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(54) Title: METHODS FOR PRODUCING BIOPROTEIN TUBES AND USES THEREOF

(57) Abstract: The present invention provides methods for the production of bioprotein tubes and thereof, (e.g., for synthetic vasculature or food products).

METHODS FOR PRODUCING BIOPROTEIN TUBES AND USES THEREOF

RELATED APPLICATIONS

This application claims priority to U.S. Provisional Patent Application No. 62/022,940, filed on July 10, 2014, the contents of which are incorporated herein in their entirety.

BACKGROUND OF THE INVENTION

Although tremendous progress in the fields of tissue engineering and regenerative medicine has been made in recent years, the preparation of large amounts of transplantable tissue remains challenging. In particular, lack of appropriate vascularization strategies for use in cultured tissues has hindered the preparation of tissues having a thickness greater than about 0.2 millimeters due to limited oxygen and nutrient diffusion in such tissues resulting in cell and tissue death. Methods to fabricate synthetic vasculature have been developed however, due tolack of sufficient elasticity, and strength such vasculature readily becomes infiltrated or exhibits structural or mechanical failure (e.g., collapse or bursting) leading to failure and cell death in thick tissues. Accordingly, there is a need in the art for methods to fabricate synthetic vasculature that has sufficient mechanical strength and resists blockage.

SUMMARY OF THE INVENTION

The present invention is based, at least in part, on the discovery of methods to produce bioprotein tubing. The tubes are biocompatible and structurally stable in culture and during extended perfusion in culture and are, therefore, not susceptible to collapse or mechanical failure such as bursting during tissue, (*e.g.,* muscle), contraction or deformation. The tubes prepared according to the methods of the invention support liquid perfusion, and are therefore suitable for use as, among other things, synthetic vasculature and for the culture of three-dimensional tissues in tissue engineering applications. Perfused bioprotein tubes exchange cell culture medium within three-dimensional (3D) tissues and supply embedded cells with nutrients required for long-term survival in 3D tissue culture. The tubes can also remove cellular and metabolic waste products from tissues thereby

maintaining tissue homeostasis and health. Acellular or cell-laden synthetic vasculature can be produced using the methods herein described. Accordingly, in one aspect, the present invention provide methods for producing a bioprotein tube.

In some embodiments, the methods of producing a bioprotein tube include contacting at least a portion of an elongate body with a first solution include alginate and passing the at least a portion of the elongate body that was in contact with the first solution through an aperture to remove excess alginate adhered to the elongate body. The methods also include exposing the at least a portion of the elongate body with adhered alginate material to a second solution including $CaCl_2$ to gel the adhered alginate, thereby forming a tube on the at least a portion of the elongate body. The methods also include positioning a first end of the tube in contact with a third solution including gelatin and/or a biogenic polymer and drawing the third solution into the tube by at least partially withdrawing the elongate body from a second end of the tube and advancing the elongate body toward the first end of the tube to expel excess third solution material.

In some embodiments, after expelling excess third solution material, the method includes positioning the first end of the tube in contact with a fourth solution including a cross-linking agent and drawing the fourth solution into the tube by at least partially withdrawing the elongate body from the second end of the tube. In some embodiments, the tube is positioned in a bath of the fourth solution to complete cross-linking. In some embodiments, the cross-linking agent comprises microbial transglutaminase. In some embodiments, the cross-linking agent comprises genipin. In some embodiments, the cross-linking agent comprises riboflavin.

In some embodiments, the method includes forming a plurality of perforations or holes in the tube. In some embodiments the plurality of perforations or holes in the tube are formed prior to positioning the first end of the tube in contact with the third solution. In some embodiments, the tube is at least partially removed from the elongate body prior to forming the plurality of perforations or holes in the tube and the tube is positioned back on the elongate body prior to drawing the third solution into the tube. In some embodiments, a diameter or width of each of the plurality of perforations or holes falls in a range of about 1 micrometer to about half an inner diameter of the tube.

In some embodiments, contacting the at least a portion of the elongate body with the first solution includes advancing the at least a portion of the elongate body into a first

reservoir holding the first solution. In some embodiments, exposing the at least a portion of the elongate body with adhered alginate material to the second solution comprising $CaCl_2$ to gel the adhered alginate includes extending the at least a portion of the elongate body with adhered alginate material into a second reservoir containing the second solution.

