric, such as the urethra implant. While not wishing to be bound by any particular theory, but collagen biological fabric of the present invention may be closed by increasing the pressure on the urethra and urine for passive resistance to the outflow of biological collagen fabric having enhanced therapeutic utility of the present invention can be Orthotics in orthopedic used in the program. Scheme 20 In certain embodiments, the fabric may be used for biological collagen orthopedic defects, such as congenital or acquired defects. Local defects caused by trauma or a bone tumor in the surgical recovery or reconstruction of the orthopedic and maxillofacial surgery is a major problem. Typically, it has been used a synthetic bone substitutes or collagen membrane, partly due to their induction of bone formation activity (see e.g., Rao, et al., 1995, J Biomater Sci Polymer Edn 7 (7)...: 623-45). However, common to ask 25 questions due to the implanted material caused by systemic infection. However, the biological tissue

collagen material of the present invention than in the art used is advantageous, particularly because as a bone substitute for orthopedic defects, low immunogenicity. In some embodiments, the collagen may be used as bio-fabric rebuild tendons, ligaments and cartilage prosthesis. In other embodiments, the collagen biological fabric of the present invention can be used as a bone substitute prosthesis.

Collagen biological fabric 30 and Cardiovascular Surgery of the present invention can be used in cardiovascular surgery procedures, such as using false body to be used to build large and small blood vessels; for the repair of congenital vascular malformations and biological valve of the present invention is sick fabrics are particularly useful as vascular graft surgery, such as a vein or artery grafts. Biological fabric utility in vascular surgery is partly due to its non-toxic; no immunogenicity; stability; easy to operate; antithrombin properties; minimum porosity graft vessel wall 5; available in a variety of sizes; reproduction of the material. In some embodiments, the fabrics of the present invention, the biological valve may be used as an alternative to drug delivery biological collagen fabric of the invention may be used as a medicament for controlled delivery of therapeutic agents such as drug delivery plant body. In some embodiments, the collagen bio-fabric delivering one or more therapeutic agents to the subject 10, preferably a human. It included within the scope of the present invention the therapeutic agent is a protein, peptides, polysaccharides, polysaccharide conjugates, based on the genetic vaccine, live attenuated vaccines, whole cell. Non-limiting examples of the method of the present invention is the use of antibiotic drugs, anti-cancer agents, antimicrobial agents, antiviral agents, vaccines, anesthetics, analgesics, anti-asthmatic agents, antiinflammatory agents, antidepressants, joint inflammatory agents, anti-diabetic agents, antipsychotics, central nervous system excitation thorn 15 agents, hormones, immunosuppressive agents, muscle relaxants, prostaglandins.

Collagen fabric may be used as a biological preferably a human subject to a controlled delivery of one or more species of small molecule delivery vectors. In some embodiments, the collagen bio-fabric delivery of one or more small molecule to a subject, preferably a human. When used herein, the term "small molecule" and similar terms include peptides, peptidomimetics, amino acids,

amino acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, having low to about 10,000 g / mole molecular weight organic or inorganic compounds (i.e., including hetero organic compounds and organometallic compounds) having less than about 5, 000 g / mole molecular weight organic or inorganic compounds, having less than about 1,000 g / mole molecular weight organic or inorganic compounds, having less than about 500 g / mole molecular weight organic or inorganic compounds, having less than about 100 g / mole molecular weight organic or inorganic compounds, and salts of such compounds, esters and other pharmaceutically acceptable form, but is not limited thereto. Also included on such compounds salts, esters and other pharmaceutically acceptable form.

In certain embodiments, known in the art with respect to the other drug delivery systems, biological collagen fabric of the present invention is used as a carrier for drug delivery of the drug leads to enhanced uptake; improved pharmacokinetic characteristics; and systemic distribution of the drug. An improved pharmacokinetic said obtained when, for example using standard pharmacokinetic parameters were measured enhanced dynamics of the drug, such as reaching the maximum plasma concentration (T...); the number of maximum plasma concentration (Cm); can cause blood or plasma concentration detected (Tlag). Enhanced uptake said that when using such parameters were measured, drug absorption is improved. measuring pharmacokinetic parameters in accordance with conventional methods in the art to carry out.

6. 6.1 Example 5 Production of collagen material of biological fabrics material for the preparation of the implementation of the following biological collagen fabric. materials / equipment • Copies of birth records • Material / family health history / informed consent • Copies source barcode tag (donor ID number) • collection # (consecutive numbers assigned to the income material) • Organization processing records (file ID # ANT-19F); retention of each batch processed in detail i have recorded • human placenta (stored in the processing time is less than 48 hours at the start) • sterile surgical clip / hemostat • sterile cell scraper (Nalgene NUNC Int. R0896) • scissors •

sterile scalpel sterile • sterile gauze (non-sterile PSS 4416, sterilization) • sterile rinsing stainless steel tray • tray • disinfection process sterile plastic boxes • sterile 0. 9 percent NaCl solution (Baxter 2F7124) • sterile water (Milli Q plus 09195 or Baxter 2F7113) • sterile sample container (VWR 15704-014) • personal protective equipment (including sterile or non-sterile gloves) • decellularization solution having a certificate of clean room • prepared beforehand (D- cells); 0 01-1 percent - hydrated sodium deoxycholate disinfection tank • • rocking platform (VWR 100) • chronograph (VWR) • sterile plastic frame net • PVC wrap film • vacuum pump (Schuco-Vac 5711-130) 5 • gel dryer (ie hot dryer; BioRad 583 type) • disinfection of stainless steel • cutting board for packaging bags • sterile, lure ruler (General Tools MFG. Co 1201) • traceable digital thermometer (61161-364 type, control company) 10 • Accu-seal automatic sealer (Accu- Seal, 630-1B6 type) at the time of birth for pregnant women screened for infectious diseases, such as pathogens, HIV, HBV, HCV, HTLV, syphilis, CMV and other viruses, and other placental tissue will be collected pollution. Use only collected from such tissue donors obtained to produce collagen biological fabric of the present invention, that his mother has been tested for the above mentioned pathogens are negative or non-reactive 15 resistance.

After a normal birth, placenta, and umbilical cord blood naturally discharged from the contraction of the uterus. Collect the placenta, umbilical cord and cord blood after birth. These materials were transferred to the laboratory, where they are in a clean room having a HEPA filtration system for processing under aseptic conditions, HEPA filtration system on at least one hour before processing. In operation 20 sets of product always wear gloves (sterile or non-sterile, when appropriate). Once the time can be implemented on disposal off all of the amnion / chorion unused (excess) portion and during tissue processing resulting contaminated liquid.

Step I with sterile Steri-Wrap sheet set out a sterile field, and the following process for 25 instruments and accessories placed on the site. cell scraper • sterile tray pack • sterile • sterile scalpel • sterile processing tray 30 record under aseptic

package ID # 0 in the recording process removed the placenta from the transfer container, and placed in sterilized stainless steel tray. With surgical clips and scissors to cut the umbilical cord about 2 inches from the placenta. The umbilical cord is placed in a separate sterile container for further processing. Vessel organization ID barcode tags; presence and identification materials and storage solution (for example, type of media). In some cases, if you do not need to be used for other programs, to abandon the umbilical cord.

From the edge of the placental membrane begins to separate amniotic membrane wool and cashmere 5 by using a finger blunt dissection. This operation is carried out prior to cutting the membrane.

After the entire surface of the amnion and chorionic and placental separation surround the umbilical cord stump with scissors cutting amnion and amniotic membrane and separate the placenta. In some cases, if it is impossible without tearing tissue separating amnion and chorion, amnion and chorion as a cut cut off from the placenta, then they are peeled apart.

10 The chorionic placed in a separate sample container for use in other programs. Vessel organization ID barcode tags, identification of materials exist (for example, the type of media) and storage solutions, monogrammed standard abbreviations and superscript date.

If the amniotic sheet still adhered to the placenta, which was detached from the placenta and umbilical cord around the ring with scissors cutting down. The placental transfer back into the container for use in other programs.

15 record the appropriate data in tissue processing records. amnion with sterile 0.9 percent NaCl solution was kept in the tray. Preferably, prior to the next steps of the process, the amniotic membrane from childbirth from refrigerated storage by up to 72 hours.

Step II once removed from the sample container piece of amniotic membrane, sterilized and placed on a stainless steel tray.

20 additional slices in separate filled with sterile water, sterile stainless steel trays until they are made ready for cleaning. Remove the excess amniotic sheet from the processing tray, filled and

placed in a separate sterile water rinsing stainless steel tray.

If blood maternal or fetal liquid / material heavily contaminated, then rinsed with sterile water sheep membrane, sterile water and replaced when necessary.

25 The amniotic membrane is placed on the processing tray, and keep the parent side up. Using a sterile cell scraper carefully remove as much visible contaminants and cellular material from the mother's side of the amniotic membrane. (Note: For this step should use minimal pressure to prevent tearing the membrane). Use sterile water to help remove cells and cell debris. Amniotic further supplied in individual sterile stainless steel rinsing tray rinsed with sterile water.

30 so that the fetus amniotic flip side up, and place it back on the tray handle, and then rinsed with sterile water. Use a cell scraper to gently remove visible cellular material and debris (Note: For this step should use minimal pressure to prevent tearing the membrane). Use sterile water to help remove cells and cell debris.

In a separate sterile rinsing trays sheep rinsed with sterile water film between two cleaning cycles. In as many times as desired (the number of cleaning cycles) to clean tissue to remove most (if not all) of visible cellular material and debris from the sides of the membrane. In between 5 to wash rinse rinse tray Replace sterile water. after each cleaning cycle with sterile water rinsing tray.

All other amnion sheets are processed in the same manner, and placed in the same container. Paste organization ID bar code identification of materials and storage solutions exist (for example, the type of media), plus the initials and date.

10 in tissue processing recording records the appropriate information and dates.

Step III from the rinse tray (or from a storage vessel) removed amnion, with fingers gently squeezing out excess liquid, and the membrane was placed in a sterile sample container. D- cells loaded with container full solution to 150 ml marking, and to ensure that cover all the amnion and closed containers.

15 The vessel was placed in the box on the rocking platform. Open rocking platform, and let

the film is set in motion in D- # 6 ? cell solution for at least 15 minutes and up to 120 minutes.

In Step I in the same manner with a new sterile equipment and sterile tray set a new sterile field. In the recording process is recorded under sterile package ID #. After completion of shaking, rocking platform closed, and remove the film from the container. The membrane is placed at the bottom of 20 new stainless steel processing tray, add sterile 0. 9 percent NaCl solution to cover the tray.

With new sterile cell scraper to remove residual D-cells and material (if any) from both sides of the organization. The steps needed to be repeated as many times in order to remove from the entire surface of the visible side as much residual cell material. Between two cleaning cycles of sterile 0.9 percent NaCl solution in the membrane was rinsed with separate rinsing tray. Replace the rinse twice between 25 sterile rinsing trays 0. 9 percent NaCl solution. after the last cleaning cycle is completed, the membrane with sterile 0. 9 percent NaCl solution, rinsed, and placed on a new filled sterile 0. 9 percent NaCl solution sterile sample container.

All the rest of the amnion sheets in exactly the same manner.

When all the pieces have been processed amnion and placed in a sterile 0.9 percent NaCl solution container 30 when the vessel, the vessel was placed in the box on the rocking platform in order to set the # 6 shaken at least five minutes. After the shaking is complete, remove the film from the sample container, replace the container sterile 0. 9 percent NaCl solution, and the film back into the sample container. tissue sample container ID and bar code label marked quarantine. Identification of the presence of the material and storage solution (for example, type of media), marked on the label's first initials and date. The sample container is placed in a clean zip-lock bag and placed in the refrigerator (2-81C) in. record all appropriate data records in tissue processing.

5 When the serological results showed that available, the label put on quarantine appropriate label (serologically negative or research use only), and separate those containers with the checked.

Step IV Step IV prior to use, checks the state inspection organization to ensure that all test results were negative can be applied.

10 to step II and III in the same manner with sterile Steri-Wrap sheet set out a sterile field, and position all sterile and disinfected instruments and accessories. membrane was removed from the freezer and placed in a new sterile stainless steel processing tray. Sterile 0.9 percent NaCl solution was added to cover the bottom of the tray.

With new sterile cell scraper to gently remove all visible cellular material and debris (such as 15 if any) (Note: For this step should use minimal pressure to prevent tearing the membrane).

Using a sterile 0. 9 percent NaCl solution to help remove cells and debris.

In a separate filled with sterile 0.9 percent NaCl solution sterile stainless steel rinsing tray rinsed film. In between cleaning cycles to replace the 0.9 percent NaCl solution. The membrane was placed in a new sterile sample container, the container with fresh sterile 0.9 percent NaCl solution filled and placed on a rocking platform 20 in order to set the # 6 shaken at least 5 minutes. preceding step was repeated three times, and in between each shaking replace sterile 0. 9 percent NaCl solution.

In the tissue processing recording records the appropriate data. once removed from the sample container a film with your fingers and gently squeeze out excess liquid, and the membrane was placed on a sterile processing tray. Gently stretch film until it flattens; 25 and to ensure its fetal side down. framework prepared by cutting the plastic sheet with sterile scissors sterilized. Frame size should be smaller than the film portion of about 0. 5 cm in each direction. Rinse framework filled with sterile 0. 9 percent NaCl solution, rinsing tray. frame will be placed on the surface of the film is slightly stretched, and pressed gently on top. 30 is necessary to the smooth side of a plastic frame toward the organization. with a scalpel cutting film frame surround and retain about 0. 5 cm extending to the edge of the frame Xi Bu. Excess film back into the sample container. clip or tweezers to extend beyond the edges of the film frame package edge Dagon live frame, and temporarily placed in the same tray in the back.

Under a film in the same manner. Importantly, the total area to be dried is not more than 300 cm2 / thermal dryer. In the "open frame" this piece of film at the same time, not on five sheets should be kept in a container aseptic 0.9 percent NaCl solution.

Setting the drying temperature dryer, and use the extended use of the probe calibrated digital thermometer to check. The drying temperature is set at 5 0IC. In the tissue processing records record number noted.

Open vacuum pump.

On

10 sterile gauze placed in a thermal dryer drying platform, covering the opening of the film than the frame area slightly larger area. It is important to ensure that the total thickness of no more than one layer of gauze folded into gauze thickness of 4M.

A piece of plastic mesh cage placed on gauze. Plastic mesh edges should extend out of gauze edge about 0. 5-1. 0 cm.

15 will stand apart from the film gently lifted and placed on a plastic mesh located above the thermal dryer platform, while maintaining the film side facing up. Repeat this until the maximum number of films (no more than 300 cm2) placed on the thermal drying internet. (Note: the fetal side of the amniotic membrane up).

A piece of PVC film bag cut of sufficient size to cover the entire drying platform of the heat dryer plus an extra foot.

20 at the same time running the vacuum pump with plastic film covering the entire drying platform of the heat dryer gently, and let both sides 1/2 feet stretching out beyond the edge of the platform and dried. It should be noted that the film is pulled tightly over the film and the frame sheet (ie it is vacuum "sucked"), and there is no leak in the organization and does not wrinkle area. Then close the lid.

The pump is set at about -22 inches Hg vacuum. 2-3 after 25 minutes of the drying cycle values pump table records. The film is heat - vacuum-dried for about 60 minutes. The drying process is about 15-30 minutes, place on a new layer of sterile gauze in a hot dryer. The total thickness of the gauze layer must not exceed the thickness of a fold into a 4x4 gauze.

After the change is to be noted that the plastic film pulled tightly over the film and the frame sheet,

and there is no leak in the membrane area and no wrinkles.

30 vacuum seal integrity by checking the pump pressure meter and single timing checks. After completion of the drying process, open the hot dryer, the pump operation while allowing the film to cool for about 2 minutes. with sterile Steri-Wrap and disinfected stainless steel cutting board beneath it set a new sterile field. The use of sterile gloves at this point. The pump is still running at the same time, from the corner began to gently remove the plastic film from the film, and with a gloved hand pressure live diaphragm. That the frame and the film together and gently lifted off the drying platform and placed located 5 not sterilized stainless steel cutting sterile field above the plate, while permitting the film side up. Forming an incision with a scalpel while cutting through the diaphragm along the edges of 1-2 mm from the edge of the frame. Gloved (sterile gloves) hand holding the membrane in place. By slowly peeling the membrane and be gently lifted off the frame, and then placed on a cutting board on top of the sterile field. with a scalpel or sharp scissors to cut the membrane fragments of the specified size. In the packaging of 10 before the sterile field cut all the pieces and safekeeping. With one hand (sterile) will be placed inside the monolithic film exposing sheet bag while with the other hand (non-sterile) Hold the bag. It should be noted, do not use "sterile" hand contact with the bag. After all the pieces placed in the bag, seal them. In designated areas outside of the bag labeled with appropriate information (e.g., part #, batch #, etc.) of the label. All of the diaphragm 15 in the same manner. The labeled and sealed exposing sheet placed in waterproof bags bag zip lock bag for storage until ready to transport them to the sterilization device or dispenser. In the tissue processing records to record all appropriate data. hydration / seamable Evaluation 6.2 collagen biological fabrics in order to obtain a surgical operation on biological fabric of the present invention, the hydration period and suture of 20 qualitative and quantitative feedback, the method according to the present invention amniotic sample preparation is supplied to four experienced, very well-known eye surgeon to evaluate surface. Samples were evaluated by a surgeon by the following method, that the purposes of tissue transplantation in pigs eye surgical specimen to determine the operating

characteristics of the present invention the fabric and stitching biological sex.

Each surgeon may use the following method: 25 (1) biological fabric in a dry condition to be cut to fit the individual pig eyes quadrant; (2) to cut out the biological fabric placed on the surface of pig eyes on; (3) on the pig eyes with sterile saline solution will allow hydration biological fabric graft that is activated by rehydration, hydration of time 2,5,10 and 20 minutes; 30 (4) biological fabric through several 9-0 Vicryl suture stitched onto the bite pig eyes epithelium; and (5) with respect to the surgeon made by hydration of amniotic tissue quality, consistency and suturing of qualitative commentary. equivalents: not to use particular embodiments described herein to limit the scope of the present invention. In fact, from the foregoing description and drawings, in addition to a variety other than those of the present invention to repair 5 ornaments for the skilled artisan will be apparent. Such modifications will fall within the scope of the appended claims. here refers to a variety of publications, patents and patent applications, the disclosures of which were incorporated by reference in its entirety.

Iraq a secluded beach known: Continued destroy surface 2B, 3 accompanying drawings

Cited by: CN103006349 A ; CN103751842 A ; CN103861151 A ; CN103861151 B ; CN105246495 A ; CN105311057 A ;

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Application date: 20090930
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Title: COMPOSITIONS AND METHODS FOR TISSUE REPAIR WITH EXTRACELLULAR MATRICES

Title: 用于组织修复的使用细胞外基质的组合物和方法

Abstract:

Described herein are compositions comprising decellularized cardiac extracellular matrix and therapeutic uses thereof. Methods for treating, repairing or regenerating defective, diseased damaged or ischemic cells, tissues or organs in are subject preferably a human using a decellularized cardiac extraceollular matrix of the of the invention are provided. Methods of preparing caidiomyocyte culture surfaces and culturing cells with absorbed decellurized cardiac extracellular matrix are provided.

Abstract:

文中描述的是含有脱细胞化心脏细胞外基质的组合物及其治疗用途。本发明提供使用本发明的脱细胞化心脏细胞外基质用于治疗、修复或再生受治疗者中、优选人体中缺陷的、病态的、受损的或缺血的细胞、组织或器官的方法。本发明提供制备心肌细胞培养表面以及用吸收的脱细胞化心脏细胞外基质培养细胞的方法。

This paper describes comprising decellularized cardiac extracellular matrix compositions and their therapeutic uses. The present invention provides the use of the present invention de extracellular matrix for the treatment of cardiac cells, repair or regeneration in a subject, preferably human defects, morbid, damaged or ischemic cells, tissues, organs, or method. The present invention

provides a cultured myocardial cells was prepared, and an outer surface with absorbed decellularized cardiac extracellular matrix of cultured cells.

Claims:

一种组合物，其含有心脏组织来源的脱细胞化细胞外基质。

A composition comprising decellularized cardiac tissue-derived extracellular matrix.

2.如权利要求1所述的组合物，其中所述组合物是可注射的。

2. The composition as claimed in claim 1, wherein, wherein the composition is injectable.

3.如权利要求1所述的组合物，其中所述组合物在温度20。C～25。C时为形式，在温度 高于25。C时为凝胶形式。

3. The composition as claimed in claim 1, wherein, wherein the composition is at a temperature of 20. C~25. Form C, at a temperature higher than 25. Gel form C.

4.如权利要求2所述的组合物，其中所述组合物配制成在心肌梗塞后注射到梗塞壁 中。

4. The composition as claimed in claim 2, wherein the composition is formulated after myocardial infarction injected into the wall.

5.如权利要求1所述的组合物，其中所述组合物保留天然的葡胺聚糖。

5. The composition as claimed in claim 1, wherein, wherein said composition is left natural meglumine glycans.

6.如权利要求1所述的组合物，其中所述组合物含有天然发生的将细胞募集到组合物 中的因子。

6. The composition as claimed in claim 1, wherein, wherein the composition contains naturally occurring cell recruitment will factor into the composition.

7.如权利要求1所述的组合物，其中所述组合物还含有非天然发生的将细胞募集到组 合物中的因子。

7. The composition as claimed in claim 1, wherein, wherein said composition further comprises a non-naturally occurring cell recruitment into the composition of the factor.

8.如权利要求1所述的组合物，其中所述组合物被设定为通过27G或更小的针输送到 组织中。

8. The composition as claimed in claim 1, wherein, wherein said composition is set through 27G or smaller needle delivered to the tissue.

9.如权利要求1所述的组合物，其中所述组合物还含有细胞。

9. The composition as claimed in claim 1, wherein, wherein the composition further comprises cells.

10.如权利要求9所述的组合物，其中所述细胞是多潜能或多能干细胞或心肌祖细胞。

10. The composition as claimed in claim 9 wherein, wherein the cell is a pluripotent or stem cells or cardiac progenitor cells.

11.如权利要求2所述的组合物，其中所述可注射的组合物还含有外源的治疗制剂。

11. The composition as claimed in claim 2, wherein said injectable composition further comprises exogenous therapeutic agent.

12.如权利要求2所述的组合物，其中所述可注射的组合物被设定为无损的传导阻滞。

12. A composition as claimed in claim 2, wherein said injectable composition is set to be non-destructive conduction block.

13.如权利要求2所述的组合物，其中所述可注射的组合物被配制为涂覆组织培养板 以培养心肌细胞或其它心脏细胞祖细胞。

14.如权利要求1所述的组合物，其中所述组合物含有治疗上可接受的成分。

15. 一种制备含有脱细胞化心脏细胞外基质的组合物的方法，其包括：(a)获取具有细胞外基质组分和非细胞外基质组分的心脏组织样品；(b)处理心脏组织样品以去除非细胞外基质组分，从而得到脱

细胞化的心脏细胞外基 质；并 (C)对脱细胞化心脏细胞外基质进行灭菌。

16.如权利要求15所述的方法，其中所述方法还包括将脱细胞化心脏细胞外基质冻干 和碾碎的步骤。

17.如权利要求15所述的方法，其中所述方法还包括对脱细胞化心脏细胞外基质进行 酶处理的步骤。

18.如权利要求15所述的方法，其中所述脱细胞化心脏细胞外基质在低于7的HI中以 胃蛋白酶进行消化。

19.如权利要求15所述的方法，其中所述方法还包括在盐缓冲溶液中悬浮和中和所述 脱细胞化心脏细胞外基质的步骤。

20.如权利要求19所述的方法，其中所述溶液通过高规格针注射到心肌内。

21.如权利要求19所述的方法，其中所述溶液在体温自发形成凝胶。

22.如权利要求19所述的方法，其中所述溶液还含有细胞或其它治疗制剂。

23.如权利要求19所述的方法，其中所述溶液被置于组织培养板或孔内，在37。C孵育 以形成凝胶。

24. 一种在吸收基质上培养细胞的方法，其包括下列步骤： 2 CN (a)在组织培养装置内提供含有心脏组织来源的脱细胞化细胞外基质的溶液； (b)孵育所述组织培养装置以吸收至少一部分所述脱细胞化细胞外基质到装置上； (C)去除所述溶液；并 (d)在吸收基质上培养细胞。

25.如权利要求24所述的方法，其中所述细胞是心肌细胞或心脏细胞祖细胞。

26. 一种用于受治疗者的心脏修复的治疗方法，其包括注射或植入治疗量的含有心脏 组织来源的脱细胞化细胞外基质的组合物。

27. 一种用于受治疗者的心脏修复的治疗方法，其包括注射或植入含有心脏组织来源 的脱细胞化细胞外基质的组合物。

28. 一种组合物，其含有骨骼肌组织来源的脱细胞化细胞外基质。

29.如权利要求28所述的组合物，其中所述组合物是可注射的。

13. The composition as claimed in claim 2, wherein the injectable composition is formulated as a coated tissue culture plates in cultured myocardial cells or other cardiac progenitor cells.

14. The composition as claimed in claim 1, wherein, wherein the composition contains a therapeutically acceptable ingredients.

15 A solution containing de-cardiac cells of extracellular matrix composition of methods, which include:. (A) to obtain an outer extracellular matrix component and non-extracellular matrix component heart tissue sample; (b) processing the cardiac tissue sample to remove non-extracellular matrix component to obtain decellularized cardiac extracellular matrix; and (C) of decellularized cardiac extracellular matrix is sterilized.

16. The method of claim 15, wherein said method further comprises the decellularized cardiac extracellular matrix lyophilization and crushed step.

17. The method of claim 15, wherein said method further comprises the step of enzymatically treated decellularized cardiac extracellular matrix were.

18. The method of claim 15, wherein said decellularized cardiac extracellular matrix is less than HI 7 to pepsin digestion.

19. The method of claim 15, wherein said method further comprises the step and the decellularized cardiac extracellular matrix was suspended in a saline buffered solution.

20. The method of claim 19, wherein the solution is injected into the myocardium through a high gauge needle.

21. A method as claimed in claim 19, wherein the solution spontaneously form gels at body temperature.

22. The method of claim 19, wherein said solution further comprises cells or other therapeutic agents.

23. The method of claim 19, wherein the solution is placed in tissue culture plates or wells, at 37. Incubate C to form a gel.

Method

30.如权利要求28所述的组合物，其中所述组合物在室温中为液体，在高于室温的温 度中为凝胶形式。

31.如权利要求28所述的组合物，其中所述组合物保留天然的葡胺聚糖。

32.如权利要求28所述的组合物，其中所述组合物含有天然发生的将细胞募集到组合 物中的因子。

33.如权利要求28所述的组合物，其中所述组合物含有非天然发生的将细胞募集到组 合物中的因子。

34.如权利要求28所述的组合物，其中所述组合物还含有细胞。

35.如权利要求28所述的组合物，其中所述细胞是干细胞。

36.如权利要求28所述的组合物，其中所述组合物被配制为涂覆组织培养表面或支架 以培养与骨骼肌修复相关的细胞。

37. 一种制备含有脱细胞化骨骼肌细胞外基质的组合物的方法，其包括： 从受治疗者获取具有细胞外基质组分和非细胞外基质组分的骨骼肌组织样品；处理骨骼肌组织样品以去除非细胞外基质组分，从而得到脱细胞化骨骼肌细胞外基 质、细胞外蛋白质和多糖；并 对脱细胞化骨骼肌细胞外基质进行灭菌。

38.如权利要求37所述的方法，其中所述方法还包括将脱细胞化骨骼肌细胞外基质冻 干和碾碎的步骤。

39.如权利要求37所述的方法，其中所述方法还包括对脱细胞化骨骼肌细胞外基质进 行酶处理、溶解或悬浮的步骤。

40.如权利要求37所述的方法，其中所述脱细胞化骨骼肌细胞外基质在低ra中以胃蛋 白酶进行消化。

41.如权利要求37所述的方法，其中所述方法还包括将所述脱细胞化骨骼肌细胞外基 质在溶液中悬浮并中和或改变ra的步骤。

42.如权利要求39所述的方法，其中所述溶液是PBS、盐水或其它缓冲溶液，其设定为 通过小直径针注射到心肌中。

43.如权利要求39所述的方法，其中所述溶液在体温形成凝胶。

24 A cultured stromal cells on the absorber, comprising the steps of: 2 CN (a) within the means provided in tissue culture decellularized extracellular matrix containing cardiac tissue sources solution; (b) incubating said tissue culture means to absorb at least a portion of the decellularized extracellular matrix to the device; (C) removing the solution; and (d) culturing the cells on the absorption matrix.

25. The method of claim 24, wherein said cell is a myocardial cells or cardiac progenitor cells.

26. A method for treating a subject for cardiac repair, which includes a therapeutic amount of injected or implanted decellularized extracellular matrix composition containing cardiac tissue sources.

27. A subject method of treatment for cardiac repair, including injection or implantation of decellularized extracellular matrix composition comprising cardiac tissue origin.

28. A composition comprising a source of skeletal muscle tissue decellularized extracellular matrix.

29. The composition as claimed in claim 28, wherein, wherein the composition is injectable.

30. The composition as claimed in claim 28, wherein, wherein the composition is a liquid at room temperature, at higher than room temperature in a gel form.

31. The composition as claimed in claim 28, wherein, wherein said composition is left natural meglumine glycans.

32. The composition as claimed in claim 28, wherein, wherein the composition contains naturally occurring cell recruitment will factor into the composition.

33. The composition as claimed in claim 28, wherein, wherein the composition contains a non-naturally occurring cell recruitment into the composition of the factor.

34. The composition as claimed in claim 28, wherein, wherein the composition further comprises cells.

35. The composition as claimed in claim 28, wherein, wherein said cell is a stem cell.

RegenX Science Exhibit 1004
Page 973

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0973

44.如权利要求39所述的方法，其中所述溶液还含有可输送到内部并在凝胶作用之 3 CN 前、之中或之后附着于以上材料的细胞、药物、蛋白质或多糖。

45.如权利要求39所述的方法，其中所述溶液被置于组织培养板或孔内，在37。C或高 于室温的温度中孵育，以形成用于细胞培养的凝胶。

46. 一种在吸收基质上培养细胞的方法，其包括下述步骤： (a)在组织培养装置内提供含有骨骼肌组织来源的脱细胞化细胞外基质的溶液； (b)孵育所述组织培养板装置； (C)去除所述溶液；并 (d)在吸收基质上培养细胞。

47.如权利要求48所述的方法，其中所述细胞是骨骼肌成肌细胞、干细胞或其它与骨 骼肌修复相关的细胞类型。

48. 一种用于受治疗者的骨骼肌修复的治疗方法，其包括植入含有骨骼肌组织来源的 脱细胞化细胞外基质的组合物。

4 CN A

36. The composition as claimed in claim 28, wherein the composition is formulated as a stent coated tissue culture surfaces or repair related to culture and skeletal muscle cells.

37 A method comprising decellularized skeletal muscle extracellular matrix composition was prepared, comprising: obtaining cells with extracellular matrix components and non extracellular matrix components from the subject skeletal muscle tissue sample points; treatment of skeletal muscle tissue sample to remove non-extracellular matrix components, resulting in decellularized skeletal muscle extracellular matrix, extracellular proteins and polysaccharides; skeletal muscle cells and decellularized extracellular matrix off bacteria.

38. The method of claim 37, wherein said method further comprises the decellularized extracellular matrix freeze-dried and crushed step in skeletal muscle cells.

39. The method of claim 37, wherein said method further comprises decellularized skeletal muscle extracellular matrix performing the enzyme treatment, the step of dissolving or suspending.

40. The method of claim 37, wherein said decellularized extracellular matrix of skeletal muscle cells in low ra to pepsin digestion.

Step

41. The method of claim 37, wherein, wherein the method further comprises the decellularized skeletal muscle extracellular matrix suspended in solution and neutralized or change of ra.

42. The method of claim 39, wherein the solution is a PBS, saline or other buffer solutions, which is set to the injection through a small diameter needle into the myocardium.

43. The method of claim 39, wherein said solution form a gel at body temperature.

44. The method of claim 39, wherein said solution further containing the gel delivered to the interior and the role of the 3 CN before, during, or after the material is attached to more cells, drugs, proteins or polysaccharides.

45. The method of claim 39, wherein the solution is placed in tissue culture plates or wells, at 37. C

or higher incubated at room temperature, to form a gel for cell culture.

Method

46 A cultured stromal cells on the absorber, comprising the steps of: (a) in tissue culture within the device provides decellularized extracellular matrix containing skeletal muscle tissue sources solution; (b) incubating said tissue culture plates device; (C) removing the solution; and (d) culturing the cells on the absorption matrix.

47. The method of claim 48, wherein said cells are skeletal myoblasts, stem cells or skeletal muscle repair and other related cell types.

48. A method of treatment of skeletal muscle repair of a subject, comprising implanted decellularized extracellular matrix composition containing skeletal muscle tissue sources.

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Description:

说明书 10/17 页 用于组织修复的使用细胞外基质的组合物和方法 [0001] 相关申请的交叉引用 [0002] 本申请要求2008年9月30日提交的美国临时申请第61/101332的优先权，其全 部内容通过引用并入本文中。

Manual page 10/17 use of extracellular matrix compositions and methods for tissue repair

[0001] Cross-Reference to Related Applications

[0002] This application claims priority to U.S. Provisional Application September 30, 2008 No. 61 / 101,332, filed its entirety by reference herein.

[0003] 政府权益声明 [0004] 本发明是在美国国立卫生研究院（NIH)授予的批准号为0D004309的美国政府支 持下完成的。政府对本发明有一定权利。

Approval number

[0003] The Government stated interests

[0004] The present invention is in the United States National Institutes of Health (NIH) grant to 0D004309 United States Government support under completion. The Government has certain rights in this invention.

背景技术 [0005] 本说明书通篇引用了多种出版物，包括专利、公开的申请、技术文章和学术文章。

各引用的出版物通过引用的方式整体并入本文中。

BACKGROUND

[0005] Throughout this specification refers to a variety of publications, including patents, published applications, technical articles and scholarly articles. each cited publications are incorporated by reference in its entirety herein.

[0006] 心血管疾病是美国死亡的主要原因。心血管疾病最普遍的起因是发生于冠状动脉 堵塞时的心肌梗塞（MI)。MI导致心肌细胞死亡和细胞外基质（ECM)降解，继而引发瘢痕组 织沉积。最终心脏衰竭发作，接着心脏扩张，导致泵血效率降低。由于心脏中只有极少量的 心脏祖细胞，且这些祖细胞不易分裂，因此心脏组织的再生不会自然发生。目前对心脏衰竭 的治疗严重依赖于创伤性的外科手术，对受损心脏组织的修复几乎无益。

[0007] 最近研究的方法利用将健康细胞注射到左心室（LV)梗塞壁，试图使心肌再生，然 而研究显

示，仅有少量的注射细胞存活。通常在涂覆有一种或几种细胞外基质蛋白的表面 或支架上培养细胞，包括成体和胚胎干细胞、诱导多潜能干细胞和如心肌细胞的分化细胞。

但是，在体内，这些细胞处在高度复杂的胞外环境中。

[0008] 目前正对一些天然来源的材料进行研究，以用于心肌注射，包括纤维蛋白、胶 原、藻酸盐、基质胶和明胶。这些均不提供显著量的心肌细胞外基质的天然组分。对于 心率不齐，非侵入性（non-ablative)治疗类型包括注射纤维蛋白和细胞。现有的用 于心肌细胞、干细胞和其它心脏相关细胞的体外细胞培养基质包括胶原、层粘连蛋白、SUREC0AT(CELUTR0N,胶原和层粘连蛋白的混合物）以及明胶。

[0009] 目前防止心肌梗塞继发心力衰竭的工作集中于细胞移植以取代坏死的心肌细胞， 防止负性左心室重构，以及使心脏组织再生。然而由于没有合适的基质，心肌细胞的体外生 长率和体内存活率低。存在改进的用于心脏修复、心率失调治疗和心肌细胞培养的组合物 的需求。同样地，也存在改进的用于骨骼肌修复、再生和细胞培养的组合物的需求。

发明内容 [0010] 一方面，本发明提供一种组合物，其含有心脏组织来源的脱细胞化细胞外基质。在 一些实例中该心脏组织为心肌组织,而在另一些实例中该心脏组织为心包组织。该组合物 可以是可注射的。该组合物可在室温中、典型地在20°c至25°C中形成液体形式，并可在高 于室温或高于35 °C的温度中形成凝胶形式。

[0011] 一些实例中，上述心脏组织选自人类心脏、灵长类心脏、猪心脏、牛心脏、或任意其 它哺乳动物或动物心脏，包括但不限于山羊心脏、小鼠心脏、大鼠心脏、兔心脏和鸡心脏。

5 CN [0012] 一些实例中，该组合物设置为在心肌梗塞后注射进梗塞壁。一些实施例中，该组合 物设置为通过小规格针(small gauge needle)(例如27规格(gauge)或更小)输送到组 织。一些实例中，上述组合物适合植入到患者中。

[0013] 一些实例中，该组合物含有天然发生的将细胞募集到组合物中的趋化因子、生长 因子和刺激因子。一些实例中，该组合物含有天然葡胺聚糖。一些实例中，该组合物还含有 非天然发生将细胞募集到组合物中的因子。

[0006] Cardiovascular disease is the main cause of death in the United States. The most common cause of cardiovascular disease, myocardial infarction (MI) occurs when coronary artery blockage. MI lead to myocardial cell death and extracellular matrix (ECM) degradation, sparking scar tissue deposition. Final onset heart failure, heart dilation followed, resulting in reduced pumping efficiency. Since only a very small number of the heart cardiac progenitor cells, and is not easy to divide these progenitor cells, and therefore regeneration of cardiac tissue do not occur naturally. Current treatment for heart failure relies heavily on traumatic surgery, to repair damaged heart tissue is almost useless.

[0007] Recent studies using the method of healthy cells were injected into the left ventricle (LV) infarction wall, trying to make myocardial regeneration, however research shows that only a small number of injected cells survived. Usually on cultured cells coated with one or several extracellular matrix protein outer surface or stand, including adult and embryonic stem cells, induced pluripotent stem cells and differentiated cells such as cardiomyocytes.

However, in vivo, these cells are in a highly complex extracellular milieu.

[0008] currently natural sources of some materials to study for myocardial injection, including fibrin, collagen, alginates, gum and gelatin matrix. These are not natural components provide a significant amount of myocardial extracellular matrix. For arrhythmia, non-invasive (non-ablative) type of treatment includes injection of fibrin and cells. Existing use in myocardial cells, in vitro stem cells and other heart-related cell culture substrate including collagen, laminin, SUREC0AT (mixture CELUTR0N, collagen and laminin) and gelatin.

[0009] currently prevent secondary heart failure, myocardial infarction work has focused on cell transplantation to replace the necrotic myocardial cells and prevent negative left ventricular remodeling, and the heart tissue regeneration. However, the absence of a suitable substrate, in vitro and in vivo growth rate of cardiomyocytes low survival rate. Improved exist for cardiac repair, heart disorders and compositions for the treatment of myocardial cell culture needs. Likewise, there is a need for an improved skeletal muscle repair, regeneration and cell culture composition.

[0014] 一些实例中，该组合物还含有外源的治疗性细胞。这些细胞可以是干细胞或其它 心肌细胞的前体或其它心脏相关的细胞。

[0015] 一些实施例中，该组合物还含有治疗制剂，而其本身设置为给药载体。一些实施例 中，该组合物被设置成无损的传导阻滞来治疗例如心律不齐。一些实施例中，该组合物设置 成涂覆例如组织培养板或支架的表面，以培养心肌细胞或其它心脏修复相关的细胞类型。

[0016] 一方面，本发明提供一种制备含有脱细胞化心脏细胞外基质的组合物的方法，其 包括：获取具有细胞外基质组分和非细胞外基质组分的心脏组织样品；处理该心脏组织样 品以去除非细胞外基质组分，从而得到脱细胞化心脏细胞外基质，包括细胞外蛋白和多糖； 并对脱细胞化心脏外基质进行灭菌。一些实例中，上述方法还包括冻干和碾碎脱细胞化心 脏细胞外基质的步骤。一些实例中，上述方法还包括对脱细胞化心脏细胞外基质进行酶处 理、溶解或悬浮的步骤。一些实例中，上述脱细胞化心脏外基质在低PH中以胃蛋白酶进行 消化。

[0017] 一些实例中，上述方法还包括在溶液中悬浮和中和上述脱细胞化心脏细胞外基质 的步骤。一些实例中，上述溶液为可通过高规格针注射到心肌层中的磷酸缓冲溶液（PBS) 或盐溶液。一些实例中，上述组合物在体温形成凝胶。一些实例中，上述组合物还含有可在 凝胶之前、之中或之后输送到组合物中或附着其上的细胞、药物、蛋白或其它治疗制剂。

[0018] 一些实例中，上述溶液被置于组织培养板或孔内，在35°C以上或约37°C孵育，从 而形成用于细胞培养的凝胶。一方面，本发明提供一种在吸收基质上培养细胞的方法，其步 骤包括：在组织培养装置内提供含有心脏组织来源的脱细胞化细胞外基质的溶液；孵育上 述组织培养板装置；去除上述溶液；并在吸收基质上培养细胞。一些实例中，上述细胞为心 肌细胞或其它心脏修复相关的细胞。

[0019] 一方面，本发明提供用于受治疗者内心脏修复的治疗方法，其包括将治疗有效量 的心脏组织来源的含有脱细胞化细胞外基质的组合物注射或植入到有需要的受治疗者中。

[0020] 另一方面，文中的组合物含有骨骼肌组织来源的脱细胞化细胞外基质。该组合物 可以是可注射的。该组合物在室温中为液态，在高于室温的温度中为凝胶形式。一些实例 中，该组合物被设置为在心肌梗塞后注射到梗塞壁中。一些实例中，该组合物被设置为通过 27规格或更小的针输送到组织。

Summary of the Invention

[0010] In one aspect, the present invention provides a composition, decellularized extracellular matrix comprising cardiac tissue sources. In some instances, the myocardial tissue of the heart tissue, while in other instances in the heart tissue of pericardial tissue. The composition may be injectable. The composition may be at room temperature, a liquid form is typically formed in 20 degrees c to 25 degrees C, and can be formed at a high gel form at room temperature or above a temperature of 35 degrees centigrade

[0011] some examples, the above-described heart tissue is selected from human heart, primate heart, pig heart, bovine heart, or any other mammal or animal heart, including but not limited to, goat heart, mouse heart, large rat heart, rabbit heart and chicken heart.

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[0012] In some examples, the composition is set in post-myocardial infarction injected into the wall. Some embodiments, the composition is set to delivery to tissue through a small gauge needle (small gauge needle) (such as 27 gauge (gauge) or less). Some examples, the above composition is suitable for implantation into a patient.

[0013] In some examples, the composition contains naturally occurring cells were recruited to the composition of chemokines, growth factors and stimulating factors. Some examples, the composition contains natural meglumine glycans. Some instances, the composition further comprises a non-naturally occurring cells were recruited to the composition factor.

[0014] some examples, the composition further comprises a therapeutic extracellular sources. These cells may be a precursor stem cells or other cardiac myocytes or other heart-related cells.

[0015] In some embodiments, the composition further comprises therapeutic agent, which itself is set to be administered the carrier. Some embodiments, the composition is arranged to lossless conduction block to treat, for example arrhythmias. Some embodiments, the composition is arranged such as tissue culture plates coated stent surface or to cultured myocardial cells or other cell types associated cardiac repair.

RegenX Science Exhibit 1004
Page 977

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0977

[0021] 一些实施方式中，文中含有骨骼肌组织来源的脱细胞化细胞外基质的组合物，其 保留天然葡胺聚糖。一些实例中，该组合物含有天然发生的将细胞募集到该组合物中的因 子。一些实例中，该组合物含有非天然发生的将细胞募集到该组合物中的因子。一些实例 中，该组合物被设置为涂覆组织培养表面或支架以培养骨骼肌修复相关的细胞。

[0022] 一方面，文中公开了一种制备含有脱细胞化骨骼肌细胞外基质的组合物的方法， 其包括：从受治疗者获取具有细胞外基质和非细胞外基质组分的骨骼肌组织样品；处理骨 6 CN 骼肌组织样品以去除非细胞外基质组分，从而得到脱细胞化骨骼肌细胞外基质、细胞外蛋 白和多糖；对脱细胞化骨骼肌细胞外基质进行灭菌。一些实例中，上述方法还包括将骨骼肌 细胞外基质冻干和碾碎的步骤。一些实例中，上述方法还包括对脱细胞化骨骼肌细胞外基质进行酶处理、溶解或悬浮。一些实例中，该脱细胞化骨骼肌细胞外基质在低PH中以胃蛋 白酶进行消化。一些实例中，上述方法还包括在溶液中悬浮和中和或改变上述脱细胞化心 脏细胞外基质的PH的步骤。一些实例中，上述溶液为PBS、盐水或其它缓冲溶液，设置为通 过小直径针注射到心肌层。该溶液可在体温中形成凝胶。该溶液可以还含有可输送到内部 并在凝胶作用之前、之中或之后输送附着于上述材料的细胞、药物、蛋白质或多糖。一些实 例中，溶液被置于组织培养板或孔内，在37°C或高于室温的温度中孵育，以形成用于细胞培 养的凝胶。

[0023] 一方面，本发明提供一种在吸收基质上培养细胞的方法，其包括下述步骤：在组织 培养装置内含有骨骼肌组织来源的脱细胞化细胞外基质的溶液；孵育上述组织培养板装 置；去除上述溶液；并在吸收基质上培养细胞。一些实例中，上述细胞为骨骼肌成肌细胞、 干细胞或其它与骨骼肌修复相关的细胞类型。

[0024] 一方面，本发明提供一种用于受治疗者的骨骼肌修复的治疗方法，其包括植入含 有骨骼肌组织来源的脱细胞化细胞外基质的组合物。

附图说明 [0025] 图1图示由输送本发明组合物的方法（上）得到的示例性心脏或由标准治疗方法(下）得到的示例性心脏。

[0026] 图2图示生长在心脏ECM上的人类胚胎干细胞来源的心肌细胞的平均肌纤维区 域。

[0027] 图3图示生长在心肌ECM上的每肌纤维区域的人类胚胎干细胞来源的心肌细胞核 的平均数量。

[0016] In one aspect, the present invention provides a method comprising decellularized cardiac extracellular matrix composition was prepared, comprising: obtaining cardiac tissue sample having an extracellular matrix component and non-extracellular matrix component; processing the cardiac tissue sample to remove non-extracellular matrix component to obtain decellularized cardiac extracellular matrix, including extracellular proteins and polysaccharides; and decellularized cardiac extracellular matrix is sterilized. Some examples, said method further comprising the step of lyophilization and crushed decellularized cardiac extracellular matrix. Some examples, the above method further comprises the step of decellularized cardiac extracellular matrix enzyme treatment, dissolved or suspended. Some examples, the above-described decellularized cardiac extracellular matrix in low PH to pepsin digestion.

[0017] some examples, said method further comprising the step suspended in the solution and neutralizing said decellularized cardiac extracellular matrix. Some examples, the solution to be injected into the myocardium in a phosphate buffer solution (PBS) by high-gauge needle or a salt solution. Some examples, the above composition forms a gel at body temperature. Some examples, the above-described composition further contains a gel may be performed before, during, or after delivery to the composition or its cell attachment, drug, protein or other therapeutic agents on.

[0018] some instances, the solution is placed in tissue culture plates or wells, about 37 degrees centigrade incubation at 35 degrees centigrade or above, a gel for cell culture formed thereby. In one aspect, the present invention provides a method of culturing cells in the absorbent substrate, comprising the steps of: providing the tissue decellularized cardiac extracellular matrix in a solution containing the cultured tissue source means; incubating said tissue culture plate means; removing said solution; and culturing the cells in the absorption matrix. Some examples, these cells of the heart muscle cells or other cells associated cardiac repair.

[0019] In one aspect, the present invention provides a method of treatment by therapy for cardiac repair, which comprises administering a

[0028] 图4图示心肌ECM上的平均桥粒斑（desmosome plaque)尺寸。

[0029] 图5图示培养于骨骼肌基质上的骨骼肌成肌细胞。

[0030] 图6图示骨骼肌成肌细胞特异性地向骨骼肌基质迁移。

具体实施方式 [0031] 在某些优选实施方式中，本发明提供一种脱细胞化心脏细胞外基质（ECM)组合物，其可用于例如，在心肌梗塞后输送治疗制剂，包括细胞，到心壁中。本发明的ECM可以来 源于哺乳动物心脏组织原生或天然的基质。本文所述的组合物包括可用于注射到需要治疗 处理的心脏组织的心脏细胞外基质。ECM还可用于将细胞募集进受损组织或用作给药载体。

组合物还可用于支持受损细胞或改变机械性能。本发明的另一个用处是作为无损的传导阻 断来治疗例如心律不齐。一些实例中，文中所述的心脏或心脏细胞外基质来源于心肌组织。

另一些实例中，文中所述的心脏或心脏细胞外基质来源于心包组织。

[0032] 文中所述的含有脱细胞化心脏ECM的组合物可以帮助缺陷或缺失的心肌再生并 恢复心脏功能。该ECM组合物可以从动物或合成来源获得。文中的细胞外基质组合物可 以还含有一种或多种添加组分，例如但不限于：外源细胞、肽、多肽或蛋白质、表达生物活性 7 CN 分子的DNA的载体和其它治疗制剂如药物、细胞生长因子、养分、抗生素或其它生物活性分 子。因此，在某些优选的实施方式中，该ECM可以还含有外源的细胞群例如下文所述的心肌 细胞前体。

[0033] 一些实例中描述了输送方法，使组合物接触缺陷的、病态的或缺失的心肌，使心肌 组织再生并使心肌恢复收缩性、传导性或健康功能。一些实例中，该组合物可以在受体内募 集内源细胞，并可协调新募集或添加的细胞的功能，允许细胞在组合物内增殖或迁移。

[0034] 目前，阻止心肌梗塞（MI)后心力衰竭的研究集中于细胞移植，以取代受体内坏死 的心肌，阻止负性左心室重构，和再生心脏组织。已探究多种细胞类型作为细胞移植治疗， 包括心肌细胞、成肌细胞、间叶细胞和胚胎干细胞。不幸的是，由于没有合适的基质，体内细 胞存活量低。本领域中已用于尝试在注射时帮助细胞停留和存活的一些天然来源的基质包 括纤维素、胶原、基质胶、藻酸盐和明

therapeutically effective amount of a cardiac tissue-derived composition comprising decellularized extracellular matrix of injection or implantation into a subject in need of treatment.

[0020] On the other hand, the text of the composition containing the skeletal muscle tissue-derived decellularized extracellular matrix. The composition may be injectable. The composition is liquid at room temperature, at temperatures above room temperature in a gel form. Some examples, the composition is set in post-myocardial infarction injected into the wall. Some examples, the composition is delivered to the set by a 27 gauge or smaller needle tissue.

[0021] In some embodiments, the text contained in skeletal muscle tissue-derived decellularized extracellular matrix composition, which retain the natural meglumine glycans. Some examples, the composition contains naturally occurring cells were recruited to the composition of factors. Some examples, the composition comprises a non-naturally occurring cells were recruited to the composition factor. Some examples, the composition is provided as a coated stent culture surface or skeletal muscle tissue repair related to culture cells.

[0022] In one aspect, a method disclosed herein is prepared containing the removal of skeletal muscle cells extracellular matrix composition, comprising: obtaining from a subject having an outer extracellular matrix and acellular matrix component skeletal muscle tissue samples; handle iliac bone 6 CN muscle tissue sample to remove non-extracellular matrix components, resulting in decellularized skeletal muscle extracellular matrix, extracellular proteins and polysaccharides; skeletal muscle cells of decellularized extracellular matrix off bacteria. Some examples, said method further comprising the skeletal muscle extracellular matrix is freeze-dried and crushed step. Some examples, the above method further comprising decellularized extracellular matrix in skeletal muscle cytoplasmic enzyme treatment, dissolved or suspended. Some examples, the decellularized extracellular matrix in skeletal muscle in the low PH to pepsin digestion. Some examples, said method further comprising the steps in the solution or suspension and neutralizing said decellularized alter cardiac extracellular matrix of PH. Some examples, the solution is PBS, saline or other buffer solutions, is

RegenX Science Exhibit 1004
Page 979

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0979

胶。但是，这些材料均不能适当地模拟特别是发现于心 脏细胞外基质中的天然组分。

[0035] 现有的治疗心脏后ffl (heart post-MI)的可注射支架不能提供细胞生长所需的细 胞外基质所有的需求组分。因此，在这些支架中细胞存活受到限制。在某些实施方式中，本 发明提供一种天然心脏ECM脱细胞化和凝胶化方法以制造用于细胞移植的原位支架。文中 提供了可以产生纳米纤维凝胶的恰当的消化和制备方法。该凝胶溶液能够注射到心肌或梗 塞中，从而显示了其作为原位凝胶支架的可能性。由于脱细胞化心脏ECM最佳地模拟自然 的心脏环境，它使注射时心肌梗塞部位的细胞存活量和停留量提高，从而促进心肌组织再 生。

[0036] 图1图示了文中的组合物的示例性输送方法。左边表示健康心脏。在图中部表示 心肌梗塞后的心脏，如右侧底部示意图所示，没有现有的标准治疗，例如仅使用现成可用的 药物和医用装置，能够有效避免心肌细胞死亡、负性LV重构、LV肥大和心力衰竭。本发明 通过输送文中所述的可注射的组合物改善了这个问题。在右侧上部示意图表示向LV输送 文中的组合物而使重建增强、梗塞尺寸减小、LV重构减弱和心脏功能改善的心脏。

[0037] 本发明特征在于脱细胞化心脏外基质以及生产和使用该基质的方法。特别地，本 发明设计一种生物相容性的组合物，其含有从心脏组织直接获得的脱细胞化心脏细胞外基 质，用于通过注射或植入生物相容性的含有脱细胞化心脏细胞外基质的组合物到受治疗者 中而治疗受治疗者的缺陷、病态、受损或缺血的组织或器官，优选人类心脏。本发明的其它 实施方式涉及脱细胞化骨骼肌、细胞外基质组合物、使用方法和生产方法。

[0038] 一些实例中，脱细胞化心脏细胞外基质来源于选自人类、猪、牛、羊、小鼠、大鼠、鸡 或任意其它的哺乳或动物心脏的天然心脏组织。一些实施方式中，含有脱细胞化心脏细胞 外基质的生物相容性组合物为可注射的凝胶或溶液形式，并可通过在心肌梗塞后移植或输 送其含有的细胞到梗塞壁中或将患者自体的细胞募集进受损心脏组织而用于心脏修复。在 其它实例中，含有脱细胞化心脏ECM的生物相容性材料为，例如碎片（patch)、乳状液、粘性 液体、片段（fragment)、颗粒、微珠或纳米珠。

[0039] 一些实例中，本发明提供生物相容性的材料用于在研究实验室中或在临床环境中 移植之前培养心肌细胞或其它心脏相关细胞，以及用于心脏修复。还提供了制造和以脱细 胞化心脏细胞外基质涂覆例如组织培养板或孔的表面的方法。本发明的生

set to a small diameter needle through the injection into the myocardium. The solution may be in the form a gel at body temperature. The solution may also be delivered to contain internal gelation and before, during, or after delivery to the cell attachment, drugs, proteins or polysaccharides of the above materials. Some instances, the solution is placed in tissue culture plates or wells and incubated at 37 degrees centigrade or above room temperature to form a gel for cell culture.

[0023] In one aspect, the present invention provides a method of culturing cells in absorbing substrate, comprising the steps of: tissue decellularized extracellular matrix solution containing the source of the apparatus skeletal muscle tissue culture; incubate said tissue culture plate means; removing the solution; and culturing the cells in the absorbent matrix. Some instances, these cells to skeletal muscle cells, stem cells or other skeletal muscle repair and related cell types.

[0024] In one aspect, the present invention provides a source of skeletal muscle tissue containing decellularized extracellular matrix composition for the treatment of skeletal muscle repair healer of a subject comprising implanted.

BRIEF DESCRIPTION

[0025] Figure 1 illustrates the composition of the present invention by the conveying method (a) obtained in an exemplary heart or by a standard method of treatment (lower) obtained in an exemplary heart.

[0026] Figure 2 illustrates the growth of average muscle fiber area in the heart of the ECM of human embryonic stem cell-derived cardiomyocytes.

[0027] The average number of Figure 3 illustrates the growth of human embryonic cardiac ECM on every muscle fiber area of stem cell-derived cardiomyocytes nucleus.

[0028] The average desmoplakin (desmosome plaque) Figure 4 illustrates the size of the myocardial ECM.

[0029] Figure 5 illustrates the culture substrate in skeletal muscle skeletal myoblasts.

RegenX Science Exhibit 1004
Page 980

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0980

物相容性材料还 适用于植入患者，无论是人类还是动物。

8 CN [0040] 本发明还提供高生产含有本发明的脱细胞化心脏细胞外基质的生物相容性材料。

这样的方法包括下列步骤：(a)获取具有细胞外基质组分和非细胞外基质组分的心脏组织 样品；(b)处理心脏组织细胞以去除至少一部分或大致所有的非细胞外基质组分，从而得 到脱细胞化心脏细胞外基质；并（C)对脱细胞化心脏细胞外基质进行灭菌。某些实施方式 中，心脏组织样品从哺乳动物如非灵长类（如牛、猪、马、猫、狗、大鼠等等）或灵长类（如猴 和人类），或鸟类来源（如鸡、鸭等等）分离得到。心脏组织样品的脱细胞过程使用本领域和文中所述的一种或多种物理、化学和/或生物技术进行。

[0041] 对于人类治疗，已有许多用于心脏细胞外基质材料的潜在来源：人类心脏（包括 自体同源的、同种异体的或尸体的）、猪心脏、牛心脏、羊心脏、大鼠心脏、小鼠心脏、兔心脏、 鸡心脏和其它动物来源。与全心脏移植不同，一个供体心脏可用于治疗许多人。非人类动 物是一类不要求人类供体的心脏细胞外基质的来源。非人类动物来源可以用作研究试剂。

[0042] 一些实施方式中，处理心脏细胞外基质的方法如下所述。首先对心脏组织脱细胞 化，只保留细胞外基质。例如，脱细胞化可通过十二烷酸硫酸钠和磷酸缓冲溶液灌注法进 行。然后冻干心脏细胞外基质，碾碎，并在低pH、约在pHl～6或pHl～4之间以胃蛋白酶、 或以其它基质降解酶如基质金属蛋白酶进行消化。

[0043] 为了生产凝胶状的心脏细胞外基质用于体内治疗，接着以PBS/盐中和含有心脏 细胞外基质的溶液，并调至所需温度、浓度和粘度。某些实施方式中，ECM的浓度可为1～ 20mg/ml，或2～ 8mg/ml。然后含有心脏细胞外基质的溶液可通过高规格针，如27规格或更 高规格，注射进心肌。在体温中，如36.8°C 士0.7°C，该溶液形成凝胶。细胞、药物、蛋白或 其它治疗制剂也可被输送到心脏ECM凝胶中。

[0044] 为了生产凝胶状的心脏细胞外基质用于体外用途，以PBS/盐中和含有心脏细胞 外基质的溶液并调至所需浓度。一些实施方式中，ECM浓度可为1～20mg/ml，或2～8mg/ ml。接着，该溶液可被放置于任何固体表面上从而进入组织培养板/孔。一旦置于37°C或 室温以上的培养箱，该溶液形成可用于细胞培养的凝胶。

[0030] Figure 6 illustrates skeletal myoblasts into skeletal muscle-specific migration matrix.

DETAILED DESCRIPTION

[0031] In certain preferred embodiments, the present invention provides a decellularized cardiac extracellular matrix (ECM) composition, which can be used, for example, in the delivery of therapeutic agents after myocardial infarction, including cell to the heart wall. ECM of the present invention may be derived from mammalian heart tissue native or natural matrix. The compositions described herein can be used for injection into the heart comprising extracellular matrix requires therapeutic treatment of cardiac tissue. ECM also be used to cell recruitment into the damaged tissue or as administration support. compositions may also be used to support damaged cells or alter mechanical properties. Another use of the present invention is a lossless conduction blocking to treat conditions such as arrhythmias. Some examples, the text of the heart or cardiac extracellular matrix derived from cardiac tissue. other examples, the text outside the heart or cardiac extracellular matrix derived from pericardial tissue.

[0032] containing the decellularized heart ECM compositions described herein can help defects or missing and restore cardiac function in myocardial regeneration. The ECM composition can be obtained from animal or synthetic origin. The text of the extracellular matrix composition may also contain one or more additional components, such as, but not limited to: foreign cells, peptide, polypeptide, or protein, DNA expression vectors 7 CN bioactive molecules such as drugs and other therapeutic agents, cell growth factors, nutrients, antibiotics, or other bioactive molecules. Thus, in certain preferred embodiments, the ECM may also contain a cell population such as myocardial cell precursors exogenous described below.

[0033] Some examples are described in the delivery method, the composition contact defects, morbid or missing myocardium, myocardial tissue regeneration and recovery of myocardial contractility, conductivity or health function. Some examples, the composition may be in the body Raised by endogenous cells and can coordinate to raise or add new functional cells, allowing cell proliferation or migration within the composition.

[0034] At present, to prevent myocardial infarction (MI) after failure focused on cell transplantation to

RegenX Science Exhibit 1004
Page 981

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0981

[0045] 本发明还提供一种用于受治疗者的心脏修复的治疗方法，其包括注射或植入部分 或整体本发明的生物相容性心脏细胞外基质到患者中。本发明还提供一种用于治疗受治疗 者的心律不齐或其它缺陷、病态、受损或缺血的组织或器官的治疗方法，包括原位注射或植 入本发明的生物相容性材料。

[0046] 文中的组合物可含有心脏组织来源的脱细胞化ECM和另一种或多种组分。一些实 例中，全部组合物中ECM的量以重量计高于组合物的90 %或95 %或99 %。一些实施方式中， 全部组合物中的ECM以重量计高于组合物的1%、5%、10%、20%、30%、40%、50%、60%、 70%或 80%。

[0047] 这样制备脱细胞化细胞外基质以保持大部分用于心肌组织再生的生物活性。文中 组合物示例性的生物活性包括但不限于：控制或启动细胞粘附、细胞迁移、细胞分化、细胞 成熟、细胞组织化（cell organization)、细胞增殖、细胞死亡（凋亡）、血管生成刺激、蛋白 水解活性、酶活性、细胞运动、蛋白和细胞调节、转录激活、供应转录、一些生物活性的抑制 例如抑制凝结、干细胞诱导（attraction)、趋化性以及MMP或其它酶活性。

[0048] 该组合物含有实质上脱细胞化细胞外基质。一些实例中，脱细胞化基质不含有可 使ECM自然形成的非活天然细胞。一些实例中，实质上脱细胞化基质含有以重量计少于 9 CN 1%、2%、3%、4%、5%、6%、7%、8%、9%或 10% 的天然细胞。

[0049] 如文中所述，组合物可含有脱细胞化心脏ECM和不同组织脱细胞化ECM或合成 或天然形成的聚合物。文中示例性聚合物包括但不限于：聚对苯二甲酸二乙醇酯纤维 (DACRON)、聚四氟乙烯（PTFE)、戊二醛交联的心包膜、聚乳酸（PLA)、聚乙二醇（PGA)、透 明质酸（HA)、聚乙二醇（PEG)、聚乙烯、镍钛诺（nitinol)和来自动物和非动物来源的胶 原（如植物或合成胶原）。一些实例中，组合物的聚合物为生物相容性的和生物可降解的 和/或生物可吸收的。示例性的生物降解或生物可吸收的聚合物包括但不限于：聚丙交酯 (polylactide)、聚乙交酯、聚己内酯、聚二噁烷和它们的无规和嵌段共聚物。生物可降解和 /或生物可吸收的聚合物可包括选自乙交酯、丙交酯（lactide)、二噁烷、己内酯、三亚甲基 碳酸酯、乙二醇和赖氨酸的单体。

[0050] 聚合物材料可以是无规共聚物、嵌段共聚物或单体混合物、含有这些单体的均聚 物、共聚物和/或杂聚物。生物可降解和/或生物可吸收的聚合物可包含生物可吸收和 生物可降解的线性脂肪族聚

replace myocardial necrosis by the body, preventing negative left ventricular remodeling, and regeneration of heart tissue. We have explored a variety of cell types for cell transplantation therapy, including myocardial cells, myoblasts, mesenchymal cells and embryonic stem cells. Unfortunately, the absence of a suitable substrate, a low amount of in vivo cell survival. The art has been used to try to inject some of residence helps cells survive natural sources and a matrix comprising cellulose, collagen, Matrigel, alginate and gelatin. However, these materials are not properly simulate especially found in cardiac extracellular matrix natural ingredients.

[0035] Existing treatments after heart ffl (heart post-MI) can not provide an injectable scaffold needed for cell growth extracellular matrix components all needs. Thus, these scaffolds in cell survival are limited. In certain embodiments, the present invention provides a natural decellularized cardiac ECM and methods to produce in situ gelling scaffold for cell transplantation. This paper provides the proper digestion and preparation methods can produce nanofiber gel. The gel solution can be injected into the myocardium or infarction, thus showing its potential as in situ gel scaffold. Since the decellularized heart ECM best simulate the natural environment of the heart, it makes the amount of viable cells in myocardial infarction injection sites and increase the amount of travel, thereby promoting myocardial tissue regeneration.

[0036] FIG. 1 illustrates an exemplary text delivery method composition. Left shows a healthy heart. In the middle of the figure shows the heart after myocardial infarction, as shown in the bottom right side of the schematic, there is no existing standard therapy, e.g., using only readily available drugs and medical devices, can effectively prevent myocardial cell death, negative remodeling LV, LV hypertrophy and heart failure. The invention described herein conveyed injectable composition to improve this problem. In the upper right side of the schematic representation LV leaving reconstruction to convey the text composition enhancements reduce infarct size, LV remodeling weakened heart and heart function improved.

Characteristics

RegenX Science Exhibit 1004
Page 982

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0982

酯如聚乙交酯（PGA)和其无规共聚物聚（乙交酯-丙交酯）(PGA-CO-PLA)。其它适合的生物相容性聚合物的实例为包括甲基丙烯酸乙酯的聚羟基烷基甲基丙烯酸酯和如聚乙烯吡咯烷酮和聚丙烯酰胺的水凝胶。其它合适的生物可吸收性材料 为生物聚合物，其包括胶原、明胶、海藻酸、壳多糖、壳聚糖、纤维蛋白、透明质酸、葡聚糖、聚 氨基酸、聚赖氨酸和这些材料的共聚物。根据本发明考虑了以上实例的任意组合、共聚物、 聚合物或其混合物以供使用。这些生物可吸收材料可根据已知方法制备。

[0051] 因此，文中描述了制备含有心肌组织来源的脱细胞化ECM的组合物的方法。本发 明还提供了以相似的工序得到的骨骼肌组织来源的ECM组合物和方法。还提供了相关组合 物、生产装置和方法。

[0052] 一些实施方式中，当在室温以上包括生理温度接近37°C受热时，组合物的粘性增 加。根据一个非限制性的实施方式，ECM来源的组合物在室温和35°C以下的其它温度下为 可注射溶液。在另一个非限制性的实施方式中，该凝胶可在体温以上约37°C或接近体温时 注射，但在升高的温度中凝胶化更迅速。凝胶在37°C生理温度中约15～20分钟后形成。提 供了制备ECM来源的凝胶的原理的总体设定以及以下实例中的制备凝胶的优选特定方案， 实施例可应用于且适用于多种组织包括但不限于心脏和骨骼肌。

[0053] 可含有细胞或其它治疗制剂的组合物可通过许多方法植入到患者、人类或动物 中。一些实例中，该组合物以液体注射到患者需要的部位。

[0054] 市售的ECM制剂也可结合到文中所述的方法、装置和组合物中。其中一个实施方 式中，ECM从小肠粘膜下层（SIS)获得。市售制剂包括但不限于SURGISIS™、SURGISIS-ES™、STRATASIS™ 和 STRATASIS_ES™(Cook Urological Inc. ； Indianapolis, Ind.)及GRAFTPATCH™(Organogenesis Inc. ； Canton，Mass.)。另一个实例中，ECM 从真皮获得。市 售制剂包括但不限于PELVIC0L™(为在欧洲以PERMAC0L™销售；Bard，Covington, GA.)、REPLIF0RM™(Microvasive ； Boston，Mass.)和ALL0DERM™(LifeCell ； Branchburg, N. J.)o

[0055] 一些实例中，本发明的心脏细胞外基质的溶液、凝胶形式和吸收形式提供与体内 发现的呈相近比例的所有组分。对于心律不齐治疗，可输送本发明的细胞外基质，其允许解 决心律不齐后的心脏组织再生。对于体外心肌细胞和其它心脏相关细胞的细胞培养，与通 常使用的胶原、层粘连蛋白、SURECOAT(CELLUTR0N，胶原和层粘连蛋白的混

[0037] The present invention resides in the heart of decellularized extracellular matrix as well as methods for producing and using the matrix. In particular, the compositions of the present invention is a biocompatible design, comprising decellularized cardiac extracellular matrix is available directly from the cardiac tissue, by injection or implantation for biocompatibility comprising decellularized cardiac cells The composition of the extracellular matrix to the subject rather treating a defect, morbid, damaged or ischemic tissue or organ, preferably a human heart. Other embodiments of the present invention relates to a decellularized skeletal muscle, extracellular matrix composition, usage, and production methods.

[0038] some instances, decellularized cardiac extracellular matrix is selected from humans, pigs, cattle, sheep, mouse, rat, chicken, or any other mammal or animal tissue heart of the natural heart. Some embodiments, contain decellularized cardiac extracellular matrix composition is biocompatible gel or injectable solution form, and through transplantation after myocardial infarction, or which contains cells transported into the infarct wall or Patients with autologous cell recruitment into the damaged heart tissue for cardiac repair. In other examples, a biocompatible material comprising decellularized cardiac ECM as, e.g., fragments (patch), emulsion, viscous liquid, fragment (fragment), particles, microbeads or nanobeads.

[0039] some instances, provide a biocompatible material of the present invention is used in a research laboratory or in a clinical setting before transplantation of cultured cardiomyocytes or other heart-related cells, as well as for cardiac repair. Also provided are methods of manufacturing and to de-cells of the cardiac extracellular matrix coating such as tissue culture plate or bore surface. Biocompatible material of the present invention is also applicable to implant patient, whether human or animals.

8 CN

[0040] The present invention also provides a high production decellularized cardiac extracellular matrix containing biocompatible material of the present invention.

Such a method comprises the steps of: (a) obtain the cardiac tissue sample having an extracellular

合物）和明胶， 10 CN 本发明的心脏细胞外基质的凝胶和吸收形式含有所有或许多细胞体内识别的相同的细胞 外基质信号。

[0056] 文中的组合物提供心脏细胞外基质的凝胶或溶液形式，以及心脏细胞外基质的这 些形式用于例如如心脏修复、心律不齐治疗和细胞培养的使用。一个实施方式中，首先将心 脏组织脱细胞化，只保留细胞外基质。然后将基质冻干、碾碎或研磨成细粉，并以胃蛋白酶 或其它酶、例如但不限于基质金属蛋白酶、胶原酶和胰蛋白酶使之溶解。

[0057] 对于凝胶治疗，接着使用PBS/盐将溶液中和并调至适合的浓度。一个实施方式 中，该溶液可通过针注射到心肌层（或通过导管经由肋骨，或在开胸过程中）。针的规格可 以是但不限于22g、23g、24g、25g、26g、27g、28g、29g、30g或更小。一个实施方式中，注射溶 液所用的针的规格为27g。也可通过气囊导管或其它无针导管进行输送。可基于伤损组织 和患者个人的不同情况而常规地决定剂量和频率。体温中，该溶液可接着形成凝胶。在又 一实施方式中，凝胶可与戊二醛、甲醛、双-NHS分子或其它交联剂交联。

[0058] 在又一实施方式中，ECM可与其它治疗制剂，如细胞、肽、蛋白质、药物、养分、抗生 素、促存活添加剂、蛋白多糖和/或糖胺聚糖结合。在又一个实施方式中，ECM可与合成的 聚合物结合和/或交联。合成聚合物的实例包括但不限于：聚对苯二甲酸乙二醇酯纤维 (DACR0N™)、聚四氟乙烯（PTFE)、聚乳酸（PLA)、聚乙二醇（PGA)、聚乙二醇（PEG)、聚乙二醇 二丙烯酸酯（P' EGDA)、聚乙烯、聚苯乙烯和镍钛诺（nitinol)。

[0059] 在又一实施方式中，可将ECM溶液或凝胶单独或与上述组分组合而注射到梗塞部 位、边缘区或心肌层，以使内源细胞向内生长、血管生成和再生。在又一实施方式中，组合物 可单独或与上述组分组合而使用，作为基质，以改变心脏的机械性能和/或阻止负性左心 室重构。在又一实施方式中，该组合物可单独或与上述组分组合，与细胞输送，用于再生心 肌层。在又一实施方式中，该组合物可单独或与上述组分组合而使用，以产生传导阻断从而 治疗心律不齐。

[0060] 在一制造可溶试剂的实施方式中，在包括但不限于0.5M、0. IM或0. OIM的乙酸或 0. IM HCl的低pH溶液中将溶液调至所需浓度，然后置于组织培养板/孔、盖玻片、支架或其 它用于组织培养的表面。在培养箱中37°C放置1小时后，或在室温中过夜后，去除过量溶 液。以PBS漂洗表面后，可在吸收基质上培养细胞。该溶液可在注射/植入之前、之

matrix component and non-extracellular matrix component; (b) processing the cardiac tissue cells to remove at least a portion or substantially all of the non-extracellular matrix component to obtain decellularized cardiac extracellular matrix; and (C) of decellularized cardiac extracellular matrix is sterilized. Some embodiments, the cardiac tissue sample from a mammal such as non-primate (such as cattle, pigs, horses, cats, dogs, rats, etc.) or primates (such as monkeys and humans), or avian origin (such as chickens, ducks, etc.) was isolated. Acellular process of cardiac tissue samples using art and culture as described in one or more physical, chemical and / or biological techniques.

Potential sources

[0041] For human therapy, there are many external cardiac extracellular matrix material used: the human heart (including autologous, allogeneic or body), pig heart, heart of cattle, sheep heart, rat heart, heart mouse, rabbit heart, chicken heart and other animal sources. With full heart transplant is different a donor heart can be used to treat many people. Non-human animal is a class of extracellular matrix of the heart cells do not require human donor sources. Non-human animal sources may be used as research reagents.

[0042] some embodiments, the method for processing cardiac extracellular matrix as described below. First decellularized heart tissue, leaving only the extracellular matrix. For example, the decellularized can be carried out by dodecyl sulphate and phosphoric acid buffer solution perfusion. Cardiac extracellular matrix is then freeze-dried, crushed, and at low pH, about pepsin, or other matrix degrading enzymes such as matrix metalloproteinases digested between pHI~6 or pHI~4.

[0043] In order to produce the outer gelatinous matrix used for in vivo treatment of heart cells, followed by PBS / salt solution containing the heart and the extracellular matrix, and adjusted to the desired temperature, concentration and viscosity. Certain embodiments, the ECM concentration can be 1~ 20mg / ml, or 2~8mg / ml. Then cardiac extracellular matrix containing a solution by high gauge needle, such as a 27 gauge or higher specification, injected into the myocardium. In body temperature, as 36.8 degrees centigrade with disabilities 0.7 degrees C, and the solution to

RegenX Science Exhibit 1004
Page 984

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0984

中或之 后，预先与肽、蛋白质、DNA、药物、养分、促存活添加剂、蛋白多糖和/或糖胺聚糖结合。

[0061] 本发明基于治疗组合物和吸收细胞培养组合物形式的体外心脏细胞外基质，提供 增强的细胞粘附和存活。与标准平板涂层相比，可溶的细胞培养试剂形式的心脏细胞外基 质诱导心肌细胞更迅速地扩散、更快成熟和/或存活改进。

[0062] 之前的研究显示，难以将从心肌细胞获得的人类胚胎干细胞（hESC)用于心肌梗 塞的治疗。一些实例中，有效分化和体内成熟心室心肌细胞的产率阻碍了治疗的有效性。以 前，分化调控主要在体外实行，例如在细胞培养基中添加可溶性因子。该处理过程受胎儿表 型以外的分化难度所限。

[0063] 除了可溶性因子，细胞外基质也可在细胞分化中起重要作用。对于成体细胞，包括 成体祖细胞，已研究了一些含有化学信号的基质，但仅进行了少量关于ECM对ESCs的效果、 特别是对hESCs的效果的研究。许多实例中，由hESC得到的心肌细胞是在含有可溶性因子 和明胶的促存活培养基中输送的。

11 CN [0064] 在文中的一个实施方式中，公开了从天然心脏中获得的仿生基质。一些实例中，基 质与体内心脏环境相似，其含有许多或所有在自然心脏ECM中发现的天然化学信号。一些 实例中，通过交联或添加或其它材料，也可模仿健康成体或胚胎的心肌层的机械性能。如文 中所述，可用简单经济的方法将心脏ECM分离并加工到凝胶中，其适合按比例扩大用于临 床转化。

[0065] 一些实例中，文中提供的组合物可含有基质和外源添加的或募集的细胞。该细胞 可以是任意各种细胞。一些实例中，该细胞为各种心脏或心脏血管细胞，包括但不限于：干 细胞、祖细胞、心肌细胞、血管细胞和自体或同种异体来源的成纤维细胞。

[0066] 本发明由此提供由天然脱细胞化心脏细胞外基质制得的凝胶的使用，以支持分离 的新生心肌细胞或体内的心肌细胞来源的干细胞祖细胞并起作原位凝胶支架的作用，提供 了基质以改进细胞在左心室壁的停留和存活。由于从心脏ECM产生的支架比目前可用的材 料更准确接近于体内环境，其适于在心肌层中用于细胞移植。

[0067] 文中含有心脏ECM和外源添加的细胞的组合物可通过在ECM中培养而制得。此 外，可在细胞外基中添加如生长因子的蛋白质，也可在组合物中添加蛋白质，或蛋白质分子 可与基质中的分子共价

form a gel. Cells, drugs, proteins or other therapeutic agents may be delivered to cardiac ECM gel.

[0044] In order to produce the outer gelatinous matrix of cardiac cells in vitro for use in PBS / salt and a solution containing the extracellular matrix of cardiac cells and adjusted to the desired concentration. Some embodiments, ECM concentration can 1~20mg / ml, or 2~8mg / ml. Subsequently, the solution may be placed on any solid surface to enter the tissue culture plate / aperture. Once placed above 37 degrees centigrade incubator or room temperature, the solution can be used to form a gel cell culture.

[0045] The present invention also provides a method for treating cardiac repair healer subject, comprising injecting or implanting the outer part or the whole of the present invention, the biocompatible matrix cardiac cells into the patient. The present invention further provides a method for treating a cardiac arrhythmia or other defects, pathological, or treating tissue damage or organ ischemia, including in-situ injection or implantation of a biocompatible material of the present invention,.

Decellularized ECM

[0046] text of the composition can contain cardiac tissue origin and another one or more components. Some examples, the amount of the total composition of the ECM, by weight more than 90 percent or 95 percent or 99 percent of the composition. Some embodiments, the ECM in the total composition is higher than 1 percent by weight of the composition, 5 percent, 10 percent, 20 percent, 30 percent, 40 percent, 50 percent, 60 percent, 70 percent or 80 percent.

[0047] Thus prepared decellularized extracellular matrix in order to maintain the biological activity of the majority for cardiac tissue regeneration. Text composition Exemplary biological activities include, but are not limited to: control or initiate cell adhesion, cell migration, cell differentiation, cell maturation, cell organization (cell organization), cell proliferation, cell death (apoptosis), angiogenesis assassination shock, proteolytic activity, enzymatic activity, cell motility, and cell regulatory protein, transcriptional activation, transcriptional supply some inhibit the biological activity of inhibition of coagulation e.g., stem cells (attraction), chemotaxis and MMP or other activity.

RegenX Science Exhibit 1004
Page 985

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0985

或非共价地连接。蛋白质与基质分子的共价连接可通过本领域已知 的标准共价蛋白质连接方法完成。蛋白质可与一种或多种基质分子共价连接。

[0068] 在一个实施方式中，当输送含有脱细胞化心脏ECM和外源细胞的组合物时，这些 细胞可以是用于治疗心肌层的细胞来源，包括同种来源、异种来源、自体来源。因此，可通过 文中的组合物输送胚胎干细胞、胎儿或成体来源的干细胞、诱导多潜能干细胞、心肌细胞祖 细胞、胎儿和新生心肌细胞、成肌纤维细胞、成肌细胞、间叶细胞、实质细胞、上皮细胞、内皮 细胞、间皮细胞、成纤维细胞、造血细胞、骨髓来源祖细胞、骨骼细胞、巨噬细胞、脂肪细胞和 自移植的扩张的心肌细胞。一些实例中，文中的细胞可在体外培养且可在培养皿环境中直 接向心肌细胞或可成为心肌细胞的骨髓细胞分化。然后将培养的细胞与组合物或接触支架 和其它组分而移植到哺乳动物中。

[0069] 成体干细胞也是另一种可成为文中组合物一部分的细胞。据认为成体干细胞通过 在新部位产生其它干细胞（如适于心肌层的细胞）而起作用，或在体内直接分化为心肌细 胞。引导至器官如心脏后它们还可分化成其它类型细胞。成体哺乳动物由循环内皮前体细 胞、骨髓来源细胞、脂肪组织、或特殊器官的细胞为成体干细胞提供来源。已知从骨髓抽取 液分离的单核细胞在体外分化成内皮细胞，并可在肌肉注射后在新形成的血管中检测到。

因此，采用来自骨髓抽取液的细胞可在体内产生内皮细胞作为组合物的组分。其它可用于 本发明的细胞为受激活的细胞因子调控的间叶干细胞。已知间叶细胞的亚群当在体外暴露 于细胞因子时分化为生肌细胞系。

[0070] 人类胚胎干细胞来源的心肌细胞可在文中含有心脏基质的组合物上生长。一些实 例中，存在文中的组合物时生长的hESC来源的心肌细胞表现出与体内更相似的形态学。一 些实例中，存在文中组合物时生长的hESC来源的心肌细胞表现出增加的成熟标记物。

[0071] 本发明还着目于药物输送系统，其包括用于输送细胞、药物、分子或蛋白质到受治 疗者中以治疗缺陷、病态、受损或缺血组织或器官的脱细胞化心脏细胞外基质。一个实施方 式中，单独或与其它组分结合的本发明的含有脱细胞化心脏细胞外基质的生物相容性材料 用作无损的传导阻断以治疗心律不齐。因此，本发明的生物相容性材料可用来移植细胞、或 12 CN 单独注射以募集天然细胞或其它细胞因子内源治疗制剂、或充当外源治疗制剂输送载体。

[0048] The composition contains substantially decellularized extracellular matrix. Some examples, decellularized matrix does not contain a natural form of ECM can make non-living natural cells. Some examples, substantially acellular matrix comprising by weight of less than 9 CN 1 percent, 2 percent, 3 percent, 4 percent, 5 percent, 6 percent, 7 percent, 8 percent, 9 percent or 10 percent of natural cells.

[0049] As described herein, the composition may contain polymer decellularized heart ECM and different organizations decellularized ECM or synthetic or natural form. Wen exemplary polymers include, but are not limited to: polyethylene terephthalate ethanol ester fiber (DACRON), polytetrafluoroethylene (PTFE), glutaraldehyde cross-linked pericardium, polylactic acid (PLA), polyethylene glycol alcohol (PGA), hyaluronic acid (HA), polyethylene glycol (PEG), polyethylene, nitinol (nitinol) and collagen from animal and non-animal origin (such as plants or synthetic collagen). Some examples of the polymer composition is biocompatible and biodegradable and / or bioabsorbable. Exemplary polymers biodegradable or bioabsorbable include, but are not limited to: polylactide (polylactide), polyglycolide, polycaprolactone, poly-dioxane and their random and block copolymers. Biodegradable and / or bioabsorbable polymer may include those selected from glycolide, lactide (lactide), dioxane, caprolactone, trimethylene carbonate, ethylene glycol and lysine monomers.

[0050] polymeric material may be a random copolymer, a block copolymer or a monomer mixture containing these monomers are homopolymers, copolymers and / or heteropolymers. Biodegradable and / or bioabsorbable polymer may contain bioabsorbable and biodegradable linear aliphatic polyesters such as polyglycolide (PGA) and their random copolymers of poly (glycolide - lactide) (PGA-CO-PLA). Examples of other suitable biocompatible polymers include poly hydroxy ethyl methacrylate alkyl methacrylate and as polyvinylpyrrolidone and polyacrylamide hydrogel. Other suitable bioabsorbable material is a biopolymer, which include collagen, gelatin, alginate, chitin, chitosan, fibrin, hyaluronic acid, dextran, poly amino acids, polylysine and these materials The copolymer. Considering any combination of the above examples, copolymers, polymers or mixtures thereof for use according to the present invention.

RegenX Science Exhibit 1004
Page 986

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0986

[0072] 本发明的组合物可还含有细胞、药物、蛋白质或其它生物材料，例如但不限于：促 红细胞生成素（EPO)、干细胞因子（SCF)、血管内皮生长因子（VEGF)、转化生长因子（TGF)、 成纤维细胞生长因子（FGF)、表皮生长因子（EGF)、软骨生长因子（CGF)、神经生长因子 (NGF)、角质细胞生长因子（KGF)、骨骼生长因子（SGF)、造血细胞生长因子（BDGF)、肝细胞 生长因子（HGF)、胰岛素样生长因子（IGF)、细胞因子生长因子（cytokine growth factor, CGF)、干细胞因子（SCG)、血小板源生长因子（PDGF)、内皮细胞生长添加剂（EGGS)、集落刺 激因子（CSF)、生长分化因子（GDF)、整合素调节因子（IMF)、钙调蛋白（CaM)、胸腺嘧啶核苷 激酶（TK)、肿瘤坏死因子（TNF)、生长激素（GH)、骨形态发生蛋白（BMP)、基质金属蛋白酶 (MMP)、基质金属蛋白酶组织抑制因子（TIMP)、干扰素、白介素、细胞因子、整合素、胶原、弹 性蛋白、微纤维蛋白、纤粘蛋白、层粘连蛋白、糖胺聚糖、血结素、血小板反应蛋白、硫酸乙酰 肝素、皮肤素（dermantan)、硫酸软骨素（CS)、透明质酸（HA)、玻连蛋白、蛋白多糖、转铁蛋 白、腱生蛋白（cytotactin)、生腱蛋白(tenascin)和淋巴因子。

[0073] 组织培养板可以用本发明细胞外基质的可溶性配体或凝胶形式、或本发明的细胞 外基质的吸收形式涂覆，以培养心肌细胞或其它与心脏修复相关的细胞。其可用作培养这 些细胞的研究试剂或移植之前用于培养细胞的临床试剂。细胞外基质试剂可与其它组织基 质和细胞组合。

[0074] 对于凝胶试剂组合物，接着使用PBS/盐或其它缓冲液将溶液中和并调至合适的 浓度，然后置于组织培养板和/或孔中。当置于37°C培养箱时，溶液形成可用于细胞培养用 的任何2D或3D培养基材的凝胶。在一个实施方式中，凝胶组合物可与戊二醛、甲醛、双-NHS 分子或其它交联物交联，或与细胞、肽、蛋白质、DNA、药物、养分、促存活添加剂、蛋白多糖和 /或糖胺聚糖结合，或与合成的聚合物结合和/或交联以备将来使用。

[0075] 本发明还提供一种在吸收脱细胞化心脏细胞外基质上培养细胞的示例性方法，其 包括以下步骤：（a)在包括但不限于0. 5M或0. OlM的乙酸或0. lM HCl的低pH溶液中提供 含有脱细胞化ECM的生物相容性材料的溶液，达到所需浓度，(b)将上述溶液置于组织培养 板或孔中，（C)在室温以上温度如37°C中孵育上述培养板或孔1小时并过夜（或在室温到 40°C中），（d)除去过量溶液，(e)以PBS漂洗组织培养板或孔，并（f)在吸收基质上培养细胞。可在含有本发明的心脏细胞外基质的吸收基质

These bioabsorbable materials may be prepared according to known methods.

[0051] Thus, this paper describes the preparation of decellularized ECM composition comprising cardiac tissue source approach. The present invention also provides compositions and methods for ECM in a similar process to get the skeletal muscle tissue sources. Also it provides related compositions, apparatus and methods of production.

[0052] In some embodiments, when at physiological temperature above room temperature include heat approaching 37 degrees C, the viscosity of the composition increases. In accordance with one non-limiting embodiment, the composition of ECM sources at room temperature and other temperatures below 35 degrees centigrade for injectable solutions. In another non-limiting embodiment, the gel may be injected or near the temperature at about 37 degrees centigrade above body temperature, but at elevated temperatures and more rapid gelation. Gel at physiological temperature of 37 degrees centigrade for about 15 ~ 20 minutes after the formation. Provides a preferred source of certain aspects of the preparation of ECM gels general principles set and the following examples of preparation of the gel, the embodiments may be applied and is applicable to a variety of tissues, including but not limited to heart and skeletal muscle.

[0053] The composition may contain cells or other therapeutic agents can be implanted into a patient, human or animal in many ways. Some examples, the composition is a liquid injected into the site of a patient in need.

[0054] ECM commercially available formulations may also be incorporated into the method described herein, devices and compositions. One embodiment in which, ECM small intestinal submucosa (SIS) to obtain. Commercially available formulations include, but are not limited to SURGISIS ◆, SURGISIS-ES ◆, STRATASIS ◆ and STRATASIS_ES ◆ (Cook Urological Inc.;. Indianapolis, Ind) and GRAFTPATCH ◆ (Organogenesis Inc.; Canton, Mass.). Another example, ECM obtained from the dermis. Commercially available formulations include, but are not limited to PELVIC0L ◆ (as in Europe with PERMAC0L ◆ sales;. Bard, Covington, GA),

上培养的细胞包括心肌细胞或与心脏修 复相关的其它细胞类型，包括干细胞和心脏祖细胞。

[0076] 一些实例中，组合物包括交联剂，包括但不限于普通的胶原交联剂、透明质酸交联 剂或其它具有改变的降解与机械性能的蛋白交联剂。

[0077] 一个实例中，制造文中的组合物的方法包括静电纺丝法。一些实例中，设置本文的 方法以控制纳米纤维的尺寸、形状或厚度。

[0078] 一些实例中，可使用例如细胞或体外起搏术向组合物引入收缩性。收缩性可产生 周期应力从而促进更天然的心肌层。

[0079] 一些实例中，当组合物含有交联基团或嵌入因子，如血管生成因子时，可向组合物 引入细胞浸润和血管生成。

[0080] 一些实例中，本文的组合物可含有微珠。微珠可以是组合物的一部分或输送到组 合物中。示例性微珠可以是任意种类的材料，例如天然的或合成的。一些实例中，微珠可具 13 CN 有不同降解性能或含有例如MMP抑制剂、生长因子或小分子。

[0081] 一些实例中，本文的组合物可包括可在输送组合物中作为粘合剂或锚定物的生物 基团 (biological group)。

[0082] 一个实例中，组合物可以是生物粘合剂，例如用于创伤修复。一些实例中，本文的 组合物可设置为细胞粘附剂。例如，本文组合物可在医用装置上涂覆或与含有或不含有细 胞的生物混合。例如，本文组合物可以是合成聚合物血管移植用的涂层。一些实例中，该组 合物含有抗菌剂或抗菌剂包含于其中。

[0083] 文中的方法包括输送组合物作为创伤修复装置。例如心脏损伤（cardiac ablation)后可输送组合物以促进愈合。

[0084] 一个实例中，组合物含有以本文所述的ECM组合物涂覆的藻酸盐珠。

[0085] 一些实例中，该组合物是可注射的。可注射的组合物可以是但不限于粉末、液体、 颗粒、碎片、凝胶或乳液。该可注射的组合物可注射到心脏中，或许多实例中，注射到左心 室、右心室、左心房、右心房或心脏瓣膜。本文的组合物可募集例如但不限于内皮、平滑肌、 心脏祖细胞、成肌纤维细胞、干细胞和心肌细胞。

REPLIF0RM ◆ (Microvasive;. Boston, Mass) and ALL0DERM ◆ (LifeCell; Branchburg, NJ) o

[0055] some instances, the heart of the extracellular matrix of the present invention, a solution, gel form and absorb all the components provided in the form of the body was found in a similar proportion. For the treatment of arrhythmias, it can deliver the extracellular matrix of the present invention, which allows the solution determined arrhythmia after heart tissue regeneration. For cardiomyocytes in vitro cell culture and other heart-related cells and collagen commonly used, laminin, SUREC0AT (mixture CELLUTR0N, collagen and laminin) and gelatin heart of the extracellular matrix of the present invention is a gel 10 CN and absorbed form of extracellular matrix containing the same signal to all or many cells in the body identification.

Gel or solution form

[0056] The compositions provide text cardiac extracellular matrix, and these form the heart of the extracellular matrix, such as for example, cardiac repair, arrhythmia therapy and cell culture. One embodiment, the first cardiac tissue decellularized, leaving only the extracellular matrix. The matrix is then freeze-dried, crushed or ground into a fine powder, and pepsin or other enzymes, such as, but not limited to, matrix metalloproteinases, collagenase and trypsin to dissolve.

[0057] For treatment gel, then use the PBS / salt solution was neutralized and adjusted to the appropriate concentration. In one embodiment, the solution may be injected into the myocardium (or through a conduit through the ribs, or the thoracotomy procedure) through the needle. Needle size may be, but is not limited to 22g, 23g, 24g, 25g, 26g, 27g, 28g, 29g, 30g or less. One embodiment, the injection needle used solution specifications for 27g. It can also be delivered through a balloon catheter or other non-needle catheter. Flaw may be based on different situations and patient organizations and individuals routinely determine the dose and frequency. Temperature, and the solution may then form a gel. In yet one embodiment, the gel with glutaraldehyde, formaldehyde, bis -NHS molecules or other crosslinking agent.

[0086] 制造本文组合物的方法可包括根据领域内我们已知的方法对任意年龄的动物或 人类组织进行脱细胞化。

[0087] 一些实例中，本文的组合物含有ECM和天然或合成的聚合物。例如，本文的组合物 含有天然聚合物如胶原、壳聚糖、藻酸盐、纤维蛋白或透明质酸。另一个实例中，本文的组合 物含有合成的聚合物、例如但不限于聚乙二醇、聚乙醇酸、聚乳酸、聚羟基酸、聚二噁烷酮、 聚己内酯、聚原酸酯、聚酸酐、聚磷腈、聚氨基酸、拟聚氨基酸、导电聚合物（如聚乙炔、聚吡 口各、聚苯胺）或聚氨基甲酸酯或它们可能的共聚物。一些实例中，这里的组合物含有ECM和 天然的以及合成的两类聚合物。文中的组合物可以是通过ECM和另一种聚合物材料连接的 多物料，例如通过与胺类、游离硫醇或短肽反应，与ECM自组装。

[0088] 文中的方法包括含有ECM的组合物的输送。示例性方法包括但不限于：外科手术 中直接注射；通过胸壁直接注射；通过导管穿过心内膜输送到心肌层；通过冠状血管输送； 以及通过灌注气囊导管输送。组合物还可以固态输送，如移植物（graft)、碎片（patch)或 与细胞支架联结。剂量和频率将根据患者需求和内科医生的判断而不同。

[0089] 一些实例中，本文的组合物为涂层。该涂层可含有来自任意组织如心肌、骨骼肌、 心包膜、肝、脂肪组织和脑的ECM。涂层可用于组织培养用途，研究和临床。涂层可用来涂覆 例如但不限于合成或其它生物学的支架/材料或植入物。一些实例中，使涂层具有某种纹 理或图案。一些实例中，制造涂层的方法包括吸收或化学交联。薄凝胶或吸收涂层可使用 组合物的ECM溶液形式形成。一些实例中，设置文中的组合物以封闭心脏如隔膜缺损中的 洞。

[0090] 本文的组合物还可从其它组织如骨骼肌、心包膜、肝、脂肪组织和脑形成。该组合 物可用作生物制剂、医用装置或给药装置的涂层。

[0091] 缺少功能性替代物限制了经伤损、肿瘤切除、各种肌病而失去的骨骼肌的重建。外 科手术治疗如肌肉移植和移位技术已有一些成功；然而，依然存在替代疗法的需求。组织工 程方法提供了潜在的新解决方案；然而，当前可选方案提供不完全再生。已经研究了许多天 然来源的和合成的材料作骨骼组织工程的支架，但均不能综合模拟具有用于细胞生存、分 14 CN 化和迁移的重要信号的天然骨细胞细胞外基质。

[0058] In yet another embodiment, ECM with other therapeutic agents, such as cells, peptides, proteins, drugs, nutrients, antibiotics, pro-survival additives, proteoglycans, and / or glycosaminoglycan binding. In yet another embodiment, ECM can be combined with synthetic polymers and / or crosslinked. Examples of synthetic polymers include, but are not limited to: polyethylene terephthalate fibers (DACR0N ◆), polytetrafluoroethylene (PTFE), polylactic acid (PLA), polyethylene glycol (PGA), polyethylene glycol alcohol (PEG), polyethylene glycol diacrylate (P 'EGDA), polyethylene, polystyrene, and Nitinol (nitinol).

[0059] In yet another embodiment, the ECM may be used alone or in solution or gel in combination with the above components and injected into the infarct site, or an edge region of the myocardium, such that endogenous cellular ingrowth, angiogenesis and regeneration. In still another embodiment, the compositions may be used alone or used in combination with the above-described components, as a substrate, to alter the mechanical properties of the heart and / or prevent negative left ventricular remodeling. In still another embodiment, the composition may be used alone or in combination with the above components, and cell delivery, for reproducing the heart muscle. In still another embodiment, the composition may be used alone or in combination with the above components used to produce conduction block thereby treating arrhythmia.

[0060] In a manufacturing soluble agent embodiment, including but not limited to 0.5M, 0. The solution was adjusted to the desired low pH solution or 0. OIM IM acetic acid or 0. IM HCI in concentration, and then placed in tissue culture plates / apertures, coverslip, other scaffold for tissue culture or surface. 37 degrees centigrade in an incubator After standing for 1 hour, or overnight at room temperature, the excess solution is removed. After rinsing with PBS surface, can be cultured cells absorbing matrix. The solution may be prior to injection / implantation, during or after, and pre-peptides, proteins, DNA, drugs, nutrients, survival promoting additives, proteoglycans, and / or glycosaminoglycan binding.

[0061] Based on the therapeutic composition and absorption cell culture composition in the form of extracellular matrix in vitro cardiac cells of the present invention to provide enhanced cell

[0092] 细胞外基质由复杂的组织特异性的蛋白质和多糖网络组成，其帮助调节细胞生 长、生存和分化。尽管天然ECM特性复杂，体外细胞研究传统上评价单个ECM组分涂层上 的细胞行为，因此对从体外细胞研究到体内环境的转化研究结果产生限制。通常，来自各种 动物来源的纯化的基质蛋白被细胞培养基材吸收，为细胞粘附提供蛋白底物并修饰细胞行 为。但是，这些方法不能提供准确的复杂微环境表征。曾用过更复杂的涂层，如单蛋白结合，显示这些结合的信号影响细胞行为，但是不像体内那样完整。对于更天然的基质，使用 了细胞来源的基质。基质胶是一个复杂的系统；但它来源于小鼠肉瘤，不能模拟任何天然组 织。许多ECM组分是相似的，但是各组织或器官具有独特的组合物，天然获得的组织特异性 来源可能是更好的细胞微环境模仿者。

[0093] 骨骼肌包括高度取向的致密肌纤维束，各复核细胞由成肌细胞得到。天然骨骼肌 中的肌纤维在细胞外三维基质中密集在一起，形成具有高细胞密度和细胞取向的有序组 织，产生纵向收缩。骨骼肌受损后可产生瘢痕组织而导致功能丧失。体外肌肉组织工程作 为备选方案对受体进行肌肉移植，为治疗骨骼肌缺损带来希望。组织工程组合物必须是生物相容性的，并可被血管化和神经支配。

[0094] 细胞外基质由复杂的组织特异性的蛋白质和多糖网络组成，其帮助调节细胞生 长、生存和分化。尽管天然ECM特性复杂，体外细胞研究传统上评价单个ECM组分涂层上的 肌肉细胞行为，因此对从体外细胞研究到体内环境的转化研究结果产生限制。克服此限制 对细胞介导的治疗很重要，其依赖于随着时间推移培养和扩增的细胞保持天然细胞行为。

[0095] 一方面，文中的组合物包括从猪的骨骼肌和心肌获得的ECM。可发展该组合物形成 基材涂层用于不同用途。一些实例中，组合物的ECM可溶化后保留肌肉特异性的ECM组分 的复杂混合物。一些实例中，文中的涂层可在体外更恰当地效仿天然肌肉ECM。

[0096] 与接种于涂覆传统的I型胶原的基材上的细胞相比时，接种于骨骼肌基质上的成 肌细胞在i)肌球蛋白重链正极肌管的数量、Ü)每肌管的核数和iii)肌管宽度呈现明显 的增长。当接种于涂覆传统明胶的基材上的细胞相比，接种于心肌基质上的人类胚胎干细 胞（HES2)来源的心肌细胞在以下方面呈现明显的增长：i)肌原纤维区域、ii)每肌原纤维 区域的心肌细胞核数量和iii)桥粒斑尺寸，其凸显出桥粒细胞一细胞连接蛋白、桥粒斑蛋 白更大更成熟的闰盘定位。一些实例中，设置组合物以提供体内重建肌

adhesion and survival. Compared with the standard tablet coating, a soluble form of heart cell culture reagents extracellular matrix induced myocardial cells spread more rapidly, faster maturation and / or survival improvements.

Studies

[0062] Before the show, it is difficult from human embryos to obtain stem cells cardiomyocytes (hESC) for the treatment of myocardial infarction. Some examples, the effective yield in vivo differentiation and maturation of ventricular cardiomyocytes hinder the effectiveness of the treatment. In the former, the regulation of differentiation in vitro is mainly implemented, for example, adding soluble factors in the cell culture medium. The process that is difficult to differentiate the fetal phenotype beyond those limits.

[0063] In addition to soluble factors, extracellular matrix may also play an important role in cell differentiation. For adult cells, including adult progenitor cells, we have studied a number of chemical signals containing matrix, but only in small amounts, in particular studies on the effect of ECM ESCs effect of hESCs. In many instances, the hESC cardiomyocytes obtained is containing the soluble factor promoting survival and gelatin medium conveyed.

11 CN

[0064] In one embodiment, the article discloses a bionic matrix obtained from the natural heart. Some examples, similar to the matrix in vivo cardiac environment, which contains many or all of the native cardiac ECM chemical signals found in nature. Some examples, or by crosslinking or addition of other materials, also mimic the mechanical properties of healthy adult myocardium or embryos. As described herein, it can be simple and economical method to separate cardiac ECM and processed into the gel, which is suitable for scaled up for clinical transformation.

[0065] Some examples, the compositions provided herein may contain matrix and exogenously added or collection of cells. The cells may be any of various cells. Some examples, the cells for a variety of heart or cardiovascular cells, including but not limited to: stem cells, progenitor cells, cardiac cells, vascular cells and autologous or allogeneic origin of fibroblasts.

肉ECM的能力。该 组合物可提供评价和保持体内肌肉和干细胞的行为与自然状态相似的工具，并可提供用于 体内细胞介导的治疗的工具。

[0097] 图2显示与标准明胶涂层相比，生长在心脏ECM的心肌细胞的平均肌原纤维区域 显著增大。图3显示与标准明胶涂层相比，心肌细胞的平均数量明显高于心脏ECM。如图4 所示，在心脏ECM上培养时，桥粒斑蛋白、一细胞内的连接蛋白，在112天时特异地定位于心 肌细胞之间和形成有序的桥粒，而在明胶上培养时不出现上述现象。

[0098] 文中所述的骨骼肌基质可以采用与心脏ECM同样或相似的方式制备。可将该骨骼 肌基质注射到骨骼肌中而用于骨骼肌组织工程。图5显示培养于骨骼肌基质上的骨骼肌成 肌细胞，如文中所述，其显示增加的肌管大小、增强的分化，且每肌管具有比胶原上培养的 成肌细胞更高的平均核仁数。采用体外Transwell迁移检测，如图6所示，骨骼肌成肌细胞 特异向骨骼肌基质迁移。

15 CN [0099] 通过以下实施例进一步说明本发明，其不应以任意方式理解为对本发明的领域产 生限制。显而易见，对于技术人员而言，能够并且考虑在本发明的领域内进行各种修改和变 动。

[0100] 实施例1 [0101] 对直接注射细胞到梗塞壁以治疗MI已经进行了各种研究，尽管许多研究显示较低的存活率。本研究的目的在于测试采用凝胶作生长平台，用于细胞粘附、生长、成熟和体 内运输。提供了由天然心脏细胞外基质组织构成的凝胶，可通过促进细胞存活而帮助心脏 组织再生。

[0102]使 Sprague Dawley 雌鼠安乐死，采用由 Ott 等（Nature Medicine，14 (2)，213， 2008)改进的方案对它们的心脏进行脱细胞化。接着将脱细胞化的心脏冻干、再水化、研磨 成粉并再次冻干，以形成干粉。根据Freytes等(Biomaterials 29： 1630，2008)所改进的 方案，接着以胃蛋白酶最低限度消化该ECM并中和。

[0103] 更特别地，对成年Sprague Dawley雌鼠进行肝素化并用戊巴比妥腹腔内麻醉。横 切主动脉和肺动脉并去除心脏。将导管插入主动脉并系到改装的LangendorfT装置上。

[0104] 用改良的、之前已公开的技术对心脏进行脱细胞化。简而言之，以十二烷基硫 酸钠（SDS)和PBS溶液对心脏的冠状血管逆向灌流24小时，然后以的triton PBS溶液 逆向灌流30分钟。一旦脱细胞化完成，则用去洗离子水清洗心脏并在冻干机中冻干。

[0066] The present invention thus provides the use of natural decellularized cardiac extracellular matrix gels prepared to support the separation of neonatal cardiomyocytes or in vivo myocardial cells derived from stem cells and progenitor cells for in situ Role gel scaffold provided stromal cells remain and to improve survival in left ventricular wall. Since the ECM from the heart than the current generation of stents available material more accurate closer to the in vivo environment, which is suitable for the muscle heart cell transplantation.

