

THREE-DIMENSIONAL POROUS HYBRID SCAFFOLD AND MANUFACTURE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application claims the benefit of priority of US provisional application No. 60/872,800, filed December 05, 2006, the contents of it being hereby incorporated by reference in its entirety for all purposes.

FIELD OF THE INVENTION

[002] The present invention refers to a three-dimensional porous hybrid scaffold for tissue engineering and methods of its manufacture and use.

BACKGROUND OF THE INVENTION

[003] Biological materials have been used in many tissue engineering applications to control the function and structure of engineered tissue by interacting with transplanted/host cells. These materials include naturally derived materials (e.g. collagen and alginate) and acellular tissue matrices (e.g. small intestinal submucosa) among others. Biological materials have been proven to support cell ingrowth and regeneration of damaged tissues with no evidence of immunogenic rejection, and encourage the remodeling process by stimulating cells to synthesize extracellular matrix (ECM) proteins and secrete ECM to aid in the healing process. Extracellular matrix components preserved in these biological materials are also able to influence the phenotypic differentiation of cells through specific interactions with cell surface markers.

[004] However, since such isolated biological materials normally show different mechanical properties upon re-implantation, their use is limited since they do not resemble the same mechanical properties as the original tissue they are derived from.

[005] Thus, it is an object of the present invention to improve the properties of biological materials for their use in tissue engineering.

SUMMARY OF THE INVENTION

[006] In a first aspect the present invention is directed to a three-dimensional hybrid scaffold for tissue engineering comprising:

- a first layer made of a decellularized biological material;
- a second porous layer connected to the surface of the first layer, wherein the second layer is a porous bioadhesive; and
- a third porous layer connected to the surface of the second layer which is located opposite the surface to which the first layer is connected, wherein the third layer is a three-dimensional porous polymer scaffold.

[007] In another aspect, the present invention refers to a method of manufacturing a three-dimensional hybrid scaffold according to any of the preceding claims, the method comprising:

- providing a first layer made of a biological material which has been decellularized;
- applying a second layer to the surface of the first layer, wherein the second layer is a bioadhesive and wherein the second layer is applied to the first layer at a temperature of the environment which is below the freezing temperature of the bioadhesive; and
- applying a third layer which is a three-dimensional porous scaffold to a side of the second layer which is not the side facing the first layer.

[008] In another aspect, the present invention is directed to the use of a three-dimensional hybrid scaffold of the present invention or a hybrid scaffold manufactured according to the method of the present invention for autologous, allogenic, xenogenic transplantation of tissue.

[009] In another aspect, the present invention is directed to the use of a three-dimensional hybrid scaffold of the present invention or a hybrid scaffold manufactured according to the method of the present invention for the manufacture of or use as a medicament.

BRIEF DESCRIPTION OF THE DRAWINGS

[010] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

[011] **Fig. 1** shows SEM micrographs of the hybrid scaffold of the present invention. Fig. 1a-b and d-e indicate different layers of a hybrid scaffold. Fig. 1a shows a cross section of a hybrid scaffold obtained by electrospinning onto fibrin coated porcine esophageal tissue maintained at subzero temperatures. It consists of an open cryogenic electrospun scaffold, a porous fibrin interface and a decellularized porcine esophageal tissue, (b) the porous fibrin interface of the hybrid scaffold, (c) top surface of the cryogenic electrospun scaffold. Fig. 1(d) shows a cross section of a hybrid scaffold obtained by electrospinning onto fibrin coated mucosa at ambient temperature, (e) the non-porous fibrin interface corresponding to (d), (f) top surface of the electrospun scaffold. In Fig. 1 a hybrid scaffold of the present invention (Fig. 1 a-c) is compared to a hybrid scaffold in which the bioadhesive layer was applied to the first layer at ambient temperature (Fig. 1 d-e). Fig. 1(b) shows the three different layers comprised in the hybrid scaffold of the present invention. In this example, the layer on the left side of the picture is a decellularized porcine esophageal tissue (ECM). It appears as a dense but porous collagenous layer preserved in its native histo-architecture. The bonding interface between the ECM and the bioadhesive is indicated with a dotted-line. The bioadhesive interface shown in Fig. 1(b) is porous with pores ranging between about 10 to 200 μm . In addition, Fig. 1f shows an example of a third layer which was electrospun without lowering the temperature of the surrounding atmosphere below the freezing point of water vapour in the atmosphere. In contrast, a hybrid scaffold in which the third layer was composed using cryogenic electrospinning as described herein provides a much larger pore size in the third layer (Fig. 1c).

[012] **Fig. 2a** shows a schematic illustration of the basic setup for conventional electrospinning. The insets show a drawing of the electrified Taylor cone and a typical SEM image of a non-woven mat deposited on a collector. **Fig. 2b** shows the setup for electrospinning using a rotating mandrel as collector. The first layer of the hybrid scaffold already connected to the second layer can be connected to the surface of this

collector so that the second layer acts as the collector for electrospinning a scaffold on the surface of the bioadhesive layer.

[013] **Fig. 3** shows a process flow of cryogenic electrospinning process (CHIEF). **Fig. 3** illustrates the principal of electrospinning used in an example of the present invention for applying the third layer at the surface of the second layer. In **Fig. 3** it is shown that the surface of the collector is chilled to a temperature below the freezing temperature of the water comprised in the surrounding atmosphere. In the method of the present invention, the chilled mandrel is coated with the first and the second layer of the hybrid scaffold. At those low temperatures ice crystals form on the surface of the chilled mandrel. After the first ice crystals have been formed electrospinning of the polymer solution starts. A fibrous scaffold forms with ice crystals embedded. After electrospinning is finished, a hybrid scaffold connected to the mandrel is detached from the chilled mandrel and freeze-dried to remove the ice crystals and to obtain the three-dimensional porous hybrid scaffold.

[014] **Fig. 4** shows SEM micrographs of (a) conventionally electrospun scaffold, (b) cryogenic electrospun scaffold (CES) and (c) oblique view showing three-dimensional structure of a pore in a cryogenic electrospun scaffold. Polymer scaffolds produced via conventional electrospinning (**Fig. 4a**) are characterized by a random polymer fiber mesh. However, the pore size between the fibers in such a polymer fiber mesh is only between several nanometer to a few micrometer. In contrast, the large pores obtained using the cryogenic method for electrospinning described herein can measure between about 50 to 500 μm in size (**Fig. 4b**). However, pores obtained with this method can exceed 500 μm . These pores are bounded by bundles of fibers that form a strut-like support. As can be seen from a SEM picture taken at an oblique view (**Fig. 4c**), the large pores have a three-dimensional spatial structure and are interconnected via their thin fibrous walls, which itself are porous like conventionally electrospun fibers.

[015] **Fig. 5** shows SEM micrographs of the cryogenic electrospun scaffolds collected at different relative humidity of the chamber. (a) 25%, (b) 40% and (c) 55%. It can be observed that as humidity increases from 25% to 55%, the pores of the cryogenic electrospun scaffolds become larger and more defined.

[016] **Fig. 6** shows the SEM micrographs of cryogenic electrospun scaffolds collected at different temperatures to illustrate the effect of the temperature on the pore size of an electrospun scaffold. The two pictures in the top row show conventional electrospun scaffolds which were electrospun at 23 °C. The left picture in the top row shows the mandrel interface whereas the right picture shows the air interface. For definition purposes it should be mentioned that the “mandrel interface” is the side of the scaffold facing the mandrel and the side of the scaffold facing the air is called the “air interface”. In the middle row of pictures, the left picture shows the mandrel interface of a scaffold electrospun at -15 °C whereas the right picture shows the air interface of this scaffold. In the bottom row of pictures, the left picture shows the mandrel interface of a scaffold electrospun at -30 °C whereas the right picture shows the air interface of this scaffold. It can be observed that a conventional dense electrospun scaffold is obtained when the mandrel temperature is kept at 23 °C. When the mandrel temperatures are -15°C and -30°C, large pore structures (> 5 µm) can be observed on both the mandrel and air interfaces of the scaffold. As illustrated in Fig. 3, ice crystals formed on the mandrel at sub-zero temperatures are embedded within the electrospun mesh. The subsequent removal of the ice crystals through freeze-drying forms these pore structures within the electrospun mesh.

[017] **Fig. 7 (a), (c) and (e)** are SEM micrographs showing the pore structures of cryogenic electrospun scaffold after 5, 10 and 15 minutes of spinning without any time interval in between spinning respectively. **Fig. 7 (b), (d) and (f)** are SEM micrographs showing the pore structures of cryogenic electrospun scaffold after 5, 10 and 15 minutes of spinning with a time interval of 5 minutes in between each cycle. From Figure 7, both Samples A (**Fig. 7 (a), (c) and (e)**) & B (**Fig. 7 (b), (d) and (f)**) are porous throughout the thickness of the scaffold, as observed by the slow building up over the 5, 10 and 15 minutes. However, pore sizes are smaller in the early stage, becoming bigger as the spinning proceeds. Sample B has pore structures that are larger in diameter and shallower, as compared to Sample A.

[018] **Fig. 8** shows the results of *in vivo* experiments in which decellularized porcine esophageal ECM, CES and conventional dense electrospun scaffolds were implanted in rats. **Fig. 8** shows the *in vivo* cell infiltration and vascularization in scaffolds obtained

from those rats. Fig. 8 shows hematoxylin and eosin staining of scaffolds implanted subcutaneously for 14 days. (a) Decellularized porcine esophageal ECM at 100 x magnification, (b) Decellularized porcine esophageal ECM at 400 x magnification, (c) CES at 400 x magnification and (d) Conventional dense electrospun scaffold at 400 x magnification. (S: Scaffold, B: Capillaries).  represents 200 μm in (a) and 50 μm in (b), (c) and (d). The pictures lying next to each other in Fig. 8 are identical but the left hand picture is a black & white print of a color picture whereas the right hand picture is a grey scale version of the color picture on the left hand side. Areas of cell infiltration are circled with dotted lines.

DETAILED DESCRIPTION OF THE INVENTION

[019] A method of manufacturing a three-dimensional hybrid scaffold according to any of the preceding claims, the method comprising:

- providing a first layer made of a biological material which has been decellularized;
- applying a second layer to the surface of the first layer, wherein the second layer is a bioadhesive and wherein the second layer is applied to the first layer at a temperature of the environment which is below the freezing temperature of the bioadhesive; and
- applying a third layer which is a three-dimensional porous scaffold to a side of the second layer which is not the side facing the first layer.

[020] This hybrid scaffold has been shown to positively influence cell-scaffold interaction such as cell attachment, migration, proliferation and function. The hybrid scaffolds manufacture by this method can be used to (1) tailor the mechanical properties of the treated ECMs to comply with host tissues, (2) replace excised tissue segments by providing a thick fibrous scaffold with large pores for cell infiltration and vascularization and (3) organize cells attachment and proliferation along aligned fibers.

[021] Another important aspect of the third layer is that it can be used to deliver drugs, growth factors and proteins. These components can be applied together with the scaffold and the scaffold can serve as carriers to these drugs or molecules. Some possible applications are as follows. For example, the hybrid scaffold can be used to make

vascular grafts for replacement of blocked coronary artery. This vascular graft consists of a decellularized blood vessel (first layer), porous bioadhesive (second layer) and electrospun fibers containing an anti-coagulant drug (e.g. heparin) (scaffold forming third layer). The release of the anti-coagulant reduces the thrombogenic reaction at the implant site and this release can be controlled by the pore size of the scaffold and the diameter of the fibers of the scaffold. The graft can be used to replace diseased coronary artery with a drug-releasing third layer on the adluminal side and the decellularized vascular ECM on the abluminal side. Another possible application is in promoting better wound healing. Transforming growth factor beta-3 (TGF- β 3) has been shown to improve scarring during wound healing (Ferguson, M.W.J., O'Kane, S., 2004, vol.359(1445), p.839-850). TGF- β 3 can be incorporated into the electrospun fibers in the scaffold forming the third layer. Controlled release of TGF- β 3 during wound healing will reduce scar formation when the hybrid scaffold is implanted. Hence, the incorporation of a third layer is advantageous to the hybrid design.