In some embodiments, the first reservoir comprises a proximal end and a distal end with the aperture disposed at the distal end and contacting the at least a portion of the elongate body with the first solution includes advancing the at least a portion of the elongate body into the proximal end of the first reservoir. Passing the at least a portion of the elongate body that was in contact with the first solution through the aperture to remove excess alginate material includes advancing the at least a portion of the elongate body through the first reservoir and out the aperture of the distal end of the first reservoir.

In some embodiments, a plug at a distal end of the elongate body prevents the first solution from leaving the first reservoir through the aperture prior to advancing the at least a portion of the elongate body through the proximal end of the first reservoir. In some embodiments the method also includes positioning the elongate body extending through the first reservoir with the plug at the distal end of the elongate body blocking the reservoir aperture and adding the first solution to the first reservoir prior to advancing the at least a portion of the elongate body into the proximal end of the first reservoir.

In some embodiments, the portion of the elongate body on which the tube is formed is called the tube forming portion of the elongate body, and a diameter or width of the elongate body throughout the tube-forming portion falls in a range of about 10 micrometers to about 5 centimeters. In some embodiments, a diameter or width of the elongate body throughout the tube-forming portion falls in a range of about 500 micrometers to about 5 millimeters.

In some embodiments, a diameter or width of the aperture is in a range of about 10 micrometers to about 5 millimeters larger than a diameter or width of the tube forming portion of the elongate body.

In some embodiments, the third solution comprises gelatin. In some embodiments, the third solution comprises extracellular matrix proteins. In some embodiments, the third solution comprises one or more of collagen, fibronectin, laminin, and combinations thereof. In some embodiments, the third solution comprises one or more of bioproteins, peptides, nucleic acids, and combinations thereof.

In other embodiments, the methods include extruding a stream of a bioprotein precursor solution comprising alginate through an orifice into a gelation solution comprising calcium chloride to initiate gelation at an outer surface of the extruded stream, thereby forming a gelled outer surface of the extruded stream, maintaining the extruded stream in the gelation solution while gelation proceeds forming a gelled front advancing from the gelled outer surface toward a central axis of the extruded stream, and exposing the extruded stream to a wash solution to cease gelation and remove the un-gelled bioprotein precursor solution from the extruded stream prior to the gelled front reaching the central axis of the extruded stream, thereby producing a bioprotein tube having an outer surface at the gelled outer surface, an inner surface at the gelled front, and a lumen.

In some embodiments, cell adhesion to the tubing can be controlled by adjusting bioprotein precursor concentrations prior to tube formation.

In one embodiment, the orifice is an orifice of a needle.

In another aspect, the present invention provides methods of producing a bioprotein tube. The methods include extruding an annular stream of a bioprotein precursor solution comprising alginate through a nozzle into a gelation solution comprising calcium chloride to initiate cross-linking and gelation at an outer surface of the extruded annular stream and at an inner surface of the extruded annular stream, and maintaining the extruded annular stream in the gelation solution while cross-linking and gelation proceeds throughout the extruded annular stream, thereby producing a bioprotein tube including an outer surface, an inner surface, and a lumen.

In one embodiment, the bioprotein precursor solution is in a reservoir and the nozzle is associated with an orifice.

In another aspect, the present invention provides methods of producing one or more bioprotein tubes. The methods include rotating a bioprotein precursor solution about an axis of rotation to cause extrusion of the solution in one or more annular streams, generating a gelation solution vortex in a collection device for collecting the one or more streams of the solution, the liquid vortex including a central air gap, and collecting the one or more streams of the solution in the collection device, wherein the one or more streams are initially extruded through the air gap of the gelation solution vortex and subsequently through the gelation solution in the gelation solution vortex of the collection device, wherein the extrusion of the solution into the air gap and subsequently into the gelation solution in the collection device causes formation of one or more bioprotein tubes.

In one embodiment, an extrusion rate is constant during extrusion of the stream. In another embodiment, the extrusion rate varies during extrusion of the stream. In another embodiment, the extrusion rate is periodically varying during extrusion of the stream.

In one embodiment, the gelation solution comprises about 1% to about 5% calcium chloride.