ECM composition and exogenously added heart cells

[0067] may be contained in the text prepared by culturing in the ECM. In addition, the base may be added in the extracellular proteins such as growth factors, may be added in the composition of proteins, or protein molecules may be covalently or non-matrix molecules covalently linked. Protein covalent connection matrix molecules known in the art may be standard protein covalently complete connection method. Protein can be covalently linked with one or more matrix molecules.

[0068] In one embodiment, when the conveying comprising decellularized cardiac ECM and exogenous cell composition, these cells can be used in the treatment of the myocardium derived cells, including isotype sources, xenogeneic sources, autologous sources. Therefore, conveyed through the text of the composition of embryonic stem cells, fetal or adult-derived stem cells, induced pluripotent stem cells, cardiac progenitor cells, fetal and neonatal cardiac cells, myofibroblasts, myoblasts, mesenchymal cells, parenchymal cells, epithelial cells, endothelial cells, mesothelial cells, fibroblasts, hematopoietic cells, bone marrow-derived progenitor cells, bone cells, macrophages, adipocytes and self-expanding transplanted cardiomyocytes. Some instances, the text of the cells can be cultured in vitro and may be in a petri dish environment directly into cardiomyocytes or bone marrow cells can become heart cells. Then the cultured cells with the composition or contacting mounts and other components and transplanted into a mammal.

[0069] Adult stem cells also can be a text in another part of the composition of cells. It is

[0105] 用水将冰冻的心脏再水化，然后将其浸没到液氮中。一旦冻结，则将心脏在杯球装 置中以70psi系统碾压10秒。然后将研磨的心脏颗粒冻干。一旦干燥，则将冻干的心脏组 织与的胃蛋白酶结合，并与0. OlM的HCl混合至浓度为lOmg/ml。室温下搅拌溶液48 小时，以使细胞外基质增溶溶解。48小时后，在冰上将该HCl溶液等分到Eppendorf管中， 并以0. IN的NaOH中和至pH7. 4。

[0106] 通过上述方法，形成天然大鼠心脏ECM凝胶。如增强的材料粘性所证实地，2. 5～ 8mg/mL凝胶的成功凝胶化在15分钟内发生。随着更高密度的凝胶可观察到增加的刚度。

[0107] 以1 XPBS稀释中和的溶液，以每孔50 ii L接种于96孔板，然后转移到37°C和5% CO2的培养箱中。凝胶形成后，使用移液器将100 y L分离的2d新生心肌细胞移到凝胶上部， 每孔60000细胞。几天后，检测细胞对凝胶的粘附性。

[0108] 细胞外基质组织脱细胞化、磨碎和消化后，一旦溶液处于生理条件（pH = 7.4， 37°C)时，凝胶形成。溶液中的ECM组织以较高浓度形成的凝胶与以较低浓度ECM形成的 凝胶相比，更硬更不透明。接种于凝胶上的细胞能够粘附到凝胶上并且在其上存活。

[0109] 在心脏ECM凝胶上以1 X IO4接种的心肌细胞表现出对ECM的成功的粘附和细胞 存活。在ECM上培养这些细胞至四天。

[0110] 通过30G针将IOOmL的心脏ECM溶液（7mg/mL)注射到麻醉鼠的LV游离壁中。本 研究显示，当处于生理pH和温度时，天然心脏细胞外基质可被分离、溶解并自组装进入凝 胶。由于凝胶含有所有的天然细胞外基质组分，尽管有些杂乱，但其使该基质允许心肌细胞 在体外以及一旦注入体内的成功粘附和生长。并且相信，由于由最初来源于心室的基质组 成的凝胶更准确地模拟体内心脏环境，它能更成功地支持心肌细胞生长，而不是其它基质如胶原或纤维蛋白凝胶。

[0111] 可注射的凝胶可潜在地适应任意三维形状并促进心脏内的细胞移植存活。注射的 16 CN 心肌细胞或可分化为心肌细胞的细胞可帮助心脏组织再生，促进心输出量。该方法发展为 产生不同浓度和硬度的天然心脏ECM凝胶平台，还为细胞生长提供体外平台，并作为原位 工程支架用于再生。注射到体内时，天然ECM为心肌组织工程提供合适的复杂环境以增加 细胞停留并促进组织再生。

believed that adult stem cells generated by the new parts of the other stem cells (such as cells of the myocardium adapted) and work, or directly in vivo differentiation into cardiac cells. After booting to organs such as the heart they can also differentiate into other cell types. Adult mammal by the circulating endothelial precursor cells, bone marrow-derived cells, adipose tissue, or organ specific cells are adult stem cells provide a source. Known isolated from bone marrow aspirate monocytes differentiate into endothelial cells in vitro, and after intramuscular detected in newly formed blood vessels.

Accordingly, the use of cells derived from bone marrow aspirate of endothelial cells in vivo can be produced as components of the composition. Other cells can be used in the present invention is that the regulation of cytokine activation of mesenchymal stem cells. Known mesenchymal cell subpopulations in vitro when exposed to cytokines division into myogenic cells.

[0070] Human embryonic stem cell-derived cardiomyocytes grown on composition may contain the heart of the substrate in the text. Some examples, the presence in the composition of the text hESC-derived cardiomyocytes grown cells exhibit morphology more similar to the body. Some examples, the presence of text composition hESC-derived cardiomyocytes grown cells exhibited increased maturation markers.

[0071] The present invention is also the head in a drug delivery system, which includes means for transporting cells, drugs, molecules or proteins to a subject to treat defects, morbid, damaged or ischemic tissue or organ decellularized cardiac extracellular matrix. In one embodiment of formula, comprising decellularized cardiac extracellular matrix of biocompatible material alone or in combination with other components of the present invention is used as a non-destructive conduction block to treat arrhythmias. Thus, the biocompatible material of the present invention may be used to transplant cells, or 12 CN injected alone to raise natural cells or other endogenous cytokine therapeutic agent, or therapeutic agents acting as exogenous delivery vehicle.

[0072] The compositions of the invention may also contain cells, drugs, proteins or other biological material, such as, but not limited to: erythropoietin (EPO), stem cell factor (SCF), vascular endothelial

[0112] 实施例2 [0113] 心肌细胞通常培养于涂覆有一个或可能多种细胞外基质（ECM)蛋白质的表面上。

但是，在体内，心肌细胞处于高度复杂的胞外环境；更准确模拟该天然环境的ECM可利于培 养的心肌细胞的存活。这里，报导了已增溶溶解而形成涂层、用于新生心肌细胞的细胞培养 的天然心脏ECM。

[0114] 将心脏从Sprague-Dawley大鼠除去并使用修改的Langendorff方案（由Ott等 人改进，2008)对心脏进行脱细胞化。将脱细胞化的心脏冻干、再水化，并在液态N2中冷冻 后碾碎成粉。以胃蛋白酶的0. OIM HCl最低限度消化ECM。48小时后，加入0. OIM的乙酸 使终浓度为lmg/ml。

[0115] 使用分离试剂盒（Cellutron，Highland Park, NJ)从新解剖的1至2天大的 Sprague-Dawley大鼠的心室获取心脏心肌细胞。弃掉最初的上清液，但随后20分钟的消 化物用含17% M199、10%马血清、5%胎牛血清和青霉素/链霉素的DMEM终止并悬浮。

分离后，将上清预接种于苯乙烯组织培养皿上，通过成纤维细胞的选择性粘附提高心肌细 胞的纯度。

[0116] 在37°C中以1小时使lmg/ml天然心脏ECM或胶原I (Sigma，St. Louis, MO)吸收 至盖玻片上。以200000/cm3的密度接种分离后的新生心肌细胞，且在24小时后将培养基更 换成低血清培养液（DMEM，18. 5% M199，5% HS, 1% FBS及抗生素）。细胞培养维持在37°C 和5% CO2，每日监测，并每2～3天更换新鲜的培养液。

[0117] 心肌细胞粘附到吸收的天然ECM上，并形成局部融合层。起初，心肌细胞以相似的 密度粘附到胶原涂层上。

[0118] 细胞培养物均在接种后第3天开始自发搏动。培养于胶原上的心肌细胞在第12 天开始分离，并在第14天停止搏动。但培养于天然心脏ECM的心肌细胞形成了清晰界定的 纤维，其以同样的频率搏动直至第28天。

[0119] 本研究证明，由于天然心脏ECM更准确的模拟体内条件，其用于心肌细胞培养是 有益的。本研究还证明，新生心肌细胞粘附到天然心脏ECM上并且比在典型的胶原涂层上 作用更持久。该新的涂层对培养干细胞来源的心肌细胞以及心脏祖细胞是有用的。

growth factor (VEGF), transforming growth factor (TGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), cartilage growth factor (CGF), nerve growth factor (NGF), keratinocyte growth factor (KGF), skeletal growth factor (SGF), hematopoietic growth factor (BDGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), a cytokine growth factor (cytokine growth factor, CGF), stem cell factor (SCG), platelet-derived growth factor (PDGF), endothelial cell growth supplement (EGGS), colony stimulating factor (CSF), growth differentiation factor (GDF), integrin regulatory factor (IMF), calmodulin (CaM), thymidine kinase (TK), tumor necrosis factor (TNF), growth hormone (GH), bone morphogenetic protein (BMP), matrix metalloproteinase (MMP), tissue inhibitor of matrix metalloproteinase (TIMP), interferons, interleukins, cytokines, integrins, collagen, elastin, fibrillin, fibronectin, laminin, glycosaminoglycans, blood tuberculin, thrombospondin, heparan sulfate, dermatan (dermantan), chondroitin sulfate (CS), hyaluronic acid (HA), vitronectin, proteoglycan, transferrin, tenascin (cytotactin), tenascin (tenascin) and lymphokines.

[0073] tissue culture plates may be soluble ligand or gel form the extracellular matrix of the present invention, the coating or absorbed form the extracellular matrix of the present invention to cultured myocardial cells or other cells associated with heart repair. It can be used as agents of these cell culture studies or clinical reagents for cell culture prior to transplantation. Extracellular matrix and cell-matrix agents may be combined with other organizations.

[0074] The reagent composition of the gel, and then using PBS / salt or other buffer solution and adjusted to an appropriate concentration and then placed in tissue culture plates and / or in the holes. When placed in an incubator at 37 degrees C, the solution to form any 2D or 3D gel may be used in cell culture with culture substrate. In one embodiment, the gel composition with glutaraldehyde, formaldehyde, bis -NHS crosslinked molecules or other cross-linking, or cells, peptides, proteins, DNA, drugs, nutrients, survival promoting additives, proteoglycans and / or glycosaminoglycan binding, or binding and / or cross-linked to future use of synthetic polymers.

[0075] The present invention also provides a method of absorption of cardiac extracellular

RegenX Science Exhibit 1004
Page 993

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0993

[0120] 实施例3 [0121] 这里，研究了已脱细胞化和增溶溶解的成年心室的天然心脏细胞外基质的细胞涂 覆用途。该优势在于，天然心脏ECM比传统的细胞表层具有更多组分，并且比预处理其它细 胞型更易于使用。

[0122] 将心脏从Sprague-Dawley大鼠除去并使用修改的Langendorff方案（由Ott等 人改进，2008)对心脏进行脱细胞化。将脱细胞化的心脏冻干、再水化，并在液态N2中冷冻 后碾碎成粉。以胃蛋白酶的0. OIM HCl最低限度消化ECM。48小时后，加入0. OIM的乙酸 使终浓度为lmg/ml。

[0123] 对天然心脏ECM的胃蛋白消化物在使用DTT的还原性条件进行心室凝胶电泳，并 17 CN 与层粘连蛋白（BD biosciences)和牛皮胶原（calf skin collagen) (Sigma)比较。使用 Imperial Protein Stain (Pierce)对凝胶染色。与胶原和层粘连蛋白相比，天然心脏ECM 可显示更复杂的ECM组分的混合物。

[0124] 使用分离试剂盒（Cellutron，Highland Park, NJ)从1至2天大的 Sprague-Dawley大鼠的心室获取心脏心肌细胞。弃掉最初的上清液，但随后20分钟的消 化物用含17% M199、10%马血清、5%胎牛血清和青霉素/链霉素的DMEM终止并悬浮。

分离后，将上清预接种于苯乙烯组织培养皿上，通过成纤维细胞的选择性粘附提高心肌细 胞的纯度。

[0125] 在37°C中以1小时使lmg/ml天然心脏ECM或胶原I (Sigma，St. Louis,MO)吸收至 48孔板上。以200000/cm2的密度接种分离后的新生心肌细胞，且在24小时后将培养基更 换成低血清培养液（DMEM，18. 5% M199,5% HS, 1% FBS及抗生素）。细胞培养维持在37°C 和5% CO2，每日监测，并每2～3天更换新鲜的培养液。在第2天、第4天和第7天对细胞 进行固定，并对a辅肌动蛋白、连接蛋白43(connexin 43)、pan-cadherin、肌动蛋白和细 胞核染色。第2天心肌细胞在培养基中开始自发搏动。培养于胶原上的细胞在第8天开始 从平板分离。培养于天然心脏ECM的一组细胞持续搏动直至第45天。培养于胶原上的所 有细胞均在第14天停止搏动。

[0126] 现有的细胞培养涂层通常是吸收到组织培养板或支架上的简单蛋白。采用更复杂 的环境有利于细胞存活和成熟。本研究显示，天然心脏ECM比其它标准细胞培养涂层含有 更多的复杂组分。新生鼠心肌细胞粘附到作为细胞培养用的涂层的天然心脏ECM上，并自 发地开始搏动。随时间推移，培养

matrix cell culture off on an exemplary method, comprising the steps of: (a) including, but not limited to 0. 5M or 0. OIM of low pH solution of acetic acid or 0. IM HCl provided comprising decellularized ECM biocompatible material solution, the desired concentration, (b) The above solution was placed in tissue culture plates or wells, (C) in above room temperature such as 37 degrees centigrade above culture plates were incubated for 1 hour and overnight or holes (or at room temperature to 40 degrees centigrade in), (d) removing the excess solution, (e) tissue culture plate was rinsed with PBS or holes, and (f) culturing the cells in the absorption matrix. Absorbing matrix in the heart of the extracellular matrix of the present invention contains the cultured cells include other cell types associated with cardiac myocytes or repair, including stem cells and cardiac progenitor cells.

[0076] some embodiments, the composition includes a crosslinking agent, including but not limited to conventional collagen crosslinking agent, a crosslinking agent, hyaluronic acid cross-linking agents or other protein degradation and mechanical properties have changed.

[0077] In one example, a method of manufacturing paper compositions include electrospinning. Some examples, provided herein a method to control the nanofiber size, shape, or thickness.

[0078] some examples, may be used, for example a cell or in vitro contractility pacing is introduced to the composition. Shrinkage stress can be generated so as to promote a more natural cycle of the myocardium.

[0079] some instances, when the composition contains a crosslinking group or embed factors, such as when angiogenic factors, may be introduced into cell infiltration and angiogenesis to the composition.

[0080] some embodiments, the compositions herein can contain beads. Microbeads or may be part of the composition fed to the compositions. Exemplary microbeads may be any kind of material, such as natural or synthetic. Some examples, the microbeads having 13 CN can have different properties or degradation e.g. containing MMP inhibitors, growth factors or small molecules.

[0081] some embodiments, the compositions herein may comprise as a binder in the

于天然心脏ECM上的心肌细胞显示增强的辅肌动蛋白、连 接蛋白43(connexin 43)、pan-cadherin染色。并且，与胶原相比，新生的心肌细胞在天然 心脏ECM上具有提高的存活能力和粘附能力。

[0127] 实施例4 [0128] 这里，研究了文中所述的凝胶的使用，其中凝胶由天然脱细胞心脏ECM制得。该凝 胶可作为原位凝胶支架，提供天然心脏基质，促进细胞LV壁中细胞停留和存活。

[0129] 对Sprague Dawley雌鼠心脏和猪心脏进行脱细胞化。将心脏组织切至～2mm厚 并以去离子水漂洗，然后在十二烷基硫酸钠（SDS)中搅拌4～5天直至脱细胞化。在 1 % Triton X-100中30分钟的附加的搅拌步骤确保完全脱细胞化，随后在去离子水中过夜 搅拌并在去离子水中最后漂洗。

[0130] 然后将脱细胞化的心脏冻干、碾碎成粉或研磨，并再次冻干以形成干粉。然后在胃 蛋白酶中消化该ECM并中和。

[0131] 然后通过添加氢氧化钠和10X PBS将溶解的心脏ECM调至生理pH或pH 8。然后 以PBS稀释中和后的心脏细胞外基质溶液至所需浓度并使其在37°C中在96孔板中凝胶化。

目测该材料证实了 2. 5～8mg/ml凝胶的成功凝胶化。随着更高密度的凝胶可观察到增加 的刚度。

[0132] 测试了不同实验条件从而为心脏ECM支架凝胶化确定不同的消化。进行心室凝胶 电泳以比较消化条件的内容物，并比较ECM内容物与鼠尾胶原。确定初始pH在心脏ECM的 消化和凝胶化中起重要作用。消化进行48～72小时。

[0133] 凝胶电泳显示0. OIM HCl对天然心脏ECM的不完全消化。在0. IM HCl中消化心 18 CN 脏ECM显示增强的降解。由此显示更强的酸性条件促进心脏ECM溶液的消化和凝胶化。对 比心脏ECM和鼠尾胶原说明心脏ECM中存在各种额外的胃蛋白酶。

[0134] 采用扫描电镜观察心脏细胞外基质凝胶形式的结构。以2. 5%戊二醛固定凝胶2 小时，接着进行一系列乙醇漂洗（30～100% ），并在临界点干燥。样品在成像前用铬进行 溅射镀膜(sputter coated)。

[0135] 通过30G针将浓度为6mg/ml心脏ECM的溶解的天然ECM成功注射到鼠LV游离壁 这，产生原位凝胶支架，心肌细胞粘附并在其上增殖。

[0136] 实施例5 [0137] 使用市售的迁移测定试剂盒进行测定心脏脱细胞化ECM溶液的体外化学趋化特

composition or delivery composition of biological anchoring groups (biological group).

[0082] one example, the composition may be bioadhesive, e.g., for wound repair. Some examples, the compositions herein may be provided as cell adhesion agent. For example, the compositions herein may be coated or mixed in the medical device and biological or may not contain cells. For example, the compositions herein may be a synthetic vascular graft with a polymer coating. Some examples, the composition containing an antibacterial agent or antibacterial agent contained therein.

[0083] text of ways including delivery composition as a wound healing device. Such as heart damage (cardiac ablation) can be delivered after the composition to promote healing.

[0084] one example, the composition comprising alginate bead compositions described herein to ECM coated.

[0085] some examples, the composition is injectable. Injectable compositions can be, but is not limited to, powders, liquids, granules, chips, gel or lotion. The injectable composition may be injected into the heart, or in many instances, is injected into the left ventricle and right ventricle, left atrium, right atrium or heart valves. The compositions herein may be raised such as, but not limited to, endothelial, smooth muscle, cardiac progenitor cells, myofibroblasts, stem cells and cardiomyocytes.

The method of manufacturing the compositions herein

[0086] According to the field may include methods known to us for any age, decellularized animal or human tissue.

[0087] some examples, the compositions herein contain ECM and natural or synthetic polymers. For example, the compositions herein contain natural polymers such as collagen, chitosan, alginate, fibrin or hyaluronic acid. Another example, the compositions herein contain synthetic polymers, such as, but not limited to, polyethylene glycol, polyglycolic acid, polylactic acid, polyglycolic acid, polydioxanone, polycaprolactone, polyorthoesters, polyanhydrides, polyphosphazenes, poly-amino acids, amino acids intended to gather, conductive

性（chemoattractive properties)。简单地说，将人类冠状动脉内皮细胞（HCAECs)和鼠 主动脉平滑肌细胞（RASMCs)血清饥饿，并评价向基质、胶原、胃蛋白酶和胎牛血清的迁移。

RASMCs显示向基质显著的迁移，而HCAECs显示趋势。因此，基质的生化信号具有能够在体 内促进细胞浸润的化学趋化特性。

[0138] 在体内，在注射区域内定量微动脉的形成以评价新生血管的形成。与注射后4h相 比，微动脉的密度在注射后11天显著更大。

[0139] 实施例6 [0140] 当在MI后注射到心肌壁中时，数个细胞类型已经显示保护心脏功能。然而，无细 胞处理能够消除用细胞疗法时普遍的低细胞存活率和免疫反应的并发症。

[0141] 心肌梗塞是在大鼠中使用25min缺血一再罐注模型通过闭合左前降动脉诱导的。

在MI后一周，从MRI图计算基线函数。

[0142] 将猪心肌ECM以小块在1% SDS中脱细胞数天，随后通过DI漂洗过夜、冻干并研磨 以产生粉末。消化在0. IM HCl中以胃蛋白酶进行，从而生成可溶形式的材料。

[0143] 注射前，使用IM NaOH将溶解性的ECM调至PH 7. 4并以PBS稀释至为6mg/ml。MI 手术后，将动物随机分为2组，梗塞手术后两周，将ECM或盐水通过30G针注射到Sprague Dawley雌鼠的LV游离壁中。

[0144] 注射手术后4周（MI后6周），使用MRI再次评价心脏功能。

[0145] 以ECM注射的动物在6周显示保持的功能（基于注射部分进行的评价），而盐水注 射的动物没有维持心脏功能。心脏舒张末期和心脏收缩末期体积在ECM注射的动物中也得 到了保持。

[0146] 实施例7 [0147] 当前，干细胞和其它细胞类型正在用于治疗心力衰竭的临床试验，其通过穿过27G 的导管输送到心肌壁中。将猪心室组织使用SDS洗涤剂进行脱细胞化，并加工以形成可溶 形式的基质，中和至生理PH并稀释至6mg/ml用于注射。

[0148] 2只Yorkshire猪接受线圈诱导的心肌梗塞（coil-induced myocardial infarction)，并在梗塞后2个月用单独的心肌基质或与细胞一起进行注射。

polymers (such as polyacetylene, polypyrrole mouth each, polyaniline) or polyurethane copolymers or they may. Some examples, the compositions herein contain two types of ECM and natural and synthetic polymers. The text of the composition may be a multi-material connected by ECM and another polymer material, for example by reaction with amines, peptides or free thiols, and ECM self-assembly.

[0088] The method comprises delivering text containing ECM composition. Exemplary methods include, but are not limited to: direct injection surgery; direct injection through the chest wall; delivered to the myocardium by catheter through endocardium; delivered through the coronary vessels; and by perfusion balloon catheter delivery. The composition may also solid-state transport, such as graft (graft), fragment (patch) or coupling with the cytoskeleton. The dosage and frequency will be based on the needs of patients and physician judgment and different.

[0089] some instances, the coating compositions herein. The coating may contain ECM from any tissue such as heart, skeletal muscle, pericardium, liver, adipose tissue and the brain. Coating can be used for tissue culture purposes, research and clinical practice. Coatings used to coat, for example, but not limited to, synthetic or other biological scaffold / or implant material. Some examples, the coating having a certain texture or pattern. Some examples, the method of manufacturing the coating comprises an absorption or chemical crosslinking. Forming a thin gel or absorptive coating compositions may be used in the form of a solution of ECM. Some examples, set the text of the composition as a closed heart septum defect hole.

[0090] The compositions herein also from other tissues such as skeletal muscle, pericardium, liver, adipose tissue and the brain is formed. The compositions are useful as coatings biologics, medical devices or dosing apparatus.

[0091] lacking a functional substitute limits by trauma, tumor resection, reconstruction of various skeletal muscle myopathy and lost. Surgical transplant surgery such as muscle and shift technology has been some success; however, there are still alternative therapies needs. Tissue engineering approach provides a potential new solutions; however, the current options provided

[0149] 将胎牛心脏外植物来源的物质用DiI、一种cyotoplasmic染料进行预先标记用于 组织学鉴定。使用显示在啮齿动物模型中增强hESC存活的促存活的鸡尾酒（cocktail)。

[0150] 通过NOGA定位引导，将单独的基质或与细胞一起以临床相关速率0. 2mL每30s通 过导管进行注射。5次每次0. ImL的注射由单独的基质或与细胞一起进行，注射到梗塞的缘 19 CN 带区域。

[0151] 单独的基质或与细胞一起的基质能够成功地注射到猪的心脏内，最低限度侵袭地 且不阻塞细导管。

[0152] 实施例8 [0153] 这里，将脱细胞化的心包组织来源的凝胶的研究和使用描述为具有通过促进体内 新生血管形成以改进在LV壁的细胞保留和存活的自体疗法的潜能。

[0154] 猪和人的心包均进行过脱细胞。使Juvenile农场雄猪安乐死，通过由Ott等 (Nature Medicine, 14(2)，213，2008)改良的步骤将它们的心包脱细胞。具体而言，将心包 简单地在DI水中漂洗，在十二烷基硫酸钠（SDS)中搅拌24h，然后在DI中搅拌近5h。

人心包组织样品从接受心胸手术的患者中收集。这些样品以相似的方式进行脱细胞：简短 的DI漂洗，随后在1 % SDS中3天，随后DI漂洗过夜。在这两种情况下，完整的脱细胞化均 用组织学染色进行验证。

[0155] 下述的步骤对于人和猪的心包ECM样品均是有效的。

[0156] 接着将脱细胞心包或心包ECM冷冻、冻干，并研磨以形成精细干燥粉末。然后通过 由Freytes等(Biomaterials 29 =1630,2008)改良的方法，将ECM粉末以溶解于HCl的胃 蛋白酶消化并中和。

[0157] 凝胶电泳（SDS-PAGE)表明与胃蛋白酶消化的胶原相比的更大的复杂性，其显示 心包样品中较小的带的宽度范围。

[0158] 复杂性通过用质谱法分析样品进行确认以确定蛋白质碎片。确定的ECM蛋白质的 碎片包括胶原、弹性蛋白、纤维蛋白和各种蛋白聚糖。

[0159] 当将150 U 1的中和溶液加载到96孔板内并使其位于培养箱中，在2～3h后观察 凝胶作用。

[0160] 体内凝胶作用通过注射60 U 1的中和ECM溶液到Sprague Dawley雄鼠的左心室 (LV)壁进行观

incomplete regeneration. Have studied many natural sources and synthetic materials for bone tissue engineering scaffold, but are not an important integrated analog signal for cell survival, points 14 CN and migration of osteoblasts natural extracellular matrix.

[0092] extracellular matrix is composed of a complex tissue-specific network of proteins and polysaccharides, which help regulate cell growth, survival and differentiation. Despite the complex nature of the natural ECM evaluate cell behavior on single ECM component of the coating on the in vitro study of traditional, and therefore the conversion from in vitro studies to produce results in vivo environment restrictions. Typically, the purified matrix proteins from various animal sources is absorbed by the cell culture substrate, providing protein substrates and modified cell line is a cell adhesion. However, these methods do not provide an accurate characterization of complex microenvironment. He had used a more complex coating, such as a single protein binding, indicating that these combined signals affect cell behavior, but not as a complete body. For a more natural substrate, using a stromal cell origin. Matrigel is a complex system; but it is derived from mouse sarcoma, can simulate any natural tissue. Many ECM components are similar, but each tissue or organ has a unique composition of tissue-specific sources might be natural to get a better cellular microenvironment imitators.

[0093] skeletal muscle include highly oriented dense muscle fiber bundles, each review cells obtained by the myoblasts. Natural skeletal muscle muscle fibers close together in a three-dimensional extracellular matrix to form an orderly tissue cells with high cell density and orientation, produce longitudinal shrinkage. After skeletal muscle damage can produce scar tissue caused by loss of function. In vitro muscle tissue engineering as an alternative to transplant muscle receptors, for the treatment of skeletal muscle defect hope. Tissue engineering composition must be biocompatible, and may be blood vessels and nerves.

[0094] extracellular matrix is composed of a complex tissue-specific network of proteins and polysaccharides, which help regulate cell growth, survival and differentiation. Despite the complex nature of the natural ECM evaluate muscle cell behavior on single ECM component of the coating

RegenX Science Exhibit 1004
Page 997

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0997

察。来自注射后45min处死的动物的切片心脏的组织学染色显示在LV壁可 见的凝胶的ECM区域。

[0161] 在相同的实验中，将动物培养2周，之后将它们处死，收集它们的心脏用于切片。

在这个时间点，ECM注射剂仍然可见，但已经被细胞浸润。

[0162] 免疫组化在组织切片上进行，以确定平滑肌细胞和内皮细胞，指示血管。在ECM注 射剂区域内大量血管的存在显示该材料促进新生血管形成。

[0163] 实施例9 [0164] 用于体外细胞培养的表面涂层通常由一种或几种细胞外基质蛋白质形成。其提供 细胞粘着表面，但它并不能模仿体内细胞外微环境。文中提出了制备各种组织来源的细胞 外基质的吸收涂层的方法，这些组织包括心脏、骨骼肌、肝脏、心包、脂肪组织和大脑。

[0165] 取出来自猪和鼠源的组织并进行脱细胞化。使用各种洗涤剂对猪和鼠源的心脏组 织、骨骼肌和肝脏以及猪源的大脑、脂肪和心包进行脱细胞化。将心脏、骨骼肌和肝脏组织 切片至为～2mm厚，以去离子水漂洗，然后在PBS的十二烷基硫酸钠（SDS)中搅拌直至 脱细胞化。用于脱细胞化的时间取决于组织类型。将大脑切成两半，并在PBS的0.001% SDS中缓慢搅拌。将心包组织在PBS的1 % SDS中进行脱细胞化，将脂肪组织在2. 5mM脱氧 胆酸钠中进行脱细胞化，然后以脂肪酶进一步处理。液测试了其它脱细胞剂。然后将脱细 20 CN 胞化组织在去离子水中漂洗以保证洗涤剂的去除，然后冻干。

[0166] 除了脱细胞化的大脑和脂肪ECM，对脱细胞化ECM进行研磨以形成干燥粉末。然后 将ECM使用胃蛋白酶在低酸性条件下进行消化以形成用作涂层的可溶形式，然后使用0. IM 乙酸稀释至希望的浓度lmg/ml。使用垂直聚丙烯酰胺凝胶电泳证实在各个组织类型里肽碎 片的复杂的混合物，其在不同组织各不相同。这证实了在脱细胞化ECM中存在组织特异性 组分。

[0167] 这些涂层可以以如通常的单蛋白质涂层相同的方式应用于表面。通过在模拟体内 细胞外基质微环境的组织特异性涂层上培养细胞，具有对存活和细胞形态更好的控制，且 能增强分化。

[0168] 将鼠皮质神经元在大脑基质上培养并与聚-1-赖氨酸的标准涂层比较。鼠皮质神 经元在大脑基质涂层上存活，且与标准涂层相比，保持它们的分枝状形态更为持久。当骨骼 成肌细胞在骨骼肌基质涂

on the in vitro study of the traditional, and therefore the conversion from in vitro cell research to produce results in vivo environment restrictions. To overcome this limitation of cell-mediated therapy is very important, it depends on the culture over time, and the expanded cells to maintain the natural cell behavior.

[0095] In one aspect, the composition includes text obtained from pig skeletal muscle and cardiac ECM. The composition can be developed for different purposes are formed coated substrate. Some examples, ECM compositions may be melted to retain a complex mixture of muscle-specific ECM components. Some instances, the text of the coating can be more appropriately ECM emulate natural muscle in vitro.

[0096] when compared to cells seeded on the coated substrate is traditional on type I collagen, seeded in the number of myoblasts in skeletal muscle matrix i) positive muscle myosin heavy chain tube, ue) nuclei per myotube and iii) myotubes width showed significant growth. When the cells were seeded in traditional gelatin-coated on the substrate as compared to inoculated in human embryonic stem cells on myocardial substrate (HES2) derived cardiomyocytes showed significant growth in the following areas: i) myofibrils area, ii) each the number of nuclear cardiac muscle fiber area and iii) desmoplakin size, which highlights the desmosomal cell-cell junction protein, desmoplakin larger and more mature intercalated disk positioning. Some examples set composition to provide the ability to muscle in vivo ECM reconstruction. The composition can provide comments and keep the body muscle and stem cell behavior and natural state similar tools, and provides tools for in vivo cell-mediated therapy.

[0097] compared to standard gelatin coating, significantly increased average muscle fiber area in the heart of ECM growing cardiomyocytes Figure 2 shows. Figure 3 shows a comparison with a standard gelatin coating, the average number of myocardial cells was significantly higher than the cardiac ECM. As shown, when cultured on cardiac ECM, desmoplakin, a connexin intracellular, in the 112 day 4 specifically positioned between cardiomyocytes and form an ordered desmosomes, and cultured on gelatin The above phenomenon does not occur.

RegenX Science Exhibit 1004
Page 998

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0998

层上培养时，与标准的胶原涂层相比，还观察到增加的分化百分 率和增加的肌管宽度。最后，当接种于心脏基质涂层时，与通常的凝胶涂层相比，心肌来源 的人胚胎干细胞显示增加的组织形成和成熟，包括细胞间连接的形成。

[0169] 实施例10 [0170] 这里，研究文中所述的凝胶的使用，其中，所述凝胶是由天然脱细胞的骨骼肌ECM 形成。凝胶可以作为原位凝胶支架起作用，提供天然骨骼肌基质以提高在腿部损伤模型中的组织再生。其优势在于，骨骼肌ECM具有与体内发现的基质相似的组分，且可以提供合适 的用于组织工程和再生、细胞募集和细胞输送的平台。

[0171] 对猪骨骼肌进行脱细胞化。将组织切片为～2mm厚并以去离子水漂洗，然后在PBS 的1%十二烷基硫酸钠（SDS)中搅拌直至脱细胞化。然后将脱细胞组织在去离子水中漂洗 以保证洗涤剂的去除。将多块脱细胞化组织切片并使用苏木精和伊红染色以保证细胞的去 除。然后将脱细胞组织冻干并研磨形成精细粉末。

[0172] 然后将骨骼肌ECM在蛋白酶在低酸性条件下消化，然后通过加入氢氧化钠和IOX PBS中和至生理或近生理PH。然后将中和的骨骼肌ECM溶液以PBS稀释至希望的浓度6mg/ ml，并使其在37°C在96孔板内凝胶化。目视检查材料确定了成功的凝胶化。

[0173] 将浓度6mg/ml溶解的天然骨骼肌ECM通过25G针成功注射到大鼠大腿股肌中，形 成凝胶支架。凝胶作用在10～15min内发生。将肌肉和ECM切除，切片并使用苏木精和伊 红染色以确认骨骼肌ECM在骨肌肉中成功的凝胶作用。

[0174] 骨骼肌ECM也可以用于输送细胞，比如在ECM中的骨骼成肌细胞或其它肌肉相关 细胞类型。

[0175] 文中展示和描述了本发明的优选实施方式，但这些实施方式只是以例子的方式提 供，这对本领域的技术人员来说是显而易见的。本领域的技术人员能够在不脱离本发明的 范围内进行多种的变动、改变和替换。应当理解的是可以在实践中采用文中描述的本发明 的实施方式的各种替代对象。因此其包括所述权利要求所限定本发明的范围，以及这些权 利要求范围内的方法和结构及它们的等价形式。

21 CN 图1 22 明胶 心脏ECM O HI 明胶 心脏ECM 35天 112天 * p<0.05 ”p<0.01 心肌细胞的平均肌纤维区域 4 1 5 ？ 3 4 3 2 籁逛异if M2 IND CO 2/S Cz S2227225 > O Z 本本 HI

[0098] skeletal muscle matrix described herein can be prepared with heart ECM uses the same or similar manner. The matrix can be injected into skeletal muscle and skeletal muscle for skeletal muscle tissue engineering. Figure 5 shows the culture substrate in skeletal muscle skeletal myoblasts, as described in the text, which shows increased muscle tube size, enhanced differentiation, and each tube has a ratio of collagen muscle cells cultured myoblasts higher average Number of nucleoli. Transwell migration in vitro testing, as shown in Figure 6, skeletal myoblasts into skeletal muscle-specific migration matrix.

15 CN

[0099] by the following examples further illustrate the present invention, which should not in any way be construed as limiting the field of producing the present invention. Clearly, the skilled person can and consider various modifications within the field of the invention and variations of movement.

[0100] Example 1

[0101] The direct injection of the cells into the infarcted wall MI to treat various studies have been carried out, although many studies have shown a lower survival rate. The purpose of this study was to test the use of the gel as growth platform for cell adhesion, growth, maturation and endosomal transport. Providing the gel extracellular matrix by the native cardiac tissue formed, by promoting cell survival and helps the heart tissue regeneration.

[0102] Sprague Dawley female rats were euthanized so, use the Ott et (Nature Medicine, 14 (2), 213, 2008) to improve the program for their hearts were decellularized. Then lyophilized decellularized heart, rehydrated, and pulverized again lyophilized to form a dry powder. According Freytes etc. (Biomaterials 29: 1630,2008) The improved scheme, followed by a minimum pepsin digestion of the ECM and neutralized.

[0103] More specifically, adult Sprague Dawley female rats were heparinized and anesthetized by intraperitoneal pentobarbital. Cross-cut aorta and pulmonary artery and removing the heart. The catheter is inserted into the aorta and tie onto LangendorfT device modified.

RegenX Science Exhibit 1004
Page 999

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0999

明胶 心脏ECM * p<0.05 “p<0.01 112天 . 1 心脏ECM O 35天 心肌细胞的平均数量 S 1 籁堪長ip M3 IND 3/S > OOOO 3 2 1 (En) 弼辋 _ 明胶 5 15o 1.ei M4 组织化桥粒 心脏ECM 明胶 35天 培养于骨豁肌基质上的骨骼肌成肌细胞 %分化 * 胶原 骨骼肌基质 求本 * p<0.05 ** p<0.01 心脏ECM 112天 每肌管的核数 * 胶原骨骼肌基质 c . . . 2 1 O 親逛長班 Cz > 筠S 击耄S 4/S 6 4 2 O if 骨豁肌成肌细胞朝向骨骼肌基质的特异性迁移 上 胶原 心脏ECM 骨骸肌 ECM FBS 脑 趋化物 Cz A 筠S 击耄S 5/

[0104] using a modified previously disclosed techniques were decellularized heart. Briefly, to sodium dodecyl sulfate (SDS), and PBS solution reverse coronary vessels of the heart perfusion 24 hours, then a solution of triton PBS reverse perfused 30 minutes. Once decellularized completed, de-ionized water wash clean heart and lyophilization lyophilizer.

[0105] The frozen water cardiac rehydration, and then immersed in liquid nitrogen. Once frozen, the ball will be in the heart of the cup means to 70psi system rolling 10 seconds. The milled particles are then freeze-dried heart. Once dried, then freeze-dried heart tissue with pepsin combined and mixed with 0. OIM of HCl to a concentration of lOmg / ml. The solution was stirred at room temperature for 48 hours, so that solubilized extracellular matrix. After 48 hours, the ice HCl solution aliquoted in Eppendorf tubes, and 0. IN NaOH was neutralized to pH7. 4.

[0106] by the method described above, forming a natural rat heart ECM gel. As confirmed by the viscosity enhancing material to, 2. 5~ 8mg / mL of gel occurred successfully gelled within 15 minutes. With the higher density gel can be observed increased stiffness.

[0107] In 1 XPBS neutralized solution was diluted to 50 ii L per well were seeded in 96-well plates, and then transferred to 37 degrees centigrade and 5 percent CO2 incubator. After gel formation, using a pipette 100 y L 2d isolated neonatal cardiomyocytes moved to the upper portion of the gel, 60,000 cells per well. A few days later, to detect cell adhesion gel.

[0108] extracellular matrix of acellular tissue, ground and digested, once the solution in physiological conditions (pH = 7.4, 37 degrees C), the gel formation. Gel solution to a higher concentration of ECM tissue formation compared to gel at lower concentrations of ECM formation, harder and more opaque. Cells were seeded onto the gel and the gel can be adhered to survive thereon.

[0109] In the cardiac ECM gel seeded with 1 X IO4 cardiomyocytes showed the successful ECM adhesion and cell survival. These cells were cultured to four days in the ECM.

[0110] 30G needle through the heart of the IOOmL ECM solution (7mg / mL) was injected into the

anesthetized rat LV free wall. This study shows that, when in the physiological pH and temperature, natural cardiac extracellular matrix may be separated, dissolved and self-assembled into the gel. Since the gel contains all natural extracellular matrix components, although some messy, but so that the matrix allows cardiomyocytes in vitro and once injected into the body of the adhesion success and growth. And we believe that, since the first group from the ventricle into the gel matrix to more accurately simulate the heart in vivo environment, it can be more successfully supported the growth of cardiac cells, rather than other substrates such as collagen or fibrin gels.

[0111] Injectable gel could potentially be adapted to any three-dimensional shape and promotes cell survival within the heart transplant. Injection of 16 CN myocardial cells or differentiate into cardiac cells can help the heart tissue regeneration, promote cardiac output. The method developed to produce different concentrations and hardness of natural cardiac ECM gel platform, as well as provide in vitro cell growth platform, and as a scaffold for regeneration in situ. When injected into the body, natural ECM provide a suitable environment for complex cardiac tissue engineering to increase the cell stays and promote tissue regeneration.

[0112] Example 2

[0113] in cultured myocardial cells typically may be coated with one or more surface proteins of the extracellular matrix (ECM).

However, in vivo, cardiomyocytes in a highly complex extracellular environment; more accurately simulate the natural environment can be conducive to the ECM cultured myocardial cells survived. Here, reported has been solubilized form a coating, for newborn cardiac cells cultured natural heart ECM.

[0114] The heart was removed from Sprague-Dawley rats using a modified Langendorff program (Ott et al., By the improvement, 2008), the heart is decellularized. The decellularized heart lyophilized, rehydrated, and frozen in liquid N2, crushed into powder. To 0. OIM HCl minimum pepsin digestion ECM. After 48 hours, acetic acid was added 0. OIM final concentration of lmg / ml.

RegenX Science Exhibit 1004
Page 1001

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1001

[0115] ventricle using isolation kit (Cellutron, Highland Park, NJ) the new anatomy of 1-2 day old Sprague-Dawley rats to obtain cardiac myocytes. The initial supernatant was discarded, but the subsequent 20 minutes of digestion was containing 17 percent M199,10 percent horse serum, 5 percent fetal bovine serum and penicillin / streptomycin DMEM and suspended terminated.

After separation, the supernatant was pre-seeded in tissue culture dishes on styrene, improve the purity of myocardial cells to selectively adhered by fibroblasts.

[0116] at 37 degrees centigrade for 1 hour to lmg / ml natural heart ECM or collagen I (Sigma, St. Louis, MO) absorbed onto the coverslip. To 200000 / cm3 density inoculation separated neonatal cells, and in the medium after 24 hours and more replaced with low serum-free medium (DMEM, 18. 5 percent M199,5 percent HS, 1 percent FBS and antibiotics). Cell culture was maintained at 37 degrees centigrade and 5 percent CO2, daily monitoring, and replace with fresh medium every 2 to 3 days.

[0117] myocardial cells adhered to absorb the natural ECM, and the formation of local integration layer. Initially, a similar density cardiomyocytes adhered to the collagen-coated.

[0118] cell cultures were the first three days after vaccination began beating spontaneously. Cultured on collagen of myocardial cells begin to separate in the first 12 days, and stop beating at 14 days. But the heart of the culture in the natural form of ECM fibers cardiomyocytes clearly defined, which beat the same frequency until the first 28 days.

[0119] This study demonstrates that, due to the natural cardiac ECM more accurately simulate in vivo conditions, which is advantageous for the culture of cardiomyocytes. This study also demonstrated that neonatal cardiomyocytes adhered to ECM natural heart and more durable than a typical effect on collagen-coated. The new coating for culturing stem cell-derived cardiomyocytes and cardiac progenitor cells are useful.

[0120] Example 3

[0121] Here, the study of the natural heart of the extracellular matrix is acellular and solubilization of

adult ventricular cells coated purposes. The advantage is that the natural heart than traditional cell surface ECM has more components, and easier to use than other cell type pretreatment.

[0122] The heart was removed from Sprague-Dawley rats using a modified Langendorff program (Ott et al., By the improvement, 2008), the heart is decellularized. The decellularized heart lyophilized, rehydrated, and frozen in liquid N2, crushed into powder. To 0. OlM HCl minimum pepsin digestion ECM. After 48 hours, acetic acid was added 0. OlM final concentration of lmg / ml.

[0123] natural heart ECM stomach digests protein using DTT reductive conditions ventricle gel electrophoresis, and 17 CN with laminin (BD biosciences) and leather collagen (calf skin collagen) (Sigma) Compare. Use Imperial Protein Stain (Pierce) gel was stained. Compared with collagen and laminin, natural cardiac ECM can display a mixture of more complex ECM components.

[0124] using isolation kit (Cellutron, Highland Park, NJ) to obtain ventricular cardiac myocytes from 1-2 day old Sprague-Dawley rats. The initial supernatant was discarded, but the subsequent 20 minutes of digestion was containing 17 percent M199,10 percent horse serum, 5 percent fetal bovine serum and penicillin / streptomycin DMEM and suspended terminated.

After separation, the supernatant was pre-seeded in tissue culture dishes on styrene, improve the purity of myocardial cells to selectively adhered by fibroblasts.

[0125] at 37 degrees centigrade for 1 hour to lmg / ml natural heart ECM or collagen I (Sigma, St. Louis, MO) absorbed onto 48-well plates. In 200000 / cm2 seeded at a density of neonatal cardiac cells separated, and in the medium after 24 hours and more replaced with low serum-free medium (DMEM, 18. 5 percent M199,5 percent HS, 1 percent FBS and antibiotics). Cell culture was maintained at 37 degrees centigrade and 5 percent CO2, daily monitoring, and replace with fresh medium every 2 to 3 days. In the first two days, the first 4 days and 7 days the cells were fixed, and a actinin, connexin 43 (connexin 43), pan-cadherin, actin and nuclei staining. The first two days of myocardial cells in culture began beating spontaneously. Cultured on collagen cells begin to separate from the plate in the first eight

days. Training in the natural heart of a group of cells ECM beating continued until the first 45 days. Cultured on collagen that there are in the first 14 days the cells to stop beating.

[0126] existing cell culture coating is usually absorbed into simple protein plate or bracket of tissue culture. A more complex environment conducive to cell survival and maturation. This study shows that native cardiac ECM coating than other standard cell culture containing more complex components. Neonatal rat myocardial cells adhered to a cell culture coating of natural heart ECM, and spontaneously began to throb. Over time, the natural cardiac myocytes cultured in ECM on exhibit enhanced actinin, connecting protein 43 (connexin 43), pan-cadherin staining. And, compared with collagen, newborn cardiomyocytes having increased viability and adhesion on the natural heart ECM.

[0127] Example 4

[0128] Here, we studied the use of the text of the gel, wherein the gel made from natural decellularized heart ECM system. The gel can be used as in-situ gel scaffold, providing natural heart matrix, promoting cell LV wall and viable cells remain.

[0129] Sprague Dawley female on heart and pig hearts were decellularized. The cut to the heart tissue ~2mm thick and rinsed with deionized water, followed by stirring sodium dodecyl sulfate (SDS) in 4 to 5 days until the decellularized. In 1 percent Triton X-100 in 30 minutes of additional stirring to ensure complete decellularized step, followed by stirring overnight in deionized water and finally rinsed in deionized water.

[0130] and then lyophilized decellularized heart, crushed or ground into powder and freeze-dried to form a dry powder again. The ECM then digested in pepsin and neutralization.

[0131] and then by adding sodium hydroxide and cardiac ECM 10X PBS was adjusted to pH or dissolved in physiological pH 8. Cardiac extracellular matrix solution was then diluted in PBS and the cells to a desired concentration and after it in the 37 degrees centigrade in 96 well plates gelation.

The material was visually confirmed 2. 5~8mg / ml gel gelled success. With the higher density gel can

be observed increased stiffness.

[0132] tested different experimental conditions in order to determine the different digestion cardiac ECM scaffold gelled. Conducted ventricular gel electrophoresis to compare the contents of the digestive conditions, and compare the contents of ECM and rat tail collagen. Determining an initial pH plays an important role in the heart of the ECM in digestion and gelling. Digestion 48~72 hours.

[0133] gel electrophoresis 0. OlM HCl natural heart ECM incomplete digestion. Heart in 0. IM HCl digestion in 18 CN dirty show enhanced degradation of ECM. Thereby displaying a stronger heart ECM solution acidic conditions promote digestion and gel. The existence of a variety of additional pepsin heart than the heart ECM ECM and rat tail collagen instructions.

[0134] using scanning electron microscopy of extracellular matrix gel form of heart cell structure. With 2.5 percent glutaraldehyde gel 2 hours, followed by a series of ethanol rinse (30~100 percent), and dried at the critical point. Sample imaging performed before sputtering (sputter coated) chromium.

[0135] 30G needle by dissolving a concentration of 6mg / ml native cardiac ECM is ECM successfully injected into mice LV free wall that generates in situ gel scaffold, and proliferated in the myocardial cell adhesion thereon.

[0136] Example 5

[0137] using a commercially available assay kit migration was measured in vitro chemical decellularized cardiac ECM solution chemotactic properties (chemoattractive properties). Simply put, the human coronary artery endothelial cells (HCAECs) and rat aortic smooth muscle cells (RASMCs) serum starvation, and to evaluate the migration to the matrix, collagen, pepsin and fetal calf serum.

RASMCs show significant migration to the substrate, while HCAECs show trends. Thus, the matrix of biochemical signals having chemical can promote cell infiltration in vivo chemotactic properties.

[0138] In vivo, in the form of quantitative micro arterial injection area to evaluate angiogenesis. And 4h after injection compared to the density of

RegenX Science Exhibit 1004
Page 1005

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1005

arterioles in 11 days after injection was significantly greater.

[0139] Example 6

[0140] When injected into the myocardial wall after MI, several cell types have been shown to protect the heart function. However, no cell treatment can eliminate complications when using cell therapy generally low cell viability and immune response.

[0141] Myocardial infarction is the use of 25min ischemia-tank NOTE model in rats induced by closing of the left anterior descending artery.

In the week after MI, baseline function calculated from MRI FIG.

[0142] The pig myocardium small acellular ECM to the number of days in 1 percent SDS, followed by DI rinse overnight, lyophilized and milled to produce powder. Digested in 0. IM HCl to pepsin, thereby generating a soluble form of the material.

[0143] Prior to injection, the use of IM NaOH solubility ECM adjusted to PH 7. 4 and diluted with PBS to a 6mg / ml. MI after surgery, the animals were randomly divided into two groups, two weeks after infarction surgery, ECM or saline through 30G needle injection into Sprague Dawley female rats in LV free wall.

[0144] 4 weeks after injection surgery (six weeks after MI), the use of MRI evaluation of cardiac function again.

[0145] In ECM injected animals display hold function (based on the injection portion of the evaluation) in six weeks, and brine injection of animals do not maintain cardiac function. End-diastolic and end-systolic volume of the heart in animals injected ECM also get to the holding.

[0146] Example 7

[0147] Currently, the treatment of heart failure in clinical trials of stem cells and other cell types are used, which fed through a catheter into the heart through 27G wall. The pig ventricular tissues using SDS detergent decellularized and processed to form a soluble form of a matrix, and to physiological PH and diluted to 6mg / ml for injection.

RegenX Science Exhibit 1004
Page 1006

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1006

[0148] 2 ? Yorkshire pigs receiving coil induced myocardial infarction (coil-induced myocardial infarction), and two months after the infarct myocardium with a separate matrix or injected with cells.

[0149] The fetal bovine heart outside plant origin material was DiI, one kind of dye cyotoplasmic previously marked for histology identification. Use show enhanced survival of pro-survival of hESC cocktail (cocktail) in rodent models.

[0150] by NOGA positioning guide, the matrix alone or with the cells in a clinically relevant rate of 0. 2mL per 30s through catheter injection. 5 times a 0. ImL injection or by a separate matrix together with cells injected into infarcted edge 19 CN band area.

[0151] matrix alone or with the cells of the matrix can be successfully injected into the pig's heart, minimally invasive manner and without obstruction thin catheter.

Gel

[0152] Example 8

[0153] Here, the decellularized pericardial tissue sources described as having studied and used in vivo by promoting angiogenesis to improve cell retention in the LV wall and viable autologous therapy potential.

[0154] pig and human acellular pericardium are carried out. Juvenile male pig farm to make euthanasia, by the modified step Ott et (Nature Medicine, 14 (2), 213,2008) to their decellularized pericardium. Specifically, the pericardium was rinsed briefly in DI water, followed by stirring at sodium dodecyl sulfate (SDS) in 24h, then stirred at DI nearly 5h. heart tissue samples collected from the patient package accepted in cardiothoracic surgery. These samples were carried out in a similar manner decellularized: DI brief rinse, followed by three days in 1 percent SDS, followed by overnight DI rinse. In both cases, the complete decellularized were verified by histological staining.

[0155] The following steps ECM for pericardial and porcine samples are effective.

[0156] Next, the acellular pericardium or pericardial ECM frozen, freeze-dried and ground

RegenX Science Exhibit 1004
Page 1007

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1007

to form a fine, dry powder. Then by the Freytes et (Biomaterials 29 = 1630,2008) modified method, the ECM powder was dissolved in HCl pepsin digestion and neutralized.

[0157] gel electrophoresis (SDS-PAGE) showed that compared to the pepsin digested collagen greater complexity, the pericardium samples showing smaller width of the belt.

[0158] to confirm the complexity of the analysis to determine the protein fragments by mass spectrometry sample. Fragments of ECM proteins identified include collagen, elastin, fibrin, and various proteoglycans.

[0159] When the Loading and solution 150 U 1 to 96 and to be located within an incubator, in 2~3h observed after gelation.

[0160] by injection in vivo gelation and ECM solution 60 U 1 to Sprague Dawley male rats of left ventricular (LV) wall were observed. Slice cardiac histological staining from 45min after injection animals were sacrificed displayed in the ECM area LV wall visible gel.

[0161] In the same experiment, the animals were cultured for 2 weeks, after which they were sacrificed, their hearts were collected for sectioning.

At this point in time, ECM injection is still visible, but had been infiltrated cells.

[0162] Immunohistochemistry on tissue sections to determine the smooth muscle cells and endothelial cells, indicating that the blood vessels. ECM in the presence of a large number of injections vessels in the area of the material to promote the neovascularization.

[0163] Example 9

[0164] In vitro cell culture surface coating for generally formed of one or more extracellular matrix proteins. Which provides cell adhesion surface, but it does not mimic the in vivo cellular microenvironment. This paper presents a method for preparing absorbing coating various tissues derived extracellular matrix, these organizations include heart, skeletal muscle, liver, pericardium, adipose tissue and brain.

RegenX Science Exhibit 1004
Page 1008

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1008

[0165] removing tissue from pigs and murine and decellularized. Use various detergents for pigs and murine cardiac tissue, brain, liver, fat and skeletal muscle and pericardium and porcine were decellularized. The heart, skeletal muscle and liver tissue slices to the ~2mm thick, rinsed with deionized water, then stirred in PBS sodium dodecyl sulfate (SDS) until decellularized. Decellularized time to depend on the tissue type. The brain was cut in half and slowly stirred in a 0.001 percent SDS in PBS. The pericardial tissue in PBS, 1 percent SDS decellularized performed, will be decellularized adipose tissue in 2. 5mM sodium deoxycholate, followed by lipase further processing. Other acellular fluid testing agents. 20 CN is then de-fine cellular organization rinsed in deionized water to ensure removal of the detergent, and then lyophilized.

[0166] In addition to removal of the brain and fat cells ECM, of decellularized ECM grinding to form a dry powder. Then ECM pepsin digestion at low acidic conditions to form a soluble form as a coating, and then use 0. IM acetic acid diluted to the desired concentration of Img / ml. Using vertical polyacrylamide gel electrophoresis demonstrated a complex mixture of peptide fragments in various tissue types, with their varied in different tissues. This confirms the presence of acellular tissue-specific ECM components.

[0167] These coatings may be generally as a single protein coating applied to the surface in the same manner. By culturing the cells in a simulated in vivo extracellular matrix microenvironment tissue-specific coating having better survival and cell morphology control, and can enhance the differentiation.

[0168] The cultured rat cortical neurons and compared with standard poly-1-lysine coating on the substrate in the brain. Rat cortical neurons in the brain matrix coating survival, and compared with a standard coating, ramified keep their shape more durable. When skeletal myoblasts are cultured on a matrix coating of skeletal muscle when compared to standard collagen coating, also observed an increase in the percentage of differentiation and increased muscular tube width. Finally, when inoculated in the heart matrix coating when compared with the conventional gel coat, cardiac sources of human embryonic stem cells show increased tissue formation and maturation, including the formation of intercellular connections.

[0169] Example 10

[0170] Here, the use of the gel study described herein, wherein the gel is a natural ECM off the skeletal muscle cell formation. Gel scaffold can act as an in-situ gel provides natural matrix to increase skeletal muscle in the leg injury model of tissue regeneration. Its advantage is that skeletal muscle has a matrix similar to the ECM component and the body was found, and may provide a suitable platform for tissue engineering and regeneration, cell recruitment and cell delivery.

[0171] porcine decellularized skeletal muscle. Tissue sections as ~2mm thick and rinsed with deionized water, then stirred in PBS 1 percent sodium dodecyl sulfate (SDS) until decellularized. Then acellular tissue was rinsed in deionized water to ensure removal of the detergent. The multi-block decellularized tissue sections using hematoxylin and eosin staining to ensure that cell removal. Acellular tissue was then freeze-dried and ground to form a fine powder.

[0172] and then the skeletal muscle ECM protease digestion at low acidic conditions, and then by adding sodium hydroxide and IOX PBS and to physiological or near physiological PH. Then in the skeletal muscle and ECM solution PBS diluted to the desired concentration 6mg / ml, and allowed 37 degrees centigrade in a 96-well plate gelled. Visually inspect the materials to determine the success of gelation.

[0173] The natural skeletal muscle ECM concentration 6mg / ml dissolved success through 25G needle injection into the thigh muscle in rats share, forming a gel scaffold. Gelation occurs within 10~15min. The muscles and ECM cut, sliced and using hematoxylin and eosin to confirm the success of ECM gel skeletal muscle in bone muscle.

[0174] ECM in skeletal muscle cells can also be used for transport, such as the bones in the ECM muscle myoblasts or other related cell types.

[0175] text shown and described preferred embodiments of the present invention, but these embodiments are merely provided by way of example, that this skill is obvious. Those skilled in the art that various changes can be made without departing from the scope of the invention, changes, and substitutions. It should be

RegenX Science Exhibit 1004
Page 1010

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1010

understood that various alternatives to the object can use text described embodiments of the present invention in practice. So that includes the claims define the scope of the present invention, as well as methods and structures within the scope of these claims and their equivalents.

21 CN Figure 122 gelatin heart ECM O HI gelatin heart ECM 35 ? 112 days * p and lt; 0.05 "p and lt;? 0.01 average muscle fiber area cardiomyocytes 415 3432 Salar visiting different if M2 IND CO 2 / S Cz S2227225 and gt; OZ books HI gelatin heart ECM * p and lt; 0.05 "p and lt;. 0.01 112 day average number of 1 cardiac ECM O 35 ? myocardial cells S 1 scoundrel worthy of long-ip M3 IND 3 / S and gt; OOOO 3 2 1 (En) Bute rim _ gelatin 5 15o 1.ei M4 organization Desmosomes heart ECM gelatin 35 days cultured muscle to the bone matrix exclusion on differentiation of skeletal myoblasts percent * Collagen skeletal muscle matrix Shui present * p and lt; 0.05 ** p and lt; 0.01 heart ECM 112 days a muscular tube auditor * collagen skeletal muscle matrix c 2 1 O affinity stroll long class Cz and... gt; hit Cha Yun S S 4 / S 6 4 2 O if bone exempt muscle myoblasts migration toward skeletal muscle-specific Shang collagen matrix ECM bones heart muscle ECM FBS brain chemoattractant Cz A strike Cha Yun S S 5 /

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RegenX Science Exhibit 1004
Page 1011

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1011

PubMed

Format: Abstract

Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi. **2010** Sep;24(9):**1052**-7.

[Preliminary study on porous scaffold prepared with decellularized artery].

[Article in Chinese]

Duan H[1], Wu X, Gu Y, Wu Y, Li J, Chen B, Zhang S, Wang Z, Liu Z.

Author information

Abstract

OBJECTIVE: To investigate the feasibility of preparing the porous extracellular matrix (ECM) by use of some chemicals and enzymes to decellularize the porcine carotid artery.

METHODS: The porcine carotid artery was procured, and warm ischemia time was less than 30 minutes. The porcine carotid artery was decellularized with 1% sodium dodecyl sulfate (SDS) for 60 hours to prepare common ECM; then common ECM was treated with 0.25% trypsin (for 6 hours) and 0.3 U/mL collagenase (for 24 hours) to prepare porous ECM. The common ECM and porous ECM were stained with HE, Masson's trichrome, and Orcein to evaluate the histological features. Then the mechanical property, cytotoxicity, and pore size of ECMs were determined. After 4 weeks of subcutaneous implantation in dogs, the histological examination was used for the study.

RESULTS: Histological observation confirmed that 2 kinds of ECMs were decellularized completely and more porous structure was observed in porous ECM. Scanning electron microscope showed the pores in porous ECM were greater and the length of shorter axis in porous ECM ranged from 5 to 30 microm, the length of longer axis from 40 to 100 microm. The porosity of porous ECM (99.25%) was greater than that of common ECM (91.50%). The burst pressure of porous ECM decreased when compared with common ECM, showing significant difference [(0.154 3 +/- 0.0127) MPa vs. [0.305 2 +/- 0.015 7) MPa, $P < 0.05$]. There was no significant difference in suture retention strength between 2 kinds of ECMs ($P > 0.05$). The cytotoxicity test showed no obvious cytotoxicity in 2 kinds of ECMs. In vivo implantation test showed that the deeper host cells infiltration and more neo-microvessels in porous ECM were observed than in common ECM.

CONCLUSION: SDS and some enzymes can be used to prepare porous ECM as the scaffold for tissue engineered blood vessels.

RegenX Science Exhibit 1004
EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1012

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Publication types, MeSH terms

Publication types

English Abstract
Research Support, Non-U.S. Gov't

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Animals
Bioprosthesis*
Blood Vessel Prosthesis*
Carotid Arteries/cytology
Cell Line
Dogs
Extracellular Matrix
Fibroblasts/cytology
Materials Testing
Mice
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RegenX Science Exhibit 1004
EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1013

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《Chinese Journal of Ocular Trauma and Occupational Eye Disease(With Ophthalmic Surgery)》 2000-01

An experimental study of extracellular matrix of conjunctiva.

MENG Xin,ZHAO Guangxi,ZHANG Qinwei,et al.Department of Ophthalmology,Beijing Jishuitan Hospital,P.R.China 100035

Objective To investigate the characteristic of extracellular matrix (ECM) of conjuntiva and the function of it in conjunctivoplasty.Methods Pieces of conjuntival ECM were made and then the conjunctival defective region was repaired with it.All of them were photographed on 3rd day,the 1st,2nd,4th,and 8th week after neoplasty,and were examined by optical microscope,electron microscope (EM),immunohistochemical examination and lymphocytotoxicity test.Results The grafts approximated normal conjunctiva on the 4th week.Under optical microscope:epithelia covered all of grafting region on 4th week,and it approximated normal conjunctival epithelia.EM:the ultra structure of regenerative conjunctival epithalia was basically same as normal on 8th week.Immunohistochemical examination verified that ECM was type I and IV collagen both pre and post transplantation.Lymphercytotoxicity test showed that no obvious humoral immunitive reaction existed.Conclusion conjunctival ECM has the characteristic of tissue activity and low antigenicity.It is an ideal conjunctival succedaneum in conjunctiviplasty.

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RegenX Science Exhibit 1004
Page 1014

EX-2021
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RegenX Science Exhibit 1004
Page 1015

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1015

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Glutaraldehyde Treatment Elicits Toxic Response Compared to Decellularization in Bovine Pericardium

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Abstract

Go to: Go to:

Glutaraldehyde-stabilized bovine pericardium is used for clinical application since 1970s because of its desirable features such as less immunogenicity and acceptable durability. However, a propensity for calcification is reported on account of glutaraldehyde treatment. In this study, commercially available glutaraldehyde cross-linked bovine pericardium was evaluated for its *in vitro* cytotoxic effect, macrophage activation, and *in vivo* toxic response in comparison to decellularized bovine pericardium. Glutaraldehyde-treated bovine pericardium and its extract were observed to be cytotoxic and it also caused significant inflammatory cytokine release from activated macrophages. Significant antibody response, calcification response, necrotic, and inflammatory response were noticed in glutaraldehyde-treated bovine pericardium in comparison to decellularized bovine pericardium in a rat subcutaneous implantation model. Glutaraldehyde-treated bovine pericardium also failed in acute systemic toxicity testing and intracutaneous irritation testing as per ISO 10993. With respect to healing and implant remodeling, total lack of host tissue incorporation and angiogenesis was noticed in glutaraldehyde-treated bovine pericardium compared to excellent host fibroblast incorporation and angiogenesis within the implant in decellularized bovine pericardium. In conclusion, using *in vitro* and *in vivo* techniques, this study has demonstrated that glutaraldehyde-treated bovine pericardium elicits toxic response compared to decellularized bovine pericardium which is not congenial for long-term implant performance.

Keywords: Calcification, decellularized bovine pericardium, glutaraldehyde-treated bovine pericardium, immunogenicity, toxic response, tissue incorporation

INTRODUCTION

Go to: Go to:

Glutaraldehyde-stabilized bovine pericardium is commercially available and used for medical application in the form of patches and bioprosthetic valves on account of its low immunogenicity and excellent durability.[1] Glutaraldehyde treatment produces stable cross-links in cellular and extra-cellular matrix proteins which substantially reduced graft immunogenicity.[2] However, it was also shown that such tissue had altered mechanical property, early mechanical failure, cytotoxicity, and incomplete suppression of immunological recognition.[2–4] Besides this severe calcification was noticed in glutaraldehyde-treated

bovine pericardium in pediatric patients.[4] An emerging alternative to glutaraldehyde treatment for reducing xenograft immunogenicity is decellularization.[5,6] Use of decellularized xenograft is an approach where acellular tissue matrices are produced by selective removal of cellular components that are believed to promote immunogenicity and calcification. These acellular matrices promote induced regeneration through remodeling of the prosthesis by neo-vascularization, re-cellularization, and laying of new extracellular matrix by the host. The remodeled decellularized xenografts may functionally and structurally integrate into the host tissue.[6]

In this study, the *in vitro* and *in vivo* toxic effects of glutaraldehyde-treated bovine pericardium in comparison to decellularized bovine pericardium are reported. The *in vivo* effects of this toxic response such as inflammatory potential, immunogenicity, calcification potential, and lack of tissue incorporation are discussed as this may have effect on the long-term implant performance in clinical situation.

MATERIALS AND METHODS

Go to: Go to:

Glutaraldehyde-treated bovine pericardium

Commercially available glutaraldehyde-treated bovine pericardium (Glut BP) approved for clinical use in India was used in this study. This treatment group was named as Glut BP.

Decellularized bovine pericardium

Decellularization of bovine pericardium (Dcl BP) was achieved using a proprietary process based on a nondetergent method. Decellularization is confirmed by demonstrating the absence of nuclear remnants using routine HE staining as well as by the use of specific nuclear stain such as Hoechst 33258 on 5 μm paraffin embedded sections (n = 10). Besides this, residual DNA was extracted from 100 mg decellularized BP using DNA-XPress Kit (Hi-Media) and concentration was estimated spectrophotometrically (n = 3). This was followed by demonstration of extractable DNA by 1% agarose gel electrophoresis. The structural content was evaluated using histochemical staining of 5 μm paraffin embedded sections using Movats Pentachrome staining (n = 10). Decellularized bovine pericardium was sterilized by a proprietary process, and sterility was confirmed by routine sterility testing.

Collagenase susceptibility

This study was conducted to assess the ability of the graft to undergo *in vivo* degradation which will pave the way for laying the host extracellular matrix. In this test, the susceptibility of the test material to Collagenase II activity (from *Clostridium histolyticum*, 125 μm/mg, Sigma Aldrich) was studied. Approximately 50 mg (wet weight in triplicates) of glutaraldehyde-treated bovine pericardium (Glut BP) and decellularized bovine pericardium (Dcl BP) were weighed. To the weighed samples, 0.5 mL of 0.1 M Tris chloride (pH 7.4) containing 0.005 M calcium chloride and 0.05 mg/mL sodium azide was added. The samples were incubated at 37°C for 1 h with constant shaking. Collagenase enzyme was prepared in 0.1 M Tris chloride (pH 7.4) at 37°C and added in to the vials so as to make the final concentration to 2 U/mg of tissue. The vials were incubated at 37°C for 24 h, 72 h, and 7 days, at the end of which, the samples were centrifuged at 12,000 rpm for 20 min at 4°C and the remaining tissue samples are blotted in filter papers for 5 min to dry them. They were weighed using Afcoset Electronic Weighing Balance ER120A. The weight loss was determined by paired comparison before and after treatments.

In vitro response

xi toxic responses of Glut BP and Dcl BP were assessed by cytotoxicity studies using L929 fibroblast cells by a direct contact method as well as by studying the effect of the extract on L929 fibroblast metabolism by MTT assay. *In vitro* inflammatory response was studied by macrophage activation studies.

Cytotoxicity testing: a direct contact method

Cytotoxic potential of Glut BP and Dcl BP groups on account of their direct contact or due to leachables was evaluated by a direct contact method (ISO 10993-5). The test samples were rinsed thrice with normal saline before cytotoxicity evaluation. Test samples, negative control (high density poly ethylene, USP), and positive control (copper) in triplicate were placed on a subconfluent monolayer of L-929 mouse fibroblast cells. After incubation of cells with test samples at 37 ± 2°C for 24 ± 1 h, the cell culture was examined microscopically for cellular response around the samples. Cellular responses were expressed as noncytotoxic, mildly cytotoxic, moderately cytotoxic, and severely cytotoxic.

Cytotoxicity testing on extract

In vitro MTT assay on extract of Glut BP and Dcl BP in physiological saline was performed to measure the effect of extract on the metabolic activity of L929 fibroblast cells. The ability of the fibroblast cells to metabolize yellow-colored tetrazolium salt 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide to purple-colored formazan was measured as an indication of its activity. Different dilutions (50% and 25%) of extracts of Glut BP sample, Dcl BP, positive control (dilute phenol), negative control (high density poly ethylene (USP)), and reagent control in triplicate were placed on the subconfluent monolayer of L929 cells. After incubation of cells with the extract at 37 ± 2°C for 2 h, extract and control medium were replaced with 50 μL MTT solution (1 mg/mL in the medium without supplements) wrapped with aluminum foils and were incubated at 37 ± 2°C for 2 h. After discarding the MTT solution, 100 μL of isopropanol was added to all wells and swayed the plates. The color developed was quantified by measuring absorbance at 540 nm using a microplate reader. The data obtained were compared with the negative control.

Macrophage activation

Macrophage activation was studied by estimating inflammatory cytokine released by adhered macrophages on contact with materials belonging to different groups. THP-1 (human acute monocytic leukemia cell lines) cells were grown in RPMI 1640 (Sigma) containing 10 % v/v fetal bovine serum (Gibco), 160 U/mL benzylpenicillin, and 100 U/mL streptomycin (Sigma). The cells were maintained at 37°C in an atmosphere of 95% air and 5% CO_2 at 90% relative humidity. The differentiation into macrophages was induced by treating THP-1 cells in a 24-well plate for 24 h with RPMI-1640 containing 20 ng/mL phorbol 12-myristate 13- acetate (PMA). Thereafter, a fresh medium was added and cells were grown for another 24 h under similar conditions. Release of proinflammatory cytokines (IL-1b, IL-6, and TNF-a) from THP 1 cells upon contact with triplicate samples of Glut BP and Dcl BP and positive control (bacterial lipopolysaccharide) at 20 pg were carried using ELISA. The concentration of cytokines in the culture supernatant was quantified by a specific ELISA kit (U-CyTech biosciences, Netherlands) as per manufacturer instructions.

In vivo response

In vivo toxic response was studied by conducting an acute systemic toxicity testing (ISO 10993-11) and an intracutaneous (intradermal) reactivity test (ISO 10993-10). *In vivo* response such as immune response, calcification response, and tissue response was studied by juvenile rat subcutaneous implantation for 60

days. The animal test protocols were approved by Institutional Animal Ethics Committee and animal care and maintenance were done as per CPCSEA guidelines.

Acute systemic toxicity testing (ISO 10993-11)

This test was done to evaluate the systemic response of mice following intraperitoneal and intravenous injection of cotton seed oil and physiological saline extract of the test samples, respectively. The test samples were prepared by washing the samples in sterile normal saline for three wash cycles, with each cycle lasting for 10 min. The samples were dried at 37°C for 24 h. Two grams each of Glut BP and Dcl BP samples were extracted with 10 mL each of physiological saline and cotton seed oil for 72 h at 50°C. Then, 50 mL/kg body weight of the above extracts were injected through intravenous ($n = 5$) and intraperitoneal routes ($n = 5$), respectively, in separate groups of randomly selected health active mice. Injection of plain extraction media in another set of animals served as control. The animals were observed immediately and after 4, 24, 48, and 72 h for evidence of abnormalities such as convulsions, prostrations, loss of body weight more than 2 g and death.

Intracutaneous (intradermal) reactivity test (ISO 10993-10)

This test was done to assess the potential of the material to produce irritation following intradermal injection of the material extract. The test samples were prepared by washing the samples in sterile normal saline for three wash cycles, with each cycle lasting for 10 min. The samples were dried at 37°C for 24 h. Two grams each of Glut BP and Dcl BP samples were extracted with 10 mL each of physiological saline and cotton seed oil for 72 h at 50°C. Then, 0.2 mL (per each site) of the extract was injected into five sites with appropriate controls in randomly selected healthy, active New Zealand white rabbits. The animals were observed for erythema and edema which was scored as 0 (nil), 1 (very slight), 2 (well-defined edema/erythema), 3 (moderate), and 4 (severe). The requirement of the test is met if the difference between the test sample mean score and control sample mean score is 1.0 or less. All the procedures were done aseptically.

Implantation study

This study was done to evaluate calcification potential, immunogenic potential, and tissue response by subcutaneous implantation for 60 days in 3-week-old male Wistar rats. Healthy animals were randomly assigned into Glut BP and Dcl BP groups with each group consisting of five animals. The animals were subcutaneously implanted under the dorsal skin with evenly placed six implants of the same treatment group. The animals were observed daily for 60 days and at the end of which they were killed. The serum was collected for estimating antibody response against bovine pericardial proteins. One set of explants ($n = 15$) were used for calcium estimation and another set ($n = 15$) was processed for histopathological evaluation.

Antibody response

The antibody response (IgG, IgM, and IgA) elicited in different groups of animals implanted with Glut BP and Dcl BP samples was assessed using indirect ELISA. For this, extractable protein of fresh bovine pericardium was coated in the 96 well ELISA immuno plate (F96 MAXISORP, NUNC) in PBSN (2 μg protein per well). Serum samples at 1:1000 dilution prepared in blocking buffer from animals belonging to the above groups ($n = 5$) was used as primary antibody. Serum from animals which is immunized with extractable protein from fresh bovine pericardium was treated as positive control. Rabbit polyclonal to rat IgG + IgM + IgA conjugated with HRP (Abcam ab8521) was used as secondary antibody. After the

reaction, the ELISA plates were read with microplate reader Expert Plus (ASYS Hitech GmbH, Austria). OD readings of micro wells without secondary antibody were taken as the negative control. OD values subtracted from the mean negative control values were used for comparison.

Calcification response

Calcium estimations from explanted samples were done as per the procedure reported earlier.[7] In brief, the explanted Glut BP and Dcl BP samples (n = 15, three samples from each animal) were digested in 2 mL of 2 N HCl for 48 h at 60°C. The supernatant was neutralized with 2 N NaOH followed by the estimation of calcium using the colorimetric method of O-Cresolphthalein complexone obtained as a standard kit. OD readings were taken at 578 nm using a Shimadzu UV-1700 spectrophotometer, Japan.

Tissue response

The explanted Glut BP and Dcl BP samples (n = 15, three samples from each animal) were fixed in 10% buffered formalin, embedded in paraffin, and 5 μm sections were made. The sections were stained by hematoxylin and eosin and Von Kosa stain. A qualitative assessment was made for peri-implant necrosis, inflammatory response, peri-implant fibrosis, tissue incorporation, angiogenesis in the implanted material and calcification.

Statistics

All quantitative estimations are expressed in mean ± SD. Significance testing for differences of means between groups is carried out using the unpaired Student-t test for equal or unequal variance depending on the homogeneity of variance tested using the F-test.. Statistically significant difference between means was assumed whenever P-value was less than 0.05.

RESULTS

Go to: Go to:

Confirmation of decellularization

Nuclear remnants could not be demonstrated both by hematoxylin and eosin as well as by Hoechst 33258 staining [Figure 1], indicating satisfactory decellularization. DNA extraction yielded a residual DNA content of 7 ± 3.5 μg/mL in decellularized bovine pericardium compared to 97 ± 2 μg/mL in fresh bovine pericardium (triplicate samples). Extractable DNA from decellularized tissue could not be demonstrated in 1% agarose gel electrophoresis probably because of extensive DNA fragmentation [Figure 2]. Structurally, decellularized pericardium mainly consisted of well-separated collagen bundles with a few elastin fibers as demonstrated by Movats Pentachrome staining.

Collagenase susceptibility

Data on residual weight of different groups following Collagenase II digestion are given in Table 1 and data on degradation are given in Table 2.

Following Collagenase type II digestion for 24 h, Dcl BP samples had 90.2% degradation with a residual weight of 4.9 ± 0.4 mg. Glut BP group had maximum Collagenase resistance with minimum weight loss of around 1 mg (2%). At 72 h residual weight Dcl BP and Glut BP groups showed a residual weight of 1.8 ± 0.3 mg (96.5% degradation) and 49 ± 1 mg (2% degradation), respectively. At 7 days, the residual weights were 1.3 ± 0.25 mg and 48 ± 1 mg for Dcl BP and Glut BP groups, respectively. At this period, the rate of weight loss has slowed down in the Dcl BP group with only an additional 1% weight loss was noticed in

about 7 days. The Glut BP group continued to lose weight at very low rate reaching about 4% at 7 days.

In vitro response

Cytotoxicity: A direct contact Dcl BP samples were seen noncytotoxic to L929 fibroblasts by a direct contact method [Figure 3]. The Glut BP sample was seen moderately cytotoxic probably because of residual glutaraldehyde. The positive control was severely cytotoxic, and the negative control was noncytotoxic.

Cytotoxicity testing on extract The MTT assay of L929 cells after contact with 50% and 25% extracts of Dcl BP showed 96% and 97% metabolic activities, respectively, compared to Glut BP which had 14% and 15.1% metabolic activities, respectively. This clearly showed that the extract of Glut BP samples has significantly affected the metabolic activity of fibroblast cells and hence it is toxic in nature compared to Dcl BP.

Macrophage activation Cytokine release profile of activated macrophage in response to exposure to different groups is given in Table 2. The positive control group showed the highest cytokine response. With respect to the TNFα and IL1β, highest macrophage activation was seen in the Glut BP group. This was seen statistically significant compared to the Dcl BP group. In the case of IL6, the Dcl BP group showed a higher response when compared to the Glut BP group ($P = 0.004$).

In vivo response

Acute systemic toxicity testing (ISO 10993-11) Administration of cotton seed oil extract of the Glut BP sample through an intraperitoneal route did not produce a systemic toxic effect evidenced as loss of body weight more than 2 g, the presence of any abnormalities or death. However, intravenous administration of physiological saline extract of the Glut BP sample produced abnormalities and death immediately after injection in three mice, indicating systemic toxicity of this sample. The control animals did not show any signs of systemic toxicity. The cotton seed oil extract and physiological saline extract of the Dcl BP sample did not produce any abnormalities, loss of weight or death following intraperitoneal and intravenous administration, respectively. This indicated that the Dcl BP extract do not cause systemic toxicity.

Intracutaneous (intradermal) reactivity test (ISO 10993-10)

The physiological saline extract of the Glut BP sample produced an average irritation score of 1.18 which is a positive reactivity for this test. At the same time, the cotton seed oil extract showed 0 score of irritation indicating no intracutaneous irritation. The physiological saline and cotton seed oil extract of Dcl BP samples produced irritation scores of 0.3 and 0.1, respectively, which indicated that the samples are nonirritating as per the set criteria for this test.

Implantation study

Animal implantation was uneventful and all animals in the groups Glut BP and Dcl BP survived the duration of the study uneventfully.

Antibody response

Antibody response against extractable protein from fresh bovine pericardium elicited in animals implanted with different treatment groups is given in Table 2. The Dcl BP group had significantly lower antibody response against fresh bovine pericardial protein compared to the Glut BP group ($P < 0.05$). This indicated

that glutaraldehyde treatment which is done to reduce immune response of xenografts has failed to reduce it in comparison to decellularization.

Calcification response

The result of calcification experiment is given in Table 2. The Dcl BP group explant had significantly lower calcium content of 157 ± 31 μg/100 mg dry weight of tissue in comparison to 434 ± 84 μg/100 mg of dry weight of the Glut BP explant.

Tissue response

The tissue response studied on hematoxylin and eosin stained paraffin sections is presented in Figure 4. The sections were qualitatively assessed for peri-implant necrosis, inflammatory response, calcification, peri-implant fibrosis, tissue incorporation, and angiogenesis into the implanted material. The Dcl BP implant did not show any peri-implant necrosis. A thin fibrous capsule could be appreciated around the implant. Mild-to-moderate diffuse infiltration of mononuclear cells into the implant is noticed indicating chronic inflammation. Macrophages could be identified at the site of implant degradation. Implant calcification could not be observed. Implant showed excellent ingrowth of cells with fibroblast morphology with evidence of newly laid collagen. Angiogenesis is noticed in the implant as well at the periphery.

Glut BP explants showed a thick capsule around the implant. Certain areas showed peri-implant necrosis. Angiogenesis was seen only in the periphery of the implant. Focal mild-to-moderate chronic inflammation observed as mononuclear cell infiltration could be seen at the periphery of the implant. The implant interior was remarkably acellular. Calcium deposit could be observed in many sections.

DISCUSSION

Go to: Go to:

In this study, it is attempted to demonstrate that glutaraldehyde treatment of bovine pericardium induces toxic response which will ultimately affect its long-term performance in clinical situation. This is because of its cytotoxicity, persistent inflammation, immunogenicity, calcification, and lack of remodeling. It is reasoned that decellularization of bovine pericardium will bring about positive remodeling of the implant which is brought about by its susceptibility to matrix metalloproteinase which promotes intraimplant host tissue incorporation, new matrix deposition, and angiogenesis besides its low immunogenicity, inflammatory potential, and calcification potential. In this study, a commercially available clinically used glutaraldehyde-treated bovine pericardium was studied and hence it was not further characterized. Decellularized tissue produced by a nondetergent based decellularization method was used for comparison. The decellularization method employed was found adequate as nuclear remnants could not be detected by routine HE staining or by nuclear stain Hoechst 33258. Polyacrylamide gel electrophoresis of residual DNA could not demonstrate DNA bands indicating extensive DNA fragmentation.

The data clearly indicated that the glutaraldehyde-treated bovine pericardium is resistant to Collagenase which is a major enzyme in the body responsible for extracellular matrix remodeling. In the rat subcutaneous implantation model, this was seen as acellular areas in the implants indicating the absence of tissue penetration into the implant. In comparison decellularized bovine pericardium was seen susceptible to Collagenase enzyme and correspondingly the *in vivo* data showed excellent host tissue incorporation into the implant.

In vitro data such as direct cytotoxicity as well as toxicity of extract clearly suggested cytotoxic nature of

the glutaraldehyde-treated bovine pericardium. This is clearly correlated in rat implantation studies where it was observed as areas of peri-implant necrosis around Glut BP samples. Whereas Dcl BP samples or its extracts were seen to be noncytotoxic and subsequently peri-implant necrosis was absent in the rat study. The intracutaneous irritation study and systemic toxicity also showed that the physiological saline extract of the glutaraldehyde-treated bovine pericardium is of toxic nature. Interestingly, only the water-soluble extract was observed as toxic whereas as the cotton seed oil extract was seen nontoxic. Earlier studies have reported on the cytotoxic nature of glutaraldehyde-treated tissue without indicating which fraction is cytotoxic.[8] Macrophages as inflammatory cells as well as antigen-presenting cells have an important role in *in vivo* response against biomaterials. Hence a macrophage activation study was conducted. This study showed a definite increase in the release of inflammatory cytokines such as TNFα and IL1β in Glut BP samples compared to Dcl BP samples. This clearly indicated that the glutaraldehyde-treated bovine pericardium activates macrophages significantly in comparison to decellularized bovine pericardium. Increased inflammatory response noticed in Glut BP samples of a rat implantation study might be a reflection of what is observed in *in vitro*. Interestingly, IL6 release was seen higher in the Dcl BP sample compared to Glut BP samples. In contrast to the above observations, a study showed no significant variation between *in vitro* cytokine release of inflammatory and anti-inflammatory cytokines in response to different polymeric materials.[9]

In vivo data such as antibody response, calcification response, and tissue response demonstrated better biocompatibility for decellularized bovine pericardium compared to glutaraldehyde-treated one. For studying the immune response, focus was given on antibody response elicited against extractable protein from fresh pericardium as it was assumed that in processed bovine pericardium the residual protein is the major immunogen. Since there was complete fragmentation of DNA following decellularization, immunogenicity on this account was not anticipated. DNA fragments less than 300 bp is reported to be nonimmunogenic.[10] Decellularized bovine pericardium had significantly reduced antibody response compared to glutaraldehyde-treated one. This indicated that glutaraldehyde treatment is not necessarily immunosuppressive. A study on antigenicity of glutaraldehyde-treated bovine serum albumin (BSA) reported that glutaraldehyde caused intermolecular cross-linkages forming soluble aggregates; such modified proteins were seen antigenic and produced antibodies against antigenic determinant of BSA as well as against newly acquired groups arising from this modification.[11] The increased antibody response seen in the glutaraldehyde-treated samples might be due to formation of soluble aggregates of BSA. *In vivo* calcification was seen directly linked to the extent of glutaraldehyde treatment. Glutaraldehyde-treated samples were seen most calcifying as previously reported.[12] Decellularized pericardium had minimum calcification. A previous study has reported direct relation between calcification and antibody response.[13] This relationship was noticed here also. Decellularized pericardium and glutaraldehyde-treated pericardium presented two extreme tissue responses. Glutaraldehyde-treated pericardium showed no host cell incorporation into implant and a thicker fibrous tissue capsule separated the implant from the host tissue. The presence of mild-to-moderate chronic inflammation is noticed in the implant periphery. There is calcification as well as significant antibody response in this group. These conditions may not favor a positive tissue remodeling.[10] Similar observation was previously reported for glutaraldehyde-treated tissue.[8] Decellularized bovine pericardium showed contrasting and desirable tissue response such as thin fibrous capsule, undetectable calcification, implant degradation with laying of new collagen, and mild chronic inflammation. All the above observations point to better implant remodeling.[14] It is assumed that the presence of mononuclear cells in this group should not be of concern since the antibody response elicited in this group was significantly lower compared to the Glut BP group. In contrast the observations noted in the glutaraldehyde-treated group such as thicker fibrous capsule, implant

calcification, moderately intense mononuclear cell infiltration in the periphery and angiogenesis restricted to implant periphery and visible lack of host cell incorporation into implant are not conducive for the sustained implant function. Hence the observations made in glutaraldehyde-treated bovine pericardium are not congenial for an acceptable tissue compatibility as well as implant remodeling which is essential for successful long-term clinical performance of a surgical implant.

CONCLUSION

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Thus, it is concluded that glutaraldehyde-treated bovine pericardium incites significant toxic, inflammatory, immune, and calcification responses which is not desirable for a long-term function as an implant. Furthermore, glutaraldehyde also prevents tissue remodeling on account of its resistance to matrix metalloproteinase resulting in the implant remaining as a separate entity in the body without physiologically integrating into the host.

ACKNOWLEDGMENTS

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Footnotes

Go to: Go to:

Source of Support: Department of Biotechnology, Government of India

Conflict of Interest: No conflict of interest.

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Go to: Go to:

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Figures and Tables

Go to: Go to:

Figure 1

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Confirmation of decellularization with Hoechst 3328 staining. (a) Decellularized pericardium with no nuclear remnants. (b) Normal pericardium showing intact nucleus (Hoechst 33258, 400×)

Figure 2

Open in a separate window

Extractable DNA from decellularized bovine pericardium: first lane: 100 bp DNA ladder, second and third lanes: DNA from decellularized bovine pericardium, fourth lane: DNA from fresh bovine pericardium

Table 1

Residual weights in different groups with time following Collagenase type II digestion

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Table 2

Toxic response (*in vitro* and *in vivo*) of Glut BP compared to Del BP

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Figure 3

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(a) Glut BP sample showing moderate cytotoxicity (b) Dcl BP sample showing noncytotoxic nature

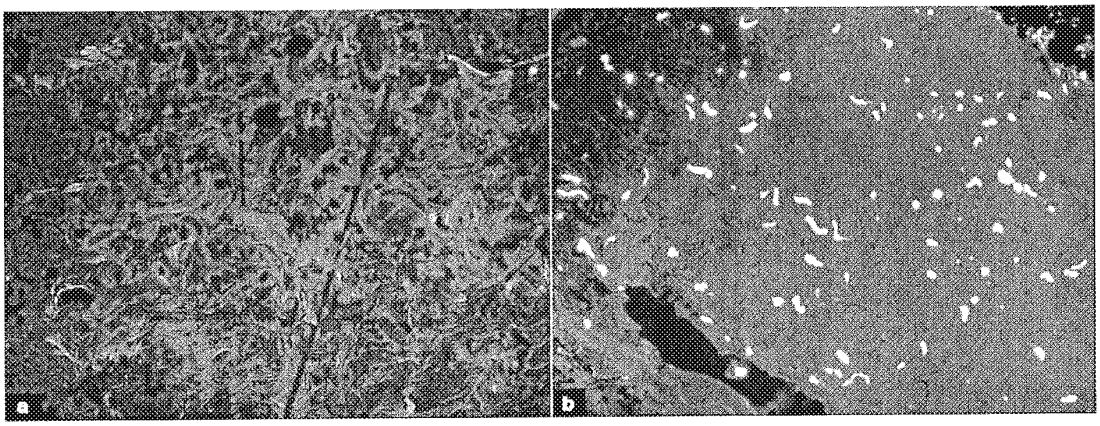
Figure 4

Open in a separate window

Tissue response to different implants at 60 days in rat subcutaneous implantation. (a) Dcl BP group minimum inflammatory response and uniform host cell incorporation and angiogenesis (arrow) within implant H and E, ×400. (b) Glut BP group, inflammatory response (arrow) in the interphase and remarkably acellular interior (H and E, ×200)

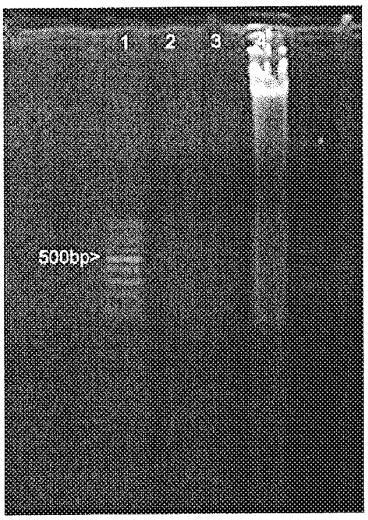
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Figure 1



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Figure 2



Extractable DNA from decellularized bovine pericardium; first lane: 100 bp DNA ladder, second and third lanes: DNA from decellularized bovine pericardium, fourth lane: DNA from fresh bovine pericardium

Table 1

Residual weights in different groups with time following Collagenase type II digestion

Residual weight (mg)	24 h	72 h	7 days
Dcl BP	4.9 ± 0.4*	1.8 0.3*	1.3 ± 0.2
Glut BP	49 ± 1*	49 1	48 ± 1

* Statistically significant at P < 0.05. Comparison was made between groups and between different time periods of the same group

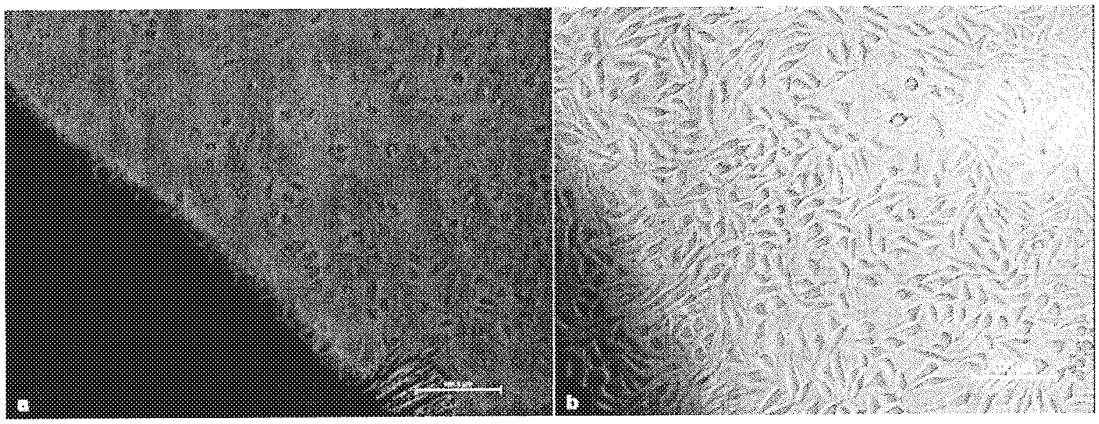
Table 2

Toxic response (*in vitro* and *in vivo*) of Glut BP compared to Dcl BP

Treatment groups	Dcl BP	Glut BP
Susceptibility to Collagenase type 2 (*in vitro*) %	90.2% degradation in 24 h; 96.5% degradation in 72 h; 97.4% in 7 days	2% degradation in 24 h, 2% in 72 h; 4% in 7 days
In vitro cytotoxicity to L929 fibroblast cells	Noncytotoxic	Moderately cytotoxic
In vitro cytotoxicity of extract	Noncytotoxic	Cytotoxic
In vitro Macrophage activation* *		
IL1 beta (pg)	82.4 ± 4.1*	144 ± 7.4*
IL6 (pg)	59.1 ± 3.6*	46 ± 0.72*
TNF alpha (pg)	45.3 ± 6.6*	267 ± 22.9*
Systemic toxicity		
Intravenous route	Nontoxic	Toxic
Intraperitoneal route	Nontoxic	Nontoxic
Intracutaneous irritation		
Cotton seed oil extract		
Physiological saline extract	Nontoxic	Nontoxic
	Nontoxic	Toxic
In vivo antibody response to BP proteins (OD)	0.83 ± 0.08*	1.11 ± 0.16*
In vivo calcification response (μg/100 mg dry weight)	157 ± 31*	434 ± 84*
Tissue response		
Necrosis	Absent	Present
Inflammation	Mild to moderate, diffuse	Focal, mild to Moderate Thick
Fibrosis (peri-implant capsule)	Thin	
Tissue incorporation	Present	Absent
Angiogenesis within implant	Present	Focal deposit
Calcification	Not detectable	seen

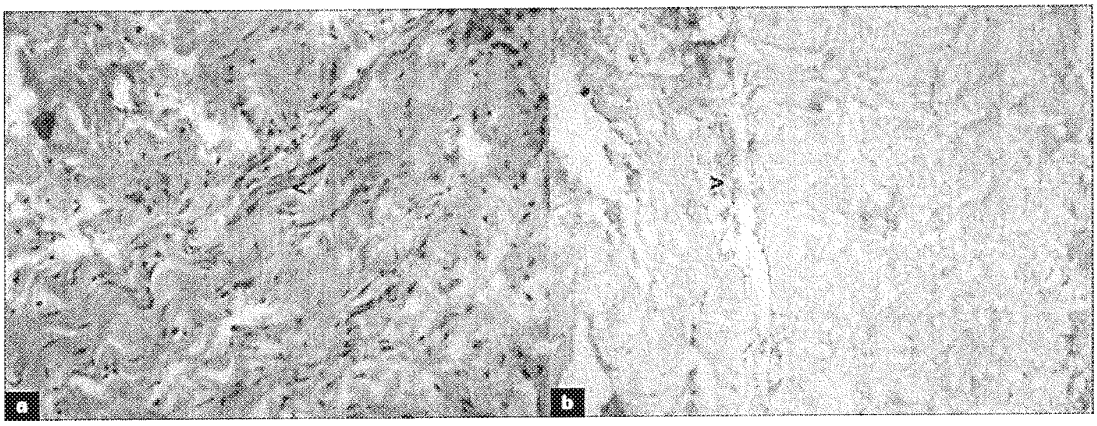
*Statistically significant difference at $P < 0.05$. ** LPS activation caused cytokine release at, IL1β: 255 pg; IL6: 280 pg, and TNFα: 346 pg.

Figure 3



(a) Glut BP sample showing moderate cytotoxicity (b) Dcl BP sample showing noncytotoxic nature

Figure 4



Tissue response to different implants at 60 days in rat subcutaneous implantation. (a) Dcl BP group minimum inflammatory response and uniform host cell incorporation and angiogenesis (arrow) within implant H and E, ×400. (b) Glut BP group, inflammatory response (arrow) in the interphase and remarkably acellular interior (H and E, ×200)

Doc Code: TRAN.LET
Document Description: Transmittal Letter

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TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission	

Application Number	14/759,895-Conf. #3358
Filing Date	July 8, 2015
First Named Inventor	Ryanne Early
Art Unit	1651
Examiner Name	R. A. Davis
Attorney Docket Number	S1860.70000US01

ENCLOSURES (*Check all that apply*)

- [x] Fee Transmittal Form
- [ ] Fee Attached
- [x] Amendment/Reply
- [ ] After Final
- [ ] Affidavits/declaration(s)
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Request for Continued Examination
FORM PTO-1449/A and B
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Cited References

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Edward R. Gates/		
Printed name	Edward R. Gates		
Date	October 22, 2018	Reg. No.	31,616

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Complete if known	
Application Number	14/759,895-Conf. #3358
Filing Date	July 8, 2015
First Named Inventor	Ryanne Early
Examiner Name	R. A. Davis
Art Unit	1651
Practitioner Docket No.	S1860.70000US01

[x] Applicant asserts small entity status. See 37 CFR 1.27.

[ ] Applicant certifies micro entity status. See 37 CFR 1.29. Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

TOTAL AMOUNT OF PAYMENT ($)1,350.00

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[ ] Check [x] Credit Card [ ] Money Order [ ] None [ ] Other (please identify): _____

[x] Deposit Account Deposit Account Number: 23/2825 Deposit Account Name: Wolf, Greenfield & Sacks, P.C.

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1. BASIC FILING, SEARCH, AND EXAMINATION FEES (U = undiscounted fee; S = small entity fee; M = micro entity fee)

	FILING FEES			SEARCH FEES			EXAMINATION FEES			
Application Type	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**Fees Paid ($)**
Utility	300	150*	75	660	330	165	760	380	190	
Design	200	100	50	160	80	40	600	300	150	
Plant	200	100	50	420	210	105	620	310	155	
Reissue	300	150	75	660	330	165	2,200	1,100	550	
Provisional	280	140	70	0	0	0	0	0	0	

* The $150 small entity status filing fee for a utility application is further reduced to $75 for a small entity status applicant who files the application via EFS-Web.

2. EXCESS CLAIM FEES

Fee Description	**Undiscounted Fee ($)**	**Small Entity Fee ($)**	**Micro Entity Fee ($)**
Each claim over 20 (including Reissues)	100	50	25
Each independent claim over 3 (including Reissues)	460	230	115
Multiple dependent claims	820	410	205

Total Claims		**Extra Claims**	**Fee ($)**		**Fee Paid ($)**
_____	- 20 or HP =	_____	x _____	=	_____

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims		**Extra Claims**	**Fee ($)**		**Fee Paid ($)**
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Multiple Dependent Claims

Fee ($)	**Fee Paid ($)**

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If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $400 ($200 for small entity) ($100 for micro entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

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_____	- 100 =	_____	/50 =	_____ (round **up** to a whole number) x	_____	= _____

4. OTHER FEE(S)

	Fees Paid ($)
Non-English specification, $130 fee (no small or micro entity discount)	
Non-electronic filing fee under 37 CFR 1.16(t) for a utility application, $400 fee ($200 small or micro entity)	
Other (e.g., late filing surcharge): 2253 Extension for response within third month	700.00
2801 Request for continued examination (RCE) - 1st ...	650.00

SUBMITTED BY					
Signature	/Edward R. Gates/	Registration No. (Attorney/Agent)	31,616	Telephone	617.646.8000
Name (Print/Type)	Edward R. Gates			Date	October 22, 2018

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PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)	Docket Number (Optional) S1860.70000US01
Application Number 14/759,895-Conf. #3358	Filed July 8, 2015
For DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE	
Art Unit 1651	Examiner R. A. Davis

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above-identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

	Fee	Small Entity Fee	Micro Entity Fee	
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☐ Two months (37 CFR 1.17(a)(2))	$600	$300	$150	$
☒ Three months (37 CFR 1.17(a)(3))	$1,400	$700	$350	$ 700.00
☐ Four months (37 CFR 1.17(a)(4))	$2,200	$1,100	$550	$
☐ Five months (37 CFR 1.17(a)(5))	$3,000	$1,500	$750	$

☒ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29.
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☒ Payment by credit card. ~~Form PTO-2038 is attached.~~

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☒ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 23/2825 .

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☒ attorney or agent of record. Registration number 31,616 .

☐ attorney or agent acting under 37 CFR 1.34. Registration number _____.

/Edward R. Gates/	October 22, 2018
Signature	Date
Edward R. Gates	617.646.8000
Typed or printed name	Telephone Number

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PTO/SB/06 (09-11)
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/759,895	Filing Date 07/08/2015	☐ To be Mailed

ENTITY: ☐ LARGE ☒ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT **10/22/2018**

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 62	Minus	** 59	= 3	X $50 =	150
Independent (37 CFR 1.16(h))	* 11	Minus	***11	= 0	X $230 =	0
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	**150**

AMENDMENT

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
YOLANDA CHADWICK

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Docket No.: S1860.70000US01
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Ryanne Early
Application No.:	14/759,895
Confirmation No.:	3358
Filed:	July 8, 2015
For:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
Examiner:	R. A. Davis
Art Unit:	1651

Certificate of Electronic Filing under 37 C.F.R. § 1.8
I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: December 7, 2018 Electronic Signature for Nicole M. Forgrave: /Nicole M. Forgrave/

SUPPLEMENTAL RESPONSE AND SUBMISSION OF DECLARATION

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In the documents submitted on October 22, 2018, the undersigned attorney inadvertently and through administrative error filed a declaration that was prepared in connection with a set of claims that was not filed. (The Declaration had been prepared in connection with a draft set of claims which Applicant ultimately decided not to pursue in this RCE.) The Declaration makes certain statements and arguments which do not make sense in the context of the presently pending claims. Therefore, Applicant requests that the Examiner disregard the Declaration filed on October 22, 2018, as it refers to and addresses claim limitations that are not present in the claims as currently pending. Applicant expressly does not rely on that Declaration.

In the October 22, 2018 filing, the undersigned attorney referred to a declaration that was to be submitted in the future, as follows: "Satoh teaches (and Applicant will shortly file a Declaration

RegenX Science Exhibit 1004
Page 1035

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1035

that confirms that Satoh teaches) use of **glutaraldehyde** and Satoh does not remove sufficient cellular components or reduce antigenicity to an extent where the matrix would be considered **non-toxic as a xenograft**." [Emphasis added.] That Declaration is provided herewith. It is by the same author as the Declaration submitted October 22, 2018, but is directed to the claims as presently pending. Applicant believes no fee is due with this response. However, if a fee is due, please charge Deposit Account No. 23/2825 under Docket No. S1860.70000US01 from which the undersigned is authorized to draw.

Applicant believes no fee is due with this response. However, if a fee is due, please charge Deposit Account No. 23/2825 under Docket No. S1860.70000US01 from which the undersigned is authorized to draw.