[022] An additional advantage is that the bioadhesive layer of the scaffold of the present invention is porous. Normally, bioadhesives are applied to connect different components with each other. For example, in US 2004/0005297 A1 fibrin glue has been used as bioadhesive to seal different layers with each other. However, the fibrin glue is applied in a way that seals and separates the different layers from each other not allowing ingrowth of tissue in this sealant layer. However, in the method and the hybrid scaffold of the present invention the bioadhesive layer is porous which allows cells to attach, migrate and proliferate through and over this layer. Thus, the hybrid scaffold of the present invention provides a continuous structure allowing development of new tissue in every section as will be explained in more detail in the following.

[023] The term "three-dimensional porous scaffold" as used herein, refers to an artificial structure capable of supporting a three-dimensional tissue formation. Scaffolds are supposed to resemble the connective tissue in an extracellular matrix. Thus, scaffolds allow for cell attachment, migration and growing of cells and synthesis of extracellular matrix components and biological molecules specific to the tissue targeted for replacement. To achieve those objects, a scaffold ideally provides a high porosity and proper pore size, a high surface area, biodegradability, proper degradation rate to match

the rate of neotissue formation and it should provide a sufficient mechanical integrity to maintain the predesigned tissue structure. A scaffold should also not be toxic to the cells (i.e. biocompatible) and should positively interact with cells including enhanced cell adhesion, growth, migration, and differentiated function (Ma, P.X., May 2004, *Materials Today*, p.30-40).

[024] "Biological material" composed of naturally occurring extracellular matrix (ECM) has received significant attention for their potential therapeutic application in tissue engineering. In general, the biological material has been isolated from a part of the body of an individual. The ECM is custom designed and manufactured by the resident cells of each tissue and organ and is in a state of dynamic equilibrium with its surrounding microenvironment and thus the ideal source for the construction of hybrid scaffolds. The structural and functional molecules of the ECM provide the means by which adjacent cells communicate with each other and with the external environment. The ECM is obviously biocompatible since host cells produce their own matrix.

[025] The ECM is obviously biocompatible since host cells produce their own matrix. The ECM also provides a supportive medium or conduit for blood vessels, nerves and lymphatics and for the diffusion of nutrients from the blood to the surrounding cells. In other words, the ECM possesses all of the characteristics of the ideal tissue engineered scaffold or biomaterial. Various forms of intact ECM have already been used as biological scaffolds to promote the constructive remodeling of tissues and organs (Meyer, T., Chodnewska, I., et al., 1998, *Transplant Proc*, vol.30, p.354 et seqq.; Robinson, K.A., Matheny, R.G., 2003, *J Am Coll Cardiol*, vol.41, no.6, suppl. 2, p.514 et seqq.; Schenke-Layland, K., Vasilevski, O., et al., 2003, *J Struct Biol*, vol.143(3), p.201-208; Sutherland, R.S., Baskin, L.S., et al., 1996, *J Urol*, vol.156, p.571-577; Valentin, J.E., Badylak, J.S., et al., 2006, *J Bone Joint Surg Am*, vol.88(12), p.2673-2686).

[026] The constructive remodeling induced by ECM scaffold materials and their widespread use across many clinical applications are a consequence of their bio-inductive properties, mechanical and material properties, the host tissue response to naturally occurring ECM, and the degradation properties of the material.

[027] In general, a "biological material" can be from any tissue of the body of an individual or different individuals. Related cells joined together are collectively referred

to as biological tissue. The cells in a tissue are not identical, but they work together to accomplish specific functions. A sample of tissue removed for examination under a microscope (biopsy) contains many types of cells. Connective tissue is the tough, often fibrous tissue that binds the body's structures together and provides support. It is present in almost every organ, forming a large part of skin, tendons, and muscles. The characteristics of connective tissue and the types of cells it contains vary, depending on where it is found in the body.

[028] The body's functions are conducted by organs. Each organ is a recognizable structure – for example, the heart, lungs, liver, eyes, and stomach – that performs specific functions. An organ is made of several types of tissue and therefore several types of cells. For example, the heart contains muscle tissue that contracts to pump blood, fibrous tissue that makes up the heart valves, and special cells that maintain the rate and rhythm of heartbeats. The eye contains muscle cells that open and close the pupil, clear cells that make up the lens and cornea, cells that produce the fluid within the eye, cells that sense light etc. Even an organ as apparently simple as the gallbladder contains different types of cells, such as those that form a lining resistant to the irritative effects of bile, muscle cells that contract to expel bile, and cells that form the fibrous outer wall holding the sac together.

[029] The biological tissue referred to in the present invention has been isolated from a part of the body of an individual and can be from any tissue or organ or part of an organ of an individual. It can for example be derived from the small intestine, liver, pancreas, urinary bladder, stomach, bladder, vascular system, bile duct, alimentary canal, respiratory tract, kidney, spleen, heart, heart valve, bone, skin or fragments or parts thereof. In one example, it is derived from the gastrointestinal tract, namely the esophagus mucosa.

[030] The term "hybrid scaffold" refers to a combination or composition comprising a "biological material" and a "scaffold" wherein these two components are connected to each other via a bioadhesive layer.

[031] The individual from which the biological tissue is derived can be a mammal, reptile or insect. In general it can be taken from any animal which tissue or organs are suitable to be implanted in a host and which are not rejected by the immune system of the

host or show at least only little rejection which could be suppressed by commercial drugs. In one example, the biological tissue is derived from a mammal which can be of porcine, bovine or ovine origin or can be a rabbit, monkey or human.

[032] In one example, the biological material is decelluralized, i.e. the material is completely acellular. Methods for decelluralizing biological material are known in the art and are described, for example in a review by Gilbert, T.W., Sellaro, T.L., et al. (2006, *Biomaterials*, vol.27, p.3675-3683), the content of it is incorporated herein by reference in its entirety. Thus, in one example of the method of the present invention, the method further comprises decellularizing the biological material before applying the bioadhesive second layer at the surface of the biological material forming the first layer.

[033] The goal of a decellularization protocol is to efficiently remove all cellular and nuclear material while minimizing any adverse effect on the composition, biological activity, and mechanical integrity of the remaining ECM of the biological material which forms the first layer in the hybrid scaffold of the present invention.

[034] Any processing step intended to remove cells will alter the native three-dimensional architecture of the ECM. The most commonly utilized methods for decellularization of tissues or parts of it involve a combination of physical and chemical treatments. The physical treatments can include agitation or sonication, mechanical massage or pressure, or freezing and thawing. These methods disrupt the cell membrane, release cell contents, and facilitate subsequent rinsing and removal of the cell contents from the ECM. These physical treatments are generally insufficient to achieve complete decellularization and must be combined with a chemical treatment. Enzymatic treatments, such as trypsin, and chemical treatment, such as ionic solutions and detergents, disrupt cell membranes and the bonds responsible for intercellular and extracellular connections.

[035] As previously mentioned, tissues are composed of both cellular material and ECM arranged in variable degrees of compactness depending on the source of the tissue. The ECM must be adequately disrupted during the decellularization process to allow for adequate exposure of all cells to the chaotropic agents and to provide a path for cellular material to be removed from the tissue leaving behind only the ECM which is used as biological material in the hybrid scaffold. The intent of most decellularization

processes is to minimize the disruption and thus retain native mechanical properties and biological properties. The most robust and effective decellularization protocols include a combination of physical, chemical, and enzymatic approaches. A decellularization protocol as described in the examples of the present application generally begins with lysis of the cell membrane using physical treatments or ionic solutions, followed by separation of cellular components from the ECM using enzymatic treatments, solubilization of cytoplasmic and nuclear cellular components using detergents, and finally removal of cellular debris from the tissue. These steps can be coupled with mechanical agitation to increase their effectiveness. Following decellularization, all residual chemicals should be removed to avoid an adverse host tissue response to the chemical. The efficiency of decellularization and preservation of the ECM can be assessed by several methods. The mechanisms of physical, enzymatic, and chemical decellularization for a variety of tissues are reviewed and displayed in Table 1 of the article of Gilbert, T.W., Sellaro, T.L., et al. (2006, *supra*).

[036] In an example of the present invention decellularization was carried out using a multistep method. Firstly, the biological material isolated from the body of an individual was rinsed in saline solution to remove cellular debris. Secondly, the biological material is left to immerse in deionized water at about 1 to 6 degrees Celsius for 30 min to 1 hour. Thirdly, a decellularizing agent comprising hydrogen peroxide, glacial acetic acid and deionized water in a ration of 20:40:40 vol.% is prepared. The biological tissue was immersed in this solution at ambient temperatures from 4 °C to 40 °C. Afterwards, the biological material is decellularized mechanically. Then the biological material was neutralized. The next step is the treatment with TritonX-100 solution in concentrations ranging from 0.05% to 5%. In another example, concentrations of 0.5% to 3 % have been used. The biological material was immersed in TritonX-100 for about 6 to 48 hours. In one example, it has been treated for 12 to 24 hours. Gentle stirring helped to remove the remaining cells from the biological material. Afterwards, the biological material is rinsed to remove all traces of TritonX-100. A more detailed description of an illustrative example can be found in the experimental section of this application. This, method allows for example also decellularization of whole organs, like kidney, liver, esophagus etc.

[037] Thus, the advantages of fabricating tissue engineering scaffolds by decellularization of tissues are the preservation of native topography of ECMs, inherent histo-architectures of structural collagen and original configurations of functional proteins. These are components necessary in promoting cell attachment and proliferation. Acellular matrices are biodegradable and they are generally resorbed during tissue remodeling processes. In a particular case, it has been reported that the degraded products of acellular matrices promoted migration of endothelial cells, hence inducing angiogenesis within the matrices (Li, F., et al., 2004, Endothelium, vol.11, p.199-206).

[038] These decellularizing processes can weaken the mechanical properties of the treated biological material. In addition, tissue segments such as muscles, which are not intended for decellularization, may be removed during the processes. These tissues segments may need to be replaced with the acellular ECMs in the reconstruction of the entire tissue. This is only one example why the scaffold and the method of the present invention connect a third layer via a second layer to the first layer, namely to compensate the structural deficiencies of the first layer weakened, for example, in a process of decellularization or upon removal of the biological material from the body of the individual.

[039] The second layer of the hybrid scaffold of the present invention is made of a bioadhesive. In general, an adhesive is a material capable of fastening two other materials together by means of surface attachment. The words glue, mucilage, mastic, and cement are synonymous with adhesive. In a generic sense, the word adhesive implies any material capable of fastening by surface attachment, and thus will include inorganic materials. In a practical sense, however, adhesive implies the broad set of materials composed of organic compounds, mainly polymeric, which can be used to fasten two materials together. The materials being fastened together by the adhesive are the adherents, and an adhesive joint or adhesive bond is the resulting assembly. Adhesion is the physical attraction of the surface of one material for the surface of another.

[040] Examples of organic adhesives which can be used in the method of the present invention for the manufacture of the scaffold of the present invention are, for example, fibrin glue, polyvinylpyrrolidone, polyvinylpyrrolidone/vinyl acetate copolymers,

cyanoacrylate gel, platelated gel, chitosan or gelatin-resorcin-formaldehyde (GRFG). In one example, fibrin glue is used to connect the first and third layer of the hybrid scaffold.

[041] For example, cyanoacrylate gel, fibrin glue and GRFG are known as bioadhesives and are described, for example, in the article of Albes, J.M., Krettek, C. et al. (1993, *The annals of Thoracic Surgery*, vol.56, p.910-915).