In one embodiment, the gelation solution further comprises a microbial transglutaminase.

In one embodiment, the bioprotein precursor solution further comprises gelatin.

In one embodiment, the ratio of alginate to gelatin is about 10:1. In another embodiment, the ratio of alginate to gelatin is about 5:1. In another embodiment, the ratio of alginate to gelatin is about 1:2.

In one embodiment, the bioprotein precursor solution further comprises a biogenic polymer, such as a biogenic polymer selected from the group consisting of silk, a keratin, an elastin, a fibrillin, a fibrinogen, a fibrin, a thrombin, a fibronectin, a laminin, a collagen, a vimentin, a neurofilament, an amyloid, an actin, a myosin, a chitosan, and a titin.

The present invention also provides bioprotein tubes prepared according to any one of methods of the invention.

In one embodiment, the methods of the invention further comprise seeding cells on the bioprotein tube, such as endothelial cells or smooth muscle cells. In one embodiment, the cells are cultured under appropriate conditions to produce a vascular tissue.

The present invention also provides vascular tissues prepared according to the methods of the invention.

In one aspect, the present invention provides methods for treating a subject having a vascular disease. The methods include providing the vascular tissues if the invention, and implanting the tissue in the subject, thereby treating the subject having a vascular disease.

In one aspect, the present invention provides methods for preparing a three-dimensional tissue. The methods include embedding a bioprotein tube prepared according to the methods of the invention between two stacked polymeric tissue scaffolds comprising a plurality of nanometer dimension polymeric fibers comprising alginate to form a tissue scaffold assembly, contacting the assembly with a plurality of cells, and culturing the cells under appropriate conditions, thereby preparing a three-dimensional tissue.

In one embodiment, the cells are myocytes.

In one embodiment, the plurality of nanometer dimension polymeric fibers further comprise gelatin. In one embodiment, the ratio of alginate to gelatin is about 10:1. In

another embodiment, the ratio of alginate to gelatin is about 5:1. In another embodiment, the ratio of alginate to gelatin is about 1:2.

In one embodiment, the plurality of nanometer dimension polymeric fibers further comprise a biogenic polymer, such as a biogenic polymer selected from the group consisting of silk, a keratin, an elastin, a fibrillin, a fibrinogen, a fibrin, a thrombin, a fibronectin, a laminin, a collagen, a vimentin, a neurofilament, an amyloid, an actin, a myosin, a chitosan, and a titin. In one embodiment, the polymeric fiber is a biohybrid fiber.

In one aspect, the present invention provides three-dimensional tissues prepared according to the methods of the invention.

In one aspect, the present invention provides edible three-dimensional muscle tissues prepared according to the methods of the invention.

In one aspect, the present invention provides edible tubular food products comprising the bioprotein tubes prepared according to the methods of the invention.

In one aspect, the present invention provides methods for identifying a compound that modulates a tissue function. The methods include providing a vascular tissue prepared according to the methods of the invention or the three-dimensional tissue prepared according to the methods of the invention, contacting the tissue with a test compound, and determining the effect of the test compound on a tissue function in the presence and absence of the test compound, wherein a modulation of the tissue function in the presence of the test compound as compared to the tissue function in the absence of the test compound indicates that the test compound modulates a tissue function, thereby identifying a compound that modulates a tissue function.

In another aspect, the present invention provides methods for identifying a compound useful for treating or preventing a tissue disease. The methods include providing a vascular tissue prepared according to the methods of the invention or the three-dimensional tissue prepared according to the methods of the invention, contacting the tissue with a test compound, and determining the effect of the test compound on a tissue function in the presence and absence of the test compound, wherein a modulation of the tissue function in the presence of the test compound as compared to the tissue function in the absence of the test compound indicates that the test compound modulates a tissue function, thereby identifying a compound useful for treating or preventing a tissue disease.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a flow diagram of a method of making a bioprotin tube employing an elongate body, in accordance with some embodiments.

Figure 2A schematically depicts a side view of a system for performing some steps in the method of Figure 1, in accordance with some embodiments.

Figure 2B is an image corresponding to a detail of a prototype corresponding to the system of Figure 2A, in accordance with some embodiments.

Figure 3A schematically depicts a side view of a guide used in the system of Figure 2A.