Dated: December 7, 2018

Respectfully submitted,

By /Edward R. Gates/
Edward R. Gates
Registration No.: 31,616
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RegenX Science Exhibit 1004
Page 1036

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1036

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICANT	: ISE Professional Testing & Consulting Services, : Inc., and Inventor Ryanne Early
TTILE OF THE INVENTION	: Decellularized Biomaterial From Non-Mammalian : Tissue
SERIAL NUMBER	: 14/759,895
FILING DATE	: PCT Filed Jan. 9, 2014; 371 date: Jul. 8, 2015 : Priority Date Jan. 9, 2013 Prov. 61/750,555
EXAMINER	: Davis, Ruth A.
GROUP ART UNIT	: 1651
ATTNY. DOCKET NO.	: S1860.70000US01
CONFIRMATION NO.	: 3358

SECOND EXPERT DECLARATION UNDER 37 C.F.R. 1.132

THE COMMISSIONER OF PATENTS & EXAMINER DAVIS:

1. I hereby declare that I am the same Ashley W. Seifert who has previously executed an Expert Declaration, on 02-15-2018, and which has been previously filed in support of patentability of the subject matter claimed in the subject patent application.

2. I am aware that, nonetheless, in an Office Action dated 04/20/2018, the Examiner has maintained the rejection of certain claims as lacking novelty, in light of Satoh et a1. (2012)[1], and as being obvious in light of Seifert in combination with Asem. I am aware of the contents of that Office Action and I am aware of the contents of the papers entitled "RESPONSE TO ACCOMPANY A REQUEST FOR CONTINUED EXAMINATION ("RCE")", filed on October 22, 2018 in connection with the above identified application. I submit this Declaration in support of the positions taken in the October 22, 2018 filing.

NOVELTY OVER SATOH:

3. In my previously-filed Declaration, I expressed my expert opinion that the processes disclosed and used by Satoh, the "Satoh Process", (described in greater detail below), to produce

[1] Satoh, A., Hirata, A. & Makanae, A. Collagen reconstitution is inversely correlated with induction of limb regeneration in Ambystoma mexicanum. *Zoological science* **29**, 191-197 (2012).

RegenX Science Exhibit 1004
Page 1037

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1037

a product, the "Satoh Product", are different from the process and product as disclosed in the subject patent application. I understand that the Examiner is requesting objective evidence to establish those differences.

4. In what follows, I provide direct experimental evidence respecting the Satoh Process and Satoh Product. In sum, following the Satoh Process, I produced and examined the Satoh Product, which, is cross-linked by virtue of glutaraldehyde treatment, and which retains significant residues of cellular structures and intact cells.

5. In what follows, use of the term "I" or "me" includes work conduced directly by myself or work conducted by others under my direction.

6. While the Satoh Process results in substantial removal of the epidermis from a piece of native axolotl skin, it does not result in removal of all cells from the piece of native axolotl skin, and the resultant Satoh Product retains cells and cellular fragments in the dermis.

7. The product as claimed in the present application requires decellularization to produce a decellularized extracellular matrix, which is defined as a material sufficiently free of cellular components to eliminate or reduce antigenicity of the extra cellular matrix to an extent where the matrix would be considered non-toxic.

8. As I show below, the Satoh Product is not a decellularized extracellular matrix as claimed in this application, because it still contains significant cellular components making it antigenic to an extent that it would be considered unsuitable as a xenograft material. In addition, in my opinion the glutaraldehyde treatment carried out per Satoh and the residual cellular material would render the thus-treated native Urodele extracellular matrix toxic and unsuitable as a xenograft material.

9. By contrast, the product produced according to the subject patent application as claimed is nontoxic, being free of cellular components which significantly reduces antigenicity of the extra cellular matrix to an extent where the matrix is non-toxic, being free of glutaraldehyde cross-linking, which would render the native material toxic.

RegenX Science Exhibit 1004
Page 1038

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1038

10. Experiments were undertaken by me (or under my direction) to determine the characteristics of a product produced as disclosed in Satoh et al., (2012).

11. Following the Satoh Process, I produced the Satoh Product. On examination of the Satoh Product, I found that:

i. The Satoh Process resulted in substantial removal of the epidermal layer of the native tissue, but the Satoh Product retained cells throughout the dermis, see Figure 1 below.

ii. Cells were visible with a simple H&E stain, which is in contrast to the product as described and claimed in the present application, as shown in Figure 5 of the published patent application, US2015/0352257, which shows H&E staining and no retention of cells.

iii. Histological analysis of the Satoh Product also revealed the retention of gland fragments in the dermis, see Figure 1, again, in contrast to the product as described and claimed in the present application, e.g. see Figure 5 of the published patent application, US2015/0352257, which shows that the disruption of the glands of the dermis, along with the all cellular constituents.

iv. Using the Hoechst nuclear stain, which binds to DNA, cells and nuclei can be seen throughout the dermis of the Satoh Product (Figure 1). This is in contrast to the product as described and claimed in the present application.

v. In order to control for possible differences caused by normal fixation, we also analyzed unfixed salamander skin recovered from the Satoh process (Figure 1). The Satoh process, even as modified in the absence of glutaraldehyde treatment, did not remove cells and cellular debris.

12. I employed the following methods:

i. Sample Collection and Decellularization

Following Satoh et al. (2012), an 8x6mm^2 biopsy was harvested from albino *Ambystoma mexicanum* dorsal skin (n=3) and immediately placed in PBS containing 0.05% EDTA. Samples

RegenX Science Exhibit 1004
Page 1039

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1039

were incubated and agitated at room temperature for 4 hours. Each sample was then rinsed with distilled H_2O for 5 minutes. A solution of distilled H_2O, 0.01% Tween-20, 1.74ug/mL PMSF, and 20.18ug/mL glutaraldehyde was prepared and added to each tube (5mL solution per sample). Samples were incubated and agitated for 48 hours at room temperature. After incubation, samples were rinsed in distilled H_2O for 5 minutes. A DNase solution was prepared according to the protocol from Promega and the samples were incubated and agitated at 37°C for 1 hour. The DNase stopper solution was added and tissue was incubated for 10 minutes at 60°C. Samples were then rinsed with distilled H_2O for 5 minutes. Each sample was then cut in half using a razor blade, one half being dehydrated in 70% ethanol (3x10min) and prepared for histological-processing, and the other was placed into 10% neutral buffered formalin overnight at 4°C. The fixed samples were then rinsed with distilled water for 3x10 minutes each and 70% ethanol for 3x10 minutes each. Samples were then embedded in paraffin using a Histos5 processor, cut at 5um thickness, and stained using standard hematoxylin and eosin (H&E) protocol (see below). A second set of sections were deparaffinized, stained using Hoechst nuclear stain at a concentration of 1:10,000 for 5 minutes, and mounted with Prolong Gold (Life Technologies).

ii. Hematoxylin and Eosin Staining

Slides containing three samples each were deparaffinized by heating at 37°C for 10 minutes, submerging in 2x xylenes for 5 minutes each, 2x 100% ethanol for 5 minutes each, 95% ethanol for 2 minutes, 70% ethanol for 3 minutes, and 2x distilled water for 1 minute each. Samples were then stained in modified Mayers hematoxylin (American Mastertech) for 8 minutes, rinsed in running tap water for 30 minutes to darken the stain, dunked in 95% ethanol 10x, stained in eosin Y/phloxine B (American Mastertech) for 45 seconds, and cleared in 95% ethanol for 2 minutes. Lastly, samples were dehydrated in 100% ethanol 2x for 2 minutes each and cleared in 3x xylenes for 2 minutes each. Samples were mounted wet using Cytoseal XYL (Thermo Fisher).

RegenX Science Exhibit 1004
Page 1040

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1040

13. The results are shown in the following Figure 1.

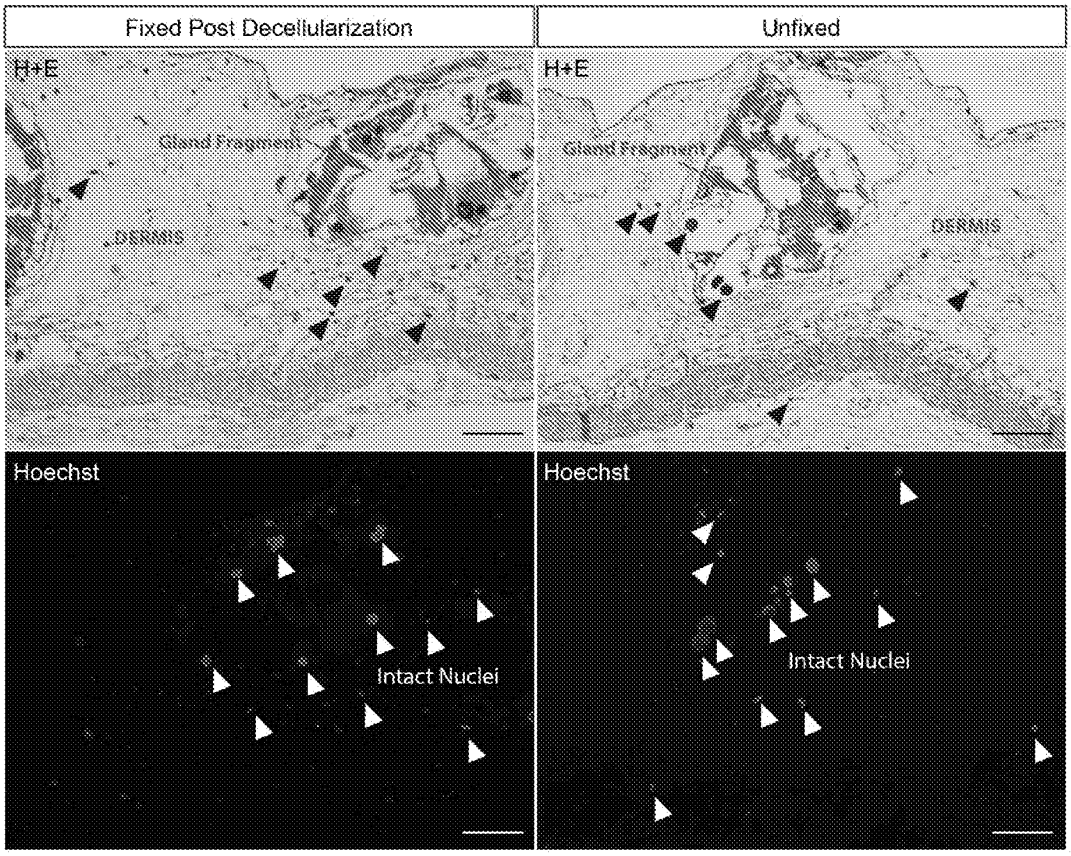


Figure 1. *Ambystoma mexicanum* tissue decellularized according to a procedure published by Satoh et al., 2012 and stained with hematoxylin/eosin and Hoechst nuclear stain. Multiple nuclear bodies can be seen in the washed tissue samples of both the fixed and unfixed dorsal skin sections (black and blue arrows), including fragments of axolotl dermal glands. Intact nuclei are clearly visible within the glands and throughout the dermis. Scale = 100um.

14. In contrast to the Satoh Product, the product described and claimed in the present application requires and provides evidence of a decellularized product which has no intact cells, glands or nuclei. Such cells and cellular debris are antigenic and would render the resulting graft antigenic/immunogenic if they were to remain, as in the Satoh Product. The product as described and claimed in the present application also excludes the use of glutaraldehyde cross-linking, which cross-linking would render the product toxic.

RegenX Science Exhibit 1004
Page 1041

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1041

15. One of ordinary skill in the art, reading the present application, would understand that the thrust of the invention is the production of Urodele extracellular matrix of such reduced antigenicity/immunogenicity as to be highly desirable as a xenograft material. One of ordinary skill in the art would understand that the present application teaches removing the immunogenic cells and cellular debris to an extent where the remaining extracellular material no longer contains such antigenic/immunogenic cells and cellular debris. One of ordinary skill in the art would also be able to achieve such a product based on the teachings of the present application, by applying decellularization techniques as taught until such cells and cellular debris are removed.

16. One of ordinary skill in the art would understand from the teachings in the present application that one should not employ cross-linking during the decellularization process, as described in Satoh, because doing so would capture and retain the very materials one is trying to remove. One of ordinary skill in the art would understand from the teachings in the present application that one should not employ cross-linking during or after the decellularization process, because doing so would render the native material) immunogenic and toxic.

17. It was unknown to me and it was, to my knowledge, unknown in the prior art as of the priority date of the present application that Urodele extracellular matrix could be produced which would have such reduced antigenicity/immunogenicity so as to be useable as a xenograft material.

18. As discussed and shown experimentally, the product and process of producing the product disclosed in Satoh is neither the same as nor even similar to the product and process of producing as described and claimed in the present application.

19. I am further aware that the Examiner is maintaining the rejection of certain pending Claims under 35 U.S.C. 103 as being unpatentable over Asem et al. (US 6485969) in view of Seifert et al. (2012); that Asem is being cited as providing a teaching of decellularized ECM comprising basement membranes (col.1 line 66 - col.2 line 1-18, col. 5 line 23-39), derived from amphibians (col. 4, line 35-40); that the Asem ECM is said to be in the form of sheets that are adhered together (or laminated) (col.6 line 20-32), powders (or particles) or reconstituted gels, made under alkaline conditions (col, 6 line 43-64); that the ECM compositions may be used as tissue grafts constructs (col. 4 line 30-35) or with cell culture comprising growth factors for culturing human (co1. 11 line 40-45) cells (or are infused with, coated with, or attached to a growth factor) (col. 7-8) or as a coating wherein it is preferably sterilized prior to use (col.7 line 30-45). I further understand that

RegenX Science Exhibit 1004
Page 1042

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1042

the Examiner has conceded that "Asem does not teach the ECM wherein the amphibian is a Urodele, or axolotl." However, that notwithstanding, I further understand that the Examiner is asserting that "at the time the instant claims were filed, in following the teachings of Asem, one of ordinary skill in the art would have been motivated to use any known functionally equivalent amphibian ECM in the compositions of Asem with a reasonable expectation for successfully obtaining a decellularized ECM. At the time the instant claims were filed, axolotls were known amphibian sources of ECM and components derived therefrom. In support, Seifert teaches axolotl ECM and its basement membrane are useful for regenerative therapies and wound repair (abstract) as well as blueprints for healing in vertebrates (entire document). As such, at the time the claims were filed, it would have been obvious to one of ordinary skill in the art to use ECM from axolotl in the compositions of Asem, since it was a known, functionally equivalent amphibian ECM and because of its known benefit in cellular regeneration. Thus, the claims are rendered obvious."

20. I am the principal author of the Seifert publication which the Examiner is citing. I have previously provided my expert opinion, that the combination of Seifert with Asem, as proposed by the Examiner in rejecting the claims, does not render the claimed subject matter unpatentable for obviousness. In my opinion, the combination does not even make sense as proposed by the Examiner.

21. In my opinion, Asem clearly teaches that follicular tissue is uniquely suited for the purposes carried out in the Asem process. There is nothing in Seifert or Asem that suggests that the Seifert material could be a suitable substitute for the Asem follicular tissue. The Examiner has not provided any citation to any aspect of Seifert or Asem which would support the position that the Seifert material is a suitable substitute for the Asem follicular tissue. As the author of the Seifert reference, it would never have occurred to me to use the Seifert material in the process of Asem because the Seifert material is not the functional equivalent of follicular tissue. It also never would have occurred to me to try to obtain follicular tissue from a Urodele to practice the Asem method, as the potential yield of follicular tissue from a Urodele would be completely impractical to work with. Finally, as Seifert was working only within a single species, i.e. the axolotl itself, there would have been no basis for believing that the Seifert material was non-antigenic/immunogenic and therefore suitable for use as a xenograft.

RegenX Science Exhibit 1004
Page 1043

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1043

22. In my opinion, even if one were to substitute the Seifert material for the Asem material, one would still not obtain the product as described and claimed in the present application. The Seifert material was not processed adequately to remove cells and cellular debris to a degree where the material would be immunoprivileged and the Seifert process involves a cross-linking step, further compromising the immunoprivileged state of the extracellular matrix and rendering that material toxic.

23. In my opinion, Seifert, if anything, teaches away from the combination with Asem proposed by the Examiner. Seifert involved same species experiments. Seifert showed extracellular matrix enhanced scarring in the same species experiment. If anything, one of ordinary skill in the art would expect a worse outcome in a different species.

24. In my opinion, much as with the Satoh Product produced according to the Satoh Process, as described above, the Seifert material fails to meet the present claims' requirements of removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial to make it suitable for use as a xenograft.

25. I further note that in Asem, it is disclosed at column 8, lines 4-14, that:

> "The present basal lamina compositions may be sterilized using conventional sterilization techniques including tanning with **glutaraldehyde, formaldehyde** tanning at acidic pH, ethylene oxide treatment, propylene oxide treatment, gas plasma sterilization, gamma radiation, and peracetic acid sterilization. A sterilization technique which does not significantly weaken the mechanical strength and biotropic properties of the material is preferably used. For instance, it is believed that strong gamma radiation may cause loss of strength in the basal lamina."

Accordingly, Asem, like Satoh and Seifert, teaches a cross-linked material, which process is not suitable to create a xenograft that is non-toxic as claimed.

26. I attach a publication, Toxicol. Int. 2012, Jan.-Apr.; 19(1):51-58, (see https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3339246/?report=printable), entitled:

RegenX Science Exhibit 1004
Page 1044

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1044

"Glutaraldehyde Treatment Elicits Toxic Response Compared to Decellularization in Bovine Pericardium", Umashankar et al., it is established that glutaraldehyde treatment renders a graft material toxic. It is reported that (bolded emphasis added):

"Glutaraldehyde-stabilized bovine pericardium is used for clinical application since 1970s because of its desirable features such as less immunogenicity and acceptable durability. However, a propensity for calcification is reported on account of glutaraldehyde treatment. In this study, commercially available glutaraldehyde cross-linked bovine pericardium was evaluated for its in vitro cytotoxic effect, macrophage activation, and in vivo toxic response in comparison to decellularized bovine pericardium. Glutaraldehyde-treated bovine pericardium and its extract were observed to be cytotoxic and it also caused significant inflammatory cytokine release from activated macrophages. **Significant antibody response, calcification response, necrotic, and inflammatory response were noticed in glutaraldehyde-treated bovine pericardium** in comparison to decellularized bovine pericardium in a rat subcutaneous implantation model. Glutaraldehyde-treated bovine pericardium also failed in acute systemic toxicity testing and intracutaneous irritation testing as per ISO 10993. With respect to healing and implant remodeling, total lack of host tissue incorporation and angiogenesis was noticed in glutaraldehyde-treated bovine pericardium compared to excellent host fibroblast incorporation and angiogenesis within the implant in decellularized bovine pericardium. In conclusion, using in vitro and in vivo techniques, this study has demonstrated that glutaraldehyde-treated bovine pericardium elicits toxic response compared to decellularized bovine pericardium which is not congenial for long-term implant performance."

Accordingly, the novel, immunopriviledged, decellularized material according to the present invention provides, in my opinion, a significant alternative to existing products, including mammalian and non-mammalian cross-linked ECM based products. The same cannot be said for the Satoh product, the Seifert Product, the Asem Product, their related processes, or any reasonable combination of the teachings in those references.

The signatory, being warned that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and that such willful false statements and the like may jeopardize the validity of the application or submission or any registration resulting therefrom, declares that all statements made of his/her own knowledge are true and all statements made on information and belief are believed to be true.

RegenX Science Exhibit 1004
Page 1045

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1045



12-06-2018

Ashley W. Seifert Date

RegenX Science Exhibit 1004
Page 1046

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1046

Electronic Acknowledgement Receipt

EFS ID:	34521881
Application Number:	14759895
International Application Number:	
Confirmation Number:	3358
Title of Invention:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
First Named Inventor/Applicant Name:	Ryanne Early
Customer Number:	23628
Filer:	Edward R. Gates/Nicole Forgrave
Filer Authorized By:	Edward R. Gates
Attorney Docket Number:	S1860.70000US01
Receipt Date:	07-DEC-2018
Filing Date:	08-JUL-2015
Time Stamp:	15:30:29
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	S186070000US01-TRN-ERG.pdf	26268 492c84385ab343fd53153f70a51cb7f1e6b218d2	no	1

Warnings:

Information:					
2	Supplemental Response or Supplemental Amendment	S186070000US01-SUPAMN-ERG.pdf	28548 b7a3bb634f4a10c24523bd5c6621a0466f6c3df6	no	2
Warnings:					
Information:					
3	Affidavit-traversing rejectns or objectns rule 132	S186070000US01-AFF-ERG.pdf	746077 31ce11aa85b7e56a79a26d728baaccf51bd9c776	no	10
Warnings:					
Information:					
		Total Files Size (in bytes):	800893		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM *(to be used for all correspondence after initial filing)*			
		Application Number	14/759,895-Conf. #3358
		Filing Date	July 8, 2015
		First Named Inventor	Ryanne Early
		Art Unit	1651
		Examiner Name	R. A. Davis
Total Number of Pages in This Submission		Attorney Docket Number	S1860.70000US01

ENCLOSURES (*Check all that apply*)

☐ Fee Transmittal Form	☐ Drawing(s)	☐ After Allowance Communication to TC
☐ Fee Attached	☐ Licensing-related Papers	☐ Appeal Communication to Board of Appeals and Interferences
☐ Amendment/Reply	☐ Petition	☐ Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
☐ After Final	☐ Petition to Convert to a Provisional Application	☐ Proprietary Information
☐ Affidavits/declaration(s)	☐ Power of Attorney, Revocation Change of Correspondence Address	☐ Status Letter
☐ Extension of Time Request	☐ Terminal Disclaimer	☒ Other Enclosure(s) (please Identify below):
☐ Express Abandonment Request	☐ Request for Refund	Supplemental Response and Submission of Declaration Seifert Declaration
☐ Information Disclosure Statement	☐ CD, Number of CD(s) _____	
☐ Certified Copy of Priority Document(s)	☐ Landscape Table on CD	
☐ Reply to Missing Parts/ Incomplete Application	Remarks	
☐ Reply to Missing Parts under 37 CFR 1.52 or 1.53		

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Edward R. Gates/		
Printed name	Edward R. Gates		
Date	December 7, 2018	Reg. No.	31,616

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: December 7, 2018 Electronic Signature for Nicole M. Forgrave: /Nicole M. Forgrave/

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICANT	: ISE Professional Testing & Consulting Services, : Inc., and Inventor Ryanne Early
TTILE OF THE INVENTION	: Decellularized Biomaterial From Non-Mammalian : Tissue
SERIAL NUMBER	: 14/759,895
FILING DATE	: PCT Filed Jan. 9, 2014; 371 date: Jul. 8, 2015 : Priority Date Jan. 9, 2013 Prov. 61/750,555
EXAMINER	: Davis, Ruth A.
GROUP ART UNIT	: 1651
ATTNY. DOCKET NO.	: S1860.70000US01
CONFIRMATION NO.	: 3358

SECOND EXPERT DECLARATION UNDER 37 C.F.R. 1.132

THE COMMISSIONER OF PATENTS & EXAMINER DAVIS:

1. I hereby declare that I am the same Ashley W. Seifert who has previously executed an Expert Declaration, on 02-15-2018, and which has been previously filed in support of patentability of the subject matter claimed in the subject patent application.

2. I am aware that, nonetheless, in an Office Action dated 04/20/2018, the Examiner has maintained the rejection of certain claims as lacking novelty, in light of Satoh et a1. (2012)[1], and as being obvious in light of Seifert in combination with Asem. I am aware of the contents of that Office Action and I am aware of the contents of the papers entitled "RESPONSE TO ACCOMPANY A REQUEST FOR CONTINUED EXAMINATION ("RCE")", filed on October 22, 2018 in connection with the above identified application. I submit this Declaration in support of the positions taken in the October 22, 2018 filing.

NOVELTY OVER SATOH:

3. In my previously-filed Declaration, I expressed my expert opinion that the processes disclosed and used by Satoh, the "Satoh Process", (described in greater detail below), to produce

[1] Satoh, A., Hirata, A. & Makanae, A. Collagen reconstitution is inversely correlated with induction of limb regeneration in Ambystoma mexicanum. *Zoological science* **29**, 191-197 (2012).

a product, the "Satoh Product", are different from the process and product as disclosed in the subject patent application. I understand that the Examiner is requesting objective evidence to establish those differences.

4. In what follows, I provide direct experimental evidence respecting the Satoh Process and Satoh Product. In sum, following the Satoh Process, I produced and examined the Satoh Product, which, is cross-linked by virtue of glutaraldehyde treatment, and which retains significant residues of cellular structures and intact cells.

5. In what follows, use of the term "I" or "me" includes work conduced directly by myself or work conducted by others under my direction.

6. While the Satoh Process results in substantial removal of the epidermis from a piece of native axolotl skin, it does not result in removal of all cells from the piece of native axolotl skin, and the resultant Satoh Product retains cells and cellular fragments in the dermis.

7. The product as claimed in the present application requires decellularization to produce a decellularized extracellular matrix, which is defined as a material sufficiently free of cellular components to eliminate or reduce antigenicity of the extra cellular matrix to an extent where the matrix would be considered non-toxic.

8. As I show below, the Satoh Product is not a decellularized extracellular matrix as claimed in this application, because it still contains significant cellular components making it antigenic to an extent that it would be considered unsuitable as a xenograft material. In addition, in my opinion the glutaraldehyde treatment carried out per Satoh and the residual cellular material would render the thus-treated native Urodele extracellular matrix toxic and unsuitable as a xenograft material.

9. By contrast, the product produced according to the subject patent application as claimed is nontoxic, being free of cellular components which significantly reduces antigenicity of the extra cellular matrix to an extent where the matrix is non-toxic, being free of glutaraldehyde cross-linking, which would render the native material toxic.

RegenX Science Exhibit 1004
Page 1051

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1051

10. Experiments were undertaken by me (or under my direction) to determine the characteristics of a product produced as disclosed in Satoh et al., (2012).

11. Following the Satoh Process, I produced the Satoh Product. On examination of the Satoh Product, I found that:

i. The Satoh Process resulted in substantial removal of the epidermal layer of the native tissue, but the Satoh Product retained cells throughout the dermis, see Figure 1 below.

ii. Cells were visible with a simple H&E stain, which is in contrast to the product as described and claimed in the present application, as shown in Figure 5 of the published patent application, US2015/0352257, which shows H&E staining and no retention of cells.

iii. Histological analysis of the Satoh Product also revealed the retention of gland fragments in the dermis, see Figure 1, again, in contrast to the product as described and claimed in the present application, e.g. see Figure 5 of the published patent application, US2015/0352257, which shows that the disruption of the glands of the dermis, along with the all cellular constituents.

iv. Using the Hoechst nuclear stain, which binds to DNA, cells and nuclei can be seen throughout the dermis of the Satoh Product (Figure 1). This is in contrast to the product as described and claimed in the present application.

v. In order to control for possible differences caused by normal fixation, we also analyzed unfixed salamander skin recovered from the Satoh process (Figure 1). The Satoh process, even as modified in the absence of glutaraldehyde treatment, did not remove cells and cellular debris.

12. I employed the following methods:

i. Sample Collection and Decellularization

Following Satoh et al. (2012), an 8x6mm^2 biopsy was harvested from albino *Ambystoma mexicanum* dorsal skin (n=3) and immediately placed in PBS containing 0.05% EDTA. Samples

RegenX Science Exhibit 1004
Page 1052

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1052

were incubated and agitated at room temperature for 4 hours. Each sample was then rinsed with distilled H_2O for 5 minutes. A solution of distilled H_2O, 0.01% Tween-20, 1.74ug/mL PMSF, and 20.18ug/mL glutaraldehyde was prepared and added to each tube (5mL solution per sample). Samples were incubated and agitated for 48 hours at room temperature. After incubation, samples were rinsed in distilled H_2O for 5 minutes. A DNase solution was prepared according to the protocol from Promega and the samples were incubated and agitated at 37°C for 1 hour. The DNase stopper solution was added and tissue was incubated for 10 minutes at 60°C. Samples were then rinsed with distilled H_2O for 5 minutes. Each sample was then cut in half using a razor blade, one half being dehydrated in 70% ethanol (3x10min) and prepared for histological-processing, and the other was placed into 10% neutral buffered formalin overnight at 4°C. The fixed samples were then rinsed with distilled water for 3x10 minutes each and 70% ethanol for 3x10 minutes each. Samples were then embedded in paraffin using a Histos5 processor, cut at 5um thickness, and stained using standard hematoxylin and eosin (H&E) protocol (see below). A second set of sections were deparaffinized, stained using Hoechst nuclear stain at a concentration of 1:10,000 for 5 minutes, and mounted with Prolong Gold (Life Technologies).

ii. Hematoxylin and Eosin Staining

Slides containing three samples each were deparaffinized by heating at 37°C for 10 minutes, submerging in 2x xylenes for 5 minutes each, 2x 100% ethanol for 5 minutes each, 95% ethanol for 2 minutes, 70% ethanol for 3 minutes, and 2x distilled water for 1 minute each. Samples were then stained in modified Mayers hematoxylin (American Mastertech) for 8 minutes, rinsed in running tap water for 30 minutes to darken the stain, dunked in 95% ethanol 10x, stained in eosin Y/phloxine B (American Mastertech) for 45 seconds, and cleared in 95% ethanol for 2 minutes. Lastly, samples were dehydrated in 100% ethanol 2x for 2 minutes each and cleared in 3x xylenes for 2 minutes each. Samples were mounted wet using Cytoseal XYL (Thermo Fisher).

RegenX Science Exhibit 1004
Page 1053

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1053

13. The results are shown in the following Figure 1.

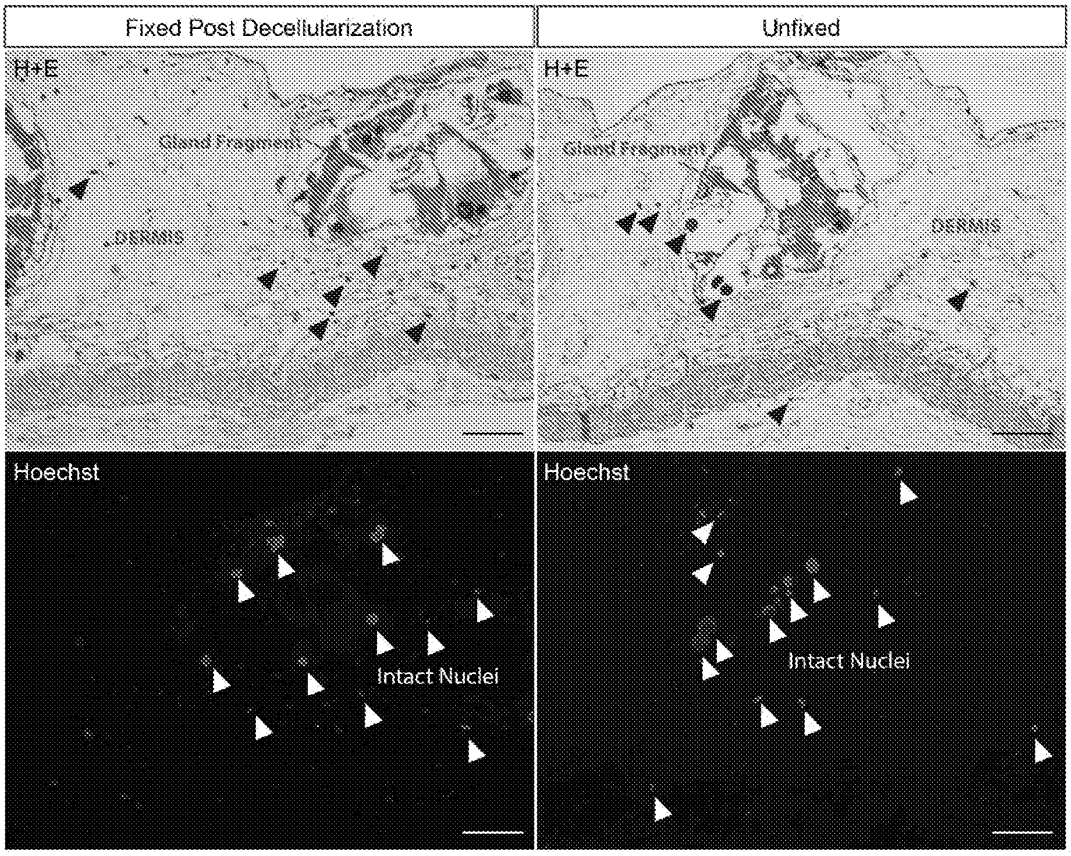


Figure 1. *Ambystoma mexicanum* tissue decellularized according to a procedure published by Satoh et al., 2012 and stained with hematoxylin/eosin and Hoechst nuclear stain. Multiple nuclear bodies can be seen in the washed tissue samples of both the fixed and unfixed dorsal skin sections (black and blue arrows), including fragments of axolotl dermal glands. Intact nuclei are clearly visible within the glands and throughout the dermis. Scale = 100um.

14. In contrast to the Satoh Product, the product described and claimed in the present application requires and provides evidence of a decellularized product which has no intact cells, glands or nuclei. Such cells and cellular debris are antigenic and would render the resulting graft antigenic/immunogenic if they were to remain, as in the Satoh Product. The product as described and claimed in the present application also excludes the use of glutaraldehyde cross-linking, which cross-linking would render the product toxic.

RegenX Science Exhibit 1004
Page 1054

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1054

15. One of ordinary skill in the art, reading the present application, would understand that the thrust of the invention is the production of Urodele extracellular matrix of such reduced antigenicity/immunogenicity as to be highly desirable as a xenograft material. One of ordinary skill in the art would understand that the present application teaches removing the immunogenic cells and cellular debris to an extent where the remaining extracellular material no longer contains such antigenic/immunogenic cells and cellular debris. One of ordinary skill in the art would also be able to achieve such a product based on the teachings of the present application, by applying decellularization techniques as taught until such cells and cellular debris are removed.

16. One of ordinary skill in the art would understand from the teachings in the present application that one should not employ cross-linking during the decellularization process, as described in Satoh, because doing so would capture and retain the very materials one is trying to remove. One of ordinary skill in the art would understand from the teachings in the present application that one should not employ cross-linking during or after the decellularization process, because doing so would render the native material) immunogenic and toxic.

17. It was unknown to me and it was, to my knowledge, unknown in the prior art as of the priority date of the present application that Urodele extracellular matrix could be produced which would have such reduced antigenicity/immunogenicity so as to be useable as a xenograft material.

18. As discussed and shown experimentally, the product and process of producing the product disclosed in Satoh is neither the same as nor even similar to the product and process of producing as described and claimed in the present application.

19. I am further aware that the Examiner is maintaining the rejection of certain pending Claims under 35 U.S.C. 103 as being unpatentable over Asem et al. (US 6485969) in view of Seifert et al. (2012); that Asem is being cited as providing a teaching of decellularized ECM comprising basement membranes (col.1 line 66 - col.2 line 1-18, col. 5 line 23-39), derived from amphibians (col. 4, line 35-40); that the Asem ECM is said to be in the form of sheets that are adhered together (or laminated) (col.6 line 20-32), powders (or particles) or reconstituted gels, made under alkaline conditions (col, 6 line 43-64); that the ECM compositions may be used as tissue grafts constructs (col. 4 line 30-35) or with cell culture comprising growth factors for culturing human (co1. 11 line 40-45) cells (or are infused with, coated with, or attached to a growth factor) (col. 7-8) or as a coating wherein it is preferably sterilized prior to use (col.7 line 30-45). I further understand that

RegenX Science Exhibit 1004
Page 1055

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1055

the Examiner has conceded that "Asem does not teach the ECM wherein the amphibian is a Urodele, or axolotl." However, that notwithstanding, I further understand that the Examiner is asserting that "at the time the instant claims were filed, in following the teachings of Asem, one of ordinary skill in the art would have been motivated to use any known functionally equivalent amphibian ECM in the compositions of Asem with a reasonable expectation for successfully obtaining a decellularized ECM. At the time the instant claims were filed, axolotls were known amphibian sources of ECM and components derived therefrom. In support, Seifert teaches axolotl ECM and its basement membrane are useful for regenerative therapies and wound repair (abstract) as well as blueprints for healing in vertebrates (entire document). As such, at the time the claims were filed, it would have been obvious to one of ordinary skill in the art to use ECM from axolotl in the compositions of Asem, since it was a known, functionally equivalent amphibian ECM and because of its known benefit in cellular regeneration. Thus, the claims are rendered obvious."

20. I am the principal author of the Seifert publication which the Examiner is citing. I have previously provided my expert opinion, that the combination of Seifert with Asem, as proposed by the Examiner in rejecting the claims, does not render the claimed subject matter unpatentable for obviousness. In my opinion, the combination does not even make sense as proposed by the Examiner.

21. In my opinion, Asem clearly teaches that follicular tissue is uniquely suited for the purposes carried out in the Asem process. There is nothing in Seifert or Asem that suggests that the Seifert material could be a suitable substitute for the Asem follicular tissue. The Examiner has not provided any citation to any aspect of Seifert or Asem which would support the position that the Seifert material is a suitable substitute for the Asem follicular tissue. As the author of the Seifert reference, it would never have occurred to me to use the Seifert material in the process of Asem because the Seifert material is not the functional equivalent of follicular tissue. It also never would have occurred to me to try to obtain follicular tissue from a Urodele to practice the Asem method, as the potential yield of follicular tissue from a Urodele would be completely impractical to work with. Finally, as Seifert was working only within a single species, i.e. the axolotl itself, there would have been no basis for believing that the Seifert material was non-antigenic/immunogenic and therefore suitable for use as a xenograft.

RegenX Science Exhibit 1004
Page 1056

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1056

22. In my opinion, even if one were to substitute the Seifert material for the Asem material, one would still not obtain the product as described and claimed in the present application. The Seifert material was not processed adequately to remove cells and cellular debris to a degree where the material would be immunoprivileged and the Seifert process involves a cross-linking step, further compromising the immunoprivileged state of the extracellular matrix and rendering that material toxic.

23. In my opinion, Seifert, if anything, teaches away from the combination with Asem proposed by the Examiner. Seifert involved same species experiments. Seifert showed extracellular matrix enhanced scarring in the same species experiment. If anything, one of ordinary skill in the art would expect a worse outcome in a different species.

24. In my opinion, much as with the Satoh Product produced according to the Satoh Process, as described above, the Seifert material fails to meet the present claims' requirements of removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial to make it suitable for use as a xenograft.

25. I further note that in Asem, it is disclosed at column 8, lines 4-14, that:

> "The present basal lamina compositions may be sterilized using conventional sterilization techniques including tanning with **glutaraldehyde, formaldehyde** tanning at acidic pH, ethylene oxide treatment, propylene oxide treatment, gas plasma sterilization, gamma radiation, and peracetic acid sterilization. A sterilization technique which does not significantly weaken the mechanical strength and biotropic properties of the material is preferably used. For instance, it is believed that strong gamma radiation may cause loss of strength in the basal lamina."

Accordingly, Asem, like Satoh and Seifert, teaches a cross-linked material, which process is not suitable to create a xenograft that is non-toxic as claimed.

26. I attach a publication, Toxicol. Int. 2012, Jan.-Apr.; 19(1):51-58, (see https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3339246/?report=printable), entitled:

RegenX Science Exhibit 1004
Page 1057

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1057

"Glutaraldehyde Treatment Elicits Toxic Response Compared to Decellularization in Bovine Pericardium", Umashankar et al., it is established that glutaraldehyde treatment renders a graft material toxic. It is reported that (bolded emphasis added):

"Glutaraldehyde-stabilized bovine pericardium is used for clinical application since 1970s because of its desirable features such as less immunogenicity and acceptable durability. However, a propensity for calcification is reported on account of glutaraldehyde treatment. In this study, commercially available glutaraldehyde cross-linked bovine pericardium was evaluated for its in vitro cytotoxic effect, macrophage activation, and in vivo toxic response in comparison to decellularized bovine pericardium. Glutaraldehyde-treated bovine pericardium and its extract were observed to be cytotoxic and it also caused significant inflammatory cytokine release from activated macrophages. **Significant antibody response, calcification response, necrotic, and inflammatory response were noticed in glutaraldehyde-treated bovine pericardium** in comparison to decellularized bovine pericardium in a rat subcutaneous implantation model. Glutaraldehyde-treated bovine pericardium also failed in acute systemic toxicity testing and intracutaneous irritation testing as per ISO 10993. With respect to healing and implant remodeling, total lack of host tissue incorporation and angiogenesis was noticed in glutaraldehyde-treated bovine pericardium compared to excellent host fibroblast incorporation and angiogenesis within the implant in decellularized bovine pericardium. In conclusion, using in vitro and in vivo techniques, this study has demonstrated that glutaraldehyde-treated bovine pericardium elicits toxic response compared to decellularized bovine pericardium which is not congenial for long-term implant performance."

Accordingly, the novel, immunopriviledged, decellularized material according to the present invention provides, in my opinion, a significant alternative to existing products, including mammalian and non-mammalian cross-linked ECM based products. The same cannot be said for the Satoh product, the Seifert Product, the Asem Product, their related processes, or any reasonable combination of the teachings in those references.

The signatory, being warned that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and that such willful false statements and the like may jeopardize the validity of the application or submission or any registration resulting therefrom, declares that all statements made of his/her own knowledge are true and all statements made on information and belief are believed to be true.

RegenX Science Exhibit 1004
Page 1058

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1058



12-06-2018

Ashley W. Seifert Date

RegenX Science Exhibit 1004
Page 1059

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1059

FORM PTO-1449/A and B (modified PTO/SB/08)	APPLICATION NO.: 14/759,895	ATTY. DOCKET NO.: S1860.70000US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT	FILING DATE: July 8, 2015	CONFIRMATION NO.: 3358
	FIRST NAMED INVENTOR: Ryanne Early	
Sheet 1 of 1	GROUP ART UNIT: 1651	EXAMINER: R. A. Davis

U.S. PATENT DOCUMENTS

Examiner's Initials #	Cite No.	U.S. Patent Document Number	Kind Code	Name of Patentee or Applicant of Cited Document	Date of Publication or Issue of Cited Document MM-DD-YYYY
		6,485,969	B1	Asem et al.	11-26-2002

FOREIGN PATENT DOCUMENTS

Examiner's Initials #	Cite No.	Foreign Patent Document Office/ Country	Number	Kind Code	Name of Patentee or Applicant of Cited Document	Date of Publication of Cited Document MM-DD-YYYY	Translation (Y/N)
		CN	1791331	A	Anthrogenesis Corp	06-21-2006	Y – Machine Translation
		CN	102227225	A	Univ California	10-26-2011	Y – Machine Translation

OTHER ART -- NON PATENT LITERATURE DOCUMENTS

Examiner's Initials #	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	Translation (Y/N)
		DUAN et al., Preliminary study on porous scaffold prepared with decellularized artery. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi. 2010 Sep;24(9):1052-7.	Y – Abstract Only
		MENG et al., An experimental study of extracellular matrix of conjunctiva. Journal of Injusries and Occupational Diseases of the Eye with Ophthalmic Surgeries. 2007 May;1:26-8.	Y – Abstract Only
		UMASHANKAR et al., Glutaraldehyde Treatment Elicits Toxic Response Compared to Decellularization in Bovine Pericardium. Toxicol Int 2012;19(1):51-8.	

[NOTE – No copies of U.S. patents, published U.S. patent applications, or pending, unpublished patent applications stored in the USPTO's Image File Wrapper (IFW) system, are included. See 37 CFR § 1.98 and 1287OG163. Copies of all other patent(s), publication(s), unpublished, pending U.S. patent applications, or other information listed are provided as required by 37 CFR § 1.98 unless 1) such copies were provided in an IDS in an earlier application that complies with 37 CFR § 1.98, and 2) the earlier application is relied upon for an earlier filing date under 35 U.S.C. § 120.]

EXAMINER:	DATE CONSIDERED:
/RUTH A DAVIS/	01/02/2019

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /R.A.D/

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/759,895	07/08/2015	Ryanne Early	S1860.70000US01	3358

23628 7590 01/10/2019
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
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EXAMINER	
DAVIS, RUTH A	

ART UNIT	PAPER NUMBER
1651	

NOTIFICATION DATE	DELIVERY MODE
01/10/2019	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Patents_eOfficeAction@WolfGreenfield.com
WGS_eOfficeAction@WolfGreenfield.com

Office Action Summary	**Application No.** 14/759,895	**Applicant(s)** Early, Ryanne	
	Examiner RUTH A DAVIS	**Art Unit** 1651	**AIA Status** Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 10/22/2018.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1-33,36-38 and 40-65 is/are pending in the application.

5a) Of the above claim(s) 1-4,6-19,23-25 and 27-33 is/are withdrawn from consideration.

6) ☑ Claim(s) 5,20-22,36-38,40-47 and 49-61 is/are allowed.

7) ☑ Claim(s) 26,48 and 62-65 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

DETAILED ACTION

A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on October 22, 2018 and December 7, 2018 have been entered.

Claims 62 – 65 are added; claims 1 – 33, 36 – 38 and 40 – 65 are pending; claims 1 – 4, 6 – 19, 23 – 25 and 27 – 33 are withdrawn; claims 5, 20 – 22, 26, 36 – 38 and 40 – 65 have been considered on the merits. All arguments and the affidavit have been fully considered.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of the first paragraph of pre-AIA 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person

skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 62 – 65 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention.

Claims 62 – 65 recite a tissue graft that is decellularized without cross-linking. While the specification teaches methods of decellularizing while maintaining natural properties, the specification fails to identify the process without crosslinking. It is noted that mere absence of a positive recitation is not the same as a basis for an exclusion (MPEP 2173.05 (i)).

Claim Rejections - 35 USC § 112

Rejections under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, are withdrawn due to amendment.

Claim Rejections - 35 USC § 102

Rejections under 35 U.S.C. 102a1 are withdrawn due to applicant's amendment and reply.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

Claims 26 and 48 are rejected under 35 U.S.C. 103 as being unpatentable over Asem et al. (US 6485969) in view of Seifert et al. (2012).

Asem teaches decellularized ECM comprising basement membranes (col.1 line 66 - col.2 line 1-18, col.5 line 23-39), derived from amphibians (col.4 line 35-40) that are adhered together (or laminated) (col.6 line 20-32)

Asem does not teach the ECM wherein the amphibian is a Urodele, or axolotl. However, at the time the instant claims were filed, in following the teachings of Asem, one of ordinary skill in the art would have been motivated to use any known functionally equivalent amphibian ECM in the compositions of Asem with a reasonable expectation for successfully obtaining a decellularized ECM. At the time the instant claims were filed, axolotls were known amphibian sources of ECM and components derived therefrom. In support, Seifert teaches axolotl ECM and its basement membrane (abstract). As such, at the time the claims were filed, it would have been obvious to one of ordinary skill in the art to use ECM from axolotl in the in the compositions of Asem, since it was a known, functionally equivalent amphibian ECM.

Thus, the claims are rendered obvious.

Response to Arguments

Applicant argues that the references do not teach Urodele or axolotl ECM; that Seifert teaches away from using Urodele ECM by way of disclosing models only; that the references do not teach a decellularized ECM with reduce antigenicity; that there is no teaching or suggestion to modify or substitute one source for the other, that the examiner uses inappropriate hindsight; and that the prior art crosslinks the ECM.

However, these arguments fail to persuade for the following reasons. It is first noted that the rejection is based on the combination of references and not any one alone. Asem teaches decellularized ECM derived from amphibians in the form of sheets that can be adhered together (or laminated) while Seifert is relied upon to demonstrate that Urodele, specifically axolotl, was a known amphibian source from which a decellularized ECM may be obtained. Moreover, Seifert provides evidence of a functionally equivalent amphibian source of ECM that can be decellularized and used in the compositions of Asem with a reasonable expectation for success.

Regarding a teaching away, it is acknowledged that Seifert teaches different outcomes than applicant. However a different outcome is not a teaching away from functionality of the ECM. Seifert is evidence that Urodele/axolotl was a known source of amphibian ECM that can be decellularized and used as the amphibian ECM in the compositions of Asem with a reasonable expectation for success. Please note that the instant claims do not exclude or require any step for producing the decellularized ECM, nor do they require or exclude any particular type or source of ECM other than being any tissue from a Urodele. Thus, the arguments are not commensurate in scope with the claimed invention.

In response to applicant's argument that the examiner's conclusion of obviousness is based upon improper hindsight reasoning, it must be recognized that any judgment on

obviousness is in a sense necessarily a reconstruction based upon hindsight reasoning. But so long as it takes into account only knowledge which was within the level of ordinary skill at the time the claimed invention was made, and does not include knowledge gleaned only from the applicant's disclosure, such a reconstruction is proper. See In re McLaughlin, 443 F.2d 1392, 170 USPQ 209 (CCPA 1971).

Allowable Subject Matter

Claims 5, 20 – 22, 36 – 38, 40 – 47, 49 – 61 are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to RUTH A DAVIS whose telephone number is (571)272-0915. The examiner can normally be reached on Monday - Friday (8am - 4pm).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Renee Claytor can be reached on 571-272-8394. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

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/RUTH A DAVIS/
Primary Examiner, Art Unit 1651

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14/759,895	Early, Ryanne
	Examiner	Art Unit
	RUTH A DAVIS	1651

CPC - Searched*		
Symbol	Date	Examiner
A61K 35/65	01/03/2019	rad

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
east, pubmed, google patent	01/03/2019	rad
A61K 35/65	01/03/2019	rad

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

	/RUTH A DAVIS/ Primary Examiner, Art Unit 1651

To: Patents_eOfficeAction@WolfGreenfield.com,WGS_eOfficeAction@WolfGreenfield.com,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 23628

Jan 10, 2019 04:16:38 AM

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600 ATLANTIC AVENUE
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UNITED STATES

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Application	Document	Mailroom Date	Attorney Docket No.
14759895	1449	01/10/2019	S1860.70000US01
	CTNF	01/10/2019	S1860.70000US01

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Document Description: Power of Attorney

PTO/AIA/82A (07-13)
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Application Number	14/759,895
Filing Date	July 8, 2015
First Named Inventor	Ryanne Early
Title	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
Art Unit	1651
Examiner Name	Ruth A. Davis
Attorney Docket Number	S1860.70000US01

SIGNATURE of Applicant or Patent Practitioner

Signature	/sima singadia kulkarni/	Date (Optional)	June 12, 2019
Name	Sima Singadia Kulkarni	Registration Number	43732
Title (if Applicant is a juristic entity)			
Applicant Name (if Applicant is a juristic entity)			

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. If more than one applicant, use multiple forms.

☐ *Total of _____ forms are submitted.

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LIMITED POWER OF ATTORNEY BY APPLICANT

I hereby appoint the Practitioners named below as attorneys or agents, as necessary, for the limited purpose of communicating with Examiner(s) in the United States Patent and Trademark Office about rejoinder of restricted claims connected with US Application No. 14/759,895:

T. Daniel Christenbury	Reg. No. 31,750
Paul A. Taufer	Reg. No. 35,703
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Sima Singadia Kulkarni	Reg. No. 43,732
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Lisa M. Lint	Reg. No. 60,856
Deborah Grove	Reg. No. 61,091
Priya Narahari	Reg. No. 71,526
Joseph W. Wolfe	Reg. No. 73,173

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035811

I am the Applicant:

☐Inventor or Joint Inventor

☐Legal Representative of a Deceased or Legally Incapacitated Inventor

■ Assignee or Person to Whom the Inventor is Under an Obligation to Assign

☐Person Who Otherwise Shows Sufficient Proprietary Interest (e.g. a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document)

ISE PROFESSIONAL TESTING &
CONSULTING SERVICES, INC.
(The undersigned is authorized to act on behalf of Applicant.)

Date: 06/11/19

By: Ryanne Early

Name:

Title: President

EAST\52436798.1

Electronic Acknowledgement Receipt

EFS ID:	36274208
Application Number:	14759895
International Application Number:	
Confirmation Number:	3358
Title of Invention:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
First Named Inventor/Applicant Name:	Ryanne Early
Customer Number:	23628
Filer:	Sima Singadia Kulkarni/Stephanie De marco
Filer Authorized By:	Sima Singadia Kulkarni
Attorney Docket Number:	S1860.70000US01
Receipt Date:	12-JUN-2019
Filing Date:	08-JUL-2015
Time Stamp:	11:36:37
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Power of Attorney	SYG_19_1252_Limited_POA.pdf	394549 d0ed19d97986213f932f062ab745ea62266c648b	no	2

Warnings:

Information:	
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New International Application Filed with the USPTO as a Receiving Office

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/759,895	07/08/2015	Ryanne Early	S1860.70000US01

CONFIRMATION NO. 3358

35811
IP GROUP OF DLA PIPER LLP (US)
ONE LIBERTY PLACE
1650 MARKET ST, SUITE 4900
PHILADELPHIA, PA 19103
UNITED STATES OF AMERICA

POA ACCEPTANCE LETTER

OC000000108909888

Date Mailed: 06/14/2019

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/qtran/

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Application	Document	Mailroom Date	Attorney Docket No.
14759895	N570	06/14/2019	S1860.70000US01

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/759,895	07/08/2015	Ryanne Early	SYG-19-1252/426329-002	3358

35811 7590 07/03/2019

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PHILADELPHIA, PA 19103

EXAMINER	
DAVIS, RUTH A	

ART UNIT	PAPER NUMBER
1651	

NOTIFICATION DATE	DELIVERY MODE
07/03/2019	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

pto.phil@dlapiper.com

Applicant-Initiated Interview Summary	**Application No.** 14/759,895	**Applicant(s)** Early, Ryanne		
	Examiner RUTH A DAVIS	**Art Unit** 1651	**AIA (First Inventor to File) Status** Yes	**Page** **1 of** 2

All participants (applicant, applicants representative, PTO personnel):

1. RUTH A DAVIS (Primary Examiner); Telephonic
2. Sima Kulkarni (Attorney of Record); Telephonic
3. Gerard Bencen (Attorney); Telephonic

Date of Interview: 28 June 2019

Claims Discussed: Proposed amendments were made as appended here to.

Amendment proposed: See attached.

Brief Description of the main topic(s) of discussion: Applicant's representative proposed amendments to the claims so that additional products and process claims may be rejoined with the allowable products.

Issues Discussed:

Proposed Amendments:
Applicant proposed amendments to the claims so as to add method claims that may be eligible for rejoinder.

Attachment(s): Proposed Amendments,

	/RUTH A DAVIS/ Primary Examiner, Art Unit 1651

Applicant is reminded that a complete written statement as to the substance of the interview must be made of record in the application file. It is the applicants responsibility to provide the written statement, unless the interview was initiated by the Examiner and the Examiner has indicated that a written summary will be provided. See MPEP 713.04
Please further see:
MPEP 713.04
Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews, paragraph (b)
37 CFR § 1.2 Business to be transacted in writing

U.S. Patent and Trademark Office
PTOL-413/413b (Rev. 01/01/2015) **Interview Summary** Paper No. 20190628

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

PROPOSED CLAIMS

Cancel claims 1-4.

5. (Allowed) A tissue graft comprising extracellular matrix components derived from a Urodele, said tissue graft derived by:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix; and

b. decellularizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft.

Cancel claims 6-19.

20. (Allowed) A sterile medical implant comprising sterile, isolated, decellularized Urodele Extracellular Matrix (ECM) or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM produced by a process comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing and sterilizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and sterilizing said tissue.

21. (Allowed) The sterile medical implant of claim 20, wherein the implant is a biocompatible sheet, mesh, gel, graft, tissue or device.

22. (Allowed) A material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM wherein said isolated, decellularized Urodele ECM is produced by a process comprising:

RegenX Science Exhibit 1004
Page 1080

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1080

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and

sterilizing said tissue.

Cancel claims 23-25.

26. (Currently amended) A device comprising a tissue graft according to claim 5 comprising at least two sheets of isolated Urodele decellularized ECM laminated to one another, wherein said extracellular matrix components are derived from a Urodele, said tissue graft derived by:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix; and

b. decellularizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft.

Cancel claims 27-33.

Claims 34-35 were previously cancelled.

36. (Allowed) The tissue graft of claim 5, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment.

37. (Allowed) The tissue graft of claim 36, wherein said process further comprises subjecting said sample to sterilization.

38. (Allowed) The tissue graft of claim 5, wherein the Urodele is an Axolotl.

39. (Previously canceled)

40. (Allowed) The sterile medical implant of claim 20, wherein the Urodele ECM includes basement membrane.

41. (Allowed) The sterile medical implant of claim 20, wherein the Urodele ECM is derived from Axolotl tissue.

42. (Allowed) The sterile medical implant of claim 20, wherein the Urodele ECM is infused with, coated with, or attached to an agent xenogenic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug.

43. (Allowed) The sterile medical implant of claim 20, further comprising a human cell and wherein the Urodele ECM is infused with, coated with, or attached to the human cell.

44. (Allowed) The sterile medical implant of claim 20, wherein the Urodele extracellular matrix (ECM) or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM is a sheet.

45. (Allowed) The sterile medical implant of claim 20, wherein the Urodele ECM is in the form of Urodele ECM particles.

46. ((Allowed) The sterile medical implant of claim 20, wherein the Urodele ECM is a reconstituted gel.

47. ((Allowed) The material of claim 22, wherein the Urodele ECM is Axolotl ECM.

48. (Allowed) The device of claim 26, wherein the Urodele ECM is Axolotl ECM.

49. (Allowed) A tissue graft according to claim 5 comprising sterile, isolated, decellularized Urodele extra cellular matrix (ECM), wherein the ECM is a powder, a paste, a gel,

a sheet, or a mesh, and wherein the ECM is parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM.

50. (Allowed) The tissue graft of claim 49, wherein the ECM is a powder.

51. (Allowed) The tissue graft of claim 50, wherein the powder has a particle size between 125 and 850 microns.

52. (Allowed) The tissue graft of claim 50, wherein the powder has a particle size of less than 250 microns.

53. (Allowed) The tissue graft of claim 49, wherein the ECM is a paste.

54. (Allowed) The tissue graft of claim 49, wherein the ECM is a gel.

55. (Allowed) The tissue graft of claim 54, wherein the gel comprises a synthetic polymer.

56. (Allowed) The tissue graft of claim 49, wherein the ECM is a mesh.

57. (Allowed) A tissue graft according to claim 5 comprising sterile, isolated, decellularized Urodele extra cellular matrix (ECM), wherein the ECM is a sheet, wherein the ECM is parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM, and the sheet of ECM is infused with, coated with, or attached to an agent xenogeneic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug.

58. (Allowed) The tissue graft of claim 57, wherein the agent is an anti-microbial agent.

59. (Allowed) The tissue graft of claim 57, wherein the agent is a growth factor.

60. (Allowed) The tissue graft of claim 57, wherein the sheet is at least two sheets of isolated Urodele decellularized ECM laminated to one another.

61. (Allowed) The tissue graft according to claim 49 wherein the Urodele ECM is Axolotl ECM and is in a form selected from the group consisting of: a powder; a paste; a gel; a mesh; a sheet of parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM, and wherein the sheet of ECM is infused with, coated with, or attached to an agent xenogeneic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug; and at least two sheets of isolated Urodele decellularized ECM laminated to one another.

Cancel claims 62-65.

66. (New) A method for making tissue graft comprising extracellular matrix components derived from a Urodele, said method comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix; and

b. decellularizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft.

67. (New) A method for making a sterile medical implant comprising sterile, isolated, decellularized Urodele Extracellular Matrix (ECM) or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM, said method comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing and sterilizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and sterilizing said tissue.

68. (New) The method according to claim 67, wherein the implant is a biocompatible sheet, mesh, gel, graft, tissue or device.

69. (New) A method for making a material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM wherein said isolated, decellularized Urodele ECM is produced by a process comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and

sterilizing said tissue.

70. (New) The method according to claim 66, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment, wherein said process further comprises subjecting said sample to sterilization, wherein the Urodele is an Axolotl.

71. (New) A method of using the tissue graft according to claim 66, comprising administering said graft to a xenograft recipient in need thereof which comprises providing said tissue graft to said recipient in a sufficient amount and in a form adapted to the need of said recipient.

72. (New) The method according to claim 71, wherein said form is a biocompatible sheet, mesh, gel, graft, tissue, device, or is a material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM.

73. (New) The method according to claim 67, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment, wherein said process further comprises subjecting said sample to sterilization, wherein the Urodele is an Axolotl.

74. (New) A method of using the sterile medical implant according to claim 67 comprising administering said implant to a xenograft recipient in need thereof which comprises providing said tissue graft to said recipient in a sufficient amount and in a form adapted to the need of said recipient.

75. (New) The method according to claim 74, wherein said Urodele ECM is infused with, coated with, or attached to an agent xenogenic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug.

76. (New) The method according to claim 75, wherein said ECM is sterile.

77. (New) The method according to claim 74, wherein said ECM is in the form of a powder, a paste, a gel, a sheet, or a mesh.

78. (New) The method according to claim 77, wherein the ECM is parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM.

79. (New) The method according to claim 77, wherein the powder has a particle size between 125 and 850 microns.

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FAX NUMBER	15712738300
FROM	Henke, Stephanie
DATE	2019-06-28 11:07:27 EST
RE	US Patent Appln. No. 14/759,895; Attorney Docket No. SYG-19-1252/426329-002; Our Ref.: SYG-19-1252

COVER MESSAGE

Examiner Davis:

In preparation for our conversation today at 1:30pm, please see the attached proposed claim set setting forth amendments, and new claims for inclusion (pursuant to a request for rejoinder).

I look forward to speaking with you.

Stephanie Henke on behalf of Sima Singadia Kulkarni
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USSN 14/759,895
DLA Ref.: SYG-19-1252

PROPOSED CLAIMS

Cancel claims 1-4.

5. (Allowed) A tissue graft comprising extracellular matrix components derived from a Urodele, said tissue graft derived by:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix; and

b. decellularizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft.

Cancel claims 6-19.

20. (Allowed) A sterile medical implant comprising sterile, isolated, decellularized Urodele Extracellular Matrix (ECM) or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM produced by a process comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing and sterilizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and sterilizing said tissue.

21. (Allowed) The sterile medical implant of claim 20, wherein the implant is a biocompatible sheet, mesh, gel, graft, tissue or device.

22. (Allowed) A material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM wherein said isolated, decellularized Urodele ECM is produced by a process comprising:

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a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and

sterilizing said tissue.

Cancel claims 23-25.

26. (Currently amended) A device comprising a tissue graft according to claim 5 comprising at least two sheets of isolated Urodele decellularized ECM laminated to one another, wherein said extracellular matrix components are derived from a Urodele, said tissue graft derived by:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix; and

b. decellularizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft.

Cancel claims 27-33.

Claims 34-35 were previously cancelled.

36. (Allowed) The tissue graft of claim 5, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment.

37. (Allowed) The tissue graft of claim 36, wherein said process further comprises subjecting said sample to sterilization.

38. (Allowed) The tissue graft of claim 5, wherein the Urodele is an Axolotl.

39. (Previously canceled)

PAGE 4/4 * RCVD AT 6/28/2019 12:08:00 PM [Eastern Daylight Time] * SVR:W-PTOFAX-002/37 * DNIS:2738300 * CSID:DLA Piper LLP * ANI:9712751300 * DURATION (mm-ss):01-53

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a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and

sterilizing said tissue.

Cancel claims 23-25.

26. (Currently amended) A device comprising a tissue graft according to claim 5 comprising at least two sheets of isolated Urodele decellularized ECM laminated to one another, wherein said extracellular matrix components are derived from a Urodele, said tissue graft derived by:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix; and

b. decellularizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft.

Cancel claims 27-33.

Claims 34-35 were previously cancelled.

36. (Allowed) The tissue graft of claim 5, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment.

37. (Allowed) The tissue graft of claim 36, wherein said process further comprises subjecting said sample to sterilization.

38. (Allowed) The tissue graft of claim 5, wherein the Urodele is an Axolotl.

39. (Previously canceled)

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40. (Allowed) The sterile medical implant of claim 20, wherein the Urodele ECM includes basement membrane.

41. (Allowed) The sterile medical implant of claim 20, wherein the Urodele ECM is derived from Axolotl tissue.

42. (Allowed) The sterile medical implant of claim 20, wherein the Urodele ECM is infused with, coated with, or attached to an agent xenogenic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug.

43. (Allowed) The sterile medical implant of claim 20, further comprising a human cell and wherein the Urodele ECM is infused with, coated with, or attached to the human cell.

44. (Allowed) The sterile medical implant of claim 20, wherein the Urodele extracellular matrix (ECM) or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM is a sheet.

45. (Allowed) The sterile medical implant of claim 20, wherein the Urodele ECM is in the form of Urodele ECM particles.

46. ((Allowed) The sterile medical implant of claim 20, wherein the Urodele ECM is a reconstituted gel.

47. ((Allowed) The material of claim 22, wherein the Urodele ECM is Axolotl ECM.

48. (Allowed) The device of claim 26, wherein the Urodele ECM is Axolotl ECM.

49. (Allowed) A tissue graft according to claim 5 comprising sterile, isolated, decellularized Urodele extra cellular matrix (ECM), wherein the ECM is a powder, a paste, a gel,

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a sheet, or a mesh, and wherein the ECM is parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM.

50. (Allowed) The tissue graft of claim 49, wherein the ECM is a powder.

51. (Allowed) The tissue graft of claim 50, wherein the powder has a particle size between 125 and 850 microns.

52. (Allowed) The tissue graft of claim 50, wherein the powder has a particle size of less than 250 microns.

53. (Allowed) The tissue graft of claim 49, wherein the ECM is a paste.

54. (Allowed) The tissue graft of claim 49, wherein the ECM is a gel.

55. (Allowed) The tissue graft of claim 54, wherein the gel comprises a synthetic polymer.

56. (Allowed) The tissue graft of claim 49, wherein the ECM is a mesh.

57. (Allowed) A tissue graft according to claim 5 comprising sterile, isolated, decellularized Urodele extra cellular matrix (ECM), wherein the ECM is a sheet, wherein the ECM is parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM, and the sheet of ECM is infused with, coated with, or attached to an agent xenogeneic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug.

58. (Allowed) The tissue graft of claim 57, wherein the agent is an anti-microbial agent.

59. (Allowed) The tissue graft of claim 57, wherein the agent is a growth factor.

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60. (Allowed) The tissue graft of claim 57, wherein the sheet is at least two sheets of isolated Urodele decellularized ECM laminated to one another.

61. (Allowed) The tissue graft according to claim 49 wherein the Urodele ECM is Axolotl ECM and is in a form selected from the group consisting of: a powder; a paste; a gel; a mesh; a sheet of parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM, and wherein the sheet of ECM is infused with, coated with, or attached to an agent xenogeneic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug; and at least two sheets of isolated Urodele decellularized ECM laminated to one another.

Cancel claims 62-65.

66. (New) A method for making tissue graft comprising extracellular matrix components derived from a Urodele, said method comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix; and

b. decellularizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft.

67. (New) A method for making a sterile medical implant comprising sterile, isolated, decellularized Urodele Extracellular Matrix (ECM) or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM, said method comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing and sterilizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and sterilizing said tissue.

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68. (New) The method according to claim 67, wherein the implant is a biocompatible sheet, mesh, gel, graft, tissue or device.

69. (New) A method for making a material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM wherein said isolated, decellularized Urodele ECM is produced by a process comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and

sterilizing said tissue.

70. (New) The method according to claim 66, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment, wherein said process further comprises subjecting said sample to sterilization, wherein the Urodele is an Axolotl.

71. (New) A method of using the tissue graft according to claim 66, comprising administering said graft to a xenograft recipient in need thereof which comprises providing said tissue graft to said recipient in a sufficient amount and in a form adapted to the need of said recipient.

72. (New) The method according to claim 71, wherein said form is a biocompatible sheet, mesh, gel, graft, tissue, device, or is a material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM.

73. (New) The method according to claim 67, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment, wherein said process further comprises subjecting said sample to sterilization, wherein the Urodele is an Axolotl.

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68. (New) The method according to claim 67, wherein the implant is a biocompatible sheet, mesh, gel, graft, tissue or device.

69. (New) A method for making a material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM wherein said isolated, decellularized Urodele ECM is produced by a process comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and

sterilizing said tissue.

70. (New) The method according to claim 66, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment, wherein said process further comprises subjecting said sample to sterilization, wherein the Urodele is an Axolotl.

71. (New) A method of using the tissue graft according to claim 66, comprising administering said graft to a xenograft recipient in need thereof which comprises providing said tissue graft to said recipient in a sufficient amount and in a form adapted to the need of said recipient.

72. (New) The method according to claim 71, wherein said form is a biocompatible sheet, mesh, gel, graft, tissue, device, or is a material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM.

73. (New) The method according to claim 67, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment, wherein said process further comprises subjecting said sample to sterilization, wherein the Urodele is an Axolotl.

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74. (New) A method of using the sterile medical implant according to claim 67 comprising administering said implant to a xenograft recipient in need thereof which comprises providing said tissue graft to said recipient in a sufficient amount and in a form adapted to the need of said recipient.

75. (New) The method according to claim 74, wherein said Urodele ECM is infused with, coated with, or attached to an agent xenogenic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug.

76. (New) The method according to claim 75, wherein said ECM is sterile.

77. (New) The method according to claim 74, wherein said ECM is in the form of a powder, a paste, a gel, a sheet, or a mesh.

78. (New) The method according to claim 77, wherein the ECM is parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM.

79. (New) The method according to claim 77, wherein the powder has a particle size between 125 and 850 microns.

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40. (Allowed) The sterile medical implant of claim 20, wherein the Urodele ECM includes basement membrane.

41. (Allowed) The sterile medical implant of claim 20, wherein the Urodele ECM is derived from Axolotl tissue.

42. (Allowed) The sterile medical implant of claim 20, wherein the Urodele ECM is infused with, coated with, or attached to an agent xenogenic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug.

43. (Allowed) The sterile medical implant of claim 20, further comprising a human cell and wherein the Urodele ECM is infused with, coated with, or attached to the human cell.

44. (Allowed) The sterile medical implant of claim 20, wherein the Urodele extracellular matrix (ECM) or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM is a sheet.

45. (Allowed) The sterile medical implant of claim 20, wherein the Urodele ECM is in the form of Urodele ECM particles.

46. ((Allowed) The sterile medical implant of claim 20, wherein the Urodele ECM is a reconstituted gel.

47. ((Allowed) The material of claim 22, wherein the Urodele ECM is Axolotl ECM.

48. (Allowed) The device of claim 26, wherein the Urodele ECM is Axolotl ECM.

49. (Allowed) A tissue graft according to claim 5 comprising sterile, isolated, decellularized Urodele extra cellular matrix (ECM), wherein the ECM is a powder, a paste, a gel,

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a sheet, or a mesh, and wherein the ECM is parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM.

50. (Allowed) The tissue graft of claim 49, wherein the ECM is a powder.

51. (Allowed) The tissue graft of claim 50, wherein the powder has a particle size between 125 and 850 microns.

52. (Allowed) The tissue graft of claim 50, wherein the powder has a particle size of less than 250 microns.

53. (Allowed) The tissue graft of claim 49, wherein the ECM is a paste.

54. (Allowed) The tissue graft of claim 49, wherein the ECM is a gel.

55. (Allowed) The tissue graft of claim 54, wherein the gel comprises a synthetic polymer.

56. (Allowed) The tissue graft of claim 49, wherein the ECM is a mesh.

57. (Allowed) A tissue graft according to claim 5 comprising sterile, isolated, decellularized Urodele extra cellular matrix (ECM), wherein the ECM is a sheet, wherein the ECM is parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM, and the sheet of ECM is infused with, coated with, or attached to an agent xenogeneic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug.

58. (Allowed) The tissue graft of claim 57, wherein the agent is an anti-microbial agent.

59. (Allowed) The tissue graft of claim 57, wherein the agent is a growth factor.

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60. (Allowed) The tissue graft of claim 57, wherein the sheet is at least two sheets of isolated Urodele decellularized ECM laminated to one another.

61. (Allowed) The tissue graft according to claim 49 wherein the Urodele ECM is Axolotl ECM and is in a form selected from the group consisting of: a powder; a paste; a gel; a mesh; a sheet of parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM, and wherein the sheet of ECM is infused with, coated with, or attached to an agent xenogeneic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug; and at least two sheets of isolated Urodele decellularized ECM laminated to one another.

Cancel claims 62-65.

66. (New) A method for making tissue graft comprising extracellular matrix components derived from a Urodele, said method comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix; and

b. decellularizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft.

67. (New) A method for making a sterile medical implant comprising sterile, isolated, decellularized Urodele Extracellular Matrix (ECM) or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM, said method comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing and sterilizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and sterilizing said tissue.

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PAGE 1/5 * RCVD AT 6/28/2019 11:52:41 AM [Eastern Daylight Time] * SVR:W-PTOFAX-001/16 * DNIS:2738300 * CSID:DLA Piper LLP * ANI:9712751300 * DURATION (mm-ss):02-23

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PAGE 2/5 * RCVD AT 6/28/2019 11:52:41 AM [Eastern Daylight Time] * SVR:W-PTOFAX-001/16 * DNIS:2738300 * CSID:DLA Piper LLP * ANI:9712751300 * DURATION (mm-ss):02-23

USSN 14/759,895
DLA Ref.: SYG-19-1252

PROPOSED CLAIMS

Cancel claims 1-4.

5. (Allowed) A tissue graft comprising extracellular matrix components derived from a Urodele, said tissue graft derived by:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix; and

b. decellularizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft.

Cancel claims 6-19.

20. (Allowed) A sterile medical implant comprising sterile, isolated, decellularized Urodele Extracellular Matrix (ECM) or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM produced by a process comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing and sterilizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and sterilizing said tissue.

21. (Allowed) The sterile medical implant of claim 20, wherein the implant is a biocompatible sheet, mesh, gel, graft, tissue or device.

22. (Allowed) A material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM wherein said isolated, decellularized Urodele ECM is produced by a process comprising:

PAGE 3/5 * RCVD AT 6/28/2019 11:52:41 AM [Eastern Daylight Time] * SVR:W-PTOFAX-001/16 * DNIS:2738300 * CSID:DLA Piper LLP * ANI:9712751300 * DURATION (mm-ss):02-23

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and

sterilizing said tissue.

Cancel claims 23-25.

26. (Currently amended) A device comprising a tissue graft according to claim 5 comprising at least two sheets of isolated Urodele decellularized ECM laminated to one another, wherein said extracellular matrix components are derived from a Urodele, said tissue graft derived by:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix; and

b. decellularizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft.

Cancel claims 27-33.

Claims 34-35 were previously cancelled.

36. (Allowed) The tissue graft of claim 5, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment.

37. (Allowed) The tissue graft of claim 36, wherein said process further comprises subjecting said sample to sterilization.

38. (Allowed) The tissue graft of claim 5, wherein the Urodele is an Axolotl.

39. (Previously canceled)

PAGE 4/5 * RCVD AT 6/28/2019 11:52:41 AM [Eastern Daylight Time] * SVR:W-PTOFAX-001/16 * DNIS:2738300 * CSID:DLA Piper LLP * ANI:9712751300 * DURATION (mm-ss):02-23

40. (Allowed) The sterile medical implant of claim 20, wherein the Urodele ECM includes basement membrane.

41. (Allowed) The sterile medical implant of claim 20, wherein the Urodele ECM is derived from Axolotl tissue.

42. (Allowed) The sterile medical implant of claim 20, wherein the Urodele ECM is infused with, coated with, or attached to an agent xenogenic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug.

43. (Allowed) The sterile medical implant of claim 20, further comprising a human cell and wherein the Urodele ECM is infused with, coated with, or attached to the human cell.

44. (Allowed) The sterile medical implant of claim 20, wherein the Urodele extracellular matrix (ECM) or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM is a sheet.

45. (Allowed) The sterile medical implant of claim 20, wherein the Urodele ECM is in the form of Urodele ECM particles.

46. ((Allowed) The sterile medical implant of claim 20, wherein the Urodele ECM is a reconstituted gel.

47. ((Allowed) The material of claim 22, wherein the Urodele ECM is Axolotl ECM.

48. (Allowed) The device of claim 26, wherein the Urodele ECM is Axolotl ECM.

49. (Allowed) A tissue graft according to claim 5 comprising sterile, isolated, decellularized Urodele extra cellular matrix (ECM), wherein the ECM is a powder, a paste, a gel,

PAGE 5/5 * RCVD AT 6/28/2019 11:52:41 AM [Eastern Daylight Time] * SVR:W-PTOFAX-001/16 * DNIS:2738300 * CSID:DLA Piper LLP * ANI:9712751300 * DURATION (mm-ss):02-23

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FAX COVER SHEET

TO	
COMPANY	
FAX NUMBER	15712738300
FROM	Henke, Stephanie
DATE	2019-06-28 10:52:23 EST
RE	US Patent Appln. No. 14/759,895; Attorney Docket No. SYG-19-1252/426329-002; Our Ref.: SYG-19-1252

COVER MESSAGE

Examiner Davis:

In preparation for our conversation today at 1:30pm, please see the attached proposed claim set setting forth amendments, and new claims for inclusion (pursuant to a request for rejoinder).

I look forward to speaking with you.

Stephanie Henke on behalf of Sima Singadia Kulkarni
IP Administrative Specialist

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PAGE 1/1 * RCVD AT 6/28/2019 12:03:14 PM [Eastern Daylight Time] * SVR:W-PTOFAX-002/24 * DNIS:2738300 * CSID:DLA Piper LLP * ANI:9712751300 * DURATION (mm-ss):00-44

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Art Unit	: 1651	**Customer No. 035811**
Examiner	: Davis, Ruth A.	
Appl. No.	: 14/759,895	Docket No.: SYG-19-1252US/ 426329-002
Appl. Filed	: July 8, 2015	
First Named Inventor	: Ryanne Early	Confirmation No.: 3358
Title	: Decellularized Biomaterial from Non-Mammalian Tissue	Dated: July 2, 2019

RESPONSE

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In response to the Non-Final Office Action dated January 10, 2019, (the "Office Action"), the Applicant amends the application as follows:

In the Claims

1-4. (Cancelled)

5. (Previously Presented) A tissue graft comprising extracellular matrix components derived from a Urodele, said tissue graft derived by:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix; and

b. decellularizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft.

6-19. (Cancelled)

20. (Previously Presented) A sterile medical implant comprising sterile, isolated, decellularized Urodele Extracellular Matrix (ECM) or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM produced by a process comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing and sterilizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and sterilizing said tissue.

21. (Previously Presented) The sterile medical implant of claim 20, wherein the implant is a biocompatible sheet, mesh, gel, graft, tissue or device.

22. (Previously Presented) A material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM wherein said isolated, decellularized Urodele ECM is produced by a process comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing said tissue sample to produce decellularized extracellular matrix by

removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and
sterilizing said tissue.

23-25. (Cancelled)

26. (Currently Amended) A device comprising a tissue graft according to claim 5 comprising at least two sheets of isolated Urodele decellularized ECM laminated to one another, wherein said extracellular matrix components are derived from a Urodele, said tissue graft derived by:
a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix; and
b. decellularizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft.

27-35. (Cancelled)

36. (Previously Presented) The tissue graft of claim 5, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment.

37. (Previously Presented) The tissue graft of claim 36, wherein said process further comprises subjecting said sample to sterilization.

38. (Previously Presented) The tissue graft of claim 5, wherein the Urodele is an Axolotl.

39. (Cancelled)

40. (Previously Presented) The sterile medical implant of claim 20, wherein the Urodele ECM includes basement membrane.

41. (Previously Presented) The sterile medical implant of claim 20, wherein the Urodele

ECM is derived from Axolotl tissue.

42. (Previously Presented) The sterile medical implant of claim 20, wherein the Urodele ECM is infused with, coated with, or attached to an agent xenogenic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug.

43. (Previously Presented) The sterile medical implant of claim 20, further comprising a human cell and wherein the Urodele ECM is infused with, coated with, or attached to the human cell.

44. (Previously Presented) The sterile medical implant of claim 20, wherein the Urodele extracellular matrix (ECM) or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM is a sheet.

45. (Previously Presented) The sterile medical implant of claim 20, wherein the Urodele ECM is in the form of Urodele ECM particles.

46. (Previously Presented) The sterile medical implant of claim 20, wherein the Urodele ECM is a reconstituted gel.

47. (Previously Presented) The material of claim 22, wherein the Urodele ECM is Axolotl ECM.

48. (Previously Presented) The device of claim 26, wherein the Urodele ECM is Axolotl ECM.

49. (Previously Presented) A tissue graft according to claim 5 comprising sterile, isolated, decellularized Urodele extra cellular matrix (ECM), wherein the ECM is a powder, a paste, a gel, a sheet, or a mesh, and wherein the ECM is parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM.

50. (Previously Presented) The tissue graft of claim 49, wherein the ECM is a powder.

51. (Previously Presented) The tissue graft of claim 50, wherein the powder has a particle size between 125 and 850 microns.

52. (Previously Presented) The tissue graft of claim 50, wherein the powder has a particle size of less than 250 microns.

53. (Previously Presented) The tissue graft of claim 49, wherein the ECM is a paste.

54. (Previously Presented) The tissue graft of claim 49, wherein the ECM is a gel.

55. (Previously Presented) The tissue graft of claim 54, wherein the gel comprises a synthetic polymer.

56. (Previously Presented) The tissue graft of claim 49, wherein the ECM is a mesh.

57. (Previously Presented) A tissue graft according to claim 5 comprising sterile, isolated, decellularized Urodele extra cellular matrix (ECM), wherein the ECM is a sheet, wherein the ECM is parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM, and the sheet of ECM is infused with, coated with, or attached to an agent xenogeneic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug.

58. (Previously Presented) The tissue graft of claim 57, wherein the agent is an anti-microbial agent.

59. (Previously Presented) The tissue graft of claim 57, wherein the agent is a growth factor.

60. (Previously Presented) The tissue graft of claim 57, wherein the sheet is at least two sheets of isolated Urodele decellularized ECM laminated to one another.

RegenX Science Exhibit 1004
Page 1114

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1114

61. (Previously Presented) The tissue graft according to claim 49 wherein the Urodele ECM is Axolotl ECM and is in a form selected from the group consisting of: a powder; a paste; a gel; a mesh; a sheet of parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM, and wherein the sheet of ECM is infused with, coated with, or attached to an agent xenogeneic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug; and at least two sheets of isolated Urodele decellularized ECM laminated to one another.

62-65. (Cancelled)

66. (New) A method for making the tissue graft of claim 5, comprising extracellular matrix components derived from a Urodele, said method comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix; and

b. decellularizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft.

67. (New) The method according to claim 66, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment, and wherein said process further optionally comprises subjecting said sample to sterilization, and wherein the Urodele is an Axolotl.

68. (New) A method of using the tissue graft according to claim 5 , providing said tissue graft to a xenograft recipient in need thereof in a sufficient amount and in a form adapted to the need of said recipient.

69. (New) The method according to claim 68, wherein said form is a biocompatible sheet, mesh, gel, graft, tissue, device, or is a material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM.

RegenX Science Exhibit 1004
Page 1115

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1115

70. (New) A method for making the sterile medical implant of claim 20, comprising sterile, isolated, decellularized Urodele Extracellular Matrix (ECM) or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM, said method comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing and sterilizing said tissue sample, to produce the sterile decellularized extracellular matrix comprised within said sterile medical implant, by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial, making it suitable for use as a xenograft; and sterilizing said tissue.

71. (New) The method according to claim 70, wherein the implant is a biocompatible sheet, mesh, gel, graft, tissue or device, or is a material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM.

72. (New) The method according to claim 70, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment, wherein said process further comprises subjecting said sample to sterilization, wherein the Urodele is an Axolotl.

73. (New) A method of using the sterile medical implant according to claim 20 which comprises providing said tissue graft to a xenograft recipient in need thereof in a sufficient amount and in a form adapted to the need of said recipient.

74. (New) The method according to claim 73, wherein said form is a biocompatible sheet, mesh, gel, graft, tissue, device, or is a material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM.

75. (New) The method according to claim 74, wherein said Urodele ECM is infused with, coated with, or attached to an agent xenogenic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug.

RegenX Science Exhibit 1004
Page 1116

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1116

76. (New) The method according to claim 75, wherein said ECM is sterile.

77. (New) The method according to claim 76, wherein said ECM is in the form of a powder, a paste, a gel, a sheet, or a mesh.

78. (New) The method according to claim 77, wherein the ECM is parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM.

79. (New) The method according to claim 77, wherein the ECM is in the form of a powder, a paste or a gel, in which said ECM has a particle size between 125 and 850 microns.

80. (New) A method for making the material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM of claim 22, wherein said isolated, decellularized Urodele ECM is produced by a process comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and sterilizing said tissue.

RegenX Science Exhibit 1004
Page 1117

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1117

Remarks

Following entry of the present amendment, the status of the claims is as follows: claims 1-4, 6-19, 23-25, and 62-65 canceled herein, claims 34-35 and 39 previously canceled, Claim 26 currently amended, Claims 5, 20-22, 26, 27-33, 36-38, and 40-61 allowed, and Claims 66-80 newly added.

New method claims 66-80 are introduced herein as being commensurate with the allowed product claims, which are produced and used by the method as claimed in the newly introduced method claims. Support for the new method claims can be found throughout the specification, including for example: paragraphs [0022] and [0038] Claims 66-69 method for making and using a tissue graft (biologic material); paragraph [0031] Claims 70-78 method for making a sterile medical implant; and paragraphs [0029], [0032] and [0035] Claim 79 method for making a material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM. Please also note that the allowed product by process claims themselves provide substantial support for the method of making claims.

In view of the foregoing amendments and following remarks, reconsideration and allowance are respectfully requested.

I. Examiner Interview

Applicant thanks Examiner Davis for her time and consideration of the pending claims during a telephone interview on June 28, 2019. During the telephone interview, Applicant's representatives requested rejoinder of previously restricted claims. The subject matter of the rejoinder claims include "method of making" and "method of use" claims that correspond to the device/product claims which the Examiner has indicated to be allowable in the January 10, 2019, office action.

II. Rejection under 35 U.S.C. §112(a)

The Office Action rejected claims 62-65 under 35 U.S.C. §112(a), as failing to comply with the written description requirement. By this Amendment, claims 62-65 are canceled thereby rendering the rejection moot. Reconsideration and withdrawal of the rejection is respectfully requested.

III. Rejection under 35 U.S.C. §112(b)

Applicant acknowledges withdrawal of the rejections based on 35 U.S.C. §112(a).

IV. Rejection under 35 U.S.C. §102(a)(1)

Applicant acknowledges withdrawal of the rejections based on 35 U.S.C. §102(a)(1).

V. Rejections Under 35 U.S.C. §103

The Office Action rejects Claims 26 and 48 under 35 U.S.C. §103 as being unpatentable over Asem et al. (US 6485969) in view of Seifert et al. (2012). According to the Office Action, Asem teaches decellularized ECM comprising basement membranes derived from amphibians that are adhered together (or laminated). Without acquiescing to the rejection, and solely in the interest of advancing prosecution, Applicant has herein amended Claim 26 to incorporate the subject matter of the allowed claims, and, since Claim 48 depends upon Claim 26, the amendment is considered to carry through to Claim 48. Reconsideration and withdrawal of the rejection is therefore respectfully requested.

VI. Allowable Subject Matter

Claims 5, 20-22, 36-38, 40-47, and 49 -61 were allowed in the January 10, 2019 Office Action.

VII. Request for Rejoinder

As stated above, during the telephone interview on June 28, 2019, Applicant requested rejoinder of previously restricted subject matter, which the Examiner indicated she felt appropriate in light of allowance of the relevant product claims. Accordingly, Applicant submits herewith new claims 66-80, which include all the limitations of the allowed claims.

RegenX Science Exhibit 1004
Page 1119

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1119

Conclusion

In view of the foregoing, it is respectfully submitted that this application is in condition for allowance. Favorable reconsideration and prompt allowance of the application are earnestly solicited.

If the Examiner has any questions or believes that any outstanding issues may be resolved with minor amendments or a phone discussion, the Examiner is invited to contact the Applicant's representative, Sima Singadia Kulkarni, at (215) 656-2478 or sima.kulkarni@dlapiper.com.

Respectfully submitted,

/sima singadia kulkarni/

Sima Singadia Kulkarni
Reg. No. 43,732

RegenX Science Exhibit 1004
Page 1120

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1120

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Art Unit	: 1651	**Customer No. 035811**
Examiner	: Davis, Ruth A.	
Appl. No.	: 14/759,895	Docket No.: SYG-19-1252US/ 426329-002
Appl. Filed	: July 8, 2015	
First Named Inventor	: Ryanne Early	Confirmation No.: 3358
Title	: Decellularized Biomaterial from Non-Mammalian Tissue	Dated: July 2, 2019

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

The Applicant encloses Form PTO-1449 together with a copy of each of the two (2) listed non-US publications. A copy of the Second Office Action from counterpart Chinese Patent Appln. No. 201480013359.0 dated May 22, 2019, and its English translation, is also enclosed. The documents are submitted under 37 CFR §1.56. The two (2) enclosed publications are believed related in view of their mention in the Search Report for the reasons stated.

Applicant certifies that each item of information contained in the Information Disclosure Statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months ago.

The Applicant respectfully requests that this Information Disclosure Statement be officially entered into the file and that appropriate notification be made that it was considered by the Examiner.

Respectfully submitted,

/sima singadia kulkarni/

Sima Singadia Kulkarni
Reg. No. 43,732

SSK/sh
(215) 656-2478

EAST\167753946.3

Form PTO-1449 US DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTY. DOCKET NO. SYG-19-1252US/426329-002	SERIAL NO. 14/759,895
	APPLICANT ISE Professional Testing & Consulting Services, Inc.	
LIST OF PUBLICATIONS CITED BY APPLICANT *(Use several sheets if necessary)*	FILING DATE July 8, 2015	GROUP 1651

US PATENT DOCUMENTS

EXAMINER INITIAL*		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
	AA	2006/153797 A1 (corr. to CN '039)	07/13/06	Bortolotto et al.			
	AB						
	AC						
	AD						
	AE						
	AF						
	AG						
	AH						
	AI						
	AJ						
	AK						

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO
	AL	1969039 A (corr. to US '797)	05/23/07	CN			X	
	AM	101337089 A	01/07/09	CN			Abstract	
	AN							
	AO							
	AP							

OTHER PUBLICATIONS (Including Author, Title, Date, Pertinent Pages, Etc.)

	AR	Second Office Action from counterpart Chinese Patent Appln. No. 201480013359.0 dated May 22, 2019, along with its English Translation.
	AS	
	AT	

EXAMINER	DATE CONSIDERED

*EXAMINER: Initial if publication considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

EAST\47689080.1
EAST\167753423.1

Espacenet

Bibliographic data: CN101337089 (A) — 2009-01-07

Muscle regeneration factor

Inventor(s): MENGZHI WANG [CN] ± (WANG MENGZHI)

Applicant(s): MENGZHI WANG [CN] ± (WANG MENGZHI)

Classification: **- international:** ***A61K8/98; A61L27/38; A61Q19/08***
- cooperative:

Application number: CN200710122922 20070704

Priority number(s): CN200710122922 20070704

Abstract of CN101337089 (A)

The invention relates to a muscle regeneration agent, which belongs to the field of the plastic surgery. The muscle regeneration agent is an injection-type muscle regeneration agent which is prepared by the following steps: under the protection of the cell wall-degraded fusion agent, newt cells with regeneration ability in the animal kingdom are fused with the wall-degraded cells of tested mice to realize the growth of bulgy histiocytes, the histiocytes growing after the fusion of degraded cell walls of newt cells are further accepted, and are prepared into the injection-type muscle regeneration agent with the cell wall-degraded fusion agent and the injection solvent after in vitro expansion. The muscle regeneration agent can be used for nose augmentation, chin augmentation and breast augmentation by the injection method (in one or more steps) in the plastic surgery. Muscle tissues can grow and bulge at the injection positions to the designed effect after injection. The injection method can also be applied to eliminating wrinkles and avoiding wrinkles from appearing in a long term.