[042] Fibrin sealants or fibrin glues are a type of surgical tissue adhesive derived from human and animal blood products (Thorn, J.J., Sørensen, H., et al., 2004, *Int. J. Oral Maxillofac. Surg.*, vol.33, p.95-100). The ingredients in these sealants interact during application to form a stable clot composed of a blood protein called fibrin. Fibrin sealants are known since World War II. All fibrin sealants in use as of 2003 have two major ingredients, purified fibrinogen (a protein) and purified thrombin (an enzyme) derived from human or bovine (cattle) blood. Many sealants have two additional ingredients, human blood factor XIII and a substance called aprotinin, which is derived from cows' lungs. Factor XIII is a compound that strengthens blood clots by forming cross-links between strands of fibrin. Aprotinin is a protein that inhibits the enzymes that break down blood clots.

[043] For preparation and application of fibrin sealants the thrombin and fibrinogen are freeze-dried and packaged in vials that must be warmed before use. The two ingredients are then dissolved in separate amounts of water. Next, the thrombin and fibrinogen solutions are loaded into a double-barreled syringe that allows them to mix and combine as they are sprayed on the surface.

[044] As the thrombin and fibrinogen solutions combine, a clot develops in the same way that it would form during normal blood clotting through a series of chemical reactions known as the coagulation cascade. At the end of the cascade, the thrombin breaks up the fibrinogen molecules into smaller segments of a second blood protein called fibrin. The fibrin molecules arrange themselves into strands that are then cross-linked by a blood factor known as Factor XIII to form a lattice or net-like pattern that stabilizes the clot. An example of a commercially obtainable fibrin sealant is TISSEEL (Baxter International Inc.).

[045] Recent developments of fibrin sealants include a delivery system that forms a fibrin sealant from the individuals own blood within a 30-minute cycle. The use of the

individuals own blood lowers the risk of allergic reactions to blood products derived from animal or donated blood. Platelet gel is a similar autologous glue where a platelet concentrate prepared from the individuals plasma substituted the fibrinogen concentrate (Hood, A.G., Hill, A.G., 1993, Proceedings of the American Academy of cardiovascular perfusion, vol.14, p.126-129).

[046] Another suitable group of adhesives is organic polymeric compositions represented by the group of alkyd resins, polyvinyl acetaldehydes, polyvinyl alcohols, polyvinyl acetates, poly(ethylene oxide), polyacrylates, ketone resins, polyvinylpyrrolidone, polyvinylpyrrolidone/vinyl acetate copolymer, polyethylene glycols of 200 to 1000 molecular weight and polyoxyethylene/polyoxopropylene block copolymers (Polyox), silicone resins and silicone based pressure sensitive adhesives such as those available from Dow Corning Company under the trade designation BIO-PSA. The pressure sensitive adhesive (PSA) are well known in the art and many are commercially available.

[047] To achieve porosity of this bioadhesive second layer it is important to apply the bioadhesive at a temperature which is at or below the freezing temperature of the solvent of the bioadhesive. If the bioadhesive layer is applied at ambient temperature, i.e. a temperature above the freezing temperature, a dense layer is formed which does not allow cell infiltration (see Fig. 1 d and e). Such a non-porous film also acts as a barrier to nutrient diffusion.

[048] However, applying bioadhesives at a temperature at or below the freezing temperature of the bioadhesive results in the formation of crystals (from the solvent of the bioadhesive) in the bioadhesive layer which form the porous system upon removal of the crystals. Such crystals can be removed by sublimation which means that the solid crystals pass directly into the gas phase without becoming liquid beforehand. A method for achieving sublimation can be freeze-drying. The pore size within the bioadhesive layer can be in a range of about 1 nm to about 500 μm . In another example, the pore size is about 50 μm to about 500 μm or 100 μm to about 300 μm . Which range will be chosen depends also on the desired tissue which is supposed to be replaced. Thus, further ranges which can be used will be mentioned further below. The pore size of the second and third porous layer can be the same or different.

[049] Fig. 1 a and b depict hybrid scaffolds in which the bioadhesive second layer has been formed at or below the freezing temperature of the bioadhesive. As can be seen, the bioadhesive layer forms a porous network with pores having a size of about 10 to 200 μm . This is a positive step towards attaining a wholly porous hybrid scaffold that would be advantageous in nutrient diffusion and cell infiltration in *in vitro* and *in vivo* systems. Another advantage of the porous interface is that it will promote cell infiltration and vascularization and thus facilitates remodeling of the scaffold construct *in vivo*.

The bioadhesive layer can be applied using any method which allows freezing the bioadhesive layer upon application. Examples of such methods include for example spraying, electrospraying, electrospinning, spin coating, dip coating, casting and brushing.

[050] Electrospraying is a method in which a liquid (in this case, a solution of bioadhesive) is atomized into a mist of droplets by electrical forces and is described, for example, in the article of Huang, J., Jayasinghe, S.N., et al. (2004, Journal of Materials Sciences, vol.39(3), p.1029-1032). Dip coating is a method in which the substrate (e.g. ECM) can be dipped into a solution of bioadhesive in a process similar to that described, for example, in the article of Liu, Z.F., Jin, Z.G., et al. (2006, Journal of Sol-Gel Science and Technology, vol.40(1), p.25-30). In dip coating, the thickness of the bioadhesive coating depends on the viscosity of the solution, the speed of substrate withdrawal from the solution and the time which the substrate is immersed in the solution, all of which are known in the prior art. Spin coating is a method in which a dilute solution of the bioadhesive is applied on the ECM and the ECM is spun at high speeds (e.g. 3000 to 5000 rpm) to obtain a thin coat of bioadhesive on the surface of the ECM. The process is described, for example, in the article of Dupont-Gillain, C.C. and Rouxhet, P.G. (2001, Nano Letters, vol.1(5), p.245-251). Casting is a method in which the bioadhesive is spread on the substrate (e.g. ECM) to obtain a uniform layer of bioadhesive on the ECM in a process similar to that described, for example, in the article of Smith, S., Stolle, D. (2000, vol.40(8), p.1870-1877).

[051] In general, the third layer needs to be applied before the bioadhesive is fully set/crosslinked in order to ensure a strong connection between the different layers of the hybrid scaffold. The surface of the scaffold of the third layer facing the bioadhesive layer

should be wetted with the bioadhesive in order to form sufficient bonding with the setting and freezing bioadhesive. A person skilled in the art can easily determine the point of time before the bioadhesive is fully set/crosslinked because the bioadhesives turn from a liquid phase into a gelling liquid and eventually to a solid film when cured. The third layer should be applied during the initial or gelling phase of the bioadhesive layer.

[052] For example, the components of the bioadhesive fibrin which are fibrinogen and thrombin are reconstituted separately into solutions according to procedures described in the instruction for use, for example, of the Tisseel Kit (Baxter International Inc.). Both of these solutions should be clear or slightly opalescent. On mixing the two solutions, the mixture turns cloudy and curing begins. After change of the phase, from solution form to a gelling liquid the scaffold should be applied. When electrospinning is used for the third layer (see further below) the fibers can be deposited during the solution and gelling liquid phase, when bonding between the bioadhesive and fibers may occur. The curing time of the bioadhesive can be shortened by either increasing the thrombin concentration, decreasing the aprotinin concentration or increasing the temperature of the mixture, all of which are known in the prior art.

[053] In another aspect, the method of the present invention further comprises controlling the degradation time of the bioadhesive layer by controlling the pore size of the bioadhesive layer which is achieved by increasing or decreasing the temperature in the environment when applying the bioadhesive layer, as long as the temperature is still at or below the freezing point of the bioadhesive. Lower temperatures result in faster rate of crystal formation and smaller solid crystals. That means that at a lower pore size within the bioadhesive layer, the degradation of the bioadhesive takes longer because the enzymes and cells growing in the area surrounding the scaffold, which is seeded with cells and possibly already implanted into the body of an individual, take longer to degrade the bioadhesive.

[054] Controlling the degradation time of the bioadhesive can be an advantage, when one wished to synchronize the degradation rate of the bioadhesive layer to the rate of remodeling by the infiltrating host cells. If the bioadhesive degrades too fast, the first layer might delaminate from the third layer in the host; conversely, if the bioadhesive or the entire hybrid scaffold degrades too slowly, remodeling takes place with scarring

around and within the construct. The hybrid scaffold might not be integrated with the host for its intended application due to the formation of excessive collagenous scar.

[055] In one example, the pore size of a bioadhesive can be changed by varying the water content in the bioadhesive solution. For example for fibrin glue, the pore size can be influenced by changing the concentration of fibrinogen and thrombin in the aqueous medium, thus changing the amount of ice within the fibrin layer. The more water in the bioadhesive solution the larger the ice crystals grow.

[056] Another factor influencing the degradation time of the bioadhesive layer is the thickness of the bioadhesive second layer. The thicker the layer the longer the degradation of the whole layer takes.

[057] In one example, the thickness of the bioadhesive second layer can be about 10 to 1000 μm or 20 to 600 μm before the third layer is applied onto the second layer. In another example the thickness is about 20 to 300 μm . However, bioadhesive layers having a thickness that exceeds 1000 μm , namely 2, 3 or even more millimeter up to 1 cm would also be possible.

[058] The third layer is a porous three-dimensional scaffold. Scaffolds are widely known in the area of tissue engineering and every scaffold which can be connected to the first layer via the bioadhesive layer can be used in for the hybrid scaffold and in the method of the present invention. The scaffolds used in the present invention comprise a reticulated structure of interconnected pores. The third layer can be for example a scaffold which is obtained via electrospinning, i.e. an electrospun scaffold.

[059] Depending on the use of the claimed method, scaffold material can be biodegradable. To use biodegradable material is especially advantageous, e.g., for tissue engineering wherein the scaffolds containing the cells are used to repair defect sites in living tissue, e.g. bone. A high variety of scaffolds can be used dependent on the application. Scaffolds comprise or can be made from agarose, polycaprolactone (Endres, M. et al., Tissue Engineering, 2003, Vol.9, No.4, P.689-702), niobium coated carbon, chitosan, hydroxyapatite-tricalcium phosphate (Harris, C.T. and Cooper, L.F., Comparison of matrices for hMSC delivery, 2004, P.747-755), collagen, hyaluronic acid, calcium phosphate, starch, hydroxyapatite, fibrin, alginate, poly-glycolic acid, carbon nano fibres, polytetrafluoroethylene, polylactic acid (Moran, J. et al., Tissue Engineering,

2003, Vol.9, No.1, P.63-70) and mixtures thereof, for example. Foam scaffold as those described in US Patent 6,231,879 which are based on thermoplastic elastomers such as polyamide, polyester, polyethylene polyvinylidene fluoride, polyethyurethane or silicone can also be used in the present invention.

[060] The scaffold can have a regular or an irregular (outer) shape. If the scaffolds are, e.g., used in tissue engineering the shape of the scaffold will fit the shape of the defect side in which the scaffold will be implanted or at least will fill up the part not already covered by the biological material of the hybrid scaffold. A scaffold with a regular shape can be rectangular, a square, or of polyhedric or spherical shape. Scaffold of a rectangular shape usually have a length in their largest dimension of about 1 mm to about 5 cm or even 20 cm.

[061] The shape of the scaffold forming the third layer depends in particular on the shape and form of the biological material forming the first layer. The third layer advantageously rounds out the missing characteristics of the first layer. For example, the scaffold of the third layer provides the mechanical stability within a defect side which the biological material of the first layer cannot provide any more. The scaffolds made of the materials listed above can be manufactured in any specific size and form depending of the application.

[062] Even though any commercially available scaffold can be used as third layer, it is also possible to create the scaffold directly on the surface of the bioadhesive second layer which is already attached to the first layer. Thus, in one aspect of the present invention, the scaffold is created by direct electrospinning on the surface of the bioadhesive second layer.