Figure 3B schematically depicts a top view of the guide.

Figure 3C is a perspective image of a prototype guide corresponding to that shown in Figures 3A and 3B, in accordance with some embodiments.

Figure 4A schematically depicts a side view of a first reservoir used in the system of Figure 2A.

Figure 4B schematically depicts a top view of the first reservoir showing an aperture.

Figure 4C is a perspective image of a prototype first reservoir corresponding to that shown in Figures 4A and 4B, in accordance with some embodiments.

Figure 5A schematically depicts a side view of an elongate body with a plug used in the system of Figure 2A.

Figure 5B is a perspective image of a prototype elongate body with a plug corresponding to that shown in Figure 5a in accordance with some embodiments.

Figure 6A schematically illustrates assembling the guide, first reservoir and elongate body for the system shown in Figure 2A.

Figure 6B is a perspective image of the prototype guide, first reservoir and elongate body with plug assembled, in accordance with some embodiments.

Figure 7 schematically depicts a side view of the system of Figure 2A as the elongate body is advanced through the first reservoir and the aperture and into a second reservoir in accordance with some embodiments.

Figure 8 schematically depicts a side view of the system of Figure 2A after the elongate body has been advanced through the first reservoir and the aperture and into a second reservoir and alginate adhered to the elongate body is being gelled in accordance with some embodiments.

Figure 9A schematically depicts a side view of forming a plurality of perforations or holes in a gelled alginate tube in accordance with some embodiments,

Figure 9B is an image of an alginate tube before perforation.

Figure 9C is an image of an alginate tube after forming perforations.

Figure 10 schematically depicts drawing a third solution including gelatin or a biogenic polymer into the gelled alginate tube in accordance with some embodiments.

Figure 11 schematically depicts expelling excess third solution from the gelled alginate tube in accordance with some embodiments.

Figure 12 schematically depicts drawing a fourth solution including a cross-linking agent into the tube in accordance with some embodiments.

Figure 13 schematically depicts soaking the tube the fourth solution in accordance with some embodiments

Figure 14A depicts one aspect of the invention in which a bioprotein precursor solution comprising alginate (*e.g.*, a precursor solution comprising a 5% alginate solution and a 5% gelatin solution mixed at a ratio of about 3:1 is extruded through an orifice (*e.g.*, an orifice having an inner diameter of about 0.84 mm) into a gelation solution comprising calcium chloride (*e.g.*, a solution comprising about 2.5% calcium chloride for about 30 seconds) to produce a bioprotein tube.

Figure 14B is a photomicrograph of a section of the bioprotein tube prepared in Figure 1A.

Figure 14C is a photomicrograph of a cross-section of the bioprotein tube prepared in Figure 1A depicted the gelled inner and outer surfaces and the lumen of the bioprotein tube. The tubes so produced have an outer diameter of about 1.2 mm and an inner diameter of about 0.4 mm.

Figure 15 depicts a single frame from a movie showing perfusion of dyed water (black) through a bioprotein tube comprising alginate that was prepared using a bioprotein

precursor solution comprising alginate (*e.g.*, a precursor solution comprising a 5% alginate solution and a 5% gelatin solution mixed at a ratio of about 3:1) which was extruded through an orifice (*e.g.*, an orifice having an inner diameter of about 0.84 mm) into a gelation solution comprising calcium chloride (*e.g.*, a solution comprising about 2.5% calcium chloride for about 10 seconds) to produce a bioprotein tube. Dyed water was loaded into a syringe and the needle tip was inserted in to the tube. Perfusion demonstrates that (i) tubes are hollow throughout their length and (ii) they do not leak through their side walls. The tubes have an outer diameter of about 1.2 mm and an inner diameter of about 0.8 mm.