[19] 中华人民共和国国家知识产权局

[51] Int. Cl.
A61L 27/38 (2006.01)
A61K 8/98 (2006.01)
A61Q 19/08 (2006.01)

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[22] 申请日 2007.7.4
[21] 申请号 200710122922.X
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[72] 发明人 王梦芝
权利要求书 1 页 说明书 5 页

[54] 发明名称

肌肉再生素

[57] 摘要

本发明肌肉再生素，属于整形外科领域。本发明涉及将动物界具有再生能力的蝾螈细胞在脱髓融合剂的保护下，与受试鼠细胞脱髓融合长出隆起的组织细胞，再次接受蝾螈细胞脱髓融合长出的组织细胞，体外扩增后，与脱髓融合剂，注射溶剂，制成注射剂型的肌肉再生素。本发明在整形外科中，可以用注射法(一次或多次)进行隆鼻、隆颏、隆胸。注射后能在注射处，长出隆起达到设计效果的肌肉组织。也可以用注射法消除皱纹，并使皱纹长期不再出现。

1、本发明肌肉再生素，是用动物蝾螈细胞，在脱襞融合剂的保护下，与实验鼠细胞脱襞融合合并隆起的组织细胞，再次接受蝾螈细胞脱襞融合长出隆起的组织细胞，体外扩增后与脱襞融合剂，注射溶剂，制成的注射剂型的肌肉再生素。

2、根据权利要求 1 所述的肌肉再生素，在整形外科中，用来隆鼻、隆颏、隆胸等，将肌肉再生素一次或多次注射进鼻、颏、乳中，就能长出达到美容设计的隆起组织。

3、根据权利要求 1 所述的肌肉再生素，在美容外科中，将肌肉再生素，注射入皱纹组织中并顺纹路抹平，就能清除皱纹，原来的皱纹将长期不会再现。

4、根据权利要求 1 所述的肌肉再生素，可以用来填补人体任何部位的缺（凹）陷，修复需要隆起（平）的部位。

RegenX Science Exhibit 1004
Page 1125

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1125

肌肉再生素

技术领域 本发明肌肉再生素属于整形外科领域。

本发明涉及在整形外科中，将肌肉再生素注射入需要隆起的部位。让它生长隆起达到美容设计效果的完整的部位。如：隆鼻、隆颏、隆胸等。本发明肌肉再生素是整形外科的新材料。

背景技术 爱美是人类的天性，人类对美的追求是社会进步的象征。19 世纪晚期性病流行于世，严重的并发症。使很多“风流人物“发生了塌鼻畸形。使原来的容貌变得特别丑陋，一些鞍鼻畸形的患者，也和梅毒鼻一样，受到社会的歧视，他们迫切要求治疗.以摆脱困境。

20 世纪以来，以隆鼻术开始，隆鼻、隆颏、隆胸的整形外科，应运而生，整形外科首先遇到的是用什么来增高需要增高的部位的难题。整形外科一开始就采用过五花八门的材料如：凡士林、石蜡、塑料、象牙、有机玻璃、马来树胶、铂、金、银、动物骨、人工骨、异体骨，自体骨、液体硅胶等等。始于 1904 年曾经风行一时的注射石蜡油，不久就出现了问题。石蜡油不但刺激性太大，组织反映强烈，而且冬天怕火烤，夏天怕暴晒，塑形能力差，由于组织反应，长出石蜡瘤，须开刀才能取出，注射石蜡油后，有的随重力向下流向腹部、大腿、甚至足踝，沿途形成多发性石蜡瘤，留下严重的后遗症，造成终生遗憾，致使许多受术者破相毁容，注射石蜡油很快就被淘汰了。此后有人认为：隆鼻、隆颏、用自体骨最好，不会被排斥吸收，无毒副作用，安全放心，能和组织长在一起，保持终生。但在实践中，自体骨也有不少缺点，人类可供使用的主要是髋骨、肋骨、和肋软骨。

RegenX Science Exhibit 1004
Page 1126

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1126

要从人体上动手术取出一块骨来，手术相对痛苦大，时间长，而且髋骨、肋骨，骨质硬，不好塑形，肋软骨厚度不够，塑形困难。不能达到手术效果。很多材料都经不起实践使用而被淘汰。

随着化学工业的发展，1963 年，人工合成的高科技有机化合物硅橡胶出现了，经过动物实验，临床试验，硅胶可根据不同需要制成不同性状的（如固体块状、成形假体）硅胶制品，植入人体需要隆起的美容部位。高分子硅胶理化性能 稳定，生物相容性好。对人体无毒、无害不吸收、不变形、来源丰富，可以批量生产。

1963 年以来，隆鼻、隆颏、隆胸的材料就只有硅胶了。使用硅胶假体进行整形美容。必须通过手术才能进行：隆鼻从一侧鼻小柱边缘；隆颏从口内或口外颏下；隆乳有五个部位选择，乳晕边缘，乳房外侧缘，乳房下皱裂反折处，腋前皱裂处，腋窝顶部（各部都有优缺点）；作小切口，通过血管钳顺切口深入鼻端、鼻背、鼻根（隆鼻）；颏部（隆颏），乳房中心区的乳腺后或胸大肌下（隆乳）；进行潜行分离出容纳假体的腔隙，通过切口将假体植入，摆正、缝合切口，这样手术才能完成。

手术不能不是流血的过程，是痛苦的，有风险的。再者植入的毕竟是假体，是异物、时间越长，越容易发生假体移位，老化、破裂、需要更换率就越大。

为了寻找非手术的整形外科手段，人们也做过种种尝试。比如：注射自体脂肪颗粒，这本来是一举两得的好办法，从局部脂肪堆积的部位抽吸出来脂肪颗粒，一面达到减肥美容，一面废物利用，简单易

RegenX Science Exhibit 1004
Page 1127
EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1127

行，安全，无异物反应，创伤小、恢复快、无切口，不留手术痕迹。但是植入的颗粒不能存活的，液化坏死后会逐渐吸收排出体外，吸收率好的是 30-60%，不好的是 70-80%。又如注射肉毒素，有效期只有 4-8 个月；注射玻尿酸，有效期也只有 6-12 个月。想要维持效果，就必须间歇性施注，承受一次又一次的风险。

座落在上海闵行紫竹高科技园区的“国家组织工程中心”；首席科学家曹谊林教授说，这里将建立一家专门的生物医学工厂，在这工厂里人们可以预购自己所需要的任何组织器官，专家从正常人体中取一小块组织细胞，让它在体外大量扩增，再回植到需要修复的部位。即把“种子细胞”注射到鼻子、下巴等需要隆起的美容部位，让它长出需要的组织。

在整形外科中，人们一直在追求一种材料注射入需要隆起的美容部位，让它长出需要的组织，可惜迄今始终未果。

本发明肌肉再生素正是人们百多年来苦苦寻求的最理想的材料。

发明内容 本发明在于用动物界具有再生能力的蝾螈细胞，二次与实验鼠细胞脱髓融合长出的隆起组织细胞，经体外扩增后，与脱髓融合剂、注射溶液，制成注射剂型的肌肉再生素。然后将其注射入人体需要隆起的部位，让它长出达到设计效果的组织，本发明肌肉再生素，在整形外科中，可以用注射法达到隆鼻、隆颏、隆乳等美容效果。

将本发明肌肉再生素注射入皱纹部位，并顺纹路抹平，去除了皱纹，并使原来的皱纹长期不会再现，做到受术者青春长驻、容颜不衰。

用本发明肌肉再生素，可以弥补或修复人体任何部位的缺（凹）

RegenX Science Exhibit 1004
Page 1128

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1128

陷。再塑美丽的形体。

本发明肌肉再生素的特征在于注入人体的本发明肌肉再生素，能与受体的肌肉细胞融合，是细胞外面的鞭溶掉才使细胞合并，脱了鞭的细胞的合并即是原生质体的合并，原生质体的合并产生了新的细胞，从而使本发明肌肉再生素与受体的细胞进行一系列的脱鞭融合，合并长出新的肌肉组织。从而使受体隆起的部位是受体自身的肌肉组织。区别于以往的整形隆起的材料，绝大多数是异物假体。

本发明的特征还在于，用动物界具有再生能力的蝾螈细胞二次与实验鼠细胞脱鞭融合长出隆起的细胞，体外扩增后，与脱鞭融合剂，注射溶剂，制成注射剂型肌肉再生素，其中配有清除排异作用的脱鞭融合剂（不是具有奇特抗排异能力的目前器官移植中常用的环孢霉素）。从而使本发明能与任何种族的人体细胞脱鞭融合，合并成新的细胞。没有任何排异性，本发明适合于任何种族的人体和任何动物的畜体细胞融合合并生成新的组织。

具体实施方式 本发明的目的是这样实现的：本发明受世界科技成果（1999 年 12 月，美国科学家发现小鼠肌肉组织的成体干细胞可以“横向分化”为血液细胞，这一发现立刻被世界各地的科学家证实后，进一步的研究发现人类成体干细胞同样具有“横向分化”的功能；2000 年 9 月，意大利科学家用成年动物的神经干细胞成功地培育出肌肉细胞；2001 年 2 月，中国科学家曹谊林在兔耳上培育出人耳；2005 年 9 月，在剑桥大学举行的一次科学研讨会上，来自美国威斯塔研究所的专家披露：世界上第一只具有超强再生能力的被称为：“MRL”鼠种的

RegenX Science Exhibit 1004
Page 1129

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1129

“奇迹老鼠”，能重新长出身上包括心脏在内的“几乎所有器官”，当把“奇迹老鼠”身上提取的胎儿肝细胞注射到普通老鼠体内后，也能奇迹般地获得了再生能力）启发。在脱髮融合剂的保护下将动物界具有肢体再生的蝾螈细胞，与实验鼠肌肉细胞融合长出隆起的组织，再次接受蝾螈细胞，脱髮融合长出隆起组织细胞，体外扩增后，配合脱髮融合剂、注射溶剂、制成本发明注射剂型的肌肉再生素。

将本发明肌肉再生素，涂在受试鼠的约 $2cm^2$ 的创面上，伤口能在 42h 内完全愈合脱痂，但愈合后的创面上留下以创面一样大小的光洁的凸起的肌肉组织。

将本发明肌肉再生素注射到受试鼠身上，3 天内注射点长出了光洁的肉柱，将长出的肉柱解剖，以受试鼠身上其它部位的肌肉组织完全一样。

作为创伤药在人体上试用，将肌肉再生素涂在伤口上，能加速伤口的愈合，愈合脱痂后的创面都凸出周围的部位。

将肌肉再生素，注射入人的小腿部位，结果也隆起了预期的效果。在重复试验时，排除（或单独使用）脱髮融合剂，则所有试验毫无结果。说明脱髮溶合剂具有超强消除排异作用和促进两种细胞脱髮融合合并的功能。

本发明将经过严格的动物试验和临床试验后，可用注射法（一次或多次），进行隆鼻、隆颏、隆胸。它定将成为整形美容的最理想的材料，也将成为人类爱美天性的品牌，留给人类美丽的青春永驻的面孔。

RegenX Science Exhibit 1004
Page 1130

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1130

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权利要求书 3 页 说明书 27 页 附图 4 页

[54] 发明名称

组织材料和肌肉来源的基质

[57] 摘要

本发明一般涉及对促进或利于细胞和组织的生长、发育和分化有用的组织制剂，包括组织细胞及其提取物。更具体而言，本发明提供了包括完整的或提取的细胞外基质和/或细胞以及细胞因子、生长因子和其他组分的肌肉来源的材料。本发明的肌肉制剂类似基底膜并且来源于基于细胞的材料。该肌肉制剂可以在体外或体内使用，尤其是在各种组织工程应用中作为细胞支架并在其他细胞培养系统用于营养和富集一系列细胞类型，包括但不限于，前体细胞和干细胞例如前脂肪形成细胞。该肌肉制剂还在例如化妆品和局部治疗工业中用作乳膏的基础并且在食品工业中用作基质或添加剂。

1. 一种用于促进细胞效应的物质组合物，所述组合物包括基于细胞的或无细胞的肌肉组织制剂提取物，所述制剂提供了下列组分的来源，所述组分例如但不限于，层粘连蛋白、胶原蛋白 I、胶原蛋白 IV、触觉蛋白/巢蛋白、硫酸乙酰肝素蛋白聚糖以及其他组分，包括细胞因子和生长因子，例如但不限于，EGF、bFGF、NGF、PDGF、IGF-1、TGF-β、VEGF 和 TNF-α或其同源物中的一种或多种。

2. 权利要求 1 的组合物，其中所述制剂是层粘连蛋白、触觉蛋白/巢蛋白、硫酸乙酰肝素蛋白聚糖、胶原蛋白 IV、bFGF、PDGF、TGF-β、VEGF 和 TNF-α的来源。

3. 权利要求 1 的组合物，其中所述细胞来自肌肉组织。

4. 权利要求 3 的组合物，其中所述肌肉组织来自人、非人灵长类、家畜动物、实验室试验动物、伴侣动物、鸟类、爬行动物或两栖动物。

5. 权利要求 3 的组合物，其中所述肌肉组织来自人、猪或大鼠。

6. 权利要求 1 的组合物，其中所述制剂是基于细胞的制剂。

7. 权利要求 1 的组合物，其中所述制剂包括完整的细胞外基质。

8. 权利要求 1 的组合物，其中所述制剂包括提取的细胞外基质。

9. 权利要求 1 的组合物，其中所述制剂包含约 1μg/ml－约 100 mg/ml 的总蛋白含量。

10. 权利要求 1－9 中任何一项的组合物，其中所述制剂聚合成凝胶。

11. 权利要求 1 的组合物，其进一步包括一种或多种外源性细胞因子、抗生素、生长强化因子、基因表达增强子、增殖抑制剂和/或干细胞分化促进因子。

12. 权利要求 1 的组合物，其中所述组合物促进细胞效应，所述细胞选自干细胞、上皮细胞、皮肤细胞、器官细胞和内皮细胞。

13. 权利要求 1 的组合物，其中存在的 bFGF 为约 0.3ng/ml－约 4ng/ml；PDGF 为约 1pg/ml－约 3000pg/ml；TFG-β为约 1pg/ml－约

RegenX Science Exhibit 1004
Page 1132

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1132

2000pg/ml；EGF 为约 0 ng/ml－约 100ng/ml；VEGF 为约 1pg/ml－约 3000pg/ml；以及 TNF-α为约 0 pg/ml－约 1000pg/ml。

14. 权利要求 1－13 中任何一项的组合物在对某些材料的脂肪形成潜力进行生物测定中的用途。

15. 权利要求 14 的用途，用于体外测定调节脂肪形成的组分、提取物或细胞系统，所述测定包括筛查肌肉制剂以鉴别具有脂肪细胞分化倾向的细胞群，产生或获得潜在调节脂肪形成的组分或提取物或细胞系统，将所述具有脂肪细胞分化倾向的细胞群接种在所述组分或提取物上，将所述细胞孵育足够时间使脂肪形成发生并随后在所述细胞中检查脂肪细胞的分化。

16. 权利要求 15 的用途，用于产生适合移植到受体内的供体血管化组织的方法，所述方法包括制造包含功能循环系统的血管蒂，所述血管蒂有组织或组织提取物或其组分注入、附着或以其他方式与所述血管蒂相联；将所述血管蒂结合在支持基质内部和/或上面；在所述支持基质中接种被分离的细胞或组织碎片，所述细胞或组织碎片利用体外测定鉴别为促进脂肪形成；或某些其他有用的端点，将包含所述血管蒂的所述支持基质植入到受体某个部位，在所述部位所述功能循环系统与局部的动脉或静脉吻合；并将所述支持基质留在所述植入部位足够的时期以允许血管化新组织生长，其中根据特定的标准选择所述注入材料或接种材料，例如，通过测定法测定时它促进脂肪形成，所述测定法包括检查组织或组织提取物以鉴别具有脂肪细胞分化倾向的细胞群，产生或获得潜在促进脂肪形成的组分或提取物，将具有脂肪细胞分化倾向的细胞群接种在所述组分或提取物上，将所述细胞孵育足够时间使脂肪形成发生并随后在所述细胞中检查脂肪细胞的分化。

17. 权利要求 16 的方法，其中所述血管蒂包括附着的脂肪或其他脂肪组织或包含下列细胞的组织，所述细胞为成肌细胞、成纤维细胞、前脂肪细胞和脂肪细胞、心肌细胞、角质化细胞、内皮细胞、平滑肌细胞、软骨细胞、周细胞、骨髓来源的基质前体细胞、胚胎干细胞、间充质干细胞或造血干细胞、许旺细胞及外周和中枢神经系统的其他

RegenX Science Exhibit 1004
Page 1133

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1133

细胞、嗅细胞、肝细胞及其他肝脏细胞、肾小球系膜及其他肾细胞、胰岛β细胞及导管细胞、甲状腺细胞、其他内分泌器官的细胞以及上述细胞的球状体。

18. 权利要求 17 的方法，其中所述血管蒂构成对于促进脂肪形成的组分或提取物的体外测定，所述测定包括在容器表面产生或获得来自肌肉基质的潜在脂肪形成提取物层，在所述层上接种具有脂肪细胞分化倾向的细胞群，将所述细胞孵育足够时间使脂肪形成发生并随后在所述细胞中检查脂肪细胞的分化。

RegenX Science Exhibit 1004
Page 1134

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1134

组织材料和肌肉来源的基质

发明背景

发明领域

总的来说，本发明涉及对促进或利于细胞和组织的生长、发育和分化有用的组织制剂，包括组织细胞及其提取物。更具体而言，本发明提供了包括完整的或提取的细胞外基质和/或细胞以及细胞因子、生长因子及其他组分的肌肉来源的材料。本发明的肌肉制剂类似基底膜并且来源于基于细胞的材料。该肌肉制剂可以在体外或体内使用，尤其是在各种组织工程应用中作为细胞支架并在其他细胞培养系统中用于营养并富集一系列细胞类型，包括但不限于，前体和干细胞例如前脂肪形成细胞（pre-adipogenic ceu）。该肌肉制剂还在例如化妆品和局部治疗剂工业中用作乳膏的基础并在食品工业中用作基质或添加剂。

现有技术描述

本说明书中的参考文献的目录细节也列在本说明书的结尾处。

本说明书中对任何现有技术的参考不是也不应被视为承认或任何形式的暗示所述现有技术构成任何国家的公知常识的一部分。

基底膜是将上皮细胞与基质及周围的神经、肌纤维、平滑肌细胞以及脂肪细胞分开的连续的薄片。电子显微镜分析表明基底膜的组分是网状结构的细丝，所述细丝相互作用形成膜。这个网状结构部分地由胶原 IV 分子的存在引起，所述胶原 IV 分子由分子间的二硫键互相连接（Inoue 等人，*J Cell Biol*, 97:1524-1539, 1983）。

已知基底膜的各种组分彼此相互作用。例如，基底膜的组分之一，层粘连蛋白，与胶原蛋白 IV 以及硫酸乙酰肝素蛋白聚糖结合。

基底膜制剂可以提供具有细胞生长、发育和分化特征的生理学相关环境。这些制剂通常在组成和活性上是异质的。例如，某些制剂是

RegenX Science Exhibit 1004
Page 1135

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1135

可溶性的并缺乏作为细胞基质的适合性（Terranova 等人，*Cell* 22:719-726，1980）。

一种来源于 Engelbreth Holm-Swarm（EHS）鼠肉瘤的制剂是以商品名“Matrigel”[商标，BD Biosciences]出售的富含基底膜的基质并由 Kleinman 等人，*Biochem* 21:8188-6193，1982 描述。Matrigel 已成为促进细胞生长、发育和分化的有用产品。然而，在某些情况下，某些细胞与鼠类来源的 Matrigel 在互相作用的水平上可能存在种属特异性差异，这使得这种产品不适合用于非鼠科动物细胞例如人细胞。它还可以在非鼠科动物宿主中不正当的引发免疫应答。

依照本发明，提供在一系列细胞包括人细胞中具有特别有用的生长、形态学和分化促进活性的新型富含基底膜的组织制剂。

发明概述

本说明书自始至终，除非上下文以别的方式要求，单词“包括”或“包含”将被理解为暗示包含所陈述的元件或整体或一组元件或整体但不排除其他任何元件或整体或一组元件或整体。

本发明提供细胞的以及完整的和提取的细胞外基质材料，它用作支架以支持细胞的生长、发育和分化并支持或实现细胞的形态学改变。组织材料优选来源于肌肉组织并包括包含基底膜组分的制剂。这些组分包括一种或多种下列成分，所述成分例如但不限于，层粘连蛋白、胶原蛋白 I、胶原蛋白 IV、触觉蛋白/巢蛋白、硫酸乙酰肝素蛋白聚糖以及一种或多种的细胞因子或生长因子，所述细胞因子或生长因子例如但不限于，表皮生长因子（EGF）、碱性成纤维细胞生长因子（bFGF）、神经生长因子（NGF）、血小板来源的生长因子（PDGF）、胰岛素样生长因子-1（IGF-1）、转化生长因子-β（TGF-β）、血管内皮生长因子（VEGF）和肿瘤坏死因子-α（TNF-α）。一般地，组织材料包括基于细胞的制剂或完整的或提取的细胞外基质。一般利用例如尿素或 SDS 提取或冷冻/融解或冷冻干燥随后洗涤的方法来制备完整的或提取的无细胞制剂。一般利用例如切碎、戊二醛固定和/或在二甲基亚砜

RegenX Science Exhibit 1004
Page 1136

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1136

（DMSO）或其他冷冻保存剂中冷冻的技术来制备基于细胞的制剂。冷冻干燥不能保存完整的细胞但临界点干燥可以。

为了方便起见，组织材料在本文中也称为肌肉基质、肌肉基底膜基质、myomatrix、myotrix、肌肉支架、myogel 和细胞培养组合物。术语“肌肉基质”为了方便简洁而使用，应理解它涵盖了基于细胞的制剂和无细胞的制剂。无细胞制剂包括完整的和提取的细胞外基质。

本发明的肌肉基质具有各种用途，例如在组织工程中促进大量组织产生用于组织修复、增加和/或替代疗法。该肌肉基质也用作工程化组织的支架，所述组织例如但不限于肌肉和脂肪。它也用作富集并营养来自适当干细胞部位的适当前脂肪形成细胞的手段。作为研究工具，本发明的肌肉基质用于细胞的生长、发育和分化研究，所述细胞例如内皮细胞、上皮细胞、神经胶质、神经元、肌细胞和前脂肪细胞。在化妆品和食品工业，该肌肉制剂用作乳膏的基础和作为食品添加剂以及在治疗上作为细胞修复组合物。

本发明的肌肉基质特别优于其他基底膜制剂，因为它诱导或以其他方式促进更加广泛范围的细胞活性并可以种类保守方式应用。

本文所使用的缩写定义于表 1 中。

表 1

缩写

缩写	描述
bFGF	碱性成纤维细胞生长因子
DMEM	达尔伯氏（Dulbecco）改良基础培养基
EGF	表皮生长因子
FCS	胎牛血清
HUVEC	人脐静脉内皮细胞
IBMX	异丁基甲基黄嘌呤
IGF-1	胰岛素样生长因子 1
NaCl	氯化钠
NGF	神经生长因子
PDGF	血小板衍生生长因子
SDS-PAGE	十二烷基硫酸钠－聚丙烯酰胺凝胶电泳
TGF-β	转化生长因子-β
TIPS	热诱导相分离法
TNF-α	肿瘤坏死因子-α
VEGF	血管内皮生长因子

RegenX Science Exhibit 1004
Page 1137

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1137

附图简述

附图 1 的照片显示了 Matrigel 与各种其他组织基质的 SDS PAGE 比较。（从左往右）泳道 1 和 2 代表预先染色的分子量标准；泳道 3 和 4 代表商购的 Metrigel（BD Biosciences）；泳道 5 代表大鼠肌肉基质；泳道 6 代表猪肌肉基质；泳道 7 代表人肌肉基质。所有样品均以大约 10μg 总蛋白浓度上样。

附图 2 的照片显示了（a）来自人骨骼肌的肌肉基质制剂。图（b）和（c）举例说明了肌肉基质的一种其它形式，即肌肉基质经 TIPS 处理形成的海绵状物。图（c）是附图 2（b）中海绵状物的扫描电子显微照片。

附图 3 的照片是（a）猪肌肉来源的粗肌肉基质和（b）Matrigel。

附图 4 的显微照片显示了组织(包括脂肪组织)在大鼠中的成功产生。附图 4（a）和（b）是来自大鼠组织工程腔室模型的代表性切片，所述模型在植入前用 MyoGel 覆盖。

附图 5A 的照片显示了在组织培养塑料制品上的对照前脂肪细胞，显示有分化。5B 显示在 Myogel 上的前脂肪细胞，显示脂质积聚以及向成熟脂肪细胞的分化。插入的图显示单个细胞积聚脂质的高倍放大图像。5C 显示小鼠腔室的低倍显微照片，所述腔室已充满诱导脂肪形成的肌肉提取物。

附图 6 的照片是不同 MyoGel 种类中各种 ECM 组分的蛋白质印迹的代表性实例。所有的箭头和数字代表分子量（MW）水平。6A 涉及大鼠肌肉提取物中胶原蛋白 I 的免疫印迹，标记 3 条链（100、200、300kD）。泳道 1－10 是十种不同的大鼠肌肉提取物制剂。6B 显示大鼠肌肉提取物中的层粘连蛋白 α4（180kD）和 α2（80kD 片段以及在 300kD 处的少许弥散）。泳道 1 为 Matrigel 对照而泳道 2－11 为相同的大鼠肌肉提取物制剂，显示特别是关于 α4 链的强带。6C 显示在大鼠 MyoGel 硫酸乙酰肝素蛋白聚糖（HSPG）凝胶中的硫酸乙酰肝素蛋白聚糖结合，泳道 1 为分子量标准，泳道 2 为 Matrigel，在 200kD 条带上有基底膜

RegenX Science Exhibit 1004
Page 1138

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1138

蛋白聚糖染色而泳道 3－11 为大鼠肌肉提取物。6D 显示在大鼠肌肉提取物样品中结合的纤连蛋白片段，泳道 1 为 Matrigel 对照而泳道 2－11 为相同的大鼠肌肉提取物。

优选实施方案的详细描述

本发明提供了尤其是对脂肪形成应用以及与组织工程、增加、修复和研究相关的其他应用有用的组织制剂。该制剂还在局部治疗剂、化妆品和食品工业中有着应用。材料的一种优选形式包括溶解的、提取的来源于肌肉的基底膜材料。另一形式包括含溶解的细胞和基底膜材料的完整基质。再一优选形式包括完整组织和细胞。因此，组织材料以不同的方式被称为肌肉提取物材料、肌肉基质、myomatrix、myotrix、肌肉基底膜基质、肌肉支架、myogel 和细胞组合物或制剂。这些术语在本说明书自始至终互换使用但由术语“肌肉基质”涵盖。

因此，肌肉基质制剂可以是基于细胞的或完整的或提取的细胞外基质。一般利用例如尿素或 SDS 提取方法来制备无细胞提取物。一般通过冷冻/融解随后洗涤来制备完整的细胞提取物材料，但可以包括提取后的残渣。一般利用例如切碎、戊二醛固定和/或冷冻干燥技术来制备基于细胞的制剂。还可采用其他类似方法并且所有此类方法均由本发明涵盖。这些方法包括临界点干燥、利用除戊二醛以外的固定剂的蛋白质交联以及机械破碎新鲜肌肉。此外，存在各种提取前提取后技术，所述技术可以用于进一步增强产品或可以单独使用。这些技术包括通过各种物理化学程序（例如研磨、粉碎、TIPS 等）处理材料、在冷冻且漂洗后和改变的冷冻后洗涤固定组织。甚至可以改变起始组织例如使用平滑肌或心肌。所有此类改变均在本发明的范围之内。

本发明的优选组织材料一般包括一种或多种下列组分，所述组分例如但不限于，层粘连蛋白、胶原蛋白 I、胶原蛋白 IV、触觉蛋白/巢蛋白、硫酸乙酰肝素蛋白聚糖以及其他组分，包括细胞因子和生长因子例如，但不限于，一种或多种 EGF、bFGF、NGF、PDGF、IGF-1、TGF-β、VEGF 和 TNF-α。组织材料富含肌肉基底膜组分。本发明的组

RegenX Science Exhibit 1004
Page 1139

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1139

织材料具有一系列的效用，包括特别是组织研究和组织工程，所述组织包括但不限于脂肪、肌肉、肝脏和胰腺。它还为体外生物测定来源材料的脂肪形成潜力提供了基础，所述材料即来自不同部位的脂肪和脂肪前体细胞。该制剂进一步在局部治疗剂、化妆品和食品工业中有着应用。

相应地，本发明提供了物质组合物用于促进细胞或组织中的细胞生长，包括分化、增殖、分裂和/或形态学改变，所述组合物包括基于细胞的或无细胞提取物的肌肉组织制剂，这种制剂提供了下列组分的来源，所述组分例如但不限于，层粘连蛋白、胶原蛋白 I、胶原蛋白 IV、触觉蛋白/巢蛋白、硫酸乙酰肝素蛋白聚糖以及其他组分，包括细胞因子和生长因子例如，但不限于，一种或多种 EGF、bFGF、NGF、PDGF、IGF-1、TGF-β、VEGF 和 TNF-α或其同源物。

在一个优选实施方案中，组织材料包含层粘连蛋白、触觉蛋白/巢蛋白、硫酸乙酰肝素蛋白聚糖、胶原蛋白 IV、bFGF、PDGF、TGF-β、VEGF 和 TNF-α。

为了方便起见，术语“细胞效应”将用于涵盖细胞分化的生长和分裂、增殖以及形态学改变。

组织材料的来源可以来自任何动物并优选哺乳动物，例如，但不限于，人、非人灵长类（例如大猩猩（gorilla）、狨猴（marmoset）或猩猩（orangoutang））、家畜类动物（例如奶牛、绵羊、猪、马、驴、山羊、骆驼）、实验室试验动物（例如小鼠、大鼠、兔、豚鼠、仓鼠）或伴侣动物（例如狗、猫）。本发明还扩展至鸟类来源，例如鸡、鸭、鹅、火鸡以及其他家禽或猎禽，爬行动物来源例如蛇和蜥蜴以及两栖动物来源例如青蛙和蟾蜍。

在一个特别优选的实施方案中，使用来自猪、小鼠、大鼠或人的肌肉组织。

相应地，本发明的另一方面提供了包括来自哺乳动物的肌肉制剂的物质组合物，所述组合物包括：

（i）基于细胞或无细胞的材料；

RegenX Science Exhibit 1004
Page 1140

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1140

（ii）选自列表的组分，所述列表包括一种或多种，但不限于，层粘连蛋白、胶原蛋白 IV、触觉蛋白/巢蛋白、硫酸乙酰肝素蛋白聚糖；

（iii）选自列表的细胞因子或生长因子，所述列表包括一种或多种，但不限于，EGF、bFGF、NGF、PDGF、IGF-1、TGF-β、VEGF 和 TNF-α或其同源物；以及

（iv）约 1μg/ml－约 100 mg/ml 的总蛋白含量。

提到上述组分例如层粘连蛋白、胶原蛋白 IV、触觉蛋白/巢蛋白、硫酸乙酰肝素蛋白聚糖或 EGF、bFGF、NGF、PDGF、IGF-1、IGF-2、TGF-β、VEGF 和 TNF-α应被认为是那些组分，但不必限于那些组分，换句话说，该制剂可以包含未列出的其他组分。

组织材料的组分可以完全来源于肌肉组织或者可以在制备过程中加入额外因子，例如但不限于，额外的胶凝剂（例如盐溶质和/或糖）、细胞因子、抗生素、生长强化因子、基因表达增强子、增殖抑制剂和/或干细胞分化促进因子。

在一个实施方案中，组织材料可以被认为是在体外或体内促进细胞培养、细胞分化、去分化或生长的组合物。

在一个特别优选的实施方案中，组织材料促进细胞生长和分化，所述细胞选自特别是干细胞、上皮细胞、皮肤细胞、器官细胞和内皮细胞。

“无细胞材料”包括单独的提取基质或细胞已被溶解并大部分去除的制剂。

本发明进一步提供细胞培养组合物用于促进细胞生长和分化或实现细胞或组织形态学改变，其中所述细胞培养组合物包括一种或多种下列组分，所述组分例如但不限于，层粘连蛋白、胶原蛋白 I、胶原蛋白 IV、触觉蛋白/巢蛋白、硫酸乙酰肝素蛋白聚糖以及其他组分，包括细胞因子和生长因子，例如但不限于，一种或多种 EGF、bFGF、NGF、PDGF、IGF-1、IGF-2、TGF-β、VEGF 和 TNF-α或其同源物，并且细胞培养组合物聚合成凝胶。

RegenX Science Exhibit 1004
Page 1141

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1141

“细胞因子”包括选自所提供列表的单个或多个细胞因子。当然，也可存在或包括其它细胞因子。同样地，可以存在层粘连蛋白、胶原蛋白 IV、触觉蛋白/巢蛋白和/或硫酸乙酰肝素蛋白聚糖之一或可以存在这些组分的两种或多种。还可存在或添加其它细胞外基质材料。

聚合作用一般发生在约 15℃ - 约 50℃的温度，例如 15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49 或 50℃。还可以采用波动温度。

术语“凝胶”以其最广泛的含义使用并包括半流体、半硬式材料、可变形的材料、稠密的液体、乳膏、固体支持体或其组合，包括适合用作食品添加剂的材料。

在一个优选的实施方案中，存在的细胞因子的量如下:

bFGF: 约 0.3 ng/ml - 约 4 ng/ml 例如 0.3、0.4、0.5、0.6、0.7、0.8、0.9、1.0、1.1、1.2、1.3、1.4、1.5、1.6、1.7、1.8、1.9、2.0、2.1、2.2、2.3、2.4、2.5、2.6、2.7、2.8、2.9、3.0、3.1、3.2、3.3、3.4、3.5、3.6、3.7、3.8、3.9、4.0 ng/ml。

PDGF: 约 1 pg/ml - 约 3000 pg/ml 例如 1、2、3、4、5、6、7、8、9、10、20、30、40、50、60、70、80、90、100、110、120、130、140、150、160、170、180、190、200、210、220、230、240、250、260、270、280、290、300、310、320、330、340、350、360、370、380、390、400、410、420、430、440、450、460、470、480、490、500、510、520、530、540、550、560、570、580、590、600、610、620、630、640、650、660、670、680、690、700、710、720、730、740、750、760、770、780、790、800、810、820、830、840、850、860、870、880、890、900、910、920、930、940、950、960、970、980、990、1000、1010、1020、1030、1040、1050、1060、1070、1080、1090、1100、1110、1120、1130、1140、1150、1160、1170、1180、1190、

RegenX Science Exhibit 1004
Page 1142

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1142

1200、1210、1220、1230、1240、1250、1260、1270、1280、1290、1300、1310、1320、1330、1340、1350、1360、1370、1380、1390、1400、1410、1420、1430、1440、1450、1460、1470、1480、1490、1500、1510、1520、1530、1540、1550、1560、1570、1580、1590、1600、1610、1620、1630、1640、1650、1660、1670、1680、1690、1700、1710、1720、1730、1740、1750、1760、1770、1780、1790、1800、1810、1820、1830、1840、1850、1860、1870、1880、1890、1900、1910、1920、1930、1940、1950、1960、1970、1980、1990、2000、2010、2020、2030、2040、2050、2060、2070、2080、2090、2100、2110、2120、2130、2140、2150、2160、2170、2180、2190、2200、2210、2220、2230、2240、2250、2260、2270、2280、2290、2300、2310、2320、2330、2340、2350、2360、2370、2380、2390、2400、2410、2420、2430、2440、2450、2460、2470、2480、2490、2500、2510、2520、2530、2540、2550、2560、2570、2580、2590、2600、2610、2620、2630、2640、2650、2660、2670、2680、2690、2700、2710、2720、2730、2740、2750、2760、2770、2780、2790、2800、2810、2820、2830、2840、2850、2860、2870、2880、2890、2900、2910、2920、2930、2940、2950、2960、2970、2980、2990、3000 pg/ml。

TGF-β：约 1 pg/ml－约 2000 pg/ml 例如 1、2、3、4、5、6、7、8、9、10、20、30、40、50、60、70、80、90、100、110、120、130、140、150、160、170、180、190、200、210、220、230、240、250、260、270、280、290、300、310、320、330、340、350、360、370、380、390、400、410、420、430、440、450、460、470、480、490、500、510、520、530、540、550、560、570、580、590、600、610、620、630、640、650、660、670、680、690、700、710、720、730、740、750、760、770、780、

RegenX Science Exhibit 1004
Page 1143

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1143

790、800、810、820、830、840、850、860、870、880、890、900、910、920、930、940、950、960、970、980、990、1000、1010、1020、1030、1040、1050、1060、1070、1080、1090、1100、1110、1120、1130、1140、1150、1160、1170、1180、1190、1200、1210、1220、1230、1240、1250、1260、1270、1280、1290、1300、1310、1320、1330、1340、1350、1360、1370、1380、1390、1400、1410、1420、1430、1440、1450、1460、1470、1480、1490、1500、1510、1520、1530、1540、1550、1560、1570、1580、1590、1600、1610、1620、1630、1640、1650、1660、1670、1680、1690、1700、1710、1720、1730、1740、1750、1760、1770、1780、1790、1800、1810、1820、1830、1840、1850、1860、1870、1880、1890、1900、1910、1920、1930、1940、1950、1960、1970、1980、1990、2000 pg/ml。

EGF: 约 0 ng/ml－约 100 ng/ml 例如 0、1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、50、51、52、53、54、55、56、57、58、59、60、61、62、63、64、65、66、67、68、69、70、71、72、73、74、75、76、77、78、79、80、81、82、83、84、85、86、87、88、89、90、91、92、93、94、95、96、97、98、99、100 ng/ml。

VEGF: 约 1 pg/ml－约 3000 pg/ml 例如 1、2、3、4、5、6、7、8、9、10、20、30、40、50、60、70、80、90、100、110、120、130、140、150、160、170、180、190、200、210、220、230、240、250、260、270、280、290、300、310、320、330、340、350、360、370、380、390、400、410、420、430、440、450、460、470、480、490、500、510、520、530、540、550、560、570、580、590、600、610、620、630、640、650、660、670、680、

RegenX Science Exhibit 1004
Page 1144

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1144

690、700、710、720、730、740、750、760、770、780、790、800、810、820、830、840、850、860、870、880、890、900、910、920、930、940、950、960、970、980、990、1000、1010、1020、1030、1040、1050、1060、1070、1080、1090、1100、1110、1120、1130、1140、1150、1160、1170、1180、1190、1200、1210、1220、1230、1240、1250、1260、1270、1280、1290、1300、1310、1320、1330、1340、1350、1360、1370、1380、1390、1400、1410、1420、1430、1440、1450、1460、1470、1480、1490、1500、1510、1520、1530、1540、1550、1560、1570、1580、1590、1600、1610、1620、1630、1640、1650、1660、1670、1680、1690、1700、1710、1720、1730、1740、1750、1760、1770、1780、1790、1800、1810、1820、1830、1840、1850、1860、1870、1880、1890、1900、1910、1920、1930、1940、1950、1960、1970、1980、1990、2000、2010、2020、2030、2040、2050、2060、2070、2080、2090、2100、2110、2120、2130、2140、2150、2160、2170、2180、2190、2200、2210、2220、2230、2240、2250、2260、2270、2280、2290、2300、2310、2320、2330、2340、2350、2360、2370、2380、2390、2400、2410、2420、2430、2440、2450、2460、2470、2480、2490、2500、2510、2520、2530、2540、2550、2560、2570、2580、2590、2600、2610、2620、2630、2640、2650、2660、2670、2680、2690、2700、2710、2720、2730、2740、2750、2760、2770、2780、2790、2800、2810、2820、2830、2840、2850、2860、2870、2880、2890、2900、2910、2920、2930、2940、2950、2960、2970、2980、2990、3000 pg/ml。

TNF-α：约 0 pg/ml－约 1000 pg/ml 例如 0、1、2、3、4、5、6、7、8、9、10、20、30、40、50、60、70、80、90、100、110、120、130、140、150、160、170、180、190、200、210、220、

RegenX Science Exhibit 1004
Page 1145

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1145

230、240、250、260、270、280、290、300、310、320、330、340、350、360、370、380、390、400、410、420、430、440、450、460、470、480、490、500、510、520、530、540、550、560、570、580、590、600、610、620、630、640、650、660、670、680、690、700、710、720、730、740、750、760、770、780、790、800、810、820、830、840、850、860、870、880、890、900、910、920、930、940、950、960、970、980、990、1000 pg/ml。

在一个实施方案中，缺少 IGF-1。

因此本发明提供了基于细胞或无细胞制剂形式的组织来源的材料，用于细胞生长和发育并用于实现细胞或组织形态学改变，所述材料来源于人、非人灵长类、家畜动物、伴侣动物、鸟类、爬行动物或两栖动物的肌肉并包括一种或多种选自列表的细胞因子和/或生长因子，所述列表包括，但不限于，约 1.0 ng/ml－约 4 ng/ml bFGF、约 1 pg/ml－约 3000 pg/ml PDGF、约 1 pg/ml－约 2000 pg/ml TGF-β、约 1 pg/ml－约 3000 pg/ml VEGF、约 0 pg/ml－约 1000 pg/ml TNF-α以及约 0 ng/ml－约 100 ng/ml EGF，所述材料进一步包括一种或多种组分，所述组分选自，但不限于，层粘连蛋白、胶原蛋白 IV、触觉蛋白/巢蛋白和/或硫酸乙酰肝素蛋白聚糖。

组织材料优选为具有类似基底膜和/或其组分的层状结构的凝胶形式。组织材料可以是凝胶形式或者它可以是可聚合为凝胶形式的“前体”形式或者它可以是基于细胞的。便利地，当组织材料是前体形式时，它是可重建为凝胶或基质形式的。甚至更便利地，重建前体的基质形式在本文中被称为“肌肉基质”。凝胶形式或前体形式（包括基于细胞制剂）的本发明肌肉基质也可被制成或掺合到珠、海绵、乳膏等中。尽管凝胶形式是制剂的一种优选形式，本发明还预期了具有与凝胶类似“胶凝作用”特征的基于细胞的制剂。

相应地，本发明的肌肉基质用于促进各种细胞的细胞生长和分化并实现细胞或组织形态学改变。上皮细胞、内皮细胞、神经细胞和干

RegenX Science Exhibit 1004
Page 1146

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1146

细胞特别响应肌肉基质而生长和分化。它还帮助细胞粘附以及帮助细胞生长、发育、分化和/或增殖，所述细胞选自，但不限于，神经元、肝细胞、支持细胞、毛囊、甲状腺细胞等。如上所述，“肌肉基质”应被理解为涵盖基于细胞的制剂和无细胞的制剂。

细胞可以在体外的肌肉基质上进行培养并随后回到它们所源自的动物或免疫抑制或组织相容的动物中。在这个上下文中，“动物”包括人、非人哺乳动物、家畜动物、伴侣动物或鸟类、爬行类或两栖类。同样地，肌肉基质可在特定位点或在腔室内或其他植入身体的支架中单独用于体内促进细胞生长或组织生长。

本发明的肌肉基质一般在低温下制备，例如约 1℃ - 约 10℃例如 1、2、3、4、5、6、7、8、9、10℃或在这个范围内的非整数温度。4℃的温度特别有用。收集新鲜肌肉。约 10 - 约 100g 便于处理。从肌肉上剪去可见脂肪并让组织在缓冲液中（例如 NaCl 缓冲液）接触蛋白酶抑制剂。为了制备无细胞提取物，对所得到的组织进行匀浆处理并随后进行离心以去除上清液。将组织沉淀重悬浮在缓冲液（例如 NaCl）中并重新进行匀浆处理。重复这两个洗涤步骤。在最后一次洗涤后，在尿素缓冲液中重悬浮沉淀。组织匀浆搅拌过夜并完成一系列洗涤，每次收集上清液。随后用纱布过滤上清液以去除游离的漂浮脂肪。随后对溶剂（例如氯仿）透析过夜以灭菌材料。将溶剂换成缓冲液并继续透析以去除溶剂。最后一次透析后，使用的最终缓冲液是 DMEM 或其等同物。将所得到的前基质材料进行分配并保存在 - 20℃。

需要时，取回前基质材料的样品并在约 20 - 约 50℃例如大约 37 - 42℃进行孵育，在所述温度下基质聚合成凝胶状形式。还可以加入帮助胶凝作用的添加剂，包括但不限于血浆。

利用 SDS 也可制备提取物。可替代地，可以利用冷冻干燥/融解随后洗涤来制备制剂。这被称为完整基质。利用切碎、冷冻干燥和/或戊二醛固定可以很方便地制备基于细胞的制剂（即完整组织）。

本发明的组织提取物材料或肌肉基质可以被包装为前基质或基质的形式出售，并且可以与关于如何使用的说明书一起供应。附加组分

RegenX Science Exhibit 1004
Page 1147

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1147

可以在包装或试剂盒内并且在使用前被包括或在使用时被混合。

如上所述，肌肉基质特别适合于培养各种细胞，所述细胞包括但不限于脂肪细胞、3T3-L1 细胞、HUVEC、MCF-7 和 MDA-MB-231 乳腺癌细胞、PC-12 细胞和 NG-108 神经细胞以及一系列通过标准程序分离的前脂肪细胞。

肌肉基质还用于体外生物测定来源材料的脂肪形成潜力。该制剂还具有体外生物测定其他基底膜响应细胞类型的分化以及许多发病状态例如癌症和糖尿病的能力，所述细胞例如上皮细胞、神经元、内皮细胞。

根据这个方面，对肌肉基质实施体外测定以测定诱导脂肪形成的组成成分，包括细胞和/或分子组分。测定包括用一层或多层待测的潜在脂肪形成组分例如肌肉基质的提取物覆盖在容器表面，接种具有潜力以经历脂肪形成分化的细胞并随后检查脂肪形成。可替代地，将肌肉基质覆盖在表面上并添加其他化合物并随后检查该系统中增强或减少的脂肪形成。在另一实施方案中，将具有潜力经历脂肪形成分化的细胞维持在悬浮培养中并向培养基中补充待测的潜在脂肪形成组分例如肌肉基质的提取物或级分。细胞悬浮的优点是允许从培养基中快速分离细胞以测定它们是否已经历分化。可替代地，测定包括产生包含潜在脂肪形成组分或提取物的三维支架，接种具有潜力经历脂肪形成分化的细胞并随后检查脂肪形成。因此，本发明的测定系统还可具有附加优点，所述优点为提供或选择或开发用于组织工程的最优化前脂肪细胞群体。测定可以方便地在体外适当的容器中进行。在这种情况下，可以用细胞材料制剂覆盖在基质的表面。容器还可与使用说明书一起被包装出售。

相应地，本发明预期体外测定调节脂肪形成的组分、提取物或细胞系统，所述测定包括检查肌肉制剂以鉴别具有脂肪细胞分化倾向的细胞群，产生或获得潜在调节脂肪形成的组分或提取物或细胞系统，将所述具有脂肪细胞分化倾向的细胞群接种在所述组分或提取物上，将所述细胞孵育足够时间使脂肪形成产生，并随后在所述细胞中检查

RegenX Science Exhibit 1004
Page 1148

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1148

脂肪细胞的分化。在这个上下文中的“细胞系统”具有与基于细胞的制剂相同的含义。

在某些实施方案中，潜在调节脂肪形成的组分或提取物或细胞系统促进脂肪形成。在其他实施方案中，调节脂肪形成的组分或提取物或细胞系统抑制脂肪形成。

“调节”是指直接或间接地增加或减少脂肪形成水平。

可以获得或产生潜在调节脂肪形成的组分或提取物或细胞系统层。可替代地，可以获得或产生包含潜在调节脂肪形成的组分或提取物或细胞系统的三维支持基质。在某些实施方案中，可以将具有潜力经历脂肪细胞分化的细胞维持在悬浮培养中并向培养基中补充潜在调节脂肪形成的组分或提取物。术语“层”包括两层或多层。本方法扩展至向肌肉基质中加入潜在脂肪形成促进剂。

本发明的再一方面提供了产生适合移植到受体内的供体血管化组织的方法，所述方法包括制造包含功能循环系统的血管蒂并且有组织或组织提取物或其组分注入、附着或以其他方式与所述血管蒂关联；将血管蒂结合在支持基质内部和/或上方；在支持基质中接种被分离的细胞或组织碎片，所述细胞或组织利用体外测定鉴别为促进脂肪形成；或某些其他有用的端点，将包含血管蒂的支持基质植入到受体某个部位，在所述部位上功能循环系统与局部的动脉或静脉吻合；并将支持基质留在植入部位足够的时期以允许血管化新组织生长，其中根据特定的标准选择注入材料或接种材料，例如，通过测定法测定时它促进脂肪形成，所述测定法包括检查组织或组织提取物以鉴别具有脂肪形成分化倾向的细胞群，产生或获得潜在促进脂肪形成的组分或提取物，将具有脂肪细胞分化倾向的细胞群接种在所述组分或提取物上，将所述细胞孵育足够时间使脂肪形成发生并随后在所述细胞中检查脂肪细胞的分化。

在一个优选实施方案中，血管蒂包括附着脂肪或其他脂肪组织或包含下列细胞的组织，所述细胞有成肌细胞、成纤维细胞、前脂肪细胞和脂肪细胞、心肌细胞、角质化细胞、内皮细胞、平滑肌细胞、软

RegenX Science Exhibit 1004
Page 1149

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1149

骨细胞、周细胞、骨髓来源的基质前体细胞、胚胎干细胞、间充质干细胞或造血干细胞、许旺（Schwann）细胞及外周和中枢神经系统的其他细胞、嗅细胞、肝细胞及其他肝脏细胞、肾小球系膜及其他肾细胞、胰岛β细胞及导管细胞、甲状腺细胞、其他内分泌器官的细胞以及上述细胞的球状体。在选择之前，所有这些细胞在体外测试其在基质上生长/存活的潜力或分化成其他有用组织的能力例如脂肪形成潜力。血管蒂上附着组织的存在进一步促进新脂肪组织在支持基质内部或周围生长。在一个替代实施方案中，组织提取物或组织的重组、合成或纯化组分与血管蒂相联。例如，在一个优选实施方案中，这些组分和提取物来源于基质材料并在体外检查脂肪形成潜力。

基质被允许设定并且在完全培养基（例如含 FCS 的 DMEM）或分化培养基（补充有 1μM 地塞米松、胰岛素、吲哚美辛和 IBMX 的完全培养基）的存在下将有脂肪细胞分化能力的细胞覆盖在单层基质上。经过 14 天的时间段观察到脂肪形成。适当的脂肪细胞的实例包括 3T3-L1 细胞或通过标准程序分离的前脂肪细胞。

因此，本发明预期体外测定促进脂肪形成的组分或提取物，所述测定包括在容器表面产生或获得来自肌肉基质的潜在脂肪形成提取物层，在所述层上接种具有脂肪细胞分化倾向的细胞群，将所述细胞孵育足够时间使脂肪形成发生并随后在所述细胞中检查脂肪细胞的分化。

在另一实施方案中，测定在三维支持基质中进行，所述三维支持基质可以基本上由肌肉基质构成或包含由肌肉基质覆盖的支架，关于这点执行本领域技术人员已知的三维细胞培养技术，例如转瓶技术（Mueller-Klieser *J Cancer Res Clin Oncol 13*: 101-122,1986）、流体镀层技术（Yuhas,等人，*Cancer Res 37*: 3639-3643,1977）。三维细胞培养技术的另一实例是经过特别改造的旋转培养瓶，通过围绕水平轴旋转流体填充的培养瓶以使重力矢量随机化，同时用最小的流体剪切力悬浮细胞和细胞团块。这些装置已在美国专利号 5,153,131; 5,153,132; 5,153,133; 5,153,034,和 5,155,035 中得

RegenX Science Exhibit 1004
Page 1150

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1150

到描述。

三维基质的优点之一是它维持培养细胞活性增殖的时期比单层系统更长。这可能部分由于三维基质的面积增加导致细胞活性增殖周期延长。基质提供维持培养细胞长期活性增殖所需的支持物、生长因子和调节因子。通过向支持物本身加入蛋白质、糖蛋白、糖胺聚糖、细胞基质以及其他材料或通过用这些材料覆盖支持物可进一步增强在该支持物存在下的细胞生长。基质的三维性允许形成有助于细胞成熟和迁移的微环境。当生长在这种三维系统中时，增殖细胞成熟并适当地分离以形成类似于体内的成熟组织组分。

为了使三维结构能够维持活细胞的活性，三维基质应当展示适当的空间和组成特性。此类基质包括水凝胶或多孔基质例如基于纤维的或海绵样基质。三维基质中常用材料为天然聚合物或“生物基质”、合成聚合物以及无机合成物。在本实施方案中，该方法预期三维基质用于体外测定时，基质的生物相容性不是特别重要。

生物基质的优选实例是从肌肉基质提取的那些或与肌肉基质相似的那些或具有包含肌肉基质的细胞系统。

本发明进一步预期了肌肉基质在促进细胞生长的组合物产品中的用途。本发明的肌肉制剂还有利于基于所选择的对肌肉制剂特异的生长和形态学特征对复杂的细胞混合物选择性纯化具体细胞类型（例如前脂肪细胞）。

冠词“a”和“an”在本文中用来指超过一个（即至少一个）语法目标的物品。例如，“组分”指一个和超过一个的组分。

通过下列非限制性实施例来进一步描述本发明。

实施例 1

基质提取 I

收集来自新近宰杀的动物（大鼠和猪）或来自经历整形外科手术的患者的肌肉样品。所有的样品均在适当的伦理委员会批准下并完全征得同意进行收集。所有程序步骤均在冰上或在 4℃进行。

RegenX Science Exhibit 1004
Page 1151

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1151

肌肉被收集、称重并在提取基质前剪去脂肪。样品随后被洗涤并在加入蛋白酶抑制剂（0.5mM PMSF、2mM EDTA、0.1M EACA、2mM NEM）的冰冷的 3.4M NaCl 缓冲液中匀浆。组织匀浆随后在 4℃ 10,000 rpm 离心 15 分钟，随后弃去上清液并且在 3.4M NaCl 缓冲液中重悬浮沉淀。这个步骤重复 2－3 次。

沉淀随后在与原始组织体积相等体积的 2M 尿素缓冲液中重悬浮，匀浆并在 4℃搅拌过夜。这之后，提取物在 4℃ 14,000 RPM 离心 30 分钟并保存上清液。沉淀在原始体积一半的 2M 尿素缓冲液中重新匀浆随后重复离心步骤。随后将上清液与以前保存的上清液合并并进行过滤。

提取物随后在 0.05M Tris/0.15M NaCl 缓冲液（TBS）中 4℃对 0.5% v/v 氯仿透析过夜作为灭菌步骤。提取物随后只对 TBS 进行几次透析，随后为 DMEM 进行透析。等分量的提取物保存于－20℃。

实施例 2

基质提取 II

所有步骤均在 4℃或在冰上进行。收集尽可能新鲜的肌肉组织（优选最少约 20－30g）并称重。在钢制解剖盘中尽可能快地从肌肉上剪去所有可见脂肪。以 2:1 的体积比（例如 100ml 缓冲液对 50g 肌肉 ）往冰上的烧杯中加入含蛋白酶抑制剂的冷的 3.4M NaCl 缓冲液并加入肌肉。样品随后彻底匀浆。肌肉组织匀浆在 4℃ 10,000 RPM 离心 15 分钟。随后弃去上清液并在等量的 3.4M NaCl 缓冲液中将沉淀重悬浮且重新匀浆。这个步骤重复 2 次，使得在 NaCl 中总共洗涤 3－4 次。第 3 次洗涤后沉淀在 1:1 体积含 0.5M NaCl、蛋白酶抑制剂的 50mM Tris-HCl（pH 7.4）中重悬浮并匀浆（例如 50ml 对 50mg 肌肉）。在 4℃利用磁力搅拌器将样品旋转过夜。样品随后在 4℃ 14,000 RPM 离心 30 分钟，弃去上清液并保存样品。沉淀随后在含 2.0M 盐酸胍）、0.2mM 二硫苏糖醇的 50mM Tris-HCl（pH 7.4）中进行匀浆。将组织匀浆搅拌过夜并在 4℃ 14,000 rpm 离心 30 分钟并去除上清液并保存。

RegenX Science Exhibit 1004
Page 1152

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1152

所有下述步骤均在两种上清液中进行，所述两种上清液在这些步骤中自始至终保持分离。

通过纱布过滤上清液以去除游离的漂浮脂肪等。提取物在磁力搅拌器上 4℃对 1-2 升的 TBS 缓冲液（5mls/升）中的 0.2% v/v 氯仿透析过夜。这是一个灭菌步骤。将缓冲液换成干净的 TBS 并透析至少 8 小时。这个步骤重复 3 次。更换透析缓冲液时，将管末端旋转数次以确保混匀。最后一次 TBS 透析后，将缓冲液换成 DMEM 并透析过夜。较稠的溶液例如猪肌肉基质需要更长时间。随后将这两种提取物混合在一起产生不同结构的基质以便改进胶凝作用。随后将样品等分至无菌试管中。在流通橱内在冰上执行这个操作。样品随后保存于 -20℃。

实施例 3

SDS-PAGE

通过二喹啉甲酸（BCA 蛋白质测定试剂盒）测量蛋白质浓度。基质样品在 SDS-PAGE 凝胶上分离之前在 Laemmli 溶液(Laemmli, Nature 227:680-685,1970）中制备成 0.5-1.0 mg/mL 并煮沸 5 分钟。在 4-12% w/v 梯度的聚丙烯酰胺凝胶（Invitrogen）上，将 15μL 体积的样品加入泳道中并通过 SDS-聚丙烯酰胺凝胶电泳分离。凝胶在 200V 的恒压下电泳 50 分钟，这之后取下凝胶并用考马斯亮蓝（Brilliant Blue）染色或转移用于免疫印迹分析。

实施例 4

免疫印迹分析

如实施例 3 所述在 SDS-PAGE 凝胶上分离蛋白质并将未经染色的凝胶转移至硝酸纤维素膜片。依照湿法转移程序通过施加 100V 恒压 1 小时，起始电流为 220mA，进行转移。首先在含 5% w/v 脱脂干乳粉、0.1% v/v Tween 20 的 Tris 缓冲盐水（TBS）（TTBS）中将印迹孵育过夜以减少非特异性反应。第一抗体在室温下孵育 1 小时，用 TTBS 漂洗 3 次并随后用结合过氧化物酶的第二抗体（1:5000-1:10000）孵

RegenX Science Exhibit 1004
Page 1153

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1153

育 1 小时。在免疫活性蛋白通过增强化学发光型免疫印迹检测系统（Amersham Pharmacia）显现之前，印迹再次在 TTBS 中漂洗 3 次。使用六种第一抗体：HSPG 1:5000（Seikagaku Corporation）、层粘连蛋白 1:5000（Dako）、巢蛋白 1:10000（Chemicon）、以及胶原蛋白 IV 1:10000（Dako）、纤连蛋白 1:10 000 以及 SPARC 1:10 000。

实施例 5

肌肉基质分析

对肌肉基质与来自 BD Biosciences 的制剂（Matrigel）进行组分比较分析且结果显示于表 2、3 和 4 中。

生长因子的鉴定：

利用 QuantikineELISA 试剂盒（R&D System）并根据试剂盒说明书测量生长因子/细胞因子水平。待测的生长因子包括血管内皮生长因子（VEGF）、血小板衍生生长因子（PDGF）、转化生长因子β（TGF-β）、碱性成纤维细胞生长因子（bFGF）、肿瘤坏死因子α（TNF-α）、表皮生长因子（EGF）、胰岛素样生长因子 1（IGF-1）、白血病抑制因子（LIF）以及神经生长因子（Chemicon 试剂盒）（NGF）。简言之，将提取物进行稀释（1:5-1:10）并与标准和对照一起在抗体涂层板中孵育 2－3 小时。对板进行洗涤并加入结合辣根过氧化物酶（HRP）的多克隆第二抗体。孵育后，去除多余结合物并且用颜色底物对板进行第 3 次孵育。加入终止溶液后，利用微板阅读器（Axion, Mutliskan, USA）来读板，所述微板阅读器的吸光度设定在λ450nm 处、校正读数设定在λ550nm 处。利用 Genesis 2.0 板阅读软件（详细的）执行所有的测量和计算，并将数值转化成 pg/mg 基质。表 2 包含如厂商所报告的以及按照 ELSA 测量的 Matrigel（注册商标）中的生长因子水平。

RegenX Science Exhibit 1004
Page 1154

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1154

表 2

	BD Full (样品片)	BD GRF (样品片)	BD Full (ELISA)	BD GFR (ELISA)	KK Full (ELISA)	KK GFR (ELISA)
bFGF	0.1 pg/ml	0.1 pg/ml	48 pg/ml	-	304 pg/ml	0 pg/ml
PDGF	12 pg/ml	<5 pg/ml	0 pg/ml	0 pg/ml	12 pg/ml	0 pg/ml
TGFβ	2.3 ng/ml	1.7 ng/ml	2.3 ng/ml	1.8 ng/ml	4.5 ng/ml	1.6 ng/ml
EGF	0.5-1.3 ng/ml	<0.5-1.3 ug/ml	0 ng/ml	0 ng/ml	0 ng/ml	0 ng/ml
VEGF	NR	NR	8.2 ng/ml	2.9 ng/ml	8.3 ng/ml	0.8 ng/ml
TNFα	NR	NR	0 pg/ml	0 pg/ml	0 pg/ml	0 pg/ml

BD Full = 商购 Matrigel（注册商标）

BD GFR = 生长因子减少的 Matrigel（注册商标）

KK Full = 我们自己的 Matrigel（注册商标）制剂

KK GFR = 我们自己的生长因子减少的制剂

NR = 未记录

表 3 和表 4 包含按照 ELISA 测量的由大鼠、猪和人制成的肌肉基质中的生长因子水平。表 3 代表数值范围。表 4 代表数据的平均值和平均标准误（SEM）。

表 3

生长因子 (pg/mg)	大鼠提取物 (n=10)	猪提取物 (n=10)	人提取物 (n=6)	小鼠提取物 (n=3) ##	Matrigel (n=3-7)
FGF2	27-297	14-1281g#	41-250		0-18
VEGF	7-64	NM	NM	34-45	801-1703
PDGF	2-45	1-8	5-12	NM	0-2.7
NGF	2-100	13-22	18-71	NM	
TGF-β	0-9	NB	0-45	NM	32-88
TNFα	3-21	3-12	2-11	NM	2-6
EGF	NB	NB	2-18	NM	
LIF	NB	NB	NB	3-9	

RegenX Science Exhibit 1004
Page 1155

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1155

表 4

基质来源	人	猪	大鼠	小鼠	Matrigel
	(n=6)	(n=10)	(n=10)	(n=3)	***
bFGF	131.16±36	217.9±121	113.5±32.6		6.17±2.3
VEGF	1.05±0.4	0.58±0.25	23.15±5.8	41.3±	1145±165
TGF-β	24.75±8	NB	1.7±94		68.5±17.8
TNF-α	7.38±1.3	7.15±1.14	7.1±1.7		2.8±1.7
PDGF	7.90±0.8	3.50±0.6	10.56±4.1		0.9±0.9
NGF	33.9±7.9	18.2±1.9	28.5±9.1		15.03
EGF	9.3±2.4	NB	NB		NB
LIF	NB	NB	NB		56.7

= 一个非常高的读数 - 其他所有读数均低于 339pg/mg。

对这些样品进行检验并察看跨种结合是否存在

NB: 使用这些 ELISA 在这些样品中没有观察到结合

NM: 未测量

总蛋白浓度的测量

利用二喹啉甲酸测定（Amersham Biosciences Corporation New Jersey, USA）来测量基质的总蛋白水平。样品以 1:10 稀释进行测量并依照试剂盒说明书对照一系列 BSA 标准进行测定。孵育后利用分光光度计 t 在 562 nm 波长处分析样品。大鼠和猪肌肉基质每种分析 10 个样品，而人肌肉基质则分析 6 个。表 5 显示按照 BCA 总蛋白测定测量的肌肉基质和 Matrigel（注册商标）中的总蛋白水平

表 5

基质来源	猪肌肉	大鼠肌肉	人肌肉	Matrigel
总蛋白	4-15 mg/ml	6-12 mg/ml	4-10 mg/ml	8-10 mg/ml

RegenX Science Exhibit 1004
Page 1156

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1156

附图 1 至 4 中也描述了肌肉基质。附图 1 提供了 Matrigel 与其他组织基质的 SDS-PAGE 比较。附图 2 是来自骨骼肌的肌肉基质的照片。附图 3 是猪肌肉来源的肌肉基质的照片。附图 4 的显微照片显示了大鼠中组织的成功产生。附图 5。

ECM 组分的测量

1,9-二甲基亚甲基蓝、Direct Red 80（Sirius Red）、硫酸软骨素 A、木瓜蛋白酶、二硫苏糖醇和胶原蛋白 I 全部购自 Sigma-Aldrich。硫酸化糖胺聚糖（GAGs）通过与染料 1,9-二甲基亚甲基蓝（DMMB）的沉淀作用在 MyoGel 样品中被定量。通过加入等体积的包含 0.6 mg/ml 木瓜蛋白酶、2mM EDTA 和 4 mM DTT 的 40 mM 磷酸钠缓冲液（pH 6.8）来消化干扰蛋白，随后在 60℃孵育 60 分钟。（Farndale 等人 *Biochimica ET Biophysica Acta 882*: 173-177, 1986）。等分量（100 uL）的每种样品随后与 1 mL 的 DMMB 溶液（在 0.2 M 盐 GuHCl、1g/L 甲酸钠和 1ml/L 甲酸中的 16 mg/L DMMB）孵育 30 分钟，并在旋转轮上连续混合。GAG-DMMB 复合物沉淀后，通过离心（10,000 × g 10 分钟）将不可溶解的材料与上清液分开并去除上清液。通过添加 1mL 的解络缓冲液（50mM 乙酸钠缓冲液，pH 6.8，含 10％正丙醇和 4M GuHCl）从沉淀中释放出染料（Barbosa 等人 *Glycobiology 13*: 647-653, 2003）。随后在微板阅读器（Multiskan RC; Labsystems）中测定缓冲液的吸光度（λ 650 nm）。采用硫酸软骨素 A 作为标准。

将层粘连蛋白从 1mL 肝素亲和层析柱（Amersham Biosciences; Uppsala, Sweden）中洗脱后对其进行评估（Talts 等人 *EMBO J 18*: 863-870, 1999）（Talts 等人 *J Biol Chem 275*: 35192-35199, 2000）。简言之，通过与等体积的含 4M GuHCl + 2mM DTT 的 50 mM Tris-HCl（pH 7.4）在 4℃孵育 24 小时来溶解 MyoGel 样品。对 50 mM Tris-HCl（pH 7.4）+0.15M NaCl 透析过夜后，将 1 mL 的等分量应用于亲和层析柱。用 5 mL 的 50 mM Tris-HCl（pH 7.4）+0.15M NaCl

RegenX Science Exhibit 1004
Page 1157

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1157

从柱中冲洗出非层粘连蛋白，并用 5 mL 的含 0.5M NaCl 的 50 mM Tris-HCl（pH 7.4）洗脱出层粘连蛋白。随后用 Bio-Rad 蛋白质测定法利用如上所述的微板微测定程序来评估层粘连蛋白

通过其与多偶氮染料 Sirius Red 的沉淀作用来测定胶原蛋白（Marotta and Martino *Analytical Biochemistry 150*: 86-90, 1985）。等分量的 MyoGel（100 μL）与 1mL 的含 50μM Sirius Red 的 0.5M 乙酸在室温下孵育 30 分钟。离心（10,000×g 10 分钟）后，在微板阅读器中测定上清液的吸光度（λ 550 nm）。使用来自大鼠尾部的胶原蛋白 I 作为标准。

通过 ELISA（Echelon, UT, USA）按照试剂盒说明书来测量透明质素的水平。

表 6 和 7 显示由大鼠、猪和人制成的肌肉基质的细胞外基质组分。表 6 代表数值范围，而表 7 代表数据的平均值和平均标准误（SEM）。

表 6

基质	大鼠提取物	猪提取物	人提取物
GAG	0-5 μg/mg	0.5-22 μg/mg	0.6-1.8 μg/mg
层粘连蛋白	223-700 μg/mg	62-1000 μg/mg	375-1000 μg/mg
胶原蛋白	49-532 μg/mg	45-1000 μg/mg	54-1249 μg/mg
透明质素	5-10 ng/mg	0-5 ng/mg	0-5 ng/mg

表 7

μg/mg 基质	人提取物	猪提取物	大鼠提取物	百分比
GAG	0.86 ± 0.2	6 ± 2.8	2.45 ± 0.6	0.4%
层粘连蛋白	865.57 ± 150	442 ± 120	422.8 ± 67	45%
胶原蛋白	437.61 ± 175	486.75 ± 133	254.28 ± 46.7	27%
ng/mg 基质				
透明质素	5-10	<5	<5	NS

RegenX Science Exhibit 1004
Page 1158

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1158

SDS-PAGE/免疫印迹分析

基质样品在 SDS-PAGE 凝胶上分离之前在 Laemmli 溶液(Laemmli, 1970,)中制备成 0.5-1.0 mg/mL 并煮沸 5 分钟。在 3-8％或 4-12％梯度的聚丙烯酰胺凝胶(Invitrogen, Carlsbad, CA, USA)上，将 10 μL 体积的样品加入泳道中并通过 SDS-聚丙烯酰胺凝胶电泳分离。凝胶在 200V 的恒压下电泳 45 分钟，这之后取下凝胶并用考马斯亮蓝染色或转移用于免疫印迹分析。

对于免疫印迹，如上所述在 SDS-PAGE 凝胶上分离蛋白质并将未经染色的凝胶转移至硝酸纤维素膜片。通过湿法转移程序施加 30V 恒压 1 小时进行转移。转移后，在含 5％脱脂干乳粉(Homebrand, Safeways AUS]、0.1％ Tween 20 的磷酸盐缓冲盐水(PBS)(TPBS)中将印迹孵育过夜以减少非特异性结合。印迹在室温下在第一抗体中孵育 1 小时，用 TPBS 漂洗 3 次并随后与适合于所用一抗的使用适当的红外标记的第二抗体(Molecular Probes, UT, USA 或 Rocklands, CA, USA)(1:10 000)孵育 1 小时。在免疫活性蛋白通过 Odyssey 红外检测系统(Licor Biosciences, USA)显现之前，印迹再次在 TPBS 中漂洗 3 次。使用的第一抗体包括抗 HSPG 1:5000(Seikagaku Corporation, Japan)、层粘连蛋白α4 和α2 1:1000(Dr Lydia Soroken 友情赠与)、巢蛋白 1:3000(Chemicon, USA)、纤连蛋白 1:5000、胶原蛋白 I 1:10000、胶原蛋白 IV 1:10000 以及 SPARC 1:10 000(Dr H Kleinman 友情提供, NIH USA)。

附图 6 是不同 MyoGel 种类中各种 ECM 组分的蛋白质印迹的代表性实例。所有的箭头和数字代表分子量水平。6A 涉及大鼠肌肉提取物中胶原蛋白 I 的免疫印迹，标记 3 条链(100、200、300kD)。泳道 1－10 是十种不同的大鼠肌肉提取物制剂。6B 显示大鼠肌肉提取物中的层粘连蛋白α4(180kD)和α2(80kD 片段以及在 300kD 处的少许弥散)。泳道 1 为 Matrigel 对照而泳道 2－11 为相同的大鼠肌肉提取物制剂，显示特别是关于α4 链的强带。6C 显示在大鼠 MyoGel HSPG 凝胶中结合的硫酸乙酰肝素蛋白聚糖，泳道 1 为分子量标准，泳道 2 为

RegenX Science Exhibit 1004
Page 1159

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1159

Matrigel，在200kD条带上有基底膜蛋白聚糖染色而泳道3－11为大鼠肌肉提取物。6D显示大鼠肌肉提取物样品中结合的纤连蛋白片段，泳道1为Matrigel对照而泳道2－11为相同的大鼠肌肉提取物。

体外细胞分化测定

利用大鼠附睾的前脂肪细胞开展测定。在24孔板中执行用于形态学分析的测定。在24孔板中加入300μl细胞外基质（ECM）/孔。基质处于37℃ 20－30。随后，向每个孔中加入细胞（对于24孔培养板为0.3×106细胞/孔）。37℃/5％CO_2下过夜允许细胞附着在基质上。经过14天的时间段（伴随每4－5天照相）观察到分化。使用单独接种在组织培养塑料制品上的细胞作为对照。

附图5显示了在组织培养塑料制品上的对照前脂肪细胞，显示无分化。5B显示在MyoGel上的前脂肪细胞，显示脂质积聚以及向成熟脂肪细胞的分化。插入的图显示单个细胞积聚脂质的高倍放大图像。5C显示小鼠腔室的低倍显微照片，所述腔室已充满诱导脂肪形成的肌肉提取物。

本领域技术人员将理解本文所述的本发明容许除具体描述的那些之外的改变和修改。还应当理解本发明包括所有此类改变和修改。本发明还包括本说明书中个别或共同提及或暗示的所有步骤、特征、组合物和化合物，以及任何及所有任何两种或多种所述步骤或特征的组合。

RegenX Science Exhibit 1004
Page 1160

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1160

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Yuhas, *et al., Cancer Res 37*: 3639-3643 1977

RegenX Science Exhibit 1004
Page 1161

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1161

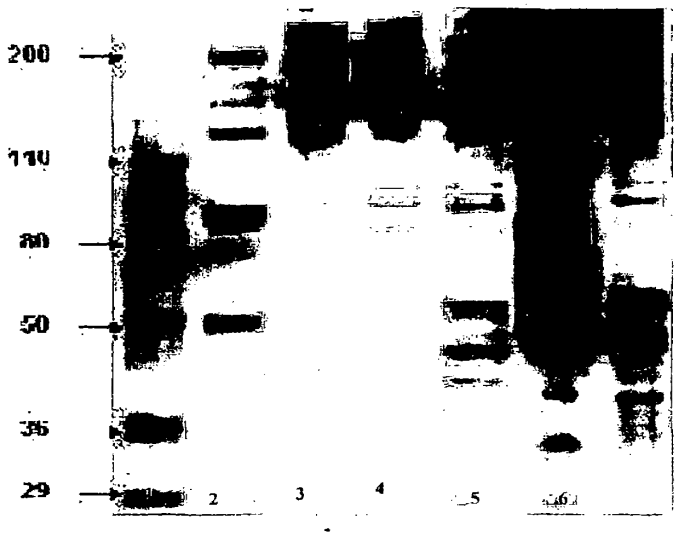


图1

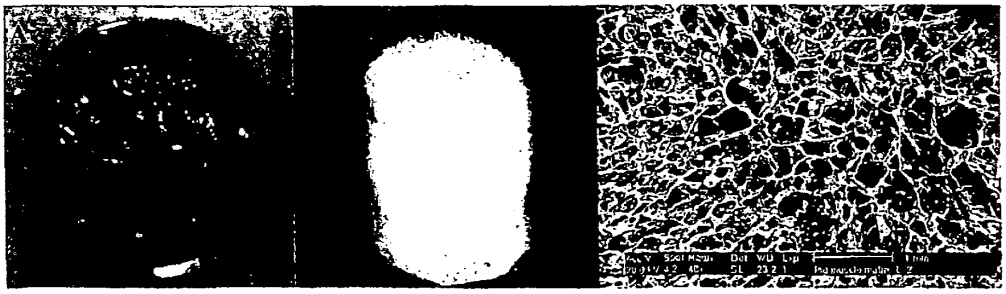


图2

RegenX Science Exhibit 1004
Page 1162

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1162

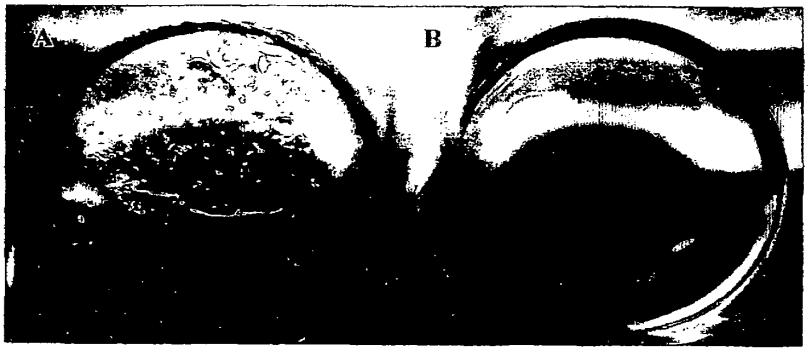


图 3

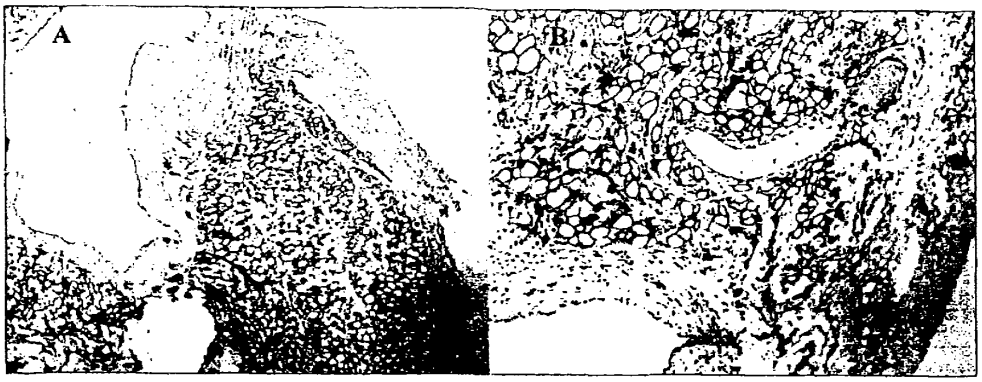


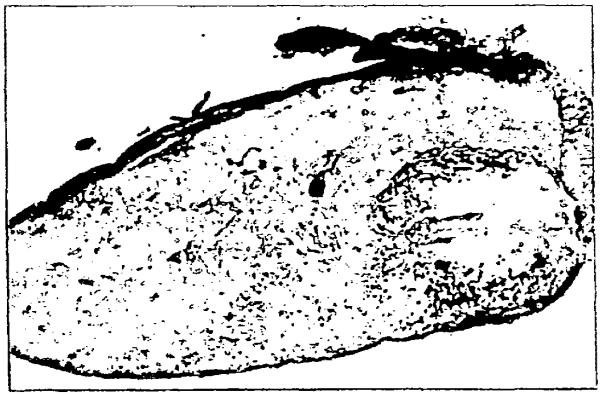
图 4

RegenX Science Exhibit 1004
Page 1163

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1163



A B



C

图5

RegenX Science Exhibit 1004
Page 1164

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1164

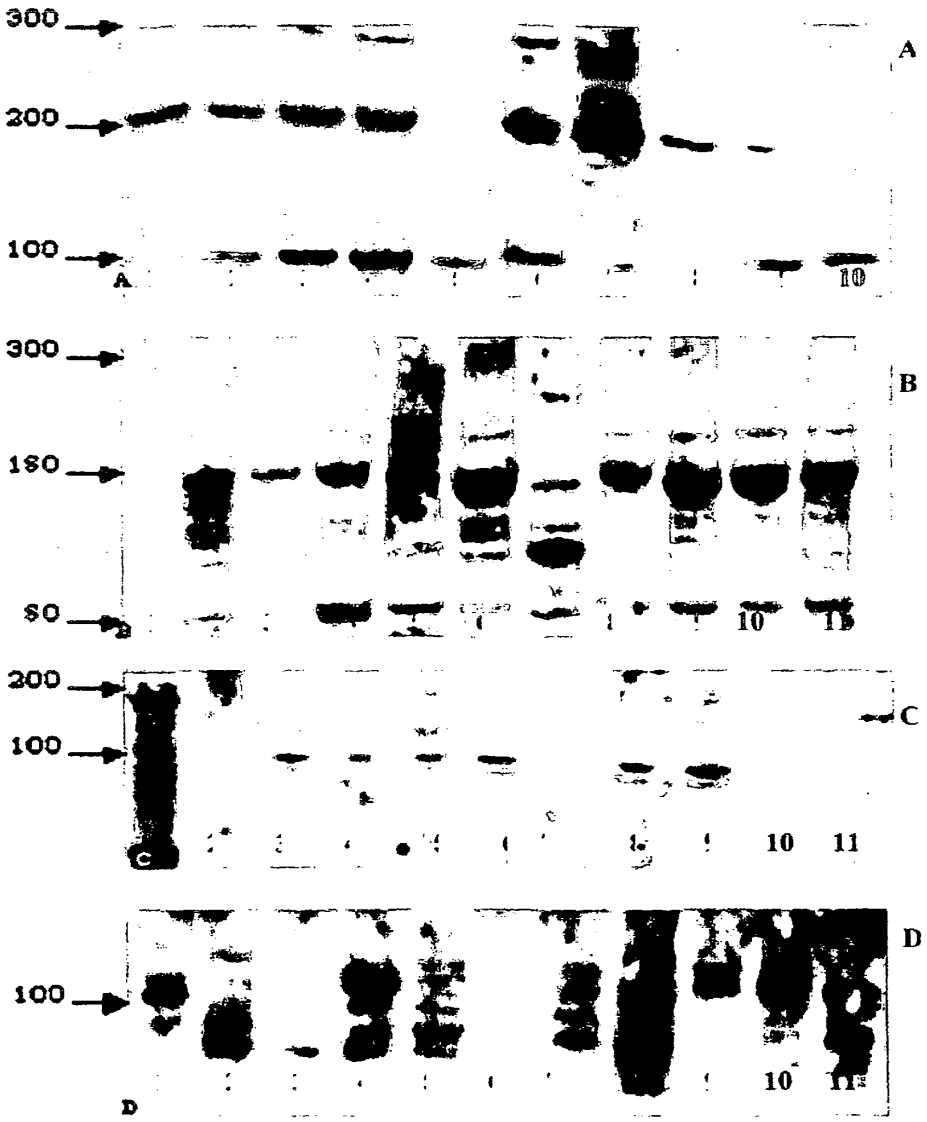


图6

RegenX Science Exhibit 1004
Page 1165
EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1165

Your Ref.: RE-1CN
Our Ref.: IP1566965P

CHINA NATIONAL INTELLECTUAL PROPERTY ADMINISTRATION

Post Code: 100004 7th FLOOR, SCITECH PLACE, NO. 22 JIAN GUO MEN WAI AVENUE, CHAOYANG DISTRICT, BEIJING. Unitalen Attorneys At Law ZHENG Bin ((8610) 59208888), PENG Kunpeng ((8610) 59208888)	Issuing Date May 22, 2019

Application No. or Patent No.: **201480013359.0** Issue No.: **2019051701641410**

Applicant or Patentee: ISE PROFESSIONAL TESTING & CONSULTING SERVICES, INC.

Title of the Invention: DECELLULARIZED BIOMATERIAL FORM NON-MAMMALIAN TISSUE

The Second Office Action

1. ☒ The examiner has received the Observations submitted by the applicant on January 16, 2019, and on this basis continued to conduct examination as to substance of the captioned patent application.

☐On the basis of the Reexamination Decision made by the Reexamination Board of the Chinese Patent Office on____, the examiner has continued to conduct examination as to substance of the captioned patent application.

2. ☐ The amended application document submitted by the applicant on____is not in conformity with the provision of Rule 51, Para. 3 of the Implementing Regulations of the Patent Law and is not acceptable.

3.Further examination has been conducted in the light of the following application document(s):

☐ the amended application document(s) attached to the said observations.

☒ the application document(s) at which the previous Office Action is directed, and the replacement sheet(s) of the amended application document(s) attached to the said Observations.

☐ the application document(s) at which the previous Office Action is directed.

☐the application document(s) confirmed in the said Reexamination Decision.

4. ☐ In this Office Action no new reference documents have been cited.

☒The following reference documents are cited in this Office Action. (Their serial numbers shall come after those previously cited and will continue to be used throughout the examination procedure):

Serial No.	Numbers or Titles of Reference Documents	Date of Publication (or filing date of interfering application)
3	CN 1969039A	May 23, 2007
4	CN 101337089A	January 07, 2009

5. Concluding comments of the examiner:

On the description:

☐ The content of the application comes within the scope where no patent right shall be granted as prescribed in Article 5 of the Patent Law.

RegenX Science Exhibit 1004
Page 1166

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1166

☐ The description is not in conformity with the provision of Article 26, Para. 3 of the Patent Law.
☐ The amendment to the description is not in conformity with the provision of Article 33 of the Patent Law.
☐ The drafting of the description is not in conformity with the provision of Rule 17 of the Implementing Regulations.

On the claims:
☐ Claim(s) _____ is/are not in conformity with the provision of Article 2, Para. 2 of the Patent Law.
☐ Claim(s) _____ is/are not in conformity with the provision of Article 9, Para. 1 of the Patent Law.
☐ Claim(s) _____ possess(es) no novelty as prescribed in Article 22, Para. 2 of the Patent Law.
☒ Claims 1-24, 26-33 possess no inventiveness as prescribed in Article 22, Para. 3 of the Patent Law.
☐ Claim(s) _____ possess(es) no practical applicability as prescribed in Article 22, Para. 4 of the Patent Law.
☐ Claim(s) _____ come(s) within the scope where no patent right shall be granted as prescribed in Article 25 of the Patent Law.
☒ Claim 25 is not in conformity with the provision of Article 26, Para. 4 of the Patent Law.
☐ Claim(s) _____ is/are not in conformity with the provision of Article 31, Para. 1 of the Patent Law.
☐ The amendment to Claim ____ is not in conformity with the provision of Article 33 of the Patent Law.
☐ Claim(s) _____ is/are not in conformity with the provisions of Rule 19 of the Implementing Regulations.
☐ Claim(s) _____ is/are not in conformity with the provisions of Rule 20 of the Implementing Regulations.
☐ Claim(s) _____ is/are not in conformity with the provisions of Rule 21 of the Implementing Regulations.
☐ Claim(s) _____ is/are not in conformity with the provisions of Rule 22 of the Implementing Regulations.
☐ ____

☐ The application is not in conformity with the provision of Art. 26, Para. 5 of the Patent Law or Rule 26 of the Implementing Regulations.
☐ The application is not in conformity with the provision of Rule 20.1 of the Implementing Regulations.
☐ The divisional application(s) is/are not in conformity with the provision of Rule 43.1 of the Implementing Regulations.

See the text portion of this Office Action for a detailed analysis of the above concluding comments.

6. In view of the above concluding comments, the examiner deems that:

RegenX Science Exhibit 1004
Page 1167

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1167

☐ the applicant should make amendment to the application document(s) according to the requirements raised in the text portion of this Office Action.

☐ the applicant should expound in his/its observations the reason why the captioned patent application is patentable and make amendment to what is not in conformity with the provisions as pointed out in the text portion of this Office Action, otherwise the said application will be rejected.

☒ the patent application has no substantive content(s) for which the patent right may be obtained, if the applicant has no sufficient reason to demonstrate that the captioned application may be granted a patent right, the said application will be rejected.

7. **The applicant should pay attention to the following matters:**

(1) According to the provision of Article 37 of the Patent Law, the applicant should submit his/its observations within **two (2)** months from the date of receipt of this Office Action; if, without any justified reason(s), the time limit for making a response is not met, the said application shall be deemed to have been withdrawn.

(2) The amendment(s) made by the applicant to the application should be in conformity with the provisions of Article 33 of the Patent Law that they shall not go beyond the original description and claims; in addition, the amendment(s) made by the applicant to the application should be in conformity with the provisions of Rule 51.3 of the Implementing Regulations of the Chinese Patent Law. The amendment(s) should be made in accordance with the requirements in the notification.

(3) The observations and/or amended text of the applicant should be submitted to the Receiving Section of the Chinese Patent Office by mail or by personal delivery; if not submitted to the Receiving Section by mail or by personal delivery, the document(s) will have no legal effect.

(4) If no appointment is made in advance, the applicant and/or the agent shall not come to the Chinese Patent Office to hold an interview with the examiner.

8. This Office Action consists of the text portion totaling 6 pages and of the following attachment(s):

☐ ___copy (copies) of the reference document(s) cited, totaling__page(s).

RegenX Science Exhibit 1004
Page 1168

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1168

Partial Text of the Second Office Action

Application No.: 201480013359.0

...

I. Claims 1-24, 26-33 do not involve an inventive step, and thus do not comply with Article 22.3 of the Chinese Patent Law.

1. Claim 1 seeks to protect a decellularized biomaterial produced by the process. D3 (CN1969039A, published on May 23, 2007 and *also published as US2006153797A1*), being the closest prior art, discloses the following contents: "the muscle matrix preparation, therefore, may be either cell-based or an intact or extracted extracellular matrix. The cell-free extract is generally prepared using methods such as urea or SDS extraction" (see paragraph 3 on page 5 of the description, *corresponding to paragraph [0024] of the description of US2006153797A1*); "one preferred form of the material comprises solubilized, extracted basement membrane material derived from muscle. Another form comprises intact matrix with lysed cells and basement membrane material" (see paragraph 2 on page 5 of the description, *corresponding to paragraph [0023] of the description of US2006153797A1*); "the present invention provides cellular and intact and extracted extracellular matrix material which is useful as a scaffold to support the growth, development and differentiation of cells and to support or effect morphological changes to cells" (see paragraph 5 on page 2 of the description, *corresponding to paragraph [0012] of the description of US2006153797A1*). As can be known, D3 has already disclosed a muscle matrix produced by decellularizing, and that the muscle matrix can be processed by decellularizing from animal tissues, and an intact matrix is retained for use as a biomaterial.

After analysis, the distinguishing features of claim 1 over D1 lie in the following: (1) sufficient cellular components are removed and structural and functional integrity

RegenX Science Exhibit 1004
Page 1169

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1169

is retained; (2) the biomaterial claimed in the present application is derived from a urodele. Based on the distinguishing features, it can be determined that the technical problem actually solved by the present application is to determine an appropriate animal tissue source and to eliminate antigenicity of material while retaining material's functions.

Regarding the distinguishing feature (1), D3 further discloses the following content: "the muscle tissue is from an organism selected from the group consisting of a: human, non-human, primate, livestock animal, laboratory test animal, companion animal, avian species, reptile and amphibian (including a urodele)" (see claim 4, *corresponding to claim 4 of US2006153797A1*). Meanwhile, D4 (CN101337089A, published on January 07, 2009) indicates a muscle regeneration factor used for smoothing wrinkle tissues and generating new tissues, and particularly discloses the following contents (see paragraph 3 on page 4 of the description): "features of the present invention also lie in that the axolotl cells having the regeneration ability in animals are used to fuse with experimental mouse cells in an exine-detached manner for two times to develop bulged cells. After being expanded in vitro, the bulged cells, together with the exine-detaching fusion agent and injection solvent, are prepared into muscle regeneration factor of an injection type, wherein the exine-detaching fusion agent with a clear rejection effect is involved ... The present invention is suitable for the fusion and combination of the body cells of any human race and any animal to form a new tissue". As can be known, D4 discloses the application of axolotl as a urodele amphibian with strong regeneration ability in regeneration hospitals, and that axolotl can promote regeneration of new tissues and wound healing. Based on the enlightenment of the selectable tissue source disclosed by D3 and axolotl as a regeneration medical material disclosed by D4, those skilled in the art can be motivated to select a tissue of urodele amphibians with good regeneration ability to prepare and obtain a corresponding biomaterial. Moreover, the description does not have evidences to demonstrate that the above means has any unexpected technical effect.

RegenX Science Exhibit 1004
Page 1170

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1170

Regarding the distinguishing feature (2), D1 (WO2007124127A, published on November 01, 2007) discloses a bioscaffold (i.e., a biomaterial), and particularly discloses the following contents: "a bioscaffold produced by the process of chemically treating a natural animal tissue, wherein an extracellular matrix component of said bioscaffold has an average pore size that is at least 20% greater than the average pore size of said extracellular matrix component of said natural animal tissue prior to said treating (which demonstrates that the sample comprises extracellular matrix); and wherein said natural animal tissue is selected from the group consisting of skin, muscle, tendon, bone, meniscus, cartilage, intervertebral discs, and ligament tissue" (see claim 21); and "an architecturally-optimized bioscaffold can be created that retains the majority of its original biomechanical integrity" (see paragraph 4 on page 7 of the description); as well as "improved bioscaffolds disclosed herein are: 1) derived from natural tissue and amenable to host-cell mediated remodeling in vivo, 2) decellularized to minimize inflammatory potential and host immune response (equivalent to reducing, via decellularizing, antigenicity of the biomaterial as a xenograft), 3) biocompatible ..." (see paragraph 5 on page 7 of the description). It follows that, D1 has disclosed a bioscaffold produced by decellularizing, and has disclosed that immunogenicity of the bioscaffold is reduced by decellularizing treatment. Based on this, those skilled in the art can be motivated to prepare a biomaterial with low immunogenicity by decellularizing as much as possible on the basis on D3.

Therefore, it is obvious for those skilled in the art to obtain the technical solution claimed in claim 1 on the basis of D3 in combination with D1, D4. That is, claim 1 neither possesses any prominent substantive features nor represents any notable progress, and thus does not involve an inventive step under Article 22.3 of the Chinese Patent Law.

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II. Claim 25 is unclear, and thus does not comply with Article 26.4 of the

RegenX Science Exhibit 1004
Page 1171

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1171

Chinese Patent Law.

Claim 25 seeks to protect a tissue culture medium conditioned with isolated Urodele decellularized ECM or a Urodele fraction derived from isolated, decellularized Urodele ECM. Features of claim 25 only define the ECM and do not define the culture medium. Claim 25 does not have technical features suitable for the subject matter, that is, claim 25 does not **define properties such as specific components, composition** of the culture medium. According to the disclosure manner, those skilled in the art cannot determine what kind of a culture medium the culture medium claimed in the claim is at all. Meanwhile, a relevant paragraph of the description of the present application describes the culture medium as follows: "numerous liquid tissue culture media are commercially available and well known to those of ordinary skill in the art" (see paragraph [0038] of the description, *corresponding to paragraph [0034] of the description in English version*). Thus, the description does not clearly disclose what kind of a commercially available or well-known culture medium the culture medium is at all. Therefore, according to disclosure of claim 25, those skilled in the art cannot determine the protection scope of claim 25. That is, claim 25 is unclear, and thus does not comply with Article 26.4 of the Chinese Patent Law.

Even if it is deemed that the culture medium is "commercially available and well known to those of ordinary skill in the art" as disclosed in the description, the culture medium is already disclosed by the prior art according to the disclosure, and thus claim 25 does not involve an inventive step or possess novelty.

III. Applicant's observations are unconvincing.

The applicant argues that: (1) the technical problem actually solved by the present application over D1 is how to obtain a biomaterial having a particularly favorable form suitable for xenograft, whereas D1 and other prior art do not provide the enlightenment to select a urodele; (2) D1 only superficially mentions amphibians,

RegenX Science Exhibit 1004
Page 1172

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1172

but amphibians contain three orders, and thus D1 does not provide the enlightenment to further select amphibians; (3) D1 provides opposite teaching to remove foreign materials and increase the pore size, whereas the ECM claimed in the present application also contains extracellular matrix such as the growth factor profile and connective tissue matrix constituents.

In this regard, the examiner deems that: (1) examples of the present application for demonstrating the beneficial effect of the invention only describe good biocompatibility of the ECM obtained using the axolotl tissue, and structural similarity of the ECM obtained using the axolotl tissue with the human amniotic membrane tissue, and the preparation method. No evidences shows that the ECM obtained using tissue derived from the animal is better than the ECM prepared from other animal tissue, that is, no evidences show the ECM from a urodele has particularly favorable properties stated by the applicant. Thus, according to the distinguishing technical features, it is determined that the technical problem actually solved is to select an appropriate ECM animal tissue source.

(2) As can be known from the preceding comments, both D1 and D3 disclose that the shown extracellular matrix material can be derived from amphibians which only contain animals of three orders, which can be determined through limited experiments. In addition, the Office Action cites new D4 which clarifies that axolotl is selected as the regeneration medical material. Based on the information, those skilled in the art would readily select axolotl (that is, a urodele) as the ECM tissue source. Moreover, the description does not have data showing that the regeneration promotion ability of ECM from this source is better than the ECM derived from other animals, that is, no unexpected technical effect is produced.

(3) The Office Action cites a new reference document — D3. D3 discloses that the matrix contains a complete matrix structure, and discloses the following contents (see paragraph 5 on page 2 of the description, *corresponding to paragraph [0012] of the description in English version*): "the present invention provides cellular and intact

RegenX Science Exhibit 1004
Page 1173

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1173

and extracted extracellular matrix material which is useful as a scaffold to support the growth, development and differentiation of cells and to support or effect morphological changes to cells. The tissue material is preferably derived from muscle tissue and comprises a preparation comprising basement membrane components. These components comprise one or more of, but not limited to, laminin, collagen I, collagen IV, entactin/nidogen, heparan sulfate proteoglycan as well as one or more of, but not limited to, EGF, bFGF, NGF, PDGF, IGF-1, TGF-[beta], VEGF and TNF-[alpha]". As can be known, the ECM disclosed in D3 contains complete extracellular matrix and corresponding growth factor profile, muscle basement membrane components, etc. That is, D3 discloses that extracellular matrix nutrient substances need to be retained. Therefore, the feature does not make the present application involve an inventive step.

To sum up, the applicant's observations are unconvincing.

...

Examiner: QUAN Hongyang

Code: 559309

RegenX Science Exhibit 1004
Page 1174

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1174

Your Ref.: RE-1CN
Our Ref.: IP1566965P

China National Intellectual Property Administration

Address: Receiving Section of the China National Intellectual Property Administration, No. 6 Tucheng Road West, Haidian District, Beijing.

Search Report

Patent Application No.: 201480013359.0	Filing Date: January 09, 2014	Supplementary Search
Applicant: ISE PROFESSIONAL TESTING & CONSULTING SERVICES, INC.	Earliest Priority Date: January 09, 2013	
No. of Claims: 33	Paragraphs of Description: 125+6	
IPC No. Determined by Examiner: A61K38/39		
Search Record Information: CN1969039A: 6 CNABS, (有尾目(urodele) or 两栖(amphibia) or 蝾螈(axolotl)) and (ECM OR 胞外基质(extracellular matrix) or 脱细胞(decellularize)); CN101337089A: 21 CNTXT, 蝾螈(axolotl) and 组织(tissue) and (再生(regenerate) s 材料(material)).		

Relevant Patent Documents

Type	Country and Document No. Given by Code [11]	Date Given by Code [43] or [45]	IPC No.	Relevant Paragraphs and/or Figure No.	Relevant to Claim No.
Y	CN1969039A	May 23, 2007	C12N5/00	1-24, 26-33	Claim 4, paragraph 5 on page 2, paragraphs 2-3 on page 3, and paragraphs 2-3 on page 5 of the description
Y	CN101337089A	January 07, 2009	A61L27/38	1-24, 26-33	Paragraph 3 on page 4 of the description

Relevant Non-Patent Documents

Type	Title of Book (Including Edition and Volume)	Date of Publication	Name of Author and Name of Publisher	Relevant Page(s)	Relevant to claim No.
Type	Title of Periodical or Abstract (Including Volume and Issue)	Date of Issue	Name of Author and Title of Article	Relevant Page(s)	Claim(s) Involved

Directions for Filling out the Form:

1. The IPC number of the field actually searched by the examiner should be filled in terms of the class and/or the subclass.
2. For the titles of periodicals or other periodic publications, abbreviations conforming to generally acknowledged international practice may be used.
3. Categories of relevant documents:

   X: a document that prejudices novelty or inventiveness;

   Y: the document that when combined with other such documents cited in the search report, prejudices inventiveness;

   A: the document defining the general state of the art;

   R: the patent or patent application document belonging to identical invention-creation that was submitted to the Patent Office by any entity or individual on the date of filing;

   P: intermediate document, that is the document published on dates falling between the filing date of the application being examined and the claimed priority date;

   E: Conflicting application.

Examiner: QUAN Hongyang
Examination Department: Pharmaceutical and Biological Invention Examination Department of Patent Examination Cooperation Center of the Patent Office, CNIPA, Sichuan
Date: May 14, 2019

Articles and Rules and Relevant Regulations of Guidelines

Cited by the Examiner in the Second Office Action

Article 22.3 of the Chinese Patent Law: Inventiveness means that, as compared with the prior art, the invention has prominent substantive features and represents a notable progress, and that the utility model has substantive features and represents progress.

Article 26.4 of the Chinese Patent Law: The claims shall be supported by the description and shall define the extent of the patent protection sought for in a clear and concise manner.

Article 33 of the Chinese Patent Law: An applicant may amend his or its application for a patent, but the amendment to the application for a patent for invention or utility model may not go beyond the scope of disclosure contained in the initial description and claims, and the amendment to the application for a patent for design may not go beyond the scope of the disclosure as shown in the initial drawings or photographs.

Rule 51.3 of the Implementing Regulations of the Chinese Patent Law: Where the applicant amends the application after receiving the notification of opinions of the examination as to substance of the patent administration department under the State Council, he or it shall make the amendment directed to the defects pointed out by the notification.

国 家 知 识 产 权 局

100004

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郑斌((8610) 59208888) 彭鲲鹏((8610) 59208888)

发文日：

2019 年 05 月 22 日

申请号或专利号：**201480013359.0**　　发文序号：**2019051701641410**

申请人或专利权人： ISE 专业检测与咨询服务有限公司

发明创造名称： 来自非哺乳动物组织的脱细胞化的生物材料

第 二 次 审 查 意 见 通 知 书

1. ☒审查员已经收到申请人于 2019 年 1 月 16 日提交的意见陈述书，在此基础上审查员对上述专利申请继续进行实质审查。

☐根据国家知识产权局于_____年_____月_____日作出的复审决定，审查员对上述专利申请继续进行实质审查。

☐_____

2. ☐经审查，申请人于_____提交的修改文件，不符合专利法实施细则第 51 条第 3 款的规定，不予接受。

3. 继续审查是针对下列申请文件进行的：

☐上述意见陈述书中所附的经修改的申请文件。

☒前次审查意见通知书所针对的申请文件以及上述意见陈述书中所附的经修改的申请文件替换文件。

☐前次审查意见通知书所针对的申请文件。

☐上述复审决定所确定的申请文件。

☐_____

4. ☐本通知书未引用新的对比文件。

☒本通知书引用下列对比文件(其编号续前，并在今后的审查过程中继续沿用)：

编号	文 件 号 或 名 称	公开日期（或抵触申请的申请日）
3	CN 1969039A	20070523
4	CN 101337089A	20090107

5. 审查的结论性意见：

关于说明书：

☐申请的内容属于专利法第 5 条规定的不授予专利权的范围。

☐说明书不符合专利法第 26 条第 3 款的规定。

☐说明书的修改不符合专利法第 33 条的规定。

☐说明书的撰写不符合专利法实施细则第 17 条的规定。

☐_____

纸件申请，回函请寄：100088 北京市海淀区蓟门桥西土城路 6 号　国家知识产权局专利局受理处收

电子申请，应当通过电子专利申请系统以电子文件形式提交相关文件。除另有规定外，以纸件等其他形式提交的文件视为未提交。

关于权利要求书：

☐权利要求_____不符合专利法第 2 条第 2 款的规定。

☐权利要求_____不符合专利法第 9 条第 1 款的规定。

☐权利要求_____不具备专利法第 22 条第 2 款规定的新颖性。

☒权利要求 1-24, 26-33 不具备专利法第 22 条第 3 款规定的创造性。

☐权利要求_____不具备专利法第 22 条第 4 款规定的实用性。

☐权利要求_____属于专利法第 25 条规定的不授予专利权的范围。

☒权利要求 25 不符合专利法第 26 条第 4 款的规定。

☐权利要求_____不符合专利法第 31 条第 1 款的规定。

☐权利要求_____的修改不符合专利法第 33 条的规定。

☐权利要求_____不符合专利法实施细则第 19 条的规定。

☐权利要求_____不符合专利法实施细则第 20 条的规定。

☐权利要求_____不符合专利法实施细则第 21 条的规定。

☐权利要求_____不符合专利法实施细则第 22 条的规定。

☐_____

☐申请不符合专利法第 26 条第 5 款或者实施细则第 26 条的规定。

☐申请不符合专利法第 20 条第 1 款的规定。

☐分案申请不符合专利法实施细则第 43 条第 1 款的规定。

上述结论性意见的具体分析见本通知书的正文部分。

6.基于上述结论性意见，审查员认为：

☐申请人应当按照通知书正文部分提出的要求，对申请文件进行修改。

☐申请人应当在意见陈述书中论述其专利申请可以被授予专利权的理由，并对通知书正文部分中指出的不符合规定之处进行修改，否则将不能授予专利权。

☒专利申请中没有可以被授予专利权的实质性内容，如果申请人没有陈述理由或者陈述理由不充分，其申请将被驳回。

☐_____

7. 申请人应注意下列事项：

（1）根据专利法第 37 条的规定，申请人应在收到本通知书之日起的 2 个月内陈述意见，如果申请人无正当理由逾期不答复，其申请被视为撤回。

（2）申请人对其申请的修改应当符合专利法第 33 条的规定，不得超出原说明书和权利要求书记载的范围，同时申请人对专利申请文件进行的修改应当符合专利法实施细则第 51 条第 3 款的规定，按照本通知书的要求进行修改。

（3）申请人的意见陈述书和/或修改文本应当邮寄或递交国家知识产权局专利局受理处，凡未邮寄或递交给受理处的文件不具备法律效力。

（4）未经预约，申请人和/或代理人不得前来国家知识产权局与审查员举行会晤。

8．本通知书正文部分共有 8 页，并附有下列附件：

☐引用的对比文件的复印件共_____份_____页。

☐_____

审查员：全弘扬　　联系电话：028-62968531　　审查部门：专利审查协作四川中心医药生物发明审查部

国 家 知 识 产 权 局

第 二 次 审 查 意 见 通 知 书

申请号:2014800133590

申请人于 2019 年 1 月 16 日提交了意见陈述书和经过修改的申请文件，审查员在阅读了上述文件后，对本案继续进行审查，再次提出如下审查意见。

一、权利要求 1-24,26-33 不具备创造性，不符合专利法第 22 条第 3 款的规定。

1、权利要求 1 要求保护一种通过以下方法产生的脱细胞化的生物材料。对比文件 3（CN1969039A，公开日为 2007 年 5 月 23 日）是其最接近的现有技术，其中公开了以下内容："因此，肌肉基质制剂可以是基于细胞的或完整的或提取的细胞外基质。一般利用例如尿素或 SDS 提取方法来制备无细胞提取物"（参见说明书第 5 页第 3 段)；"材料的一种优选形式包括溶解的、提取的来源于肌肉的基底膜材料。另一形式包括含溶解的细胞和基底膜材料的完整基质"（参见说明书第 5 页第 2 段)；"本发明共细胞的以及完整的和提取的细胞外基质材料，它用作支架以支持细胞的生长、发育和分化并支持或实现细胞的形态学改变"（参见说明书第 2 页第 5 段)。由此可知，对比文件 3 已经公开了一种脱细胞化产生的肌肉基质，且该肌肉基质可以从动物组织脱细胞化处理，并保留完整的基质以用作生物材料。

经分析，权利要求 1 与对比文件 1 相比，其区别特征在于：（1）充分去除细胞成分且保留结构功能完整性；（2）本申请所述的生物材料来源为有尾目动物。基于所述区别特征，可以确定本发明实际解决的技术问题是确定合适的动物组织来源并且保持材料功能的同时消除材料抗原性。

针对区别特征（1），对比文件 3 还公开了："所述肌肉组织来自人、非人灵长类、家畜动物、实验室试验动物、伴侣动物、鸟类、爬行动物或两栖类(包含有尾目)"（参见权利要求 4)。同时，对比文件 4(CN101337089A，公开日为 2009 年 1 月 7 日）指出了一种能够用于皱纹组织平顺和生成新组织的肌肉再生素，具体为（参见说明书第 4 页第 3 段)："本发明的特征还在于，用动物界具有再生能力的蝾螈细胞二次与实验鼠细胞脱髓融合长出隆起的细胞，体外扩增后，与脱髓融合剂，注射溶剂，制成注射剂型肌肉再生素，其中配有清楚排异作用的脱髓融合剂……本发明适合于任何种族的人体和任何动物的畜体细胞融合合并生成新的组织"。由此可知，对比文件 4 公开了蝾螈作为再生能力强大的有尾目两栖类动物在再生医院中的应用，且能够促进新组织的再生和伤口的愈合。基于对比文件 3 公开的可选择组织来源和对比文件 4 公开的蝾螈作为再生医学材料的启示，本领域技术人员有动机选择具有良好再生能力的两栖类有尾目动物组织以制备获得相应的生物材料，且说明书中没有证据表明其具有预料不到的技术效果。

针对区别特征（2），对比文件 1（WO2007124127A，公开日为 2007 年 11 月 1 日）中公开了一种生物支架（即一种生物材料)，其具体公开了以下内容："通过化学处理自然动物组织获得的生物支架，所述生物支架的细胞外基质成分具有大于未采用化学处理前组织细胞外基质（表明所述样品包含细胞外基质）孔径 20%的平均孔径；所述自然动物组织可选择皮肤、肌肉、腱、骨、半月板、软骨、椎间盘以及韧带"（参见权利要求

RegenX Science Exhibit 1004
Page 1180
EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1180

21)；“一种人工优化的生物支架可以通过保留其主要的原始生物力学完整性以制备得到”(参见说明书第 7 页第 4 段)；“本发明公开的优化的生物支架具有：(1) 从自然组织中获取且有利于宿主细胞介导的体内重构；(2) 脱细胞化以最小化减少验证潜能和宿主免疫反应(相当于通过脱细胞化降低生物材料作为异种移植物的抗原性)；(3) 生物相容性……”(参见说明书第 7 页第 5 段)。由此可知，对比文件 1 已经公开了一种通过脱细胞化产生的生物支架，且公开了以脱细胞化处理使得其减低了免疫原性。基于此，本领域技术人员有动机在对比文件 3 的基础上尽可能的脱细胞化以制备出免疫原性低的生物材料。

因此，在对比文件 3 基础上结合对比文件 1、4 获得权利要求 1 要求保护的技术方案对于本领域技术人员来说是显而易见的，即权利要求 1 不具有突出的实质性特点和显著的进步，不具备专利法第 22 条第 3 款规定的创造性。

2、权利要求 2-4 是权利要求 1 的从属权利要求，其进一步限定了所述生物材料。对比文件 3 公开了相应的内容：“提取物随后在 0.05M Tris/0.15M NaCl 缓冲液（TBS）中 4℃对 0.5% v/v 氯仿透析过夜作为灭菌步骤”(参见说明书第 18 页第 3 段)；“术语‘肌肉基质’为了方便简洁而使用，应理解它涵盖了基于细胞的制剂和无细胞的制剂”(参见说明书第 3 页第 2 段)。对比文件 1 公开了相应的内容：“所述的处理过程可以包括采用包含有去污剂的碱溶液处理”(参见说明书第 15 页第 1 段)。因而，对比文件 3 公开了所述生物材料要进行灭菌处理、并且可以包含细胞的制剂；对比文件 1 公开了可以用碱溶液处理，且明确了脱细胞化以减低免疫原性。基于这些信息，本领域技术人员容易想到其还包含有失活（失去免疫原性）的细胞。因此，在其引用的权利要求 1 不具备创造性的情况下，权利要求 2-4 也不具有突出的实质性特点和显著的进步，不具备专利法第 22 条第 3 款规定的创造性。

3、权利要求 5 要求保护一种组织移植物，其包含来源于有尾目动物的胞外基质成分。参见权利要求 1 的意见评述可知，通过脱细胞化处理的有尾目动物胞外基质成分的生物材料是不具备创造性的。同时，对比文件 3 还公开了（参见说明书第 3 页第 3 段)：“本发明的肌肉基质具有各种用途，例如在组织工程中促进大量组织产生用于组织修复、增加和/或替代疗法。该肌肉基质也用作工程化组织的支架，所述组织例如但不限于肌肉和脂肪”，即公开了该基质可以用作组织工程支架移植物；基于此，本领域技术人员也容易想到将所述来源于有尾目动物的胞外基质作为组织移植物，即获得含有所述胞外基质的组织移植物，且其技术效果可以预期。因此，权利要求 5 也不具有突出的实质性特点和显著的进步，不具备专利法第 22 条第 3 款规定的创造性。

4、权利要求 6 要求保护一种分离的脱细胞化的有尾目动物 ECM。参见权利要求 1 的意见评述可知，通过脱细胞化处理的有尾目动物胞外基质成分的基质是不具备创造性的，而根据前述对比文件 3 公开的制备方法可知这种基质包含一种分离的脱细胞化的动物 ECM。因此，基于与权利要求 1 相同的理由，权利要求 6 也不具有突出的实质性特点和显著的进步，不具备专利法第 22 条第 3 款规定的创造性。

5、权利要求 7-10 是权利要求 6 的从属权利要求，其进一步限定了所述的 EMC。参见前述意见可知，由

RegenX Science Exhibit 1004
Page 1181
EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1181

于对比文件 3 已经公开了所述组织可以是来源于两栖类动物的组织，对比文件 4 公开了将蝾螈应用于再生医学，在此基础上，本领域技术人员容易进一步选择容易获得的两栖类蝾螈作为组织的提供者，且其技术效果可以预期。同时，对比文件 1 还公开了，脱细胞化也需要保持原组织的生物力学完整性，基于此，本领域技术人员也容易想到其必定需要包含基膜以保证其生物力学的完整性。此外，对比文件 1 还公开了（参见说明书第 9 页第 4 段）："蛋白（诸如生长因子）或其他添加物（诸如抗生素、抗炎性因子和其他免疫反应的调节因子）可以随时被添加入细胞或生物支架中。同时，各种处理都可以用于提高细胞与底物之间的附着度。合适的处理于 US 专利号为 5613982 的专利中记载。所述处理包括采用如生长因子或细胞外基质胶蛋白处理生物支架底物或生长中的结构。例如，胶原蛋白、弹性蛋白、纤粘蛋白、层粘蛋白或蛋白聚糖可应用于生物支架。所述生物支架可以被生长因子如 NGF，aFGF，bFGF，PDGF，TGF β，VEGF，GDF-5/6/7，BMP-1/2/3/4/5/6/7/13/12/14，IGF-1 等灌满，这些试剂可以被添加入培养基中"。由此可知，对比文件 1 已经公开了将异种的物质注入所述 EMC，所述物质可以是生长因子、蛋白质、糖、抗生素等，在此基础上，本领域技术人员容易进一步想到也可以采用这些物质进行包被、附着等处理，且进一步选择添加细胞因子、趋化因子、碳水化合物、糖、类固醇、合成聚合物、粘合剂、药物等其他免疫调节分子的物质，且其附着的技术效果可以预期。参见前述意见可知，对比文件 1 还公开了可以将哺乳动物细胞种植于所述的生物支架上面，即公开了可以添加异种的细胞，且据此也容易想到添加需要移植的宿主——人类的细胞。因此，在其引用的权利要求 6 不具备创造性的情况下，权利要求 7-10 也不具有突出的实质性特点和显著的进步，不具备专利法第 22 条第 3 款规定的创造性。

6、权利要求 11-14 是权利要求 6 或在先权利要求的从属权利要求，其进一步限定了 ECM 的形态。对比文件 2（WO2005/009498A，公开日为 2005 年 2 月 3 日）中公开了 ECM 能够制备成为的形态，具体为："在一个实施例中，如前所述的骨髓细胞外基质悬液或溶液可以被干燥后用以制备成为 ECM 薄片或吸收于其表面"（参见说明书第 26 页第 3 段）；"在另一个实施例中，所述的脱细胞化的骨髓细胞外基质可以被制备成为凝胶"（参见说明书第 13 页最后一段）；"这些物质可以采用溶液、悬浮液、乳液、片剂、药丸、胶囊、干粉、缓释制剂的形式"（参见说明书第 16 页第 3 段）。由此可知，对比文件 2 已经公开了可以将 ECM 制备成为片、干粉、凝胶的形式，而重构凝胶也是凝胶制剂中一种常见的剂型，同时参见前述记载对比文件 1 中记载的所述生物支架具有孔。因此，在其引用的权利要求不具备创造性的情况下，权利要求 11-14 也不具有突出的实质性特点和显著的进步，不具备专利法第 22 条第 3 款规定的创造性。

7、权利要求 15 是权利要求 6-13 的从属权利要求，其进一步限定了 ECM 是无菌的。参见前述意见可知，对比文件 3 已经公开了所述的肌肉基质是无菌的。因此，在其引用的权利要求不具备创造性的情况下，权利要求 15 也不具有突出的实质性特点和显著的进步，不具备专利法第 22 条第 3 款规定的创造性。

8、权利要求 16 要求保护一种包装，其包含无菌的分离的脱细胞化的有尾目动物 ECM 或来源于所述无菌

RegenX Science Exhibit 1004
Page 1182
EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1182

的分离的脱细胞化的有尾目动物 ECM 的有尾目动物级分。参见前述意见可知，所述的无菌的分离的脱细胞化的有尾目动物 ECM 或所述动物级分是没有创造性的。同时，对比文件 1 还公开有（参见权利要求 32）：“如权利要求 21 所述的生物支架无菌地包装于无菌容器中”，即对比文件 1 已经公开了可以将脱细胞化的生物支架制备成为一个包装。基于此，本领域技术人员有动机将无菌的分离的脱细胞化的有尾目动物 ECM 或所述无菌的分离的脱细胞化的有尾目动物 ECM 的动物级分也采用无菌容器进行包装，并获得所述包装，且其技术效果可以预期。因此，在对比文件 1 的基础上结合本领域的常规技术手段获得权利要求 16 要求保护的技术方案对于本领域技术人员来说是显而易见的，即权利要求 16 不具有突出的实质性特点和显著的进步，不具备专利法第 22 条第 3 款规定的创造性。

9、权利要求 17-19 是权利要求 16 的从属权利要求，其附加技术特征限定了 ECM 的剂型。可以获知，权利要求 17-19 的附加技术特征与权利要求 11、13-14 的附加技术特征相同，参见前述意见这些特征已经为对比文件 2 所公开。因此，在其引用的权利要求 16 不具备创造性的情况下，权利要求 17-19 也不具有突出的实质性特点和显著的进步，不具备专利法第 22 条第 3 款规定的创造性。

10、权利要求 20 要求保护一种无菌的医学植入物，其包含无菌的分离的脱细胞化的有尾目动物 ECM 或来源于所述无菌的分离的脱细胞化的有尾目动物 ECM 的有尾目动物级分。参见前述意见可知，所述的无菌的分离的脱细胞化的有尾目动物 ECM 或所述动物级分是没有创造性的。同时，对比文件 1 还公开了（参见说明书第 4 页第 4 段）：“本发明还涉及了将所述生物支架用作组织移植物或生物移植材料进行治疗的方法”，即公开了该生物支架可以用作医学植入物。基于此，本领域技术人员有动机将无菌的分离的脱细胞化的有尾目动物 ECM 或所述无菌的分离的脱细胞化的有尾目动物 ECM 的动物级分也用作医学植入物，并获得所述植入物，且其技术效果可以预期。因此，在对比文件 1 的基础上结合本领域的常规技术手段获得权利要求 20 要求保护的技术方案对于本领域技术人员来说是显而易见的，即权利要求 20 不具有突出的实质性特点和显著的进步，不具备专利法第 22 条第 3 款规定的创造性。