[063] Unlike other methods for generating nanostructures, the formation of a thin fiber for a scaffold via electrospinning is based on the uniaxial stretching (or elongation) of a viscoelastic jet derived from a polymer solution or melt. This technique is similar to the commercial processes for drawing microscale fibers except for the use of electrostatic repulsions between surface charges (rather than a mechanical or shear force) to continuously reduce the diameter of a viscoelastic jet or a glassy filament. Compared with mechanical drawing, electrostatic spinning is better suited for generating fibers with much thinner diameters, since the elongation can be accomplished via a contactless

scheme through the application of an external electric field. Like mechanical drawing, electrospinning is also a continuous process and therefore should work well for high-volume production (Li, D. & Xia, Y.N., 2004, *supra*).

[064] In electrospinning, a solid fiber is generated as the electrified jet (composed of a highly viscous polymer solution, see further below) is continuously stretched due to the electrostatic repulsions between the surface charges and the evaporation of solvent. As the fiber travels toward the surface of the collector, evaporation of the solvent in which the polymer is dissolved occurs and the fiber is typically dry when arriving at the surface of the collector (see Fig. 2 a and b).

[065] Therefore, the terms “electrospinning” or “electrospun” as used herein refer to any method where materials are streamed, sprayed, sputtered or otherwise transported in the presence of an electric field. The electrospun solution comprising at least one polymer can be deposited from the direction of a charged container towards a grounded collector, or from a grounded container in the direction of a charged collector.

[066] Thus, the third layer can be made of a scaffold which is directly electrospun on the bioadhesive second layer. However, it should be noted that also the second layer can be applied on the first layer using electrospinning. Thus, the following arguments regarding the options of modifying electrospun scaffolds can also be transferred to the electrospinning of the bioadhesive second layer.

[067] However, polymer scaffolds produced via conventional electrospinning, which can certainly be used herein as third layer, are often characterized by a random polymer fiber mesh as illustrated by the SEM picture of such a mesh in Fig. 2a and Fig. 4a. However, the pore size between the fibers in such a polymer fiber mesh is only between several nanometer to a few micrometer.

[068] But some applications require a bigger pore size in order to enable cells seeded in the hybrid scaffold to grow and develop the tissue they are supposed to resemble. For example, Klawitter et al. reported (1976, J Biomed Mater Res, vol.10(2), p.311-323) that for adequate bone regeneration to occur in a scaffold, scaffold pore size needs to be at least 100 micrometer. It is generally known in the art that optimal bone regeneration occurs for pore sizes between 300 to 600 micrometer. For other applications the following pore sizes have been reported to be optimal for the specific applications:

about 5 μm for neovascularization, between about 5 to about 15 μm for fibroblast ingrowth, about 20 μm for hepatocyte ingrowth, between about 20 to 125 μm for skin regeneration, between about 70 to 120 μm for chondrocyte ingrowth, between about 40 to 150 μm for fibroblast binding, between about 45 to 150 μm for liver tissue regeneration, between about 60 to 150 μm for vascular smooth muscle cell binding, between about 100 to 300 μm for bladder smooth muscle cell adhesion and ingrowth, between about 100 to 400 μm for bone regeneration and between about 200 to 350 μm for osteoconduction.

[069] Thus, the pore size of the two layers which are not natural, namely the bioadhesive second layer and the third layer made of an artificial three-dimensional scaffold should match or come close to the pore size required for replacing a certain type of tissue or whole organ.

[070] For example, the dense ECM (first layer) can be used as a barrier for cells (e.g. epithelial cells, endothelial cells) that need a basal membrane for attachment, proliferation and differentiation. The open porous structure of the porous electrospun (third layer) can be used to promote cell infiltration and vascularization and is important in reconstruction of thick tissues (e.g. muscle). Some examples of cells or combination of cells that can be grown on the hybrid for tissue replacement are described in the following. Esophagus – Epithelial cells on decellularized esophageal ECM (first layer) and smooth muscle cells in porous electrospun scaffold (third layer). Blood vessels – Endothelial cells on decellularized blood vessel (first layer) and smooth muscle cells in porous electrospun scaffold (third layer). Skin – Keratinocytes on decellularized dermis and dermal fibroblasts in porous electrospun scaffold (third layer). Bladder – Urothelial cells on decellularized bladder (first layer) and smooth muscle cells in porous electrospun scaffold (third layer).

[071] Obtaining pore sizes as described in paragraph 68 using electrospinning is possible due to the following method. In this method a three-dimensional scaffold for tissue engineering is manufactured using an apparatus for electrospinning comprising a high-voltage power supply; at least one spinneret connected to at least one container comprising a solution with at least one polymer dissolved therein; and a collector which can already be connected to the first and second layer of the hybrid scaffold which are already connected to each other. The method comprises:

- forming crystals from a molecule or group of molecules in vapor phase comprised in the surrounding atmosphere at the surface of the second layer, wherein the atmosphere in the reaction chamber of the electrospinning apparatus comprising the collector or the first and second layer already connected to each other has a temperature which allows formation of crystals at the surface of the second layer;
- electrospinning the solution comprising at least one polymer dissolved therein around the crystals;
- continuing the formation of crystals and the electrospinning simultaneously; and
- removing the crystals by sublimation.

[072] In case the reaction chamber already contains the first and second layer which are already connected to each other, the bioadhesive second layer will form the collector for electrospinning.

[073] It should be noted that this specific technique of obtaining an electrospun scaffold, which can be used as third layer, can also be carried out separately to obtain the cryogenic scaffold. After obtaining such a cryogenic scaffold, the scaffold can be connected to the surface of said second layer.

[074] Formation of solid crystals in the above method can be achieved by either cooling the collector of the electrospinning apparatus itself to create a temperature gradient above the first and second layer connected to the collector or by cooling the atmosphere in the reaction chamber of the electrospinning apparatus to an extent that solid crystals are formed at the surface of the second layer.

[075] This method of electrospinning provides for the design of electrospun scaffolds at the surface of the bioadhesive second layer having different distributions of pore sizes to cater for different requirements in basal membrane formation (nano-scale), tissue remodeling and regeneration (nano to micro scale), vascularization (micro scale) and cell in-growth (micro-scale).

[076] When referring to "pores" in connection with the pores of the electrospun scaffold, it is not referred to the pores which might be formed in the fibers which are spun but the size of the three-dimensional pores formed by the fibers as can be seen, for

example, in Fig. 1a, 3, 4b, 4c, 5a, 5b and 5c. A conventional electrospun scaffold as shown, for example, in Fig. 1f and 4a shows a highly porous network of non-woven submicron fibers in a planar orientation. The pores are bounded by individual fibers, and measure only between several nanometer to a few micrometer. In contrast, the large pores obtained using the above method for electrospinning of a scaffold on the second layer of the hybrid scaffold measure in this illustrative example between about 50 to 500 μm in size (see Fig. 4b). However, it should be noted that such pores can exceed 500 μm . These pores are bounded by bundles of fibers that form a strut-like support. As can be seen from a SEM picture taken at an oblique view (Fig. 4c), the large pores have a three-dimensional spatial structure and are interconnected via their thin fibrous walls, which itself are porous like conventionally electrospun fibers.

[077] As can be seen from Fig. 3, this method of electrospinning involves in this example a reaction chamber cooled to subzero temperatures. In one example, a hollow rotating mandrel (see Fig. 2b) is used as collector. The first layer is directly connected to the collector and the bioadhesive second layer is applied on the first layer and cooled to provide a porous structure. In one example, the temperature used for freezing the bioadhesive second layer is the same temperature which is used for the cryogenic electrospinning as long as the temperature is equal or below the freezing temperature of the bioadhesive as well as the solvent in the polymer solution used for electrospinning. Subsequently, solid crystals made, for example, of water (H_2O) from the surrounding atmosphere of the collector form on the surface of the second layer and serve as a negative template around which electrospun fibers are deposited. After the scaffold reaches the desired form, the scaffold can be subsequently freeze-dried to remove ice crystals, leaving behind a cryogenic electrospun scaffold (CES) with large pores (see Fig. 4b and 4c). Further details for this illustrative example are given in the experimental section of this application.

[078] Thus, this cryogenic electrospinning technique enables the fabrication of an electrospun scaffold with large pores, while retaining the nanofibrous structure that mimics the physical environment of the targeted tissue or organ to be replaced and which is required for cell growth, vascular ingrowth and tissue development.

[079] The crystals which form at the surface of the second layer can be made of any molecule or group of molecules in vapor phase which is/are comprised in the atmosphere surrounding the collector and which is/are deposited at the surface of the second layer in form of solid crystals when the temperature at the surface of the second layer is lowered to or below the freezing point of the molecule or group of molecules which is/are comprised in the atmosphere surrounding the second layer. Since the question of forming crystals depends also on the pressure in the surrounding atmosphere, the pressure can be increased or decreased to support formation of crystals even at higher temperatures. Which pressure and temperature is most suitable to ease the freezing step of a certain group of molecules can be easily determined by a person skilled in the art when looking at the phase diagram of the molecule which shall form crystals at the surface of the collector.

[080] In one example, the crystals are ice crystals formed from water (H_2O) comprised in the surrounding atmosphere. After the scaffold reaches its final dimensions the crystals are removed by freeze-drying. Instead of water D_2O can also be used. In another example, the crystals are formed from CO_2 comprised in the surrounding atmosphere. Other than the water ice crystals which are removed by freeze-drying, the CO_2 crystals are removed by sublimation. The sublimation temperature of carbon dioxide (CO_2) is -78.5°C at atmospheric pressure. If the electrospinning environment is filled with vapor CO_2 , deposition of solid CO_2 crystals on the surface of the collector can be achieved if the collector is maintained at temperatures below -78.5°C . After formation of the electrospun scaffold the atmosphere surrounding the crystals are removed from the hybrid scaffold through sublimation of CO_2 into vapor phase at room temperature.

[081] The shape and thus the size of crystals depends also on the temperature at the surface of the second layer or the surrounding of the layer. For example, water has a freezing point of 0°C at atmospheric pressure. Just below freezing, at temperatures near $T = -2^\circ\text{C}$, the growth of ice crystals is plate-like, with thick plates at lower supersaturations, thinner plates at intermediate supersaturations, and plate-like dendritic structures at high supersaturations. For temperatures near $T = -5^\circ\text{C}$, the growth is columnar, with stout columns at the lower supersaturations, more slender, often hollow columns at intermediate supersaturations, and clusters of thin, needle-like crystals at higher

supersaturations. Colder still, near $T = -15^{\circ}\text{C}$, the growth again becomes plate-like, and again one sees increasing structure with increasing supersaturation. Finally, at the lowest temperatures the growth becomes a mixture of thick plates at low supersaturations and columns at higher supersaturations. Growth of heavy water (D_2O) crystals from the vapour phase produces similar morphologies as a function of temperature, except shifted by approximately four degrees, in keeping with the isotopic shift in the freezing point between D_2O and H_2O (Libbrecht, K.G., 2005, Reports on Progress in Physics, vol.68, p.855-895). This principle regarding the shape and the size of crystals applies also to the structure of the porous network of the bioadhesive second layer.

[082] Thus, this method also comprises increasing or decreasing the temperature of the second layer or the surrounding atmosphere of the layer as long as the temperature allows freezing of the molecules or group of molecules from the surrounding atmosphere at the surface of the second layer.

[083] In one example, crystals can be formed by depositing water at the surface of the second layer at a temperature of the second layer of about -15°C or -30°C . When the molecule to be deposited at the surface of the second layer is water then the temperature should be below 0°C at atmospheric pressure or between about 0°C to about -100°C . In another example, the temperature is between about 0°C or -1°C to about -30°C .

[084] It is also important to note that the crystal growth in this method can be enhanced when one let flow air over a growing surface of crystals, a phenomenon called the ventilation effect.

[085] From the previous comments it also becomes obvious that not only the temperature can influence the crystal formation and thus the size and structure of the pores formed in the scaffold but also the saturation of the atmosphere with the elements or group of elements which are to be deposited on the surface of the collector.