Figures 16A-16C are photomicrographs of bioprotein tubes comprising alginate and gelatin demonstrating that the methods of the invention are suitable to prepare tubes having various inner and outer diameters. Outer diameters of the tubes are determined by the dimensions of the extrusion nozzle (*e.g.*, needle gauge). Wall thickness is determined by gelation time and gelation agent concentration. The bioprotein tubes were prepared by extruding a bioprotein precursor solution comprising alginate (*e.g.*, a precursor solution comprising a 5% alginate solution and a 5% gelatin solution mixed at a ratio of about 3:1) through an orifice (*e.g.*, an orifice having an inner diameter of about 0.84 mm(A), about 0.514 mm (B), or about 0.34 mm) into a gelation solution comprising calcium chloride (*e.g.*, a solution comprising about 2.5% calcium chloride for about 10 seconds (A), about 5 seconds (B), or about 2 seconds (C)). Bioprotein tube outer and inner diameters, respectively, were 1.2 mm and 0.8 mm (A), 0.65 mm and 0.4 mm (B), 0.4 mm and 0.3 mm (C).

Figure 17A is a photomicrograph of a cross-section of a bioprotein tube comprising alginate and gelatin and ***Figure 17B*** is a photomicrograph of a longitudinal section of a bioprotein tube comprising alginate and gelatin depicting an air bubble trapped inside of the bioprotein tube. The bioprotein tubes were prepared by extruding a bioprotein precursor solution comprising alginate (*e.g.*, a precursor solution comprising a 5% alginate solution and a 5% gelatin solution mixed at a ratio of about 3:1) through an orifice (*e.g.*, an orifice having an inner diameter of about 1.0 mm) into a gelation solution comprising calcium chloride (*e.g.*, a solution comprising about 2.5% calcium chloride for about 10 seconds). Tube outer and inner diameters were 2 mm and 1.6 mm, respectively.

Figures 18A-18C are photomicrographs of bioprotein tubes comprising alginate and gelatin having an outer diameter of about 0.65 mm and inner diameter of about 0.4 mm. Three tubes are shown in Figure 5C to emphasize dimensional reproducibility of the

extrusion process. The bioprotein tubes were prepared by extruding a bioprotein precursor solution comprising alginate (*e.g.,* a precursor solution comprising a 5% alginate solution and a 5% gelatin solution mixed at a ratio of about 3:1) through an orifice (*e.g.,* an orifice having an inner diameter of about 0.514 mm) into a gelation solution comprising calcium chloride (*e.g.,* a solution comprising about 2.5% calcium chloride for about 5 seconds).

Figure 19A depicts the use of an immersed rotary jet spinning (iRJS) device for the production of water insoluble, three-dimensional bio-functional polymeric scaffolds comprising alginate, shown in ***Figures 19B and 19C***, and the use of the scaffolds for *in vitro* tissue culturing (***Figure 19D***).

Figures 20A-20J are scanning electron micrographs (SEM) of alginate:gelatin polymeric scaffolds. These scaffolds were produced using a wide range of alginate:gelatin ratios (indicated above), with (Figures 20A-20E) and without (Figures 20F-20J) subsequent crosslinking by microbial transglutaminase (mTG). Each of these scaffolds can be used for cell culture and have variable dissolution rates.

The fibers in the scaffold were prepared using a bioprotein precursor solution comprising alginate and gelatin (*e.g.,* a precursor solution comprising a 5% solution of alginate and a 5% solution of gelatin mixed together at the ratios indicated above the panels of the figure) subjected to rotational motion using, *e.g.,* an iRJS device, operated at about 30,000 rpm, and having an orifice diameter of about 0.2 mm.

Scaffolds containing less than about 20% gelatin are stable in standard cell culture conditions (saline solution containing low-CaCl2 concentrations, 37 °C, 5% CO_2) for about one week. Scaffolds containing at least 20% gelatin that were cross-linked with mTG (by incubation with 5% mTG for at least two hours) are stable for at least about two weeks in standard cell culture conditions.

All scaffolds containing 50% or more alginate are stable for at least about two weeks in standard cell culture conditions. All scaffolds containing at least 25% gelatin that are cross-linked with mTG (by incubation with 5% mTG for at least about two hours) are stable for at least about two weeks in standard cell culture conditions.

Figure 21 is a photomicrograph of a water insoluble, three-dimensional bio-functional polymeric scaffold comprising alginate cultured with moue myoblasts (C2C12 myoblasts) and fluorescently stained for α-actinin, F-actin, and nuclear DNA (DAPI staining).