11、权利要求 21 是权利要求 20 的从属权利要求，其进一步限定了植入物。参见前述意见可知，对比文件 1 还公开了所述的生物支架具有生物相容性。同时，参见对比文件 2 公开了的内容可知可以将所述的生物材料制备成为各种剂型。基于此，本领域技术人员容易想到将所述的医学植入物制备成为片、网、凝胶、移植物、组织或装置等医学上常见的用于移植治疗的物质进行应用，且说明书中也没有记载这些形态能够使得所述的医学植入物产生预料不到的技术效果。因此，在其引用的权利要求 20 不具备创造性的情况下，权利要求 21 也不具有突出的实质性特点和显著的进步，不具备专利法第 22 条第 3 款规定的创造性。

12、权利要求 22 要求保护一种材料，其被分离的脱细胞化的有尾目动物 ECM 或来源于所述分离的脱细胞化的有尾目动物 ECM 的有尾目动物级分包被、浸渍、封装和分离。参见前述意见可知，所述的无菌的分离的脱细胞化的有尾目动物 ECM 或所述动物级分是没有创造性的。同时，对比文件 1 还公开有（参见说明书第 9

RegenX Science Exhibit 1004
Page 1183
EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1183

页第 4 段）：“蛋白（诸如生长因子）或其他添加物（诸如抗生素、抗炎性因子和其他免疫反应的调节因子）可以随时被添加入细胞或生物支架中。同时，各种处理都可以用于提高细胞与底物之间的附着度。合适的处理于 US 专利号为 5613982 的专利中记载。所述处理包括采用如生长因子或细胞外基质胶蛋白处理生物支架底物或生长中的结构。例如，胶原蛋白、弹性蛋白、纤粘蛋白、层粘蛋白或蛋白聚糖可应用于生物支架。所述生物支架可以被生长因子如 NGF，aFGF，bFGF，PDGF，TGF β，VEGF，GDF-5/6/7，BMP-1/2/3/4/5/6/7/13/12/14，IGF-1 等灌满，这些试剂可以被添加入培养基中”，即对比文件 1 已经公开了可以将脱细胞化的生物支架浸渍于其他物质的材料。基于此，本领域技术人员有动机将无菌的分离的脱细胞化的有尾目动物 ECM 或所述无菌的分离的脱细胞化的有尾目动物 ECM 的动物级分也采用包被、浸渍、封装和分离等常规与其他物质联合的方式制备成为相应的医用材料，并获得所述材料，且其技术效果可以预期。因此，在对比文件 1 的基础上结合本领域的常规技术手段获得权利要求 22 要求保护的技术方案对于本领域技术人员来说是显而易见的，即权利要求 22 不具有突出的实质性特点和显著的进步，不具备专利法第 22 条第 3 款规定的创造性。

13、权利要求 23 要求保护一种组织培养系统，其包括分离的脱细胞化有尾目动物 ECM 和组织培养基以及对所述有尾目动物来说为异种的细胞。参见前述意见可知，所述的分离的脱细胞化的有尾目动物 ECM 是没有创造性的。同时，对比文件 1 还公开有（参见说明书第 9 页第 4 段）：“所述生物支架可以被生长因子如 NGF，aFGF，bFGF，PDGF，TGF β，VEGF，GDF-5/6/7，BMP-1/2/3/4/5/6/7/13/12/14，IGF-1 等灌满，这些试剂可以被添加入培养基中”，即对比文件 1 已经公开了异种含有所述脱细胞化生物支架和培养基的系统；同时，参见前述意见可知，对比文件 1 还公开了可以对该支架种植异种的哺乳动物细胞。基于此，本领域技术人员有动机将人类（一种哺乳动物）细胞也种植于分离的脱细胞化的有尾目动物 ECM 中，为了使得种植的异种细胞生长也容易想到采用组织培养基的方式进行培养，即形成一种含有分离的脱细胞化有尾目动物 ECM、组织培养基以及异种的细胞的组织培养系统。因此，在对比文件 1 的基础上结合本领域的常规技术手段获得权利要求 23 要求保护的技术方案对于本领域技术人员来说是显而易见的，即权利要求 23 不具有突出的实质性特点和显著的进步，不具备专利法第 22 条第 3 款规定的创造性。

14、权利要求 24 是权利要求 23 的从属权利要求，其进一步限定了所述细胞为人细胞。参见前述意见可知，对比文件 1 公开了种植的细胞可以为哺乳动物细胞，而人是一类医用移植需求最大的宿主哺乳动物，本领域技术人员容易想到向其中种植的细胞为人细胞，且说明书中没有记载种植人细胞能够产生预料不到的技术效果。因此，在其引用的权利要求 23 不具备创造性的情况下，权利要求 24 也不具有突出的实质性特点和显著的进步，不具备专利法第 22 条第 3 款规定的创造性。

15、权利要求 26 要求保护一种装置，其包含分离的脱细胞化的有尾目动物 ECM 至少两个彼此层压的片。参见前述意见可知，所述的分离的脱细胞化的有尾目动物 ECM 是没有创造性的。同时，参见前述意见可知，对比文件 2 还公开了可以将 ECM 制备成为片状。基于此，本领域技术人员有动机将分离的脱细胞化的有尾目

RegenX Science Exhibit 1004
Page 1184

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1184

动物 ECM 制备成为片状进行使用；而选择几层的片状是根据实际需要能够选择和设计，因而获得这种含有至少两个彼此层压的片的装置对于本领域技术人员来说是容易想到的，且其技术效果可预期。因此，在对比文件 1 的基础上结合对比文件 2 和本领域的常规技术手段获得权利要求 26 要求保护的技术方案对于本领域技术人员来说是显而易见的，即权利要求 26 不具有突出的实质性特点和显著的进步，不具备专利法第 22 条第 3 款规定的创造性。

16、权利要求 27 要求保护一种产品，其通过从有尾目动物分离有尾目动物 ECM、使所述 ECM 脱细胞化并对脱细胞化的 ECM 灭菌来制备。参见前述意见可知，所述的分离的脱细胞化的有尾目动物 ECM 是没有创造性的。同时，对比文件 1 还公开了所述的生物支架为无菌的，并将其应用于医学治疗移植中。基于此，本领域技术人员有动机将分离的脱细胞化的有尾目动物 ECM 制备移植产品；而选择采用灭菌的方法来制备得到无菌产品也是无菌操作中的常规手段。因此，在对比文件 1 的基础上结合本领域的常规技术手段获得权利要求 27 要求保护的技术方案对于本领域技术人员来说是显而易见的，即权利要求 27 不具有突出的实质性特点和显著的进步，不具备专利法第 22 条第 3 款规定的创造性。

17、权利要求 28 要求保护一种制备生物材料的方法。参见权利要求 1 的意见评述可知，对比文件 1 已经公开了一种制备生物支架（生物材料）的方法，其包括了从动物资源中获得组织样品，且所述的组织样品包含有胞外基质；是缩水样品脱细胞化从而获得所述材料，并减少所述样品的免疫原性。

经分析，权利 28 与对比文件 1 相比其区别特征为：本申请所述的生物材料确定了动物组织的来源为有尾目动物。基于所述区别特征，可以确定本发明实际解决的技术问题是确定一种合适的动物组织来源。

针对该区别特征，参见权利要求 1 的评述，在对比文件 3 和对比文件 4 的启示下，本领域技术人员有动机选择蝾螈（一种有尾目两栖类动物）作为组织的来源，且其没有产生预料不到的技术效果。

因此，在对比文件 1 的基础上结合对比文件 3-4 获得权利要求 28 要求保护的技术方案对于本领域技术人员来说是显而易见的，即权利要求 28 不具有突出的实质性特点和显著的进步，不具备专利法第 22 条第 3 款规定的创造性。

18、权利要求 29-33 是权利要求 28 或在先权利要求的从属权利要求，其进一步限定了所述方法。参见前述意见可知，对比文件 1 已经公开了生物支架可以通过保留其主要的原始生物力学完整性以制备得到，基于此，本领域技术人员容易想到需要保证期结构和功能的完整性。同时，根据记载，权利要求 30-33 的附加技术特征与权利要求 9、13、14、15 的附加技术特征或部分附加技术特征相同，即已经为对比文件 1-2 公开或为在公开内容之上所容易进一步所想到的内容。因此，在其引用的权利要求都不具备创造性的情况下，权利要求 29-33 也不具有突出的实质性特点和显著的进步，不具备专利法第 22 条第 3 款规定的创造性。

二、 权利要求 25 不清楚，不符合专利法第 26 条第 4 款的规定

RegenX Science Exhibit 1004
Page 1185
EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1185

权利要求 25 要求保护经分离的脱细胞化的有尾目动物 ECM 或来源于所述分离的脱细胞化的有尾目动物 ECM 的有尾目动物级分条件化的组织培养基，其特征部分仅仅对 ECM 进行了限定了，没有限定所述培养基。权利要求 25 没有与该主题相适应的技术特征，即没有对该培养基的具体成分、组成等性质做出限定，根据该记载方式本领域技术人员无法确定其要求保护的培养基究竟是一种什么样的培养基。同时，本申请说明书相关段落对于该培养基的解释为“许多液体组织培养基是市售的并且是本领域普通技术人员公知的”（参见说明书第[0038]段），因而说明书中也没有清楚的记载究竟是哪一种市售或公知的培养基。因此，根据权利要求 25 的记载，本领域技术人员无法确定权利要求 25 要求保护的范围，即权利要求 25 不清楚，不符合专利法第 26 条第 4 款的规定。

即使认为所述培养基为其说明书中记载的“市售的并且是本领域普通技术人员公知的”，那么根据该记载其也已经是现有技术公开的培养基，或将不具备创造性或新颖性。

三、申请人的意见陈述不具有说服力。

申请人认为：（1）本申请相对于对比文件 1 实际解决的技术问题是如何获得具有特别有利的形式从而适用于异种移植的生物材料，而对比文件 1 和其他现有技术都没有给出该启示选择有尾目动物；（2）对比文件 1 也只是泛泛提及了两栖类动物，而两栖类动物含有三种目，没有启示进一步选择两栖类；（3）对比文件 1 给出了相反教导去除外来物质增加孔径，而本申请要求保护的 ECM 还含有生长因子谱、结缔组织基质成分等胞外基质。

对此，审查员认为：（1）本申请用以证明其发明具有有益效果的实施例中，仅仅描述了采用蝾螈组织获得的 ECM 具有良好的生物相容性、与人羊膜组织的结构相似性以及制备方法，没有证据表明采用该动物来源的组织所得的 ECM 优于其他动物组织制备的 ECM，即没有证据表明有尾目动物来源的 ECM 具有申请人所声称的特别有利的性质。因而，根据区别技术特征确定的实际解决的技术问题是选择一种合适的 ECM 动物组织来源。

（2）参见前述意见可知，对比文件 1 和 3 都公开了所示胞外基质材料可以来源于两栖类动物，而两栖类动物中只包含三个目的动物，通过有限的试验即可确定。此外，通知书引入新的对比文件 4，该对比文件明确了选择蝾螈作为再生医学的材料。基于这些信息，本领域技术人员容易进一步选择蝾螈（即有尾目动物）作为 ECM 的组织来源，且说明书中并没有数据表明该来源的 ECM 的促再生能力优于其他动物来源 ECM，即没有产生预料不到的技术效果。

（3）通知书引用了新的对比文件——对比文件 3，对比文件 3 中公开了所述基质包含完整的基质结构，且公开了以下内容（参见说明书第 2 页第 5 段）：“本发明提供细胞的以及完整的和提取的细胞外基质材料，它用作支架以支持细胞的生长、发育和分化并支持或实现细胞的形态学改变。组织材料优选来源于肌肉组织

RegenX Science Exhibit 1004
Page 1186
EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1186

并包括包含基底膜组分的制剂。这些组分包括一种或多种下列成分，所述成分例如但不限于，层粘连蛋白、胶原蛋白 I、胶原蛋白 IV、触觉蛋白/巢蛋白、硫酸乙酰肝素蛋白聚糖以及一种或多种的细胞因子或生长因子，所述细胞因子或生长因子例如但不限于，表皮生长因子(EGF)、碱性成纤维细胞生长因子(bFGF)、 神经生长因子(NGF)、血小板来源的生长因子(PDGF)、胰岛素样生长因子-1(IGF-1)、转化生长因子-β(TGF-β)、血管内皮生长因 子(VEGF)和肿瘤坏死因子-α(TNF-α)”。由此可知，对比文件 3 中公开的 ECM 包含完整的胞外基质以及相应的生长因子谱、肌肉基底膜成分等，即公开了需要保持胞外基质营养物质。因而，该特征没有使得本发明具备创造性。

综上所述，申请人的意见陈述不具有说服力。

基于上述理由，本申请不能被授予专利权，而且本申请的说明书中也没有记载其它任何可获得专利权的实质性内容，因而即使对申请文件进行修改，本申请也不具备被授予专利权的前景。如果申请人不能在本通知书规定的答复期限内提出具有说服力的理由，本申请将视为撤回或被驳回。申请人对申请文件的修改应当符合专利法第 33 条的规定，不得超出原说明书和权利要求书记载的范围。申请人提交的修改文件应当符合专利审查指南第二部分第八章第 5.2.4.1 节的要求，提交重新打印的替换页和在原文复制件上作出修改的对照页，或者提交重新打印的替换页和修改对照表。

审查员姓名:全弘扬　　审查员代码:559309

国 家 知 识 产 权 局

检 索 报 告

申请号：2014800133590	申请日：20140109	补充检索
申请人：ISE 专业检测与咨询服务有限公司	最早的优先权日：20130109	
权利要求项数：33	说明书段数：125+6	
审查员确定的 IPC 分类号：A61K38/39		
检索记录信息：CN1969039A: 6 CNABS, (有尾目 or 两栖 or 蠑螈) and (ECM OR 胞外基质 or 脱细胞)； CN101337089A: 21 CNTXT, 蠑螈 and 组织 and (再生 s 材料)。		

相 关 专 利 文 献

类型	国别以及代码[11]给出的文献号	代码[43]或[45]给出的日期	IPC 分类号	相关的段落和／或图号	涉及的权利要求
Y	CN1969039A	20070523	C12N5/00	1-24,26-33	权利要求 4、说明书第 2 页第 5 段，第 3 页第 2-3 段，第 5 页第 2-3 段
Y	CN101337089A	20090107	A61L27/38	1-24,26-33	说明书第 4 页第 3 段

RegenX Science Exhibit 1004
Page 1188

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1188

相 关 非 专 利 文 献					
类型	书名（包括版本号和卷号）	出版日期	作者姓名和出版者名称	相关页数	涉及的权利要求
类型	期刊或文摘名称 （包括卷号和期号）	发行日期	作者姓名和文章标题	相关页数	涉及的权利要求

表格填写说明事项：

1．审查员实际检索领域的 IPC 分类号应当填写到大组和 / 或小组所在的分类位置。

2．期刊或其它定期出版物的名称可以使用符合一般公认的国际惯例的缩写名称。

3．相关文件的类型说明：

X：一篇文件影响新颖性或创造性；

Y：与本报告中的另外的 Y 类文件组合而影响创造性；

A：背景技术文件；

R：任何单位或个人在申请日向专利局提交的、属于同样的发明创造的专利或专利申请文件。

P：中间文件，其公开日在申请的申请日与所要求的优先权日之间的文件；

E：抵触申请。

审 查 员：全弘扬　　审查部门：专利审查协作四川中心医药生物发明审查部

2019 年 05 月 14 日

Electronic Patent Application Fee Transmittal

Application Number:	14759895
Filing Date:	08-Jul-2015
Title of Invention:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
First Named Inventor/Applicant Name:	Ryanne Early
Filer:	Sima Singadia Kulkarni/Stephanie De marco
Attorney Docket Number:	SYG-19-1252/426329-002

Filed as Small Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 3 months with $0 paid	2253	1	700	700
Miscellaneous:				
		Total in USD ($)		**700**

Electronic Acknowledgement Receipt

EFS ID:	36478065
Application Number:	14759895
International Application Number:	
Confirmation Number:	3358
Title of Invention:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
First Named Inventor/Applicant Name:	Ryanne Early
Customer Number:	35811
Filer:	Sima Singadia Kulkarni/Stephanie De marco
Filer Authorized By:	Sima Singadia Kulkarni
Attorney Docket Number:	SYG-19-1252/426329-002
Receipt Date:	02-JUL-2019
Filing Date:	08-JUL-2015
Time Stamp:	14:08:32
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$700
RAM confirmation Number	070319INTEFSW00000420502719
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Extension of Time	SYG_19_1252US_AMTL.pdf	10500 b9aceab19cf13a4e3ccc06341dfd5343a1fb7a28	no	1
Warnings:					
Information:					
2		SYG_19_1252US_Response.pdf	48209 39f8840496cbf48f8b66bea4ccf97fd9fc8466d8	yes	11

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment/Req. Reconsideration-After Non-Final Reject	1	1
	Claims	2	8
	Applicant Arguments/Remarks Made in an Amendment	9	11

Warnings:					
Information:					
3	Transmittal Letter	SYG_19_1252US_SIDS.pdf	4750 039fc0f9de2640c652147f769a45aff573a5986e	no	1
Warnings:					
Information:					
4	Information Disclosure Statement (IDS) Form (SB08)	SYG_19_1252US_1449.pdf	8863 12fb2d8be42f4086431e7dcb43ee264386e703ac	no	1
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					

5	Foreign Reference	CN101337089A.pdf	493381 3ce64572729cb36f29ed8aa46e233b3094be28e2	no	8
Warnings:					
Information:					
6	Foreign Reference	CN1969039A.pdf	1837414 9341b5ca085390032a0cc1fef7236b67cd48f8d1	no	35
Warnings:					
Information:					
7	Non Patent Literature	CN_OA.pdf	4883172 f61c483644bbf09f89c7b6e38df1dc1d90049f1f	no	24
Warnings:					
The page size in the PDF is too large. The pages should be 8.5 x 11 or A4. If this PDF is submitted, the pages will be resized upon entry into the Image File Wrapper and may affect subsequent processing					
Information:					
8	Fee Worksheet (SB06)	fee-info.pdf	30998 4459a4bd5e8bfdb8c4de031cb80c92c945648fe6	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	7317287		

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Attorney Docket No.: SYG-19-1252US/426329-002

In re Application of ISE Professional Testing & Consulting Services, Inc.
Serial No.: 14/759,895
Filed: July 8, 2015
For: DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

A response to the outstanding official action in the above-identified application is enclosed.

☐ Small entity status of this application under 37 CFR §1.9 and §1.27 has been established.

☒ This is a Petition for an Extension of Time for the period noted below, as well as for any additional period necessary to render this submission timely.

☐ No additional fee is required.

					SMALL ENTITY		OTHER THAN SMALL ENTITY	
TIME EXTENSION PETITION FEE			No. of month(s): 3		$700.00		$0.00	
Subtract time extension fee previously paid			No. of month(s): 0		($0.00)		($0.00)	
TOTAL EXTENSION FEE DUE					$700.00		$0.00	
CLAIM FEE	CLAIM(S) REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	EXTRA CLAIM(S) PRESENT	RATE	ADD'L CLAIM FEE	RATE	ADD'L CLAIM FEE
TOTAL	45	MINUS	62	= 0	x 50=	$0.00	x 100=	$
INDEPENDENT	3	MINUS	11	= 0	x 230=	$0.00	x 460=	$
☐	FIRST PRESENTATION OF MULTIPLE CLAIM(S)				+ 410=	$	+ 820=	$
				TOTAL ADDITIONAL CLAIM FEE DUE		$0.00		$
								TOTAL FEE DUE $700.00

☒ Please charge Deposit Account No. 50-2719 in the amount of $700.00.

☒ The Commissioner is authorized to charge payment of the following fees associated with this communication or credit any overpayment to Deposit Account No. 50-2719.

☒ Any filing fees under 37 CFR §1.16 for the presentation of extra claims.

☒ Any patent application processing fees under 37 CFR §1.17.

Respectfully submitted,

/sima singadia kulkarni/

Date: July 2, 2019

Sima Singadia Kulkarni, Reg. No. 43,732

SSK/sh
(215)656-2478

EAST\167746869.1

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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/759,895	Filing Date 07/08/2015	☐ To be Mailed

ENTITY: ☐ LARGE ☑ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $40 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $210 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	06/28/2019	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 35	Minus	** 59	= 0		x $50 =	0
	Independent (37 CFR 1.16(h))	* 11	Minus	*** 11	= 0		x $230 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	SLIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/DEIDRE C KING-WOODWARD/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Dear PAIR Customer:

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UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 35811 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

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Application	Document	Mailroom Date	Attorney Docket No.
14759895	INTV.SUM.APP	07/03/2019	SYG-19-1252/426329-002
	EXIN	07/03/2019	SYG-19-1252/426329-002

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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/759,895	Filing Date 07/08/2015	☐ To be Mailed

ENTITY: ☐ LARGE ☑ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $40 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $210 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	07/02/2019	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 45	Minus	** 62	= 0		x $50 =	0
	Independent (37 CFR 1.16(h))	* 3	Minus	*** 11	= 0		x $230 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/CAROL A BARNES/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Application Data Sheet

Inventor Information

Inventor Number::	1
Name Prefix::	Ms.
Given Name::	Ryanne
Family Name::	Early
City of Residence::	Fleming Island
State or Province of Residence::	FL
Country of Residence::	US
Street of mailing address::	1909 Hickory Trace Dr.
City of mailing address::	Fleming Island
State or Province of mailing address::	FL
Postal or Zip Code of mailing address::	32003

Correspondence Information

Correspondence Customer Number::	~~23628~~ 035811

Application Information

Application Type::	Regular
Subject Matter::	Utility
CD-ROM or CD-R?::	None
Sequence submission?::	None
Computer Readable Form (CRF)?::	No

Title::	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
Attorney Docket Number::	S1860.70000US01
Request for Early Publication?::	No
Request for Non-Publication?::	No
Total Drawing Sheets::	6
Small Entity?::	Yes
Petition included?::	No
Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2::	No
Authorization to Permit Access to the Instant Application by the Participating Offices::	No
This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013::	Yes

Representative Information

Representative Customer Number::	23628

RegenX Science Exhibit 1004
Page 1200

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1200

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This Application	National Stage of	PCT/US2014/010890	01/09/14
PCT/US2014/010890	An application claiming the benefit under 35 USC 119(e)	61/750555	01/09/13

Foreign Priority Information

Applicant Information

Applicant Number::	1
Applicant Type::	Assignee
Organization Name::	ISE Professional Testing & Consulting Services, Inc.
Street of mailing address::	2007 NW 5th Street
City of mailing address::	Gainesville
State or Province of mailing address::	FL
Postal or Zip Code of mailing address::	32603

Assignee Information Including Non-Applicant Assignee Information

Assignee Number::	1
Organization Name::	ISE Professional Testing & Consulting Services, Inc.
Street of mailing address::	2007 NW 5th Street
City of mailing address::	Gainesville

State or Province of mailing address::	FL
Postal or Zip Code of mailing address::	32603

RegenX Science Exhibit 1004
Page 1202

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1202

Signature:

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications.			
Signature	/sima singadia kulkarni/	Date	2019-07-10
Name	Sima Singadia Kulkarni	Registration Number	43,732

Electronic Acknowledgement Receipt

EFS ID:	36545721
Application Number:	14759895
International Application Number:	
Confirmation Number:	3358
Title of Invention:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
First Named Inventor/Applicant Name:	Ryanne Early
Customer Number:	35811
Filer:	Sima Singadia Kulkarni/Stephanie De marco
Filer Authorized By:	Sima Singadia Kulkarni
Attorney Docket Number:	SYG-19-1252/426329-002
Receipt Date:	10-JUL-2019
Filing Date:	08-JUL-2015
Time Stamp:	15:29:54
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Oath or Declaration filed	SYG_19_1252US_Dec.pdf	283578 a6838dee5473d685e758f6b26c7c547400ae8de9	no	4

Warnings:

Information:					
2	Application Data Sheet	SYG_19_1252US_SADS.pdf	111865 86c9facee7875ba0d7778bcbfc02a2e0dc228a39	no	5
Warnings:					
Information:					
This is not an USPTO supplied ADS fillable form					
			Total Files Size (in bytes):	395443	

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Art Unit	: 1651	**Customer No. 035811**
Examiner	: Davis, Ruth A.	
Appl. No.	: 14/759,895	Docket No.: SYG-19-1252US/ 426329-002
Appl. Filed	: July 8, 2015	
First Named Inventor	: Ryanne Early	Confirmation No.: 3358
Title	: DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE	Dated: July 10, 2019

TRANSMITTAL LETTER

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Applicant acknowledges receipt of the Notice of Acceptance of Application under 35 U.S.C 371 and 37 CFR 1.495 dated August 31, 2015. Applicant submits an executed Declaration and requests that it be formally entered in the official file.

The Commissioner is authorized to charge any insufficiency or credit any overpayment to Deposit Account No. 50-2719.

Respectfully submitted,

/sima singadia kulkarni/

Sima Singadia Kulkarni
Reg. No. 43,732

SSK/sh
(215) 656-2478

EAST\167866028.2

Doc Code: Oath
Document Description: Oath or declaration filed

PTO/AIA/08 (11-15)
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DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

☐ Declaration Submitted With Initial Filing OR ☒ Declaration Submitted After Initial Filing (surcharge (37 CFR 1.16(f)) required)

Attorney Docket Number	RE-US1
First Named Inventor	RYANNE EARLY
COMPLETE IF KNOWN	
Application Number	14/759895
Filing Date	01/09/2014
Art Unit	1651
Examiner Name	RUTH DAVIS A.

DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE

(Title of the Invention)

As a below named inventor, I hereby declare that:

This declaration is directed to:

☐ The attached application,

OR

☒ United States Application Number or PCT International application number 14/759895

filed on 01/09/2014.

The above-identified application was made or authorized to be made by me.

I believe I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

Direct all correspondence to: ☐ The address associated with Customer Number: _____ OR ☒ Correspondence address below

Name
GERARD H. BENCEN, ESQ.

Address
10 E. UNIVERSITY AVENUE

City	State	Zip
GAINESVILLE	FLORIDA	32608

Country	Telephone	Email
UNITED STATES	352-214-0385	GERARD@PATENT-ARTS.COM

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 21 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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PTO/AIA/08 (11-15)
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DECLARATION — Utility or Design Patent Application

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available. Petitioner/applicant is advised that documents which form the record of a patent application (such as the PTO/SB/01) are placed into the Privacy Act system of records DEPARTMENT OF COMMERCE, COMMERCE-PAT-7, System name: *Patent Application Files*. Documents not retained in an application file (such as the PTO-2038) are placed into the Privacy Act system of COMMERCE/PAT-TM-10, System name: *Deposit Accounts and Electronic Funds Transfer Profiles*.

LEGAL NAME OF SOLE OR FIRST INVENTOR:

(*E.g.*, Given Name (first and middle if any) and Family Name or Surname)

RYANNE EARLY

Inventor's Signature	Date (Optional)
	5/7/19

Residence: City	State	Country
Dallas	TX	USA

Mailing Address

5614 Swiss Ave

City	State	Zip	Country
Dallas	TX	75214	USA

☐ Additional inventors are being named on the _____ Supplemental sheet(s) PTO/AIA/10 attached hereto

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

UNITED STATES PATENT AND TRADEMARK OFFICE

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United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/759,895	07/08/2015	Ryanne Early	SYG-19-1252/426329-002	3358

35811 7590 08/27/2019

IP GROUP OF DLA PIPER LLP (US)
ONE LIBERTY PLACE
1650 MARKET ST, SUITE 5000
PHILADELPHIA, PA 19103

EXAMINER	
DAVIS, RUTH A	

ART UNIT	PAPER NUMBER
1651	

NOTIFICATION DATE	DELIVERY MODE
08/27/2019	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

pto.phil@dlapiper.com

Office Action Summary	**Application No.** 14/759,895	**Applicant(s)** Early, Ryanne	
	Examiner RUTH A DAVIS	**Art Unit** 1651	**AIA (FITF) Status** Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 7/2/2019.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☑ This action is **FINAL.** 2b) ☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 5,20-22,36-38,40-47,49-61 and 66-80 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☑ Claim(s) 5,20-22,36-38,40-47,49-61,66-67,70,72 and 80 is/are allowed.

7) ☑ Claim(s) 68-69,71 and 73-79 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

DETAILED ACTION

Applicant's amendment and reply filed July 2, 2019 have been received and entered into the case. Claims 1 – 4, 6 – 19, 23 – 25, 27 – 35, 62 – 65 are canceled. Claims 66 – 79 are added.

Claims 5, 20 – 22, 26, 36 – 38, 40 – 61 and 66 – 79 are pending and have been considered on the merits. All arguments and the affidavit have been fully considered.

Election/Restrictions

Claims 5, 20 – 22, 26, 36 – 38, 40 – 61 are allowable. The restriction requirement between the product and methods of making the product, as set forth in the Office action mailed on September 9, 2016, has been reconsidered in view of the allowability of claims to the elected invention pursuant to MPEP § 821.04(a). **The restriction requirement is hereby withdrawn as to any claim that requires all the limitations of an allowable claim.** Specifically, the restriction requirement of September 9, 2019 is withdrawn. Claims 66 – 80 are directed to methods of making and using the allowable product are no longer withdrawn from consideration because the claim(s) requires all the limitations of an allowable claim.

In view of the above noted withdrawal of the restriction requirement, applicant is advised that if any claim presented in a continuation or divisional application is anticipated by, or includes all the limitations of, a claim that is allowable in the present application, such claim may

be subject to provisional statutory and/or nonstatutory double patenting rejections over the claims of the instant application.

Once a restriction requirement is withdrawn, the provisions of 35 U.S.C. 121 are no longer applicable. See In re Ziegler, 443 F.2d 1211, 1215, 170 USPQ 129, 131-32 (CCPA 1971). See also MPEP § 804.01.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of the first paragraph of pre-AIA 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 68 – 69, 73 – 79 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention.

Claim 68 and 73 recite a "method of using" a tissue graft or implant, however these claims and those depending therefrom are considered genus claims that encompass a wide

variety of uses. The specification fails to set forth a representative number of examples in order to reasonably verify possession of such a potentially enormous number of uses in an undefined subject with an undefined need, an undefined condition, and an undefined "adaptation" to the needs of said patient. In addition, claim 75 recites the method wherein the implant is infused with a variety of classes of compounds and molecules.

The MPEP states that written description for a genus can be achieved by a representative number of species within a broad generic. It is unquestionable that the claims are broad generics, with respect to all uses, in all subjects that might result from any need, any adaptation of the tissue grafts and any molecule or compound encompassed by claim 75. The instant disclosure fails to identify a single use, in any specific recipient in need, with any adaptation of the grafts or implants, or with any other compound or molecule infused therein. The possible variations of "uses" are limitless with potentially millions of types of uses, treatments, subjects and adaptations.

In addition, the claims appear to merely recite an undisclosed problem to be solved without any active step to accomplish the method. It is noted that without reciting the particular structure, materials or steps that accomplish the function or achieve the result, all means or methods of resolving the problem may be encompassed by the claim. Unlimited functional claim limitations that extend to all means or methods of resolving a problem is not adequately supported by the instant specification. It is noted that a single claim reciting a functional outcome covering every conceivable means for achieving the stated result fails to satisfy the written description requirement because the specification discloses only those means known to the inventor, and is not commensurate in scope with the claim. (MPEP 2173.05(g)).

The purpose of the written description requirement is to ensure that the invention had possession, as of the filing date of the application, of the specific subject matter later claimed by him or her. A patent specification must describe an invention and do so in sufficient detail that one skilled in the art can clearly conclude that the inventor invented the claimed invention. Thus, an applicant complies with the written description requirement "by describing the invention, with all its claimed limitations" and by using "such descriptive means as words, structures, figures, diagrams, formulas, etc., that set forth the claimed invention." The specification lacks sufficient variety of species of uses, recipients or adaptations to reflect this variance in the genus since the specification does not provide any examples of such a genus of "uses". Accordingly, the specification fails to provide adequate written description for the genus of "method of use" and does not reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed had possession of the entire scope of the claimed invention. Moreover, the specification neither describes the complete structure of a representative number of species, nor describes a representative number of species in terms of partial structure and relevant identifying characteristics. Absent of such teachings and guidance as to the structure and function of these uses or adaptations of the grafts or implants, or any description of a recipient in need or "sufficient amounts" that deliver a particular response to an undefined recipient, the specification does not describe the claimed in such full, clear, concise and exact terms so as to indicate that Applicant had possession of these methods of use at the time of filing of the present application.

Thus, the written description requirement has not been satisfied.

Claims 68, 73 and their dependents are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The

claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention.

Claims 68 and 73 recite "a sufficient amount" of a xenograft and "a form adapted to" the need of a recipient. The specification fails to describe these phrases as originally filed. This is a new matter rejection.

> The following is a quotation of 35 U.S.C. 112(b):
> (b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.
>
> The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:
> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 68 – 69, 71 – 79 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

Claims 68, 73 and their dependents are drawn to a method of using a tissue graft, however are rendered vague and indefinite for reciting "method of using" because the phrase is not adequately defined by the claims or specification such that one in the art would know the scope of the claims. The claims are further indefinite for reciting "recipient in need thereof", "a sufficient amount", "form adapted to" and "need of said recipient" because the phrases have not been adequately defined by the claim language or specification. It is unclear what active steps

must be practiced, to whom, and in what capacity because neither the claims nor the define any treating population, any need that a recipient might have to determine any sufficient amount, or any form that may be adapted such that one would know the scope of the claim.

In claims 69, 71 and 74, the term "encapsulating" is grammatically confusing, rendering the claims indefinite because it is unclear if the material is being encapsulated, or acting to encapsulate something else.

In claims 69, 71 and 74, line 3, it is unclear if the recited decellularized ECM is a second and separate ECM, or it if is the same as that recited in the claims on which they depend. It is recommended to insert "the" before the recitation of "isolated" in line 3.

In claim 75, it is unclear if "an agent xenogenic to a Urodele" is specific to the Urodele ECM, or if the term is intended to relate to something that is xenogenic to another, generic Urodele.

Claim Rejections - 35 USC § 103

Previous rejections under 35 U.S.C. 103 are withdrawn due to amendment.

Allowable Subject Matter

Claims 5, 20 – 22, 36 – 38, 40 – 47, 49 – 61, 66 – 67, 70, 72, 80 are allowed.

Conclusion

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a), 821.04. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to RUTH A DAVIS whose telephone number is (571)272-0915. The examiner can normally be reached on Monday - Friday (8am - 4pm).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Renee Claytor can be reached on 571-272-8394. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/RUTH A DAVIS/
Primary Examiner, Art Unit 1651

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14/759,895	Early, Ryanne
	Examiner	Art Unit
	RUTH A DAVIS	1651

CPC - Searched*		
Symbol	**Date**	**Examiner**
A61K 35/12, 65	08/15/2019	rad
A61K 9/70	08/15/2019	rad
A61L 37/3687, 58,60,54,362	08/15/2019	rad
C12N 5/0625	08/15/2019	rad

CPC Combination Sets - Searched*		
Symbol	**Date**	**Examiner**

US Classification - Searched*			
Class	**Subclass**	**Date**	**Examiner**

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	**Date**	**Examiner**
east, pubmed, google patent	08/15/2019	rad
A61K 35/65	08/15/2019	rad

Interference Search			
US Class/CPC Symbol	**US Subclass/CPC Group**	**Date**	**Examiner**
A61L	27/54,58,60,362,3687	08/15/2019	rad
A61K	9/70	08/15/2019	rad
A61K	35/12	08/15/2019	rad
C12N	5/0625	08/15/2019	rad

	/RUTH A DAVIS/ Primary Examiner, Art Unit 1651

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14/759,895	Early, Ryanne
	Examiner	**Art Unit**
	RUTH A DAVIS	1651

	/RUTH A DAVIS/ Primary Examiner, Art Unit 1651

Form PTO-1449 US DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTY. DOCKET NO. SYG-19-1252US/426329-002	SERIAL NO. 14/759,895
	APPLICANT ISE Professional Testing & Consulting Services, Inc.	
LIST OF PUBLICATIONS CITED BY APPLICANT *(Use several sheets if necessary)*	FILING DATE July 8, 2015	GROUP 1651

US PATENT DOCUMENTS

EXAMINER INITIAL*		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
	AA	2006/153797 A1 (corr. to CN '039)	07/13/06	Bortolotto et al.			
	AB						
	AC						
	AD						
	AE						
	AF						
	AG						
	AH						
	AI						
	AJ						
	AK						

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO
	AL	1969039 A (corr. to US '797)	05/23/07	CN			X	
	AM	101337089 A	01/07/09	CN			Abstract	
	AN							
	AO							
	AP							

OTHER PUBLICATIONS (Including Author, Title, Date, Pertinent Pages, Etc.)

	AR	Second Office Action from counterpart Chinese Patent Appln. No. 201480013359.0 dated May 22, 2019, along with its English Translation.
	AS	
	AT	

EXAMINER /RUTH A DAVIS/	DATE CONSIDERED 08/08/2019

*EXAMINER: Initial if publication considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

EAST\47689080.1
EAST\167753423.1

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /R.A.D/

To: pto.phil@dlapiper.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
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Aug 27, 2019 03:25:42 AM

Dear PAIR Customer:

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Application	Document	Mailroom Date	Attorney Docket No.
14759895	CTFR	08/27/2019	SYG-19-1252/426329-002
	1449	08/27/2019	SYG-19-1252/426329-002

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Thank you for prompt attention to this notice,

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PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

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POWER OF ATTORNEY TO PROSECUTE APPLICATIONS BEFORE THE USPTO

I hereby revoke all previous powers of attorney given in the application identified in the attached statement under 37 CFR 3.73(c).

I hereby appoint:

[X] Practitioners associated with Customer Number: 27530

OR

[ ] Practitioner(s) named below (if more than ten patent practitioners are to be named, then a customer number must be used):

Name	Registration Number	Name	Registration Number

As attorney(s) or agent(s) to represent the undersigned before the United States Patent and Trademark Office (USPTO) in connection with any and all patent applications assigned only to the undersigned according to the USPTO assignment records or assignment documents attached to this form in accordance with 37 CFR 3.73(c).

Please change the correspondence address for the application identified in the attached statement under 37 CFR 3.73(c) to:

[X] The address associated with Customer Number: 27530

OR

[ ]

Firm or individual name		
Address		
City	State	Zip
Country		
Telephone	Email	

Assignee name and address: ISE Professional Testing & Consulting Services, Inc.
2785 S. Bay St. Suite G
Eustis, FL 32726

A copy of this form, together with a statement under 37 CFR 3.73(c) (Form PTO/AIA/96 or equivalent) is required to be filed in each application in which this form is used. The statement under 37 CFR 3.73(c) may be completed by one of the practitioners appointed in this form, and must identify the application in which this Power of Attorney is to be filed.

SIGNATURE of Assignee of Record

The individual whose signature and title is supplied below is authorized to act on behalf of the assignee.

Signature	Date September 13, 2019
Name Ryanne Early	Telephone 352-474-0823
Title President, ISE Professional Testing & Consulting Services, Inc.	

This collection of information is required by 37 CFR 1.31, 1.32, and 1.33. The information is required to obtain or retain a benefit by the public, which is to update (and by the USPTO to process) the file of a patent or reexamination proceeding. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 18 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

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1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

PTO/AIA/96 (08-12)
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STATEMENT UNDER 37 CFR 3.73(c)

Applicant/Patent Owner: ISE Professional Testing & Consulting Services, Inc.

Application No./Patent No.: 14/759,895 Filed/Issue Date: July 8, 2015

Titled: DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE

ISE Professional Testing & Consulting Services, Inc., a corporation

(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that, for the patent application/patent identified above, it is (choose **one** of options 1, 2, 3 or 4 below):

1. ☑ The assignee of the entire right, title, and interest.

2. ☐ An assignee of less than the entire right, title, and interest (check applicable box):

   ☐ The extent (by percentage) of its ownership interest is _____%. Additional Statement(s) by the owners holding the balance of the interest must be submitted to account for 100% of the ownership interest.

   ☐ There are unspecified percentages of ownership. The other parties, including inventors, who together own the entire right, title and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

3. ☐ The assignee of an undivided interest in the entirety (a complete assignment from one of the joint inventors was made). The other parties, including inventors, who together own the entire right, title, and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

4. ☐ The recipient, via a court proceeding or the like (*e.g.*, bankruptcy, probate), of an undivided interest in the entirety (a complete transfer of ownership interest was made). The certified document(s) showing the transfer is attached.

The interest identified in option 1, 2 or 3 above (not option 4) is evidenced by either (choose **one** of options A or B below):

A. ☑ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel 049715, Frame 0325, or for which a copy thereof is attached.

B. ☐ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: _____ To: _____

   The document was recorded in the United States Patent and Trademark Office at

   Reel _____, Frame _____, or for which a copy thereof is attached.

2. From: _____ To: _____

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This collection of information is required by 37 CFR 3.73(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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STATEMENT UNDER 37 CFR 3.73(c)

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☐ Additional documents in the chain of title are listed on a supplemental sheet(s).

☑ As required by 37 CFR 3.73(c)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (i.e., a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

/Lloyd G. Farr/	September 27, 2019
Signature	Date
Lloyd G. Farr	38,446
Printed or Typed Name	Title or Registration Number

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2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
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9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Corrected Application Data Sheet

Application Information

Application Number::	14/759,895
Filing Date::	07/08/2015
Application Type::	Nonprovisional
Subject Matter::	Utility
Title::	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
Attorney Docket Number::	~~S1860.70000US01~~ 040035/09002

Correspondence Information

Customer Number	~~035811~~ 27530
Email Address	ip@nelsonmullins.com

Representative Information

Representative Customer Number	~~23628~~ 27530

Applicant Information

Applicant Number::	1
Applicant Type::	Assignee
Organization Name::	ISE Professional Testing & Consulting Services, Inc.
Street of mailing address::	~~2007 NW 5th Street~~ 2785 S. Bay St. Suite G

RegenX Science Exhibit 1004
Page 1228

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1228

City of mailing address::	~~Gainesville~~ Eustis
State or Province of mailing address::	FL
Country of mailing address::	US
Postal or Zip Code of mailing address::	~~32603~~ 32726

Assignee Information Including Non-Applicant Assignee Information

Applicant Number::	1
Applicant Type::	Assignee
Organization Name::	ISE Professional Testing & Consulting Services, Inc.
Street of mailing address::	~~2007 NW 5th Street~~ 2785 S. Bay St. Suite G
City of mailing address::	~~Gainesville~~ Eustis
State or Province of mailing address::	FL
Country of mailing address::	US
Postal or Zip Code of mailing address::	~~32603~~ 32726

RegenX Science Exhibit 1004
Page 1229

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1229

Signature:

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).** This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants. See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	/Lloyd G. Farr/	Date (YYYY-MM-DD)	2019-09-27
Name	Lloyd G. Farr	Registration Number	38,446

RegenX Science Exhibit 1004
Page 1230

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1230

Electronic Acknowledgement Receipt

EFS ID:	37301912
Application Number:	14759895
International Application Number:	
Confirmation Number:	3358
Title of Invention:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
First Named Inventor/Applicant Name:	Ryanne Early
Customer Number:	35811
Filer:	Lloyd G. Farr/Jennifer Falcone
Filer Authorized By:	Lloyd G. Farr
Attorney Docket Number:	SYG-19-1252US/426329-002
Receipt Date:	27-SEP-2019
Filing Date:	08-JUL-2015
Time Stamp:	14:48:07
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Power of Attorney	0430035_09002_POA.pdf	278161 04f1cf4b8da32a79e1dbbc35d3771cc346e8a1ce	no	2

Warnings:

Information:					
2	Assignee showing of ownership per 37 CFR 3.73	0430035_09002_373C.pdf	134984 fd7682b43daa8a45db0ac83d1be34a83ce7e8cf0	no	3
Warnings:					
Information:					
3	Application Data Sheet	040035_09002_FSADS.pdf	71780 66a948fd711096410370cebf47074f133cae30a4	no	3
Warnings:					
Information:					
This is not an USPTO supplied ADS fillable form					
		Total Files Size (in bytes):	484925		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/759,895	07/08/2015	Ryanne Early	040035/09002

CONFIRMATION NO. 3358

27530
Nelson Mullins Riley & Scarborough LLP
IP Department
One Wells Fargo Center
301 South College Street, 23rd Floor
Charlotte, NC 28202

POA ACCEPTANCE LETTER

OC000000111578809

Date Mailed: 10/02/2019

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 09/27/2019.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/hsarwari/

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/759,895	07/08/2015	Ryanne Early	SYG-19-1252US/426329-002

CONFIRMATION NO. 3358

35811
IP GROUP OF DLA PIPER LLP (US)
ONE LIBERTY PLACE
1650 MARKET ST, SUITE 5000
PHILADELPHIA, PA 19103

POWER OF ATTORNEY NOTICE

OC000000111578799

Date Mailed: 10/02/2019

NOTICE REGARDING CHANGE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 09/27/2019.

- The Power of Attorney to you in this application has been revoked by the applicant. Future correspondence will be mailed to the new address of record(37 CFR 1.33).

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/hsarwari/

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
14/759,895	07/08/2015	1651	4590	040035/09002	33	11

CONFIRMATION NO. 3358

CORRECTED FILING RECEIPT

27530
Nelson Mullins Riley & Scarborough LLP
IP Department
One Wells Fargo Center
301 South College Street, 23rd Floor
Charlotte, NC 28202

Date Mailed: 10/02/2019

Receipt is acknowledged of this non-provisional utility patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF FIRST INVENTOR, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection.

Please verify the accuracy of the data presented on this receipt. If an error is noted on this Filing Receipt, please submit a written request for a corrected Filing Receipt, including a properly marked-up ADS showing the changes with strike-through for deletions and underlining for additions. If you received a "Notice to File Missing Parts" or other Notice requiring a response for this application, please submit any request for correction to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections provided that the request is grantable.

Inventor(s)

Ryanne Early, Fleming Island, FL;

Applicant(s)

ISE Professional Testing & Consulting Services, Inc., Eustis, FL;

Assignment For Published Patent Application

ISE Professional Testing & Consulting Services, Inc., Eustis, FL

Power of Attorney: The patent practitioners associated with Customer Number 27530

Domestic Priority data as claimed by applicant

This application is a 371 of PCT/US2014/010890 01/09/2014
which claims benefit of 61/750,555 01/09/2013

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: No

Permission to Access Search Results: No

RegenX Science Exhibit 1004
Page 1235

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1235

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 08/27/2015

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 14/759,895**

Projected Publication Date: Not Applicable

Non-Publication Request: No

Early Publication Request: No
**** SMALL ENTITY ****
Title

DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE

Preliminary Class

424

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: Yes

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific

RegenX Science Exhibit 1004
Page 1236

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1236

countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

SelectUSA

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop

technology, manufacture products, deliver services, and grow your business, visit http://www.SelectUSA.gov or call +1-202-482-6800.

RegenX Science Exhibit 1004
Page 1238

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1238

To: ip@nelsonmullins.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 27530

Oct 02, 2019 03:28:17 AM

Dear PAIR Customer:

Nelson Mullins Riley & Scarborough LLP
IP Department
One Wells Fargo Center
301 South College Street, 23rd Floor
Charlotte, NC 28202
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 27530 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

Disclaimer:
The list of documents shown below is provided as a courtesy and is not part of the official file wrapper. The content of the images shown in PAIR is the official record.

Application	Document	Mailroom Date	Attorney Docket No.
14759895	N570	10/02/2019	040035/09002
	N570	10/02/2019	040035/09002
	APP.FILE.REC	10/02/2019	040035/09002

To view your correspondence online or update your email addresses, please visit us anytime at https://sportal.uspto.gov/secure/myportal/privatepair.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

Doc Code: PET.POA.WDRW
Document Description: Petition to withdraw attorney or agent (SB83)

PTO/AIA/83 (04-13)
Approved for use through 03/31/2021. OMB 0651-0035
U.S. Patent and Trademark Office, U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

REQUEST FOR WITHDRAWAL AS ATTORNEY OR AGENT AND CHANGE OF CORRESPONDENCE ADDRESS

Application Number	14/759,895
Filing Date	July 8, 2015
First Named Inventor	Ryanne Early
Art Unit	1651
Examiner Name	Ruth A. Davis
Practitioner Docket Number	040035/09002

To: Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Please withdraw me as attorney or agent for the above-identified patent application, and

☐ all the practitioners of record;

☐ the practitioners (with registration numbers) of record listed on the attached paper(s); or

☒ the practitioners of record associated with Customer Number: 27530

NOTE: The immediately preceding box should only be marked when the practitioners were appointed using the listed Customer Number.

The reason(s) for this request are those described in 37 CFR:

☐ 11.116(a)(1) ☐ 11.116(a)(2) ☒ 11.116(a)(3)
☐ 11.116(b)(1) ☐ 11.116(b)(2) ☐ 11.116(b)(3)
☐ 11.116(b)(4) ☐ 11.116(b)(5) ☐ 11.116(b)(6)
☐ 11. 116(b)(7) Please explain below:

Certifications

Check each box below that is factually correct. WARNING: If a box is left unchecked, the request will likely not be approved.

1. ☒ I/We have given reasonable notice to the client, prior to the expiration of the response period, that the practitioner(s) intend to withdraw from employment.

2. ☒ I/We have delivered to the client or a duly authorized representative of the client all papers and property (including funds) to which the client is entitled.

3. ☒ I/We have notified the client of any responses that may be due and the time frame within which the client must respond.

Please provide an explanation, if necessary:

This collection of information is required by 37 CFR 1.36. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 15 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

PTO/AIA/83 (04-13)
Approved for use through 03/31/2021. OMB 0651-0035
U.S. Patent and Trademark Office, U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

REQUEST FOR WITHDRAWAL AS ATTORNEY OR AGENT AND CHANGE OF CORRESPONDENCE ADDRESS

Complete the following section only when the correspondence address will change. *Changes of address will only be accepted to an applicant.*

Change the correspondence address and direct all future correspondence to:

A. ☐ The address of the applicant associated with Customer Number: _____

OR

B. ■ Applicant

Address 2785 S. Bay Street, Suite G

City	State	Zip	Country
Eustis	FL	32726	US

Telephone | Email

I am authorized to sign on behalf of myself and all withdrawing practitioners.

Signature /Lloyd G. Farr/

Name Lloyd G. Farr — Registration No. 38,446

Address Nelson Mullins Riley & Scarborough LLP
One Wells Fargo Center/301 S. College Street, 23rd Floor

City	State	Zip	Country
Charlotte	NC	28202	US

Date 10/10/2019 — Telephone No. (404) 322-6165

NOTE: Withdrawal is effective when approved rather than when received.

This collection of information is required by 37 CFR 1.36. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 15 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	37427355
Application Number:	14759895
International Application Number:	
Confirmation Number:	3358
Title of Invention:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
First Named Inventor/Applicant Name:	Ryanne Early
Customer Number:	27530
Filer:	Lloyd G. Farr/Michell Graydon
Filer Authorized By:	Lloyd G. Farr
Attorney Docket Number:	040035/09002
Receipt Date:	10-OCT-2019
Filing Date:	08-JUL-2015
Time Stamp:	18:14:08
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Petition to withdraw attorney or agent (SB83)	040035-09002_PetitiontoWithdrawApplication_14759895.pdf	590257 f6d4255c10cf388d960381709cf6ab2406aafefd	no	3

Warnings:

Information:	
Total Files Size (in bytes):	590257

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: PA..
Document Description: Power of Attorney

PTO/AIA/82A (07-13)
Approved for use through 01/31/2018. OMB 0651-0035
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

TRANSMITTAL FOR POWER OF ATTORNEY TO ONE OR MORE REGISTERED PRACTITIONERS

NOTE: This form is to be submitted with the Power of Attorney by Applicant form (PTO/AIA/82B) to identify the application to which the Power of Attorney is directed, in accordance with 37 CFR 1.5, unless the application number and filing date are identified in the Power of Attorney by Applicant form. If neither form PTO/AIA/82A nor form PTO/AIA82B identifies the application to which the Power of Attorney is directed, the Power of Attorney will not be recognized in the application.

Application Number	14/759,895
Filing Date	July 8, 2015
First Named Inventor	Ryanne Early
Title	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
Art Unit	1651
Examiner Name	Ruth A. Davis
Attorney Docket Number	SYG-19-1252US/426329-002

SIGNATURE of Applicant or Patent Practitioner

Signature	/sima singadia kulkarni/	Date (Optional)	12/05/2019
Name	Sima Singadia Kulkarni	Registration Number	43,732
Title (if Applicant is a juristic entity)			
Applicant Name (if Applicant is a juristic entity)			

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. If more than one applicant, use multiple forms.

☐ *Total of _____ forms are submitted.

This collection of information is required by 37 CFR 1.131, 1.32, and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

POWER OF ATTORNEY BY APPLICANT

I hereby appoint the Practitioners named below as our agents, and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached Transmittal for Power of Attorney to One or More Registered Practitioners:

T. Daniel Christenbury	Reg. No. 31,750
Paul A. Taufer	Reg. No. 35,703
Darius C. Gambino	Reg. No. 41,472
Sima Singadia Kulkarni	Reg. No. 43,732
Richard L. Cruz	Reg. No. 52,783
William L. Bartow	Reg. No. 54,981
Lisa M. Lint	Reg. No. 60,856
Deborah Grove	Reg. No. 61,091
Priya Narahari	Reg. No. 71,526
Joseph W. Wolfe	Reg. No. 73,173

Please recognize or change the correspondence address for the application identified in the attached Transmittal for Power of Attorney to One or More Registered Practitioners to:

035811

I am the Applicant:

☐Inventor or Joint Inventor
☐Legal Representative of a Deceased or Legally Incapacitated Inventor
■Assignee or Person to Whom the Inventor is Under an Obligation to Assign
☐Person Who Otherwise Shows Sufficient Proprietary Interest (e.g. a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document)

ISE PROFESSIONAL TESTING & CONSULTING SERVICES, INC.
(The undersigned is authorized to act on behalf of Applicant.)

Date: 12/3/19

By: _____

Name: Ryanne Early

Title: President

EAST\169732488.1

Electronic Acknowledgement Receipt

EFS ID:	37943738
Application Number:	14759895
International Application Number:	
Confirmation Number:	3358
Title of Invention:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
First Named Inventor/Applicant Name:	Ryanne Early
Customer Number:	27530
Filer:	Sima Singadia Kulkarni/Stephanie De marco
Filer Authorized By:	Sima Singadia Kulkarni
Attorney Docket Number:	040035/09002
Receipt Date:	05-DEC-2019
Filing Date:	08-JUL-2015
Time Stamp:	16:13:28
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Power of Attorney	SYG_19_1252US_POA.pdf	265056 6065775b4ebfb8750c0b465ed18b76026dad1b70	no	2

Warnings:

Information:	
Total Files Size (in bytes):	265056

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/759,895	07/08/2015	Ryanne Early	040035/09002

CONFIRMATION NO. 3358

POWER OF ATTORNEY NOTICE

27530
Nelson Mullins Riley & Scarborough LLP
IP Department
One Wells Fargo Center
301 South College Street, 23rd Floor
Charlotte, NC 28202

OC000000113196152

Date Mailed: 12/10/2019

NOTICE REGARDING CHANGE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 12/05/2019.

- The Power of Attorney to you in this application has been revoked by the applicant. Future correspondence will be mailed to the new address of record(37 CFR 1.33).

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/sleutchit/

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/759,895	07/08/2015	Ryanne Early	SYG-19-1252US/426329-002

CONFIRMATION NO. 3358

35811
IP GROUP OF DLA PIPER LLP (US)
ONE LIBERTY PLACE
1650 MARKET ST, SUITE 5000
PHILADELPHIA, PA 19103

POA ACCEPTANCE LETTER

OC000000113196187

Date Mailed: 12/10/2019

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 12/05/2019.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/sleutchit/

To: pto.phil@us.dlapiper.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 35811

Dec 10, 2019 03:29:50 AM

Dear PAIR Customer:

IP GROUP OF DLA PIPER LLP (US)
ONE LIBERTY PLACE
1650 MARKET ST, SUITE 5000
PHILADELPHIA, PA 19103
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 35811 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

Disclaimer:
The list of documents shown below is provided as a courtesy and is not part of the official file wrapper. The content of the images shown in PAIR is the official record.

Application	Document	Mailroom Date	Attorney Docket No.
14759895	N570	12/10/2019	SYG-19-1252US/426329-002
	N570	12/10/2019	SYG-19-1252US/426329-002

To view your correspondence online or update your email addresses, please visit us anytime at https://sportal.uspto.gov/secure/myportal/privatepair.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

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PTO/SB/122 (04-15)
Approved for use through 03/31/2021. OMB 0651-0035
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

CHANGE OF CORRESPONDENCE ADDRESS
Application

Address to:
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Application Number	14/759,895
Filing Date	01/09/14
First Named Inventor	Ryanne Early
Art Unit	
Examiner Name	
Attorney Docket Number	

Please change the Correspondence Address for the above-identified patent application to:

☒ The address associated with Customer Number: 167127

OR

☐ Firm or Individual Name

Address

City | State | Zip

Country

Telephone | Email

This form cannot be used to change the data associated with a Customer Number. To change the data associated with an existing Customer Number use "Request for Customer Number Data Change" (PTO/SB/124).

I am the:

☒ Applicant/Inventor

☐ Assignee of record of the entire interest.
Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96).

☐ Attorney or agent of record. Registration Number _____.

☐ Registered practitioner named in the application transmittal letter in an application without an executed oath or declaration. See 37 CFR 1.33(a)(1). Registration Number _____.

Signature

Typed or Printed Name: Ryanne Early

Date: 12/11/19 | Telephone: 352-474-0823

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

☑ *Total of 1 forms are submitted.

This collection of information is required by 37 CFR 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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FAX COVER SHEET

TO	
COMPANY	
FAX NUMBER	15712730177
FROM	ChristianEarly
DATE	2019-12-11 16:14:25 GMT
RE	

COVER MESSAGE

PAGE 1/3 * RCVD AT 12/11/2019 11:14:49 AM [Eastern Standard Time] * SVR:W-PTOFAX-002/31 * DNIS:2730177 * CSID:12142796653 * ANI:5137046888 * DURATION (mm-ss):02-20
PAGE 1/3 * RCVD AT 12/11/2019 11:43:18 AM [Eastern Standard Time] * SVR:W-PTOFAX-001/18 * DNIS:2738300 * CSID:7036841409 * ANI:7036832501 * DURATION (mm-ss):01-16

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

167127 7590 01/13/2020

Ryanne Early
2785 S Bay St. G
Eustis, FL 32726

EXAMINER
DAVIS, RUTH A

ART UNIT	PAPER NUMBER
1651	

DATE MAILED: 01/13/2020

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/759,895	07/08/2015	Ryanne Early	SYG-19-1252US/426329-002	3358

TITLE OF INVENTION: DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	SMALL	$500	$0.00	$0.00	$500	04/13/2020

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Maintenance fees are due in utility patents issuing on applications filed on or after Dec. 12, 1980. It is patentee's responsibility to ensure timely payment of maintenance fees when due. More information is available at www.uspto.gov/PatentMaintenanceFees.

RegenX Science Exhibit 1004
Page 1255

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1255

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

167127 7590 01/13/2020
Ryanne Early
2785 S Bay St. G
Eustis, FL 32726

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/759,895	07/08/2015	Ryanne Early	SYG-19-1252US/426329-002	3358

TITLE OF INVENTION: DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	SMALL	$500	$0.00	$0.00	$500	04/13/2020

EXAMINER	ART UNIT	CLASS-SUBCLASS
DAVIS, RUTH A	1651	424-093100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1_____
2_____
3_____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. Fees submitted: ❑Issue Fee ❑Publication Fee (if required) ❑Advance Order - # of Copies _____

4b. Method of Payment: *(Please first reapply any previously paid fee shown above)*

❑ Electronic Payment via EFS-Web ❑ Enclosed check ❑ Non-electronic payment by credit card (Attach form PTO-2038)

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. _____

5. **Change in Entity Status** (from status indicated above)

❑ Applicant certifying micro entity status. See 37 CFR 1.29

❑ Applicant asserting small entity status. See 37 CFR 1.27

❑ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

RegenX Science Exhibit 1004
Page 1256

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1256

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/759,895	07/08/2015	Ryanne Early	SYG-19-1252US/426329-002	3358

167127 7590 01/13/2020

Ryanne Early
2785 S Bay St. G
Eustis, FL 32726

EXAMINER
DAVIS, RUTH A

ART UNIT	PAPER NUMBER
1651	

DATE MAILED: 01/13/2020

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No. 14/759,895	Applicant(s) Early, Ryanne	
	Examiner RUTH A DAVIS	Art Unit 1651	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to Interview on January 7, 2020.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☑ The allowed claim(s) is/are 5,20-22,26,36-38,40-61,66-67,70-72 and 80 . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   Certified copies:

   a) ☐ All b) ☐ Some *c) ☐ None of the:

   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☑ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☑ Interview Summary (PTO-413), Paper No./Mail Date. 01-07-2020.
5. ☑ Examiner's Amendment/Comment
6. ☐ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/RUTH A DAVIS/
Primary Examiner, Art Unit 1651

RegenX Science Exhibit 1004
Page 1259

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1259

Notice of Pre-AIA or AIA Status

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Election/Restrictions

Claims 5, 20 – 22, 26, 36 – 38, 40 – 61 are allowable. The restriction requirement between the product and methods of making the product, as set forth in the Office action mailed on September 9, 2016, has been reconsidered in view of the allowability of claims to the elected invention pursuant to MPEP § 821.04(a). **The restriction requirement is hereby withdrawn as to any claim that requires all the limitations of an allowable claim.** Specifically, the restriction requirement of September 9, 2019 is withdrawn. Claims 66 – 67, 70 – 72 and 80, directed to methods of making the allowable product are no longer withdrawn from consideration because the claim(s) requires all the limitations of an allowable claim.

In view of the above noted withdrawal of the restriction requirement, applicant is advised that if any claim presented in a continuation or divisional application is anticipated by, or includes all the limitations of, a claim that is allowable in the present application, such claim may be subject to provisional statutory and/or nonstatutory double patenting rejections over the claims of the instant application.

Once a restriction requirement is withdrawn, the provisions of 35 U.S.C. 121 are no longer applicable. See In re Ziegler, 443 F.2d 1211, 1215, 170 USPQ 129, 131-32 (CCPA 1971). See also MPEP § 804.01.

EXAMINER'S AMENDMENT

An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment was given in an interview with Sima Kulkarni on January 7, 2020.

The application has been amended as follows:

In claim 71, line 2, the first recitation of "or" is deleted and replaced with --,--.

In claim 71, line 2, the term "encapsulating" is deleted and replaced with --encapsulated with--.

In claim 71, line 3, --, the-- has been inserted after the recitation of "thereto".

Claim 68 – 69 and 73 – 79 are canceled.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to RUTH A DAVIS whose telephone number is (571)272-0915. The examiner can normally be reached on Monday - Friday (8am - 4pm).

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Renee Claytor can be reached on 571-272-8394. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/RUTH A DAVIS/
Primary Examiner, Art Unit 1651

Examiner-Initiated Interview Summary	**Application No.** 14/759,895	**Applicant(s)** Early, Ryanne		
	Examiner RUTH A DAVIS	**Art Unit** 1651	**AIA (First Inventor to File) Status** Yes	**Page** **1 of** 2

All participants (applicant, applicants representative, PTO personnel):

1. RUTH A DAVIS (Primary Examiner); Telephonic 2. Sima Kulkarni (Attorney of Record); Telephonic

Date of Interview: 07 January 2020

Claims Discussed: 68 - 69, 73 - 79.

Amendment proposed: See Examiner's amendment.

Brief Description of the main topic(s) of discussion: Applicant's representative requested an Examiner Amendment to cancel claims 68 - 69 and 73 - 79 in response to the previous office action. Additional amendments are included to address 112,B of record, relative to claim 71.

Issues Discussed:

Proposed Amendments:
See attached Examiner's amendment.

Attachment(s): Proposed Amendments,

	/RUTH A DAVIS/ Primary Examiner, Art Unit 1651

Applicant is reminded that a complete written statement as to the substance of the interview must be made of record in the application file. It is the applicants responsibility to provide the written statement, unless the interview was initiated by the Examiner and the Examiner has indicated that a written summary will be provided. See MPEP 713.04
Please further see:
MPEP 713.04
Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews, paragraph (b)
37 CFR § 1.2 Business to be transacted in writing

Applicant recordation instructions: It is not necessary for applicant to provide a separate record of the substance of interview .

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14/759,895	Early, Ryanne
	Examiner	**Art Unit**
	RUTH A DAVIS	1651

CPC							
Symbol						**Type**	**Version**
A61L	/	27	/	3687		F	2013-01-01
A61K	/	9	/	70		I	2013-01-01
A61L	/	27	/	58		I	2013-01-01
A61L	/	27	/	60		I	2013-01-01
A61L	/	27	/	54		I	2013-01-01
A61L	/	27	/	362		I	2013-01-01
A61L	/	27	/	3691		I	2013-01-01
A61K	/	38	/	1703		I	2013-01-01
A61K	/	35	/	12		I	2013-01-01
A61L	/	27	/	3625		I	2013-01-01
A61L	/	27	/	3604		I	2013-01-01
A61L	/	27	/	3633		I	2013-01-01
A61L	/	27	/	38		I	2013-01-01
A61K	/	35	/	65		I	2013-01-01
A61L	/	27	/	52		I	2013-01-01
C12N	/	5	/	0625		I	2013-01-01
A61L	/	2430	/	40		A	2013-01-01
A61L	/	2430	/	34		A	2013-01-01
A61L	/	2300	/	64		A	2013-01-01
A61L	/	2300	/	606		A	2013-01-01
A61L	/	2300	/	604		A	2013-01-01
A61L	/	2300	/	412		A	2013-01-01
A61L	/	2300	/	252		A	2013-01-01
C12N	/	2533	/	90		A	2013-01-01
C12N	/	2533	/	92		A	2013-01-01

CPC Combination Sets								
Symbol					**Type**	**Set**	**Ranking**	**Version**
	/		/					

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	36	
/RUTH A DAVIS/ Primary Examiner, Art Unit 1651	07 January 2020	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	NONE

U.S. Patent and Trademark Office — Part of Paper No.: 20190815

RegenX Science Exhibit 1004
Page 1265

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1265

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14/759,895	Early, Ryanne
	Examiner	**Art Unit**
	RUTH A DAVIS	1651

INTERNATIONAL CLASSIFICATION				
CLAIMED				
A61L	/	27	/	36
A61K	/	9	/	70
A61L	/	27	/	58
A61K	/	35	/	12
C12N	/	5	/	071

NON-CLAIMED				
	/		/	

US ORIGINAL CLASSIFICATION	
CLASS	**SUBCLASS**

CROSS REFERENCES(S)						
CLASS	**SUBCLASS (ONE SUBCLASS PER BLOCK)**					

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 36	
/RUTH A DAVIS/ Primary Examiner, Art Unit 1651 (Primary Examiner)	07 January 2020 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure NONE

U.S. Patent and Trademark Office

Part of Paper No.: 20190815

RegenX Science Exhibit 1004
Page 1266

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1266

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14/759,895	Early, Ryanne
	Examiner	**Art Unit**
	RUTH A DAVIS	1651

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIMS

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
1	5		15		25		35	27	45	11	55		65		75
	6		16	35	26	2	36	28	46	12	56	18	66		76
	7		17		27	3	37	33	47	14	57	19	67		77
	8		18		28	4	38	36	48	15	58		68		78
	9		19		29		39	5	49	16	59		69		79
	10	20	20		30	22	40	6	50	17	60	29	70	34	80
	11	21	21		31	23	41	7	51	13	61	30	71		
	12	32	22		32	24	42	8	52		62	31	72		
	13		23		33	25	43	9	53		63		73		
	14		24		34	26	44	10	54		64		74		

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	36	
/RUTH A DAVIS/ Primary Examiner, Art Unit 1651	07 January 2020	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	NONE

U.S. Patent and Trademark Office — Part of Paper No.: 20190815

RegenX Science Exhibit 1004
Page 1267

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1267

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14/759,895	Early, Ryanne
	Examiner	Art Unit
	RUTH A DAVIS	1651

CPC - Searched*		
Symbol	**Date**	**Examiner**
A61K 35/12, 65	01/07/2020	rad
A61K 9/70	01/07/2020	rad
A61L 37/3687, 58,60,54,362	01/07/2020	rad
C12N 5/0625	01/07/2020	rad

CPC Combination Sets - Searched*		
Symbol	**Date**	**Examiner**

US Classification - Searched*			
Class	**Subclass**	**Date**	**Examiner**

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	**Date**	**Examiner**
inventor	01/07/2020	rad
A61K 35/65	01/07/2020	rad

	/RUTH A DAVIS/ Primary Examiner, Art Unit 1651

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14/759,895	Early, Ryanne
	Examiner	Art Unit
	RUTH A DAVIS	1651

Interference Search			
US Class/CPC Symbol	**US Subclass/CPC Group**	**Date**	**Examiner**
A61L	27/54,58,60,362,3687	01/07/2020	rad
A61K	9/70	01/07/2020	rad
A61K	35/12	01/07/2020	rad
C12N	5/0625	01/07/2020	rad

	/RUTH A DAVIS/ Primary Examiner, Art Unit 1651

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	5	early near ryanne	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2020/01/07 11:20
L2	4	early near ryanne.in.	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2020/01/07 11:20
L3	4	early.in. and ryanne.in.	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2020/01/07 11:21

1/ 7/ 2020 11:29:57 AM
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Form PTO-1449 US DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTY. DOCKET NO. SYG-19-1252US/426329-002	SERIAL NO. 14/759,895
	APPLICANT ISE Professional Testing & Consulting Services, Inc.	
LIST OF PUBLICATIONS CITED BY APPLICANT *(Use several sheets if necessary)*	FILING DATE July 8, 2015	GROUP 1651

US PATENT DOCUMENTS

EXAMINER INITIAL*		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
	AA	2006/153797 A1 (corr. to CN '039)	07/13/06	Bortolotto et al.			
	AB						
	AC						
	AD						
	AE						
	AF						
	AG						
	AH						
	AI						
	AJ						
	AK						

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO
	AL	1969039 A (corr. to US '797)	05/23/07	CN			X	
	AM	101337089 A	01/07/09	CN			Abstract	
	AN							
	AO							
	AP							

OTHER PUBLICATIONS (Including Author, Title, Date, Pertinent Pages, Etc.)

	AR	Second Office Action from counterpart Chinese Patent Appln. No. 201480013359.0 dated May 22, 2019, along with its English Translation.
	AS	
	AT	

EXAMINER	/RUTH A DAVIS/	DATE CONSIDERED 08/08/2019

*EXAMINER: Initial if publication considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

EAST\47689080.1
EAST\167753423.1

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /R.A.D/

PTO/AIA/122 (08-12)
Approved for use through 03/31/2021. OMB 0651-0035
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CHANGE OF CORRESPONDENCE ADDRESS
Application

Address to:
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Alexandria, VA 22313-1450

Application Number	14/759,895
Filing Date	July 8, 2015
First Named Inventor	Ryanne Early
Art Unit	1651
Examiner Name	Ruth A. Davis
Attorney Docket Number	SYG-19-1252US/426329-002

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I am the:

☐ Applicant

☒ Attorney or agent of record. Registration Number 43,732.

☐ Registered practitioner named in the application transmittal papers who acts in a representative capacity under 37 CFR 1.34. See 37 CFR 1.33(a)(1). Registration Number _____.

Signature /sima singadia kulkarni/

Typed or Printed Name Sima Singadia Kulkarni

Date	Telephone
January 21, 2020	(215) 656-2478

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

☐ *Total of _____forms are submitted.

This collection of information is required by 37 CFR 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

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9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	38345129
Application Number:	14759895
International Application Number:	
Confirmation Number:	3358
Title of Invention:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
First Named Inventor/Applicant Name:	Ryanne Early
Customer Number:	167127
Filer:	Sima Singadia Kulkarni/Stephanie De marco
Filer Authorized By:	Sima Singadia Kulkarni
Attorney Docket Number:	SYG-19-1252US/426329-002
Receipt Date:	21-JAN-2020
Filing Date:	08-JUL-2015
Time Stamp:	10:24:27
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Change of Address	SYG_19_1252US_CoA.pdf	223606 08331f782108b5e420a40d03ab5af98e53e6a01b	no	2

Warnings:

Information:	
Total Files Size (in bytes):	223606

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Form PTO-1449 US DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTY. DOCKET NO. SYG-19-1252US/426329-002	SERIAL NO. 14/759,895
	APPLICANT ISE Professional Testing & Consulting Services, Inc.	
LIST OF PUBLICATIONS CITED BY APPLICANT *(Use several sheets if necessary)*	FILING DATE July 8, 2015	GROUP 1651

US PATENT DOCUMENTS

EXAMINER INITIAL*		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
	AA						
	AB						
	AC						
	AD						
	AE						
	AF						
	AG						
	AH						
	AI						
	AJ						
	AK						

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO
	AL	2009-510091 A (corr. to WO '686)	03/12/09	JP			X	
	AM	2007/038686 A2 (corr. to JP '091)	04/05/07	WO				
	AN	2004-521935 A (corr to. WO '720)	04/22/04	JP			X	
	AO	02/078720 A2 (corr. to JP '935)	10/10/02	WO				
	AP	2007-532153 A (corr. to WO '219)	11/15/07	JP			X	

OTHER PUBLICATIONS (Including Author, Title, Date, Pertinent Pages, Etc.)

	AR	Notice for Reasons of Refusal from counterpart Japanese Patent Appln. No. 2018-224475 dated February 3, 2020, and its English translation.
	AS	
	AT	

EXAMINER	DATE CONSIDERED

*EXAMINER: Initial if publication considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

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Form PTO-1449 US DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTY. DOCKET NO. SYG-19-1252US/426329-002	SERIAL NO. 14/759,895
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	AK						

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO
	AL	2005/097219 A1 (corr. to JP '153)	10/20/05	WO				
	AM	2009-513269A	04/02/09	JP			X	
	AN							
	AO							
	AP							

OTHER PUBLICATIONS (Including Author, Title, Date, Pertinent Pages, Etc.)

	AR	
	AS	
	AT	

EXAMINER	DATE CONSIDERED

*EXAMINER: Initial if publication considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

PATENT TERM ADJUSTMENT STATEMENT UNDER 37 CFR 1.704(d)

Application Number	14/759,895
Filing Date	July 8, 2015
First Named Inventor	Ryanne Early
Art Unit	1651
Examiner Name	Ruth A. Davis
Practitioner Docket No.	SYG-19-1252US/426329-002

APPLICANT HEREBY STATES THE FOLLOWING (please review 37 CFR 1.704(d) before filing this form):

☒ Each item of information contained in the information disclosure statement was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office, and this communication was not received by any individual designated in 37 CFR 1.56(c) more than thirty days prior to the filing of the information disclosure statement.

AND/OR

☒ Each item of information contained in the information disclosure statement is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office, and this communication was not received by any individual designated in 37 CFR 1.56(c) more than thirty days prior to the filing of the information disclosure statement.

INSTRUCTIONS:

- **This form will not satisfy the requirement of 37 CFR 1.97(e).** The present statement is filed under 37 CFR 1.704(d) and will not substitute for compliance with any of the requirements of 37 CFR 1.97 and 1.98. For an information disclosure statement to comply with 37 CFR 1.97(c) or (d), the information disclosure statement must be accompanied by a statement under 37 CFR 1.97(e) notwithstanding any statement filed under 37 CFR 1.704(d).
- The present form (PTO/SB/133) should be filed concurrently with the information disclosure statement to derive benefit under 37 CFR 1.704(d).

Signature	/sima singadia kulkarni/	Date	February 26, 2020
Typed or Printed Name	Sima Singadia Kulkarni	Practitioner Registration Number	43,732

***Note**: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required in accordance with 37 CFR 1.33 and 11.18. Please see 37 CFR 1.4(d) for the form of the signature. If necessary, submit multiple forms for more than one signature, see below*.*

☐ *Total of _____ forms are submitted.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Notice of Reasons for Refusal

Application number: Japanese Patent Application No. 2018-224475
Date of Drafting: Reiwa 2(2020) January 28
Patent examiner: DATE, Rina 3960 Four B00
Representative/Applicant: KUZUWA, Kiyoshi
Applied Provisions: The first item of Article 29, Article 29(2), Article 36, Article 37

This application should be refused for the reason mentioned below. If the applicant has any opinion(s) against the reason, a written opinion should be submitted within 3 months from the date on which this notification was dispatched.

Reason

1. (Unity of Invention) This patent application does not fulfill the requirement of the provision of Patent Act Article 37 in terms of the item(s) listed below.

2. (Clarity) This patent application does not fulfill the requirement of the provision of Patent Act Article 36(6)(ii) in terms of the item(s) listed below regarding the statement of the scope of claims.

3. (Support Requirement) This patent application does not fulfill the requirement of the provision of Patent Act Article 36(6)(i) in terms of the item(s) listed below regarding the statement of the scope of claims.

4. (Novelty) The claimed invention(s) in the each claim listed below of this patent application shall not be granted a patent under the provision of Patent Act Article 29 (1)(iii) for the reason that the claimed invention(s) has/have been deemed to be identical with the invention(s) described in the distributed publication(s) listed below or made available to the public through electric telecommunication lines in Japan or other foreign countries prior to the filing of the patent application.

5. (Inventive step) The claimed invention(s) listed below of this patent application shall not be granted a patent under the provision of Patent Act Article 29 (2) for the reason that the claimed invention(s) could have easily been made by persons who have common knowledge in the technical field to which the claimed invention(s) pertains, on the basis of the invention(s) described in the distributed publication listed below or made available to the public through electric telecommunication lines in Japan or other foreign countries prior to the filing of the patent application.

Note (For the cited documents, please refer to the list of cited documents below.)

- Reason 1 (Unity of Invention)

- Claim 11-55

1. Determination of the subject of examination based on special technical features

Invention according to claim 1, "to obtain a tissue sample comprising extracellular matrix from Urodela, to retain the structural and functional integrity of the tissue sample, and to remove the cellular components of the sample sufficiently to reduce or eliminate the antigenicity of the biomaterial as a xenograft in a human in a human."The special technical feature of the use of Urodela extracellular matrices (ECM) for use in humans or animals produced by processes involving the invention was found. Therefore, the invention according to Claim 1 and the invention according to Claim 2-10 having the same or corresponding special technical feature as the said special technical feature of Claim 1 are the subject of examination.

2. Determination of the subject of examination based on the efficiency of examination

The invention according to Claim 11-55 is not an invention of the same category including all the matters specifying the invention of the invention according to Claim 1. Moreover, as a result of examining the invention subject to examination, the invention according to Claim 11-55 is not an invention that is capable of performing examination without substantially requiring an additional prior art search or determination, and there is no other circumstances in which it can be said that it is efficient to conduct examination together with the invention.

Therefore, the invention according to Claim 11-55 is not a subject to examination concerning a requirement other than the provisions of Article 37 of the Patent Act.

Since the application violates the provisions of Article 37 of the Patent Act, no examination on a requirement other than that of Article 37 of the same Act is conducted for the inventions according to claims other than Claim 1-10.

- Reason 2 (Clarity)

- Claim 1-10
<Concerning Claim 1, the invention according to Claim 1 is "Urodela extracellular matrix (ECM)" (the invention of the product). However, the claim contains "(A) Obtaining a tissue sample containing extracellular matrix from the Urodela ; and, in the Claim, obtaining a tissue sample containing extracellular matrix. (B) (I) maintaining the structural and functional integrity of the tissue sample, and (ii) removing cellular components of the sample sufficient to reduce or eliminate the antigenicity of the biomaterial as a xenograft in a human ; andIt is recognized that a process for the preparation of the product is described, which is produced by a method involving decellularizing the sample by a method involving the use of the

compound. Here, in a case where the claim concerning the invention of the product describes a method for producing the product, it can be said that the statement of the claim complies with the requirement of "the invention is clear" specified in Article 36 (6) (2) of the Patent Act. It is reasonable to understand that it is limited to the case where it is impossible to directly specify the product by its structure or characteristics at the time of the filing of the application, or that it is not practically practical (the "impossible / impractical circumstances") (2), (2015), (5), (2012), (1204), and (2658) of the Supreme court (s). However, there is no statement in the description, etc. about the existence of an impossible / impractical situation, and the applicant has not been allegation and proved. Therefore, the reason for finding the existence is not found. Therefore, the invention according to Claim 1 is not clear. The invention according to Claim 2-10, which is dependent on Claim 1, is not clear.
<Suggestions of the amendments etc.>
In order to overcome the reasons for refusal, the applicant is considered to take the following measures. Also, refer to part II, chapter 2, 2203-2205 of Examination Handbook for Patent and Utility Model in Japan.
A. Dismissal of the Claims
B. An amendment to make the invention according to the claim an invention of a method for producing a product
C. Correction D. Regarding an invention of a product which does not include a manufacturing method, and allegation and proof by a written opinion or the like concerning an impossible / impractical situation
E. In case of the counterargument that does not fall under "a method of manufacturing the product," it should be noted that it must be performed within the scope of the matters described in the Description, the scope of claims, or drawings (the "original description, etc.") at the time of filing the application. In particular, it should be noted that an amendment to delete the statement of a manufacturing method simply in the above C is likely to be not within the scope of the matters described in the Originally attached description, etc. In dealing with the above D, a situation where "directly identifies the product at the time of filing by its structure or characteristics" or a circumstance which is almost impractical is specifically stated.

- Claim 1-10
It is described that the Urodela extracellular ECM described in Claim 1 is produced by a method including "maintaining structural and functional integrity" from a tissue sample from a Urodela. However, Claim 8, which is an invention dependent on Claim 1, describes ECM, which is a "dry powder," and Claim 9 describes an ECM, which is a "reconstituted gel.". Since it is obvious that the dry powder and the reconstituted gel do not have structural integrity and functional integrity of the tissue sample from the Urodela, the statements in Claim 1 and the statements in Claim 8 and Claim 9 are inconsistent. Therefore, it is unclear what the inventions according to Claim 1 and Claims 8 and 9 are. Claims 2-7 and 10 which are dependent on Claim 1 are similar to Claim 1. Therefore, the invention according to Claim 1-10 is not clear.

- Claim 4,10
In Claim 4, the ECM is "coupled to this." However, since it is unclear what "this" means in the description, the invention claimed in Claim 4 is unclear. Claim 10 which is dependent on Claim 4 is similar to Claim 1.

- Claim 5,10
In Claim 5, "a pharmaceutical" is described, and Claim 5 refers to Claim 1. However, since Claim 1 does not describe "a pharmaceutical agent," it is unclear what the "pharmaceutical agent" refers to in Claim 5. Hence, the invention according to Claim 10 which depends from Claim 5 and Claim 5 is not clear.

- Reason 3 (Support Requirement)

- Claim 1-10
The problem to be solved by the Invention as understood from the description in the description is easily available and is recognized to provide a biological material suitable for transplantation that maintains the function of the natural tissue. Moreover, the means for solving the problem disclosed in the description is to decellularize a tissue sample derived from Urodela while maintaining the structural integrity and the functional integrity and to prepare an ECM sheet suitable for use in humans. However, Claim 1 describes ECM for use in "human or animal", and describes ECM for use in animals other than humans. In the description, although it is considered that the ECM sheet from Urodela is suitable for the use of human amnion or the like, it is not considered that it is suitable for transplantation in animals other than humans. Since the structure of a biological tissue such as the dermis cannot be said to be common regardless of the animal species, it is not found that an ECM sheet suitable for use in a human is suitable for use in any other animal species. Therefore, the invention according to Claim 1 is not found to solve the problem of the present application. Claim 2-10 which is dependent on Claim 1 is similar to Claim 1. Hence, the invention according to Claim 1-10 is not described in the detailed description of the invention.

- Claim 8-10
Claim 8 describes ECM which is a "dry powder". In the description, it is merely stated that the ECM sheet of the Urodela is suitable for use in humans from its structural features, and it is not shown that it was suitable for use in humans even if it is a powder. It cannot be said that the common general technical knowledge of using Urodela's ECM for human regenerative medicine, etc. existed at the time of the filing of the present application. Consequently, it is not recognized that the matters disclosed in the description can be expanded to or generalized to the scope of the invention according to Claim 8. In the same manner, the ECM which is the "reconstituted gel" described in Claim 9 does not have the structural features of the ECM sheet of the Urodela, and thus the same deficiency as Claim 8 exists. Hence, the inventions according to Claims 8 and 9 and Claim 10 which depends therefrom are not described in the detailed description of the invention.

- Reason 4 (Novelty) , Reason 5 (Inventive step)

- Claim 1-3,6
- Cited Document, etc. 1

- Remarks

Cited Document 1 discloses that a decellularized ECM sheet produced by decellularizing the skin of a Ambystoma mexicanum (corresponding to the "Acarbose") was implanted at the wound site (p. 192, the last paragraph to the right column, l. 1, p. 194, the right column, l. 2, and Fig. 6). Hence, the invention according to Claim 1-3,6 is the invention described in the cited document 1. Even if there is a part that is not so, it could have been easily conceived by a person skilled in the art from the description in the cited document 1.

- Reason 5 (Inventive step)

- Claim 4,5,7-10
- Cited Document, etc. 1,2

- Remarks

Cited Document 2 describes a collagen membrane produced from amniotic membranes, which has the structural integrity of natural untreated amniotic membranes, is completely decellularized, and is used in the medical field as a wound dressing or the like. The membrane is prepared in a sterile solution, dried, impregnated with a substance such as a therapeutic agent or a scaffold, containing a hydrogel composition, and is used in the treatment of humans. (Full text, especially Claim 1) 76)
Both cited documents 1 and 2 describe an invention relating to an ECM sheet used for medical purposes such as treatment of wound wounds.
It could have been easily conceived by a person skilled in the art to apply similar processing to the ECM sheet described in Cited Document 1 by referring to the processing method described in Cited Document 2, in which various medications are impregnated with the ECM sheets, as a gel composition, and dried or sterilized. In the description, it is not shown that the effects of the inventions according to Claims 4,5,7 to 10 are remarkable. In particular, there is no statement that the ECM sheets of the present application were actually powder dried or reconstituted as a gel, and the effects obtained by these operations were not examined. Therefore, the effects of the inventions claimed in Claims 8 and 9 are not indicated in the description. Therefore, the inventions according to Claims 4,5,7 to 10 could have been easily invented by a person skilled in the art based on the inventions described in Cited Documents 1 and 2 and the common general technical knowledge before the priority date of the present application.

<The list of cited documents etc.>
1. ZOOLOGICAL SCIENCE, 2012, Vol.29, pp.191-197
2. JP 2006-507851A

Record of the result of prior art search etc.

- Fields searched :

IPC
C12N 5/00
DB name PubMed

- Prior art documents :

International Publication No. WO 2008/129997
JP 2004-521935A
National Publication of International Patent Application No. 2009 - 513269, The Journal of Experimental Zoology, 1982, Vol.220, pp.243 - 250
JP 2009-510091A
National Publication of International Patent Application No. 2007 - 532153 The recording of the results of the prior art document does not constitute a reason for refusal.

<Suggestions in amending>
(1) When making amendment to the description or the scope of claims, the amended parts should be underlined so as to be clearly identified (please refer to Note 6 and 7 of Form 13, Regulations under the Patent Act).
(2) Amendments must not go beyond the description, scope of claims, or drawings of the present application as originally filed. In addition, for the evidence of the legality of each amendment, it is advisable to allege the legality in a written opinion with clear indication of the corresponding parts in the original description, claims and drawings.
(3) One should make sure not to perform any corrections that invoke a violation against Patent Act Article 17-2(4) when amending the scope of claims.

If there is an inquiry concerning the content of the notice of reasons for refusal, please refer to the next contact. In addition, Part 3 of the Examination is transferred from the Roppongi Temporary Building to the Principal Government Store, and starts its business in the Principal Government on April 30, 2 (wood). **. For transfer work, telephone calls and fax readers may not communicate with each other for a period of April 27, 2 (months) to April 28, 2016 (fire) (**). In addition, the fax number is changed after the transfer (the telephone number is not changed). Please confirm the FAX number by telephone in advance.
Examination No. 3, BIOENGINEERING Date, Susumu
TEL. 03 to 3581 - 1101 Extensions 3448
FAX. 03, pages 3585 to 2093 (effective until April 24, 2 (Gold)) **

A Director General(p.p.), a Director(p.p.), an examiner, an assistant examiner, UEHARA, Katsunori, DATE, Rina, and 9840 3960

拒絶理由通知書

特許出願の番号	特願２０１８－２２４４７５
起案日	令和　２年　１月２８日
特許庁審査官	伊達　利奈　　３９６０　４Ｂ００
特許出願人代理人	葛和　清司　様
適用条文	第２９条第１項、第２９条第２項、第３６条、第３７条

この出願は、次の理由によって拒絶をすべきものです。これについて意見がありましたら、この通知書の発送の日から３か月以内に意見書を提出してください。

理由

１．（発明の単一性）この出願は、下記の点で特許法第３７条に規定する要件を満たしていない。

２．（明確性）この出願は、特許請求の範囲の記載が下記の点で、特許法第３６条第６項第２号に規定する要件を満たしていない。

３．（サポート要件）この出願は、特許請求の範囲の記載が下記の点で、特許法第３６条第６項第１号に規定する要件を満たしていない。

４．（新規性）この出願の下記の請求項に係る発明は、その出願前に日本国内又は外国において、頒布された下記の刊行物に記載された発明又は電気通信回線を通じて公衆に利用可能となった発明であるから、特許法第２９条第１項第３号に該当し、特許を受けることができない。

５．（進歩性）この出願の下記の請求項に係る発明は、その出願前に日本国内又は外国において、頒布された下記の刊行物に記載された発明又は電気通信回線を通じて公衆に利用可能となった発明に基いて、その出願前にその発明の属する技術の分野における通常の知識を有する者が容易に発明をすることができたものであるから、特許法第２９条第２項の規定により特許を受けることができない。

記　（引用文献等については引用文献等一覧参照）

●理由１（発明の単一性）について

・請求項１１－５５

１．特別な技術的特徴に基づく審査対象の決定

請求項１に係る発明に、「有尾類からの細胞外マトリックスを含む組織試料を得ること、該組織試料の構造的および機能的完全性を保持すること、及び、ヒトにおける異種移植片としての生体材料の抗原性を低減または排除するために十分なように試料の細胞成分を除去すること、を含む方法により生産されるヒト又は動物への使用のための有尾類細胞外マトリックス（ＥＣＭ）。」という特別な技術的特徴が発見された。

したがって、請求項１に係る発明と、当該特別な技術的特徴と同一の又は対応する特別な技術的特徴を有する、請求項２－１０に係る発明とを、審査対象とする。

２．審査の効率性に基づく審査対象の決定

請求項１１－５５に係る発明は、請求項１に係る発明の発明特定事項を全て含む同一カテゴリーの発明ではない。そして、請求項１１－５５に係る発明は、審査対象とされた発明を審査した結果、実質的に追加的な先行技術調査や判断を必要とすることなく審査を行うことが可能である発明ではなく、当該発明とまとめて審査を行うことが効率的であるといえる他の事情も無い。

したがって、請求項１１－５５に係る発明は、特許法第３７条以外の要件についての審査対象としない。

この出願は特許法第３７条の規定に違反しているので、請求項１－１０以外の請求項に係る発明については、同法第３７条以外の要件についての審査を行っていない。

●理由２（明確性）について

・請求項１－１０

＜物の発明に係る請求項にその物の製造方法が記載されている場合の明確性要件について＞

請求項１に係る発明は、「有尾類細胞外マトリックス（ＥＣＭ）。」（物の発明）であるが、当該請求項には、「（Ａ）有尾類からの、細胞外マトリックスを含む組織試料を得ること、および（Ｂ）（ｉ）該組織試料の構造的および機能的完全性を保持すること、および（ｉｉ）ヒトにおける異種移植片としての生体材料の抗原性を低減または排除するために十分なように試料の細胞成分を除去すること、を含む方法により前記試料を脱細胞化すること、を含む方法により生産さ

れる、」という、その物の製造方法が記載されているものと認められる。

ここで、物の発明に係る請求項にその物の製造方法が記載されている場合において、当該請求項の記載が特許法第３６条第６項第２号にいう「発明が明確であること」という要件に適合するといえるのは、出願時において当該物をその構造又は特性により直接特定することが不可能であるか、又はおよそ実際的でないという事情（「不可能・非実際的事情」）が存在するときに限られると解するのが相当である（最二小判平成２７年６月５日　平成２４年（受）１２０４号、同２６５８号）。

しかしながら、不可能・非実際的事情が存在することについて、明細書等に記載がなく、また、出願人から主張・立証がされていないため、その存在を認める理由は見いだせない。

したがって、請求項１に係る発明は明確でない。請求項１を引用する請求項２－１０に係る発明も明確でない。

＜補正等の示唆＞

出願人は、上記拒絶理由を解消するため、以下の対応をとることが考えられます。また、特許・実用新案審査ハンドブック第ＩＩ部第２章２２０３～２２０５も適宜参照してください。

ア．当該請求項の削除

イ．当該請求項に係る発明を、物を生産する方法の発明とする補正

ウ．当該請求項に係る発明を、製造方法を含まない物の発明とする補正

エ．不可能・非実際的事情についての意見書等による主張・立証

オ．当該請求項は、「その物の製造方法が記載されている場合」に該当しない旨の反論

補正の際は、出願当初の明細書、特許請求の範囲又は図面（「当初明細書等」）に記載した事項の範囲内で行う必要があることに留意してください。特に上記ウにおいて単に製造方法の記載を削除する補正は、当初明細書等に記載した事項の範囲内でないものになりやすいことに留意してください。

また、上記エの対応をする際には、「出願時において当該物をその構造又は特性により直接特定すること」が不可能又はおよそ非実際的である事情を具体的に記載してください。

・請求項１－１０

請求項１に記載されている有尾類細胞外ＥＣＭは、有尾類からの組織試料の「構造的及び機能的完全性を保持する」ことを含む方法によって生産されるものであることが記載されている。しかし、請求項１を引用する発明である請求項８には、「乾燥粉末」であるＥＣＭが記載されているし、請求項９には「再構成されたゲル」であるＥＣＭが記載されている。乾燥粉末及び再構成されたゲルが、有尾類からの組織試料の構造的完全性及び機能的完全性を有していないことは明らかであるため、請求項１における記載と、請求項８及び請求項９における記載は

相容れない。
そのため、請求項１並びに請求項８及び９に係る発明がどのようなものであるのかが不明である。請求項１を引用する請求項２－７及び１０についても同様である。
したがって、請求項１－１０に係る発明は明確でない。

・請求項４，１０
請求項４において、ＥＣＭが「これに結合されている」と記載されているが、当該記載における「これ」が何を意味しているのかが不明確であるため、請求項４に係る発明が不明確となっている。請求項４を引用する請求項１０についても同様である。

・請求項５，１０
請求項５において「薬剤」が記載され、請求項５は請求項１を引用している。
しかし、請求項１には「薬剤」が記載されていないため、請求項５における「薬剤」が何を指しているのかが不明である。
したがって、請求項５及び請求項５を引用する請求項１０に係る発明は明確でない。

●理由３（サポート要件）について

・請求項１－１０
明細書の記載から理解される本願発明が解決しようとする課題は、容易に入手可能であり、天然の組織が有する材料機能性を維持した移植に適した生物材料を提供することであると認められる。
そして、明細書に開示されている上記課題を解決するための手段は、有尾類由来の組織試料を構造的完全性及び機能的完全性を維持したまま脱細胞化して、ヒトへの使用に適したＥＣＭシートを作製したことである。
しかし、請求項１には、「ヒトまたは動物」への使用のためのＥＣＭが記載されており、ヒト以外の動物への使用のためのＥＣＭが記載されている。
明細書において、有尾類由来のＥＣＭシートは、ヒト羊膜との構造的共通性からヒトへの移植等の使用に適することが考察されているものの、ヒト以外の動物への移植に適していることについては検討されていない。真皮等の生体組織の構造が動物種を問わず共通しているとはいえないため、ヒトへの使用に適したＥＣＭシートが他の任意の動物種への使用に適しているとは認められない。
そのため、請求項１に係る発明が本願の課題を解決するものとは認められない。請求項１を引用する請求項２－１０についても同様である。
したがって、請求項１－１０に係る発明は、発明の詳細な説明に記載したものでない。

・請求項８－１０

請求項８には「乾燥粉末」であるＥＣＭが記載されている。

明細書において、有尾類のＥＣＭシートはその構造的特徴からヒトへの使用に適していると記載されているだけで、粉末化したものであってもヒトへの使用に適したものであったことは示されていない。

有尾類のＥＣＭをヒトの再生医療等へ使用することについての技術常識が本願出願時に存在したとはいえない。

そうすると、明細書に開示された事項を、請求項８に係る発明の範囲まで拡張ないし一般化することができるとは認められない。

請求項９に記載されている「再構成されたゲル」であるＥＣＭについても同様に、有尾類のＥＣＭシートの構造的特徴を有していないものであるため、請求項８と同じ不備が存在する。

したがって、請求項８及び９並びにこれらを引用する請求項１０に係る発明は、発明の詳細な説明に記載したものでない。

●理由４（新規性）及び理由５（進歩性）について

・請求項１－３，６
・引用文献等１
・備考

引用文献１には、Ａｍｂｙｓｔｏｍａ　ｍｅｘｉｃａｎｕｍ（アホロートルに相当）の皮膚を脱細胞化処理して作製した脱細胞ＥＣＭシートを、創傷部位に移植した旨が記載されている（第１９２頁左欄最終段落－右欄第１段落、第１９４頁右欄第２段落、Ｆｉｇ．６）。

したがって、請求項１－３，６に係る発明は、引用文献１に記載された発明である。そうでない部分があるとしても、引用文献１の記載から当業者が容易に想到し得たものである。

●理由５（進歩性）について

・請求項４，５，７－１０
・引用文献等１，２
・備考

引用文献２には、羊膜から産生されるコラーゲン膜であって、天然未処理羊膜の構造的完全性を有し、完全に脱細胞化され、創傷包帯等として医療分野で用いられる膜が記載されている。当該膜は、滅菌溶液中で調製されること、乾燥されること、治療薬や足場剤等の物質が含浸されること、ヒドロゲル組成物を含むこと、ヒトの治療に使用されることが記載されている。（全文、特に、請求項１－

７６）
引用文献１及び２はともに創傷治療等の医療用途に使用されるＥＣＭシートに関する発明が記載されている。
引用文献２に記載されている、ＥＣＭシートに対して種々の薬剤を含浸させたり、ゲル組成物としたり、乾燥したり、滅菌したりする加工方法を参照して、引用文献１に記載されているＥＣＭシートに対しても同様の加工を行うことは、当業者が容易になし得ることである。
明細書において、請求項４，５，７－１０に係る発明の効果が格別なものであることは示されていない。特に、本願のＥＣＭシートを実際に粉末乾燥したことや、ゲルとして再構成したことは記載されていないし、それらの操作による効果についても検討されていないため、請求項８及び９に係る発明の効果は明細書に何ら示されていない。
したがって、請求項４，５，７－１０に係る発明は、引用文献１及び２に記載された発明及び本願優先日前の技術常識に基づいて、当業者が容易に発明をすることができたものである。

＜引用文献等一覧＞
１．ZOOLOGICAL SCIENCE, 2012, Vol.29, pp.191-197
２．特表２００６－５０７８５１号公報
－－

先行技術文献調査結果の記録等
・調査した分野
　　ＩＰＣ　　Ｃ１２Ｎ　５／００
　　ＤＢ名　　PubMed
・先行技術文献
国際公開第２００８／１２９９９７号
特表２００４－５２１９３５号公報
特表２００９－５１３２６９号公報
The Journal of Experimental Zoology, 1982, Vol.220, pp.243-250
特表２００９－５１００９１号公報
特表２００７－５３２１５３号公報
この先行技術文献調査結果の記録は、拒絶理由を構成するものではありません。

＜補正をする際の注意＞
（１）明細書、特許請求の範囲について補正をする場合は、補正により記載を変更した個所に下線を引いてください（特許法施行規則様式第１３備考６、７）。
（２）補正は、この出願の出願当初の明細書、特許請求の範囲又は図面に記載した事項の範囲内で行わなければなりません。また、意見書で、各補正事項につい

て補正が適法なものである理由を、根拠となる出願当初の明細書等の記載箇所を明確に示したうえで主張してください。
（３）特許請求の範囲について補正をする際には、特許法第１７条の２第４項に違反する補正とならないよう、注意してください。

この拒絶理由通知の内容に関するお問い合わせがありましたら次の連絡先までご連絡ください。
なお、審査第三部は六本木仮庁舎から本庁舎へ移転し、令和２年４月３０日（木）から本庁舎での業務を開始します。
移転作業のため、令和２年４月２７日（月）～４月２８日（火）の期間、電話、ＦＡＸでのご連絡をお受けできません。
また、移転後はＦＡＸ番号が変わります（電話番号は変わりません）。ＦＡＸ番号を事前にお電話でご確認ください。
審査第三部生命工学　伊達　利奈
ＴＥＬ．０３－３５８１－１１０１　内線　３４４８
ＦＡＸ．０３－３５８５－２０９３（令和２年４月２４日（金）まで有効）

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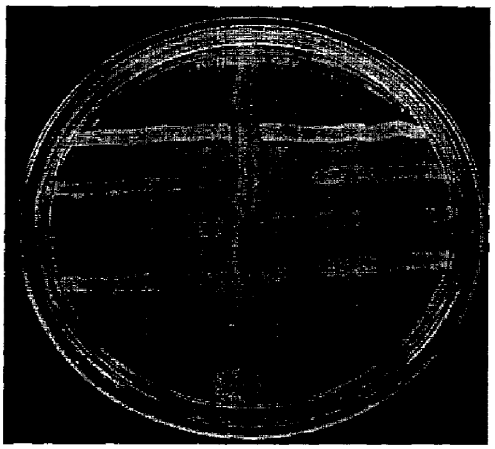
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(86)(22)出願日　平成14年1月4日 (2002.1.4)
(85)翻訳文提出日　平成15年7月7日 (2003.7.7)
(86)国際出願番号　PCT/US2002/000217
(87)国際公開番号　WO2002/078720
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(54)【発明の名称】両生類の皮膚から単離した細菌由来の抗生物質

(57)【要約】
本発明は、少なくとも部分的に皮膚呼吸をしている粘液産生生物から分離された微生物から誘導された生物学的に活性な化合物又は化合物の組合せを含む抽出物を開示する。サラマンダー及びカエルの皮膚から分離された菌は、抗ウィルス、抗腫　、抗菌及び抗真菌特性を有する化合物を産生することを見出した。これらの化合物は、カンジダ属、小胞子菌属、ブドウ球菌属、緑膿菌属、大腸菌属、及び腸球菌属を含むヒト日和見病原体、並びにＨＩＶ株及び腫　細胞系に対する阻害効果を有する。



【特許請求の範囲】
【請求項１】
少なくとも部分的に皮膚呼吸をしている生物の皮膚から分離した細菌からの抽出物であって、細菌、真菌、ウィルス、又は腫　の成長を阻害する抽出物。
【請求項２】
生物が両生類である、請求項１に定義の抽出物。
【請求項３】
生物がサラマンダー類からなる群から選ばれる、請求項１に定義の抽出物。
【請求項４】
生物がカエル類からなる群から選ばれる、請求項１に記載の抽出物。
【請求項５】
抽出物がヒト病原菌又はヒト病原真菌の成長を阻害する、請求項１に記載の抽出物。
【請求項６】
抽出物がヒトＨＩＶの成長を阻害する、請求項１に記載の抽出物。
【請求項７】
ヒト病原菌が、腸球菌属、ブドウ球菌属、大腸菌属、及び緑膿菌属からなる群から選ばれる、請求項６に記載の抽出物。
【請求項８】
抽出物が約５ＰＰｍ～約２５０ＰＰｍの範囲で効果を示す、請求項１に記載の抽出物。
【請求項９】
請求項１に記載の抽出物の治療量をヒトに投与すること。
【請求項１０】
ＤＱ００１Ｄ、ＰＯ０１４、ＰＯ０１９、ＰＯ０２６、ＰＯ０２７、ＡＣ０２１、ＡＣ０２４、ＰＣ０１７、ＰＤ０２６、ＥＧ００６、ＥＣ００９、ＥＣ０２４、及びＲＣ００８からなる群から選ばれる細菌分離株。
【請求項１１】
生物学的に活性な細菌代謝産物を確認する方法であって、請求項１０に記載の細菌分離株を１種類以上の病原微生物を含むアッセイ系で培養し、細菌分離株によって産生された代謝産物の、病原微生物の成長に及ぼす影響を対照と比較して評価することを含む方法。
【請求項１２】
生物学的に活性な抽出物、画分、又は化合物を確認する方法であって、
（ａ）請求項１０に記載の細菌分離株を培養し；
（ｂ）該培養株から代謝産物を抽出し；
（ｃ）場合により、抽出した代謝産物を分画し；そして
（ｄ）適切なアッセイ系で、抽出した又は分画した代謝産物の、病原微生物、腫　細胞、又はウィルスの成長を阻害する能力を評価する
ことを含む方法。
【請求項１３】
請求項１２に記載の方法に従って確認された生物学的に活性な抽出物、画分、又は化合物。
【請求項１４】
請求項１３に記載の一つ以上の生物学的に活性な抽出物、画分、又は化合物と、製薬上許容しうる担体とを含む医薬組成物。
【請求項１５】
組成物が患者への局所、腸内、非経口、又は静脈内投与に適している、請求項１４に記載の医薬組成物。
【請求項１６】
患者における病原微生物の成長阻害法であって、請求項１４に記載の医薬組成物の有効量を該患者に投与することを含む方法。
【請求項１７】