[086] Thus, the method also comprises increasing or decreasing the saturation of the atmosphere with the molecules or group of molecules which are to be deposited on the surface of the collector.

[087] As demonstrated in the examples (see also Fig. 5a to 5c), when increasing the saturation of the atmosphere surrounding the collector with water (i.e. humidity), the size of the three-dimensional pores is larger and more defined.

[088] The "atmosphere" surrounding the collector can be varied to contain, for example, the specific molecules or group of molecules which are to be deposited on the surface of the second layer or to vary the saturation of the atmosphere with the specific molecules or group of molecules to be deposited at the surface of the second layer. In one example, normal air forms the atmosphere surrounding the collector. In another example, mentioned herein, nitrogen (N_2) is added to the atmosphere to control the water content in the air. A pure CO_2 atmosphere would for example also be possible.

[089] Thus, the method of electrospinning can also comprise increasing or decreasing the flow rate of the solution comprising the at least one polymer dissolved therein. The flow rate of the solution or in other words the solution feeding rate can be changed by increasing or decreasing the pressure in the at least one container comprising the at least one polymer dissolved therein. In general, a higher feeding rate for the solution leads to the formation of thicker fibers.

[090] In general, the diameter of fibers affects the surface area of the fibers, which in turn affects the rate of degradation of the scaffold material. The mechanical properties of the electrospun mesh can also be affected by the diameter of the fibers. Varying solution flow rate is also in changing the pore size and porosity of the eventual scaffold. To elaborate, if one increases solution flow rate while keeping all other parameters constant, there is more throughput of polymer through the spinneret which will occupy more space with respect to the deposited crystals. In this way, the pore size and the porosity of the scaffold will decrease with increasing flow rate of the solution.

[091] In still another aspect the method can further comprise varying the time interval between electrospinning and the formation of crystals. It can be easily imagined that the rates of crystal formation and fiber deposition are two competing factors that can have an effect on the pore structure of the cryogenic electrospun scaffolds. As more time passes between different electrospinning steps as larger the crystals can grow on the collector which means that the resulting pores of the electrospun scaffold are growing larger. To demonstrate the effect of the relative rates of crystal formation and fiber deposition on the pore structure of the cryogenic electrospun scaffold, the time interval between fiber deposition has been varied in an example. A greater time interval between

spinning steps allows ice crystals to grow in size, hence resulting in larger pores in the cryogenic electrospun scaffold formed on the second layer of the hybrid scaffold.

[092] Thus, by varying the time intervals between different spinning steps, an electrospun scaffold with different layers having different pore sizes formed by the fibers can be manufactured.

[093] The method for electrospinning can also comprise varying at least one of the parameters selected from the group consisting of needle size and design, voltage and the concentration of the at least one polymer in the solution.

[094] Changing the voltage applied during electrospinning can influence the diameter of the fibers produced. As described by Megelski S. et al. (2002, *Macromolecules*, vol.35, no.22, p.8456-8466) and Pham, Q.P. et al. (2006, *Tissue Engineering*, vol.12, no.5, p.1197-1211) the diameter of a fiber can be decreased with increasing the spinning voltage, whereas decreasing the spinning voltage increases the diameter of a fiber. At low voltage of field strengths, a drop of the solution comprising the at least one polymer dissolved therein is typically suspended at the tip of the spinneret, i.e. needle tip. A jet will originate from the Taylor cone ("Taylor cone" refers to the droplet produced at the tip of the needle (see Fig. 2a) when an electric field is applied. G.I. Taylor showed 1969 that this droplet is a cone-shaped and the jets are ejected from the vertices of the cone) producing spinning (assuming that the force of the electric field is sufficient to overcome the surface tension of the solution). Using laser diffraction, it has also been shown that increased voltages can produce jets with larger diameters and ultimately lead to the formation of several jets (Demir, M.M., Yilgor, I. et al., 2002, *Polymer*, vol.43, p.3303 et seqq.)

[095] In this method of electrospinning, the voltage applied can be in a range of 0 kV to 50 kV or 7 kV up to 35 kV. In one example, a voltage between 10 and 35 kV has been applied. The voltage across the electrodes is usually varied together with other parameters of the electrospinning process. For example, when the distance between the spinneret and second layer is increased, the voltage has to be increased to sustain the Taylor cone at the spinneret.

[096] Several designs and configurations of needle tips have been investigated for the electrospinning process (Pham, Q.P., Sharma, U. et al., 2006, *supra*). For example, a

coaxial, two-capillary spinneret was designed. Using polymer feeds consisting of two immiscible liquids, it was possible to produce hollow nanofibers. With this two-capillary spinneret it was also possible to prepare blends of polymers. The use of multiple tips has also been investigated as a way to increase the throughput and production rate of electrospinning of poly(ethylene oxide) (PEO). Multiple needle tips have also been used to prepare blends of poly(vinyl alcohol) (PVA) and cellulose acetate. Using four tips and varying the number containing PVA and cellulose acetate allowed for fibers with various weight ratios of PVA and cellulose acetate to be produced. Using two tips and a collector that could move transversely, mixes of PEO and polyurethane fibers have been spun. The transverse motion of the collector allowed for more uniform distribution of each polymer.

[097] The concentration and thus the viscosity of the solution comprising the at least one polymer has also been examined. Megelski S. et al. (2002, *supra*) reported that the fiber diameter increases with increasing solution concentration. Both concentration and viscosity of the polymer solution are parameters that can be changed to provide for a steady Taylor cone and consequently, a stable electrospinning process. Changing the concentration of the solution can also affect the morphology of the electrospun fibers. In general, increasing the solution concentration while keeping all other parameters for electrospinning constant result in a slower flow rate of the solution.

[098] The method for electrospinning is not limited to a specific kind of polymer for electrospinning. Every known polymer which is suitable for electrospinning or can be made suitable for electrospinning can be used in this method for electrospinning. A list of electrospun polymers which is suitable is listed for example in Table I of the article of Subbiah, T. and Bhat, G.S. et al. (2005, Journal of Applied Polymer Science, vol.96, p.557-569).

[099] Due to the use of the scaffolds in cell biology and tissue engineering, the polymers used for electrospinning can also be biocompatible and/or biodegradable. "Biodegradable" refers to material that can be absorbed or degraded in a patient's body. "Biocompatible" refers to materials that do not have toxic or injurious effects on biological functions.

[0100] A large number of suitable biocompatible polymers is known and can be selected from the group consisting of poly(urethanes), poly(siloxanes), poly(silicones),

poly(ethylene), poly(vinyl pyrrolidone), poly(2-hydroxy ethyl methacrylate), poly(N-vinyl pyrrolidone), poly(methyl methacrylate), poly(vinyl alcohol) (PVA), poly(acrylic acid), poly(vinyl acetate), polyacrylamide, poly(ethylene-co-vinyl acetate), poly(ethylene glycol), poly(methacrylic acid), polylactic acid (PLA), polyglycolic acids (PGA), poly(lactide-co-glycolides) (PLGA), nylons, polyamides, polyanhydrides, poly(ethylene-co-vinyl alcohol) (EVOH), polycaprolactone, poly(vinyl acetate), polyvinylhydroxide, poly(ethylene oxide) (PEO) and polyorthoesters. It is also possible to use blends of different polymers listed above. In one example PLA is used which has been dissolved in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP).

[0101] In matrices composed of electrospun elastin (for elasticity), electrospun collagen (to promote cell infiltration and lend mechanical integrity), and other components, such as PLGA, PEO, PVA, or other blends, the relative ratio of the different components in the matrix can be tailored to specific applications (e.g. more elastin, less collagen depending on the tissue to be engineered).

[0102] Representative materials for forming the biodegradable material include natural or synthetic polymers, such as, collagen, poly(alpha esters) such as poly(lactate acid), poly(glycolic acid), polyorthoesters, polyanhydrides and their copolymers, which degrade by hydrolysis at a controlled rate and are reabsorbed. These materials provide the maximum control of degradability, manageability, size and configuration.

[0103] Other biodegradable materials can be selected from the group consisting of cellulose ether, cellulose, cellulose ester, chitosan, gelatin, fluorinated polyethylene, poly-4-methylpentene, polyacrylonitrile, polyamide, polyamideimide, polyacrylate, polybenzoxazole, polycarbonate, polycyanoarylether, polyester, polyestercarbonate, polyether, polyetheretherketone, polyetherimide, polyetherketone, polyethersulfone, polyethylene, polyfluoroolefin, polyimide, polyolefin, polyoxadiazole, polyphenylene oxide, polyphenylene sulfide, polypropylene, polystyrene, polysulfide, polysulfone, polytetrafluoroethylene, polythioether, polytriazole, polyurethane, polyvinyl, polyvinylidene fluoride, regenerated cellulose, silicone, urea-formaldehyde, or copolymers or physical blends of these materials. The material of the electrospun scaffold forming the third layer as well as the first and second layer of the hybrid scaffold may be

impregnated with suitable antimicrobial agents and may be colored by a color additive to improve visibility and to aid in surgical procedures.

[0104] In one example, the polymer used for the polymer solution for electrospinning can be polylactides (PLA), polyglycolides (PGA), polycaprolactones (PCL), copolymers of PLA-PGA, PGA-PCL, PLA-PCL or a terpolymer of PLA-PGA-PCL. Another group of polymers that can be used in the method of the present invention are polymers having amino acids (e.g. lysine or RGD sequence) or peptides (polypeptides or polypeptides co-polymers) added to the polymer backbone or grafted onto the surface of the polymers to promote cell material interactions. Such materials are described for example in the article of Deng, X.M., Liu., et al. (2002, European Polymer Journal, vol.38(7), p.1435-1441). One example of such a polymer would be poly(DL-lactic acid)-co-poly(ethylene glycol)-co-poly(L-lysine)copolymer.

[0105] As limited by their molecular weights and/or solubilities, some functional polymers are not suitable for use with electrospinning. One strategy for solving this problem is to blend them with polymers that are well-suited for electrospinning. Based on this approach, Kaplan and co workers (2002, Biomacromolecules, vol.3, p.1233 et seqq.) have successfully fabricated protein-carrying fibers by adding the proteins to the solution of a conventional polymer. Fibers consisting of blends between polyaniline (or polythiophene) with conventional organic polymers have also been investigated. Blending was found to be fruitful in improving some properties or applications associated with fibers. For instance, it has been demonstrated that the physical and biological properties (e.g., biodegradation rate and hydrophilicity) of PLA fibers could be finely tuned by simply controlling the compositions of polymer blend solutions used for electrospinning (Kim, K. Yu, M. et al., 2003, Biomaterials, vol.24, p.4977 et seqq.)

[0106] The use of biocompatible and/or biodegradable polymers will depend on given applications and specifications required. A more detailed discussion of such polymers and types of polymers can also be found in Brannon-Peppas, Lisa, "Polymers in Controlled Drug Delivery," Medical Plastics and Biomaterials, November 1997, which is incorporated herein by reference.

[0107] Sometimes it is necessary to dissolve a polymer before it can be used for electrospinning. Therefore, the at least one polymer used in the method of electrospinning

can be dissolved in an aqueous solvent or an organic solvent. Exemplary solvents which are known in the prior art can be selected from the group consisting of acetone, N,N-dimethylformamide (DMF), water/chloroform, water, methylethylketone, silk aqueous solution, acetic acid, formic acid, ethanol, diethylformamide, hexa-fluoro-2-propanol, methylene chloride together with dimethyl formamide, dimethyl formamide:toluene (1:9), water/ethanol or NaCl, hydrochloric acid, camphorsulfonic acid, dichloromethane mixed with trifluoroacetic acid, chloroform, dimethylacetamide, dimethyl formamide:tetrahydrofuran (1:1), dichloromethane, tetrahydrofuran (THF), N,N-dimethyl acetamide (DMAc), 1,1,1,3,3,3-hexa fluoro-2-propanol (HFIP), HFIP mixed with DMF, isopropyl alcohol (IPA), sulphuric acid, hexafluoro isopropanol, and mixtures thereof.