The fibers in the scaffold were prepared using a bioprotein precursor solution comprising alginate (*e.g.,* a precursor solution comprising a 5% solution of alginate and a 5% solution of gelatin mixed together at a ratio of about 3:1) subjected to rotational motion using, *e.g.,* an iRJS device, operated at about 30,000 rpm, and having an orifice of about 0.2 mm and extruded into a gelation solution comprising calcium chloride (*e.g.,* a solution comprising about 2.5% calcium chloride for about 5 minutes).

Figures 22A-22C are photomicrographs of a water insoluble, three-dimensional bio-functional polymeric scaffold comprising alginate cultured with moue myoblasts (C2C12 myoblasts) and fluorescently stained for α-actinin (light gray in (9B)), F-actin (light gray in (9A)), and nuclear DNA (DAPI staining; medium gray in (9A and 9B)). The photomicrograph in (9C) is an enlargement of the boxed section of the image in 9A. Sarcomeric α-actinin striations in (9C) confirmed that the scaffold supported C2C12 myoblast maturation following 10 days in culture. Alginate:gelatin ratio was about 3:1 (*i.e.,* prepared from *a* precursor solution comprising a 5% solution of alginate and a 5% solution of gelatin mixed together at a ratio of about 3:1), rotary extruder orifice (*e.g.,* an iRJS device having an orifice), diameter of orifice was about 0.2 mm, rotation speed was about 30,000 RPM, $CaCl_2$ concentration was about 2.5% and crosslinking time was about 5 minutes.

Figure 23 illustrates an exemplary immersed rotary jet spinning (iRJS) device for extruding an annular stream of bioprotein precursor solution through a nozzle associated with an orifice of a rotating reservoir into a cross-linking solution in a collection device for the formation of a bioprotein tube. By extruding a bioprotein precursor solution through an orifice in the rotating reservoir, this device water is also suitable for preparation of insoluble, three-dimensional bio-functional polymeric scaffolds comprising a plurality of nanometer dimension polymeric fibers comprising alginate.

Figure 24A is an image of a bioprotein tube of Example 2 illustrating the inner tube diameter.

Figure 24 B is an image of the bioprotein tube of Figure 24A showing the tube wall.

Figure 24C is a detail of the image of Figure 24B indicating the wall thickness.

Figure 25 is a graph of measured inner tube diameter for extruded bioprotein tubes and cast bioprotein tubes of various outer diameters.

Figure 26 is a graph of measured tube wall thickness for extruded bioprotein tubes and cast bioprotein tubes of various outer diameters.

Figure 27 schematically depicts a microfluidic platform including a tissue culture chamber, in accordance with some embodiments.

Figure 28 schematically depicts perforation of a gel tube in a microfluidic platform with a tissue culture chamber.

Figure 29 is an image of a perfused alginate tube in a microfluidic platform.

Figure 30 is an image of a system including a microfluidic platform a pump and a flow sensor.

Figure 31A is an axial cross-sectional image of an alginate tube.

Figure 31B is a side cross-sectional image of the alginate tube cultured with HUVEC cells.

Figure 31C is a detail of the image of Figure 31B.

Figure 32A is an axial cross-sectional image of a gelatin-coated alginate tube.

Figure 32B is a side cross-sectional image of the gelatin-coated alginate tube cultured with HUVEC cells.

Figure 32C is a detail of the image of Figure 32B.

Figure 33A is a side cross-sectional fluorescent image of a gelatin-coated alginate tube cultured with GFP-HUVEC cells.

Figure 33B is a detail of Figure 33A.

Figure 33C is a portion of an axial cross-sectional fluorescent image of the gelatin-coated alginate tube cultured with GFP-HUVEC cells.

Figure 34A is a side cross-sectional fluorescent image of GFP-HUVEC cells proliferating through a perforation in a wall of a gelatin-coated alginate tube.

Figure 34B is a single image of a Z-stack series of images showing GFP-HUVEC cells proliferating through a perforation in a wall of a gelatin-coated alginate tube.

Figure 35A is a perspective view of a reservoir configured for coaxial extrusion in accordance with an embodiment.

Figure 35B is a cross-sectional view of the reservoir of Figure 35A.

Figure 35C is a detail view of Figure 35B showing a coaxial extrusion orifice.

Figure 35D is a detail view of Figure 35C.showing an inner diameter and an outer diameter of the coaxial extrusion orifice or nozzle.

Figure 36A is a cross-sectional view and a detail view of a reservoir configured for coaxial extrusion in accordance with another embodiment.