患者における腫　細胞の成長阻害法であって、請求項１４に記載の医薬組成物の有効量を該患者に投与することを含む方法。
【請求項１８】
患者におけるウィルスの成長阻害法であって、請求項１４に記載の医薬組成物の有効量を該患者に投与することを含む方法。
【請求項１９】
ウィルスがヒト免疫不全ウィルスである、請求項１８に記載の方法。
【発明の詳細な説明】
【０００１】
先願
本願は、仮出願第６０／２６０，０２２号、出願日２００１年１月５日に基づく優先権を主張する。
【０００２】
背景技術
すべての両生類は、程度の差こそあれ、皮膚呼吸をしている。従って、両生類の外皮は、外部環境に対するガス透過性バリヤとしての役割を果たさねばならない［Ｌｉｌｌｙｗｈｉｔｅ，Ｈ．Ｂ．，及びＰ．Ｆ．Ａ．Ｍａｄｅｒｓｏｎ。１９８８年。外皮の構造と透過性(The structure and permeability of the integument)。Ａｍｅｒｉｃａｎ　Ｚｏｏｌｏｇｉｓｔ　２８：９４５－９６２］。皮膚が呼吸器として利用されるには水分が不可欠である［Ｆｏｘ，Ｈ．１９９４年。外皮の構造(Structure of the integument)。“Ａｍｐｈｉｂｉａｎ　Ｂｉｏｌｏｇｙ．Ｖｏｌ　１．Ｔｈｅ　Ｉｎｔｅｇｕｍｅｎｔ”の第１章、Ｈ．Ｈｅａｔｗｏｌｅ及びＧ．Ｔ．Ｂａｒｔｈａｌｍｕｓ編、Ｓｕｒｒｅｙ　Ｂｅａｔｔｙ　ａｎｄ　Ｓｏｎｓ，チッピング・ノートン］。この必要な水分は、外皮に付随する粘液産生腺による粘液の産生によって達成される（Ｄｕｅｌｌｍａｎ，Ｗ．Ｅ．，及びＬ．Ｔｒｕｅｂ。１９８６年。Ｂｉｏｌｏｇｙ　ｏｆ　ＡｍＰｈｉｂｉａｎｓ。ＭｃＧｒａｗ　Ｈｉｌｌ，米国ニューヨーク州ニューヨーク；Ｆｏｘ　１９９４年）。両生類の粘液の主成分はムコ多糖（糖タンパク質）である（Ｄｕｅｌｌｍａｎ及びＴｒｕｅｂ　１９８６年）。糖タンパク質は、ポリペプチド骨格に共有結合している一つ以上の炭水化物鎖を含有する［Ｓｃｈａｅｃｈｔｅｒ，Ｍ．，及びＩ．Ｂｒｏｃｋｈａｕｓｅｎ。１９８９年。分枝Ｏ－グリカンの生合成(The biosynthesis of branched O glycans)。粘液及び関連トピックスに収載。Ｅ．Ｃｈａｎｔｌｅｒ及びＮ．Ａ．Ｒａｔｃｌｉｆｆｅ（編）、Ｓｙｍｐｏｓｉａ　ｏｆ　ｔｈｅ　Ｓｏｃｉｅｔｙ　ｆｏｒ　ＥＸＰｅｒｉｍｅｎｔａｌ　Ｂｉｏｌｏｇｙ，ｎｏ．XLIII、ケンブリッジ大学、ケンブリッジ］。粘液は、微生物の成長及び全部ではないがほとんどの細胞化合物の合成を支える必須元素の炭素に富んでいる［Ｇｕｉｒａｒｄ，Ｂ．Ｍ．，及びＥ．Ｅ．Ｓｎｅｌｌ。１９６２年。微生物の栄養必要量(Nutritional requirements of microorganisms)。ＰＰ．３３－９８。Ｉ．Ｃ．Ｇｕｎｓａｌｉｓ及びＲ．Ｙ．Ｓｔａｎｃｉｅｒ（編）、Ｔｈｅ　Ｂａｃｔｅｒｉａに収載。Ａ　Ｔｒｅａｔｉｓｅ　ｏｎ　Ｓｔｒｕｃｔｕｒｅ　ａｎｄ　Ｆｕｎｃｔｉｏｎ。Ｖｏｌ．ＩＶ：Ｔｈｅ　ＰｈｙＳｉｏｌｏｇｙ　ｏｆ　Ｇｒｏｗｔｈ。Ａｃａｄｅｍｉｃ　ＰｒｅＳＳ、米国ニューヨーク州ニューヨーク］。従って、両生類が皮膚呼吸を達成するために必然的に産生している粘液層は、微生物にとって栄養豊富な生息環境となる［Ａｕｓｔｉｎ，Ｊｒ．，Ｒ．Ｍ．２０００年。プレトドン・ベントラリスにおける皮膚の微生物フローラと抗生作用：プレトドン科における子育てに関する推論(Cutaneous microbial flora and antibiosis in Plethodon ventralis: inferences for parental care in the Plethodontidae)。ＰＰ．４５１－４６１。Ｒ．Ｃ．Ｂｒｕｃｅ，Ｒ．Ｇ．Ｊａｅｇｅｒ及びＬ．Ｄ．Ｈｏｕｃｋ（編）、Ｔｈｅ　Ｂｉｏｌｏｇｙ　ｏｆ　Ｐｌｅｔｈｏｄｏｎｔｉｄ　Ｓａｌａｍａｎｄｅｒｓに収載、Ｋｌｕｗｅｒ　Ａｃａｄｅｍｉｃ／Ｐｌｅｎｕｍ　Ｐｕｂ．，米国ニューヨーク州ニューヨーク］。
【０００３】
ほとんどの生態系において大多数の微生物は表面に付着するので［ＷｉｍＰｅｎｎｙ，Ｊ

．Ｗ．Ｔ．，Ｓ．Ｌ．Ｋｉｎｎｉｍｅｎｔ，及びＭ．Ａ．Ｓｃｏｕｒｆｉｅｌｄ。１９９８年。バイオフィルムの生理学と生化学(The physiology and biochemistry of biofilms)。ＰＰ．２７４－３１８。Ｓ．Ｄｅｎｙｅｒ．Ｐ．Ｇｏｒｍａｎ．及びＭ．Ｓｕｓｓｍａｎ（編）、Ｍｉｃｒｏｂｉａｌ Ｂｉｏｆｉｌｍｓ：Ｆｏｒｍａｔｉｏｎ ａｎｄ Ｃｏｎｔｒｏｌに収載。Ｂｌａｃｋｗｅｌｌ Ｓｃｉｅｎｔｉｆｉｃ Ｐｕｂｌｉｃａｔｉｏｎｓ、英国ロンドン］、動物の外皮は微生物共同体の発達に適した生息環境として働くことが多い（Ａｌｅｘａｎｄｅｒ．Ｍ．１９７１年。Ｍｉｃｒｏｂｉａｌ Ｅｃｏｌｏｇｙ。Ｊｏｈｎ Ｗｉｌｅｙ ａｎｄ Ｓｏｎｓ、米国ニューヨーク州ニューヨーク）。これらのミクロ共同体は、より大きい生物の共同体（マクロ共同体）が示すのと同じ種類の共同体レベルの相互作用を示すことが多い。ミクロ共同体のメンバーは限られた資源を巡って競合し、親密関係、時に偏害作用関係を築く［Ａｔｌａｓ．Ｒ．Ｍ．，及びＲ．Ｂａｒｔｈａ。１９９８年。Ｍｉｃｒｏｂｉａｌ Ｅｃｏｌｏｇｙ。Ｆｕｎｄａｍｅｎｔａｌｓ ａｎｄ ＡＰＰｌｉｃａｔｉｏｎｓ、第３版。Ｂｅｎｊａｍｉｎ Ｃｕｍｍｉｎｇｓ、米国ニューヨーク州ニューヨーク；Ｂｕｌｌ．Ａ．Ｔ．，及びＪ．Ｈ．Ｓｌａｔｅｒ。１９８２年。Ｍｉｃｒｏｂｉａｌ Ｉｎｔｅｒａｃｔｉｏｎｓ ａｎｄ Ｃｏｍｍｕｎｉｔｉｅｓ。Ｖｏｌ．１。Ａｃａｄｅｍｉｃ Ｐｒｅｓｓ、米国ニューヨーク州ニューヨーク；Ｆｒｅｄｅｒｉｃｋｓｏｎ．Ａ．Ｇ．，及びＧ．ＳｔｅＰｈａｎｏＰｏｕｌｏｓ。１９８１年。微生物の競合(Microbial competition)。Ｓｃｉｅｎｃｅ ２１３：９７２－９７９］。このような偏害作用戦略の一つは、抗生物質の産生である。

【０００４】

抗生物質は、微生物が産生する、他の微生物を殺滅したり成長を阻害することが可能な物質である［ＷｉｌｌｉａｍＳ．Ｓ．Ｔ．１９８２年。抗生物質は土壌中に産生されるか(Are antibiotics produced in soil)。Ｐｅｄｏｂｉｏｌｏｇｙ ２３：８５－８７；Ｗｉｌｌｉａｍｓ．Ｓ．Ｔ．，及びＪ．Ｃ．Ｖｉｃｋｅｒｓ。１９８６年。抗生物質産生の生態学(The ecology of antibiotic production)。Ｍｉｃｒｏｂｉａｌ Ｅｃｏｌｏｇｙ １２：４３－５２；Ｂｒｏｃｋ．Ｔ．Ｄ．１９９４年。Ｔｈｅ Ｂｉｏｌｏｇｙ ｏｆ Ｍｉｃｒｏｏｒｇａｎｉｓｍｓ。第７版、Ｐｒｅｎｔｉｃｅ Ｈａｌｌ．Ｅｎｇｌｅｗｏｏｄ Ｃｌｉｆｆｓ、ニュージャージー州］。このような物質の産生は、自然環境中の競合を削減又は阻害するための適応とみなされてきた（Ｗｅｉｎｅｒ １９９６年）。固体及び液体培地における抗生物質産生菌と抗生物質感受性菌間の競合研究により、この理論を裏付ける証拠が提供されている［例えば、Ｒａｓｏｏｌ．Ｋ．，及びＪ．Ｗ．Ｔ．ＷｉｍＰｅｎｎｙ。１９８３年。抗生物質産生ストレプトミセス菌と大腸菌を用いた混合連続培養実験(Mixed continuous culture experiments with an antibiotic producing streptomycete and Escherichia coli)。Ｍｉｃｒｏｂｉａｌ Ｅｃｏｌｏｇｙ ８：２６７－２７７；ＴｕｒＰｉｎ．Ｐ．Ｅ．，Ｖ．Ｋ．Ｄｉｈｒ．Ｋ．Ａ．Ｍａｙｃｒｏｆｔ．Ｃ．Ｒｏｗｌａｎｄｓ．及びＥ．Ｍ．Ｈ．Ｗｅｌｌｉｎｇｔｏｎ。１９９２年。土壌中のサルモネラの生存に及ぼすストレプトミセス属の影響(The effect of Streptomyces species on the survival of Salmonella in soil)。ＦＥＭＳ Ｍｉｃｒｏｂｉｏｌ．Ｅｃｏｌ．１０１：２７１－２８０］。実験室での実験によれば、抗生物質の産生は競合菌の侵入を防止するものの、確立された抗生物質感受性微生物集団を侵害したりそれと競合する能力までは改善しないことを示している［Ｗｉｅｎｅｒ．Ｐ．１９９６年。細菌の抗生物質産生の生態学的役割に関する実験的研究(Experimental studies on the ecological role of antibiotic production in bacteria)。Ｅｖｏｌｕｔｉｏｎａｒｙ Ｅｃｏｌｏｇｙ １０：４０５－４２１］。実験室での実験では抗生物質産生に証拠があるにもかかわらず、抗生物質の自然の役割及びそれらの産生条件は不明のままである（Ｗｉｅｎｅｒ １９９６年）。

【０００５】

プレトドン科のサラマンダー(salamander)は、現存するサラマンダーの中で最大の科を構成する。その仲間は多くの森林及び流水生態系で最も数の多い脊椎動物の一つである。肺を持たないというのが最も特筆すべき特徴である。この科の示す生活史は極めて多彩であ

る［例えば、Ｔｉｌｌｅｙ，Ｓ．Ｇ．，及びＪ．Ｂｅｒｎａｒｄｏ。１９９８年。プレトドン科サラマンダーにおける生活史の進化(Life history evolution in plethodontid salamanders)。ＨｅｒＰｅｔｏｌｏｇｉｃａ ４９：１５４－１６８；Ｗａｋｅ，Ｄ．Ｂ．，及びＳ．Ｍ．Ｍａｒｋｓ。１９９８年。プレトドン科サラマンダーの発生と進化：過去の研究のレビューと将来の研究に対する展望(Development and evolution of plethodontid salamanders: a review of prior studies and a prospectus for future research)。ＨｅｒＰｅｔｏｌｏｇｉｃａ ４９：１９４－２０３］。このように多様であるにもかかわらず、それらの繁殖生物学における保守性、特に卵の世話という形態でのほぼ普遍的な子育て［Ｓａｌｔｈｅ，Ｓ．Ｎ．，及びＪ．Ｓ．Ｍｃｃｈａｍ。１９７４年。繁殖及び求愛パターン(Reproductive and courtship patterns)。ＰＰ．Ｂ．ＬｏｆｔＳ（編）、ＰｈｙＳｉｏｌｏｇｙ ｏｆ ｔｈｅ ＡｍＰｈｉｂｉａに収載。Ｖｏｌ．２：Ａｃａｄｅｍｉｃ ＰｒｅＳＳ、米国ニューヨーク州ニューヨーク］はよく報告されている［例えば、Ｆｏｒｅｓｔｅｒ，Ｄ．Ｃ．１９７８年。卵を守るマウンテンダスキーサラマンダー（両生類、有尾目、プレトドン科）の雌と可能性ある捕食者間の実験室での遭遇(Laboratory encounters between attending Desmognathus ochrophaeus(Amphibia, Urodela, Plethodontidae) females and potential predators)。Ｊｏｕｒｎａｌ ｏｆ ＨｅｒＰｅｔｏｌｏｇｙ １２：５３７－５４１，１９７９年；Ｈｉｇｈｔｏｎ，Ｒ．，及びＴ．Ｓａｖａｇｅ。１９６１年。雌のセアカサラマンダーにおける抱卵行動の機能(Functions of the brooding behavior in the female red backed salamander, Plethodon cinereus)。Ｂｕｌｌｅｔｉｎ ｏｆ ｔｈｅ Ｆｌｏｒｉｄａ Ｓｔａｔｅ Ｍｕｓｅｕｍ ６：２３５－２３７；Ｐｉｅｒｓｏｌ，Ｗ．Ｈ．１９０９年。プレトドン・エリスロノトゥスの習性と幼態(The habits and larval state of Plethodon erythronotus)。ＴｒａｎＳａｃｔｉｏｎＳ ｏｆ ｔｈｅ Ｃａｎａｄｉａｎ Ｉｎｓｔｉｔｕｔｅ ８：４６９－４９３；Ｒｉｔｔｅｒ，Ｗ．Ｅ．１９０３年。アウトダクス・ルグブリスの習性に関する追加メモ(Further notes on the habits of Autodax lugubris)。Ａｍｅｒｉｃａｎ Ｎａｔｕｒａｌｉｓｔ ３７：８８３－８８６；Ｔｉｌｌｅｙ，Ｓ．Ｇ．１９７２年。ノースカロライナ州西部のマウンテンダスキーサラマンダー（両生類：プレトドン科）の子育てと 発生の様子(Aspects of parental care and embryonic development in Desmognathus ochrophaeus(Amphibia: Plethodontidae) in western North Carolina)。Ａｍｅｒｉｃａｎ Ｍｉｄｌａｎｄ Ｎａｔｕｒａｌｉｓｔ ８９：３９４－４０７］。

【０００６】

子育ては、接合後の親による子孫への投資と定義できる（ＴｒｉｖｅｒＳ，Ｒ．Ｌ．１９８５年。Ｓｏｃｉａｌ Ｅｖｏｌｕｔｉｏｎ。Ｂｅｎｊａｍｉｎ ＣｕｍｍｉｎｇＳ、米国ニューヨーク州ニューヨーク）。プレトドン科の中では、この投資は、ほとんどの場合雌親による卵の世話という形態で現れる［ＣｒｕｍＰ，Ｍ．Ｌ．１９９５年。子育て(Parental care)。ＰＰ．５１８－５６７。Ｈ．Ｈｅａｔｗｏｌｅ及びＢ．Ｋ．Ｓｕｌｌｉｖａｎ（編）、ＡｍＰｈｉｂｉａｎ Ｂｉｏｌｏｇｙに収載。Ｖｏｌ．２：Ｓｏｃｉａｌ Ｂｅｈａｖｉｏｕｒ。ＳｕｒｒｅｙＳ Ｂｅａｔｔｙ ａｎｄ ＳｏｎＳ、豪ニューサウスウェールズ州チッピング・ノートン］。ほとんどの場合、雌は 化が起こるまでクラッチ（一腹卵）と共にとどまる。プレトドン科におけるこのような行動の適応利益は明確に確立されており、捕食者、非病原性の常在土壌真菌種による真菌感染、乾燥からの保護、及び発生異常の防止を含む（ＣｒｕｍＰ、１９９５年、包括的概説参照）。子育ては、行動レパートリー（例えば、クラッチへの身体接触、卵いじり、咽頭による脈動）からなり、これら複数の利益は明らかに発生中の子に対しても同時に授けられる。

【０００７】

プレトドン科の中での子育ての利益は明確に報告されているが、ある側面が達成されるメカニズムは最近まで不明のままであった（例えば、Ａｕｓｔｉｎ ２０００年）。この状況は、雌が寄り添うとクラッチの真菌感染が減少することに関する前世紀来の多くの観察に関して特にそうであった。こうした観察から、雌（例えば、Ｎｏｂｌｅ，Ｇ．Ｋ．１９３１年。Ｔｈｅ Ｂｉｏｌｏｇｙ ｏｆ ｔｈｅ ＡｍＰｈｉｂｉａ。ＭｃＧｒａｗ Ｈ

ｉｌｌ、米国ニューヨーク州ニューヨーク：ＰｉｅｒＳｏｌ，１９０９年）又は発生中の子（Ｈｉｇｈｔｏｎ及びＳａｖａｇｅ、１９６１年）のいずれかが、世話されているクラッチ上での真菌の成長を削減又は阻害できる抗真菌代謝産物を産生しているという仮説が導かれた。しかしながら、その後の研究でも、サラマンダーからの抗菌分泌物の存在は示されていない［Ｄａｎｉｅｌ，Ｊ．Ｃ．，Ｊｒ．，及びＲ．Ｗ．ＳｉｍＰｓｏｎ。１９５４年。抗生物質に関する否定的報告(A negative note on antibiotics)。ＨｅｒＰｅｔｏｌｏｇｉｃａ　１０：１６；Ｆｏｒｅｓｔｅｒ，Ｄ．Ｃ．１９７９年。マウンテンダスキーサラマンダー（有尾目：プレトドン科）における子育ての適応性(The adaptiveness of parental care in Desmognathus ochrophaeus(Urodela: Plethodontidae))。ＣｏＰｅｉａ　１９７９年：３３２－３４１；Ｔｉｌｌｅｙ、１９７２年；Ｖｉａｌ，Ｊ．Ｌ．，及びＦ．Ｂ．Ｐｒｉｅｂ。１９６６年。サラマンダーのプレトドン・シネレウス（グリーン）からの皮膚分泌物の抗生物質アッセイ(Antibiotic assay of dermal secretions from the salamander, Plethodon cinereus(Green))。ＨｅｒＰｅｔｏｌｏｇｉｃａ　２２：２８４－２８７；Ｖｉａｌ，Ｊ．Ｌ．，及びＦ．Ｂ．Ｐｒｉｅｂ。１９６７年。サラマンダーのプレトドン・シネレウスにおける抱卵行動の機能としての抗生作用の調査(An investigation of antibiosis as a function of brooding behavior in the salamander, Plethodon cinereus)。Ｔｒａｎｓａｃｔｉｏｎｓ　ｏｆ　ｔｈｅ　Ｍｉｓｓｏｕｒｉ　Ａｃａｄｅｍｙ　ｏｆ　Ｓｃｉｅｎｃｅ　１：３７－４０］。両生類の皮膚環境を可能性ある医学的に有用な細菌源として利用する研究者の発表報告は知られていない。両生類及び他の生物由来の医学的に有用な化合物に関する研究の多くは、両生類及び他のマクロ生物の皮膚分泌物中におけるそのような化合物の産生に焦点を当て、そのような生物の皮膚に生息する細菌フローラには目を向けてこなかった［例えば、ＧｌａｕｓｉｕｓＺ，Ｊ．１９８８年。カエルの液(The frog solution)。Ｄｉｓｃｏｖｅｒ　１９：（１１）８８－９２；Ｃａｒｔｅ，Ｂ．Ｋ．１９９６年。天然水産物の生物医学的可能性(Biomedical potential of marine natural products)。ＢｉｏＳｃｉｅｎｃｅ　４６（４）：２７１－２８７；Ｐｏｒｔ，Ｏ．２００１年。カエル由来の薬物研究における躍進(A leap in drug research from frogs)。Ｂｕｓｉｎｅｓｓ　Ｗｅｅｋ　３７５２：９９；Ｔｙｌｅｒ，Ｍ．Ｊ．１９９５年。カエルと薬物(Frogs and Drugs)。Ａｕｓｔｒａｌｉａｎ　Ｎａｔｕｒａｌ　Ｈｉｓｔｏｒｙ　２４（１２）：４６－５２；Ｃｏｇｈｌａｎ，Ａ．２０００年。バグ・バスター(Bug busters)。Ｎｅｗ　Ｓｃｉｅｎｔｉｓｔ．１６６（２２３３）：１５；Ｓｔｉｅｎｂｏｒｎｅｒ，Ｓ．Ｔ．，Ｇ．Ｊ．Ｃｕｒｒｉｅ，Ｊ．Ｈ．Ｂｏｗｉｅ，Ｊ．Ｃ．Ｗａｌｌａｃｅ，及びＭ．Ｊ．Ｔｙｌｅｒ。１９９８年。オーストラリアアマガエルのアカメアメガエルの皮膚由来の新規抗生物質カエリン１ペプチド(New antibiotic caerin 1 peptides from the skin of the Australian tree frog Litoria chloris)。ミナミアマガエル属由来のカエリン１ペプチドの活性の比較(Comparison of the activities of the caerin 1 peptides from the genus Litoria)。Ｊｏｕｒｎａｌ　ｏｆ　ＰｅＰｔｉｄｅ　Ｒｅｓｅａｒｃｈ，５１：（２），１２１－１２７；Ｒｅｎｎｉｅ，Ｊ．１９９８年。ここにはインチキ薬はない(No snake oil here)。Ｓｃｉｅｎｔｉｆｉｃ　Ａｍｅｒｉｃａｎ　２６６（３）１３６－１３７；ＭｃＮａｍｅｅ，Ｄ．１９９４年。イモリの眼、カエルの爪先(Eye of newt, toe of frog)。Ｌａｎｃｅｔ．３４４（８９３８）：１６９６－１６９８；ＥｒＳＰａｍｅｒ　１９９４年］。

【０００８】

皮膚呼吸を容易にするために産生される粘液が栄養豊富な性質を持つため、プレトドン科サラマンダーの成功に関する他の側面は、常在する微生物フローラに帰することができる。プレトドン科サラマンダーは呼吸ガスの交換を皮膚だけに頼らなければならないため、皮膚表面に生息している好気性土壌菌を積み過ぎると、呼吸ガスを通さないバリヤとなり、最終的にはサラマンダーを死に至らしめることになる。

【０００９】

両生類の栄養豊富な皮膚環境は、周囲環境のそれとは明らかに異なる微生物共同体を支持することが示されている［Ａｕｓｔｉｎ，Ｊｒ．，Ｒ．Ｍ．１９９７。トウブジグザグサ

ンショウウウオ（両生類：プレトドン科）の皮膚細菌フローラ(The cutaneous bacterial flora of the eastern zi9za9 salamander, Plethodon dorsalis Cope(Amphibia;Plethodontidae))。博士論文。ミシシッピ大学、ミシシッピ州ユニバーシティ]。微生物は、栄養豊富な環境で、抗生物質の産生（Ｆｒｅｄｅｒｉｃｋｓｏｎ及びＳｔｅＰｈａｎｏＰｏｕｌｏＳ、１９８１年）を含む偏害作用戦略を利用することが示されていることを考えれば、両生類の皮膚環境は、微生物の成長を削減又は阻害する化合物を産生できる微生物を支持し、そうすることで両生類の生態学及び生活史に競合的利益を付与することが予測できる。驚くべきことに、少なくとも部分的に皮膚呼吸をする生物の皮膚環境から分離されたある種の微生物によって産生される代謝産物は、重要なヒト病原菌及び病原真菌に対して抗菌活性を示し、また抗ウィルス及び抗腫　活性も有することが最近確認された。

【００１０】

発明の要旨

本発明は、少なくとも部分的に皮膚呼吸をしている粘液産生生物の体から分離された微生物によって産生される化合物及び化合物の組合せを含む抽出物からなる。該抽出物はヒトにおける抗腫　、抗ウィルス、抗真菌、及び／又は抗菌活性を有する。更に詳しくは、本発明は、皮膚呼吸をしている動物の粘液中で成長する細菌によって産生される抗生物質からなる。尚更に詳しくは、本発明は、両生類の皮膚から分離された細菌によって産生される、抗ウィルス活性、抗腫　活性、ヒトに対して病原性のある細菌及び／又は真菌に対する活性を含む抗菌及び／又は抗真菌活性を有する化合物及び／又は化合物の組合せからなる。具体例は、サラマンダー及びカエルの皮膚から分離された細菌によって産生される、カンジダ属(Candida sp.)、小胞子菌属(Microsporum sp.)、連鎖球菌属(Streptococcus sp.)（ペニシリン耐性連鎖球菌を含む）、ブドウ球菌属(Staphylococcus sp.)（メチシリン耐性ブドウ球菌を含む）、腸球菌属(Enterococcus sp.)（バンコマイシン耐性腸球菌を含む）、緑膿菌属(Pseudomonas sp.)、大腸菌属(Escherichia sp.)に対する活性、ヒトリンパ球のＣＥＭ及びＭＴ－４中のＨＩＶ株の細胞変性の削減を含むヒト免疫不全ウィルス（ＨＩＶ）株に対する活性、並びに腫　細胞のｆ－マウス白血病、マウス乳がん、及びヒトＴ－リンパ球細胞に対する活性を有する化合物を含む抽出物などである。

【００１１】

本発明の目的は、少なくとも部分的に皮膚呼吸をしている生物の皮膚由来の細菌分離株からの、抗菌、抗真菌、抗ウィルス、又は抗腫　効果を有する化合物又は化合物の組合せの抽出である。

【００１２】

本発明の別の目的は、少なくとも部分的に皮膚呼吸をしている生物の皮膚由来の細菌分離株の確認である（該細菌分離株によって産生され分離株から抽出される化合物は、抗菌、抗真菌、抗ウィルス、又は抗腫　効果を有する）。

【００１３】

好適な態様の詳細な説明

本発明は、少なくとも部分的に皮膚呼吸をしている動物の皮膚微生物フローラから採取した細菌分離株の確認と、該分離株からの、抗菌、抗真菌、抗ウィルス、又は抗腫　効果を有する化合物又は化合物の組合せ（抽出物）の抽出とを含む。分離株の確認法と抽出物の製造法について説明する。抽出物は、単一の活性化合物、化合物の組合せ、又は抽出物中の他の化合物との組合せで見つかったときに相乗効果を有する一つ以上の化合物を含みうる。

【００１４】

原料となる生物は、それらの野生環境で採集し隔離しておく。隔離期間後、生物の皮膚細菌フローラの試料を成長させ、形態学的特徴に基づいて分離する。細菌分離株と該分離株からの抽出物の、特定の細菌株に対する抗菌効果を培地上で試験する。液体抽出技術を用いて細菌分離株から抽出物を調製し、該抽出物の、特定の細菌及び真菌株の成長阻害能を測光評価法を用いて試験する。抽出物の成分の、細胞培養物の細胞変性を阻害する能力を試験する。抽出物の成分の、がん細胞の増殖を阻害する能力を試験する。

【００１５】
実施例１－クロハラサラマンダーの皮膚微生物フローラ及び病原菌に対する抗生作用
クロハラサラマンダー(Desmognathus quadramaculatus)種の抱卵雌をピードモント自然園（Ｐｉｅｄｍｏｎｔ　Ｎａｔｕｒｅ　Ｇａｒｄｅｎ）で採集し、採集地の落葉や土壌を詰めた３．７９リットルビニル袋に入れ、旅行用クーラーに入れ、研究室に輸送した。このサラマンダーを濡らしたろ紙を敷いたガラスペトリ皿に隔離し、１週間６～７℃に維持した。
【００１６】
隔離期間後、サラマンダーの肩帯と腰帯間の背を細菌学用ループ（白金耳）で画線して細菌を採取した。細菌試料は、トリプシン大豆酵母エキス（ＴＳＹＥ）寒天（２９のＤｉｆｃｏ　Ｂａｃｔｏトリプシン大豆ブロス、１９のＤｉｆｃｏ　Ｂａｃｔｏ酵母エキス、１リットルの水に対し１２９のＤｉｆｃｏ　Ｂａｃｔｏ精製寒天）上で培養し、室温で７２時間成長させた。形態学的に異なる細菌コロニーをＴＳＹＥ寒天上に再分離し、７２時間成長させて純培養物を得た。純培養物が得られたら、分離株をＴＳＹＥ寒天斜面培地上で培養し、室温で２週間成長させ、必要時まで５℃で保管した。
【００１７】
プレートは暗室で周囲温度でインキュベートした。翌日、プレート上で成長はさほど観察されなかった。１スリーブ当たり２０枚のＴＳＹＥプレートを３スリーブ用意した。翌日、抱卵中のクロハラサラマンダーの雌からの４枚のプレートは驚くほどわずかな成長しか観察されず、４枚のプレートに７個のコロニーが観察されただけあった。分離株を新しいＴＳＹＥプレートに塗り広げて分離し、暗室で周囲温度でインキュベートした。
【００１８】
翌日観察すると、６個のコロニーが認められたので、ＤＱ００１Ａ、ＤＱ００１Ｂ、ＤＱ００１Ｃ、ＤＱ００１Ｄ、ＤＱ００１Ｅ、及びＤＱ００１Ｆとして識別した。ＤＱ００１Ｄは、とりわけエッジが不透明で中心部が白色のコロニーであった。
【００１９】
５種類の公知のヒト日和見病原体、表皮ブドウ球菌(Staphylococcus epidermidis)、エンテロバクター・エロゲネス(Enterobacter aerogenes)、エンテロコッカス・フェカーリス(Enterococcus faecalis)、コリネバクテリウム・ゼロシース(Corynebacterium xerosis)、及びセラチア・マルセッセンス(Serratia marcescens)の平行線条をつけた６枚のプレートを用意した。プレートを３７℃で７２時間インキュベートした。次に、６枚のプレートのそれぞれに、６種類の分離株ＤＱ００１Ａ－Ｆを１種類ずつ塗り広げた。次いで、６枚のプレートを３７℃で７２時間インキュベートした。
【００２０】
次に、６枚のプレートを観察し、６種類の分離株のいずれかが５種類のヒト日和見病原体のいずれかに阻害効果を示したかどうかを判定した。分離株ＤＱ００１Ｄは、表皮ブドウ球菌（阻止帯＝３７ｍｍ）、エンテロコッカス・フェカーリス（阻止帯＝３２ｍｍ）、及びコリネバクテリウム・ゼロシース（阻止帯＝６０ｍｍ）に対して阻害を示したが、他の２種類の病原体に対しては阻害を示さなかったことが観察された。他の分離株はいずれも何の阻害も示さなかった。
【００２１】
実施例２－サラマンダーの皮膚微生物フローラと病原菌及び病原真菌に対する抗生作用
材料と方法
原料生物の採集
１種類あたり最低５個体で構成される（ｎ＝５）１６種類の両生類を、自然の固有生息地から採集した。種と採集地を表１にまとめた。
【００２２】
【表１】

表１－原料生物の採集データの概要

科	種	生物	生息地
プレトドン	デスモグナトゥス・フォルケルツィ (*Desmognathus folkertsi*)	サラマンダー	川辺
プレトドン	スポテッドダスキーサラマンダー (*Desmognathus fuscus*)	サラマンダー	川辺
プレトドン	クロハラサラマンダー (*Desmognathus quadramaculatus*)	サラマンダー	川辺
プレトドン	ユーリセア・シリゲラ(*Eurycea cirrigera*)	サラマンダー	沼地
プレトドン	ミスジサラマンダー(*Eurycea guttolineata*)	サラマンダー	川辺
プレトドン	ケイブサラマンダー(*Eurycea lucifuga*)	サラマンダー	洞穴
プレトドン	プレトドン・アウレオルス(*Plethodon aureolus*)	サラマンダー	森林地帯
プレトドン	プレトドン・チャッタフーチー (*Plethodon chattahoochee*)	サラマンダー	森林地帯
プレトドン	プレトドン・クロロブリオニス (*Plethodon chlorobryonis*)	サラマンダー	森林地帯
プレトドン	プレトドン・ドルサリス(*Plethodon dorsalis*)	サラマンダー	森林地帯
プレトドン	プレトドン・オクムルジー(*Plethodon ocmulgee*)	サラマンダー	森林地帯
プレトドン	プレトドン・オコナルフティー(*Plethodon oconaluftee*)	サラマンダー	森林地帯
トラフサンショウウオ	マーブルサラマンダー(*Ambystoma opacum*)	サラマンダー	沼地
アカガエル	ブロンズガエル(*Rana clamitans*)	カエル	沼地
アマガエル	コオロギガエル(*Acris crepitans*)	カエル	川辺
アマガエル	トリゴエアマガエル(*Pseudacris crucifer*)	カエル	川辺

【００２３】
採集したら、両生類の種を３．７９リットルのビニル袋に入れて採集地から捕獲し、旅行用クーラーに入れて研究室に輸送した。両生類の種を湿らせたろ紙を敷いた０．０９４６Ｌのプラスチック容器に個別に隔離し、Ｃｏｒｏｎモデル６０１０　Ｅｎｖｉｒｏｎｍｅｎｔａｌ　Ｃｈａｍｂｅｒに入れ、１週間１６℃に維持した。

【００２４】
隔離期間後、各種の両生類から細菌を採取し、両生類の種から分離された、表現型が異なる細菌に、該分離株を採取した個体に対応するコードを振った。各細菌分離株の表現型は、標準のコロニー形態学技術（Ｓａｌｌｅ及びＭｅａｃｈｅｍ、１９７４年）によって特徴付けした。この特徴付けは、異なる各細菌コロニーについて、コロニー全体の形状、辺縁（エッジ）の形状、隆起、色、及びフィニッシュを記録することによって達成した。細菌分離株のグラム染色は、細菌分離株の確認と分類に役立つＳａｌｌｅ，Ａ．Ｊ．１９７３年、Ｌａｂｏｒａｔｏｒｙ　Ｍａｎｕａｌ　ｏｎ　Ｆｕｎｄａｍｅｎｔａｌ　Ｐｒｉｎｃｉｐｌｅｓ　ｏｆ　Ｂａｃｔｅｒｉｏｌｏｇｙ、ＭｃＧｒａｗ　Ｈｉｌｌ，米国ニューヨーク州ニューヨークに概説してあるようなグラム染色技術によって判定した。細菌分離株の細胞形態学も記録した。コロニーと細胞の特徴付けの後、原料生物から分離した形態学的に異なる各細菌をＴＳＹＥ寒天斜面培地上で培養し、室温で２週間成長させ、必要時まで５℃で保管した。

【００２５】
寒天プレート阻害試験法
原料生物から分離された形態学的に異なる細菌分離株の、グラム陽性及びグラム陰性ヒト病原菌に対して有効な抗菌化合物産生能力を試験した。阻害試験に利用したヒト病原菌種は、エンテロコッカス・フェカーリス（ＡＴＣＣ＃２９２１２）、大腸菌(Escherichia coli)（ＡＴＣＣ＃２５９２２）、緑膿菌(Pseudomonas aeruginosa)（ＡＴＣＣ＃２７８５３）及び黄色ブドウ球菌(Staphylococcus aureus)（ＡＴＣＣ＃２９２１３）であった。
【００２６】
寒天プレート阻害試験は、原料生物から分離した形態学的に異なる各細菌の、ヒト病原菌の成長阻害能を、Ａｌｃａｍｏ，Ｉ．Ｅ．１９９４年、Ｌａｂｏｒａｔｏｒｙ Ｆｕｎｄａｍｅｎｔａｌｓ ｏｆ Ｍｉｃｒｏｂｉｏｌｏｇｙ，Ｂｅｎｊａｍｉｎ Ｃｕｍｍｉｎｇｓ、米国ニューヨーク州ニューヨーク、に記載の方法とプロトコルに従って試験することから成った。原料生物由来の細菌分離株を個々にＴＳＹＥ寒天プレート上に細菌学用ループ（白金耳）で中央画線で画線した。次に、プレートを室温で最低７２時間インキュベートして細菌を成長させた。次に、ヒト病原菌種を最初の居住細菌培養物に対して直角に単画線で画線した。病原分離株が最初の細菌画線に接触しないように注意した。次いで、プレートを４８時間再インキュベートして、ヒト病原菌種を成長させた。インキュベーション後、原料生物の細菌に隣接するヒト病原分離株の成長についてプレートを調べた。
【００２７】
阻害は、最初の居住細菌画線に隣接する、成長が観察されない地帯とみなした。観察された場合、阻害は、最初の居住細菌から最も近い０．５ｍｍまでのゼロ成長地帯の距離を測定することによって定量化した。
【００２８】
抗菌／抗真菌代謝産物阻害試験法
原料生物から分離され、寒天プレート阻害試験でヒト病原菌の成長阻害能を示した細菌を、０．５Ｌの液体ＴＳＹＥ培地で周囲温度で７日間成長させた。各分離株は、２種類の異なる抽出法に適用するために複製フラスコで成長させた。成長期間後、原料生物から分離された細菌分離株によって産生された複合分子（代謝産物）を、標準液体抽出法を利用して液体培地から抽出した。
【００２９】
抽出法は、以後の試験のために、水溶性及び非水溶性化合物の両方の抽出を目的として考案された二つの独立した抽出工程から成った。
水溶性の抽出工程は、細菌分離株の細胞培養物を含有する液体培地試料を－８０℃で急速凍結することから成った。凍結工程後、凍結試料を凍結乾燥させた。凍結乾燥後、試料を０．５Ｌの１００％メタノールに加え、１時間機械的に撹　した。試料をろ過殺菌して、全細菌細胞／細胞フラグメントを確実に除去した。次に、得られたメタノール／化合物の混合物から回転蒸発器を利用してメタノールを蒸発させ、可能性ある生物活性を有する一つ以上の化合物を含有する複数の化合物の粗抽出物だけを残した。
【００３０】
非水溶性の抽出工程は、細菌分離株の細胞培養物を含有する培地試料に、１５０ｍｌの酢酸エチルを加えることから成った。次に、この得られた混合物を１５分間機械的に撹　した。次に、酢酸エチルを混合物から分液漏斗で分離し、取り置いた。この操作を３回繰り返し、４５０ｍｌの酢酸エチル／化合物の混合物を得た。次に、得られた混合物から回転蒸発器を使用して酢酸エチルを蒸発させ、可能性ある生物活性を有する一つ以上の化合物を含有する複数の化合物の粗抽出物だけを残した。
【００３１】
阻害性の各細菌分離株を水溶性及び非水溶性抽出工程にかけて得られた各代謝産物抽出物を、１種類以上のヒト病原菌、抗生物質耐性ヒト病原菌、ヒト病原真菌、及びヒト病原ウィルスの阻害能について試験した。利用したヒト病原菌は、エンテロコッカス・フェカーリス　ＡＴＣＣ　２９２１２、バンコマイシン耐性エンテロコッカス・フェカーリス（Ｖ

ＲＥ） ＡＴＣＣ ７００２２１、黄色ブドウ球菌 ＡＴＣＣ ２９２１３、メチシリン耐性黄色ブドウ球菌（ＭＲＳＡ） ＡＴＣＣ ３３５９１、化膿連鎖球菌(Streptococcus pyogenes) ＡＴＣＣ １２３５８、ペニシリン耐性肺炎球菌(Streptococcus pneumonia) ＡＴＣＣ ７００６７４、緑膿菌 ＡＴＣＣ ２７８５３、及びネズミチフス菌(Salmonella typhimurium) ＡＴＣＣ ７００４０８であった。利用したヒト病原真菌は、カンジダ・アルビカンス(Candida albicans) ＡＴＣＣ ２４４３３、石膏状小胞子菌(Microsporum gypseum) ＡＴＣＣ １４６８３、トリコフィトン・メンタグロフィテス(Trichophyton mentagrophytes) ＡＴＣＣ ９５３３及びアスペルギルス・フミガーツス(Aspergillus fumigatus) ＩＨＥＭ ２８９５であった。

【００３２】

抗菌試験はＭｕｅｌｌｅｒ－Ｈｉｎｔｏｎブロス中で実施する。接種物は、１８～２４時間の寒天プレートから選んだ分離コロニーの直接食塩水懸濁液を製造することによって調製する。その後、接種物を５００，０００ＣＦＵ／ｍｌに標準化する。インキュベーション温度は３５℃、インキュベーション時間は１６時間である。酵母スクリーニングは、０．３ｇ／ｌのグルタミン及び３４．６ｇ／ｌのモルホリンプロパンスルホン酸（ＭＯＰＳ－緩衝液）を補給したＲＰＭＩ １６４０ブロス中で実施する。インキュベーション温度は３５℃、インキュベーション時間は２４時間である。

【００３３】

接種物は、１８～２４時間のサブロー寒天プレートから選んだ分離コロニーの直接食塩水懸濁液を製造することによって調製する。その後、接種物を標準化する（１０００～５００００ＣＦＵ／ｍｌ）。測定ツールは成長曲線下面積で、これをＢｉｏｌｉｎｋソフトウェアによって自動測定する。ブロスと試験物質（例えば２５ＰＰｍの抽出物）だけを含有する試料の面積を、接種物も含有する試料の面積から引く。各試料に５個の複製物を用いる結果、対照抗生物質、例えばアンホテリシンＢ、ペニシリン及びバンコマイシンとの比較が可能な数が得られる。結果は、試験物質を含有しない試料に対する成長率（％）として表される。

【００３４】

抗カビスクリーニングは、０．３ｇ／ｌのグルタミン及び３４．６ｇ／ｌのモルホリンプロパンスルホン酸（ＭＯＰＳ－緩衝液）を補給したＲＰＭＩ １６４０ブロス中で実施する。アスペルギルス属に対するインキュベーション温度／時間は３５℃／４８時間である。皮膚糸状菌のインキュベーション温度は２５℃、インキュベーション時間は５日間である。

【００３５】

接種物は、７日間培養した培養物を約１ｍｌの滅菌食塩水で覆うことによって調製する。懸濁液は、コロニーをパスツールピペットの先で優しくプロービングすることによって製造する。その後、接種物を標準化する（４０００～５００００ＣＦＵ／ｍｌ）。カビの場合、動力学的測定は使用せず、開始点と終点の測定だけである。測定ツールとして、ＯＤ（スタートとエンド）を使用し、これをＢｉｏｌｉｎｋソフトウェアによって自動測定する。ＯＤ－スタートをＯＤ－エンドから引く。各試料に５個の複製物を使用する。これらのデータは、対照抗生物質、例えばアンホテリシンＢとの比較が可能な数になる。結果は、試験物質を含有しない試料に対する成長率（％）としても表される。

【００３６】

ヒト病原菌及び真菌の種は、標準化濃度１００及び２５０マイクログラム／ミリリットル（ＰＰｍ）の、原料生物から分離した各細菌からの代謝産物抽出物に曝露した。代謝産物の阻害試験は、垂直測光法（光学濃度）によって細菌及び真菌種の濁度進行（すなわち成長）をモニタできる自動リーダーインキュベーターのＢｉｏｓｃｒｅｅｎ Ｃ Ａｎａｌｙｚｅｒを利用して実施した。結果を用いて、細菌分離株から得られた代謝産物抽出物に応答する成長曲線を作成した。各試験には、陽性対照又は試験生物に対するＭＩＣがわかっている対照抗生物質も含めた。

【００３７】

抗ウィルス阻害試験法

個々の代謝産物を、原料生物の皮膚環境から分離した細菌から得られた粗抽出物から、前述の２種類の抽出工程を通ってＨＰＬＣカラムを用いて抽出した。ＨＰＬＣカラムの詳細は、Ｂｅｃｋｍａｎ　Ｕｌｔｒａｓｐｈｅｒｅ（４．６ｍｍ×１５０ｍｍ）、パート番号２３５３３０、ＲＰ－１８、粒径５μｍであった。溶離液はアセトニトリル：水＝７５：２５の混合物で、流速１ｍｌ／分であった。ＵＶ検出器を２１０ｎｍにセットした。原料生物の皮膚環境から分離した細菌からの代謝産物抽出物の、ヒトリンパ球ＣＥＭ及びＭＴ－４細胞培養物中のＨＩＶ誘発細胞変性を阻害する能力を試験した。ＣＥＭ細胞を培地１ミリリットル当たり約２５０，０００個の細胞濃度で懸濁させ、１ミリリットル当たり５０％細胞培養物感染量（$CCID_{50}$）の約１００倍の野生型ＨＩＶ－１（III_B）で感染させた。次に、感染細胞懸濁液１００μｌを、適当に希釈した試験化合物１００μｌを含有する２００μｌの微量定量プレートのウェルに加えた。３７℃で４日間のインキュベーション後、細胞培養物のシンシチウム形成を顕微鏡で調べた。シンシチウム形成を５０％阻害するのに要した化合物濃度としてEC_{50}（５０％有効濃度）を測定した。

【００３８】

抗腫　試験法

原料生物の皮膚環境から分離した細菌から抽出した代謝産物の、ｆ－マウス白血病細胞（Ｌ１２１０／０）、マウス乳がん細胞（ＦＭ３Ａ）、及びヒトＴ－リンパ球細胞（Ｍｏｌｔ４／Ｃ８及びＣＥＭ／０）の各細胞の増殖阻害能を調べた。すべてのアッセイは９６穴微量定量プレートで実施した。各ウェルに５～７．５×10^4個の細胞と所定量の試験化合物を加えた。細胞は、加湿CO_2制御雰囲気下３７℃で４８時間（マウス白血病Ｌ１２１０、マウス乳がんＦＭ３Ａ）又は７２時間（ヒトリンパ球ＣＥＭ及びＭｏｌｔ）増殖させた。インキュベーション期間終了時に細胞をＣｏｕｌｔｅｒカウンターで計数した。IC_{50}（５０％有効阻害濃度）は、生細胞数を５０％削減する化合物濃度と定義された。

【００３９】

結果

皮膚の微生物フローラの試料を、１３種のサラマンダーと３種のカエルで構成される１６種の両生類（原料生物）から採取した（表１）。これらの試料から合計４１７個の形態学的に異なる細菌分離株を分離した。内訳は、サラマンダーから分離した３０６個のグラム陰性　菌、２０個のグラム陽性　菌、２５個のグラム陰性球菌、及び５個のグラム陽性球菌を含む３５６個の固有分離株、並びにカエルから分離した３７個のグラム陰性　菌、１０個のグラム陽性　菌、７個のグラム陰性球菌、及び７個のグラム陽性球菌を含む６１個の固有分離株であった（表２）。

【００４０】

【表２】

表２－両生類の皮膚環境から分離された細菌分離株の概要

	グラム陰性桿菌 分離株の数	グラム陽性桿菌 分離株の数	グラム陰性球菌 分離株の数	グラム陽性球菌 分離株の数
サラマンダーの 細菌分離株	３０６	２０	２５	５
カエルの細菌分離株	３７	１０	７	７

【００４１】

寒天プレート阻害試験

４種類のヒト病原菌、エンテロコッカス・フェカーリス（ＡＴＣＣ　２９２１２）、大腸菌（ＡＴＣＣ　２５９２２）、緑膿菌（ＡＴＣＣ　２７８５３）及び黄色ブドウ球菌（Ａ

ＴＣＣ　２９２１３）に対する４１７個の各固有細菌分離株の寒天プレート阻害試験から、１種類以上のヒト病原菌種の成長を阻害できる１３個の固有細菌分離株の存在がわかった。これら１３個の分離株のうち、９個はプレトドン科のサラマンダーから分離され、４個はアマガエル科及びアカガエル科のカエルから分離された。クロハラサラマンダーの分離株は、実験１の抱卵中の雌とは対照的に実質的阻害を示さなかった。サラマンダーの皮膚の細菌フローラは、ホルモン、環境変化、及び他の因子によって影響されることがわかっているので、実験１の個体と実験２の個体間で観察された細菌フローラに違いがあっても不思議ではない。従って、実験１の細菌分離株ＤＱ００１Ｄも本実験２で実施する試験に含めた。病原菌の成長を阻害できる１３個の固有細菌分離株のコロニー及び細胞の形態学的特徴を表３にまとめた。

【００４２】

【表３】

表３－両生類から分離された阻害能を示す細菌のコロニー及び細胞の形態学的特徴の概要

分離株番号	コロニーの形態	辺縁の形態	隆起の形態	色	フィニッシュ	グラム反応	原料生物
DQ001D	円形	完全	こぶ	白色不透明のエッジ、白色の中心部	光沢	グラム陰性桿菌	サラマンダー
PO014	円形	完全	凸	桃色の中心部、白色のエッジ	光沢	グラム陰性桿菌	サラマンダー
PO019	円形	完全	凸	黄色	光沢	グラム陰性桿菌	サラマンダー
PO026	円形	完全	凸	白色不透明	光沢	グラム陰性桿菌	サラマンダー
PO027	円形	波形	凸	白色	光沢	グラム陰性桿菌	サラマンダー
AC021	円形	波形	凸	白色不透明	光沢	グラム陰性桿菌	カエル
AC024	不規則	波形	凸	白色	光沢	グラム陰性桿菌	カエル
PC017	円形	完全	凸	白色	光沢	グラム陽性桿菌	カエル
PD026	円形	波形	凸	桃色不透明	光沢	グラム陰性桿菌	サラマンダー
EG006	不規則	切れ込み	凸	オレンジ色	光沢	グラム陰性桿菌	サラマンダー
EC009	点状	完全	凸	黄色	光沢	グラム陰性桿菌	サラマンダー
EC024	不規則	切れ込み	こぶ	白色不透明のエッジ、白色の中心部	光沢	グラム陰性桿菌	サラマンダー
RC003	円形	鋸歯状	凸	紫色	光沢	グラム陽性球菌	カエル

【００４３】

原料生物から分離した１３個の固有細菌分離株のうち８個が、試験対象のヒト病原菌の２種類以上を阻害する能力を示した。原料生物から分離した５個の固有細菌分離株は、試験対象のヒト病原菌の１種類だけの阻害に有効であった。表４に示したデータからわかるように、細菌分離株ＤＱ００１Ｄ、ＡＣ０２１、ＥＧ００６、ＥＣ００９、ＰＯ０２６、ＰＯ０２７、及びＲＣ００３は、エンテロコッカス・フェカーリス（ＡＴＣＣ　２９２１２）の成長を阻害する化合物を産生している可能性を示した。細菌分離株ＥＧ００６、ＥＣ００９、ＰＯ０１４、ＥＣ０２４、及びＡＣ０２４は、大腸菌（ＡＴＣＣ　２５９２２）の成長を阻害する化合物を産生している可能性を示した。細菌分離株ＡＣ０２１、ＰＯ０１９、ＰＯ０１４、ＰＯ０２７、ＲＣ００３、ＰＤ０２６、及びＰＣ０１７は、黄色ブドウ球菌（ＡＴＣＣ　２９２１３）の成長を阻害する化合物を産生している可能性を示した

。細菌分離株ＥＣ０２４及びＡＣ０２４は、緑膿菌（ＡＴＣＣ　２７８５３）の成長を阻害する化合物を産生している可能性を示した。

【００４４】

【表４】

表４－細菌分離株によるヒト病原菌の阻止帯

	阻止帯（mm）			
分離株	エンテロコッカス・フェカーリス	大腸菌	黄色ブドウ球菌	緑膿菌
DQ001D	37.0			
PO014		17.5	>50	
PO019			45.5	
PO026	25.0			
PO027	23.0		22.0	
AC021	27.0		30.5	
AC024		29.5		32.0
PC017			22.0	
PD026			>50	
EG006	>50	>50		
EC024		35.5		32.0
EC009	>50	>50		
RC003	44.0		45.5	

【００４５】

抗菌／抗真菌代謝産物阻害試験

原料生物の皮膚環境から分離され、寒天プレート阻害試験で病原菌の成長を阻害した１３個の細菌分離株からの代謝産物抽出物の、病原菌又は病原真菌の成長を阻害する効果について試験した。これらの代謝産物のうち、細菌分離株ＤＱ００１Ｄ、ＰＯ０２６、ＰＯ０２７及びＰＣ０１７からの抽出物は、病原菌、病原真菌、又はその両方の成長の阻害に強力な生物活性を示した。

【００４６】

分離株番号ＤＱ００１Ｄからの代謝産物抽出物は、１００ＰＰｍ（マイクログラム／ミリリットル）及び２５ＰＰｍの両方で、複数種のグラム陽性病原菌の阻害に有効であった。具体的には、ＤＱ００１Ｄからの代謝産物抽出物は、病原菌種のエンテロコッカス・フェカーリス、バンコマイシン耐性エンテロコッカス・フェカーリス、黄色ブドウ球菌、メチシリン耐性黄色ブドウ球菌、化膿連鎖球菌、及びペニシリン耐性肺炎球菌の成長を完全に阻害した。さらに、ＤＱ００１Ｄからの代謝産物抽出物は、１００ＰＰｍでヒト病原真菌のカンジダ・アルビカンスの成長の完全阻害にも有効であり、また１００ＰＰｍでヒト病原真菌の石膏状小胞子菌の成長の部分的阻害も示した。

【００４７】

分離株番号ＰＯ０２６からの代謝産物抽出物は、１００ＰＰｍで病原真菌のカンジダ・アルビカンスの成長の完全阻害を示した。

分離株番号ＰＯ０２７からの代謝産物抽出物は、２５ＰＰｍでヒト病原菌の黄色ブドウ球菌の成長の完全阻害に有効であった。さらに、ＰＯ０２７からの代謝産物抽出物は、２５０ＰＰｍで病原真菌のカンジダ・アルビカンスの成長も阻害した。

【００４８】

分離株番号ＰＣ０１７からの代謝産物抽出物は、１００ＰＰｍでヒト病原真菌のカンジダ・アルビカンスの成長阻害能を示した。各種両生類由来の他の細菌分離株からの抽出物の

抗菌／抗真菌活性についても同様の結果が最近得られており、本発明を応用すれば今後他のそのような活性の発見が期待されることを示している。

【００４９】

抗ウィルス阻害試験

原料生物の皮膚環境から分離され、寒天プレート阻害試験で病原菌の成長を阻害した１３個の細菌分離株から抽出された代謝産物の、複数株のヒト免疫不全ウィルス（ＨＩＶ）の成長を阻害する効果について試験した。これらの抽出物のうち、ＤＱ００１Ｄからの代謝産物抽出物（リテンションタイム７．２７分）は、ヒトリンパ球ＣＥＭ及びＭＴ－４細胞培養物のＨＩＶ細胞変性の阻害に顕著な能力を示した。具体的には、ＣＥＭ細胞中のＨＩＶ－１及びＨＩＶ－２のＨＩＶ細胞変性を阻害するのに要したＤＱ００１Ｄの有効代謝産物濃度は、それぞれ７ＰＰｍ及び１７ＰＰｍであった。ＭＴ－４細胞中のＨＩＶ－１のＨＩＶ誘発細胞変性を阻害するのに要したＤＱ００１Ｄの有効代謝産物濃度は９ＰＰｍであった。ＭＴ－４細胞に対するＤＱ００１Ｄからの代謝産物の確定毒性は４０ＰＰｍであった。分離株を試験したこれまでの経験に基づくと、将来的に他の分離株の抗ウィルス活性が示されるであろうことが期待される。

【００５０】

抗腫　試験

細菌分離株番号ＤＱ００１Ｄからの代謝産物抽出物は、ｆ－マウス白血病細胞（Ｌ１２１０／０）、マウス乳がん細胞（ＦＭ３Ａ）及びヒトＴ－リンパ球細胞（Ｍｏｌｔ４／Ｃ８、ＣＥＭ／０）の増殖を、それぞれ１７ＰＰｍ、１６ＰＰｍ、１７ＰＰｍ及び１７ＰＰｍで阻害することを示した。分離株を試験したこれまでの経験に基づくと、将来的に他の分離株の抗腫　活性が示されるであろうことが期待される。

【００５１】

細菌及び真菌の公知抗生物質及び抗真菌薬に対する耐性のために、そうした疾患と闘うことが可能な薬物の新規材料源が世界中で探し求められている。そのような医学的に有用な化合物の新規細菌源の発見成功率がこれまで極端に低かったため、研究者らは医学的に有用な新薬の可能性ある新規材料としてマクロ生物に焦点を当てるようになった（例えばＢｅａｔｔｉｅ　１９９２年）。本明細書中に提示したデータは、両生類が、抗菌、抗真菌、抗ウィルス、及び抗腫　代謝産物を産生可能な医学的に有用な細菌源となることに関する明白かつ実質的な証拠を提供している。

【００５２】

さらに、公知薬物に対する細菌の抗生物質耐性は、世界的に深刻な健康問題をもたらしている。本研究は、両生類の皮膚環境が、高い抗生物質耐性を持つバンコマイシン耐性エンテロコッカス・フェカーリス及びメチシリン耐性黄色ブドウ球菌を含む複数のヒト病原菌及び病原真菌の成長を阻害できる細菌種を含有することを明らかに示している。さらに、細菌分離株ＤＱ００１Ｄからの代謝産物抽出物の抗ウィルス及び抗腫　活性は、両生類の皮膚環境から分離された医学的に有用な化合物の応用も明確に示している。これ及び他の分離株の生物活性を考えると、今後の分離株も同様の抗ウィルス活性を示すであろうと考えられ、また期待される。

【００５３】

前述の説明は本発明の例示的態様を含む。本明細書中に記載した前述の態様及び方法は、当業者の能力、経験及び好みによって変動可能である。ある順序で本発明の方法のステップを単に列挙することが、必ずしも該方法のステップの順序に関して何らかの制約を構成するわけではない。前述の説明及び図面は単に本発明を説明し図示しているだけであって、本発明は、クレームでそのように制限されない限り、それに制限されることはない。開示内容を前にした当業者であれば、本発明の範囲内で多様な変形を為すことが可能であろう。

【図面の簡単な説明】

【００５４】

【図１】阻害試験の対照として用いたペトリ皿を示す写真である。縦の画線（ＡＣ０２４

）はコオロギガエル(Acris crepitans)の皮膚から分離された細菌を含み、横の画線は４種類のヒト病原菌、エンテロコッカス・フェカーリス（ＡＴＣＣ　２９２１２）、大腸菌（ＡＴＣＣ　２５９２２）、緑膿菌（ＡＴＣＣ　２７８５３）、及び黄色ブドウ球菌（ＡＴＣＣ　２９２１３）である。縦の両生類細菌画線に対する横のヒト病原菌画線の成長の近接度に基づいて阻害効果の有無を示す。

【図２】図１で説明したのと同様の阻害試験の対照として用いたペトリ皿を示す写真で、縦の画線（ＥＣ０２４）はユーリセア・シリゲラ(Eurycea cirrigera)の皮膚から分離された細菌を含む。

【図３】図１で説明したのと同様の阻害試験の対照として用いたペトリ皿を示す写真で、縦の画線（ＲＣ００３）はブロンズガエル(Rana clamitans)の皮膚から分離された細菌を含む。

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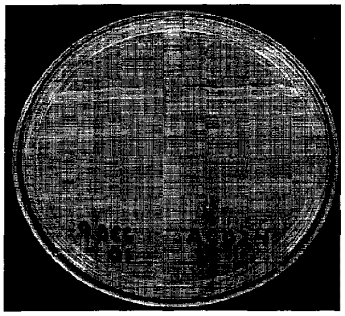
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[Continued on next page]

(54) Title: BACTERIAL ISOLATES FROM ORGANISMS THAT RESPIRE AT LEAST PARTIALLY THROUGH THEIR SKIN AND BIOLOGICALLY ACTIVE EXTRACTS DERIVED THEREFROM



(57) Abstract: Extracts including a biologically active compound or combination of compounds derived from microorganisms isolated from mucus-producing organisms that respire at least partially through their skin. Red shaded bacteria isolated from the skin of salamanders and frogs are found to produce compound(s) which have antiviral, antitumor, antibacterial and antifungal properties. These compound(s) have an inhibitory effect on opportunistic human pathogens, including *Candida* sp., *Microsporum* sp., *Staphylococcus* sp., *Pseudomonas* sp., *Escherichia* sp.,and *Enterococcus* sp., as well as on HIV strains and tumor cell lines.

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BACTERIAL ISOLATES FROM ORGANISMS THAT RESPIRE AT LEAST PARTIALLY THROUGH THEIR SKIN AND BIOLOGICALLY ACTIVE EXTRACTS DERIVED THEREFROM

Prior application

This application claims priority to provisional application Serial No. 60/260,022, filed January 5, 2001.

Background of the Invention

All amphibians respire, to varying degrees, cutaneously. As such, their integument must serve as a gas permeable barrier to their external environment (Lilleywhite, H. B., and P. F. A. Maderson. 1988. The structure and permeability of the integument. American Zoologist 28:945-962). Moisture is requisite for skin to be utilized as a respiratory organ (Fox, H. 1994. Structure of the integument. Cpt. 1 in "Amphibian Biology, Vol 1, The Integument", ed by H. Heatwole and G. T. Barthalmus, Surrey Beatty and Sons, Chipping Norton). This necessary moisture is achieved through the production of mucus via mucus-producing glands associated with the integument (Duellman, W. E., and L. Trueb. 1986. Biology of Amphibians. McGraw Hill, New York, New York, U.S.A.; Fox 1994). The primary component of mucus in amphibians is a mucopolysaccharide (glycoprotein) (Duellman and Trueb 1986). Glycoproteins contain one or more carbohydrate chains covalently linked to a polypeptide backbone (Schaechter, M., and I. Brockhausen. 1989. The biosynthesis of branched O-glycans. *In* Mucus and related topics. E. Chantler and N. A. Ratcliffe (eds). Symposia of the Society for Experimental Biology, no. XLIII, University of Cambridge, Cambridge). The mucus is rich in carbon, a necessary element to support microbial growth and synthesis of most, if not all, cellular compounds (Guirard, B. M., and E. E. Snell. 1962. Nutritional requirements of microorganisms. Pp. 33-93. *In* I. C. Gunsalis and R. Y. Stanier (Eds.), The Bacteria. A Treatise on Structure and Function. Vol. IV: The Physiology of Growth. Academic Press, New York, New York, U.S.A.). Thus, the mucus layer necessarily produced by amphibians in order to accomplish cutaneous respiration represents a nutrient rich habitat for microorganisms (Austin, Jr., R.M. 2000. Cutaneous microbial flora and antibiosis in *Plethodon ventralis*: inferences for parental care in the Plethodontidae. Pp. 451-461. *In* R.C. Bruce, R.G. Jaeger and L.D. Houck

(Eds.), The Biology of Plethodontid Salamanders, Kluwer Academic / Plenum Pub., New York, New York, U.S.A.).

The majority of microorganisms in most ecosystems are attached to surfaces (Wimpenny, J. W. T., S. L. Kinniment, and M. A. Scourfield. 1993. The physiology and biochemistry of biofilms. Pp. 274 – 318. *In* S. Denyer, P. Gorman, and M. Sussman, (Eds.), Microbial Biofilms: Formation and Control. Blackwell Scientific Publications, London, U.K.), and the integuments of animals often serve as suitable habitats for the development of microbial communities (Alexander, M. 1971. Microbial Ecology. John Wiley and Sons, New York, New York, U.S.A.). These microcommunities often exhibit the same types of community-level interactions that communities of larger organisms (macrocommunities) exhibit. Members of microcommunities compete for limited resources and form intimate, and, at times, ammensalistic relationships (Atlas, R. M., and R. Bartha. 1993. Microbial Ecology. Fundamentals and Applications, 3rd ed. Benjamin Cummings, New York, New York, U.S.A.; Bull, A. T., and J. H. Slater. 1982. Microbial Interactions and Communities. Vol. 1. Academic Press, New York, New York, U.S.A.; Frederickson, A. G., and G. Stephanopoulos. 1981. Microbial competition. Science 213:972-979). Such amensalistic strategies include, but are not limited to, the production of antibiotics.

Antibiotics are substances produced by microorganisms that are capable of killing or inhibiting growth of other microorganisms (Williams, S. T. 1982. Are antibiotics produced in soil? Pedobiology 23:85-87, Williams, S. T., and J. C. Vickers. 1986. The ecology of antibiotic production. Microbial Ecology 12:43-52; Brock, T. D. 1994. The biology of Microorganisms. 7th ed., Prentice Hall, Englewood Cliffs, New Jersey). The production of such substances has been viewed as an adaptation to reduce or inhibit competition in natural environments (Weiner 1996). Competition studies between antibiotic-producing and antibiotic sensitive bacteria in both solid and liquid media have provided evidence that supports this theory (e.g. Rasool, K., and j. W. T. Wimpenny. 1983. Mixed continuous culture experiments with an antibiotic-producing streptomycete and *Escherichia coli*. Microbial Ecology 8:267-277; Turpin, P. E., V. K. Dihr, K. A. Maycroft, C. Rowlands, and E. M. H. Wellington. 1992. The effect of *Streptomyces* species on the survival of *Salmonella* in soil. FEMS Microbiol. Ecol. 101:271-280). Laboratory experiments indicate that antibiotic production prevents invasion of competitors but does not improve ability to invade or compete with established populations of antibiotic-sensitive microbes (Wiener, P. 1996. Experimental studies on the ecological role of

antibiotic production in bacteria. Evolutionary Ecology 10:405-421). Despite the evidence for antibiotic production in laboratory experiments, the natural role of antibiotics and the conditions under which they are produced remains unclear (Wiener 1996).

Salamanders of the family Plethodontidae comprise the largest family of extant salamanders. Its members are among the most numerous vertebrates in many forest and lotic ecosystems. They are characterized most notably by their lack of lungs. Life histories exhibited within the family are extremely diverse (e.g., Tilley, S. G., and J. Bernardo. 1993. Life history evolution in plethodontid salamanders. Herpetologica 49: 154-163; Wake, D. B., and S. M. Marks. 1993. Development and evolution of plethodontid salamanders: a review of prior studies and a prospectus for future research. Herpetologica 49:194-203). Despite such variations, the conservatism in their reproductive biology, particularly the near universality of parental care in the form of egg attendance (Salthe, S. N., and J. S. Mecham. 1974. Reproductive and courtship patterns. Pp. *In* B. Lofts (Ed.), Physiology of the Amphibia. Vol 2: Academic Press, New York, New York, U.S.A.), is well documented (e.g., Forester, D. C. 1978. Laboratory encounters between attending *Desmognathus ochrophaeus* (Amphibia, Urodela, Plethodontidae) females and potential predators. Journal of Herpetology 12:537-541, 1979; Highton, R., and T. Savage. 1961. Functions of the brooding behavior in the female red-backed salamander, *Plethodon cinereus*. Bulletin of the Florida State Museum 6:235-237; Piersol, W. H. 1909. The habits and larval state of *Plethodon erythronotus*. Transactions of the Canadian Institute 8:469-493; Ritter, W. E. 1903. Further notes on the habits of *Autodax lugubris*. American Naturalist 37:883-886; Tilley, S. G. 1972. Aspects of parental care and embryonic development in *Desmognathus ochrophaeus* (Amphibia: Plethodontidae) in western North Carolina. American Midland Naturalist 89:394-407).

Parental care can be defined as post-zygotic parental investment in progeny (Trivers, R. L. 1985. Social Evolution. Benjamin Cummings, New York, New York, U.S.A.). Within the Plethodontidae this investment occurs in the form of egg attendance, most often by the female parent (Crump, M. L. 1995. Parental care. Pp. 518–567. *In* H. Heatwole and B. K. Sullivan (Eds.), Amphibian Biology. Vol. 2: Social Behaviour. Surrey Beatty and Sons, Chipping Norton, New South Wales, Australia). In most cases the female remains with the clutch until hatching occurs. The adaptive benefits of such behavior in plethodontids have been clearly established, and include protection against predators, fungal infections from nonpathogenic common soil fungal species, desiccation, and prevention of developmental abnormalities, (see

Crump, 1995, for a comprehensive review). Parental care consists of a behavioral repertoire (e.g., bodily contact with the clutch, egg manipulation, gular pulsations) which apparently confers these multiple benefits simultaneously on the developing offspring.

Although the benefits of parental care within the Plethodontidae are clearly documented, the mechanisms of how certain aspects are achieved have remained unclear until recently (e.g. Austin 2000). This situation has been particularly true with respect to the many observations over the past century regarding the reduction of fungal infections in clutches when the female is present. Such observations led to the hypotheses that either females (e.g., Noble, G. K. 1931. The Biology of the Amphibia. McGraw Hill, New York, New York, U.S.A.; Piersol, 1909) or their developing embryos (Highton and Savage, 1961) produce an antifungal metabolite capable of reducing or inhibiting fungal growth on attended egg clutches. Subsequent research has provided no indication for the existence of antimicrobial secretions from salamanders (Daniel, J. C., Jr., and R. W. Simpson. 1954. A negative note on antibiotics. Herpetologica 10:16; Forester, D. C. 1979. The adaptiveness of parental care in *Desmognathus ochrophaeus* (Urodela: Plethodontidae). Copeia 1979:332-341; Tilley, 1972; Vial, J. L., and F. B. Prieb. 1966. Antibiotic assay of dermal secretions from the salamander, *Plethodon cinereus* (Green). Herpetologica 22: 284-287; Vial, J. L., and F. B. Prieb. 1967. An investigation of antibiosis as a function of brooding behavior in the salamander, *Plethodon cinereus*. Transactions of the Missouri Academy of Science 1:37-40). No published accounts are known of researchers utilizing the cutaneous environment of amphibians as a potential source of medically-useful bacteria. Much research regarding medically useful compounds from amphibians and other organisms has focused upon the production of such compounds by amphibians and other macro-organisms in their skin secretions rather than the bacteria flora which inhabit the skin of such organisms (e.g. Glausiusz, J. 1998. The frog solution. Discover. 19: (11) 88-92; Carte, B. K. 1996. Biomedical potential of marine natural products. Bioscience. 46(4): 271-287; Port, O. 2001. A leap in drug research from frogs. Business Week. 3752:99; Tyler, M. J. 1995. Frogs and Drugs. Australian Natural History. 24(12): 46-52; Coghlan, A. 2000. Bug busters. New Scientist. 166(2233):15; Stenborner, S. T., G. J. Currie, J. H. Bowie, J. C. Wallace, and M. J. Tyler. 1998. New antibiotic caerin 1 peptides from the skin of the Australian tree frog *Litoria chloris*. Comparison of the activities of the caerin 1 peptides from the genus *Litoria*. Journal of Peptide Research, 51: (2), 121-127; Rennie, J. 1993. No snake oil here. Scientific American 266

(3) 136-137; McNamee, D. 1994. Eye of newt, toe of frog. Lancet. 344(8938):1696-1698; Erspamer 1994).

Because of the nature of the nutrient rich mucus produced to facilitate cutaneous respiration, other aspects regarding the success of plethodontid salamanders may be attributable to their resident microorganismal flora. Because plethodontid salamanders must rely solely upon their skin for the exchange of respiratory gases, excessive microbial loads of aerobic soil bacteria inhabiting the surface of the skin would provide an impenetrable barrier for respiratory gases, ultimately leading to the death of the salamander.

The nutrient rich skin environment of amphibians has been shown to support a microbial community that is distinct from those of surrounding environments (Austin, Jr., R. M. 1997. The cutaneous bacterial flora of the eastern zigzag salamander, *Plethodon dorsalis* Cope (Amphibia:Plethodontidae). Dissertation. University of Mississippi, University, MS). Given that microorganisms have been shown to utilize amensalistic strategies in nutrient rich environments, including the production of antibiotics (Frederickson and Stephanopoulos, 1981), the cutaneous environment of amphibians can be predicted to support microorganisms capable of producing compounds which reduce or inhibit microbial growth, thus imparting a competitive advantage to the ecology and life history of amphibians. Surprisingly, it has recently been determined that metabolites produced by certain of the microorganisms isolated from the cutaneous environment of organisms that respire at least partially through their skin exhibit antimicrobial activity against important human pathogenic bacteria and fungi, as well as having antiviral and antitumor activities.

Summary of the Invention

The invention consists of extracts which comprise compounds and combinations of compounds produced by microorganisms isolated from the body of mucus-producing organisms that respire at least partially through their skin, and which have antitumor, antiviral, antifungal, and/or antibacterial activity in humans. More specifically, the invention consists of antibiotics produced by bacteria growing in the mucus of animals which respire through their skin. Even more specifically, the invention consists of compounds and/or combinations of compounds produced by bacteria isolated from the skin of amphibians which have antiviral, antitumor, antibacterial and/or antifungal activity, including activity against bacteria and/or fungi which are pathogenic to humans. Specific examples include extracts which comprise compound(s)

produced by bacteria isolated from the skin of salamanders and frogs which have activity against *Candida* sp., *Microsporum* sp., *Streptococcus* sp. (including penicillin-resistant *Streptococcus*), *Staphylococcus* sp. (including methicillin-resistant *Staphylococcus*), *Enterococcus* sp. (including vancomycin-resistant *Enterococcus*), *Pseudomonas* sp., *Escherichia* sp, the Human Immunodeficiency Virus (HIV) strains including the reduction of cytopathicity of HIV strains in human lymphocytes CEM and MT-4, and the tumor cells f-murine leukemia, murine mammary carcinoma, and human T-lymphocyte cells.

An object of the invention is the extraction of a compound or combination of compounds from bacterial isolates from the skin of organisms that respire at least partially through their skin that have antibacterial, antifungal, antiviral, or antitumor effects.

Another object of the invention is the identification of bacterial isolates from the skin of organisms that respire at least partially through the skin that produce compounds that are extracted from the isolates that have antibacterial, antifungal, antiviral, or antitumor effects.

Brief Description of the Drawings

Fig. 1 is a photograph of a petri dish used as an inhibition trial control, wherein the vertical streak (AC024) includes a bacterium isolated from the skin of *Acris crepitans*, the horizontal streaks are the four human bacterial pathogens *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Staphylococcus aureus* (ATCC 29213), and which shows the presence or absence of an inhibitory effect based on the proximity of growth of horizontal human pathogen bacterial streaks to the vertical amphibian bacterial streaks.

Fig. 2 is a photograph of a petri dish used as an inhibition trial control as described with reference to Fig. 1, wherein the vertical streak (EC024) includes a bacterium isolated from the skin of *Eurycea cirrigera*.

Fig. 3 is a photograph of a petri dish used as an inhibition trial control as described with reference to Fig. 1, wherein the vertical streak (RC003) includes a bacterium isolated from the skin of *Rana clamitans*.

Detailed Description of Preferred Embodiments

The invention comprises the identification of bacterial isolates taken from the cutaneous microbial flora of animals that respire at least partially through their skin and the extraction of a

compound or combination of compounds (extract) from the isolates which has or have antibacterial, antifungal, antiviral, or antitumor effects. A methodology for identifying the isolates and a methodology for producing the extracts are described. The extract may comprise a single active compound, a combination of compounds, or one or more compounds which have a synergistic effect when found in combination with other compounds in the extract.

Source organisms are collected in their native environments and placed in isolation. After the isolation period, samples of the cutaneous microflora of the organisms are grown and isolated based on morphological characteristics. The bacterial isolates and extracts from the isolates are tested for antibacterial effects against selected bacterial strains on culture media. Liquid extraction techniques are used to prepare extracts from the bacterial isolates which are then tested for the ability to inhibit the growth of selected strains of bacteria and fungi using photometric evaluation. Components of the extracts are tested for their ability to inhibit cytopathicity in cell cultures. Components of the extracts are tested for their ability to inhibit the proliferation of carcinoma cells.

Example 1 - Cutaneous Microbial Flora and Antibiosis on Pathogenic Bacteria in *Desmognathus quadramaculatus*

A brooding female of the species *Desmognathus quadramaculatus* was collected at Piedmont Nature Garden and placed in a 3.79 liter plastic bag filled with leaf litter and soil taken from the locality of collection, placed in a travel cooler, and transported to the laboratory. The salamander was isolated in glass petri dishes lined with moist filter paper, and maintained at 6-7° C for one week.

Following the isolation period, bacteria were sampled from the salamander by streaking the dorsum between the pectoral and pelvic girdles with a bacteriological loop. Bacterial samples were cultured on Tryptic Soy Yeast Extract (TSYE) agar (2g Difco Bacto tryptic soy broth, 1 g Difco Bacto yeast extract, 12g Difco Bacto purified agar to one liter of water) and allowed to grow for 72h at room temperature. Morphologically distinct bacterial colonies were re-isolated on TSYE agar and allowed to grow for 72 h to obtain pure cultures. Once pure cultures were obtained, isolates were cultured on TSYE agar slants, allowed to grow for two weeks at room temperature, and stored at 5° C until needed.

The plates were incubated at ambient temperature in the dark. The next day, not much growth was observed on the plates. Three sleeves of 20 TSYE plates per sleeve were prepared.

The next day, the four plates from the brooding *D. quadramaculatus* female were observed to have surprisingly little growth, only seven colonies being observed on the four plates. The isolates were struck on new TSYE plates for isolation and were incubated at ambient temperature in the dark.

Upon observation the next day, six colonies were seen and identified as DQ001A, DQ001B, DQ001C, DQ001D, DQ001E, and DQ001F. DQ001D, notably, was an opaque edge, white center colony.

Six plates were prepared with parallel stripes of five known opportunistic human pathogens, *Staphylococcus epidermidis*, *Enterobacter aerogenes*, *Enterococcus faecalis*, *Corynebacterium xerosis*, and *Serratia marcescens*. The plates were incubated at 37° C for 72 hours. Each of the six plates was then struck with one of each of the six isolates DQ001A-F. The six plates were then incubated at 37° C for 72 hours.

The six plates were then observed to determine if any of the six isolates exhibited an inhibitory effect against any of the five opportunistic human pathogens. It was observed that isolate DQ001D exhibited inhibition against *S. epidermidis* (inhibition zone = 37 mm), *E. faecalis* (inhibition zone = 32 mm), and *C. xerosis* (inhibition zone = 60 mm), but no inhibition against the other two pathogens. None of the other isolates exhibited any inhibition.

Example 2 - Cutaneous Microbial Flora and Antibiosis on Pathogenic Bacteria and Fungi in Salamanders

MATERIALS AND METHODS

Collection of Source Organisms

A series of 16 amphibian species, composed of at least five individuals per species (n=5), were collected from their natural, endemic habitats. Species and collection locations are summarized in Table 1.

TABLE 1 - Collection data summary for source organisms

Family	Species	Organism	Habitat Type
Plethodontidae	*Desmognathus folkertsi*	Salamander	Stream Edge
Plethodontidae	*Desmognathus fuscus*	Salamander	Stream Edge
Plethodontidae	*Desmognathus quadramaculatus*	Salamander	Stream Edge
Plethodontidae	*Eurycea cirrigera*	Salamander	Swamp
Plethodontidae	*Eurycea guttolineata*	Salamander	Stream Edge
Plethodontidae	*Eurycea lucifuga*	Salamander	Cave
Plethodontidae	*Plethodon aureolus*	Salamander	Woodland Forest
Plethodontidae	*Plethodon chattahoochee*	Salamander	Woodland Forest
Plethodontidae	*Plethodon chlorobryonis*	Salamander	Woodland Forest
Plethodontidae	*Plethodon dorsalis*	Salamander	Woodland Forest
Plethodontidae	*Plethodon ocmulgee*	Salamander	Woodland Forest
Plethodontidae	*Plethodon oconaluftee*	Salamander	Woodland Forest
Ambystomatidae	*Ambystoma opacum*	Salamander	Swamp
Ranidae	*Rana clamitans*	Frog	Swamp
Hylidae	*Acris creptitans*	Frog	Stream Edge
Hylidae	*Pseudacris crucifer*	Frog	Stream Edge

Once collected, amphibian species were placed in 3.79 liter plastic bags and were taken from the locality of collection, placed in a travel cooler, and transported to the laboratory. Amphibian species were individually isolated in 0.0946 L plastic containers lined with moist filter paper, placed in a Coron model 6010 Environmental Chamber, and maintained at 16° C for a period of one week.

Following the isolation period, bacteria were sampled from each amphibian species, phenotypically distinct bacteria isolated from the amphibian species were assigned a code corresponding to the individual from which the isolate was obtained. Each bacterial isolate was phenotypically characterized by standard colonial morphological techniques (Salle and Meacham, 1974). This characterization was achieved by noting whole colony forms, margin (edge) forms, elevation, color, and finish, for each distinct bacterial colony. Gram stain of the bacterial isolates was determined by the Gram stain technique as outlined in Salle, A. J. 1973. Laboratory Manual on Fundamental Principles of Bacteriology. McGraw Hill, New York, New York. U.S.A. to aid in identifying and classifying bacterial isolates. Cellular morphology of the

bacterial isolates was also noted. Following colonial and cellular characterization, each morphologically distinct bacterium isolated from the source organisms was cultured on TSYE agar slants, allowed to grow for two weeks at room temperature, and stored at 5 °C until needed.

Agar Plate Inhibition Trials Methodology

Morphologically distinct bacterial isolates isolated from source organisms were tested for the ability to produce antimicrobial compounds effective against Gram positive and Gram negative human pathogenic bacteria. The human pathogenic bacterial species utilized in the inhibition trials were *Enterococcus faecalis* (ATCC #29212), *Escherichia coli* (ATCC # 25922), *Pseudomonas aeruginosa* (ATCC #27853) and *Staphylococcus aureus* (ATCC # 29213).

Agar plate inhibition trials consisted of testing each morphologically distinct bacterium isolated from source organisms for the ability to inhibit growth of the human pathogenic bacteria according to the methodology and protocol listed in Alcamo, I. E. 1994. Laboratory Fundamentals of Microbiology. Benjamin Cummings, New York, New York, U.S.A.. Bacterial isolates from the source organisms were independently streaked on TSYE agar plates in a median streak with a bacteriological loop. The plates were then incubated at room temperatures for a minimum of 72 h to allow growth of the bacteria. The human pathogenic bacterial species were then streaked in single streaks at right angles to the initial resident bacterial culture. Care was taken to ensure that the pathogenic isolates did not touch the initial bacterial streak. The plates were then re-incubated for 48 h to allow growth of the human pathogenic bacterial species. Following incubation, the plates were examined for growth of the human pathogenic bacterial isolates adjacent to the original source orgnaisms bacteria.

Inhibition was considered as a zone of unobservable growth adjacent to the original resident bacterial streak. When observed, inhibition was quantified by measuring the distance of the zero-growth zone from the initial resident bacterium to the nearest 0.5 mm.

Antibacterial/Antifungal Metabolite Inhibition Trials Methodology

Bacteria isolated from source organisms that exhibited the ability to inhibit the growth of human pathogenic bacteria in the Agar Plate Inhibition Trials were grown in 0.5 L of liquid TSYE media at ambient temperature for a period of 7 days. Each isolate was grown in duplicate flasks for subjection to two different extraction procedures. Following the growth

period, complex molecules (metabolites) produced by the bacterial isolates isolated from source organisms were extracted from the liquid media utilizing standard liquid extraction methods.

The extraction methodology consisted of two independent extraction procedures, designed to target the extraction of both water-soluble and non-water soluble compounds for further testing.

The water-soluble extraction procedure consisted of rapidly freezing the liquid media sample containing the bacterial isolate cell culture at −80 °C. Following the freezing procedure, the frozen sample was lyophilized. Following lyophilization, the sample was added to 0.5L of 100 percent methanol and mechanically stirred for one hour. The sample was filter sterilized to ensure all bacterial cell/cell fragments were removed. Methanol was then evaporated from the resulting methanol/compound mixture utilizing a rotoevaporator, leaving only the crude extract of multiple compounds containing one or more compounds with potential biological activity.

The non-water soluble extraction procedure consisted of adding 150 ml of ethyl acetate to the media sample containing the bacterial isolate cell culture. This resulting mixture was then mechanically stirred for 15 minutes. The ethyl acetate was then separated from the mixture via a separatory funnel and saved. This procedure was repeated three times, resulting in 450 ml of ethyl acetate/compound mixture. The ethyl acetate was then evaporated from the resulting mixture with the use of a rotary evaporator, leaving only the crude extract of multiple compounds containing one or more compounds with potential biological activity.

Each resulting metabolite extract obtained from both water-soluble and non-water soluble extraction procedures of each of the inhibitory bacterial isolates was then tested for the ability to inhibit one or more human pathogenic bacteria, antibiotic-resistant human pathogenic bacteria, human fungal pathogens, and a human pathogenic virus. Human pathogenic bacteria utilized were *Enterococcus faecalis* ATCC 29212, vancomycin-resistant *Enterococcus faecium* (VRE) ATCC 700221, *Staphylococcus aureus* ATCC 29213, Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 33591, *Streptococcus pyogenes* ATCC 12358, penicillin resistant *Streptococcus pneumonia* ATCC 700674, *Pseudomonas aeruginosa* ATCC 27853, and *Salmonella typhimurium* ATCC 700408. Human pathogenic fungi utilized were *Candida albicans* ATCC 24433, *Microsporum gypseum* ATCC 14683, *Trichophyton mentagrophytes* ATCC 9533 and *Aspergillus fumigatus* IHEM 2895.

Antibacterial testing are conducted in Mueller-Hinton broth. The inoculum is prepared by making a direct saline suspension of isolated colonies selected from 18 to 24 hour agar plate.

Afterwards the inoculum is standardized to 500,000 CFU/ml. The incubation temperature is 35°C and the incubation time is 16 hours. Yeast screenings are conducted in RPMI 1640 broth supplemented with 0.3g/l glutamine and 34.6g/l morpholine propane sulfonic acid (MOPS-buffer). The incubation temperature is 35°C and the incubation time is 24 hours.

The inoculum is prepared by making a direct saline suspension of isolated colonies selected from 18 to 24 hour Sabouraud agar plate. Afterwards the inoculum is standardized (1000 to 5000 CFU/ml). As measurement tool, the area under the growth curve which is automatically determined via the Biolink software. The area of the sample only containing broth and test chemical (e.g. 25 ppm of an extract) is subtracted from the area of the sample also containing inoculum. Five replicates are used for each sample, resulting in a number that can be compared with the reference antibiotic, e.g. amphotericin B, penicillin and vancomycin. Results are expressed as percentage of growth relative to the sample without test-chemical.

Antimould screenings are conducted in RPMI 1640 broth supplemented with 0.3g/l glutamine and 34.6g/l morpholine propane sulfonic acid (MOPS-buffer). The incubation temperature/time is 35°C / 48 hours for the Aspergillus sp. The dermatophytes have an incubation temperature of 25°C and the incubation time is 5 days.

The inoculum is prepared by covering a seven days old culture with approximately 1ml of sterile saline. A suspension is made by gently probing the colonies with the tip of a Pasteur pipette. Afterwards the inoculum is standardized (4000 to 50000 CFU/ml).

For the moulds, kinetic measurements are not used, but only beginning and end point measurements. As measurement tool we use the OD (start and end) which is automatically determined via the Biolink software. OD-start is subtracted from OD-end. Five replicates are used for each sample. These data result in a number that can be compared with the reference antibiotic, e.g. amphotericin B. Results are also expressed as percentage of growth relative to the sample without test-chemical.

The human pathogenic bacterial and fungi species were exposed to standardized concentrations of 100 and 25 micrograms/milliliter (ppm) of the metabolite extract from each of the bacteria isolated from source organisms. The metabolite inhibition trials were conducted utilizing a Bioscreen C Analyzer, an automated reader-incubator capable of monitoring turbidity development (i.e. growth) of bacterial and fungal species by vertical photometry (optical density). The results were utilized to generate growth curves in response to the metabolite

extracts obtained from the bacterial isolates. For each test we also included a positive control or reference antibiotic with known MIC against the tested organism

Antiviral Inhibition Trials Methodology

Individual metabolites were extracted from the crude extracts obtained from the bacteria isolated from the skin environment of source organisms via the two extraction procedures previously described using an HPLC column, specifically, a Beckman Ultrasphere (4.6 mm x 150 mm), Part No. 235330, RP-18, 5 m particle size. The eluent was a 75:25 mixture of acetonitrile and water with a flow of 1 ml/min. A UV detector was set at 210 nm. Metabolite extracts from bacteria isolated from the skin environment of source organisms, were tested for their ability to inhibit HIV-induced cytopathicity in human lymphocyte CEM and MT-4 cell cultures. CEM cells were suspended at approximately 250,000 cells per milliliter of culture medium and infected with wild-type HIV-1(III_B) at approximately 100 times the 50% cell culture infective dose ($CCID_{50}$) per milliliter. Then, 100 l of the infected cell suspensions were added to 200- l microtiter plate wells containing 100 l of an appropriate dilution of the test compounds. After 4 days incubation at 37 °C, the cell cultures were microscopically examined for syncytium formation. The EC_{50} (50% effective concentration) was determined as the compound concentration required to inhibit syncytium formation by 50%.

Antitumor Trials Methodology

Metabolites extracted from bacteria isolated from the skin environment of source organisms, were tested for their ability to inhibit the proliferation of murine leukemia cells (L1210/0), murine mammary carcinoma cells (FM3A), and human T-lymphocyte cells (Molt4/C8 and CEM/0) cells. All assays were performed in 96-well microtiter plates. To each well were added 5 - 7.5 x 10^4 cells and a given amount of the test compound. The cells were allowed to proliferate for 48 hr (murine leukemia L1210, murine mammary carcinoma FM3A) or 72 hr (human lymphocyte CEM and Molt) at 37 °C in a humidified CO_2-controlled atmosphere. At the end of the incubation period, the cells were counted in a Coulter counter. The IC_{50} (50% effective inhibitory concentration) was defined as the concentration of the compound that reduced the number of viable cells by 50%.

RESULTS

The cutaneous microbial flora was sampled from 16 amphibian species (source organisms) comprised of 13 salamander and 3 frog species (Table 1). From these samples, a total of 417 morphologically distinct bacterial isolates were isolated. These included 356 unique isolates from salamanders comprised of 306 Gram negative rods, 20 Gram positive rods, 25 Gram negative cocci, and 5 Gram positive cocci, and 61 unique isolates from frogs comprised of 37 Gram negative rods, 10 Gram Positive rods, 7 Gram negative cocci, and 7 Gram positive cocci (Table 2).

TABLE 2 - Summary of bacterial isolates isolated from the skin environment of amphibians

	Number of Gram Negative Rod Isolates	Number of Gram Positive Rod Isolates	Number of Gram Negative Coccus Isolates	Number of Gram Positive Coccus Isolates
Salamander bacterial isolates	306	20	25	5
Frog bacterial isolates	37	10	7	7

Agar Plate Inhibition Trials

Agar plate inhibition trials of each of the 417 unique bacterial isolates against the four human bacterial pathogens *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Staphylococcus aureus* (ATCC 29213), revealed the presence of 13 unique bacterial isolates capable of inhibiting the growth of one or more of the human pathogenic species. Of these 13 isolates, nine were isolated from salamanders of the family Plethodontidae and four were isolated from frogs of the families Hylidae and Ranidae. The isolates of *Desmognathus quadramaculatus*, in contrast to the gravid, brooding female of Experiment 1, did not exhibit substantial inhibition. It is known that the cutaneous microflora of salamanders are affected by hormones, environmental changes, and other factors, so it is not surprising that differences in the microflora were observed between the individual of Experiment 1 and the individuals of Experiment 2. Accordingly, the bacterial isolate DQ001D of Experiment 1 was included in the tests conducted under this Experiment 2. Colonial and cellular morphological characteristics of the 13 unique bacterial isolates capable of inhibiting pathogenic bacterial growth are summarized in Table 3.

TABLE 3 - Summary of the colonial and cellular morphology characteristics of bacteria exhibiting inhibitory capabilities isolated from amphibians

Isolate number	Colony Form	Margin Form	Elevation Form	Color	Finish	Gram Reaction	Source Organism
DQ001D	Circular	Entire	Umbonate	Opaque White Edge, White Center	Glossy	Gram Neg. Rod	Salamander
PO014	Circular	Entire	Convex	Peach Center, White Edge	Glossy	Gram Neg. Rod	Salamander
PO019	Circular	Entire	Convex	Yellow	Glossy	Gram Neg. Rod	Salamander
PO026	Circular	Entire	Convex	Opaque White	Glossy	Gram Neg. Rod	Salamander
PO027	Circular	Undulate	Convex	White	Glossy	Gram Neg. Rod	Salamander
AC021	Circular	Undulate	Convex	Opaque White	Glossy	Gram Neg. Rod	Frog
AC024	Irregular	Undulate	Convex	White	Glossy	Gram Neg. Rod	Frog
PC017	Circular	Entire	Convex	White	Glossy	Gram Positive Rod	Frog
PD026	Circular	Undulate	Convex	Opaque Peach	Glossy	Gram Neg. Rod	Salamander
EG006	Irregular	Lobate	Convex	Orange	Glossy	Gram Neg. Rod	Salamander
EC009	Punctiform	Entire	Convex	Yellow	Glossy	Gram Neg. Rod	Salamander
EC024	Irregular	Lobate	Umbonate	Opaque White Edge, White Center	Glossy	Gram Neg. Rod	Salamander
RC003	Circular	Serrate	Convex	Purple	Glossy	Gram Positive Coccus	Frog

Of the 13 unique bacterial isolates isolated from source organisms, eight exhibited the capability of inhibiting more than one of the human pathogenic species against which they were tested. Five of the unique bacterial isolates isolated from source organisms were effective in inhibiting only one of the human pathogenic species against which they were tested. As can be seen from the data presented in Table 4, bacterial isolates DQ001D, AC021, EG006, EC009, PO026, PO027, and RC003 exhibited the capability of producing compound(s) inhibiting the

growth of *Enterococcus faecalis* (ATCC 29219). Bacterial isolates EG006, EC009, PO014, EC024, and AC024 exhibited the capability of producing compound(s) inhibiting the growth of *Escherichia coli* (ATCC 25922). Bacterial isolates AC021, PO019, PO014, PO027, RC003, PD026, and PC017 exhibited the capability of producing compound(s) inhibiting the growth of *Staphylococcus aureus* (ATCC 29213). Bacterial isolates EC024 and AC024 exhibited the capability of producing compound(s) inhibiting the growth of *Pseudomonas aeruginosa* (ATCC 27853).

TABLE 4 - Inhibition zones of human pathogenic bacteria by the bacterial isolates

	Inhibition Zone (mm)			
Isolate	*Enterococcus faecalis*	*Escherichia coli*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
DQ001D	37.0			
PO014		17.5	>50	
PO019			45.5	
PO026	25.0			
PO027	23.0		22.0	
AC021	27.0		30.5	
AC024		29.5		32.0
PC017			22.0	
PD026			>50	
EG006	>50	>50		
EC024		35.5		32.0
EC009	>50	>50		
RC003	44.0		45.5	

Antibacterial/Antifungal Metabolite Inhibition Trials

Metabolite extracts from the 13 bacterial isolates isolated from the skin environment of source organisms which inhibited pathogenic bacterial growth during agar plate inhibition trials were tested for their efficacy in inhibiting pathogenic bacterial or fungal growth. Of these metabolites, extracts from bacterial isolates DQ001D, PO026, PO027 and PC017 exhibited strong biological activities in inhibiting the growth of pathogenic bacteria, pathogenic fungi, or both.

The metabolite extract from isolate number DQ001D was effective in inhibiting multiple Gram positive bacterial pathogenic species at both 100 ppm (micrograms/milliliter) and 25 ppm. Specifically, metabolic extracts from DQ001D completely inhibited the growth of pathogenic species *Enterococcus faecalis*, vancomycin-resistant *Enterococcus faecium*, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, *Streptococcus pyogenes*, and

penicillin-resistant *Streptococcus pneumonia*. Additionally, the metabolite extract from DQ001D was also effective in completely inhibiting the growth of the pathogenic human fungus *Candida albicans* at 100 ppm and showed partial growth inhibition of the human fungal pathogen *Microsporum gypseum* at 100 ppm.

The metabolic extract from isolate number PO026 showed complete growth inhibition of the pathogenic fungus *Candida albicans* at 100 ppm.

The metabolic extract from isolate number PO027 was effective in completely inhibiting the growth of the human bacterial pathogen *Staphylococcus aureus* at 25 ppm. Additionally, the metabolic extract from PO027 also inhibited the growth of the fungal pathogen *Candida albicans* at 250 ppm.

The metabolic extract from isolate number PC017 exhibited the ability to inhibit the growth of the human fungal pathogen *Candida albicans* at 100 ppm. Similar results of antibacterial/antifungal activities of extracts from other bacterial isolates from amphibian species have been recently obtained, further indicating the application of the invention, leading to the expectations of finding other such activities in the future.

Antiviral Inhibition Trials

Metabolites extracted from the 13 bacterial isolates isolated from the skin environment of source organisms which inhibited pathogenic bacterial growth during agar plate inhibition trials were tested for their efficacy in inhibiting the growth of multiple strains of the Human Immunodeficiency Virus (HIV). Of these extracts, the metabolite extract from DQ001D (retention time 7.27 min) exhibited marked abilities in inhibiting HIV cytopathicity in human lymphocyte CEM and MT-4 cell cultures. Specifically, the effective metabolite concentration of DQ001D required to inhibit HIV cytopathicity of HIV-1 and HIV-2 in CEM cells was 7 ppm and 17 ppm respectively. The effective metabolite concentration of DQ001D required to inhibit HIV-induced cytopathicity of HIV-1 in MT-4 cells was 9 ppm. The determined toxicity of the metabolite extract from DQ001D on MT-4 cells was 40 ppm. Based on the experience to date testing the isolates, it is expected that future antiviral activity of other isolates will be indicated.

Antitumor Trials

The metabolite extract from bacterial isolate number DQ001D was shown to inhibit the proliferation of murine leukemia cells (L1210/0), murine mammary carcinoma cells (FM3A)

RegenX Science Exhibit 1004
Page 1326

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1326

and human T-lymphocyte cells (Molt4/C8, CEM/0) at 17 ppm, 16 ppm, 17 ppm and 17 ppm respectively. Based on the experience to date testing the isolates, it is expected that future antitumor activity of other isolates will be indicated.

The resistance of bacteria and fungi to known antibiotics and antifungal drugs has led to a world-wide search for sources of new sources for drugs that are capable of combating such diseases. Because of the previous extreme low success rate of finding new bacterial sources of such medically useful compounds, researchers have focused upon macro-organisms as potential new sources of new medically useful drugs (e.g. Beattie 1992). The data presented here provide clear and substantial evidence as to amphibians being sources for medically useful bacteria capable of producing antibacterial, antifungal, antiviral, and antitumor metabolites.

Additionally, antibiotic resistance of bacteria to known drugs presents a serious world-wide health problem. This study provides clear indication that the skin environment of amphibians contains bacterial species which are capable of inhibiting the growth on multiple human bacterial and fungal pathogens, including those are highly antibiotic resistant such as vancomycin resistant *Enterococcus faecium* and methicillin resistant *Staphylococcus aureus*. Furthermore, the antiviral and antitumor activity of the metabolite extract from the bacterial isolate DQ001D provides clear indication of the application of medically useful compounds isolated from the skin environment of amphibians. Given the biological activity of this and other isolates, it is believed and expected that future isolates will exhibit similar antiviral activities.

The foregoing description comprise illustrative embodiments of the present inventions. The foregoing embodiments and the methods described herein may vary based on the ability, experience, and preference of those skilled in the art. Merely listing the steps of the method in a certain order does not necessarily constitute any limitation on the order of the steps of the method. The foregoing description and drawings merely explain and illustrate the invention, and the invention is not limited thereto, except insofar as the claims are so limited. Those skilled in the art who have the disclosure before them will be able to make modifications and variations therein without departing from the scope of the invention.

What is claimed is:

1. An extract from a bacterium isolated from the skin of an organism that respires at least partially through its skin that inhibits the growth of bacteria, fungi, viruses, or tumors.

2. An extract as defined in claim 1, wherein the organism is an amphibian.

3. An extract as defined in claim 1, wherein the organism is selected from the group consisting of salamanders.

4. An extract as defined in claim 1, wherein the organism is selected from the group consisting of frogs.

5. An extract as defined in claim 1, wherein the extract inhibits the growth of human pathogenic bacteria or human pathogenic fungi.

6. An extract as defined in claim 1, wherein the extract inhibits the growth of human HIV.

7. An extract as defined in claim 6, wherein the human pathogenic bacteria are selected from the group comprising *Enterococcus* sp., *Staphylococcus* sp., *Escherichia* sp, and *Pseudomonas* sp.

8. An extract as defined in claim 1, wherein the extract is effective in a range between about 5 ppm and about 250 ppm.

9. Administering to a human a therapeutic amount of an extract as defined in claim 1.

10. A bacterial isolate selected from the group consisting of DQ001D, PO014, PO019, PO026, PO027, AC021, AC024, PC017, PD026, EG006, EC009, EC024, and RC003.

11. A method of identifying biologically active bacterial metabolites, comprising culturing a bacterial isolate according to claim 10 in an assay system comprising one or more pathogenic organisms and assessing the effect of the metabolites produced by the bacterial isolate on the growth of the pathogenic organisms(s) relative to a control.

12. A method of identifying biologically active extracts, fractions, or compounds, comprising

(a) culturing a bacterial isolate according to claim 10;

(b) extracting metabolites from the culture;

(c) optionally fractionating the extracted metabolites; and

(d) assessing the ability of the extracted or fractionated metabolites to inhibit the growth of pathogenic organisms, tumor cells, or viruses in a suitable assay system.

13. A biologically active extract, fraction, or compound identified according to the method of claim 12.

14. A pharmaceutical composition comprising one or more biologically active extracts, fractions, or compounds according to claim 13 and a pharmaceutically acceptable carrier.

15. A pharmaceutical composition according to claim 14, wherein the composition is suitable for topical, enteral, parenteral, or intravenous administration to a subject.

16. A method of inhibiting the growth of pathogenic organisms in a subject comprising administering an effective amount of a pharmaceutical composition according to claim 14 to the subject.

17. A method of inhibiting the growth of tumor cells in a subject comprising administering an effective amount of a pharmaceutical composition according to claim 14 to the subject.

RegenX Science Exhibit 1004
Page 1329

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1329

18. A method of inhibiting the growth of a virus in a subject comprising administering an effective amount of a pharmaceutical composition according to claim 14 to the subject.

19. A method according to claim 18, wherein the virus is a human immunodeficiency virus.

RegenX Science Exhibit 1004
Page 1330

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
1330

FIG. 1

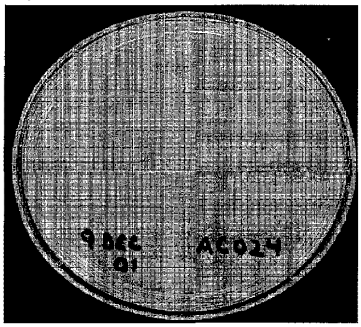


FIG. 2

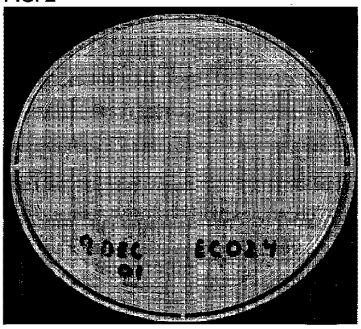
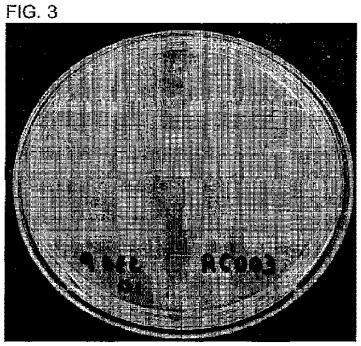


FIG. 3



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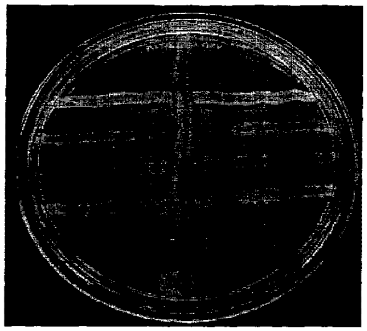
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(54) Title: ANTIBIOTICS FROM BACTERIA ISOLATED FROM AMPHIBIAN SKIN



(57) Abstract: This invention discloses extracts including a biologically active compound or combination of compounds derived from microorganisms isolated from mucous-producing organisms that respire at least partially through their skin. Rod-shaped bacteria isolated from the skin of salamanders and frogs are found to produce compound(s) that have antiviral, antitumor, antibacterial and antifungal properties. These compounds have an inhibitory effect on opportunistic human pathogens, including *Candida* sp., *Microsporum* sp., *Staphylococcus* sp., *Pseudomonas* sp., *Escherichia* sp. and *Enterococcus* sp., as well as on HIV strains and tumor cell lines.