[0108] Those solvents evaporate during electrospinning. In one example HFIP is used to dissolve PLA. In Table 1 of a review article of Huang, Z.-M., Kotaki, M. and Ramakrishna, S. (2003, Composites Science and Technology, vol.63, p.2223-2253) at page 2226-2230, a list of polymers together with a possible solvent is given. Another example is the list referred to in the article of Subbiah, T., et al. (2005, Table I, *supra*). These articles and in particular the content of Table I is incorporated by reference into the present application. It should be noted that these lists illustrate only exemplary combinations of polymers and solvents and that a person skilled in the art would know how to create further or different combinations than the one mentioned in these articles.

[0109] Organic solvents could be, for example, acetone, N,N-dimethylformamide (DMF), diethylformamide, chloroform, methylethylketone, acetic acid, formic acid, ethanol, 1,1,1,3,3,3-hexa fluoro-2-propanol (HFIP), tetrafluoroethanol, dichloromethane (DCM), tetrahydrofuran (THF), trifluoroacetic acid (TFA), camphorsulfonic acid, dimethylacetamide, isopropyl alcohol (IPA) and mixtures thereof. Examples of mixtures would be DCM with DMF, DMF:Toluene (1:9), ethanol/NaCl, DCM mixed with TFA, DMF:THF (1:1) and HFIP mixed with DMF.

[0110] Inorganic solvents could be, for example, water, hydrochloric acid, sulphuric acid and mixtures thereof. Examples of mixtures of inorganic solvents would be water/NaCl and water/chloroform.

[0111] For some applications it might be of some advantage not to use solvents. In these cases polymer melts can be used alternatively. Thus, it is also possible to use a

polymer melt for electrospinning. The use of polymer melts is not limited to a specific polymer melt. For example, polymers, such as polyethylene (PE), polypropylene (PP), nylon 12 together with PA-12, polyethylene terephthalate (PET), polyethylene naphthalate (PEN), polyethylene terephthalate together with polyethylene naphthalate, and polycaprolactone together with poly(ethylene oxide-*block-ε*-caprolactone) (PEO-b-PCL), or poly(D,L-lactide) (PLA), poly(glycolide) (PGA), poly(lactides-co-glycolides) (PLGA) and further blends or mixtures thereof can be used in melted forms.

[0112] Polymer melts are known to a person skilled in the art and are described, for example, in the article of Huang, Z.-M., Kotaki, M. and Ramakrishna, S. (2003, *supra*), Table II and Dalton, P.D., Klinkhammer, K. et al. (2006, *Biomacromolecules*, vol.7, no.3, p.686-690).

[0113] The processing temperature for polyethylene (PE) is about 200 to 220 °C, about 200 to 240 °C for polypropylene (PP), about 220 °C for nylon 12 together with PA-12, about 270 °C for polyethylene terephthalate (PET), about 290 °C for polyethylene naphthalate (PEN), about 290 °C for polyethylene terephthalate together with polyethylene naphthalate (75/25 or 25/75 (wt.%)), and about 85 °C for polycaprolactone together with poly(ethylene oxide-*block-ε*-caprolactone) (PEO-b-PCL) (20:80). The melting temperature of PLA melt is about 200°C. For example, PLA is commonly used in biomedical applications and is described for example by Zhou, H.J., Green, T.B., et al. (2006, *Polymers*, vol.47(21), p.7497-7505).

[0114] It is further possible to enhance attachment of cells to the biocompatible or biodegradable substrate of the different layers of the hybrid scaffold by coating the matrix with compounds such as basement membrane components, agar, agarose, gelatin, gum arabic, collagens, fibronectin, laminin, glycosaminoglycans, mixtures thereof, and other materials having properties similar to biological matrix molecules known to those skilled in the art of cell culture. Mechanical and biochemical parameters ensure the matrix (three-dimensional structure of the scaffold or biological material) provide adequate support for the cells with subsequent growth and proliferation. Factors, including nutrients, growth factors, inducers of differentiation or dedifferentiation, products of secretion, immunomodulators, inhibitors of inflammation, regression factors, biologically active compounds which enhance or allow vascular ingrowth or ingrowth of

the lymphatic network or nerve fibers, and drugs, can be incorporated into the matrix of the scaffold or provided in conjunction with the matrix. Similarly, polymers containing peptides such as the attachment peptide RGD (Arg-Gly-Asp) can be synthesized for use in forming matrices.

[0115] In general it is also possible to add a variety of functional components directly to the solution for electrospinning to obtain fibers with a diversified range of compositions and well-defined functionalities. In particular components which can support the ingrowth of cells into the scaffold can be incorporated. Those components can be applied either by mixing them directly with the solution or by applying the additional components simultaneously or subsequently through another container onto the scaffold. Those substances can also be applied to the first and second layer of the hybrid scaffold of the present invention.

[0116] For example, biological molecules such as fibronectin and laminin can be incorporated to promote cellular activities such as attachment and migration. The final freeze-drying step serves to lyophilize the molecules, which might help to preserve the proteins in the porous mesh. In order to integrate cells into porous mesh of the hybrid scaffold, the freeze drying step is preferably excluded. This means that the process has to use other vapor molecules as the templating crystals. For example, a technique that uses CO₂ could be used as the crystals are removed by sublimation at room temperature without freeze drying. In this way, the viability of the cells can be easily maintained.

[0117] The sublimation step is carried out to remove the solid crystals formed and which are responsible for forming the pores within the second and third layer of the hybrid scaffold. Sublimation can be carried out by freeze-drying (also known as lyophilization). Freeze drying works by freezing the material and then reducing the surrounding pressure and adding enough heat to allow the frozen compound in the material to sublime directly from the solid phase to gas.

[0118] Different kinds of freeze dryers can be used, such as rotary evaporators, manifold freeze dryers, or tray freeze dryers.

[0119] Optionally it is possible to include a further step of drying after the sublimation step to remove residual solvent which still remained in the scaffold.

[0120] Figure 2a shows a schematic illustration of the basic setup of an apparatus for electrospinning. Figure 2a shows the four major components: a high-voltage power supply, a spinneret (i.e. a metallic needle), and a collector (a grounded conductor). Direct current (DC) power supplies are usually for electrospinning although the use of alternating current (AC) potentials is also feasible.

[0121] The spinneret is connected to a syringe in which the polymer solution (or melt is hosted). It is possible to use more than one spinneret, for example, 1, 2, 3 or 4 spinnerets, which are connected to a syringe or container comprising the polymer solution or melt. More than one spinneret is of specific advantageous if several kinds of polymers are ought to be applied together and those polymers are not dissolvable in the same solvent.

[0122] With the use of a syringe pump, the solution can be fed through the spinneret at a constant and controllable rate. When a high voltage (usually in the range of 1 to 35 kV) is applied, the pendent drop of polymer solution at the nozzle of the spinneret will become highly electrified and the induced charges are evenly distributed over the surface. As a result, the drop will experience two major types of electrostatic forces: the electrostatic repulsion between the surfaces charges; and the Coulomb force exerted by the external electric field. Under the action of these electrostatic interactions, the liquid drop will be distorted into a conical object commonly known as the Taylor cone. Once the strength of the electric field has surpassed a threshold value, the electrostatic forces can overcome the surface tension of the polymer solution and thus force the ejection of a liquid jet from the nozzle. This electrified jet then undergoes a stretching and whipping process, leading to the formation of a long and thin thread. As the liquid jet is continuously elongated and the solvent is evaporated, its diameter can be greatly reduced from hundreds of micrometers to as small as tens of nanometers. Attracted by the grounded collector placed under the spinneret, the charged fiber is deposited on the collector.

[0123] The orifice of the metallic needle (spinneret) can be of different size depending on the final diameter of the fiber which is desired to deposit on the bioadhesive second layer. In general, the size of the needle used for electrospinning depends on the concentration and viscosity of the solution. A person skilled in the art is

able to select the correct needle size to obtain the optimal conditions for electrospinning. In one example, the needle sizes range from 21 to 26 gauges. In another example a needle size of 26 G is used.

[0124] This needle(s) can be connected to one or more container depending on how many polymer streams are supposed to be fed through one needle. Preferably, each needle is connected to one container comprising a polymer solution.

[0125] Fibers have also been collected using a rotating cylindrical drum rather than a stationary target (see Fig. 2b). The first and second layer of the hybrid scaffold are fixed on this drum. Thus, the collector used in the apparatus of the present invention can also be rotatable around at least one axis. In another variation, a thin, steel pin was used as a counter electrode and was placed behind a rotating, non-conductive cylindrical collector. The rotating drum can also be combined with the multiple field method.

[0126] For the method of the present invention it can be important that the hybrid scaffold provides a certain shape which resembles the shape of the defect side in which the hybrid scaffold is supposed to be implanted after manufacturing. For example, in case of bone or skin reconstruction it might be necessary to reconstruct a certain specific part of the tissue with specific measurements. When reconstructing specific tissues, such as whole organs or blood vessels, a specific structure of the scaffold will be necessary to mimic the original extracellular membrane (ECM) of the tissue like in the biological material as good as possible.

[0127] In another aspect, the present invention is directed to a three-dimensional hybrid scaffold for tissue engineering obtained by the method of the present invention. A hybrid scaffold of the present invention comprises:

- a first layer made of a biological material which has been decellularized;
- a second porous layer connected to the surface of the first layer, wherein the second layer is a porous bioadhesive; and
- a third porous layer connected to the surface of the second layer which is located opposite the surface to which the first layer is connected, wherein the third porous layer is a scaffold.

[0128] In one aspect, the scaffold of the third layer is an electrospun three-dimensional porous scaffold. In one example this electrospun scaffold has been

manufactured and applied/connected to the surface of the bioadhesive layer at a temperature which is at or below the freezing temperature of the solvent in which the polymer has been dissolved.

[0129] In a further aspect, the present invention refers to the use of a three-dimensional hybrid scaffold of the present invention which has been manufactured according to the method of the present invention for autologous, allogenic, xenogenic transplantation of tissue.

[0130] In a further aspect, the hybrid scaffold is used for the transplantation of small intestine, liver, pancreas, urinary bladder, stomach, bladder, vascular system, bile duct, alimentary canal, respiratory tract, kidney, spleen, heart, heart valve, bone, skin or fragments or parts thereof. In one example, the hybrid scaffold is used for transplantation of esophageal mucosa.

[0131] In still another aspect, the present invention is directed to the use of a three-dimensional hybrid scaffold of the present invention which has been manufactured according to the method of the present invention for the manufacture of or use as a medicament. The use as a medicament includes replacing damaged part of a tissue or organ. The hybrid scaffold is supposed to accelerate the healing process by providing a porous ECM like structure allowing ingrowth of newly formed tissue and vascularization.

[0132] By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present.

[0133] By "comprising" it is meant including, but not limited to, whatever follows the word "comprising". Thus, use of the term "comprising" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present.

[0134] The inventions illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the

features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[0135] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0136] Other embodiments are within the following claims and non-limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

EXAMPLES

[0137] In the following section the general setup of the examples for the manufacture of a hybrid scaffold of the present invention is described in more detail before referring to some specific examples carried out.

[0138] The purpose of producing a hybrid scaffold is to combine the advantages of both biocompatible polymers with that of acellular matrices for tissue engineering application.

[0139] Figs. 2a and b show a schematic diagram of the electrospinning process. In this process, long and fine threads are drawn from droplets of polymer by the application of a high voltage electric field. The resulting nano-fibers are deposited onto a rotating mandrel (collector) to form a tubular interconnected porous network. The collector can be in any geometrical shapes other than the cylindrical mandrel used in this particular embodiment. Many different materials have been electrospun alone or in combinations

using different solvents. Some examples are given in the following reviews (Huang, Z.-M., Kotaki, M. and Ramakrishna, S., 2003, *supra*; Li, D., Xia, Y.N., 2004, *Advanced Materials*, vol.16, p.1151-1170). In all examples, the polymer used is poly(D,L-lactide) (PLA) dissolved in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) in various concentrations.