Figure 36B is a detail view of Figure 36A showing an inner diameter and an outer diameter of the coaxial extrusion orifice or nozzle.

DETAILED DESCRIPTION OF THE INVENTION

I. Methods of the Invention

The present invention is based, at least in part, on the discovery of methods to produce bioprotein tubing. The tubes are biocompatible and structurally stable in culture and are, therefore, not susceptible to collapse during tissue, *e.g.,* muscle, contraction or deformation. The tubes prepared according to the method of the invention also biocompatible and support liquid perfusion. Perfused bioprotein tubes exchange cell culture medium within three-dimensional (3D) tissues and supply embedded cells with nutrients required for long-term survival in 3D tissue culture. The tubes can also remove cellular and metabolic waste products from tissues thereby maintaining tissue homeostasis and health. Cell adhesion to the tubing can be controlled by adjusting bioprotein precursor concentrations prior to tube formation. Acellular or cell-laden synthetic vasculature can be produced using the methods herein described. Therefore, the bioprotein tubes produced according to the methods disclosed herein can be, for example, used as synthetic vasculature and, together with cells, may also be used in forming engineered tissue. Such tissue is useful not only for the production of prosthetic devices and regenerative medicine, but also for investigating tissue developmental biology and disease pathology, drug discovery and toxicity testing, and for use as edible food products, *e.g.,* a tubular food product, *e.g.,* sausage casing and candy. The bioprotein tubes of the invention may also be combined with other substances, such as, therapeutic agents, in order to deliver such substances to the site of application or implantation of the bioprotein tubes and/or tissue.

Accordingly, the present application provides methods of producing a bioprotein tube.

A. Formation of Bioprotein Tubes by Casting on an Elongate Body

Figure 1 is a flow chart of a method 10 of producing bioprotein tube in accordance with some embodiments. In some aspects, the methods include contacting at least a portion of an elongate body with a first solution including alginate (step 12). The portion of the elongate body that contacts the first solution may be referred to as the tube forming portion of the elongate body. An outer diameter or cross-sectional width of the tube forming portion of the elongate body at least partially determines an inner diameter or inner cross-sectional width of the resulting bioprotein tube. In some embodiments, the diameter or cross-sectional width of the elongate body throughout the tube forming portion falls in an range of about 10 micrometers to 5 centimeter. In some embodiments, such as embodiments in which the bioprotein tubes are for use as synthetic vasculature, the diameter or cross-sectional width of the elongate body throughout the tube forming portion falls in a range of about 500 micrometers to about 5 millimeters, about 400 micrometers to about 5 millimeters, about 350 micrometers to about 5 millimeters, about 250 micrometers to about 5 millimeters, about 200 micrometers to about 5 millimeters, about 500 micrometers to about 4 millimeters, about 500 micrometers to about 3 millimeters, about 500 micrometers to about 2 millimeters, about 500 micrometers to about 1 millimeters, about 400 micrometers to about 5 millimeters, about 400 micrometers to about 4 millimeters, about 400 micrometers to about 3 millimeters, about 400 micrometers to about 2 millimeters, about 300 micrometers to about 5 millimeters, about 300 micrometers to about 4 millimeters, about 300 micrometers to about 3 millimeters, about 300 micrometers to about 2 millimeters, about 250 micrometers to about 5 millimeters, about 250 micrometers to about 4 millimeters, about 250 micrometers to about 3 millimeters, about 250 micrometers to about 2 millimeters, about 200 micrometers to about 5 millimeters, about 200 micrometers to about 4 millimeters, about 200 micrometers to about 3 millimeters, about 200 micrometers to about 2 millimeters, about 150 micrometers to about 5 millimeters, about 150 micrometers to about 4 millimeters, about 150 micrometers to about 3 millimeters, about 150 micrometers to about 2 millimeters. In other embodiments, such as embodiments in which the bioprotein tubes are for use a tubular food product, such as a sausage, the diameter or cross-sectional width of the elongate body throughout the tube forming portion falls in a range of about 10 millimeters to about 50 millimeters, such as about 10, 15, 20, 25, 30, 35, 40, or about 50 millimeters. In yest other embodiments, such

as embodiments in which the bioprotein tubes are for use a tubular food product, such as a candy, the diameter or cross-sectional width of the elongate body throughout the tube forming portion falls in a range of about 2 millimeters to about 20 millimeters, such as about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or about 20 millimeters. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