WO 02/078720 A3

WO 02/078720 A3

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

【国際調査報告】

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/00217

A. **CLASSIFICATION OF SUBJECT MATTER**
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US CL : ; G01N 424/94.26, 115; 435/7.21, 7.32, 41, 42, 93.5
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B. **FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/94.26, 115; 435/7.21, 7.32, 41, 42, 93.5

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. **DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6,310,176 B1 (BARRA et al.) 30 October 2001, column 1, line 1-column 4, line10 and Table 2.	1-2, 4-5, 7-9, 12-16
A	US 2002/0035061 A1 (KRIEGER et al.) 21 March 2002, paragraph 17, Tables 1, 3-6 and Claims 30-65.	1-9, 11-16 AND 18-19

☐ 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 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
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"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
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer DR. Kailash C. Srivastava Telephone No. (703)-308-0196

Janice Ford for

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PCT/US02/00217

Continuation of Item 4 of the first sheet:
TITLE TOO LONG (18 WORDS)

ANTIBIOTICS FROM BACTERIA ISOLATED FROM AMPHIBIAN SKIN

Continuation of B. FIELDS SEARCHED Item 3:
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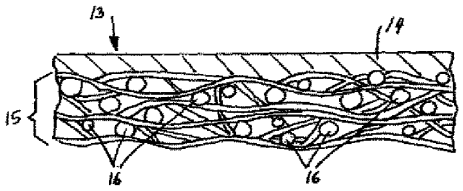
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最終頁に続く

(54)【発明の名称】ＥＣＭを基材としたグラフト材料

(57)【要約】
【解決手段】　本発明は、細胞外基質（ＥＣＭ）と治療剤を備えているグラフト材料に着眼している。本発明は、患者身体の損傷又は病変のある組織を治癒するために当該グラフト材料を使用する方法にも着眼している。
【選択図】　図２



【特許請求の範囲】
【請求項1】
細胞外基質（ＥＣＭ）と、
少なくとも１つの治療剤と、を備えているグラフト材料。
【請求項2】
前記ＥＣＭは細胞外膠原基質である、請求項１に記載のグラフト材料。
【請求項3】
前記細胞外膠原基質は、コラーゲン、グリコプロテイン、プロテオグリカン、及びグリコサミノグリカンを含んでいる、請求項２に記載のグラフト材料。
【請求項4】
前記ＥＣＭは、小腸粘膜下組織、無細胞真皮、死体筋膜、膀胱無細胞基質、及び羊膜から成る群より選択される、請求項１に記載のグラフト材料。
【請求項5】
前記ＥＣＭは、小腸粘膜下組織である、請求項４に記載のグラフト材料。
【請求項6】
前記小腸粘膜下組織は流動化している、請求項５に記載のグラフト材料。
【請求項7】
前記治療剤は、成長因子、抗生剤、抗ウイルス剤、鎮痛剤、ステロイド系抗炎症剤、非ステロイド系抗炎症剤、抗新生物剤、鎮痙剤、細胞－細胞外基質相互作用モジュレータ、酵素及び酵素阻害物質、抗凝固剤及び／又は抗血栓剤、ＤＮＡ、ＲＮＡ、ＤＮＡ又はＲＮＡ又はタンパク質合成阻害物質、ポリペプチド、細胞の移動を調整する化合物、増殖と成長を調整する化合物、及び血管拡張剤から成る群より選択される、請求項１に記載のグラフト材料。
【請求項8】
少なくとも２つの治療剤が含まれている、請求項１に記載のグラフト材料。
【請求項9】
前記治療剤は、抗生物質、抗ウィルス剤、及び抗真菌剤である、請求項８に記載のグラフト材料。
【請求項10】
前記治療剤は、ペニシリン、セファロスポリン、サイクロセリン、バンコマイシン、イミダゾール抗真菌剤、ポリミキシン、アンホテリシンＢ、クロラムフェニコール、テトラサイクリン、リファンピン、エリスロマイシン、クリンダマイシン、リファマイシン、キノロン、スルホンアミド、ジドブジン、アシクロビル、及びミノサイクリンから成る群より選択される、請求項９に記載のグラフト材料。
【請求項11】
前記少なくとも１つの治療剤は、時間経過に伴い必要に応じて組織に放出される、請求項１に記載のグラフト材料。
【請求項12】
補助剤を更に含んでいる、請求項１に記載のグラフト材料。
【請求項13】
添加物を更に含んでいる、請求項１に記載のグラフト材料。
【請求項14】
前記添加物は、安定剤、増量剤、抗酸化剤、触媒、可塑剤、顔料、及び潤滑剤から成る群より選択される、請求項１３に記載のグラフト材料。
【請求項15】
組織の治療を促進するための方法において、
治療を要する組織を、細胞外基質（ＥＣＭ）と少なくとも１つの治療剤とを含んでいるグラフト材料とともに接触させる段階、を含んでいる方法。
【請求項16】

前記組織は皮膚である、請求項１５に記載の方法。
【請求項17】
前記ＥＣＭは、小腸粘膜下組織、無細胞皮膚、死体筋膜、膀胱無細胞基質、及び羊膜から成る群より選択される、請求項１５に記載の方法。
【請求項18】
前記ＥＣＭは、小腸粘膜下組織である、請求項１７に記載の方法。
【請求項19】
前記小腸粘膜下組織は流動化している、請求項１８に記載の方法。
【請求項20】
前記治療剤は、成長因子、抗生剤、抗ウイルス剤、ステロイド系抗炎症剤、非ステロイド系抗炎症剤、抗新生物剤、鎮痙剤、細胞－細胞外基質相互作用モジュレータ、酵素及び酵素阻害物質、抗凝固剤及び／又は抗血栓剤、ＤＮＡ、ＲＮＡ、ＤＮＡ又はＲＮＡ又はタンパク質合成阻害物質、ポリペプチド、細胞の移動を調整する化合物、増殖と成長を調整する化合物、及び血管拡張剤から成る群より選択される、請求項１５に記載の方法。

【発明の詳細な説明】
【技術分野】
【0001】
本発明は、細胞外基質（ＥＣＭ）と治療薬を備えているグラフト材料に着眼している。本発明は、患者身体の損傷又は病変のある組織を治癒するためのグラフト材料の使用方法にも着眼している。
【背景技術】
【0002】
本発明は、３５Ｕ．Ｓ．Ｃ．第１１９条（ｅ）に基づき、２００４年3月３１日出願の米国仮特許出願第６０／５５８，１７５号の恩典を主張すると共に、同仮特許出願を参考文献として本願に援用する。
【0003】
慢性的又は一時的な創傷を効果的に治すのに不適切な方法及び組成は、保健医療上深刻な問題である。創傷の治癒が巧く行かないと、死亡及び病的状態に陥る可能性が高まる。皮膚の創傷は、皮膚に対する最小限の直接的外傷により生じる、連続した体組織における裂け目と定義される。創傷を被った皮膚が迅速に閉じることが、有効な反応を促進する。
【0004】
皮膚に対する最も一般的な外傷の１つに熱傷がある。熱傷は、表皮並びに深層の皮膚及び皮下組織の破壊を引き起こす。熱傷を受けた面積が広範に及んでおらず且つ汚染されていなければ、上記組織の殆どは自然な治癒反応により再生することができる。米国では、熱傷は年間２００万件を越える傷害の原因であり、重度の熱傷傷害が原因で毎年１万人以上が死亡している。
【0005】
糖尿病患者では、重症で生命を脅かすほどの創傷が体の四肢によく見受けられる。慢性糖尿病による足の潰瘍が原因で切断に到ることも多い。そのような創傷の効果的な治療が求められている。
【0006】
傷害を被った組織及び器官の効果的な修復と再生は、細胞浸潤と代謝支援に必要な血流の早期確立にかかっていることは知られている。損傷又は病変のある組織に置換するために設計される生体材料は、細胞が中に入り込める支持体（すなわち、スカフォード(scaffold)）として機能し、上記必要とされる供給を確立せねばならない（Han ZC 並びに Liu Y, Int. J. Hematol.（１９９９年））。
【0007】
これまで、１つの取り組み方として、合成誘導生体適合性ポリマーのスカフォード(scaffold)を、組織及び修復と再生のためのバックボーンとして機能させて、損傷又は病変のある組織を治療する方法があった。それら合成ポリマー・スカフォードは頑丈で、その後

の沈着が進むと所定の速度で分解する（又は全く分解しない）ように作ることができる。また、これらの合成スカフォードは、置換対象の天然組織の材料特性を真似て設計される。しかしながら、合成スカフォードを使用する場合、しばしば幾つかの臨床学的合併症に遭遇することになる。

【0008】

これらの合併症ために、別の取り組み法として、ホスト細胞の成長支援として動物組織から入手した無傷の細胞外基質（ＥＣＭ）を活用して組織の修復と再生を図るという方法もあった。

【0009】

Badylak他（Badylak他、心臓外科フォーラム#2002-72222 6(2)（２００３年））は、心筋組織の修復に関連してブタＥＣＭスカフォードの使用について研究を行った。Badylak他は、膀胱粘膜下組織（ＵＢＭ）及び小腸粘膜下組織－ＥＣＭ（ＳＩＳ－ＥＣＭ）スカフォードは、共に、外科的移植後に完全に吸収され、心筋、線維性結合組織、脂肪性結合組織、及び軟骨性結合組織を含む結合組織の混合体に置換されることを見い出した。

【0010】

Bilbo（ＷＯ０２／２２１８４号）は、天然組織である腸コラーゲン（ＩＣＬ）由来の加工された組織基質から作られた、移植対象の患者又はホストとの生体適合性を有する、組織工学による多層人工器官を教示している。

【0011】

温血脊椎動物の腸の粘膜下組織膜を含む組成物は、組織グラフト材料として使用することができる。その様な組織グラフト組成物は、高い破裂圧力を含む優れた機械的性質と有効間隙率を特徴としており、このおかげで、それら組成物は、血管グラフト並びに結合組織グラフト構造体として有効に使用することができる。そのような用途に使用される場合、グラフト構造体は、グラフト構造体で置換された組織を再成長させる基質として働くのみならず、内生組織のそのような再成長を促進又は誘導する。この改造過程の一般的な事象には、広範に及ぶ迅速な新血管形成、肉芽間葉細胞の増殖、移植された腸の粘膜下組織材料の生分解／再吸収、及び免疫拒絶の無いこと、が含まれる。

【0012】

患者身体の傷害又は病変のある組織を治癒するための新奇なグラフト材料とそれらグラフト材料を使用する方法を、ここで提案している。

【特許文献1】米国仮特許出願第６０／５５８，１７５号
【特許文献2】国際特許ＷＯ０２／２２１８４号
【特許文献3】国際特許ＷＯ０３／０２１６５号
【特許文献4】米国特許第６，３７９，７１０号
【特許文献5】米国特許第４，５０２，１５９号
【特許文献6】国際特許ＷＯ０４／２２１０７号
【特許文献7】米国特許第６，２０６，９３１号
【特許文献8】米国特許第６，３５８，２８４号
【特許文献9】米国特許第６，６６６，８９２号
【特許文献10】ＰＣＴ出願ＰＣＴ／ＵＳ９７／２２７２７号に基づくＷＯ９８／２５６３７号
【特許文献11】ＰＣＴ出願ＰＣＴ／ＵＳ９７／２２７２９号に基づくＷＯ９８／２６２９１号
【特許文献12】ＰＣＴ出願ＰＣＴ／ＵＳ９７／２３０１０号に基づくＷＯ９８／２５６３６号
【特許文献13】米国特許第５，５５４，３８９号
【特許文献14】米国特許第４，９０２，５０８号
【特許文献15】米国特許出願第０８／９１６，４９０号
【特許文献16】米国特許第５，７３３，３３７号
【特許文献17】ＰＣＴ出願／ＵＳ９７／１４８５５号に基づくＷＯ９８／２２１５８号
【特許文献18】

米国特許出願公開番号第２００４０１８００４２号
【特許文献19】米国特許第５，２８１，４２２号
【特許文献20】米国特許第５，２７５，８２６号
【特許文献21】米国特許第５，５１６，５３３号
【特許文献22】米国特許第６，２６４，９９２号
【特許文献23】米国特許第２，１２７，９０３号
【特許文献24】米国特許第５，７１１，９６９号
【特許文献25】米国特許出願第０８／９１６，４９０号
【特許文献26】米国特許第６，３５８，２８４号
【特許文献27】米国特許第５，１７６，９９６号
【特許文献28】米国特許第５，２６４，５６４号
【特許文献29】米国特許第５，２５６，７７５号
【特許文献30】米国特許第５，２０８，０３６号
【特許文献31】ＰＣＴ出願番号第ＷＯ９６／２０６９８号
【非特許文献1】Han ZC 並びに Liu Y, Int. J. Hematol.(１９９９年)
【非特許文献2】Badylak他、心臓外科フォーラム#2002-72222 6(2)（２００３年）
【非特許文献3】HoddeJ.,組織工学8(2);295-308（２００２年）
【非特許文献4】１７ネイチャーバイオテクノロジー誌１０８３（１９９９年１１月）
【非特許文献5】Medalie他、ASAIO J.42:M455(１９９６年)
【非特許文献6】Isch他、J. Pediatr. Surg. 36:266(２００１年)
【非特許文献7】Chaplin他、Newrosurgery 45:320(１９９９年)
【非特許文献8】Inoue並びにLleon, J. Reconstr. Microsurg. 12:307（１９９６年）
【非特許文献9】Walden他、Ann. Plast. Surg. 45:162（２０００年）
【非特許文献10】Dalla他、J. Pediatr. Surg. 45:162（２０００年）
【非特許文献11】Medalie他ASAIO J. 42:M455（１９９６年）
【非特許文献12】Carbone他、J. Urol. 165:1605（２００１年）
【非特許文献13】Meezan他、Life Sci. 17:1721（１９７５年）
【非特許文献14】Badylak他、J. Pediatr. Surg. 35:1097(２０００年)
【非特許文献15】Merguerian他、BJU Int.85:894（２０００年）
【非特許文献16】Wefer他、J. Urol. 165:1755（２００１年）
【非特許文献17】Reddy他、J. Urol. 164:936（２０００年）
【非特許文献18】Badylak他、J.Pediatr. Surg. 35:1097（２０００年）
【非特許文献19】Aplin他、J. Cell Sci.79:119（１９８５年）
【非特許文献20】Lei他、Biol. Reprod.60:176号（１９９９年）
【非特許文献21】Meinert他、J. Obstet. Gynecol. 184:679（２００１年）
【非特許文献22】Avila他、Cornea 20:414（２００１年）
【非特許文献23】Young他、Fertil. Steril. 55:624（１９９１年）
【非特許文献24】Gene Therapy andNeuroscience Applications Ed. Cplitt and Loewy, Academic Press, San Diego （１９９５年）
【非特許文献25】Sambrook, Fritsch及びManiatis著、Molecular Cloning A Laboratory Manual、第２版（Cold Spring Harbor Laboratory Press１９８９年）、第１６章及び１７章
【非特許文献26】Goodman & Gilman’s The Pharmacological Basis of Therapeutics, 第９版
【発明の開示】
【課題を解決するための手段】
【0013】
或る実施形態では、本発明は、細胞外基質（ＥＣＭ）と少なくとも１つの治療薬とを備えているグラフト材料を包含している。グラフト材料のＥＣＭは、細胞外膠原基質であるのが望ましい。グラフト材料に含まれる治療薬としては、成長因子、抗生剤、抗真菌剤、

鎮痛剤、抗ウイルス剤、ステロイド性抗炎症剤、非ステロイド性抗炎症剤、抗新生物剤、鎮痙剤、細胞－細胞外基質相互作用モジュレータ、酵素及び酵素阻害物質、抗凝固剤及び／又は抗血栓剤、ＤＮＡ、ＲＮＡ、ＤＮＡ又はＲＮＡ又はタンパク質合成阻害物質、ポリペプチド、細胞の移動、増殖、及び／又は成長を調整する化合物、及び血管拡張剤が挙げられる。

【0014】

別の実施形態では、本発明は、組織の治癒を促進するための方法である。この方法は、治療を必要とする組織をグラフト材料と接触させる段階を含んでいる。グラフト材料は、ＥＭＣと少なくとも１つの治療薬を含んでいる。グラフト材料のＥＣＭは、細胞外膠原基質であることが望ましい。グラフト材料に含まれる治療薬としては、成長因子、抗生剤、抗真菌剤、鎮痛剤、抗ウイルス剤、ステロイド性抗炎症剤、非ステロイド性抗炎症剤、抗新生物剤、鎮痙剤、細胞－細胞外基質相互作用モジュレータ、酵素及び酵素阻害物質、抗凝固剤及び／又は抗血栓剤、ＤＮＡ、ＲＮＡ、ＤＮＡ又はＲＮＡ又はタンパク質合成阻害物質、ポリペプチド、細胞の移動、増殖、及び／又は成長を調整する化合物、及び血管拡張剤が挙げられる。

【発明を実施するための最良の形態】

【0015】

なお、本発明は、特定の方法論、プロトコル、細胞系、動物種又は属、構造体、又は上記試薬に限定されるものではなく、本発明は様々に適用できるものと理解されたい。また、ここで使用している用語論は特定の実施形態を説明することだけを目的としたものであり、本発明の範囲を限定するものではなく、本発明の範囲は特許請求の範囲によってのみ限定されるものであることも併せて理解されたい。

【0016】

なお、本明細書及び特許請求の範囲で使用する場合、単数形の「或る」及び「当該、前記」には、特に明記しない限り、複数の対象物への言及が含まれることに注目されたい。従って、例えば、「或る細胞」という場合は、１つ又はそれ以上の細胞への言及であり、当業者には既知のそれらの等価物を包含する。

【0017】

特に定義しない限り、ここで使用する全ての技術及び科学用語は、本発明が属する分野における当業者が一般に理解しているのと同じ意味を持つ。ここで説明しているものと同様又は等価な方法、装置、及び材料は、何れも本発明の実施又は試験に使用することができるが、好適な方法、装置、及び材料をこれより説明する。

【0018】

本発明では、患者身体の損傷又は病変のある組織を治癒すると共に治療薬を患者に送り込むためのグラフト材料、及びそれらグラフト材料を使用する方法を記載している。

【0019】

或る実施形態では、本発明は、細胞外基質（ＥＣＭ）と少なくとも１つの治療薬を含んでいるグラフト材料を考えている。

【0020】

別の実施形態では、本発明は、組織の治癒を促進する方法を考えている。この方法は、治療を必要としている組織をグラフト材料と接触させる段階を含んでいる。グラフト材料は、ＥＣＭと少なくとも１つの治療薬を含んでいる。

【0021】

本発明のグラフト材料を使用することの１つの利点は、例えば、グラフト材料による治療を必要としている患者身体の一部の創傷清拭を繰り返し行う必要が少なくなることである。

【0022】

用語の定義

「グラフト」は、体の一部を修復するためにドナーからレシピエントに移植される組織又は器官の一部であり、患者がドナーでもあると同時にレシピエントでもあるという場合

もある。例えば、グラフトは、破壊された組織に置換されるか、何も無かったところに新しい組織を生成する。

【0023】

「生体適合性」という用語は、使用を意図している生体内環境において実質的に無害であって、且つ患者の生理系に実質的に拒絶されない（即ち、非抗原性である）、或る種の細胞外基質材料のようなものをいう。これは、国際標準化機構（ＩＳＯ）標準、第１０９９３号及び／又は米国薬局方（ＵＳＰ）２３及び／又は米国食品医薬品局（ＦＤＡ）ブルーブック備忘録第Ｇ９５－１の「国際標準化機構ＩＳＯ－１０９９３の使用、医療装置の生物学的評価、第１部：評価と試験」に記載されている生体適合性試験に合格する材料の能力によって測定することができる。通常、この試験では、材料の毒性、伝染力、発熱性、刺激の可能性、反応性、溶血作用、発癌性、及び／又は免疫原性が測定される。生体適合性構造体又は材料は、患者の大多数に導入した場合でも、目だって悪い生体反応又は応答が長く続いたり更に激しさを増すような事態は起こさないはずであり、手術又は生体器官への異物の移植に伴ってよく起こる軽度の一過性の炎症とは区別される。

【0024】

「生体分解性の」及び「生体侵食性の」という用語は、使用を意図している生体内環境に置かれた場合に、生体組織に通常存在する状態の下で代謝又は排泄される成分に最終的には溶解してしまうグラフト材料、移植片、被覆、又は包帯のようなものをいう。代表的な実施形態では、生態分解又は生体侵食の速度及び／又は程度は、予測可能なやり方で制御される。

【0025】

「治療用化合物」又は「治療薬」は、患者身体の損傷又は病変のある組織の治癒に有効な化合物又は薬剤を意味する。

【0026】

「治癒する」という用語は、患者身体の損傷又は病変のある組織を、置換、修復、治癒、又は治療することを意味する。

【0027】

「核酸」という用語は、リボヌクレオチド又はデオキシヌクレオチドの何れかのヌクレオチドのポリマー形態、又は上記何れかのヌクレオチドの変性形態をいう。上記用語は、等価物として、ヌクレオチド類似体から形成されたＲＮＡ又はＤＮＡの何れかの類似体を含み、また上記実施形態に適用可能なものとして、一本鎖（センス又はアンチセンスなど）及び二本鎖のポリヌクレオチドを含むものと理解されたい。

【0028】

ここで同義的に使用されている「ポリペプチド」という用語及び「タンパク」及び「ペプチド」という用語は、アミノ酸のポリマーをいう。

【0029】

「治療上有効な量」という用語は、治療を必要とする対象に投与された場合に、治療を有効にするのに十分なモジュレータ、薬剤、又は他の分子の量をいう。治療上有効な量は、治療対象者及び治療対象となる病状、対象者の体重と年齢、病状の重症度、投与法などにより様々であり、当業者には容易に決められるものである。

【0030】

ここで使用する「組織」という用語は、特定機能の性能に結び付けられた同様に特化された細胞の集合体をいう。組織は、結合組織（例えば、骨組織又は骨の様な硬質形態）並びに他の筋肉又は骨格組織を含む、硬組織及び軟組織の両方を含む全ての種類の生物組織を包含する。或る好適な実施形態では、組織は皮膚である。

【0031】

「ベクター」という用語は、結びついている別の核酸を輸送することができる核酸をいう。ここで使用する１つのタイプのベクターは、エピソーム、即ち、染色体外複製が可能な核酸である。他のベクターとしては、結びついている核酸の自動複製及び発現を行えるベクターがある。作動的に結びついている遺伝子の発現を誘導することのできるベクター

をここでは「発現ベクター」と称する。一般に、ＤＮＡ組み換え技術に有用な発現ベクターは、環状二本鎖ＤＮＡ分子を指す「プラスミド」の形態であることが多く、これはベクター形態では染色体に結合することはない。プラスミドが最も一般的に使用されているベクターの形態であることから、本明細書では、「プラスミド」と「ベクター」を同義的に使用している。しかしながら、本開示は、これ以降に当技術分野で知られることになる、等価な機能を供する発現ベクターのその様な他の形態も含むものとする。

【0032】

「条件」という用語は、対象に有害な生物学的結果を生じさせる傷害、疾病、疾患、又は効果をいう。

【0033】

本願で使用する「患者」、「対象」及び「レシピエント」という用語は、あらゆる哺乳類、特にヒト、を指す。

【0034】

治療を目的とした「哺乳類」は、人間、並びに、犬、馬、猫、牛、豚、羊など、家庭内及び農場の動物、動物園、スポーツ、又はペット用動物を含め、哺乳類として分類されるあらゆる動物をいう

【0035】

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細胞外基質（ＥＣＭ）

本発明では、グラフト材料は、細胞外基質と少なくとも１つの治療薬を含んでいる。

グラフト材料を対象の体に適用する場合、グラフト材料内のＥＣＭは改造されて、治療薬を送り出すと共に内生組織の細胞成長を誘発する。グラフト材料内のＥＣＭは、内生組織の成長を促進及び／又は誘発するためにマトリクスとして機能し、生体改造過程を経験する。この生体改造過程に関係する一般的な事象としては、広い範囲に及ぶ迅速な新血管形成、肉芽間葉細胞の増殖、移植された純化腸粘膜下組織材料の生分解／再吸収、及び免疫反応の無いこと、が含まれる。治療薬は、生体内に所望の生物学的効果（例えば、細胞分割、移動、又はアポトーシスの刺激又は抑制、免疫反応の刺激又は抑制、抗ウイルス活性など）を生み出すことにより治癒過程を前進させる。

【0036】

研究により、温血脊椎動物粘膜下組織の様なＥＣＭ材料は、下部泌尿器、体壁、腱、靭帯、骨、心臓血管組織、及び中枢神経系を含む多くの生体内微環境への移植の後、ホスト組織増殖と組織構造の生体改造及び再生を誘起する能力のあることが分かった。移植が行われると、細胞の浸潤と迅速な新血管形成が観察され、粘膜下組織材料は生体改造されて、部位特定構造と機能的特性を備えたホスト置換組織になる。これは、例えば、グリコサミノグリカン、グリコプロテイン、プロテオグリカン、及び／又は、形質転換成長因子－α、形質転換成長因子－β、及び／又は線維芽細胞成長因子２（塩基性）を含む成長因子、を含む粘膜下組織の構成要素の１つ又はそれ以上の結果として起こる。

【0037】

ＥＣＭは、組織の非細胞部分であり、残留細胞の分泌するタンパク質と炭水化物構造で構成されている。ＥＣＭは、組織の構造要素として働く。細胞外基質は、各種方法で分離され処理される。組織源から採取されてグラフト材料に作られると、ＥＣＭは、自然発生ポリマースカフォード、バイオスカフォード(bioscaffold)、バイオマトリクス、ＥＣＭスカフォード(ECM scaffold)、細胞外基質材料（ＥＣＭＭ）、又は自然発生バイオポリマーと呼ばれる。ＥＣＭ材料は、下記の幾つかの異なる身体系から採取されるが、どれもグラフト材料に加工されると類似性を共有する。特に、源動物から取り出された後は最小限の加工しか施されないため、天然状態と殆ど同じ構造と組成が維持される。ホスト細胞が取り除かれ、スカフォードを無細胞的に移植して、損傷のある組織を置換又は修復すると共に治療薬を組織に送り込む。

【0038】

グラフト材料を調製する際に使用するＥＣＭは、コラーゲン基質を含め市販されている

各種基質から選択してもよいし、各種天然コラーゲン源から調製してもよい。これら自然発生ＥＣＭの例には、組織粘膜下組織、無細胞真皮、死体筋膜、膀胱無細胞基質グラフト、及び羊膜（詳しくは、Hodde J.,組織工学8(2);295-308（２００２年）を参照）が含まれる。また、本発明のグラフト材料を調製する際に使用するものとして、温血脊椎動物の腎被膜由来のコラーゲン基材の細胞外基質を選択してもよい。温血脊椎動物の腎被膜由来の細胞外基質は、ＷＯ０３／０２１６５号に記載されており、その開示内容を参考文献としてここに援用する。

【0039】

肝基底膜から取り出した、別のタイプのＥＣＭが、米国特許第６，３７９，７１０号に記載されており、同特許を参考文献としてここに援用する。ＥＣＭは、米国特許第４，５０２，１５９号に記載されているように、心膜から取り出してもよく、同特許も参考文献としてここに援用する。

【0040】

異種生体材料の他に、自己組織も採取することができる。また、エラスチン又はエラスチン様ポリペプチド（ＥＬＰ）なども、生物活性ＥＣＭとして潜在的な可能性がある。もう１つの代替案は、採取した天然の弁組織の様な同種移植片を使用することである。そのような組織は、凍結保存状態で市販されている。

【0041】

或る例では、本発明に従って使用するためのＥＣＭには、保存性の高いコラーゲン、グリコプロテイン、プロテオグリカン、及びグリコサミノグリカン、及び／又は、形質転換成長因子－α、形質転換成長因子－β、及び／又は線維芽細胞成長因子２（塩基性）を含む成長因子を、それらの天然の構成及び天然の濃度を備えたまま有する膠原基質が含まれる。別の事例では、膠原基質には、温血脊椎動物の粘膜下組織由来の組織、例えば小腸粘膜下組織（ＳＩＳ）が含まれる。粘膜下組織は、例えば、豚、牛、羊、又は他の温血脊椎動物を含む、食肉製造用として飼育されている動物から採取された各種脊椎動物器官（腸組織など）から得ることができる。

【0042】

ブタ哺乳類の様な温血脊椎動物からの未熟な粘膜下組織も使用することができる。未熟な粘膜下組織は、ＷＯ０４／２２１０７号に記載されており、同特許の開示内容を参考文献としてここに援用する。

【0043】

ホスト細胞を取り除いた後、スカフォードを無細胞的に移植して、損傷のある組織を置換又は修復すると共に、例えば治療薬を組織に送り込む。

【0044】

グラフト材料のＥＣＭは、例えば、組織粘膜下組織である。「組織粘膜下組織」又は「粘膜下組織」とは、動物の消化管、呼吸器、泌尿器、及び生殖器官の大部分の粘膜の下に発現するコラーゲンを含んだ結合組織の層をいう。組織粘膜下組織はＥＣＭの好適な源である。望ましいタイプのＥＣＭである、純粋な組織粘膜下組織については、これまでも米国特許第６，２０６，９３１号、同第６，３５８，２８４号、及び同第６，６６６，８９２号に、損傷又は病変のあるホスト組織の修復を強化する生体適合性を有する非血栓性材料として記載されている。米国特許第６，２０６，９３１号、同第６，３５８，２８４号、及び同第６，６６６，８９２号を、参考文献としてここに援用する。粘膜下組織は、腸から誘導することができる。粘膜は、ＰＣＴ出願ＰＣＴ／ＵＳ９７／２２７２７号に基づくＷＩＰＯ公開、ＷＯ９８／２５６３７号に記載されているように脊椎動物肝組織から、又はＰＣＴ出願ＰＣＴ／ＵＳ９７／２２７２９号に基づくＷＩＰＯ公開、ＷＯ９８／２６２９１号に記載されているように消化管粘膜から、又はＰＣＴ出願ＰＣＴ／ＵＳ９７／２３０１０号に基づくＷＩＰＯ公開、ＷＯ９８／２５６３６号に記載されているように胃粘膜から、又は米国特許第５，５５４，３８９号に記載されているように膀胱粘膜から誘導することもでき、上記全文献の開示内容を明示的にここに援用する。

【0045】

粘膜下組織は、温血脊椎動物の望ましくは腸から、更に望ましくは小腸から、即ち小腸粘膜下組織（ＳＩＳ）から誘導される。ＳＩＳは、インディアナ州ウエストラファイエットのCook Biotech社から市販されている。好適な小腸粘膜下組織は、代表的には、筋層と少なくとも粘膜の管腔部分の両方から剥離した粘膜下組織膜を含んでいる。或る例では、粘膜下組織は、粘膜下組織膜と、粘膜筋と細胞層を含んでいる粘膜の基底部と、を含んでいる。腸粘膜下組織の調製は、米国特許第４，９０２，５０８号に記載されており、組織粘膜下組織の調製は、米国特許出願第０８／９１６，４９０号に記載されており、両特許文献を参考文献としてここに援用する。粘膜下組織の調製は、米国特許第５，７３３，３３７号並びに１７ネイチャーバイオテクノロジー誌１０８３（１９９９年１１月）、１９９８年５月２８日付けのＷＩＰＯ公開、ＷＯ９８／２２１５８号、これはＰＣＴ／ＵＳ９７／１４８５５号の出願公開であるが、そこにも記載されている。また、実質的に無菌状態の純度の高い剥離された組織粘膜下組織膠原基質を得るための方法は、これまでにも、米国特許出願、公開番号第２００４０１８００４２号に記載されており、その開示内容を参考文献としてここに援用する。

【0046】

実質的に滅菌され且つ加工を最小限に留めた組織粘膜下組織が製造するため、組織粘膜下組織源の剥ぎ取りは、殺菌又は滅菌されたケーシング機械を使用することにより実施されるのが望ましい。適したケーシング機械は、スエーデンのゴトボルグのAB Stridhs Maskiner社から市販されている、型番３－Ｕ－４００Stridhs Universal Machine for Hog Casingである。加工の結果として、測定された生物負担量は、最小即ち実質的にゼロである。組織粘膜下組織源を剥離するには、例えば、手で剥離することを含め、他の手段を採用することもできる。

【0047】

この方法では、脊椎動物の腸の部分は、ブタ、羊、又は牛から採取されることが望ましく、先ず縦方向に拭い取る動作を使って軽く研削して、漿膜及び筋層として識別される外層と、最も内側の層、すなわち粘膜の管腔部分の両方を取り出す。粘膜下組織は、水又は生理食塩水ですすぎ、随意的に殺菌して、水和した又は脱水した状態で保管される。筋層と粘膜の少なくとも管腔部分との両方から粘膜下組織膜を剥離して、粘膜下組織をすすぐことにより、粘膜下組織として規定された無細胞基質を得る。靭帯及び腱グラフトを形成するためのその様な材料の使用と操作処理、並びに内生結合組織の成長を誘導するためにそのような粘膜下組織構造を更に一般的に使用することは、１９９４年１月２５日発行の米国特許第５，２８１、４２２号に記載され主張されており、その開示内容を参考文献としてここに援用する。

【0048】

剥離に続き、粘膜下組織には、プロピレンオキシド又はエチレンオキシド処理及び気体プラズマ殺菌を含む従来の殺菌技法を使用して殺菌処理を施す。純化された粘膜下組織の機械的強度、構造、及び生物向性に悪影響を及ぼさない殺菌技法が好適である。好適な殺菌技法には、グラフトをエチレンオキシド処理又は気体プラズマ殺菌に曝すことも含まれる。通常、純化された粘膜下組織には、２つ又はそれ以上の殺菌処理が施される。純化された粘膜下組織に例えば化学処理によって殺菌を施した後、この基質構造をプラスチック又は箔ラップで包み、電子ビーム又はガンマ放射殺菌技法を使用して再度殺菌する。

【0049】

好適な粘膜下組織は、次の表１に記載の低汚染レベルで特徴付けられる。表１の汚染レベルは、所与のＥＣＭ試料個々について又は組み合わせについて見ることができる。表１の略語は次の通りであり、ＣＦＵ／ｇ＝グラム当たりのコロニー形成単位；ＰＦＵ／ｇ＝グラム当たりのプラーク形成単位；μｇ／ｍｇ＝ミリグラム当たりのマイクログラム；ｐｐｍ／ｋｇ＝キログラム当たりの１００万分の１、である。

【0050】

【表1】

	第１の好適なレベル	第２の好適なレベル	第３の好適なレベル
菌体内毒素	＜１２ＥＵ／ｇ	＜１０ＥＵ／ｇ	＜５ＥＵ／ｇ
生物負担量	＜２ＣＦＵ／ｇ	＜１ＣＦＵ／ｇ	＜０．５ＣＦＵ／ｇ
真菌	＜２ＣＦＵ／ｇ	＜１ＣＦＵ／ｇ	＜０．５ＣＦＵ／ｇ
核酸	＜１０μｇ／ｍｇ	＜５μｇ／ｍｇ	＜２μｇ／ｍｇ
ウイルス	＜５００ＰＦＵ／ｇ	＜５０ＰＦＵ／ｇ	＜５ＰＦＵ／ｇ
処理剤	＜100,000ppm/kg	＜1,000ppm/kg	＜100ppm/kg

【0051】
純化された粘膜下組織は、多くのやり方で更に処理され、本発明のグラフト材料に組み入れるのに適したＥＣＭを提供する。

【0052】
なお、粘膜下組織の細砕形態は、内生組織の成長を誘発するという粘膜下組織の能力を失うことなく調製できるものと理解されたい。細砕された粘膜下組織組成物は、腸粘膜下組織の溶液又は懸濁液又は粉末として調製され、機械的に手に入れた粘膜下組織又は酵素的に処理された粘膜下組織を備えている。或る事例では、粘膜下組織は機械的又は酵素的に処理され、実質的に均一で均質な溶液を形成する。別の事例では、粘膜下組織には、トリプシン又はペプシンの様なプロテアーゼ又は他の適切な酵素で、組織を溶解して実質的に均質な溶液を形成するのに十分な期間、処理が施される。

【0053】
腸粘膜下組織開始材料は、引き裂き、切断、粉砕、剪断などにより、機械的に細砕される。粘膜下組織の片々の懸濁液を高速（高剪断）ブレンダで処理して脱水処理を施すことにより、また必要に応じて、遠心分離して上澄みの余剰水分を移すことによっても良好な結果が得られるが、凍結又は凍結乾燥状態の粘膜下組織を細砕するのが好ましい。得られた流体状の腸粘膜下組織を乾燥させて粘膜下粉末を形成する。その後、水和して、即ち、水と結合させ、又は生理食塩水及び随意的に他の薬学的に許容可能な賦形剤で緩衝して、腸粘膜下組織組成物を、２５℃で約２ｃｐｓから３００，０００ｃｐｓの粘度を有する流体として形成する。より高い粘度の粘膜下組織組成物は、ゲル又はペーストの粘度を有する。流動化した組成物は、電離放射線を照射するなどの当技術で認められている殺菌技法を使用して殺菌される。腸粘膜下組織の流体形態を調製することは、米国特許第５，２７５，８２６号、同第５，５１６，５３３号、及び同第６，２６４，９９２号に記載されており、その開示内容を参考文献としてここに援用する。

【0054】
腸粘膜下組織は、粘膜下組織の粉末形態であってもよい。或る事例では、粘膜下組織の粉末形態は、腸粘膜下組織を液体窒素下で細砕することにより調製され、最大粒径が０．０１ｍｍから１ｍｍの範囲の粒子を生成する。次いで粒子組成物を一晩凍結乾燥して、再度細砕し、随意的には殺菌して、実質的に無水の粒子組成物を形成する。別の例では、粘膜下組織の粉末形態は、粘膜下組織の懸濁液又は溶液を乾燥させることにより、流体状粘膜下組織から形成される。

【0055】
腸粘膜下組織の固体及び流体の両形態は、迅速な新血管形成、肉芽間葉細胞の増殖、粘膜下組織の再吸収、及び免疫拒絶を起こさないことを含め、内生改造過程を誘発することが分かっている。生体内では、粘膜下組織は、接触しているか又は置換対象の細胞／組織の増殖と成長を誘導するために効果的であることが分かっている。

【0056】
また、米国特許第２，１２７，９０３号及び同第５，７１１，９６９号に記載の技術を使用して、２つ又はそれ以上の組織粘膜下組織部分を組み合わせることにより表面積の大きな構造体を形成することもでき、上記両特許を参考文献としてここに援用する。この様に、複数の組織粘膜下組織条片を、例えば、脱水条件下で条片の重なり合った部分を押し

付けることにより互いに融合させて、構造体を形成するのに使用された個々の条片それぞれの平坦な表面よりも広い表面積を有する全体的に平坦な構造体を形成することができる。

【0057】

上記処理手法の変型を使用して、グラフト材料のポリマーシートに組み込まれる粘膜下組織を生成することができる。例えば、組織粘膜下組織の源組織、例えば胃、腸全体、牛の子宮などは、部分的に剥離させ、消毒又は殺菌剤で処理し、その後で組織粘膜下組織の完全な剥離を施す。例示的に説明すると、付着している腸間膜層及び／又は腸全体の漿膜層は、消毒剤で処理する前に取り除いて、その後にまだ付着している組織を組織粘膜下組織から剥離させる。上記工程の後に、追加の消毒処理工程、例えば、酵素的な殺菌及び／又は核酸除去を、行っても行わなくてもよい。代わりに、組織粘膜下組織源は、消毒剤又は他のその様な薬剤で最小限の処理を施してから、筋層及び粘膜から組織粘膜下組織を剥離して、その後、所望の汚染レベルを得るために完全な消毒処理を実施する。この工程でのこの様な変型又は修正の全てが考慮されている。

【0058】

米国特許出願第０８／９１６，４９０号に記載されているように、純化された粘膜下組織を調節して、純化された粘膜下組織の粘弾性を変えることができる。純化された粘膜下組織は、伸縮、化学処理、酵素処理、又は基質構造を他の環境因子に曝すことによって調節される。或る実施形態では、純化された組織粘膜下組織の条片は、２０％以下の歪みまで縦及び／又は横方向に引き伸ばすことにより調節される。歪みは、荷重を掛けた後の材料の長さの増加率である。

【0059】

別の実施形態では、純化された粘膜下組織は、ＥＣＭが形成された純化された粘膜下組織の長さよりも長くなるまで、材料を長手方向に引き伸ばすことにより調節される。引き伸ばしにより基質を調整する１つの方法は、純化された粘膜下組織に所与の荷重を３から５サイクル加えることを含んでいる。各サイクルは、材料に５秒間荷重を加えた後、１０秒間弛緩状態に置くことから成る。３から５サイクルで、引き伸ばし調節された材料が生成される。純化された粘膜下組織は、元の大きさに直ちに戻るわけではなく、「引き伸ばされた」寸法のままである。随意的に、純化された粘膜下組織は、横寸法を引き伸ばすことにより前もって調節してもよい。

【0060】

或る実施形態では、純化された粘膜下組織は、予測最終荷重の５０％を使って引き伸ばされる。「最終荷重」とは、基質構造を損傷すること無く、純化された粘膜下組織に掛けることのできる最大荷重である（即ち、組織のブレイクポイント）。最終荷重は、材料の源及び厚さに基づいて、純化された粘膜下組織の所与の条片について予測することができる。従って、引き伸ばしによって基質構造を調節する１つの方法は、予測最終荷重の５０％を純化された粘膜下組織に３から１０サイクルに亘って加えることを含んでいる。各サイクルは、材料に５秒間荷重を加えた後、１０秒間弛緩状態に置くことから成る。その結果得られた調節済み純化粘膜下組織は、生じた歪みが３０％未満であり、普通は歪みが約２０％から約２８％である。或る好適な実施形態では、調節済み純化粘膜下組織は、歪みがわずか２０％である。得られた調節済み純化粘膜下組織は、以下の様式で使用することができる。調節工程並びに他の関連工程については、米国特許第６，３５８，２８４号に記載されており、同特許を参考文献としてここに援用する。

【0061】

グラフト材料のＥＣＭは、例えば、無細胞真皮である。無細胞真皮は、細胞を取り除いた後に残っている正常な真皮組織構造から構成されている。他の自然発生生体ポリマーと同様に、無細胞真皮はコラーゲン、タイプＩが豊富である。無細胞真皮は、天然真皮のタイプＩＶ及びタイプＶＩＩコラーゲン組成を高レベルで保有している（Medalie他、ASAIO J.42:M455(１９９６年)）。コラーゲンの他に、真皮のエラスチン含有量も処理中保持され、好ましいエラスチン特性を備えたグラフト構造が得られる（Isch他、J. Pediatr. Su

rg. 36:266（２００１年））。

【0062】

無細胞真皮は、豚又は人の死体皮膚から採取される。例えば、無細胞真皮は、Chaplin他（Chaplin他、Neurosurgery 45:320(１９９９年)）に従って調製される。簡単に言えば、表皮は、塩化ナトリウム（ＮａＣｌ）に皮膚を浸すことにより取り除く。皮膚の線維芽細胞と上皮細胞は、エチレンジアミンテトラアセテート（ＥＤＴＡ）を含んでいるデオキシコール酸内で材料を培養することにより取り除く。次いで、真皮は、マルトデキストリンとジナトリウム－ＥＤＴＡの組み合わせを用いて凍結保護を施し、使用時まで凍結乾燥される（Chaplin他、Neurosurgery 45:320(１９９９年)）。無細胞組織グラフトとして移植された場合、無細胞真皮は、修復された血管構造に内皮細胞を形成し（Inoue and Lleon, J. Reconstr. Microsurg. 12:307（１９９６年））、過剰な傷の収縮を阻止し（Walden他、Ann. Plast. Surg. 45:162（２０００年））、ホスト細胞の合体と移植部位への毛細管の成長を支援する（Dalla他、J. Pediatr. Surg. 45:162（２０００年）；及びMedalie他ASAIO J. 42:M455（１９９６年））。

【0063】

グラフト材料のＥＣＭは、例えば、死体筋膜である。大腿筋膜張筋は、脚の側方側で骨盤を膝に取り付けている結合組織の厚い帯である。その臀部における筋肉成分は、厚い結合組織に接合しており、腸脛靭帯（ＩＴ靭帯）に引張力を掛けることにより、股関節と膝関節を安定化させ落ち着かせる働きをしている。ＩＴ靭帯、即ち大腿筋膜張筋の遠位部分は、本発明のグラフト材料のＥＣＭ用に採取される。

【0064】

その天然状態では、大腿筋膜張筋は細胞外基質組織により一体に保持されているタイプ１コラーゲン繊維の重くて平行な束から構成されている。線維の束の間には線維芽細胞、神経、及び腱に栄養を供給する血管がある。死体筋膜は、エタノール抽出の後、抗生剤で高圧洗浄して得られる。次いで、抽出組織を凍結乾燥して、ガンマ照射を用いて最後に殺菌する。術中に、グラフト材料は使用前に生理食塩水に浸漬してもどす（Carbone他、J. Urol. 165:1605（２００１年））

【0065】

グラフト材料のＥＣＭは、例えば、膀胱無細胞基質でもよい。膀胱無細胞基質グラフト（ＢＡＭＧ）は、１９７５年に初めて文献に記載があるが（Meezan他、Life Sci. 17:1721（１９７５年））、これは、ＳＩＳ生体材料体を備えている粘膜下組織と類似している膀胱の層に由来する。天然の膀胱では、膀胱粘膜下組織は粘膜下構造を支え、線維芽細胞により分泌及び維持されている。ＥＣＭの正常な機能は、膀胱壁を完全な状態に保つ結合組織構造を維持すると共に、異なるタイプ粘膜下細胞の成長と分化を支援することである。しかしながら、外側筋肉層から容易に分離される腸の粘膜下組織とは異なり、膀胱の粘膜下組織は筋肉質の膀胱壁にぴったりと付着している。両層を機械的に完全に分離するのは面倒で難しいことが判明しているので、膀胱粘膜下組織を筋肉無しの状態にする試みでは、水酸化ナトリウム、デスオキシコール酸ナトリウム(sodium desoxycholate)、ドデシル硫酸ナトリウム(sodium dodecyl sulfate)（ＳＤＳ）、又はデオキシリボヌクレアーゼの様な化学薬品及び／又は酵素剤による手法を使うことが多い（Badylak他、J. Pediatr. Surg. 35:1097(２０００年)；Merguerian他、BJU Int.85:894（２０００年）；Wefer他、J. Urol. 165:1755（２００１年）；Reddy他、J. Urol. 164:936（２０００年））。

或る処理方法では、膀胱全体をトリス-EDTA(Tris-EDTA)溶液に４８時間浸した後で、トリトン-X(Triton-X)を含有するトリス-塩化カリウム－EDTA(Tris-potassium choloride-EDTA)溶液に更に浸す。その後、膀胱をSorenson社のリン酸緩衝液ですすぎ、これにデオキシリボヌクレアーゼとリボヌクレアーゼを加えて１晩培養して細胞質と核材料を取り除き、Tris及びSDSを含有する溶液中に更に抽出する。次いで、抽出された膀胱をエタノールに沈めて、残っているSDSを取り除き、リン酸緩衝液で洗浄して、使用するまでは、冷蔵された生理食塩水で保存する（Reddy他、J. Urol. 164:936（２０００年））。

【0066】

代わりに、膀胱粘膜下組織は、ＳＩＳの場合に使用される方法に従って、無細胞化及び殺菌処理してもよい(Badylak他、J.Pediatr. Surg. 35:1097（２０００年））。膀胱層を機械的に分離して、得られた粘膜下組織を水で徹底的にすすいで細胞を溶解させる。粘膜下細胞は過酢酸で処理して、水とリン酸緩衝生理食塩水を順次入れ替えながらすすいでｐＨを中性にする。次いで２．５-mRadガンマ照射を使用して殺菌し、使用まで冷蔵保存する。

【0067】

グラフト材料のＥＣＭは、例えば羊膜でもよい。羊膜は妊娠中に胎児を包み込む袋を形成している。羊膜は、極めて頑丈で、組織の厚さは２μｇから５μｇであり、幾つかの組織修復用途でグラフト材料として使用される。天然状態では、羊膜の上皮組織は、比較的細胞の無い基底膜ＥＣＭの上に乗った単一の細胞層で構成されている（Aplin他、J. Cell Sci.79:119（１９８５年））。このＥＣＭは、希薄層と、線維コラーゲン、タイプＩとＩＩＩ及び基底層コラーゲン、タイプＩＶを含む幾つかのタイプのコラーゲンで形成される稠密緻密層と、から成る微小基礎構造から構成されている（Aplin他、J. Cell Sci.79:119（１９８５年）及びLei他、Biol. Reprod.60:176号（１９９９年））。少なくとも１つのプロテオグリカン、デコリンが、当面の羊膜に同定されており（Meinert他、J. Obstet. Gynecol. 184:679（２００１年））、グリコサミノグリカン・ヒアルロン酸（Meinert他、J. Obstet. Gynecol. 184:679（２００１年））を有している。表皮性成長因子、幾つかの形質転換成長因子イソ型、塩基性線維芽細胞成長因子、ケラチノサイト成長因子、及びヘパトサイト成長因子を含む幾つかの成長因子も同定されており、加工された組織基質に維持されていることが報告されている。

【0068】

羊膜を、分娩時に得て、ペニシリン、ストレプトマイシン、アンホテリシンＢ、及びクリンダマイシンを含有する生理食塩水を用いて血液を洗い流す（Avila他、Cornea 20:414（２００１年））。羊膜を、鈍的切開により絨毛膜から分離し、滅菌水で洗浄し、トリプシン１０％溶液に３時間浸して、細胞を溶解する。次いで、膜をガンマ照射で殺菌し、臨床使用されるまで凍結する（Young他、Fertil. Steril. 55:624（１９９１年））。

【0069】

治療薬

本発明によれば、グラフト材料は少なくとも１つの治療薬を含んでいる。

先に述べたようにグラフト材料に含まれる治療薬は、生体内で所望の生物学的効果（例えば、細胞分割、移動、又はアポトーシスの刺激又は抑制、免疫反応の刺激又は抑制、抗ウイルス活性など）を発揮することができる。

【0070】

適した治療薬としては、成長因子、抗生剤、抗ウイルス剤、鎮痛剤、ステロイド系及び非ステロイド系抗炎症剤、抗新生物剤、チャネルブロッカーを含む鎮痙剤、細胞成長阻害物質及び抗接着分子を含む細胞－細胞外基質相互作用モジュレータ、酵素及び酵素阻害物質、抗凝固剤及び／又は抗血栓剤、ＤＮＡ、ＲＮＡ、ＤＮＡ又はＲＮＡ又はタンパク質合成阻害物質、細胞の移動、増殖及び／又は成長を調整する化合物、血管拡張剤、及び組織への傷害の治療に一般に使用されている他の薬剤を挙げることができる。

【0071】

上記治療薬の組み合わせも使用することができる。

【0072】

治療薬は、例えば、細胞又は組織の様々な特定の内方成長を強化又は排除する物質である。その様な物質としては、例えば、骨誘導物質、脈管形成物質、分裂促進物質、又は類似の物質があり、それらには、例えば、ＴＧＦ－アルファ、ＴＧＦ－ベータ－１、ＴＧＦ－ベータ－２、ＴＧＦ－ベータ－３の様な形質転換成長因子（ＴＧＦ）、例えば、酸性及び塩基性の線維芽細胞成長因子（ａＦＧＦとｂＦＧＦ）の様な線維芽細胞成長因子（ＦＧＦ）、血小板由来成長因子（ＰＤＧＦ）、血小板由来内皮細胞成長因子（ＰＤ－ＥＣＧＦ）、腫瘍壊死因子アルファ（ＴＮＦ－アルファ）、腫瘍壊死因子ベータ（ＴＮＦ－ｂ）、

上皮成長因子（ＥＧＦ）；結合組織活性化ペプチド（ＣＴＡＰ）、例えば、ＢＭＰ－１、ＢＭＰ－２、ＢＭＰ－３ＭＰ－４、ＢＭＰ－５、ＢＭＰ－６、ＢＭＰ－７、ＢＭＰ－８、ＢＭＰ－９の様な骨形成因子、例えば、ＩＧＦ－Ｉ及びＩＧＦ－ＩＩの様なインスリン様成長因子（ＩＧＦ）、エリスロポエチン、ヘパリン結合成長因子（ｈｂｇｆ）、血管内皮成長因子（ＶＥＧＦ）、肝細胞成長因子（ＨＧＦ）、コロニー刺激因子（ＣＳＦ）、マクロファージ－ＣＳＦ（Ｍ－ＣＳＦ）、顆粒球/マクロファージＣＳＦ（ＧＭ－ＣＳＦ）、一酸化窒素シンターゼ（ＮＯＳ）、神経成長因子（ＮＧＦ）、筋肉形態形成因子（ＭＭＰ）、インヒビン（例えば、インヒビンＡ、インヒビンＢ）、成長分化因子（例えば、ＧＤＦ－１）、アクティビン（例えば、アクティビンＡ、アクティビンＢ、アクティビンＡＢ）、アンギオゲニン(angiogenin)、アンギオテンシン、アンギオポエチン、アンギオトロピン、抗脈管形成アンチトロンビン（ａａＡＴ）、心房ナトリウム排泄因子（ＡＮＦ）、ベータセルロース、エンドスタチン；内皮細胞由来成長因子（ＥＣＤＧＦ）、内皮細胞成長因子（ＥＣＧＦ）、内皮細胞成長阻害剤、内皮単球活性ポリペプチド（ＥＭＡＰ）、内皮細胞生存維持因子、エンドセリン（ＥＴ）、内皮種由来移動因子（ＥＤＭＦ）、脈管細胞増殖の心臓由来阻害剤、造血成長因子、エリスロポエチン（Ｅｐｏ）、インターフェロン（ＩＦＮ）、インターロイキン（ＩＬ）、オンコスタチンＭ、胎盤成長因子（ＰＩＧＦ）、ソマトスタチン、トランスフェリン、トロンボスポンジン、血管活性腸管ペプチド、及びそのような成長因子の生物活性類似体、断片、及び誘導体が含まれる。

【0073】

代表的な実施形態では、治療剤は、成長因子、脈管形成因子、抗炎症剤の様な線維芽細胞組織の内方成長を選択的に阻害する化合物、及び形質転換（癌性）細胞の成長と増殖を選択的に阻害する化合物である。上記因子は、グラフト材料のＥＣＭ内又はその周囲に含まれている細胞の成長と機能を制御するために使用され、例えば、血液の内方成長、及び／又はグラフト材料の周りの線維組織の堆積と編成を制御するために使用される。

【0074】

治療薬は、例えば、ポリヌクレオチドである。治療薬として有用なポリヌクレオチドの例としては、限定するわけではないが、例えば、ＤＮＡ、ＲＮＡ、ｃＤＮＡ及び組み換え核酸；裸ＤＮＡ、ｃＤＮＡ及びＲＮＡ；ゲノムＤＮＡ、ｃＤＮＡ又はＲＮＡ；オリゴヌクレオチド；アプトメリックオリゴヌクレオチド；リボザイム；アンチセンスオリゴヌクレオチド（ＲＮＡ又はＤＮＡを含む）；アンチセンスＲＮＡをコード化するＤＮＡ；ｔＲＮＡ又はｒＲＮＡ分子をコード化するＤＮＡ（即ち、欠陥があるか欠損したな内在分子に置換するもの）；二本鎖小干渉ＲＮＡ（ｓｉＲＮＡ）；ポリヌクレオチドペプチド結合オリゴ（ＰＮＡ）；環状又は直鎖ＲＮＡ；環状一本鎖ＤＮＡ；自己複製ＲＮＡ；ｍＲＮＡ転写産物；例えば、ハンマーヘッド、ヘアピン、デルタ肝炎ウイルス、及び生体内で特定のＲＮＡ配列を狙う及び／又は切断するグループＩイントロンを含む触媒性ＲＮＡ；更にここで定義する、治療用タンパク質又はポリペプチドをコード化するポリヌクレオチド；例えば、ＤＮＡ／ＤＮＡハイブリッド、ＲＮＡ／ＲＮＡハイブリッド、ＤＮＡ／ＲＮＡハイブリッド、ＤＮＡ／ペプチドハイブリッド、及びＲＮＡ／ペプチドハイブリッドを含むキメラ核酸；ＤＮＡ凝縮剤；及び非伝染性ベクターの核酸（即ち、プラスミド）及びウイルスベクターの核酸を含む、遺伝子／ベクター系（即ち、核酸の取り込みと発現を許容するあらゆる媒体）、を含む核酸及び核酸の断片が挙げられる。

【0075】

在る代表的な実施形態では、キメラ核酸には、例えば、キメラ分子の位置を、体内、細胞内、又は細胞膜上の或る位置に方向決めするペプチド標的配列（即ち、膜転位配列（ＭＴＳ））に結合する核酸が含まれる。

【0076】

別の実施形態では、核酸は、多種多様な細胞型式で発現する構成ハウスキーピング遺伝子、又はその断片に融合する。

【0077】

或る実施形態では、非ウイルス方法により誘導されたポリヌクレオチドは、ナノキャッ

プ（例えば、ナノ粒子ＣａＰＯ４）、コロイド金、ナノ粒子合成ポリマー、及び／又はリポソームと配合され又は結びつく。或る実施形態では、ポリヌクレオチドは、ＱＤＯＴＴＭプローブ（www.qdots.com）と結びつく。

【0078】

他の実施形態では、治療薬として有用なポリヌクレオチドは、ヌクレアーゼ、例えば、エクソヌクレアーゼ及び／又はエンドヌクレアーゼなど、に対する抵抗を高めるように改質されており、従って生体内での安定性が高まる。代表的な改質は、限定するわけではないが、核酸のホスホラミデート、ホスホチオエート、及びメチルホスホネート類似体が挙げられる（米国特許第５，１７６，９９６号、同第５，２６４，５６４号、及び同第５，２５６，７７５号）。

【0079】

或る実施形態では、治療薬はベクター内に含まれるポリヌクレオチドである。本発明による使用に適したベクターとしては、例えば、アデノウイスルベクター、単体ヘルペスベクター、乳頭腫ベクター、アデノ随伴ベクター、レトロウイルスベクター、仮性狂犬病ベクター、アルファ－ヘルペスウィルスベクターなどの様なウイルスベクター又はウイルス源から誘導されたベクターが挙げられる。ウイルスベクター、特に非複製細胞を改質するのに適したウィルスベクター、及びその様なベクターを対象とするポリヌクレオチドの発現と組み合わせてどの様に使用するかということについての全体的検討は、ウイルスベクターという本、Gene Therapy and Neuroscience Applications Ed. Caplitt and Loewy, Academic Press, San Diego （１９９５年）に記載されている。

【0080】

ベクターは、例えば、非伝染性ベクター又はプラスミドでもよい。適した非伝染性ベクターは、限定するわけではないが、バクテリア内でのベクターの伝播を促進する真核配列と、真核細胞で発現される１つ又はそれ以上の真核転写単位の双方を含む哺乳類発現ベクターを含む。ｐｃＤＮＡ／ａｍｐ、ｐｃＤＮＡ／ｎｅｏ、ｐＲｃ／ＣＭＶ、ｐＳＶ２ｇｐｔ、ｐＳＶ２ｎｅｏ、ｐＳＶ２－ｄｈｆｒ、ｐＴｋ２、ｐＲＳＶｎｅｏ、ｐＭＳＧ、ｐＳＶＴ７、ｐｋｏ－ｎｅｏ、及びｐＨｙｇ由来のベクターは、真核細胞のトランスフェクションに適した哺乳類発現ベクターの例である。上記ベクターの幾つかは、原核細胞と真核細胞の両方での複製と薬物耐性選択をやり易くするために、ｐＢＲ３２２の様なバクテリアプラスミドからの配列で改質される。真核細胞にタンパク質を一過性に発現させるために、代わりに、ウシ乳頭腫ウイルス（ＢＰＶ－１）又はエプスタイン・バールウイルス（ｐＨＥＢｏ、ｐＲＥＰ由来及びｐ２０５）の様なウイルスの誘導体を使用してもよい。プラスミドの調製とホスト器官の形質転換に採用される各種方法は、当技術では周知である。原核細胞と真核細胞の両方に適した他の発現システム並びに一般的な組み換え手法については、Sambrook, Fritsch及びManiatis著、Molecular Cloning A Laboratory Manual、第２版（Cold Spring Harbor Laboratory Press１９８９年）第１６章及び１７章に記載されている。

【0081】

治療薬は、例えば、ＤＮＡ、ＲＮＡ、又はタンパク質合成の阻害薬であってもよい。

【0082】

治療薬は、例えば、生体内で生物学的効果を発揮する他の生物学的活性分子であってもよい。本発明のグラフト材料であるＥＣＭと共に使用されるそれら治療薬としては、抗生剤、抗真菌剤、抗ウイルス剤、鎮痛剤、ステロイド系及び非ステロイド系抗炎症剤、抗新生物剤、チャネルブロッカーを含む鎮痙剤、細胞成長阻害物質及び抗接着分子を含む細胞－細胞外基質相互作用モジュレータ、酵素及び酵素阻害物質（アンギオテンシン変換酵素阻害剤化合物）、抗凝固剤及び／又は抗血栓剤、ＤＮＡ、ＲＮＡ、又はタンパク質合成阻害物質、細胞の移動、増殖及び／又は成長を調整する化合物、血管拡張剤、及び組織への傷害の治療に一般に使用されている他の薬剤を挙げることができる。上記治療薬の例と詳細な説明は、GoodmanとGilmanによるThe Pharmacological Basis of Therapeutics、第９版に記載されている。

【0083】
抗生剤の具体例としては、限定するわけではないが、アミノペニシリン（アンピシリン、アモキシシリン及びそれらのコンジナー）を含むペニシリン；セファロスポリン；サイクロセリン；バンコマイシン；ポリミキシン(polymyxin)；アンホテリシンＢ(amphotericin B)；クロラムフェニコール；テトラサイクリン（クロルテトラサイクリン(Chlortetracycline)、オキシテトラサイクリン(Oxytetracycline)、デメクロサイクリン(Demeclocycline)、メタサイクリン(Methacycline)、ドキシサイクリン(Doxyclyclie)及びミノサイクリン(Minocycline)）；マクロライド（エリスロマイシン(Erythromycin)、クラリスロマイシン(Clarithromycin)、アジスロマイシン(Azithromycin)）；クリンダマイシン；リファマイシン；キノロン；スルホンアミド；リファンピン（ＲＩＦＡＤＩＮ、ＲＩＭＡＣＴＡＮＥ）を含むリファマイシン；エタンブト(ethambuto)；及び他の入手可能な抗生剤が挙げられる。
【0084】
例えば、細菌又は真菌感染の予防及び治療には、スルファジアジン銀（商品名：Silvadene、SSD、SSD AF、Thermazene）、サルファ剤を使用することができる。
【0085】
全身及び局所抗真菌剤の具体例としては、アンホテリシンＢ、フルシトシン(flucytozine)、イミダゾールとトリアゾール、ケトコナゾール、イトラコナゾール、フルコナゾール、シクロピロックスオラミン、ハロプロジン、トルナフテート、ナフチフィン、テルビナフィン、及びポリエン抗真菌抗生物質（ナイスタチン）が挙げられる。
【0086】
抗ウイルス剤の具体例としては、限定するわけではないが、抗レトロウイルス剤（ジダノシン、スタブジン、ザルシタビン、ジドブジン）、抗ヘルペスウィルス剤（アシクロビル、ファムシクロビル、フォスカーネット、トリフルリジン、ビダラビル）、及び他の抗ウイルス剤（アマンタジン、インターフェロンアルファ、リバビリン、リマンタジン）が挙げられる。
【0087】
非ステロイド系抗炎症剤の具体例としては、限定するわけではないが、サリチル酸誘導体（アスピリン、サリチル酸ナトリウム）、パラ－アミノフェノール誘導体（アセトアミノフェン）、インドール及びインデン酢酸（インドメタシン）、ヘテロアリル酢酸（トルメチン）、アリールプロピオン酸（イブプロフェン、ナプロキセン、ケトプロフェン）、アントラニル酸(anthanilic acid)（メフェナム酸）、エノール酸（ピロキシカム、フェニルブタゾン）、及びアルカノン（ナブメトン）が挙げられる。
【0088】
抗新生物剤の具体例としては、限定するわけではないが、アルキル化剤（ナイトロジェンマスタード、トリアゼン）；代謝拮抗剤（葉酸類似体、ピリミジン類似体）；天然産物（抗生物質、酵素）；ホルモン及び拮抗物質（プロゲスチン、エストロゲン、アンドロゲン）、及び他の雑多な薬剤（副腎皮質刺激抑制剤、置換尿素）が挙げられる。
【0089】
血小板凝集阻害剤、即ち抗凝固化合物及び／又は抗血栓剤の具体例としては、プロスタサイクリン、ヘパリン、ストレプトキナーゼ、ウロキナーゼ、組織プラスミノーゲン活性化剤（ＴＰＡ）及びアニソイル化プラスミノーゲン活性剤（ＴＰＡ）、及びアニソイル化プラスミノーゲン－ストレプトキナーゼ活性剤複合体（ＡＰＳＡＣ）が挙げられる。
【0090】
代表的な血栓溶解剤は、組織プラスミノゲン活性剤、ストレプトキナーゼ、ウロキナーゼ、及びヘパリンである。
【0091】
チャネルブロッカーの具体例としては、カルシウムチャネルブロッキング薬が挙げられる。
【0092】

細胞間相互作用モジュレータの具体的な例としては、インターロイキン、血小板由来成長因子、酸及び塩基性線維芽細胞成長因子（ＦＧＦ）、形質転換成長因子β（ＴＧＦ－ベータ）、表皮性成長因子（ＥＧＦ）、インスリン様成長因子、及びそれらに対する抗体が挙げられる。

【0093】

核酸の具体例としては、遺伝子及びｃＤＮＡコード化タンパク質、発現ベクター、アンチセンス、及び遺伝子発現を調整又は阻止するために使用することのできるリボザイムの様な他のオリゴヌクレオチドが挙げられる。

【0094】

他の生物活性剤の具体例としては、改質された細胞外基質化合物又はそれらのレセプタと、脂肪性及びコレステロール捕捉剤が挙げられる。

【0095】

或る種の実施形態では、治療剤は、例えば、抗生剤及び抗炎症剤を含む、小さな有機分子を含む薬学的な化合物又は製剤である。

【0096】

治療剤は、例えば、サイトカイン、インターフェロン、及びインターロイキンの様なタンパク質、ポエチン、コロニー刺激因子を含ませるための被覆と組み合わせて使用してもよい。選択用受容体に結合させると炎症を抑制できることが分かっているSialyl Lewisを含む炭水化物であってもよい。

【0097】

「引渡可能な成長因子等価物（略してＤＧＦＥ）」、即ち細胞又は組織のための成長因子を使用してもよく、それらは、成長因子、サイトカイン、インターフェロン、インターロイキン、タンパク質、コロニー刺激因子、ジベレリン、オーキシン、及びビタミンを含み、更に、ペプチド断片又は上記物質の他の活性断片を含み、更に、ベクター、即ち、形質転換又は一過性の発現によるかは問わず標的細胞内でその様な因子を合成することのできる核酸構造を含み、更に、天然の信号分子、アンチセンス、及び三重螺旋核酸などを含め、組織内でその様な因子の合成を刺激又は抑制するエフェクター、を含むものと広く解釈される。代表的なＤＧＦＥは、ＶＥＧＦ、ＥＣＧＦ、ｂＦＧＦ、ＢＭＰ、及びＰＤＧＦ、及びそれらのＤＮＡコード化である。

【0098】

治療薬は、例えば、酸化防止活性（即ち、活性酸素を破壊又はその形成を阻止する）を有する薬剤であってもよく、それらは、例えば癒着予防に有効である。例を挙げると、スーパーオキシドジスムターゼが挙げられ、又は他のタンパク質薬剤としては、カタラーゼ、ペルオキシダーゼ、及びチトクロムＰ４５０、グルタチオン・ペルオキシダーゼ、及び他の天然又は変性ヘムタンパク質のような、一般的なオキシダーゼ又は酸化酵素が挙げられる。

【0099】

治療薬は、例えば、鎮痛薬であってもよい。鎮痛薬は、痛みの緩和又は痛みの抑制に使用され、特に熱傷の治療に使用される。鎮痛薬の例としては、限定するわけではないが、先に述べた非ステロイド系抗炎症剤、並びにモルヒネ、メサドン、コデイン、エトルフィン、ナロキソン他の様なオピオイドを挙げることができる。

【0100】

治療薬は、例えば、哺乳類ストレス反応タンパク質、又は熱ショックタンパク質７０（ｈｓｐ７０）及びｈｓｐ９０の様な熱ショックタンパク質、又は、ストレス反応タンパク質又は熱ショックタンパク質発現を阻害又は抑制するように作用するそれら刺激物質、例えばフラボノイドであってもよい。

【0101】

治療剤（即ち、ポリペプチド、ポリヌクレオチド、小分子、薬剤など）は、例えば、組織内の細胞への運搬、及び／又は細胞の取り込みをやり易くする物質と混和させ、又はそれらに封入してもよい。

【0102】
或る実施形態では、ポリヌクレオチドは、核酸を細胞に導入するのに役立つカチオン脂質と混和され、それらカチオン脂質としては、限定するわけではないが、ジアシルグリセロールに結合するモノカチオンコリンのヘッド基で構成されるLIPOFECTIN™(DOTMA)（Epstein他に対する米国特許第５，２０８，０３６号）；リポスペルミンヘッド基(lipospermine head group)を持つ合成カチオン脂質であるTRANSFECTAM™（DOGS）（ウィスコンシン州マジソン、Promega社）；DMRIE及びDMRIE.HP（カリフォルニア州ラホヤ、Vical社）；DOTAP™　（インジアナ州インジアナポリス、Boehringer Mannheim 社）、及びLipofectamine（DOSPA）（メリーランド州ゲイサーズバーグ、Life Technology社）が挙げられる。
【0103】
他の実施形態では、治療薬（即ち、ポリペプチド、ポリヌクレオチド、小分子、薬剤など）は、哺乳類組織への浸透及び哺乳類細胞による取り込みを促進するマイクロスフィア又はナノスフィアと混和され又はそれらに封入される。各種実施形態では、マイクロスフィア又はナノスフィアには、随意的に他の分子が結合していてもよい。上記改質には、例えば、マイクロスフィア又はナノスフィアに、特定の組織又は細胞を狙って結合する能力、投与部位にそれらが保持されるようにする能力、組み込まれた生物活性剤を保護する能力、抗血栓効果を発揮する能力、凝集を阻害する能力、及び／又はマイクロスフィアの放出特性を変えるする能力、を与えてもよい。そのような表面改質を施したマイクロスフィアの製造については、Levy他のＰＣＴ出願番号第ＷＯ９６／２０６９８号に論じられており、その開示内容を参考文献としてここに援用する。
【0104】
代表的な実施形態では、レセプタ特定分子をマイクロスフィア内に又はその上に組み込んでレセプタ特定粒子取り込みを仲介させることが望ましく、その様なレセプタ特定分子としては、例えば、ＩｇＭ、ＩｇＧ、ＩｇＡ、ＩｇＤなどの様な抗体、又はそれらの諸部分又はサブセット、細胞因子、細胞面レセプタ、ＭＨＣ又はＨＬＡマーカー、ウイルスエンベロプタンパク質、ペプチド、又は小さな有機リガンド、それらの誘導体など、が挙げられる。
【0105】
治療薬（即ち、ポリペプチド、ポリヌクレオチド、小分子、薬剤、細胞など）は、例えば、哺乳類細胞への運搬、又は哺乳類細胞による取り込みを促進し、骨伝導特性を提供し、質量輸送に影響を及ぼす粒子と、混和させ又は複合体を形成させてもよい。適した粒子としては、ヒドロキシアパタイト（ＨＡ）の様な生体セラミクス、又はモノ-、ジ-、オクタ-、アルファ-トリ-、ベータ-トリ-、又はテトラ-カルシウム・ホスフェート、フルオロアパタイト、カルシウムサルフェート、カルシウムフルオライド、及びそれらの混合物の様な他のカルシウム含有化合物；酸化カルシウム、二酸化シリコン、酸化ナトリウム、五酸化リン、及びそれらの混合物の様な酸化金属を備えている生物活性ガラス；などが挙げられる。或る代表的な実施形態では、骨誘導及び／又は骨伝導特性を提供することから、ヒドロキシアパタイトが生体セラミック材料として使用されている。粒子の粒径は、約０．１ｎｍから約１００ｎｍが望ましく、約２ｎｍから約５０ｎｍであれば更に望ましい。
【0106】
治療薬は、例えば、日数、週数、月数、又は年数に基づき、ＥＣＭが分解され又は侵食されるにつれて、制御された放出が行われるように調合してもよい。或る代表的な実施形態では、ＥＣＭの分解は、ＥＣＭの分解及び／又は侵食の速度を、落とすか（例えばペプチド、タンパク質、又は化学プロテアーゼ、例えばアプロチニン、を介して）、又は上げるか（例えば、プロテアーゼで）する作用物質により調整される。代わりに、治療剤は、ＥＣＭに取り付けられ、又は組み込まれたマイクロスフィア組成物を含んでいてもよい。この実施形態では、ＥＣＭは、治療剤の時間放出効果を作り出すために分解する必要はない。放出特性は、マイクロスフィアの大きさと物理的特性で決まる。
【0107】
治療剤は、例えば、安定剤、増量剤、酸化防止剤、触媒、柔軟剤、顔料、潤滑剤の様な

補助剤及び添加剤を、その様な成分が本治療薬の意図される目的に対する有用性を損なわない範囲で、含んでいてもよい。

【0108】

グラフト材料の調製

本発明のグラフト材料は、治療剤を傷害の部位に送り込むために、治療剤を伴って調製される。

【0109】

治療剤は、例えば、グラフト材料調製の際に、ＥＣＭに組みこまれるか、又はＥＣＭに共有結合される。代わりに、治療剤は、例えば、浸漬、噴霧、塗装、又は他のやり方で治療剤をＥＣＭに塗布することにより、ＥＣＭの調製後にＥＣＭに添加してもよい。例えば、図１は、グラフト材料１０がＥＣＭ１１を備えている概略図である。治療剤１２は、ＥＣＭ１１の一方の側に噴霧することにより塗布されている。

【0110】

各種実施形態では、治療剤は、ＥＣＭの所望の箇所に直接塗布されるか、又は患者に用いる前に事前塗布される。

【0111】

グラフト材料は、傷害部位を覆うように皮膚に接着される平坦な膜でもよいし、プラグや楔の様な３－Ｄ構造体であってもよい。別の例では、グラフト材料は、固体シート、ストリップ、ゲル、又は粉末の形態である。

【0112】

グラフト材料は、様々な創傷に用いるのに適した標準的な形態で供給して、そのまま用いてもよいし、特定の適用部位に用いる前に切断、成形、又は何らかの方法で付形してもよい。代わりに、グラフト材料は、特定の傷害、病変、傷痕、創傷、又は創傷の種類に合わせて特別に設定した形態で製造してもよい。

【0113】

グラフト材料は、局所的用途に使用してもよい。代わりに、グラフト材料全体を使用してもよい。

【0114】

或る実施形態では、グラフト材料は、患者身体上の部位へ用いる用意が整った湿潤状態の材料として提供されている。別の実施形態では、グラフト材料は、乾燥状態の材料として供給され、体に用いる際に又は前に再水和される。

【0115】

本発明の更に別の実施形態では、治療剤は、流動化したＳＩＳの様な流動化したＥＣＭと混和させて、ＥＣＭと所望の治療剤を含んでいる実質的に均質なグラフト材料溶液を形成している。この事例では、流動化したグラフト材料は、その後患者の体に用いられる。

【0116】

本発明の別の実施形態では、治療剤は、最初に流動化したＳＩＳの様な流動化したＥＣＭと混和させて、ＥＣＭと所望の治療剤を含んでいる実質的に均質なグラフト材料を形成する。次いで、患者に用いる前に、流動化したグラフト材料を乾燥させる。流動化した又は細砕した小腸粘膜下組織を調製する方法については、後述の実施例１で説明している。

【0117】

図２は、ＥＣＭ１５を備えているグラフト材料１３の概略図であり、治療剤１６は、治療剤を流動化したＥＣＭと混和して、当該グラフト材料を乾燥させることにより、ＥＣＭ１５に組み込まれている。治療剤は、ＥＣＭの表面上に層１４を形成している。

【0118】

ある種の実施形態では、グラフト材料は、治療剤を所望の場所に送り出すことができるように、治療剤を伴って調製されている。治療剤は、医療処置に対する補助物（例えば、抗生剤）としてか、又は治療の主要物質（例えば、局所的に投与される遺伝子）として、被覆に含まれている。各種治療剤が使用され、その中には、ヒアルロン酸の様な受動的機能物質並びに成長ホルモンの様な能動的作用物質が含まれる。本発明のグラフト材料の治療

剤の具体的な事例については、先に説明済みである。
【0119】
治療法
本発明の方法は、患者身体の損傷又は病変のある組織の治癒に有用である。
【0120】
本発明に従って形成され使用されるグラフト材料は、患者身体の損傷又は病変のある組織上に載置されると、新しい内生組織の支持と成長のために迅速に血管が発達する基質として機能すると同時に、その様な治療を必要とする患者身体の障害又は病変のある部位に治療薬を送り込む機能を果たす。次いで、グラフト材料は改造され（吸収され、自家分化組織に置換され）、グラフト材料が載置部位で関係付けられる組織の特徴的な造形を作り出す。
【0121】
例えば、本発明のグラフト材料の都合のよい特性により、グラフト材料を使った治療を必要とする患者身体の一部を繰り返し清拭する必要性が減る。
【0122】
或る実施形態では、本発明は、組織の治癒を促進する方法を包含している。当該方法は、治癒する必要のある組織を、ＥＣＭと少なくとも１つの治療剤を備えているグラフト材料に接触させる段階を含んでいる。
【0123】
治療剤は特定の機能を有することが多い。例えば、本発明のグラフト材料に含まれる治療剤の量は、グラフト材料が載置される部位で内生組織の成長を促進するのに有効な量である。グラフト材料に含まれる治療剤の治療上有効な量は、一日につき体重１キログラム当たり約０．１ミリグラム（ｍｇ／ｋｇ／日）から約１００ｍｇ／ｋｇ／日まで、変わると予想される。
【0124】
ヒトの損傷又は病変のある組織を治療する技術分野の当業者であれば、ヒトを治療するためにグラフト材料に組み込む治療剤の用量は分かるはずである。一般に、有効な治療薬の量は、その様な治療を必要とする体表面積に基づいて調整される。
【0125】
本発明の治療剤の治療上有効な量は、医師又は獣医（担当臨床医）により容易に決定される。用量は、患者の要件、治療対象の症状の重傷度、及び採用された特定の作用物質によって変わる。用量を決める際には、担当臨床医は多くの要因を考慮するが、それら考慮すべき要素としては、限定するわけではないが、治療対象の具体的な組織；特定の作用物質の薬力学的特性；治療の所望の時間的進行；哺乳類の種；その大きさ、年齢、及び一般的健康状態；関与する特定の疾患；疾患の程度又は関与の度合い又は重傷度；個々の患者の反応；投与される特定の化合物；投与形態；投与される調合物の生物学的利用能特性；選択された用量処方計画；併用療法の種類；他の関連状況、が挙げられる。
【0126】
或る実施形態では、患者身体の損傷又は病変のある部分は、ＥＣＭ基質と少なくとも１つの治療剤を含んでいるグラフト材料のパッチを載置することにより修復される。
【0127】
別の実施形態では、ここで開示しているグラフト材料は、生体分化可能な創傷包帯又はバンドエイドを製作するのに使用されている。創傷包帯は、創傷治療用包帯、組織シーラント（即ち、組織又は器官を出入りする血液、尿、空気などの様な流体又は気体に曝されないようにするために組織又は器官をシールするもの）、及び／又は細胞成長スカフォードとして使用される。各種実施形態では、創傷包帯は、傷害のある組織を保護し、湿潤環境を維持し、水分に対する透過性を有し、適用が容易で、頻繁な交換を必要とせず、毒性が無く、非抗原性であり、微生物制御を維持し、及び／又は有効な化治療剤を創傷部位に送り込む。
【0128】

ここに説明するグラフト材料に従って使用される生体吸収可能なシーラント及び接着剤の例としては、例えば、Focal社により製造されているFOCALSEAL（登録商標）（キセノン光ワンドを用いた光重合を必要とする生体分解可能なエオシン-PEG-ラクチドヒドロゲル）；Adventis-Bering社により製造されているBERIPLAST（登録商標）；CanvaTec社（Bristol-Meyers-Squibb社）により製造されているVIVOSTAT（登録商標）；Baxter社により製造されているSEALAGEN™；CyoLife社により製造されているFIBRX（登録商標）（ウイルス非活性化ヒトフィブリノゲン及び抑制化ヒトトロンビンを含有）；Baxter社により製造されているTISSEEL（登録商標）（天然凝固経路の最終段階からのプラズマ誘導物から成る線維糊であり、溶解可能なフィブリノゲンは固体線維に変化している）　とTISSUCOL（登録商標）；Omrix Biopharm社により製造されているQUIXIL（登録商標）（生物活性成分とトロンビン）；Cohesion社（コラーゲン社）により製造されるPEG-コラーゲン共重合体；Davis & Geck社により製造されているHYSTOACRYL　BLUE（登録商標）(ENBUCRILATE)(シアノアクリレート)；Closure Medical社(TriPoint Medical社)により製造されているNEXACRYL™(N-ブチルシアノアクリレート)、NEXABOND™、NEXABOND™S/C、及びTRAUMASEAL™（シアノアクリレートを主材とした製品）；Dermabond社(Ethicon社)により製造されている、2-オクチルシアノアクリレートから成るDERMABOND™；Medi-West Pharma社により製造されているTISSUEGLU（登録商標）；及び３M社により製造されているn-ブチルシアノアクリレートから成るVETBONDTMが挙げられる。

【0129】

創傷包帯は、神経修復、器官修復、皮膚修復、脈管修復、筋肉修復、及び眼科用途を含む軟組織修復に使用される。代表的な実施形態では、創傷包帯は、例えば、真皮及び表皮の面、吻合、縫合、ステープル、穿刺、切開、裂傷、又は組織の付着成長の部位の表面、の様な面を治療するのに使用される。

【0130】

代表的な実施形態では、創傷包帯は、組織の被覆又は密閉が求められるあらゆる症状に関連して使用される。例えば、体液を停止又はできる限り少なくすることができ；骨盤及び腹部、心膜、脊髄及び硬膜、腱及び腱鞘の癒着を含め、術後の癒着を予防するためにバリアを用いることができる。創傷包帯は、切開、擦過傷、熱傷、炎症、及び体の外表面に対する被覆を用いることが必要とされる他の症状を修復又は治癒する際に、露出した皮膚を治療するのに有用である。本発明のグラフト材料は、皮膚を治療するのに使用されるのが望ましい。

【0131】

或る事例では、熱傷は本発明のグラフト材料を用いて治療され、グラフト材料は、ＥＣＭと、スルファジアジン銀、抗生物質、又は鎮痛剤、及び／又は上記作用物質の組み合わせのような治療剤と、を含んでいる。

【0132】

各事例では、患者身体の損傷又は病変のある組織を治療、置換、又は治癒するための損傷包帯として使用される本発明のグラフト材料には、適切な治療剤が含まれている。

【0133】

本発明を、更に、以下の実験例により説明するが、これは本発明を限定するものではない。本出願全体を通して引用した全ての文献、特許、及び公開出願を、参考文献としてここに援用する。

【0134】

実施例

実施例１：流動化したグラフト材料を調製する方法

流動化したグラフト材料は、腸粘膜下組織の溶液又は懸濁液として調製される。腸粘膜下組織開始材料は、引き裂き、切断、粉砕、剪断などにより細砕されるか、又は、組織が溶解して実質的に均質な粘膜下組織溶液を形成する十分な期間、トリプシン又はペプシンの様なプロテアーゼで消化させる。

【0135】

試料を底が平らなステンレス鋼容器に入れて、液体窒素を容器に導入し、試料を凍結して、更に細砕するために調製する。

【0136】

次いで、凍結した粘膜下組織試料を細砕して粗い粘膜下組織粉末にする。このような処置は、例えば、凍結した試料の頂部に円筒状の真鋳インゴットを載せて手動アーバプレスで行う。インゴットは、試料とプレス機のアーバの間の接触面として働く。通常、粘膜下組織試料を凍結状態に保つためには、それに周期的に液体窒素を加える必要がある。

【0137】

代わりに、粘膜下組織の懸濁液は、高速（高速剪断）ブレンダで処理して脱水し、必要に応じて、遠心分離して上澄みの余剰水分を捨てて行ってもよい。粘膜下組織の粉末が出来上がる。その後、粘膜下組織の粉末を、例えば、緩衝生理食塩水と治療剤を組み合わせたものを使って再水和し、所望の粘度、例えば２５℃で約２から約３００，０００ｃｐｓの粘度の流体化した組織のグラフト材料を形成する。

【0138】

より粘度の高いグラフト材料は、ゲル又はペースト粘性を有する。

【0139】

グラフト材料は、次いで、電離放射線に曝すなど当技術で認められている殺菌技法を使用して殺菌される。

【0140】

実施例２：創傷を治療する方法

ＳＩＳと抗生物質を含んでいるグラフト材料を使用して、全層皮膚創傷を治療する。グラフト材料は、ＳＩＳのシートであり、グラフト材料を噴射することにより一方の側に抗生物質が塗布されている。

【0141】

第２度の熱傷、裂傷、引き裂き、及び剥離を含む皮膚創傷、癌性増殖部又は自家移植片皮膚供給部の切除による外科的切除創傷、並びに静脈性潰瘍、糖尿病、圧力（床ずれ）及び他の慢性潰瘍の様な皮膚潰瘍は、ＳＩＳと治療剤を備えているグラフト材料を用いて管理される。

【0142】

グラフト材料を創傷に適用する前に、創傷層に適用のための前処理を施す。

【0143】

グラフト処置が必要な熱傷創傷を持つ患者を選択する。グラフト材料は、抗生剤を含んでいる側が創傷に面するようにして、切開された創傷層に直接載せる。

【0144】

グラフトが移植される熱傷創傷部位に、標準的な実施法に従って治療する前に清拭などの前処理を施して、皮膚の熱傷領域が完全に切開されるようにする。切開された層は、清潔で臨床学的に非感染となる。

【0145】

外科的切開を受ける患者には局所的麻酔をかける。術前領域を、抗菌／殺菌皮膚クレンザー（Hibiclens（登録商標））で洗浄して、通常の生理食塩水ですすぐ。皮膚に深い部分的な創傷を入れて、癌性でない限り、その皮膚をどこか他の場所に移植する。グラフト材料を、創傷層に当てて殺菌包帯を当てる。

【0146】

何れの創傷の事例でも、処置対象の創傷の検査、洗浄、包帯交換などの際に、適切な創傷の手当てが患者に施される。

【0147】

創傷を本発明のグラフト材料で治療すると、清拭を繰り返す必要が少なくなる。

【0148】

治療部位の状態の完全な記録が、全ての処置、必要な薬物治療、包帯交換の頻度、及び観察できたこと全てを文書に保存される。創傷層は外部環境から保護され、湿潤した状態

に保たれ、創傷の管理と治癒が助長される。

【0149】

上記詳細な説明は、本発明を限定するものではなく説明を目的としたものであり、本発明の精神と範囲を定義するのは、特許請求の範囲と全ての等価物であると理解されたい。

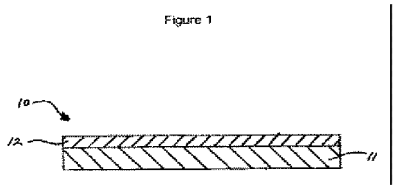
【図面の簡単な説明】

【0150】

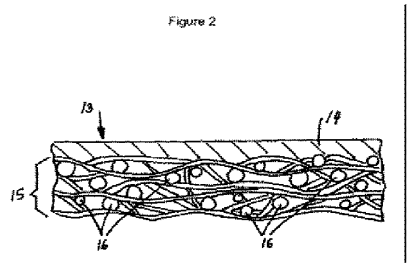
【図1】本発明のグラフト材料の概略図であり、ＥＣＭを整えた後、ＥＣＭに治療剤が添加される。

【図2】グラフト材料の概略図であり、グラフト材料のＥＣＭに治療薬が組み込まれている。

【図1】



【図2】



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2005/010905

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61L27/24 A61L27/54 A61L27/58 A61L27/60

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)
IPC 7 A61L

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 206 931 B1 (COOK WILLIAM A ET AL) 27 March 2001 (2001-03-27) column 6, line 22 - line 34 column 9, line 18 - line 28 column 10, line 51 - line 59 column 13, line 3 - line 12 column 13, line 50 - line 57	1-8, 11-20
X	US 2002/099448 A1 (HILES MICHAEL C ET AL) 25 July 2002 (2002-07-25) the whole document	1-8, 11-20

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in 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 document 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
2 August 2005	13. 10. 2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hars, J

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2005/010905

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/22158 A (COOK BIOTECH, INCORPORATED; MED INSTITUTE, INCORPORATED) 28 May 1998 (1998-05-28) page 2 page 8, last paragraph - page 9, paragraph 2 page 14, paragraph 1 page 15, last paragraph - page 16, paragraph 2 page 21, paragraph 1	1-8,11, 13-20
X	US 4 956 178 A (BADYLAK ET AL) 11 September 1990 (1990-09-11) column 3, line 25 - line 34 column 3, line 55 - line 58 column 4, line 12 - line 37	1-5,7,8, 11, 13-18,20
X	US 4 902 508 A (BADYLAK ET AL) 20 February 1990 (1990-02-20) column 3, line 25 - line 34 column 3, line 55 - line 58 column 4, line 12 - line 37	1-5,7,8, 11, 13-18,20

Form PCT/ISA/210 (continuation of second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2005/010905

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 15-20 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

5, 6, 18, 19 (entirely), 1-4, 7-17, 20 (partially)

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 5,6,18,19 (entirely); 1-4,7-17,20 (partially)

   A graft material comprising an extracellular matrix (ECM) made from small intestine submucosa (SIS), and its associated method of healing a tissue defect.

   ---

2. claims: 1-4,7-17,20 (partially)

   A graft material comprising an extracellular matrix (ECM) made from acellular dermis, and its associated method of healing a tissue defect.

   ---

3. claims: 1-4,7-17,20 (partially)

   A graft material comprising an extracellular matrix (ECM) made from cadaveric fascia, and its associated method of healing a tissue defect.

   ---

4. claims: 1-4,7-17,20 (partially)

   A graft material comprising an extracellular matrix (ECM) made from the bladder acellular matrix, and its associated method of healing a tissue defect.