[0140] For the purposes of this disclosure, the biological material used is a freeze-dried decellularized porcine esophageal mucosa (hereinafter known as decellularized mucosa). While references are made to porcine esophageal mucosa, it is understood that other naturally occurring matrices (e.g. stomach, bladder, bile duct, alimentary, respiratory), from other sources (e.g. bovine, ovine, human) are within the scope of this disclosure. The decellularized mucosa is prepared by mechanically separating the mucosal and submucosal layers from the remaining tissue (consisting of muscularis externa and serosa/adventitia).

[0141] The mucosa is treated with dispase solution to detach the epithelium from the basal membrane.

[0142] This is followed by treatment with Triton-X, peracetic acid solution and mechanical abrasion to remove cellular components from the tissue, rendering it acellular, while attempting to preserve the important structural and functional proteins. The resulting acellular ECM is then subjected to freeze drying process to remove the liquid within the hydrated scaffold via sublimation. Alternatively, the liquid may be removed by subjecting the sample to critical point drying (CPD).

[0143] Biodegradable and biocompatible adhesives, such as fibrin glue as the bonding interface between the acellular ECM forming the first layer and, for example, an electrospun scaffold as the third layer. More importantly, the the bioadhesive is made porous by applying it to the biological material which is maintained below subzero temperatures such that ice crystals formed within the bioadhesive. The hybrid scaffold is subsequently formed by electrospinning biocompatible polymer directly onto the biological material coated with the the bioadhesive. This is followed by lyophilization to remove the ice crystals, forming a wholly porous hybrid scaffold.

[0144] Bioadhesives can be applied to either side (adluminal and abluminal) of the decellularized mucosa by casting. Other methods such as spraying, electrospraying, electrospinning, spin coating, dip coating and brushing can be used. After the application

of the bioadhesive on the decellularized mucosa, electrospinning of the fibers begins before the bioadhesive is fully set/crosslinked. This is to ensure that the depositing electrospun fibers are wetted with the bioadhesive in order to form sufficient bonding with the setting bioadhesive.

[0145] In one example, the fibrin coated decellularized mucosa is attached to a chilled mandrel. Ice crystals formed on the fibrin serve as a negative template around which electrospun fibers are deposited. In addition, the water component within the fibrin freezes to form ice within the crosslinking fibrin. The hybrid scaffold is subsequently freeze-dried to remove ice crystals, leaving behind a construct consisting of cryogenic electrospun scaffold (CES) with large pores and a porous fibrin interface. This wholly porous construct can be used to support cell infiltration and vascularization within the scaffold. This is useful in replacing thick segments of the tissues that have been removed during the decellularization processes. In comparison, the non-porous fibrin film acts as a barrier to nutrient diffusion and cell infiltration. Another advantage of the porous interface is that the degradation products of fibrin are angiogenic and the porous fibrin layer will facilitate vascular ingrowth *in vivo*. By promoting cell infiltration and vascularization, the porous fibrin layer also facilitates remodeling of the construct *in vivo*. The infiltrated fibroblasts can deposit collagen and start the remodeling process while the porous fibrin layer degrades. In comparison, the non-porous fibrin interface degrades without collagen deposited from the host cells within the interface, which might result in delamination of the hybrid scaffold upon complete degradation of the fibrin. Lastly, the degradation of fibrin can be controlled by controlling the pore size of the interface. The pore size can be varied by changing the concentration of fibrinogen and thrombin in the aqueous medium, thus changing the amount of ice within the fibrin layer.

[0146] Thus, a hybrid scaffold of the present invention adds more versatility to native acellular matrices. It can be used to (1) tailor the mechanical properties of the treated ECMs to comply with host tissues, (2) replaced excised tissue segments by providing a thick fibrous hybrid scaffold with large pores for cell infiltration and vascularization and (3) organize cells attachment and proliferation along aligned fibers.

[0147] **Hybrid scaffold consisting of cryogenic electrospun scaffold and decellularized mucosa with porous fibrin layer**

[0148] **Decellularization of esophageal mucosa**

[0149] A sample of length 5 cm is harvested from the mid-section of freshly harvested porcine esophagus. It is rinsed in saline solution to remove cellular debris for 5 minutes at room temperature, and the sample slit opened longitudinally. The mucosal and submucosal layers are then mechanically separated from the remaining tissue (consisting of muscularis externa and serosa), and left to immerse in deionized water at 4 degrees Celsius for 30 minutes.

[0150] The decellularization reagent is prepared using a formulation of 31% hydrogen peroxide, glacial acetic acid and deionized water in the ratio of 20% volume: 40% volume: 40% volume. The typical concentrations of the reagent utilized in this particular invention ranged from 1% - 20%, with 1% - 10% preferred. 30 ml of the reagent is then poured into a 100 mm glass Petri dish. Individual samples of the mucosal and submucosal layers are then immersed in the reagent at ambient temperature from 4 to 40 °C, and both sides delaminated using mechanical tools including, but not limited to, gauze, brush, and the edge of microscopic slides.

[0151] Mechanical delamination cycles ranged from about 50 to about 300 cycles. 150 to about 300 cycles are also suitable. After mechanical delamination in the reagent, the samples are then neutralized to physiological pH with the use of sterile phosphate buffered saline (PBS) by placing them on the rotor shaker for 15 minutes at ambient temperature. Typically, it took 5 to 6 changes of sterile PBS before the samples are neutralized at physiological pH. The sample is then immersed in a Triton X-100 solution, typical concentrations ranging from about 0.05% to 5% or about 0.5% to 3%. Time immersion of the sample in Triton X-100 ranged from 6 hours to 48 hours or from 12 to 24 hours. Mechanical agitation was provided in the form of gentle stirring with methods including magnetic stirring, but not limited to mechanical agitation such as rotor shaking, paddle stirrer and rotation shaking. After the treatment with Triton X-100, samples are rinsed of residual detergent by mechanical stirring by the above means, with rotor shaking preferred. Typically, it takes about 6 changes of sterile PBS to remove all traces

of Triton X-100 from the samples. The sterile acellular matrices are then stored in sterile PBS supplemented with antibiotics (penicillin/streptomycin) at 4°C till further use.

[0152] **Manufacture of hybrid scaffold**

[0153] The PLA solution is placed in a 30 ml syringe fitted with a 26 gauge metal needle, which is in contact with the earthed plate (needle sizes can vary from 18G to 28G). The environmental conditions are controlled with an ambient temperature between 20 - 28°C and a relative humidity between 25 – 80%. The mandrel is chilled to subzero temperatures. The decellularized mucosa is attached to the mandrel with the abluminal surface facing up. The fibrinogen and thrombin solutions are constituted, mixed thoroughly and applied onto the top surface. Constant pneumatic pressure is applied to the syringe to sustain a droplet at the needle tip. A voltage between 10 to 35kV is applied to the mandrel until a stable Taylor cone formed and a constant polymer jet is ejected. Electrospun fibers are deposited while ice crystals simultaneously form on the chilled fibrin bioadhesive. The aqueous solvent in the fibrin layer froze, forming ice crystals within the bioadhesive. When electrospinning is completed, the hybrid scaffold is freeze-dried overnight to remove the embedded ice crystals.

[0154] Figures 1a and b depict the cross section of the hybrid scaffold obtained by electrospinning onto a fibrin coated mucosa maintained at subzero temperatures. As a comparison, Figures 1d and e show the cross section of the hybrid scaffold by electrospinning onto a fibrin coated mucosa at ambient temperature. As a result of the subzero temperatures in which cryogenic electrospinning is carried out, the formation of a porous fibrin interface is observed (Figure 1b). This is a positive step towards attaining a wholly porous hybrid scaffold that would be advantageous in nutrient diffusion and cell infiltration in *in vitro* and *in vivo* systems. In comparison, the non-porous fibrin film shown in Figure 1e acts as a barrier to nutrient diffusion and cell infiltration. Another advantage of the porous interface is that the degradation products of fibrin are angiogenic and the porous fibrin layer will facilitate vascular ingrowth *in vivo*. By promoting cell infiltration and vascularization, the porous fibrin layer also facilitates remodeling of the construct *in vivo*. The infiltrated fibroblasts can deposit collagen and start the remodeling process while the porous fibrin layer degrades. In comparison, the non-porous fibrin

interface degrades without collagen deposited from the host cells within the interface, which might result in delamination of the hybrid scaffold upon complete degradation of the fibrin. Lastly, the degradation of fibrin can be controlled by controlling the pore size of the interface. The pore size can be varied by changing the concentration of fibrinogen and thrombin in the aqueous medium, thus changing the amount of ice within the fibrin layer.

[0155] Figure 1c shows the top surface of the cryogenic electrospun scaffold which corresponds to Figure 1a and b. The large pores of these scaffolds will be useful in inducing cell ingrowth (e.g. smooth muscle cells) and vascularization when reconstructing the muscularis externa. In comparison, the dense scaffold shown in Figure 1f acts as a barrier to cell infiltration and may not be useful in constructing thick tissues.

[0156] **Method for cryogenic electrospinning**

[0157] The polymer used in this example is poly(D,L-lactide) (PLA, LASIA H100J, T_g 58 °C, T_m 165 °C), and the solvent is 1,1,1,3,3,3-hexafluoroisopropanol (HFIP, analytical grade, Merck Singapore).

[0158] Cryogenic electrospun scaffolds (CES) can be fabricated as follows. PLA is dissolved in HFIP at a concentration of 15 wt./vol.%. The PLA solution is then placed in a 30 ml syringe fitted with a 26 gauge metal needle, which is in contact with the earthed plate. The environmental conditions are controlled with an ambient temperature between 20 °C – 28 °C and a relative humidity between 25% – 80%. The mandrel is chilled to subzero temperatures. Constant pneumatic pressure is applied to the syringe to sustain a droplet at the needle tip. A voltage between 10 to 35 kV is applied to the mandrel until a stable Taylor cone is formed and a constant polymer jet is ejected towards the collector. Electrospun fibers are deposited while ice crystals simultaneously form on the chilled rotating mandrel. When electrospinning is completed, the fibrous mesh is freeze-dried (Freeze-dryer, Alpha 1-2, Germany) overnight to remove the embedded ice crystals. The scaffold is then oven-dried (Thermoline VORD-460-D, Australia) to remove residual solvent. A schematic presentation of this method is illustrated in Fig. 3.

[0159] Effect of relative humidity of the environment on pore structure of cryogenic electrospun scaffold

[0160] The effect of the relative humidity of the environment on the pore structure of cryogenic electrospun scaffold used in the hybrid scaffold of the present invention as third layer is investigated. The relative humidity of the electrospinning chamber is decreased by introducing dry gaseous nitrogen (N_2) into the chamber or is increased by introducing water vapour with a humidifier. Three data points, 25% RH, 40% RH and 55% RH (RH = relative humidity) are collected. The mandrel temperature is maintained at -30°C and all other parameters are kept constant.

[0161] Fig. 5 shows the SEM micrographs for the three data points (Fig. 5a: 25% RH, Fig. 5b: 40% RH and Fig. 5c: 55%). It can be observed that as humidity increases from 25% to 55%, the pores of the cryogenic electrospun scaffolds become larger and more defined. This accord with the observation that simple plate-like structures are formed at low saturations, while higher saturations produce dendritic ice crystals (Libbrecht, K.G., 2005, *supra*). These complex structures aggregate more readily and occupy more space, resulting in the larger pore size of the cryogenic electrospun scaffold at higher humidities. Moreover, the rate of ice crystal formation increases with humidity; hence the proportion of ice crystals to fiber is increased resulting in a more open structure.