   ---

5. claims: 1-4,7-17,20 (partially)

   A graft material comprising an extracellular matrix (ECM) made from amniotic membrane, and its associated method of healing a tissue defect.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2005/010905

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			ZA 8904551	A	28-03-1990

Form PCT/ISA/210 (patent family annex) (January 2004)

(81)指定国　　AP(BW,GH,GM,KE,LS,MW,MZ,NA,SD,SL,SZ,TZ,UG,ZM,ZW),EA(AM,AZ,BY,KG,KZ,MD,RU,TJ,TM),EP(AT,BE,BG,CH,CY,CZ,DE,DK,EE,ES,FI,FR,GB,GR,HU,IE,IS,IT,LT,LU,MC,NL,PL,PT,RO,SE,SI,SK,TR),OA(BF,BJ,CF,CG,CI,CM,GA,GN,GQ,GW,ML,MR,NE,SN,TD,TG),AE,AG,AL,AM,AT,AU,AZ,BA,BB,BG,BR,BW,BY,BZ,CA,CH,CN,CO,CR,CU,CZ,DE,DK,DM,DZ,EC,EE,EG,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KP,KR,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,MZ,NA,NI,NO,NZ,OM,PG,PH,PL,PT,RO,RU,SC,SD,SE,SG,SK,SL,SM,SY,TJ,TM,TN,TR,TT,TZ,UA,UG,US,UZ,VC,VN,YU,ZA,ZM,ZW

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A61P 17/02	(2006.01)	A61P	17/02		
A61P 43/00	(2006.01)	A61P	43/00	105	
A61P 29/00	(2006.01)	A61P	29/00		
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審査請求 未請求　予備審査請求 未請求　（全 86 頁）　最終頁に続く

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最終頁に続く

(54)【発明の名称】羊膜調製物及び精製組成物及びその使用方法

(57)【要約】
特定の生物学的成分の組み合わせからなる組成物が、哺乳動物細胞において、ＴＧＦ－βシグナル伝達、アポトーシス及び哺乳動物細胞の増殖の調節、さらにはマウスにおける炎症の抑制を含む、多くの有用な作用を発現することが分かった。これらの成分は市販されているものを入手してもよいし、胎盤組織などの生物学的組織から調製してもよい。ここに記載される胎盤羊膜（ＡＭ）調製物としては、ＡＭ小片、ＡＭ抽出物、ＡＭゼリー、ＡＭ間質及びこれら組成物と他の成分との混合物が挙げられる。この組成物は、創傷治癒、炎症及び血管新生関連疾患などの各種疾患の治療に使用することができる。

【特許請求の範囲】
【請求項1】
架橋した高分子ヒアルロン酸（ＨＡ）；
腫瘍壊死因子活性化遺伝子６（ＴＳＧ－６）；
ペントラキシン（ＰＴＸ－３）；
トロンボスポンジン（ＴＳＰ－１）を含む、精製組成物。
【請求項2】
構成成分のうち少なくとも一部が、ヒト羊膜、ヒト羊膜ゼリー、ヒト羊膜間質、又はこれらの組み合わせから選択されるヒト羊膜材料から調製される請求項１記載の精製組成物。
【請求項3】
Ｓｍａｄ７をさらに含む請求項１記載の精製組成物。
【請求項4】
ＨＡの架橋がインターα－トリプシン阻害剤の重鎖への共有結合を含む請求項１記載の精製組成物。
【請求項5】
ＨＡに対するタンパク質の比が約１００未満である請求項１記載の精製組成物。
【請求項6】
前記調製することが
凍結した、又はあらかじめ凍結したヒト胎盤を得え；
該胎盤を解凍し、その解凍した胎盤からヒト羊膜材料を単離し；
該ヒト羊膜材料を適当なバッファーの中でホモジナイズすることを含む請求項２記載の精製組成物。
【請求項7】
前記調製することが
該ヒト羊膜材料を凍結し、；
該凍結羊膜材料を粉砕することを含む請求項６記載の精製組成物。
【請求項8】
前記調製することが
該ホモジネートを凍結乾燥すること；又は
該ホモジネートを遠心分離し、その遠心分離したホモジネートから上清を分離することを含む請求項６記載の精製組成物。
【請求項9】
前記調製することが
該上清を凍結乾燥して粉末にすることを含む請求項８記載の精製組成物。
【請求項10】
薬学的に許容される非固体製剤用、又は徐放性固体製剤用担体をさらに含む請求項１記載の精製組成物。
【請求項11】
有効量の瘢痕形成抑制組成物を、瘢痕形成の抑制を必要としている被験者に提供するステップを含む被験者における瘢痕形成を抑制する方法であって、該瘢痕形成抑制組成物が、ヒト羊膜、ヒト羊膜ゼリー、ヒト羊膜間質、又は羊膜から抽出されたこれらの組み合わせから選択されるヒト羊膜材料から調製される少なくとも一成分を含む方法。
【請求項12】
少なくとも一成分が該ヒト羊膜材料から抽出されたものである請求項１１記載の方法。
【請求項13】
該ヒト羊膜材料が該ヒト羊膜間質である請求項１２記載の方法。
【請求項14】
該抽出方法が

凍結した、又はあらかじめ凍結したヒト胎盤を得るステップ；
該胎盤を解凍し、その解凍した胎盤からヒト羊膜材料を単離するステップ；
該ヒト羊膜材料を適当なバッファーの中でホモジナイズするステップ；
必要に応じて、該ホモジネートを凍結乾燥して粉末にするステップ；
該ホモジネート又は該粉末を薬学的に許容される非固体製剤用、又は徐放性固体製剤用担体と混合することを含む請求項１２記載の方法。
【請求項15】
該調製方法における該ホモジネートを凍結乾燥するステップを
該ホモジネートを遠心分離し、その遠心分離したホモジネートから上清を分離し、必要に応じてその上清を凍結乾燥して粉末にするステップに置き換える請求項１４記載の方法。
【請求項16】
有効量の瘢痕逆行組成物を、瘢痕を有する被験者に提供するステップを含む被験者における瘢痕形成を逆行させる方法であって、該瘢痕逆行組成物が、ヒト羊膜、ヒト羊膜ゼリー、ヒト羊膜間質、又は羊膜から抽出されたこれらの組み合わせから選択されるヒト羊膜材料から調製される少なくとも一成分を含む方法。
【請求項17】
少なくとも一成分が該ヒト羊膜材料から抽出されたものである請求項１６記載の方法。
【請求項18】
該ヒト羊膜材料が該ヒト羊膜間質である請求項１７記載の方法。
【請求項19】
該抽出方法が
凍結した、又はあらかじめ凍結したヒト胎盤を得るステップ；
該胎盤を解凍し、その解凍した胎盤からヒト羊膜材料を単離するステップ；
該ヒト羊膜材料を適当なバッファーの中でホモジナイズするステップ；
必要に応じて、該ホモジネートを凍結乾燥して粉末にするステップ；
該ホモジネート又は該粉末を薬学的に許容される非固体製剤用、又は徐放性固体製剤用担体と混合することを含む請求項１７記載の方法。
【請求項20】
該調製方法において、該ホモジネートを凍結乾燥するステップを
該ホモジネートを遠心分離し、その遠心分離したホモジネートから上清を分離し、必要に応じてその上清を凍結乾燥して粉末にするステップに置き換える請求項１９記載の方法。
【請求項21】
有効量の血管新生抑制組成物を、血管新生の抑制を必要としている被験者に提供するステップを含む被験者における血管新生を抑制する方法であって、該血管新生抑制組成物が、ヒト羊膜、ヒト羊膜ゼリー、ヒト羊膜間質、又は羊膜から抽出されたこれらの組み合わせから選択されるヒト羊膜材料から調製される少なくとも一成分を含む方法。
【請求項22】
少なくとも一成分が該ヒト羊膜材料から抽出されたものである請求項２１記載の方法。
【請求項23】
該ヒト羊膜材料が該ヒト羊膜間質である請求項２２記載の方法。
【請求項24】
該組成物が、
架橋した高分子ヒアルロン酸（ＨＡ）；
腫瘍壊死因子活性化遺伝子６（ＴＳＧ－６）；
ペントラキシン（ＰＴＸ－３）；
トロンボスポンジン（ＴＳＰ－１）を含む請求項２１記載の方法。
【請求項25】
該抽出方法が

凍結した、又はあらかじめ凍結したヒト胎盤を得るステップ；
該胎盤を解凍し、その解凍した胎盤からヒト羊膜材料を単離するステップ；
該ヒト羊膜材料を適当なバッファーの中でホモジナイズするステップ；
必要に応じて、該ホモジネートを凍結乾燥して粉末にするステップ；
該ホモジネート又は該粉末を薬学的に許容される非固体製剤用、又は徐放性固体製剤用担体と混合するステップを含む請求項２２記載の方法。
【請求項26】
該調製方法において、該ホモジネートを凍結乾燥するステップを
該ホモジネートを遠心分離し、その遠心分離したホモジネートから上清を分離し、必要に応じてその上清を凍結乾燥して粉末にするステップに置き換える請求項２５記載の方法。
【請求項27】
血管新生の抑制を必要としている該被験者が癌患者である請求項２１記載の方法。
【請求項28】
血管新生の抑制を必要としている該被験者が加齢黄斑変性症患者である請求項２１記載の方法。
【請求項29】
該血管新生抑制組成物が、非固体製剤又は徐放性固体製剤として提供される請求項２１記載の方法。
【請求項30】
該血管新生抑制組成物が以下の性質を有する請求項２１記載の方法：
血管形成に関与する内皮細胞のアポトーシスを引き起こす；
血管形成に関与する内皮細胞の遊走を抑制する；
血管形成に関与する内皮細胞の管腔形成を抑制する。
【請求項31】
有効量の炎症抑制組成物を、炎症の抑制又は予防を必要としている被験者に提供するステップを含む被験者における炎症を抑制又は予防する方法であって、該炎症抑制組成物が、ヒト羊膜、ヒト羊膜ゼリー、ヒト羊膜間質、又は羊膜から抽出されたこれらの組み合わせから選択されるヒト羊膜材料から調製される少なくとも一成分を含む方法。
【請求項32】
少なくとも一成分が該ヒト羊膜材料から抽出されたものである請求項３１記載の方法。
【請求項33】
該ヒト羊膜材料が該ヒト羊膜である請求項３２記載の方法。
【請求項34】
該組成物が、
架橋した高分子ヒアルロン酸（ＨＡ）；
腫瘍壊死因子活性化遺伝子６（ＴＳＧ－６）；
ペントラキシン（ＰＴＸ－３）；
トロンボスポンジン（ＴＳＰ－１）を含む請求項３１記載の方法。
【請求項35】
該抽出方法が
凍結した、又はあらかじめ凍結したヒト胎盤を得るステップ；
該胎盤を解凍し、その解凍した胎盤からヒト羊膜材料を単離するステップ；
該ヒト羊膜材料を適当なバッファーの中でホモジナイズするステップ；
必要に応じて、該ホモジネートを凍結乾燥して粉末にするステップ；
該ホモジネート又は該粉末を薬学的に許容される非固体製剤用、又は徐放性固体製剤用担体と混合するステップを含む請求項３２記載の方法。
【請求項36】
該調製方法において、該ホモジネートを凍結乾燥するステップを
該ホモジネートを遠心分離し、その遠心分離したホモジネートから上清を分離し、必要

に応じてその上清を凍結乾燥して粉末にするステップに置き換える請求項３５記載の方法。

【請求項37】

該ヒトが関節炎を患っている請求項３１記載の方法。

【請求項38】

該ヒトが少なくとも片方の目に炎症を患っている請求項３１記載の方法。

【請求項39】

該炎症抑制組成物が、非固体製剤又は徐放性固体製剤として提供される請求項３１記載の方法。

【請求項40】

該炎症抑制組成物が少なくとも以下の二つの性質を有する請求項３１記載の方法：

炎症部位におけるマクロファージのアポトーシスを引き起こす；

炎症部位におけるプロスタグランジンＥ１に対するプロスタグランジンＤ２の比を増加させる；

炎症部位におけるＴＧＦ－β１活性を抑制する；又は

炎症部位におけるインターフェロン－γシグナル伝達を阻害する。

【発明の詳細な説明】

【技術分野】

【0001】

関連出願の参照

本出願は２００５年９月２７日出願された米国仮出願第６０／７２０，７６０号の利益を主張するものであり、同仮出願の全内容は参照によって本明細書に組み入れられるものとする。

【0002】

連邦政府による資金提供を受けた研究開発に関する記載

本発明は、国立衛生研究所から与えられたグラントＮｏ．ＲＯ１　ＥＹ０６８１９に基づく米国政府の支援によってなされたもので、米国政府は本発明に一定の権利を有する。

【0003】

本発明は一般的に生物学と医薬分野に関する。特に本発明は、羊膜調製物から見いだすことができる化合物の組み合わせを使用する、細胞生理学的及び病理学的プロセッシングを調節する組成物及び方法に関する。

【背景技術】

【0004】

胎盤は妊娠期間中における胎児の周りを囲む一時的な臓器である。胎盤によって気体や栄養素が輸送され、また他の代謝や内分泌機能がもたらされる。胎盤は数種類の組織により構成される。臍帯は胎盤と胎児をつなげ、これによって酸素が胎児に運ばれる。臍帯には、二本の動脈と一本の静脈がある。ワルトンゼリーという特殊なゼリー状の結合組織物質が臍帯の周りを囲み、胎動及び胎児生育期におけるダメージから胎児を保護する。胎盤の外側の「シェル」は「絨毛膜」として知られている。胎盤のほとんどは、絨毛膜絨毛樹の伸長部である絨毛膜絨毛により構成される。このような構造によって胎児の栄養交換が行われる。羊膜（ＡＭ）は無血管膜嚢であり、羊水で満たされている。この膜が、羊膜腔中の胎児を囲んでいる一番内側に存在する膜である。この組織は上皮層及び下側に隣接する無血管の間質層からなる。

【発明の開示】

【発明が解決しようとする課題】

【0005】

本明細書は、精製組成物及び羊膜調製物（すなわち、羊膜、羊膜間質及び羊膜ゼリーを含む羊膜材料より調製される組成物）を開示する。ある態様では、精製組成物の少なくとも一成分が羊膜調製物から得られる。また本明細書は、精製組成物の少なくとも一成分がヒト胎盤と絨毛膜から得られる精製組成物を開示する。また本明細書は、上記精製組成物及び調製物のいずれかを調製する方法を開示する。また本明細書は、上記精製組成物及び

調製物のいずれかを保管及び保存する方法を開示する。また本明細書は、上記精製組成物及び調製物のいずれかを使用する方法を開示し、例えば、保存方法、細胞培養方法、組織培養方法、治療方法、予防方法、美容方法が挙げられる。

【課題を解決するための手段】

【0006】

一つの態様は、

・架橋した高分子ヒアルロン酸（ＨＡ）；

・腫瘍壊死因子活性化遺伝子６（ＴＳＧ－６）；

・ペントラキシン（ＰＴＸ－３）；

・トロンボスポンジン（ＴＳＰ－１）を含む、精製組成物である。

【0007】

さらなる実施態様では、該精製組成物の構成成分のうち少なくとも一部が、ヒト羊膜、ヒト羊膜ゼリー、ヒト羊膜間質、又はこれらの組み合わせから選択されるヒト羊膜材料から調製される。別の又はさらなる実施態様では、該精製組成物がさらにＳｍａｄ７を含む。該精製組成物のさらなる又は別の実施態様では、ＨＡの架橋がインター－α－トリプシン阻害剤の重鎖への共有結合を含む。該精製組成物のさらなる又は別の実施態様では、ＨＡに対するタンパク質の比が約１００未満である。

【0008】

該精製組成物のさらなる又は別の実施態様では、該精製組成物の調製方法が

・凍結した、又はあらかじめ凍結したヒト胎盤を得るステップ；

・該胎盤を解凍し、その解凍した胎盤からヒト羊膜材料を単離するステップ；

・該ヒト羊膜材料を適当なバッファーの中でホモジナイズするステップ、を含む。

【0009】

さらなる又は別の実施態様では、該調製方法がさらに

・該ヒト羊膜材料を凍結するステップ；

・該凍結羊膜材料を粉砕するステップ、を含む。

【0010】

さらなる又は別の実施態様では、該調製方法がさらに

・該ホモジネートを凍結乾燥するステップ；又は

・該ホモジネートを遠心分離し、その遠心分離したホモジネートから上清を分離するステップ、を含む。

【0011】

さらなる又は別の実施態様では、該調製方法がさらに

・該上清を凍結乾燥して粉末にするステップ、を含む。

【0012】

さらなる又は別の実施態様では、該精製組成物が、薬学的に許容される非固体製剤用、又は徐放性固体製剤用担体をさらに含む。

【0013】

本明細書に記載される他の態様は、有効量の瘢痕形成抑制組成物を、瘢痕形成の抑制を必要としている被験者に提供するステップを含む被験者における瘢痕形成を抑制する方法であって、該瘢痕形成抑制組成物が、ヒト羊膜、ヒト羊膜ゼリー、ヒト羊膜間質、又は羊膜から抽出されたこれらの組み合わせから選択されるヒト羊膜材料から調製される少なくとも一成分を含む方法である。

【0014】

該方法のさらなる又は別の実施態様では、少なくとも一成分が該ヒト羊膜材料から抽出されたものである。該方法のさらなる又は別の実施態様では、該ヒト羊膜材料が該ヒト羊膜間質である。

【0015】

該方法のさらなる又は別の実施態様では、該抽出方法が

・凍結した、又はあらかじめ凍結したヒト胎盤を得るステップ；

・該胎盤を解凍し、その解凍した胎盤からヒト羊膜材料を単離するステップ；
・該ヒト羊膜材料を適当なバッファーの中でホモジナイズするステップ；
・必要に応じて、該ホモジネートを凍結乾燥して粉末にするステップ；
・該ホモジネート又は該粉末を薬学的に許容される非固体製剤用、又は徐放性固体製剤用担体と混合するステップ、を含む。

【0016】
さらなる又は別の実施態様では、該調製方法において、該ホモジネートを凍結乾燥するステップを
・該ホモジネートを遠心分離し、その遠心分離したホモジネートから上清を分離し、必要に応じてその上清を凍結乾燥して粉末にするステップに置き換える。

【0017】
他の態様は、有効量の瘢痕逆行組成物を、瘢痕を有する被験者に提供するステップを含む被験者における瘢痕形成を逆行させる方法であって、該瘢痕逆行組成物が、ヒト羊膜、ヒト羊膜ゼリー、ヒト羊膜間質、又は羊膜から抽出されたこれらの組み合わせから選択されるヒト羊膜材料から調製される少なくとも一成分を含む方法である。この方法における他の又はさらなる実施態様では、少なくとも一成分が該ヒト羊膜材料から抽出されたものである。この方法における他の又はさらなる実施態様では、該ヒト羊膜材料が該ヒト羊膜間質である。

【0018】
この方法における他の又はさらなる実施態様では、該抽出方法が
・凍結した、又はあらかじめ凍結したヒト胎盤を得るステップ；
・該胎盤を解凍し、その解凍した胎盤からヒト羊膜材料を単離するステップ；
・該ヒト羊膜材料を適当なバッファーの中でホモジナイズするステップ；
・必要に応じて、該ホモジネートを凍結乾燥して粉末にするステップ；
・該ホモジネート又は該粉末を薬学的に許容される非固体製剤用、又は徐放性固体製剤用担体と混合するステップ、を含む。

【0019】
他の又はさらなる実施態様では、該調製方法において、該ホモジネートを凍結乾燥するステップを
・該ホモジネートを遠心分離し、その遠心分離したホモジネートから上清を分離し、必要に応じてその上清を凍結乾燥して粉末にするステップに置き換える。

【0020】
本明細書に記載される他の態様は、有効量の血管新生抑制組成物を、血管新生の抑制を必要としている被験者に提供するステップを含む被験者における血管新生を抑制する方法であって、該血管新生抑制組成物が、ヒト羊膜、ヒト羊膜ゼリー、ヒト羊膜間質、又は羊膜から抽出されたこれらの組み合わせから選択されるヒト羊膜材料から調製される少なくとも一成分を含む方法である。

【0021】
この方法における他の又はさらなる実施態様では、少なくとも一成分が該ヒト羊膜材料から抽出されたものである。この方法における他の又はさらなる実施態様では、該ヒト羊膜材料が該ヒト羊膜間質である。

【0022】
この方法における他の又はさらなる実施態様では、該組成物が、
・架橋した高分子ヒアルロン酸（ＨＡ）；
・腫瘍壊死因子活性化遺伝子６（ＴＳＧ－６）；
・ペントラキシン（ＰＴＸ－３）；
・トロンボスポンジン（ＴＳＰ－１）を含む。

【0023】
この方法における他の又はさらなる実施態様では、該抽出方法が
・凍結した、又はあらかじめ凍結したヒト胎盤を得るステップ；

・該胎盤を解凍し、その解凍した胎盤からヒト羊膜材料を単離するステップ；
・該ヒト羊膜材料を適当なバッファーの中でホモジナイズするステップ；
・必要に応じて、該ホモジネートを凍結乾燥して粉末にするステップ；
・該ホモジネート又は該粉末を薬学的に許容される非固体製剤用、又は徐放性固体製剤用担体と混合するステップ、を含む。

【0024】
この方法における他の又はさらなる実施態様では、該調製方法において、該ホモジネートを凍結乾燥するステップを
・該ホモジネートを遠心分離し、その遠心分離したホモジネートから上清を分離し、必要に応じてその上清を凍結乾燥して粉末にするステップに置き換える。

【0025】
この方法における他の又はさらなる実施態様では、血管新生の抑制を必要としている該被験者が癌患者である。この方法における他の又はさらなる実施態様では、血管新生の抑制を必要としている該被験者が加齢黄斑変性症患者である。

【0026】
この方法における他の又はさらなる実施態様では、該血管新生抑制組成物が、非固体製剤又は徐放性固体製剤として提供される。

【0027】
この方法における他の又はさらなる実施態様では、該血管新生抑制組成物が以下の性質を有する：
・血管形成に関与する内皮細胞のアポトーシスを引き起こす；
・血管形成に関与する内皮細胞の遊走を抑制する；
・血管形成に関与する内皮細胞の管腔形成を抑制する。

【0028】
有効量の炎症抑制組成物を、炎症の抑制又は予防を必要としている被験者に提供するステップを含む被験者における炎症を抑制又は予防する方法であって、該炎症抑制組成物が、ヒト羊膜、ヒト羊膜ゼリー、ヒト羊膜間質、又は羊膜から抽出されたこれらの組み合わせから選択されるヒト羊膜材料から調製される少なくとも一成分を含む方法。

【0029】
この方法における他の又はさらなる実施態様では、少なくとも一成分が該ヒト羊膜材料から抽出されたものである。この方法における他の又はさらなる実施態様では、該ヒト羊膜材料が該ヒト羊膜間質である。

【0030】
この方法における他の又はさらなる実施態様では、該組成物が、
・架橋した高分子ヒアルロン酸（ＨＡ）；
・腫瘍壊死因子活性化遺伝子６（ＴＳＧ－６）；
・ペントラキシン（ＰＴＸ－３）；
・トロンボスポンジン（ＴＳＰ－１）を含む。

【0031】
この方法における他の又はさらなる実施態様では、該抽出方法が
・凍結した、又はあらかじめ凍結したヒト胎盤を得るステップ；
・該胎盤を解凍し、その解凍した胎盤からヒト羊膜材料を単離するステップ；
・該ヒト羊膜材料を適当なバッファーの中でホモジナイズするステップ；
・必要に応じて、該ホモジネートを凍結乾燥して粉末にするステップ；
・該ホモジネート又は該粉末を薬学的に許容される非固体製剤用、又は徐放性固体製剤用担体と混合するステップ、を含む。

【0032】
この方法における他の又はさらなる実施態様では、該調製方法において、該ホモジネートを凍結乾燥するステップを
・該ホモジネートを遠心分離し、その遠心分離したホモジネートから上清を分離し、必

要に応じてその上清を凍結乾燥して粉末にするステップに置き換える。
【0033】
この方法における他の又はさらなる実施態様では、該ヒトが関節炎を患っている。この方法における他の又はさらなる実施態様では、該ヒトが少なくとも片方の目に炎症を患っている。この方法における他の又はさらなる実施態様では、該炎症抑制組成物が、非固体製剤又は徐放性固体製剤として提供される。
【0034】
この方法における他の又はさらなる実施態様では、該炎症抑制組成物が少なくとも以下の二つの性質を有する：
・炎症部位におけるマクロファージのアポトーシスを引き起こす；
・炎症部位におけるプロスタグランジンＥ１に対するプロスタグランジンＤ２の比を増加させる；
・炎症部位におけるＴＧＦ－β１活性を抑制する；又は
・炎症部位におけるインターフェロン－γシグナル伝達を阻害する。
【0035】
各種ＡＭ調製物が、哺乳動物細胞と無処理の哺乳動物組織において、多くの生理学的に有意な効果を発現する。そのような効果としては、ＴＧＦ－βシグナル伝達の抑制、マクロファージのアポトーシスの増加、血管内皮細胞の増殖の減少、遊走の減少とアポトーシスの増加、保存やディスパーゼ処理により引き起こされる、角膜と角膜縁の上皮細胞及び角膜実質細胞のアポトーシスからの保護、組織における炎症の抑制が挙げられる。無処理ＡＭ片の他に、本明細書に記載される他の調製物としては、ＡＭ間質片、加工された（例えば、粉砕、微粉砕）ＡＭやＡＭ間質、及び無処理ＡＭ及びＡＭ間質の様々な抽出物が挙げられる。ＡＭ抽出物は液状でも凍結乾燥による粉末状でもよい。
【0036】
また、該組成物としては、ＡＭ抽出物と一種以上の追加の基質成分（ＥＣＭ）のような増粘剤を混合して作ることができる、粘性を高めた、又はゲル状のＡＭ抽出物が挙げられる。数多くのＥＣＭ成分が知られており、例えば、コラーゲン、ヒアルロン酸（ＨＡ）及びフィブリンが挙げられる。
【発明を実施するための最良の形態】
【0037】
本明細書に記載のものに類似のあるいは同等の調製物、材料及び方法は、本発明の実施又は試験に際して使用することができるが、適切な調製物、方法及び材料をここに記載する。本明細書に記載されているすべての出版物の全内容は参照によって本明細書に組み入れられるものとする。争いが生じた場合には、定義を含む本明細書を基準とする。また、以下に示す具体的な実施の形態は、単なる説明のためのものにすぎず、発明を制限するためのものではない。
【0038】
定義
処方、組成物又は成分に関する本明細書において使用される用語「許容される」は、治療される被験者の全般的健康に対して、持続的有害な影響を及ぼさないことを意味する。
【0039】
「抗酸化剤」としては、例えば、ブチル化ヒドロキシトルエン（ＢＨＴ）、アスコルビン酸ナトリウム、アスコルビン酸、メタ重亜硫酸ナトリウム、トコフェロールが挙げられる。ある態様では、必要に応じて、抗酸化剤が化学安定性を高める。
【0040】
「結合剤」は粘着性を与えるものであり、例えば、アルギン酸及びその塩；カルボキシメチルセルロース、メチルセルロース（例えば、メトセル（登録商標））、ヒドロキシプロピルメチルセルロース、ヒドロキシエチルセルロース、ヒドロキシプロピルセルロース（例えば、クルセル（登録商標））、エチルセルロース（例えば、エトセル（登録商標））及び微結晶性セルロース（例えば、アビセル（Ａｖｉｃｅｌ、登録商標））などのセル

ロース誘導体；微結晶性デキストロース；アミロース；ケイ酸アルミニウムマグネシウム；多糖酸；ベントナイト；ゼラチン；ポリビニルピロリドン／ビニル酢酸共重合体；クロスポビドン；ポビドン；デンプン；アルファ化デンプン；トラガント；デキストリン；スクロース（例えば、Ｄｉｐａｃ（登録商標））、グルコース、デキストロース、モラセス、マンニトール、ソルビトール、キシリトール（例えば、キシリタブ（Ｘｙｌｉｔａｂ、登録商標））及びラクトースなどの糖類；アカシア、トラガント、ガティゴム、イサポール皮の粘液、ポリビニルピロリドン（例えば、ポリビドン（登録商標）、ＣＬ，Ｋｏｌｌｉｄｏｎ（登録商標）、ＣＬ，Ｐｏｌｙｐｌａｓｄｏｎｅ（登録商標）、ＸＬ－１０）、カラマツ（ｌａｒｃｈ　ａｒａｂｏｇａｌａｃｔａｎ）、ビーゴム（Ｖｅｅｇｕｍ（登録商標）などの天然又は合成ゴム；ポリエチレングリコール、ワックス類、アルギン酸ナトリウム、などが挙げられる。

【0041】

本明細書において使用される用語「担体」とは、化合物の細胞内又は組織内への取り込みを促進する比較的無毒性の化合物質又は化学剤のことをいう。

【0042】

「担体物質」には、一般的に医薬品に使用されるあらゆる賦形剤が含まれ、本明細書に開示される化合物との適合性及び目的の剤形の放出プロファイル特性に基づいて選ぶべきである。一般的な担体物質としては、例えば、結合剤、懸濁剤、崩壊剤、充填剤、界面活性剤、可溶化剤、安定化剤、潤滑剤、湿潤剤、希釈剤などが挙げられる。「医薬的に適合できる担体物質」としては、これに限定されないが、アカシア、ゼラチン、コロイド状二酸化ケイ素、グリセロリン酸カルシウム、乳酸カルシウム、マルトデキストリン、グリセリン、ケイ酸マグネシウム、ポリビニルピロリドン（ＰＶＰ）、コレステロール、コレステロールエステル、カゼイン酸ナトリウム、大豆レシチン、タウロコール酸、ホスファチジルコリン、塩化ナトリウム、リン酸三カルシウム、リン酸二カリウム、セルロース、セルロース複合体、糖類、ステアロイル乳酸ナトリウム、カラギーナン、モノグリセリド、ジグリセリド、アルファ化デンプンなどが挙げられる。例えば、Ｒｅｍｉｎｇｔｏｎ：Ｔｈｅ　Ｓｃｉｅｎｃｅ　ａｎｄ　Ｐｒａｃｔｉｃｅ　ｏｆ　Ｐｈａｒｍａｃｙ，Ｎｉｎｅｔｅｅｎｔｈ　Ｅｄ（Ｅａｓｔｏｎ，Ｐａ．：Ｍａｃｋ　Ｐｕｂｌｉｓｈｉｎｇ　Ｃｏｍｐａｎｙ，１９９５）；Ｈｏｏｖｅｒ，Ｊｏｈｎ　Ｅ．，Ｒｅｍｉｎｇｔｏｎ’ｓ　Ｐｈａｒｍａｃｅｕｔｉｃａｌ　Ｓｃｉｅｎｃｅｓ，Ｍａｃｋ　Ｐｕｂｌｉｓｈｉｎｇ　Ｃｏ．，Ｅａｓｔｏｎ，Ｐｅｎｎｓｙｌｖａｎｉａ　１９７５；Ｌｉｂｅｒｍａｎ，Ｈ．Ａ．ａｎｄ　Ｌａｃｈｍａｎ，Ｌ．，Ｅｄｓ．，Ｐｈａｒｍａｃｅｕｔｉｃａｌ　Ｄｏｓａｇｅ　Ｆｏｒｍｓ，Ｍａｒｃｅｌ　Ｄｅｃｋｅｒ，Ｎｅｗ　Ｙｏｒｋ，Ｎ．Ｙ．，１９８０；Ｐｈａｒｍａｃｅｕｔｉｃａｌ　Ｄｏｓａｇｅ　Ｆｏｒｍｓ　ａｎｄ　Ｄｒｕｇ　Ｄｅｌｉｖｅｒｙ　Ｓｙｓｔｅｍｓ，Ｓｅｖｅｎｔｈ　Ｅｄ．（Ｌｉｐｐｉｎｃｏｔｔ　Ｗｉｌｌｉａｍｓ　＆　Ｗｉｌｋｉｎｓ　１９９９）参照。

【0043】

本明細書において使用される用語「同時投与」などは、選択した複数の治療剤を一人の患者に投与することを含み、複数の治療剤を同じ又は異なる投与経路によって、あるいは同時に又は異なる時間に投与する治療レジメンを含む。

【0044】

本明細書において使用される用語「遅延放出」とは、放出が、ある一般的に予測可能な場所であって、遅延放出の変化がなかったならば達成したであろう場所よりも遠位である場所において達成できるような配送のことをいう。

【0045】

「分散剤」及び／又は「粘度調節剤」は、液状媒体又は粒状化方法もしくはブレンド方法によって薬剤の拡散と均一性をコントロールする物質を含む。ある態様では、これらの薬剤は基質をコーティング又は浸食する有効性も促進する。一般的な拡散促進剤／分散剤としては、例えば、親水性ポリマー、電解質、Ｔｗｅｅｎ（登録商標）６０又は８０、ＰＥＧ、ポリビニルピロリドン（ＰＶＰ；プラスドン（登録商標）が市販品として知られて

いる）、及び、例えば、ヒドロキシプロピルセルロース（例えば、ＨＰＣ、ＨＰＣ－ＳＬ及びＨＰＣ－Ｌ）、ヒドロキシプロピルメチルセルロース（例えば、ＨＰＭＣ　Ｋ１００、ＨＰＭＣ　Ｋ４Ｍ、ＨＰＭＣ　Ｋ１５Ｍ及びＨＰＭＣ　Ｋ１００Ｍ）、カルボキシメチルセルロースナトリウム、メチルセルロース、ヒドロキシエチルセルロース、ヒドロキシプロピルセルロース、ヒドロキシプロピルメチルセルロースフタレート、ヒドロキシプロピルメチルセルロースアセテートステアレート（ＨＰＭＣＡＳ）、非結晶セルロース、ケイ酸アルミニウムマグネシウム、トリエタノールアミン、ポリビニルアルコール（ＰＶＡ）、ビニルピロリドン／ビニル酢酸共重合体（Ｓ６３０）、エチレンオキシド付加４－（１，１，３，３－テトラメチルブチル）－フェノールポリマー、ホルムアルデヒド（チロキサポールとしても知られる）、ポロキサマー（例えば、プルロニックＦ６８（登録商標）、Ｆ８８（登録商標）及びＦ１０８（登録商標）があり、これらはエチレンオキサイドとプロピレンオキサイドとのブロックコポリマー）などの炭水化物型分散剤；ポロキサミン（例えば、テトロニック９０８（登録商標）、ポロキサミン９０８（登録商標）としても知られている。これはプロピレンオキサイド及びエチレンオキサイドをエチレンジアミン（ＢＡＳＦ社、Ｐａｒｓｉｐｐａｎｙ、ニュージャージー州）に順次的に付加することによって得られる四官能性ブロックコポリマーである）、ポリビニルピロリドンＫ１２、ポリビニルピロリドンＫ１７、ポリビニルピロリドンＫ２５、又はポリビニルピロリドンＫ３０、ポリビニルピロリドン／ビニル酢酸共重合体（Ｓ－６３０）、ポリエチレングリコール、（例えば、このポリエチレングリコールは、分子量が約３００～約６０００でもよく、約３３５０～約４０００でもよく、あるいは約７０００～約５４００でもよい）、カルボキシメチルセルロースナトリウム、メチルセルロース、ポリソルベート－８０、アルギン酸ナトリウム、ゴム（例えば、トラガカントゴム、アカシアゴム、グアーゴム、キサンタン（キサンタンゴムを含む）など）、糖類、セルロース誘導体（例えば、カルボキシメチルセルロースナトリウム、メチルセルロース、カルボキシメチルセルロースナトリウムなど）、ポリソルベート－８０、アルギン酸ナトリウム、ポリエトキシル化ソルビタンモノラウレート、ポリエトキシル化ソルビタンモノラウレート、ポビドン、カルボマー、ポリビニルアルコール（ＰＶＡ）、アルギン酸塩、キトサン、及びこれらの組み合わせが挙げられる。セルロースやトリエチルセルロースなどの可塑剤も分散剤として使用できる。分散剤は、特にリポソーム型分散液に有用であり、また自己乳化型分散液としては、ジミリストイルホスファチジルコリン、卵由来天然ホスファチジルコリン、卵由来天然ホスファチジルグリセロール、コレステロール、イソプロピルミリステートが挙げられる。

【0046】

用語「希釈剤」とは、目的の化合物を、配送に先だって希釈するために使用される化学物質のことをいう。また、希釈剤はより安定した環境を提供することができるので、化合物を安定化するために使用することもできる。当技術分野では、緩衝化した溶液（これはｐＨコントロール又は維持も提供することができる）に溶解した塩が希釈剤として利用され、これに限定されないが、リン酸緩衝食塩水が挙げられる。ある態様では、圧縮を容易にするために、又はカプセル充填において均一にブレンドするのに十分な容量を付与するために、希釈剤を用いて化合物の容量を増加させる。このような化合物としは、例えば、ラクトース、デンプン、マンニトール、ソルビトール、デキストロース、アビセル（Ａｖｉｃｅｌ、登録商標）などの微結晶性セルロース、第二リン酸カルシウム、第二リン酸カルシウム二水和物、リン酸三カルシウム、リン酸カルシウム；無水ラクトース、噴霧乾燥ラクトース；アルファ化デンプン、Ｄｉ－ｐａｃ（（登録商標）、Ａｍｓｔａｒ）などの圧縮性糖、マンニトール、ヒドロキシプロピルメチルセルロース、ヒドロキシプロピルメチルセルロースアセテートステアレート、スクロース型希釈剤、粉砂糖、第一硫酸カルシウム一水和物、硫酸カルシウム二水和物、乳酸カルシウム三水和物、デキストレート（ｄｅｘｔｒａｔｅ）、固形加水分解シリアル、アミロース、粉末セルロース、炭酸カルシウム、グリシン、カオリン、マンニトール、塩化ナトリウム、イノシトール、ベントナイトなどが挙げられる。

【0047】

「腸溶コーティング」とは、胃中では実質的に元の状態のままであり、小腸又は結腸の中で薬剤が溶解し放出される物質のことをいう。一般的には、腸溶コーティングは低いｐＨ環境である胃中での放出を抑制し、それより高いｐＨ、一般的にはｐＨ６～７ではイオン化し、その結果、小腸又は結腸中において十分に溶解し、そこで活性薬剤を放出するポリマー物質からなる。

【0048】

「充填剤」としては、ラクトース、炭酸カルシウム、リン酸カルシウム、第二リン酸カルシウム、硫酸カルシウム、微結晶性セルロース、粉末セルロース、デキストロース、デキストレート、デキストラン、デンプン、アルファ化デンプン、スクロース、キシリトール、ラクチトール、マンニトール、ソルビトール、塩化ナトリウム、ポリエチレングリコールなどの化合物が挙げられる。

【0049】

本明細書において使用される用語「有効量」又は「治療的有効量」とは、治療される疾患又は異常の一つ以上の症状をある程度軽減する、投与される薬剤又は化合物の十分な量のことをいう。その効果は、疾患の徴候、症状又は原因の軽減及び／又は緩和、あるいは生物学的システムにおける他の所望の変更でもよい。例えば、治療のために使用する「有効量」とは、過度の副作用を引き起こすことなく疾患の症状について臨床的意義のある減少をもたらすのに必要な、本明細書に開示される化合物を含む組成物の量のことをいう。任意の個々の場合における適切な「有効量」は、用量漸増試験などの技術を用いて決定することができる。用語「治療的有効量」には、例えば予防的有効量が含まれる。本明細書に開示される化合物の「有効量」は、過度の副作用を引き起こすことなく所望の薬理学的効果又は治療的改善を達成するのに有効な量である。なお、当然のことながら「有効量」又は「治療的有効量」は、被験者の該組成物の代謝におけるばらつき、年齢、体重、全身状態、治療される症状、治療される症状の重症度、及び処方医師の判断により被験者ごとに異なりうる。

【0050】

本明細書において使用される用語「増強する」又は「増強」とは、所望の効果の作用強度又は作用時間を増加させる、もしくは引き伸ばすことを意味する。従って、治療剤の効果の増強についての、用語「増強」とは、他の治療剤のシステムに対する効果の作用強度又は作用時間を増加させる、もしくは引き伸ばす能力のことをいう。本明細書において使用される「増強有効量」とは、目的とするシステムにおける他の治療剤の効果を増強するための適切な量のことをいう。

【0051】

用語「キット」及び「製造品」は同義語として用いられる。

【0052】

本明細書において使用される用語「調節する」とは、目的物の活性を変えるように、直接的又は間接的に目的物と相互に作用することを意味する。例えば、例示のためだけであるが、目的物の活性を増強すること、目的物の活性を阻害すること、目的物の活性を制限すること、目的物の活性を延長することが挙げられる。

【0053】

本明細書において使用される用語「調節物質」とは、分子の活性を変える化合物のことをいう。例えば、調節物質は、分子のある活性の大きさを、この調節物質がない場合の活性の大きさと比べて、増加させたり減少させたりすることができる。ある態様では、調節物質は分子の一つ以上の活性の大きさを減少させる阻害物質である。ある態様では、阻害物質が分子の一つ以上の活性を完全に阻止する。ある態様では、調節物質は分子の少なくとも一つの活性の大きさを増加させる活性化物質である。ある態様では、調節物質が存在することにより、その調節物質が存在しない場合には生じなかった活性が生じる。

【0054】

用語「非水溶性希釈剤」とは、一般的に医薬品の処方において使用される化合物を意味し、例えば、リン酸カルシウム、硫酸カルシウム、デンプン、変性デンプン及び微結晶性

セルロース、マイクロセルロース（例えば、約０．４５ｇ／ｃｍ^{3}の密度を有するものあり、例えば、アビセル（Ａｖｉｃｅｌ）、粉末セルロースが挙げられる）、タルクが挙げられる。

【0055】

本明細書において使用される「薬学的に許容される」という用語によって、該化合物の生物学的活性又は性質を抑制しない、かつ比較的無毒性、すなわち望ましくない生物学的作用を引き起こさない、又はその物質が含まれている組成物の成分と悪影響を及ぼすように相互作用することなく、その物質を個人に投与することができる担体又は希釈剤のような物質のことをいう。

【0056】

本明細書において使用される用語「医薬的組み合わせ」とは、一つを超える有効成分を混合又は組み合わせることにより製造される製品のことを意味し、複数の有効成分の固定の組み合わせと非固定の組み合わせの両方を含む。用語「固定の組み合わせ」とは、複数の有効成分、例えば本明細書に記載のＡＭ調製物と精製組成物、及び併用剤を、単一の実体あるいは製剤として両者を同時に患者に投与することを意味する。用語「非固定の組み合わせ」とは、複数の有効成分、例えば本明細書に記載のＡＭ調製物と精製組成物、及び併用剤を別々の実体として同時に、一斉に、あるいは順次的に、特定の時間間隔限度を設けずに、患者に投与することを意味し、このような投与により患者の体内における二つの化合物の有効濃度が提供される。また、後者の投与は、カクテル治療（例えば、三つ以上の有効成分の投与）にも適用される。

【0057】

「可塑剤」とは、マイクロカプセル封入物質又はフィルムコートを柔らかくして壊れににくくするために使用される化合物のことをいう。適切な可塑剤としては、例えば、ＰＥＧ３００、ＰＥＧ４００、ＰＥＧ６００、ＰＥＧ１４５０、ＰＥＧ３３５０及びＰＥＧ８００などのポリエチレングリコール、ステアリン酸、プロピレングリコール、オレイン酸、トリエチルセルロース、トリアセチンが挙げられる。ある態様では、可塑剤は分散剤や湿潤剤としても機能することができる。

【0058】

本明細書において使用される用語「ポリペプチド」又は「タンパク質」とは、完全長のポリペプチド、又はポリペプチドのフラグメント又はセグメントのことを意味し、また完全長のポリペプチドのうち少なくとも約８個のアミノ酸残基、一般的には少なくとも１０個のアミノ酸残基、より一般的には少なくとも２０個のアミノ酸残基、頻繁には少なくとも３０個のアミノ酸残基、より頻繁には少なくとも５０個のアミノ酸残基あるいはそれ以上のアミノ酸残基からなる配列を包含する。

【0059】

「可溶化剤」としては、トリアセチン、クエン酸トリエチル、オレイン酸エチル、カプリル酸エチル、ラウリル硫酸ナトリウム、ドキュセートナトリウム、ビタミンＥ　ＴＰＧＳ、ジメチルアセトアミド、Ｎ－メチルピロリドン、Ｎ－ヒドロキシエチルピロリドン、ポリビニルピロリドン、ヒドロキシプロピルメチルセルロース、ヒドロキシプロピルシクロデキストリン、エタノール、ｎ－ブタノール、イソプロピルアルコール、コレステロール、胆汁酸塩、ポリエチレングリコール２００～６００、グリコフロール、トランスクトール、プロピレングリコール、ジメチルイソソルバイドなどの化合物が挙げられる。

【0060】

「安定化剤」としては、抗酸化剤、緩衝剤、酸、防腐剤などの化合物が挙げられる。

【0061】

生物学的物質、羊膜の文脈、及び／又はタンパク質の文脈において使用される「実質的に純粋な」又は「精製した」とは、一般的に、混入している他のタンパク質、核酸、起源生物由来の他の生物学的物質からその物質を単離することを意味する。純度又は「単離」は、常法により測定することができ、通常は少なくとも約１０％純粋であり、より通常には少なくとも約２０％純粋であり、一般的には少なくとも約３０％純粋であり、より一般

的には少なくとも約４０％純粋であり；さらに別の態様では、少なくとも約５０％純粋であり、より頻繁には少なくとも約６０％純粋であり；さらに別の態様では、少なくとも約９５％純粋である。

【0062】

「懸濁剤」としては、ポリビニルピロリドン（例えば、ポリビニルピロリドンＫ１２、ポリビニルピロリドンＫ１７、ポリビニルピロリドンＫ２５、ポリビニルピロリドンＫ３０など）、ビニルピロリドン／ビニル酢酸共重合体（Ｓ６３０）、ポリエチレングリコール、（例えば、このポリエチレングリコールは、分子量が約３００～約６０００でもよく、約３３５０～約４０００でもよく、あるいは約７０００～約５４００でもよい）、カルボキシメチルセルロースナトリウム、メチルセルロース、ヒドロキシプロピルメチルセルロース、ヒドロキシメチルセルロースアセテートステアレート、ポリソルベート－８０、ヒドロキシエチルセルロース、アルギン酸ナトリウム、ゴム（例えば、トラガカントゴム、アカシアゴム、グアーゴム、キサンタン（キサンタンゴムを含む）など）、糖類、セルロース誘導体（例えば、カルボキシメチルセルロースナトリウム、メチルセルロース、カルボキシメチルセルロースナトリウム、ヒドロキシプロピルメチルセルロース、ヒドロキシエチルセルロースなど）、ポリソルベート－８０、アルギン酸ナトリウム、ポリエトキシル化ソルビタンモノラウレート、ポリエトキシル化ソルビタンモノラウレート、ポビドンなどの化合物を挙げることができる。

【0063】

「界面活性剤」としては、ラウリル硫酸ナトリウム、ドキュセートナトリウム、Ｔｗｅｅｎ６０又は８０、トリアセチン、ビタミンＥ　ＴＰＧＳ、ソルビタンモノオレエート、ポリオキシエチレンソルビタンモノオレエート、ポリソルベート、ポロクサマー、胆汁酸塩、モノステアリン酸グリセリン、エチレンオキサイドとプロピレンオキサイドとの共重合体（例えば、プルロニック（登録商標、ＢＡＳＦ））などの化合物が挙げられる。その他の界面活性剤としては、ポリオキシエチレン脂肪酸グリセリド及び植物油（例えば、ポリオキシエチレン（６０）硬化ヒマシ油）；ポリオキシエチレンアルキルエーテル及びアルキルフェニルエーテル（例えば、オクトキシノール１０、オクトキシノール４０）が挙げられる。ある態様では、物理的安定性を増強するために、あるいは他の目的で界面活性剤を含むことができる。

【0064】

本明細書において使用される用語「被験者」とは、動物、好ましくはヒトを含む哺乳動物を意味する。患者及び被験者という用語は、同じ意味で使うことができる。

【0065】

本明細書において使用される用語「治療する」、「治療すること」又は「治療」には、疾患又は異常の症状の緩和、寛解又は回復、随伴症状の予防、潜在的な代謝性原因による症状の寛解又は予防、疾患又は異常の抑制（例えば、疾患又は異常の発症の阻止、疾患又は異常の緩和、疾患又は異常の軽減の惹起、疾患又は異常により引き起こされる異常の緩和、あるいは疾患又は異常の症状の予防的及び／又は治療的阻止）が含まれる。

【0066】

「湿潤剤」としては、オレイン酸、モノステアリン酸グリセリン、ソルビタンモノオレエート、ソルビタンモノラウレート、オレイン酸トリエタノールアミン、ポリオキシエチレンソルビタンモノオレエート、ポリオキシエチレンソルビタンモノラウレート、ドキュセートナトリウム、オレイン酸ナトリウム、ラウリル硫酸ナトリウム、ドキュセートナトリウム、トリアセチン、Ｔｗｅｅｎ８０、ビタミンＥ　ＴＰＧＳ、アンモニウム塩などの化合物が挙げられる。

【0067】

本明細書において記載されるすべての出版物や特許出願は、それぞれ個々の出版物又は特許出願が引用により組込まれていることを特異的且つ個々に示されているかのように、引用により本明細書に組み入れられるものとする。

【0068】

本発明の新規の特徴は、特に添付の特許請求の範囲において説明される。本発明の特徴及び効果は、以下に記載の、本発明の原理を利用する実施の形態を説明する詳細な記述及び添付図面を参照することによりさらに理解されるであろう。

【0069】

組成物

本明細書に記載されているのは、哺乳動物細胞及び無処理の哺乳動物組織において、多くの生理学的に有意な効果を発現する精製組成物である。該精製組成物は、少なくとも以下の４つの構成成分を含む。

・架橋した高分子ヒアルロン酸（ＨＡ）；

・腫瘍壊死因子活性化遺伝子６（ＴＳＧ－６）；

・ペントラキシン（ＰＴＸ－３）；

・トロンボスポンジン（ＴＳＰ－１）

【0070】

これらの４つの構成成分を有する精製組成物には、さらに追加の構成成分を含むことができ、例えばＳｍａｄ７が挙げられる。

【0071】

本明細書に記載の該精製組成物における任意の又はすべての構成成分は、（本明細書に記載されているような）ヒト羊膜ゼリー調製物と抽出物、（本明細書に記載されているような）ヒト羊膜調製物と抽出物、（本明細書に記載されているような）ヒト羊膜間質調製物と抽出物を含むヒト羊膜材料から調製することができる。

【0072】

まとめると、これらの４つの構成成分（Ｓｍａｄ７の有無にかかわらず）は、ＴＧＦ－βプロモーター活性を抑制することができる。例えば、マクロファージのアポトーシスの増加；ヒト血管内皮細胞の増殖の減少、遊走の減少、アポトーシスの増加；ヒト線維芽細胞の生存率の減少、炎症の抑制；保存や傷害にさらされる上皮細胞のアポトーシスの抑制が挙げられる。

【0073】

ヒアルロン酸（ＨＡ）は天然の糖であり、滑膜・関節液、眼の硝子体液、軟骨、血管、細胞外基質、皮膚及び臍帯中に含まれている。ＨＡの架橋は、タンパク質のような他の分子と共有結合することにより達成することができる。例えば、ＨＡは、インター－α－トリプシン阻害剤の重鎖に共有結合することができる。本明細書に記載のＡＭ調製物と精製組成物中のＨＡに対するタンパク質の割合は、約２００：１未満、約１００：１未満、約５０：１未満、あるいは約１０：１未満でありうる。

【0074】

ＴＳＧ－６は、細胞外基質再構築、細胞増殖、白血球遊走に関与するヒアルロン酸結合タンパク質である。ＴＳＧ－６は、セリンプロテアーゼ阻害剤、インター－α－阻害剤と共に複合体を形成することができる。ＰＴＸ－３（ペントラキシン）は５量体の円盤状構造を持ち、血漿中に存在するCa^{2+}依存性リガンド結合タンパク質である。ＴＳＰ－１（トロンボスポンジンＩ）は、強力な血管新生阻害活性を有しかつ他の生物学的活性を有するホモ三量体の糖タンパク質である。ＴＳＰ－１は、各種細胞により細胞外基質に分泌される。

【0075】

これらの構成成分は、任意の適切なソースから得ることができる。例えば、その構成成分のうち少なくとも一つは、ヒトの組織、例えば羊膜、羊膜ゼリー、羊膜間質、又はこれらの組み合わせなどから得ることができる。その構成成分のうち少なくとも一つは、市販品から得ることができる。それらの構成成分のうち少なくとも一つは、形質転換体から単離することができる。そのタンパク質の配列は、ヒトのタンパク質の配列と少なくとも９０％、９３％、９５％、９７％、９９％又は９９．５％の類似性を有する。この構成成分は、精製、実質的に精製、又は部分的に精製されていてもよく、あるいは未精製抽出物中に存在していてもよい。また、この４つの構成成分は、それぞれ羊膜組織中に存在するの

で、哺乳動物の羊膜組織から調製してもよい。
【0076】
他の態様では、タンパク質Ｓｍａｄ７も該組成物に含まれている。Ｓｍａｄ７は、任意の適切なソース、例えば、羊膜、市販品、形質転換体からの単離物質から得ることができる。このＳｍａｄ７タンパク質は、精製、実質的に精製、又は部分的に精製されていてもよく、あるいは、未精製抽出物中に存在していてもよい。
【0077】
羊膜由来ＡＭ調製物
ある態様では、ＨＡ、ＴＳＧ－６、ＰＴＸ－３、ＴＳＰ－１、必要ならばＳｍａｄ７、の構成成分のうち少なくとも一つは羊膜の調製物から得ることができる。あるいは、ＨＡ、ＴＳＧ－６、ＰＴＸ－３、ＴＳＰ－１、必要ならばＳｍａｄ７、の組み合わせを含む、未精製の羊膜調製物を調製することができる。各種ＡＭ調製物の典型的な調製方法は本明細書に記載されている。
【0078】
ヒト胎盤材料は、例えば、Ｂｉｏ－Ｔｉｓｓｕｅ　Ｉｎｃ．（マイアミ、フロリダ州）及びＢａｐｔｉｓｔ　Ｈｏｓｐｉｔａｌ（マイアミ、フロリダ州）（ＩＲＢ承認を受けて）のようなソースから得ることができる。組織は一般的に生の状態か凍結の状態で得られる。組織は、余分な保存緩衝液、血液、あるいは混入物質を除去するために洗浄する。余分な液体は、例えば、短時間の遠心分離ステップ、あるいは他の方法により除去する。組織は、次のホモジナイズ処理を容易にするために、例えば、液体窒素や他の冷却方法により凍結してもよい。ＡＭ組織のソースはヒトとすることができる。しかし、他のソースのＡＭ組織、例えば、ウシ又はブタのＡＭ組織なども使用することができる。
【0079】
該組成物を調製するためにＡＭを使用することができる。ＡＭ調製物は、無処理のＡＭ、ＡＭ間質基質、ＨＡ、ＡＭゼリーから精製又は抽出した成分又は一部、インター－α－トリプシン阻害剤（ＨＡ－ＩＴＩ）を含んでもよい。必要ならば、ＡＭ調製物のある成分をステップ中任意の時間にその調製物から単離してもよい。例えば、特定のタンパク質あるいはＡＭタンパク質のセットを多く含む抽出物を該調製物から単離してもよい。組織のホモジナイズ処理後、大きい粒子を分離して除いてもよいし、調製物中に残したままにしてもよい。この調製物は必要ならば乾燥してもよい。典型的な調製方法は実施例１に記載される。
【0080】
該組成物はＡＭゼリーからも得ることができる。ＡＭゼリーは生のＡＭ組織から得ることができ、また凍結ステップ前又は後に得ることもできる。ＡＭゼリーは凍結することができ、本明細書に記載されているように、ＡＭ調製物の調製手順に従って凍結粉砕することもできる。このゼリーは遠心分離してもよいし、凍結乾燥してもよい。
【0081】
他の態様では、実質的に間質層から製造した組成物を調製する。この組成物を調製するために、間質層を、生、凍結、解凍、あるいは他の方法で処理されたＡＭ膜から分離する。間質は、例えば、酵素的方法、機械的方法、あるいは他の方法によって取り出すことができる。間質層材料は、生の状態か凍結状態でありうる。間質材料は、本明細書に記載されているようなＡＭ調製物の調製手順に従って粉砕又は凍結粉砕することができる。必要ならば、間質基質材料は遠心分離してもよいし、凍結乾燥してもよい。
【0082】
組織は粉砕ステップの前に凍結してもよい。凍結ステップは任意の適切な冷却方法を用いて行うことができる。例えば、液体窒素を用いて組織を瞬間凍結することができる。あるいは、その材料をイソプロパノール／ドライアイス浴に入れることもできるし、他の冷却材を用いて瞬間凍結することもできる。市販の迅速凍結方法を用いることができる。さらに、その材料を冷凍庫に入れ、瞬間凍結ではなく、よりゆっくりと保存温度と平衡になるようにすることもできる。組織は任意の所望の温度で保存することができる。例えば、

－２０℃又は－８０℃又は他の温度を保存温度として使用できる。

【0083】

凍結する前に組織を粉砕するよりも、凍結したまま組織を微粉砕することが、組織の調製方法における一つの選択肢である。あるいは、生の組織、一部解凍した組織、又は解凍した組織を用いて粉砕ステップをすることができる。次に組織（生、凍結、又は解凍の状態）は、適切な用具、例えば外科用メスなどを用いて、所望の大きさの小片にスライスし、次いで、バイオパルベライザー（ＢｉｏＰｕｌｖｅｒｉｚｅｒ）（Ｂｉｏｓｐｅｃ　Ｐｒｏｄｕｃｔｓ，Ｉｎｃ．，Ｂａｒｔｌｅｓｖｉｌｌｅ，オクラホマ州）又は他の適切な装置を用いて微粒子になるように粉砕し、次いで、ホモジナイザー、例えばＴｉｓｓｕｅ－Ｔｅａｒｏｒ（ＢｉｏＳｐｅｃ　Ｐｒｏｄｕｃｔｓ，Ｉｎｃ．，Ｄｒｅｍｅｌ，ウィスコンシン州）などを用いて適切な溶液の中でホモジナイズする。代表的な溶液としては、リン酸緩衝食塩水（ＰＢＳ）、ＤＭＥＭ、ＮａＣｌ溶液及び水が挙げられるが、これに限定されない。溶液のｐＨは必要に応じて調整する。ある態様では、ｐＨ範囲は約５．５又は６．０～約８．５である。ある態様では、凍結組織をｐＨが約６．３、約６．６又は約７．０～約７．４、約７．６又は約７．８である溶液の中で粉砕する。

【0084】

任意の適切な緩衝液又は液体を用いて製剤を調製することができる。実施例２では、調製物中の全タンパク質含有量及びＨＡに対して、各種抽出緩衝液（高塩濃度、低塩濃度、ＰＢＳなど）の使用について試験を行っている（表Ｘ）。実施例２では、いくつかの抽出方法を用いて、特定のタンパク質、ＴＳＧ－６（図１４）、ＰＴＸ－３（図１８）、ＴＳＰ－１（図１９）、Ｓｍａｄ７（図２０）の濃度について、さらに試験を行っている。

【0085】

その後、ホモジネートを任意の適切な速度、温度、又は他のパラメータにて混合することができる。混合は、例えば、約１℃又は３℃～約６℃、１０℃、１５℃又は２０℃の温度範囲で行うことができる。ある態様では、混合を約４℃で行う。ホモジネートの混合は、例えば、約１分未満、１０分、又は２０分～約１、２、３時間、あるいはそれ以上行うことができる。

【0086】

その後必要ならば、ホモジネートは遠心分離をして、任意の残存する大きい粒子を取り除くことができる。遠心分離は、必要に応じて、適切な時間、温度、タンパク質濃度、緩衝液、速度範囲を用いて行うことができる。遠心分離は、例えば、約１，０００、５，０００又は１０，０００ｘｇ～約２０，０００ｘｇの範囲で行うことができる。ある態様では、遠心分離を約１５，０００ｘｇにて行う。遠心分離は、１分未満、５分、１０分、又は２０分～約４０分、６０分、１．５時間、あるいはそれ以上の持続時間にて行うことができる。その後、上清を回収し、アリコットに分割して－８０℃で保管することができる。必要ならば、全タンパク質は、例えばＢＣＡアッセイ（Ｐｉｅｒｃｅ，Ｒｏｃｋｆｏｒｄ，イリノイ州）などの任意の適切な市販のタンパク質分析キットを用いて定量することができる。実施例２、表Ｘ、図１３は、低速又は高速遠心分離後のＡＭ調製物の分析について記述する。

【0087】

生化学的特徴づけと精製のために、上記の溶液には、プロテアーゼ阻害剤を添加することができる。プロテアーゼ阻害剤の典型的な配合物を以下に示す：１μｇ／ｍｌのアプロチニン、１μｇ／ｍｌのロイペプチン、１μｇ／ｍｌのペプスタチンＡ、１ｍＭのＰＭＳＦ。しかし、一般的には、調製物を生細胞や生組織に添加する場合は、プロテアーゼ阻害剤は調製物に添加しない。

【0088】

特定の成分又はタンパク質の存在を確認するために、製剤を試験することができる。例えば、これに限定されないが、ＨＡ、ＴＳＧ－６、ＰＴＸ－３、ＴＳＰ－１、Ｓｍａｄ７などの分子の存在について、製剤を試験することができる。また、調製ステップ中の任意の時点で病原体が存在しないことを確認するために、製剤を試験することもできる。

【0089】
ＡＭ調製物は液体、懸濁液、凍結乾燥粉末（例えば、粉砕又は微粉砕状態）又は他の形状でありうる。例えば抗生物質や抗真菌剤のような抗菌剤を添加してもよい。組成物を安定化及び／又は保存するために、その組成物に他の物質を添加してもよい。該組成物は包装し、使用前に、例えば室温で保管してもよく、あるいは、例えば－２０℃や－８０℃で保管してもよい。
【0090】
ある態様では、調製物は乾燥粉末製剤として存在する。乾燥粉末製剤は、より小さい容量で保管することができ、また、時間経過による分解から製剤を守るための同程度の低温度保管条件は必要でない。乾燥粉末製剤は、保管し、使用前に再構成すればよい。乾燥粉末製剤は、例えば、本明細書に記載されているような凍結粉砕ＡＭ組織を調製し、次いで組成物中の少なくとも水分を除去することにより調製することができる。過剰水は、任意の適切な方法により調製物から除去することができる。典型的な水の除去方法としては、市販の凍結乾燥機やフリーズドライヤーを用いた凍結乾燥の利用が挙げられる。適切な装置としては、例えば、Ｖｉｒｔｉｓ，Ｇａｒｄｉｎｅｒ，ニューヨーク州；ＦＴＳシステム，Ｓｔｏｎｅ　Ｒｉｄｇｅ，ニューヨーク州；Ｓｐｅｅｄ　Ｖａｃ（Ｓａｖａｎｔ　Ｉｎｓｔｒｕｍｅｎｔｓ　Ｉｎｃ．，Ｆａｒｍｉｎｇｄａｌｅ，ニューヨーク州）が挙げられる。除去される水分量は、約５％、１０％、２０％又は３０％～約６０、７０、８０、９０、９５又は９９％、あるいはそれ以上である。ある態様では、実質的にすべての過剰水を除去する。その後、凍結乾燥した粉末を保管する。保管温度は、約－１９６℃未満、－８０℃、－５０℃又は－２０℃～約２３℃を超える温度、と様々である。必要ならば、粉末を保管する前に特徴付けてもよい（重量、タンパク質含有量など）。
【0091】
凍結乾燥した粉末は、使用前に適切な溶液又は緩衝液を用いて再構成することができる。典型的な溶液としては、これに限定されないが、ＰＢＳ、ＤＭＥＭとＢＳＳが挙げられる。溶液のｐＨは、必要に応じて調整することができる。ＡＭの濃度は、必要に応じて変えることができる。ある手順では、より濃縮した調製物が有用であり、一方他の手順では、ＡＭ濃度が低い溶液が有用である。さらに追加の化合物を該組成物に添加することができる。再構成した製剤に添加することができる典型的な化合物としては、これに限定されないが、ｐＨ調整剤、緩衝液、コラーゲン、ＨＡ、抗生物質、界面活性剤、安定化剤、タンパク質などが挙げられる。また、この凍結乾燥したＡＭ粉末は、所望の濃度になるように、調製されたクリーム、軟膏又はローションに添加することができる。
【0092】
要求に応じて、該組成物にさらに追加の化合物を添加することができる。ある態様では、水溶性又は粉末化ＡＭ調製物をコラーゲン、フィブリン又はＨＡのようなＥＣＭ成分と混合することができる。
【0093】
コラーゲンは体内で見つかる主要な構造タンパク質である。コラーゲンは組織を支え、組織と骨をつなげ、かつ人体の構造を提供する。人体が治癒プロセッシング中の場合は、コラーゲンが、細胞構造を構築する手助けをする役割を果たす。ヒアルロン酸は、天然の糖であり、滑膜・関節液、眼の硝子体液、軟骨、血管、細胞外基質、皮膚及び臍帯中に含まれている。フィブリンは血液凝固に関係するタンパク質である。
【0094】
水溶性ＡＭ調製物は、コラーゲン、フィブリン又はＨＡと混合することができる。同様に、凍結乾燥したＡＭ調製物も、コラーゲン、フィブリン又はＨＡと混合することができる。コラーゲン、フィブリン、ＨＡは、コラーゲン又はＨＡと混合したＡＭ調製物が、ＴＧＦ－βプロモーター活性を抑制する効果を発現することが示されたので、適切な配送ビヒクルになりうる。ＡＭ調製物は、本明細書に記載の実験において、コラーゲンゲル及びＨＡゲルと混合したが、任意の可溶型（例えば、液体）のコラーゲンとＨＡ、又は他のＥＣＭ成分（例えば、フィブリン）を使用することもできる。コラーゲン、フィブリン又は

ＨＡは、任意の適切な起源生物から入手することができる。コラーゲン、フィブリン又はＨＡが添加される場合、ＡＭに対するこれらの化合物の比は、要求に応じて変えることができる。例えば、コラーゲン（又はフィブリンもしくはＨＡ）に対するＡＭの比は、約０．００１未満：１、０．０１:1、０．０５:1、又は０．１:1～約１：１、１．５：１、２：１、５：１、１０：１、１００：１又は１０００：１、あるいはそれ以上を用いることができる。

【0095】

コラーゲンゲルは、実施例１に記載されているように、例えば、原液（４ｍｇ／ｍｌ）を０．１Ｎの酢酸を用いて希釈し、それを適切な容積比の２０ｘＤＭＥＭ又は適切な緩衝液、及び１ＮのＮＡＯＨと混合することにより調製することができる。調製物中のコラーゲンは、約２ｍｇ／ｍｌ未満～約４ｍｇ／ｍｌを超える範囲で存在することができる。

【0096】

高分子ＨＡの各種希釈物は、例えば、市販のＨＡ（ヒーロン（Ｈｅａｌｏｎ、登録商標）（１０ｍｇＨＡ／ｍｌ）（Ｐｈａｒｍａｃｉａ、Ｌａｊｏｌｌａ、カリフォルニア州））をＤＭＥＭ又は適切な緩衝液を用いて希釈することにより調製することができる。凍結乾燥した粉末及び水溶型ＡＭ調製物は、ＰＢＳ、ＤＭＥＭのような溶液又は他の溶液を用いて目的のコラーゲン濃度に希釈することができる。調製物中のＨＡは、例えば、約２μｇ／ｍｌ未満～約１２９μｇ／ｍｌを超える範囲で存在することができる。

【0097】

以下の手順は、本明細書において記述されかつ使用される羊膜調製物と精製組成物を調製するための説明的な方法を示す。

【0098】

保存ヒトＡＭの調製：

ヒト胎盤は、選択的帝王切開分娩の際に採取した（Ｈｅｉｌｉｇｅｎｈａｕｓら、Ｉｎｖｅｓｔ Ｏｐｈｔｈａｌｍｏｌ．Ｖｉｓ Ｓｃｉ．４２：１９６９－１９７４、２００１、Ｌｅｅ ａｎｄ Ｔｓｅｎｇ、Ａｍ Ｊ Ｏｐｈｔｈａｌｍｏｌ．１２３：３０３－３１２、１９９７、Ｐｒａｂｈａｓａｗａｔら、Ｏｐｈｔｈａｌｍｏｌｏｇｙ １０４：９７４－９８５、１９９７、Ｔｓｅｎｇら、Ａｒｃｈ Ｏｐｈｔｈａｌｍｏｌ．１１６：４３１－４４１、１９９８）。ＡＭは、ニトロセルロース紙（ハイボンド－Ｎ＋、Ａｍｅｒｓｈａｍ、英国）の上に、上皮表面を上にして平らにして乗せた。ＡＭサンプルは、使用するまでＤＭＥＭ／グリセロール（１：２（ｖ／ｖ））の中で－８０℃で保管した。

【0099】

羊膜抽出物の調製

生のヒト胎盤及び凍結ヒト胎盤はＢｉｏ－Ｔｉｓｓｕｅ、Ｂｉｏ－Ｔｉｓｓｕｅ Ｉｎｃ．（マイアミ、フロリダ州）から入手した。全ヒトＡＭ抽出物（ＡＭＥ）の全調製手順は、次の細胞培養ベースの実験で使用できるように無菌的に行った。ＡＭは、バイオパルベライザー（ＢｉｏＰｕｌｖｅｒｉｚｅｒ）（Ｂｉｏｓｐｅｃ Ｐｒｏｄｕｃｔｓ，Ｉｎｃ．，Ｂａｒｔｌｅｓｖｉｌｌｅ，オクラホマ州）の容器に入るように、小片にスライスし、液体窒素で凍結させ、微粉末になるよう粉砕し、秤量した。プロテアーゼ阻害剤（プロテアーゼ阻害剤カクテル、Ｐ８３４０、Ｓｉｇｍａ及び１ｍＭのＰＭＳＦを含む）と、ホスファターゼ阻害剤（５０ｍＭのフッ化ナトリウム及び０．２ｍＭのバナジン酸ナトリウム）とを含む１ｘＰＢＳの冷緩衝液（ｐＨ７．４）を得られた粉末に１：１（ｍｌ／ｇ）の割合で添加した。混合物は氷上に保持し、Ｔｉｓｓｕｅ－Ｔｅａｒｏｒ（ＢｉｏＳｐｅｃ Ｐｒｏｄｕｃｔｓ，Ｉｎｃ．，Ｄｒｅｍｅｌ，ウィスコンシン州）を用いて、５回、各１分間ずつ（各回が終わるたびに２分間冷却する）ホモジナイズした。これら水溶性抽出物は「全」ＡＭ抽出物（ＡＭＥ）と命名した。

【0100】

全ＡＭ抽出物は、二つの５０ｍｌ容の円錐形遠心チューブに分注した。一つは、高速（ＨＳ、４８，０００ｘｇ）で遠心分離し、もう一つは、低速（ＬＳ、１５，０００ｘｇ）で4℃にて遠心分離した。ＨＳ及びＬＳの上清のアリコットを無菌の１．５ｍｌ容のエッ

ペンドルフチューブに移し、それぞれＡＭ／ＨＳ及びＡＭ／ＬＳと命名した。所望のＡＭ／ＨＳサンプルは、凍結乾燥前に－２０℃にて１時間凍結させた。次にそのサンプルは蓋に穴があいているＦｒｅｅＺｏｎｅ（Ｌａｂｃｏｎｃｏ、カンザスシティ、ミズーリ州）のチャンバーに入れた。サンプルは－５０℃、０．８５ｍＢａｒの真空度にて５時間凍結乾燥した。使用前にサンプルは、同じ容量の無菌蒸留水を用いて再構成した。ＡＭ間質に付着した物質から簡単にこすり落としたＡＭゼリーから抽出物を調製する際にも、同じ方法を用いた。

【0101】

全可溶性ヒト羊膜と羊膜ゼリー抽出物の調製

凍結ヒト胎盤材料は、Ｂｉｏ－Ｔｉｓｓｕｅ、Ｂｉｏ－Ｔｉｓｓｕｅ　Ｉｎｃ．（マイアミ、フロリダ州）から入手した。全ヒトＡＭ抽出物（ＡＭＥ）の全調製手順は、次の細胞培養ベースの実験で使用できるように無菌的に行った。ＡＭは、バイオパルベライザー（ＢｉｏＰｕｌｖｅｒｉｚｅｒ）（Ｂｉｏｓｐｅｃ　Ｐｒｏｄｕｃｔｓ，Ｉｎｃ．，Ｂａｒｔｌｅｓｖｉｌｌｅ，オクラホマ州）の容器に入るように、小片にスライスし、液体窒素で凍結させ、微粉末になるよう粉砕し、秤量した。プロテアーゼ阻害剤（プロテアーゼ阻害剤カクテル、Ｐ８３４０、Ｓｉｇｍａ、及び１ｍＭのＰＭＳＦを含む）及びホスファターゼ阻害剤（５０ｍＭのフッ化ナトリウム及び０．２ｍＭのバナジン酸ナトリウム）を含む１ｘＰＢＳの冷緩衝液（ｐＨ７．４）を得られた粉末に１：１（ｍｌ／ｇ）の割合で添加した。混合物は氷上に保持し、Ｔｉｓｓｕｅ－Ｔｅａｒｏｒ（ＢｉｏＳｐｅｃ　Ｐｒｏｄｕｃｔｓ，Ｉｎｃ．，Ｄｒｅｍｅｌ，ウィスコンシン州）を用いて、５回、各１分間ずつ（各回が終わるたびに２分間冷却する）ホモジナイズした。これら水溶性抽出物は「全」ＡＭ抽出物（ＡＭＥ）と命名した。

【0102】

全水溶性抽出物は４℃にて１時間混合し、次に４℃で３０分間、二つの異なる遠心分離速度（すなわち、１５０００ｘｇと４８０００ｘｇ）によって分画した。得られた上清は、それぞれＬ及びＨと命名した。それぞれの上清はアリコットに分け、－８０℃で保管した。この方法は、ＡＭ間質に付着した物質から簡単にこすり落としたＡＭゼリーから抽出物を調製する際にも用いた。

【0103】

異なる緩衝液及び分画方法による全可溶性ヒト羊膜と羊膜ゼリー抽出物

異なる抽出緩衝液を用いた調製物の試験において、上記より調製した粉末を秤量し、バッファーＡ（等張性低塩濃度）（１００ｍＭのＴｒｉｓ－ＨＣｌ（ｐＨ７．６）、１５０ｍＭのＮａＣｌ、４ｍＭのＥＤＴＡ、１％のＴｒｉｔｏｎ　Ｘ－１００）と湿重量（ｇ）のＡＭと緩衝液（ｍｌ）の比が１：１となるように混合し、４℃にて１時間撹拌した。４８０００ｘｇにて遠心分離し、次に得られたペレットをバッファーＢ（高塩濃度）（１００ｍＭのＴｒｉｓ－ＨＣｌ（ｐＨ７．６）、１ＭのＮａＣｌ、４ｍＭのＥＤＴＡ、１％のＴｒｉｔｏｎ　Ｘ－１００）を用い、４℃にて１時間撹拌することにより、抽出した。再び４８０００ｘｇにて遠心分離し、得られたペレットをバッファーＣ（４Ｍの塩酸グアニジン）（１００ｍＭの酢酸ナトリウム（ｐＨ５．８）、４Ｍの塩酸グアニジン、４ｍＭのＥＤＴＡ、１％のＴｒｉｔｏｎ　Ｘ－１００）を用い、４℃にて２４時間撹拌することにより、最終的に抽出した。上記３種の緩衝液には、全て以下のプロテアーゼ及びホスファターゼ阻害剤を添加した：１μｇ／ｍｌのアプロチニン、１μｇ／ｍｌのロイペプチン、１μｇ／ｍｌのペプスタチンＡ、０．５ｍＭのＰＭＳＦ、５０μＭのフッ化ナトリウム、０．２μＭのバナジン酸ナトリウム。得られた上清はそれぞれ抽出物Ａ、Ｂ、Ｃと命名し、０．５ｍＭのＰＭＳＦを含む透析バッファー（５０ｍＭのＴｒｉｓ－ＨＣｌ（ｐＨ７．５）、０．１５ＭのＮａＣｌ）に対して、４℃にて６時間、二回透析バッファーを交換して（それぞれ５００ｘ（透析バッファー：サンプル（体積比））を用いて）透析した。透析後、各サンプルの容量を計測し、透析バッファーと同じ容量となるように調整した。簡単にこすり落とすことができた、ＡＭ間質に付着した物質であるＡＭゼリーから抽出物を調製する際にも、同じ方法を用いた。

【0104】
ＰＢＳを用いる全可溶性ヒト羊膜抽出物の調製
全可溶性ヒトＡＭ抽出物（Ｔ）の全調製手順は、次の細胞培養ベースの実験で使用できるように無菌的に行った。凍結ヒト胎盤は、Ｂｉｏ－Ｔｉｓｓｕｅ　Ｉｎｃ．（マイアミ、フロリダ州）から入手し、そこからＡＭを回収した。ＡＭは、バイオパルベライザー（ＢｉｏＰｕｌｖｅｒｉｚｅｒ）（Ｂｉｏｓｐｅｃ　Ｐｒｏｄｕｃｔｓ，Ｉｎｃ．，Ｂａｒｔｌｅｓｖｉｌｌｅ，オクラホマ州）の容器に入るように、小片にスライスし、液体窒素で凍結させ、次いで微粉末になるよう粉砕した。得られた粉末は秤量し、プロテアーゼ阻害剤（プロテアーゼ阻害剤カクテル、Ｐ８３４０、Ｓｉｇｍａ、１ｍＭのＰＭＳＦを含む）及びホスファターゼ阻害剤（５０ｍＭのフッ化ナトリウムと０．２ｍＭのバナジン酸ナトリウム）を含む冷ＰＢＳ緩衝液（１０ｘＰＢＳ（ｃａｔ＃７００１１－０４４、Ｉｎｖｉｔｒｏｇｅｎ、Ｃａｒｌｓｂａｄ、カリフォルニア州）から蒸留水を加えて１ｘＰＢＳ（ｐＨ７．４）に調製した）と１：１（ｍｌ／ｇ）の割合で混合した。混合物は氷上に保持し、Ｔｉｓｓｕｅ－Ｔｅａｒｏｒ（ＢｉｏＳｐｅｃ　Ｐｒｏｄｕｃｔｓ，Ｉｎｃ．，Ｄｒｅｍｅｌ，ウィスコンシン州）を用いて、５回、各１分間ずつ（各回が終わるたびに２分間冷却する）ホモジナイズした。この水溶性抽出物は「全」（Ｔ）と命名した。全水溶性抽出物は４℃にて１時間混合し、次に４℃で３０分間４８０００ｘｇにて遠心分離した。得られた上清はアリコットに分け、－８０℃で保管した。

【0105】
水溶性ＡＭ間質抽出物の調製
無菌的方法により、Ｂｉｏ－Ｔｉｓｓｕｅ　Ｉｎｃ．（マイアミ、フロリダ州）から入手した凍結ヒトＡＭは、もとの保存培地を除去するために、ＨＢＳＳを用いて２～３回簡単に洗浄した。ＡＭ間質はスパチュラでこすり落とし、液体窒素の気相中で凍結させ、バイオパルベライザー（ＢｉｏＰｕｌｖｅｒｉｚｅｒ）（Ｂｉｏｓｐｅｃ　Ｐｒｏｄｕｃｔｓ，Ｉｎｃ．，Ｂａｒｔｌｅｓｖｉｌｌｅ，オクラホマ州）で微粒子になるように粉砕し、その後、ＰＢＳ（ｐＨ７．４）中で、Ｔｉｓｓｕｅ－Ｔｅａｒｏｒ（ＢｉｏＳｐｅｃ　Ｐｒｏｄｕｃｔｓ，Ｉｎｃ．，Ｄｒｅｍｅｌ，ウィスコンシン州）を用いて、氷上にて１分間ホモジナイズした。得られたホモジネートは、１時間回転することにより混合し、その後１４，０００ｘｇにて３０分間４℃で遠心分離した。得られたＰＢＳ中の上清を回収し、アリコットに分け、－８０℃で保管した。タンパク質濃度は、ＢＣＡアッセイにより測定した。この水溶性タンパク質抽出物は羊膜間質抽出物（ＡＳＥ）と命名し、本明細書に記載の実験に使用した。

【0106】
ＡＭ間質抽出物の調製
タンパク質抽出物の全調製手順は無菌的に行った。Ｂｉｏ－Ｔｉｓｓｕｅ（マイアミ、フロリダ州）から入手した凍結ヒトＡＭは、保存培地を除去するために、ＨＢＳＳ（Ｉｎｖｉｔｒｏｇｅｎ、Ｃａｒｌｓｂａｄ、カリフォルニア州）を用いて２～３回簡単に洗浄した。ＡＭ間質抽出物を調製するために、ＡＭ間質は、ＡＭの間質側からスパチュラでこすり落とした。Ｂａｐｔｉｓｔ　Ｈｏｓｐｉｔａｌ（マイアミ、フロリダ州）から入手したヒト胎盤と絨毛膜は、血液を除去するために、ＨＢＳＳを用いて３回リンスした。水溶性タンパク質抽出物を調製するために、全ＡＭ、こすり落としたＡＭ間質、間質を除去したＡＭ、胎盤と絨毛膜は、それぞれ液体窒素の気相中で凍結させ、次いで、それぞれをバイオパルベライザー（ＢｉｏＰｕｌｖｅｒｉｚｅｒ）（Ｂｉｏｓｐｅｃ　Ｐｒｏｄｕｃｔｓ，Ｉｎｃ．，Ｂａｒｔｌｅｓｖｉｌｌｅ，オクラホマ州）で微粒子になるように粉砕し、その後、ＰＢＳ（ｐＨ７．４）中で、Ｔｉｓｓｕｅ－Ｔｅａｒｏｒ（ＢｉｏＳｐｅｃ　Ｐｒｏｄｕｃｔｓ，Ｉｎｃ．，Ｄｒｅｍｅｌ，ウィスコンシン州）を用いて、氷上にて１分間ホモジナイズした。得られた各ホモジネートは、１時間かけて混合し、その後１４，０００ｘｇにて３０分間４０℃で遠心分離した。得られた各上清（ＰＢＳ中）を回収し、アリコット（０．５ｍｌ）に分け、－８０℃で保管した。ＢＣＡアッセイ（Ｐｉｅｒｃｅ、Ｒｏｃｋｆｏｒｄ、イリノイ州）を用いて、それぞれの抽出物中の全タンパク質を定量

した。

【0107】

水溶性型及び凍結乾燥粉末状のヒトＡＭ抽出物の調製

ヒトＡＭ抽出物の典型的な調製手順においては、全手順は無菌的に行われる。特に断りのない限り、手順ステップ中においてＡＭ抽出物は室温で取り扱うことができる。まず、生の又は凍結状態のヒトＡＭを、好ましくはＢｉｏ－Ｔｉｓｓｕｅ　Ｉｎｃ．（マイアミ、フロリダ州）から入手する。凍結ＡＭは、保存培地を除去するために、ＨＢＳＳ（Ｉｎｖｉｔｒｏｇｅｎ、Ｃａｒｌｓｂａｄ、カリフォルニア州）を用いて２～３回簡単に洗浄する。生のヒト胎盤又は絨毛膜は、血液を除去するために、ＨＢＳＳを用いて３回リンスする。

【0108】

水溶性型ＡＭ抽出物を調製するために、ＡＭ（例えば、ＡＭ間質、間質を除去したＡＭ、胎盤、絨毛膜）を無菌の５０ｍｌ容の遠心チューブに移し、４℃で５分間５０００ｘｇにて遠心分離し、余分な液体を除去する。得られたＡＭを秤量し、１００ｍｍ又は１５０ｍｍの無菌のペトリ皿に移し、その後のホモジナイズ処理を容易にするために、液体窒素容器の気相中で２０分間凍結させる。次に、凍結ＡＭは、使い捨てメスを用いて小片にスライスするか、バイオパルベライザー（ＢｉｏＰｕｌｖｅｒｉｚｅｒ）（Ｂｉｏｓｐｅｃ　Ｐｒｏｄｕｃｔｓ，Ｉｎｃ．，Ｂａｒｔｌｅｓｖｉｌｌｅ，オクラホマ州）又は他の適切な装置を用いて微粒子に粉砕し、Ｔｉｓｓｕｅ－Ｔｅａｒｏｒ（Ｂｉｏｓｐｅｃ　Ｐｒｏｄｕｃｔｓ，Ｉｎｃ．，Ｄｒｅｍｅｌ，ウィスコンシン州）又は他の適切な装置を用いて、リン酸緩衝食塩水（ＰＢＳ）又はＤＭＥＭ（フェノールレッド不含）（Ｉｎｖｉｔｒｏｇｅｎ、Ｃａｒｌｓｂａｄ、カリフォルニア州）中で、中性ｐＨにてホモジナイズする。生化学的特徴づけ及び精製のために、上記の溶液には、以下に記載のプロテアーゼ阻害剤を添加することができる：１μｇ／ｍｌのアプロチニン、１μｇ／ｍｌのロイペプチン、１μｇ／ｍｌのペプスタチンＡ、１ｍＭのＰＭＳＦ。しかし、抽出物を細胞培養液に直接添加する場合は、プロテアーゼ阻害剤を抽出物に添加しない。得られたホモジネートは、４℃で３０分間混合し、その後１５，０００ｘｇにて３０分間遠心分離する。上清（すなわちＡＭ抽出物）を回収し、アリコット（０．５ｍｌ）に分け、－８０℃で保管する。ＢＣＡアッセイ（Ｐｉｅｒｃｅ、Ｒｏｃｋｆｏｒｄ、イリノイ州）を用いて、それぞれのＡＭ抽出物中の全タンパク質を定量する。

【0109】

凍結乾燥粉末状のＡＭ抽出物を調製するために、凍結ＡＭをバイオパルベライザー（ＢｉｏＰｕｌｖｅｒｉｚｅｒ）（Ｂｉｏｓｐｅｃ　Ｐｒｏｄｕｃｔｓ，Ｉｎｃ．，Ｂａｒｔｌｅｓｖｉｌｌｅ，オクラホマ州）又は他の適切な装置を用いて微粒子に粉砕し、さらに、本明細書に記載されているようにホモジナイズする。約０．５ｍｌのアリコットをスピードバック（ＳｐｅｅｄＶａｃ）（Ｓａｖａｎｔ　Ｉｎｓｔｒｕｍｅｎｔｓ　Ｉｎｃ．，Ｆａｒｍｉｎｇｄａｌｅ、ニューヨーク州）を用いて、４℃にて４時間凍結乾燥し、重量を２８０ｍｇから３２ｍｇ（約８９％減少）に減少させる。得られた凍結乾燥粉末を秤量し、－８０℃で保管する。使用前には、この凍結乾燥粉末を適切な緩衝液で再構成することができる。

【0110】

ＡＭ間質抽出物を調製するために、ＡＭ間質を無処理の全ＡＭの間質表面から、基底膜と羊膜上皮に傷をつけずにこすり落とし、それを凍結し、その凍結したＡＭ間質を、本明細書に記載されているように、バイオパルベライザーを用いて粉砕する。間質は、ＰＢＳ（例えば、１．４ｍｇ／ｍｌ）を用いて、中性ｐＨにて４℃で３０分間抽出し、その後１５，０００ｘｇにて３０分間遠心分離する。その上清を回収し、アリコット（０．５ｍｌ）に分け、－８０℃で保管する。ＢＣＡアッセイ（Ｐｉｅｒｃｅ、Ｒｏｃｋｆｏｒｄ、イリノイ州）を用いて、ＡＭ間質抽出物中の全タンパク質を定量する。

【0111】

通常の分子生物学的手法に関わる方法は、本明細書に記載される。このような手法は、

一般に当技術分野で周知であり、例えば、Ｍｏｌｅｃｕｌａｒ　Ｃｌｏｎｉｎｇ：Ａ　ｌａｂｏｒａｔｏｒｙ　Ｍａｎｕａｌ，　３ｒｄ　ｅｄ．，　ｖｏｌ．１－３，ｅｄ．Ｓａｍｂｒｏｏｋら、Ｃｏｌｄ　Ｓｐｒｉｎｇ　Ｈａｒｂｏｒ　Ｌａｂｏｒａｔｏｒｙ　Ｐｒｅｓｓ，Ｃｏｌｄ　Ｓｐｒｉｎｇ　Ｈａｒｂｏｒ，Ｎ．Ｙ．，２００１；Ｃｕｒｒｅｎｔ　Ｐｒｏｔｏｃｏｌｓ　ｉｎ　Ｍｏｌｅｃｕｌａｒ　Ｂｉｏｌｏｇｙ，ｅｄ．Ａｕｓｕｂｅｌら、Ｇｒｅｅｎｅ　Ｐｕｂｌｉｓｈｉｎｇ　ａｎｄ　Ｗｉｌｅｙ－Ｉｎｔｅｒｓｃｉｅｎｃｅ，Ｎｅｗ　Ｙｏｒｋ，２００３（定期的に更新される）などの方法論に詳細に記載されている。動物細胞を培養する各種手法は、当技術分野で周知であり、Ｃｕｌｔｕｒｅ　ｏｆ　Ａｎｉｍａｌ　Ｃｅｌｌｓ：Ａ　Ｍａｎｕａｌ　ｏｆ　Ｂａｓｉｃ　Ｔｅｃｈｎｉｑｕｅ，　４ｔｈ　ｅｄ．，Ｒ．Ｉａｎ　Ｆｒｅｓｈｎｅｙ，Ｗｉｌｅｙ－Ｌｉｓｓ，Ｈｏｂｏｋｅｎ，ＮＪ，２０００，及びＡｎｉｍａｌ　Ｃｅｌｌ　Ｃｕｌｔｕｒｅ　Ｔｅｃｈｎｉｑｕｅｓ（Ｓｐｒｉｎｇｅｒ　Ｌａｂ　Ｍａｎｉａｌ），Ｍ．Ｃｌｙｎｏｓ，Ｓｐｒｉｎｇｅｒ－Ｖｅｒｌａｇ，Ｎｅｗ　Ｙｏｒｋ，ＮＹ，１９９８に記載されている。タンパク質の分析及び精製に関する方法もまた、当技術分野で周知であり、Ｐｒｏｔｅｉｎ　Ａｎａｌｙｓｉｓ　ａｎｄ　Ｐｕｒｉｆｉｃａｔｉｏｎ：Ｂｅｎｃｈｔｏｐ　Ｔｅｃｈｎｉｑｕｅｓ，２ｎｄ　ｅｄ．，Ｉａｎ　Ｍ．Ｒｏｓｅｎｂｅｒｇ，Ｂｉｒｋｈａｕｓｅｒ，Ｎｅｗ　Ｙｏｒｋ，ＮＹ，２００４に記載されている。

【0112】

医薬組成物

ＡＭ調製物は、例えば、液剤、滴剤、懸濁剤、ペースト剤、スプレー剤、軟膏剤、油剤、乳剤、エアロゾル剤、被覆包帯、パッチ剤、クリーム剤、ローション剤、ゲル剤、などの組成物を製造するために、配送ビヒクルと組み合わせることにより、非固体製剤として、投与目的で処方することができる。使用する剤形は、特定の適用に応じて決定される。ゲル剤は、導入部での有効成分の保持をより向上させ、有効成分のクリアランス前において有効成分の効果をより長く発現させるので、組成物を投与するのに有用である。あるいは、ＡＭ調製物は、徐放性固体製剤（経口製剤を含む）として処方することができる。典型的な薬学的に許容される担体又はビヒクル及び希釈剤の記載は、製剤と同様、本明細書中に提供され、また、当技術分野及びＵＳＰ／ＮＦにおける標準書である、Ｒｅｍｉｎｇｔｏｎ’ｓ　Ｐｈａｒｍａｃｅｕｔｉｃａｌ　Ｓｃｉｅｎｃｅｓ中にも見つけることができる。

【0113】

医薬組成物は、１種以上の生理学的に許容される担体（有効成分を薬学的に使用しうる調製剤に加工するのを容易にする賦形剤及び補助剤を含む）を用いて、通常の方法により処方することができる。適切な処方は選択する投与経路に応じて決定される。当技術分野において適切でかつ理解されているように、任意の公知の手法、担体及び賦形剤を使用することができる。本明細書に記載の医薬組成物の概要は、例えば、Ｒｅｍｉｎｇｔｏｎ：Ｔｈｅ　Ｓｃｉｅｎｃｅ　ａｎｄ　Ｐｒａｃｔｉｃｅ　ｏｆ　Ｐｈａｒｍａｃｙ，Ｎｉｎｅｔｅｅｎｔｈ　ｅｄ（Ｅａｓｔｏｎ，Ｐａ．：Ｍａｃｋ　Ｐｕｂｌｉｓｈｉｎｇ　Ｃｏｍｐａｎｙ，１９９５）；Ｈｏｏｖｅｒ，Ｊｏｈｎ　Ｅ．Ｒｅｍｉｎｇｔｏｎ’ｓ　Ｐｈａｒｍａｃｅｕｔｉｃａｌ　Ｓｃｉｅｎｃｅｓ，Ｍａｃｋ　Ｐｕｂｌｉｓｈｉｎｇ　Ｃｏ．，Ｅａｓｔｏｎ，Ｐｅｎｎｓｙｌｖａｎｉａ　１９７５；Ｌｉｂｅｒｍａｎ，Ｈ．Ａ．ａｎｄ　Ｌａｃｈｍａｎ，Ｌ．，Ｅｄｓ．，Ｐｈａｒｍａｃｅｕｔｉｃａｌ　Ｄｏｓａｇｅ　Ｆｏｒｍｓ，Ｍａｒｃｅｌ　Ｄｅｃｋｅｒ，Ｎｅｗ　Ｙｏｒｋ，Ｎ．Ｙ．，１９８０；Ｐｈａｒｍａｃｅｕｔｉｃａｌ　Ｄｏｓａｇｅ　Ｆｏｒｍｓ　ａｎｄ　Ｄｒｕｇ　Ｄｅｌｉｖｅｒｙ　Ｓｙｓｔｅｍｓ，　Ｓｅｖｅｎｔｈ　Ｅｄ．（Ｌｉｐｐｉｎｃｏｔｔ　Ｗｉｌｌｉａｍｓ　＆　Ｗｉｌｋｉｎｓ；１９９９）中に見つけることができ、その全内容は参照によって本明細書に組み入れられる。

【0114】

ある態様では、該組成物は、薬学的に許容される希釈剤、賦形剤、又は担体を含む。さらに、本明細書に記載のＡＭ調製物と精製組成物は、本明細書に記載のＡＭ調製物と精製

組成物を他の有効成分と混合し、例えば併用療法などの目的で、医薬組成物として投与することができる。ある態様では、該医薬組成物は、他の薬剤又は医薬的作用物質、担体、アジュバント、例えば防腐剤、安定化剤、湿潤剤又は乳化剤、溶液促進剤、浸透圧を調節するために塩、及び／又は緩衝液を含んでもよい。さらに、該医薬組成物は他の治療上役に立つ物質を含んでもよい。

【0115】

本明細書において使用される医薬組成物とは、本明細書に記載のＡＭ調製物と精製組成物と、例えば、担体、安定化剤、希釈剤、分散剤、懸濁剤、増粘剤、及び／又は賦形剤などの他の化学的構成成分との混合物のことをいう。医薬組成物は、該化合物の生物への投与を容易にする。本明細書に記載の治療方法又は使用方法を実行するに当たって、治療的有効量の本明細書に記載のＡＭ調製物と精製組成物を、治療が必要な疾患、障害又は異常を有する哺乳動物に、医薬組成物の形態で投与する。ある態様では、その哺乳動物はヒトである。治療的有効量は、疾患の重症度、被験者の年齢と相対的健康状態、使用する該化合物の力価、他の要因に応じて幅広く変わりうる。該化合物は、単独で又は他の１種以上の治療剤と組み合わせて構成成分の混合物として使用することができる。

【0116】

局所製剤

本明細書に記載のＡＭ調製物と精製組成物の製剤は、局所投与に適したものを含む。該製剤は単位剤形として便宜的に提供され、薬学分野でよく知られている任意の方法で調製することができる。単回剤形を製造するために担体物質と組み合わせることができる有効成分の量は、治療される被験者、特に剤形に応じて決定する。

【0117】

懸濁液は、懸濁剤（例えば、エトキシル化イソステアリルアルコール、ポリオキシエチレンソルビトール及びソルビタンエステル、微結晶性セルロース、アルミニウムメタ水酸化物、ベントナイト、寒天、トラガカント及びこれらの混合物など）を含むことができる。

【0118】

本明細書に記載の典型的な組成物は、多種多様な物理的形状を含む。例えば、これに限定されないが、溶液、ローション、クリーム、オイル、ゲル、スティック、スプレー、軟膏、バーム、シャンプー及びペーストが挙げられる。一般にこのような担体システムは、溶液、エマルション、ゲル、固体、エアロゾルとすることができる。該組成物は局所的に皮膚に適用することができ、また、例えば、当技術分野で周知のマイクロニードル、パッチ、包帯、又はガーゼパッドなどの経皮配送装置の形状で適用することもできる。

【0119】

このような軟膏、ペースト、クリーム、ゲルは、本明細書に記載のＡＭ調製物と精製組成物の他に、例えば、動物及び植物脂、油、ワックス、パラフィン、デンプン、トラガカント、セルロース誘導体、ポリエチレングリコール、シリコーン、ベントナイト、ケイ酸、タルク、酸化亜鉛、又はこれらの混合物などの賦形剤を含むことができる。

【0120】

粉末及びスプレーは、本明細書に記載のＡＭ調製物と精製組成物の他に、例えば、ラクトース、タルク、ケイ酸、水酸化アルミニウム、ケイ酸カルシウム、ポリアミド粉末、又はこれらの混合物などの賦形剤を含むことができる。スプレーは、さらに、通常使用される塩化フッ化炭化水素などの噴霧剤及びブタンやプロパンなどの揮発性不飽和炭化水素を含むこともできる。

【0121】

溶媒は一般に適切な局所的組成物の調製に使用される。このような溶媒は水性又は有機性のいずれでもよい。溶媒は、治療する動物に対し刺激を与えずに有効成分を分散又は溶解する能力を有する必要がある。水は全ての水性溶媒の基礎を作る。一方、適切な有機溶媒としては、プロピレングリコール、ブチレングリコール、ポリエチレングリコール、ポリプロピレングリコール、グリセロール、１，２，４－ブタントリオール、ソルビトール

エステル、１，２，６－ヘキサントリオール、エタノール、イソプロパノール、ブタンジオール、これらの混合物が挙げられる。溶媒は、全体の組成物中に０．１％～９９％、好ましくは２．０％～７５％の範囲の量を含ませることができる。ある態様では、該組成物は、皮膚軟化薬含有組成物の形態で製造される。多種多様な適切な皮膚軟化薬が知られており、本明細書において使用することができる。

【0122】

ある態様では、該組成物は、本明細書に記載のＡＭ調製物と精製組成物を約０．０１％～１０％の範囲で含むローション剤として処方される。他の態様では、該組成物はクリーム剤として、溶液担体システムの形態で処方される。好ましくは、クリーム組成物は、本明細書に記載のＡＭ調製物と精製組成物を約０．１％～１５％、好ましくは１％～５％の範囲で含む。ローション剤とクリーム剤は、エマルション及び溶液として処方することができる。該組成物は、当業界で入手できるスプレー剤とは異なる形態である、液体に懸濁したリポソームの形態を含む、液体の形態で投与することもできる。

【0123】

他の態様では、該有効成分は軟膏剤として処方される。適切な軟膏剤は、動物又は植物油又は半固形炭化水素（油性）の単純な基剤を含む。適切な軟膏剤はさらに、エマルションを形成するために水を吸収する吸収性軟膏基剤を含んでもよい。軟膏担体は同様に、水溶性である。軟膏剤は１％～９９％の皮膚軟化剤と約０．１％～９９％の増粘剤を含んでもよい。

【0124】

本明細書に記載のＡＭ調製物と精製組成物の該組成物中の割合は、約０．０１重量％～約１００重量％、より好ましくは、約０．１重量％～約９９．９重量％、特に約１．０重量％～約９９．０重量％の範囲内で変動しうる。

【0125】

「担体」又は「ビヒクル」とは、好ましくは、局所投与に適した担体物質のことをいい、無毒性であり、該組成物の他の構成成分と悪影響を及ぼすように相互作用しない当技術分野で周知の物質、例えば、液体、ゲル溶媒、液体希釈剤、可溶化剤などが挙げられる。本明細書で使用される適切な担体としては、例えば、水、シリコーン、液状の糖類、ワックス、油、ワセリン、及び各種他の物質が上げられる。

【0126】

ある態様では、担体又はビヒクルとして、１種以上の溶媒、油、界面活性剤、湿潤剤、増粘剤、抗酸化剤、キレート剤、緩衝液、防腐剤が挙げられる。

【0127】

溶媒としては、例えば、ヘキサノール、シクロヘキサノール、ベンジルアルコール、１，２－ブタンジオール、グリセロール及びアミルアルコールなどのC_2-C_{10}アルコール；ｎ－ヘキサン、シクロヘキサン、エチルベンゼンなどのC_5-C_{10}炭化水素；ヘプチルアルデヒド、シクロヘキサノン、ベンジルアルデヒドなどのC_4-C_{10}アルデヒド、ケトン；アミルアセテート及びベンジルプロピオネートなどのC_4-C_{10}エステル；ユーカリ油、ヘンルーダ油、クミン油、リモネン、チモール、１－ピネンなどのエーテル油；１－クロロヘキサン、１－ブロモヘキサン、クロロシクロヘキサンなどの２～８個の炭素原子を有するハロゲン化炭化水素が挙げられる。

【0128】

油としては、例えば、オリーブオイルや硬化油などの油脂；蜜ろう及びラノリンなどのワックス；流動パラフィンノセレシンやスクアランなどの炭化水素；ステアリン酸やオレイン酸などの脂肪酸；セチルアルコール、ステアリルアルコール、ラノリンアルコール、ヘキサデカノールなどのアルコール；イソプロピルミリステート、イソプロピルパルミテート、ブチルステアレートなどのエステルが挙げられる。

【0129】

界面活性剤としては、例えば、ステアリン酸ナトリウム、硫酸セチルナトリウム、ポリオキシエチレンラウリルエーテルホスフェート、Ｎ－アシルグルタミン酸ナトリウムなど

のアニオン性界面活性剤；塩化ステアリルジメチルベンジルアンモニウム及び塩化ステアリルトリメチルアンモニウムなどのカチオン性界面活性剤；アルキルアミノエチルグリシン塩酸塩溶液及びレシチンなどの両性界面活性剤；モノステアリン酸グリセリン、モノステアリン酸ソルビタン、ショ糖脂肪酸エステル、モノステアリン酸プロピレングリコール、ポリオキシエチレンオレイルエーテル、モノステアリン酸ポリエチレングリコール、モノパルミチン酸ポリオキシエチレンソルビタン、ポリオキシエチレンココナッツ脂肪酸モノエタノールアミド、ポリオキシプロピレングリコール（例えば、「プルロニック」という登録商標のもと市販されている物質）、ポリオキシエチレンヒマシ油、ポリオキシエチレンラノリンなどの非イオン性界面活性剤が挙げられる。

【0130】

吸湿剤としては、例えば、グリセリン、１，３－ブチレングリコール、プロピレングリコールが挙げられる。増粘剤としては、例えば、キサンタンゴム、ヒドロキシプロピルセルロース、ヒドロキシプロピルメチルセルロース、ポリエチレングリコール、カルボキシメチルセルロースナトリウムが挙げられる。抗酸化剤としては、例えば、ブチル化ヒドロキシトルエン、ブチル化ヒドロキシアニソール、没食子酸プロピル、クエン酸、エトキシキンが挙げられる。キレート剤としては、例えば、エデト酸二ナトリウム及びエタンヒドロキシ二リン酸が挙げられる。緩衝剤としては、例えば、クエン酸、クエン酸ナトリウム、ホウ酸、ホウ砂、リン酸水素二ナトリウムが挙げられる。防腐剤としては、パラオキシ安息香酸メチル、パラオキシ安息香酸エチル、ジヒドロ酢酸、サリチル酸、安息香酸が挙げられる。

【0131】

ある態様では、担体／ビヒクルは、皮膚閉塞の制御ができる上記の物質から構成され、それによって、皮膚への刺激は最小であるが、生物学的に活性な成分の皮膚への浸透の最適な増強をもたらす。ある態様では、担体／ビヒクルは、連続相に分散した有効成分の粒子相を維持するのに役立つ分散剤を含むことができる。他の態様では、ラウリン酸アルコール、プロピレングリコールモノラウリン酸、乳酸ミリスチル、乳酸ラウリルなどの非イオン性賦形剤などにより、分散が促進される。

生物学的に活性な成分の真皮表面への配送率は、真皮配送増強剤によって向上させることができる。適切な真皮配送増強剤としては、例えば、ジメチルスルホキシドやジメチルアセトアミドなどのプロトン受容溶媒が挙げられる。他の適切な真皮配送増強剤としては、例えば、２－ピロリジン、Ｎ，Ｎ－ジエチル－ｍ－トルアミド、１－ドデシルアザシクロヘプタン－２－オン、Ｎ，Ｎ－ジメチルホルムアミド、Ｎ－メチル－２－ピロリジン、テルペン、界面活性剤、チオグリコール酸カルシウムが挙げられる。

【0132】

適切な真皮浸透増強剤としては、パラアミノ安息香酸の１～５個の炭素脂肪酸エステル、イソプロピルパルミチン酸、イソプロピルミリスチン酸、エタノール、イソブチルアルコール、イソブチルアルコール、ステアリルアルコール、グリセロール、２－ピロリドン、尿素、プロピレングリコール、オレイン酸、パルミチン酸、ジメチルスルホキシド、Ｎ，Ｎ－ジメチルアセトアミド、Ｎ，Ｎ－ジメチルホルムアミド、２－ピロリドン、１－メチル－２－ピロリドン、５－メチル－２－ピロリドン、１，５－ジメチル－２－ピロリドン、１－エチル－２－ピロリドン、２－ピロリドン－５－カルボキシル酸、Ｎ，Ｎ－ジメチル－ｍ－トルアミド、尿素、エチル酢酸、１－ドデシルアザシクロヘプタン－２－オン、オレイン酸、イミダゾリン、ブチルウレア、ピロリドンカルボン酸エステルが挙げられる。

【0133】

着色剤、リリース剤、コーティング剤、甘味料、矯味矯臭剤及び香料、防腐剤、抗酸化剤同様に、湿潤剤、乳化剤、界面活性剤、潤滑剤（例えば、ラウリル硫酸ナトリウム及びステアリン酸マグネシウムなど）もまた、該組成物に含まれていてもよい。

【0134】

薬学的に許容される抗酸化剤としては、例えば、（１）水溶性抗酸化剤（例えば、アス

コルビン酸、システイン塩酸塩、硫酸水素ナトリウム、メタ硫酸水素ナトリウム、亜硫酸ナトリウムなど）；（２）油溶性抗酸化剤（例えば、パルミチン酸アスコルビル、ブチル化ヒドロキシアニソール（ＢＨＡ）、ブチル化ヒドロキシトルエン（ＢＨＴ）、レシチン、没食子酸プロピル、α－トコフェロールなど）；（３）金属キレート剤（例えば、クエン酸、エチレンジアミン四酢酸（ＥＤＴＡ）、ソルビトール、酒石酸、リン酸など）が挙げられる。

【0135】

適切な局所投与用組成物としては、局所的に投与される治療剤に通常使用される全ての組成物（例えば、クリーム剤、ゼリー剤、包帯剤、シャンプー剤、チンキ剤、ペースト剤、軟膏剤、膏薬、粉剤、液剤、半液剤、など）等が挙げられる。該組成物の適用は、エアロゾルによって行うことができ、例えば、窒素、二酸化炭素、フレオンなどの噴霧剤を用いて行うことができ、また、例えば、ポンプ、スプレー、滴剤、ローション、又はスワブを用いて適用することができる増粘した組成物などの半固体など、噴霧剤を用いることなく行うこともできる。特に、膏薬、クリーム剤、ペースト剤、ゼリー剤、軟膏剤などの組成物や半固体組成物が都合よく使用される。

【0136】

眼科用製剤

本明細書に記載のＡＭ調製物と精製組成物は、眼への局所配送のあらゆる形態を含む、様々な方法で投与することができる。さらに、本明細書に記載のＡＭ調製物と精製組成物は、経口投与や静脈投与などの全身投与も可能である。本明細書に記載のＡＭ調製物と精製組成物は、眼への局所投与することができ、溶液、懸濁液、ゲル又は軟膏などの様々な局所投与可能な眼科用組成物に処方することができる。従って、「眼への投与」は、これに限定されないが、眼内注射、網膜下注射、硝子体内注射、眼窩周囲投与、結膜下注射、眼球後注射、前房内注射（前眼房又は硝子体眼房への投与を含む）、テノン嚢下注射又は埋め込み、眼科用溶液、眼科用懸濁液、眼軟膏剤、眼内埋め込み、及び眼内挿入、眼内液剤、イオントフォレシスの使用、手術用洗浄液への混和、及び湿布（ほんの一例として、脳弓への飽和させた綿球の挿入）を包含する。

【0137】

本明細書に記載のＡＭ調製物と精製組成物からなる組成物は、例えば、薬剤が溶液中、懸濁液中、又はその両方に存在する液体の形態をとることができる。一般的に、該組成物が溶液又は懸濁液として投与されるときには、第一の割り当て分の薬剤が溶液中に存在し、第二の割り当て分の薬剤が、液体マトリックス中の懸濁液に粒子の形態で存在する。ある態様では、液体組成物にはゲル製剤が含まれる。他の態様では、液体組成物が水溶性である。あるいは、該組成物が軟膏剤の形態でありうる。

【0138】

有用な組成物は、水性溶液、懸濁液、又は溶液／懸濁液であってもよく、これは、点眼剤の形態で存在することができる。所望の投与量は、周知の数の液滴により眼に投与することができる。例えば、一滴の容量が２５μｌである場合、１～６滴の投与により、２５～１５０μｌの組成物が配送される。水性組成物は、本明細書に記載のＡＭ調製物と精製組成物を、一般的に約０．０１％～約５０％、より一般的に約０．１％～約２０％、さらにより一般的に約０．２％～約１０％、最も一般的に約０．５％～約５％（ｗ／ｖ）含む。

【0139】

一般的に、水性組成物は、眼科的に許容されるｐＨとモル浸透圧濃度を有する。製剤、組成物又は構成成分に関する「眼科的に許容される」とは、治療する眼、又はその機能、あるいは治療される被験者の健康状態に持続的な有害な影響を及ぼさないことを意味する。一時的な影響、例えば、軽い刺激や「突き刺すような」感覚などは薬剤の眼科用局所投与には一般的であり、問題となっている、「眼科的に許容される」製剤、組成物又は構成成分に整合性が取れている。

【0140】

有用な水性懸濁液もまた、１種以上のポリマーを懸濁剤として含むことができる。有用なポリマーとしては、例えば、セルロース系ポリマー（例えば、ヒドロキシプロピルメチルセルロース）などの水溶性ポリマー、及び例えば、架橋型カルボキシル基含有ポリマーなどの水不溶性ポリマーが挙げられる。また有用な組成物は、例えば、カルボキシメチルセルロース、カルボマー（アクリル酸ポリマー）、ポリ（メチルメタクリレート）、ポリアクリルアミド、ポリカルボフィル、アクリル酸／ブチルアクリレートコポリマー、アルギン酸ナトリウム及びデキストランから選択される眼科的に許容される粘膜付着性ポリマーを含むこともできる。

【0141】

また、有用な組成物は、本明細書に記載のＡＭ調製物と精製組成物の構成成分の溶解度を助けるために、眼科的に許容される可溶化剤を含むことができる。用語「可溶化剤」には、一般に、薬剤のミセル溶液や真の溶液を形成させる媒介物が含まれる。ある眼科的に許容される非イオン性界面活性剤、例えば、ポリソルベート８０は可溶化剤として有用である。眼科的に許容されるグリコール、ポリグリコール（例えば、ポリエチレングリコール４００やグリコールエーテル）も同様に有用でありうる。

【0142】

また、有用な組成物は、１種以上の眼科的に許容されるｐＨ調整剤又は緩衝剤を含み、例えば、酸（例えば、酢酸、ホウ酸、クエン酸、乳酸、リン酸及び塩酸など）；塩基（例えば、水酸化ナトリウム、リン酸ナトリウム、ホウ酸ナトリウム、クエン酸ナトリウム、酢酸ナトリウム、乳酸ナトリウム、トリス－ヒドロキシメチルアミノメタンなど）；緩衝液（例えば、クエン酸塩／デキストロース、炭酸水素ナトリウム、塩化アンモニウム）が挙げられる。このような、酸、塩基、緩衝液は、該組成物のｐＨを眼科的に許容される範囲内に維持するのに必要な量で含まれる。

【0143】

また、有用な組成物は、１種以上の眼科的に許容される塩を、組成物の重量モル浸透圧濃度を眼科的に許容される範囲内にするのに必要な量で含むことができる。このような塩としては、ナトリウムカチオン、カリウムカチオン又はアンモニウムカチオン及び塩素アニオン、クエン酸アニオン、アスコルビン酸アニオン、ホウ酸アニオン、リン酸アニオン、炭酸水素アニオン、硫酸アニオン、チオ硫酸アニオン、又は亜硫酸水素アニオンを有する塩が挙げられ、適切な塩としては、塩化ナトリウム、塩化カリウム、チオ硫酸ナトリウム、亜硫酸水素ナトリウム、硫酸アンモニウムが挙げられる。

【0144】

また、他の有用な組成物は、微生物の活性を阻害するために、１種以上の眼科的に許容される防腐剤を含むことができる。適切な防腐剤としては、例えば、メルフェン（ｍｅｒｆｅｎ）やチオマーサル（ｔｈｉｏｍｅｒｓａｌ）などの銀を含有する物質；安定化した二酸化塩素；塩化ベンザルコニウム、臭化セチルトリメチルアンモニウム、塩化セチルピリジニウムなどの四級アンモニウム化合物が挙げられる。

【0145】

さらに他の有用な組成物は、物理的安定性を増強させるため、あるいは他の目的で、１種以上の眼科的に許容される界面活性剤を含むことができる。適切な非イオン性界面活性剤としては、ポリオキシエチレン脂肪酸グリセリドや植物油（例えば、ポリオキシエチレン（６０）硬化ヒマシ油）；ポリオキシエチレンアルキルエーテルやアルキルフェニルエーテル（例えばオクトキシノール１０、オクトキシノール４０）が挙げられる。

【0146】

さらに他の有用な組成物は、必要ならば、化学的安定性を増強させるために、１種以上の眼科的に許容される抗酸化剤を含むことができる。適切な抗酸化剤としては、ほんの一例として、アスコルビン酸及びメタ重亜硫酸ナトリウムが挙げられる。

【0147】

水性懸濁性組成物は、再び密閉できない単回投与用の容器に充填することができる。あるいは、再び密閉できる複数回投与用の容器を用いることもでき、この場合、一般的に該

組成物は防腐剤を含んでいる。

【0148】

眼科用組成物は、眼と眼瞼の間、又は結膜嚢の中に挿入することができる、固形品の形状をとることもでき、その挿入された場所で本明細書に記載のＡＭ調製物と精製組成物が放出される。放出は、該固形品が一般的に密接に接触する角膜表面を覆っている涙液に対して、あるいは角膜に対し直接行われる。このような方法による、眼内埋め込みに適した固形品は、一般に主にポリマーから構成され、生分解性であっても生分解性でなくてもよい。

【0149】

注射剤

筋肉内注射、皮下注射又は静脈内注射に適した製剤としては、生理学的に許容される無菌の水性又は非水性溶液剤、分散剤、懸濁剤又はエマルションが挙げられ、また無菌の注射可能溶液剤又は分散剤に再構成するための無菌粉末剤が挙げられる。適切な水性及び非水性の担体、希釈剤、溶媒又はビヒクルとしては、水、エタノール、ポリオール（プロピレングリコール、ポリエチレングリコール、グリセロール、クレモホールなど）、それらの適切な混合物、植物油（例えば、オリーブ油など）、オレイン酸エチルなどの注射可能な有機エステルが挙げられる。適切な流動性は、例えば、レシチンなどのコーティングを利用することにより、分散液の場合は必要な粒子サイズを維持することにより、及び界面活性剤を使用することにより維持することができる。皮下注射に適した製剤は、防腐剤、湿潤剤、乳化剤、分散剤などの添加剤を含むことができる。微生物の増殖の防止は、様々な抗菌剤や抗真菌剤（例えば、パラベン、クロロブタノール、フェノール、ソルビン酸など）により確保することができる。また、糖類、塩化ナトリウムなどの等張化剤を含んでいることが好ましい。注射可能な製剤形態の持続的吸収は、モノステアリン酸アルミニウムやゼラチンなどの吸収遅延剤を用いることにより、達成することができる。

【0150】

静脈内注射剤については、例えば、ハンクス液、リンガー液、生理食塩緩衝液、又は他の適切な溶液などの生理学的に適合性のある緩衝液を用いて、本明細書に記載のＡＭ調製物と精製組成物を水性溶液に製剤化することができる。経粘膜投与の場合、該製剤には透過すべきバリアに対して適した浸透剤を使用する。このような浸透剤は当技術分野において一般的に周知である。他の非経口注射剤については、適切な製剤は、好ましくは生理学的に適合性のある緩衝液又は賦形剤を用いて、水性又は非水性溶液を含むことができる。このような賦形剤は当技術分野において一般的に周知である。

【0151】

非経口注射剤は、急速投与注射又は連続注入に関与してもよい。注射用製剤は単位剤形（例えば、アンプル）で存在してもよく、あるいは複数回投与容器（防腐剤を添加する）の形態で存在してもよい。本明細書に記載の医薬組成物は、油性又は水性ビヒクルを用いた無菌懸濁液、溶液又はエマルションとして非経口注射に適した形態をとることができ、また、懸濁剤、安定化剤及び／又は分散剤などの製剤化剤を含むこともできる。非経口投与用製剤としては、水溶性形態の有効成分の水性溶液が挙げられる。さらに、有効成分の懸濁液が、適切な油性注射懸濁剤として調製されてもよい。適切な親油性溶媒又はビヒクルとしては、ゴマ油や合成脂肪酸エステル（例えば、オレイン酸エチル、トリグリセリド、リポソームなど）などの油脂類が挙げられる。水性注射懸濁剤は、その懸濁剤の粘性を高める物質を含むことができる。この物質としては、カルボキシメチルセルロースナトリウム、ソルビトール、デキストランが挙げられる。必要ならば、該懸濁剤は、適切な安定化剤又は化合物の溶解性を高める媒介物を含んでもよく、これにより高度に濃縮された溶液の調製が可能となる。あるいは、有効成分が粉末の形態でもよく、使用前に適切なビヒクル、例えば無菌のパイロジェンフリー水を用いて再構成する。

【0152】

経皮製剤

本明細書に記載の経皮製剤は、当技術分野に記載されている各種装置を用いて投与する