[0162] Based on these findings, it can be postulated that parameters that governs ice crystal formation on the surface of the second layer, such as the temperature of the second layer, temperature and relative humidity of the environment, affects the pore structures of the cryogenic electrospun scaffold. Conditions that favor aggregation and formation of ice crystals result in higher volumes of ice crystals between bundles of electrospun fibers; hence resulting in larger pores of the cryogenic electrospun scaffold.

[0163] Effect of time interval between fiber deposition on pore structure of cryogenic electrospun scaffold

[0164]. Ice crystal formation and fiber deposition are two events that happen simultaneously. It follows that the rates of ice crystal formation and fiber deposition are two competing factors that can affect the pore structure of the cryogenic electrospun scaffolds. To demonstrate the effect of the relative rates of ice crystal formation and fiber

deposition on the pore structure of the cryogenic electrospun scaffold, the time interval (X) between fiber deposition is varied. The longer the time interval, the slower the rate of fiber deposition.

[0165] The environmental relative humidity (RH) is maintained at 55% RH, and the mandrel temperature is maintained at -30 °C. All other parameters are kept constant.

[0166] For this study, two waiting periods were used as follows:

- (i) Sample A – X = 0 minute
- (ii) Sample B – X = 5 minutes

[0167] Samples were labeled as follows:

- (i) Sample A: A-5 (spin for 5 mins, X=0), A-10 (spin for 10 mins, X=0), A-15 (spin for 15 mins, X=0)
- (ii) Sample B: B-5 (spin for 5 mins, X=5min), B-10 (spin for 10 mins, X=5 mins), B-15 (spin for 15 mins, X=5 mins)

[0168] Figure 7 illustrates the result of this experiment. Figure 7 (a), (c) and (e) are SEM micrographs showing the pore structures of cryogenic electrospun scaffold after 5, 10 and 15 minutes of spinning without any time interval in between spinning respectively. Figure 7 (b), (d) and (f) are SEM micrographs showing the pore structures of cryogenic electrospun scaffold after 5, 10 and 15 minutes of spinning with a time interval of 5 minutes in between each cycle. From Figure 6, both Samples A (Fig. 7 (a), (c) and (e)) & B (Fig. 7 (b), (d) and (f)) are porous throughout the thickness of the scaffold, as observed by the slow building up over the 5, 10 and 15 minutes. However, pore sizes are smaller in the early stage, becoming bigger as the spinning proceeds. Sample B has pore structures that are larger in diameter and shallower, as compared to Sample A. Both of these observations can be attributed to the time interval X. The waiting period between spinning allows ice crystals to grow in size, hence resulting in larger pores in the cryogenic electrospun scaffold.

[0169] **Effect of mandrel temperature on the pore structure of cryogenic electrospun scaffold**

[0170] The effect of the mandrel temperature on the pore structure of the cryogenic electrospun scaffold is investigated. The temperature of the mandrel which is used in this

experiment can be varied by, but not limited to, packing different mass of dry ice inside the hollow mandrel. Three data points (23°C, -15°C, -30°C) are collected. All other parameters are kept constant.

[0171] Fig. 6 shows the SEM micrographs of the cryogenic electrospun scaffolds collected at different mandrel temperatures. For definition purposes it should be mentioned that the “mandrel interface” is the side of the scaffold facing the mandrel and the side of the scaffold facing the air is called the “air interface”. It can be observed that a conventional dense electrospun scaffold is obtained when the mandrel temperature is kept at 23 °C. When the mandrel temperatures are -15°C and -30°C, large pore structures (> 5 µm) can be observed on both the mandrel and air interfaces of the scaffold. As illustrated in Fig. 3, ice crystals formed on the mandrel at sub-zero temperatures are embedded within the electrospun mesh. The subsequent removal of the ice crystals through freeze-drying forms these pore structures within the electrospun mesh.

[0172] In addition, for scaffolds formed at sub-zero temperatures, it can be observed that the mandrel interface of the scaffold have less defined and smaller pore structures as compared to the air interface but still much larger pores than scaffolds obtained using classical electrospinning at room temperature.

[0173] ***In vivo* cell infiltration for decellularized porcine esophageal ECM and cryogenic electrospun scaffolds (CES) implanted subcutaneously**

[0174] The decellularized sterile porcine esophageal ECM and the CES scaffold are prepared according to the procedures described above. Dense electrospun scaffold prepared by conventional electrospinning technique is used as a comparison for the CES. Prior to use, both ECM and all electrospun scaffolds are rinsed 5 times for 15 minutes with 40 ml of phosphate buffered saline (PBS) per rinse.

[0175] Wistar rats weighing 300 to 350 g are used for the subcutaneous implantation study. Implantation is performed in an aseptic manner under a laminar hood. The rats are anaesthetized with inhalational isoflurane and oxygen, administered via a facemask. A patch of skin on the dorsum is shaved and cleansed with chlorhexidine and iodine. A single 3 cm dorsal midline incision is made. Subcutaneous pockets are created by blunt dissection. The decellularized ECM and electrospun scaffolds are implanted in separate

rats. The decellularized ECM is inserted into the pocket, ensuring that placement is flat. A second pocket is left empty as a negative control for normal healing response. For the electrospun scaffolds, three subcutaneous pockets are created by blunt dissection. The cryogenic electrospun scaffolds (CES) and dense conventionally electrospun scaffolds are inserted into two of the pockets, ensuring that placement is flat and that the scaffolds remained separate from each other. The last pocket is left empty as a negative control for normal healing response. The incisions are closed with interrupted 3/0 polypropylene sutures. Postoperatively, an injection of tolfedine 0.1 ml is administered intramuscularly in the thigh for pain relief. Sutures are removed on the tenth post-operative day.

[0176] At 14 days post implantation, the rats are euthanized by carbon dioxide inhalation. The dorsum is shaved and the previous incision reopened and extended to visualize all the pockets. Each of the scaffold and the empty pocket are retrieved with the surrounding tissue. The samples are fixed overnight in 10 % buffered formalin, embedded in paraffin and 7 μ m sections obtained using a microtome (Leica RM2125RT, Germany). These are then stained with hematoxylin and eosin to assess cellular infiltration.

[0177] The entire decellularized ECM is infiltrated with host cells by Day 14 (Fig. 8 (a) and (b)). A second observation is the presence of capillaries containing intraluminal red blood cells within the ECM. Both these phenomena are important as they show that the ECM (first layer of invention) supports *in vivo* cell infiltration and vascularization such that remodeling of the scaffold can take place. Figs 8(c) and (d) show the difference between the CES and conventional dense electrospun scaffold in promoting cell infiltration. There is markedly better cell infiltration in the CES with macrophages and collagen-producing fibroblasts penetrating deep into the scaffold at Day 14 (Fig. 8(c)). In contrast, cells are limited to the periphery of the conventional dense electrospun scaffold (Fig. 8(d)). This shows that the CES, with its open porous structure, promote better cell infiltration as a third layer of the invention.

Claims

1. A three-dimensional hybrid scaffold for tissue engineering comprising:
 - a first layer made of a decellularized biological material;
 - a second porous layer connected to the surface of said first layer, wherein said second layer is a porous bioadhesive; and
 - a third porous layer connected to the surface of said second layer which is located opposite the surface to which said first layer is connected, wherein said third layer is a three-dimensional porous polymer scaffold.
2. The hybrid scaffold according to claim 1, wherein said third layer is an electrospun three-dimensional porous scaffold.
3. The hybrid scaffold according to claim 2, wherein said electrospun scaffold is obtained by
 - providing an electrospinning apparatus;
 - forming crystals from a molecule or group of molecules, which are in vapor phase comprised in the surrounding atmosphere, at the surface of a collector of said electrospinning apparatus, wherein the reaction chamber of said electrospinning apparatus has a temperature which allows formation of crystals at said surface of said collector;
 - electrospinning a solution comprising at least one polymer dissolved therein around the crystals;
 - continuing the formation of crystals and the electrospinning simultaneously; and
 - removing the crystals by sublimation.
4. The hybrid scaffold according to claim 3, wherein said collector of said electrospinning apparatus is composed of said first and second layer and thus said electrospun scaffold is electrospun directly onto the surface of said second layer.

5. The hybrid scaffold according to any of claims 1 to 4, wherein said biological material is selected from the group consisting of small intestine, liver, pancreas, urinary bladder, stomach, bladder, vascular system, bile duct, alimentary canal, respiratory tract, kidney, spleen, heart, heart valve, bone, skin or fragments or parts thereof.
6. The hybrid scaffold according to any of claims 1 to 5, wherein said biological material is esophageal mucosa.
7. The hybrid scaffold according to any of the preceding claims, wherein said biological material is derived from an individual which is selected from the group consisting of mammal, reptiles and insects.
8. The hybrid scaffold according to claim 7, wherein said mammal is selected from the group consisting of porcine, bovine, ovine, rabbit, monkey and human.
9. The hybrid scaffold according to any of the preceding claims, wherein said bioadhesive is selected from the group consisting of fibrin glue, polyvinylpyrrolidone, polyvinylpyrrolidone/vinyl acetate copolymers, polyethylene glycol, platelated gel, chitosan or gelatin-resorcin-formaldehyde.
10. The hybrid scaffold according to claim 9, wherein said bioadhesive is fibrin glue.
11. The hybrid scaffold according to any of the preceding claims, wherein the polymer for said three-dimensional porous polymer scaffold is a biodegradable and/or biocompatible polymer.
12. The hybrid scaffold according to any of the preceding claims, wherein said second porous layer and said third layer have a pore size which is between about 10 nm to about 500 μm .

13. The hybrid scaffold according to any of the preceding claims, wherein the thickness of said bioadhesive layer is about 10 to 1000 μm before said third layer is connected to said bioadhesive second layer.
14. A method of manufacturing a three-dimensional hybrid scaffold according to any of the preceding claims, the method comprising:
 - providing a first layer made of a biological material which has been decellularized;
 - applying a second layer to the surface of said first layer, wherein said second layer is a bioadhesive and wherein said second layer is applied to said first layer at a temperature of the environment which is at or below the freezing temperature of said bioadhesive; and
 - applying a third layer which is a three-dimensional porous scaffold to a side of said second layer which is not the side facing said first layer.
15. The method according to claim 14, wherein said third layer is comprised of an electrospun scaffold.
16. The method according to claim 14, wherein said third layer is comprised of an electrospun scaffold which is obtained by the process referred to in claim 3 or 4.
17. The method according to any of claims 14 to 16, wherein said bioadhesive layer is applied to said first layer using a method selected from the group consisting of spraying, electrospraying, electrospinning, spin coating, dip coating, casting and brushing.
18. The method according to any of claims 14 to 17, wherein said third layer is applied before the bioadhesive is fully set/crosslinked.
19. The method according to any of claims 14 to 18, further comprising controlling the degradation time of said bioadhesive layer by controlling the pore size of said

bioadhesive layer which is achieved by increasing or decreasing the temperature in the environment when applying the bioadhesive layer, as long as said temperature is still at or below the freezing point of said bioadhesive.

20. The method according to any of claims 14 to 19, wherein said bioadhesive is selected from the group consisting of fibrin glue, polyvinylpyrrolidone, chitosan and gelatin-resorcin-formaldehyde.
21. The method according to claim 20, wherein said bioadhesive is fibrin glue.
22. Use of a three-dimensional hybrid scaffold according to any of claims 1 to 13 or manufactured according to any of claims 14 to 21 for autologous, allogenic, xenogenic transplantation of tissue.
23. The use according to claim 22, wherein said tissue is selected from the group consisting of small intestine, liver, pancreas, urinary bladder, stomach, bladder, vascular system, bile duct, alimentary canal, respiratory tract, kidney, spleen, heart, heart valve, bone, skin or fragments or parts thereof.
24. Use of a three-dimensional hybrid scaffold according to any of claims 1 to 13 or manufactured according to any of claims 14 to 21 for the manufacture of or use as a medicament.