In some embodiments, the cross-sectional shape of the tube forming portion is circular. In some embodiments, the cross-sectional shape of the tube forming portion is non-circular (*e.g.*, oval, elliptical, square, rectangular, polygonal, *etc.*)

The length of the tube forming portion is at least as long as a length the resulting bioprotein tube. The bioprotein tubes formed by casting on an elongate body may be of any suitable length. For example, the bioprotein tubes may be about 1 nanometer, about 1 millimeter, about 1 centimeter, about a meter, or longer. Additionally, after formation, the bioprotein tubes may be cut to a desired length using any suitable instrument.

Any elongate body with suitable dimensions may be employed (*e.g.*, a rod, a mandrel, a solid tube, *etc.*). Any suitable material may be employed for the elongate body (*e.g.*, metal, glass, *etc.*).

In some embodiments, the first solution has a concentration of about 1% to about 8% alginate, *e.g.,* about 1.0, about 1.25, about 1.5, about 1.75, about 2.0, about 2.25, about 2.5, about 2.75, about 3.0, about 3.25, about 3.5, about 3.75, about 4.0, about 4.25, about 4.5, about 4.75, about 5.0, about 5.25, about 5.5, about 5.75, about 6.0, about 6.25, about 6.5, about 6.75, about 7.0, about 7.25, about 7.5, about 7.75, about 8.0%. In some embodiments, the first solution is about 3% alginate. In other embodiments, the first solution is about 5% alginate. In some embodiments, the first solution includes water as a solvent. In some embodiments, the first solution is a mixture of alginate and gelatin, *e.g.,* a mixture of about a 5% alginate solution and about a 5% gelatin solution at a ratio of about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1 about 4:1, about 3:1, about 2:1, about 1:1, or about 0.5:1. Ratios intermediate to the above recited ratios (*e.g.,* about 5.5:1, about 4.9:1, about 6:1.2) are also contemplated to be part of the invention. Amounts intermediate to the above recited amount (*e.g.,* about 2.3%) are also contemplated to be part of the invention.

The alginate adheres to the portion of the elongate body that was in contact with the first solution. The portion of the elongate body that was in contact with the first solution is passed through an aperture to remove excess alginate adhered to the elongate body (step 14). Cross-sectional dimensions of the aperture at least partially determine outer cross-sectional dimensions of the resulting bioprotein tube. Further, the difference between the diameter or cross-sectional width of the elongate body and the diameter or width of the aperture at least partially determines a wall thickness of the resulting bioprotein tube. In some embodiments, the diameter or width of the aperture is in a range of 10 micrometers to 5 millimeters larger than a diameter or cross-sectional width of the tube forming portion of the elongate body.

The method also includes exposing the portion of the elongate body with adhered alginate material to a second solution including $CaCl_2$ to gel the adhered alginate to form an alginate tube on the elongate body (step 16). In some embodiments, the second solution is a solution of about 0.5% to about 5% $CaCl_2$ (*e.g.,* about 0.5%, about 0.75%, about 1%, about 1.25%, about 1.5%, about 1.75%, about 2%, about 2.25%, about 2.5%, about 2.75%, about 3%, about 3.25%, about 3.5%, about 3.75%, about 4%, about 4.25%, about 4.5%, about 4.75%, or about 5%). In some embodiments, the second solution is about 2.5% $CaCl_2$. In some embodiments, the $CaCl_2$ is a solution of $CaCl_2$ and water. In some embodiments, the tube forming portion of the elongate body with adhered alginate remains in contact with the second solution for a period of time sufficient to gel the alginate material. In some embodiments, the period of time is about 15 minutes.

In some embodiments, contacting at least a portion of the elongate body with the first solution includes advancing the portion of the elongate body into a first reservoir holding the first solution. In some embodiments, exposing the portion of the elongate body with adhered alginate material to the second solution to gel the adhered alginate includes extending the portion of the elongate body with adhered alginate material into a second reservoir containing the second solution.

In some embodiments the first reservoir includes a proximal end and a distal end with the aperture disposed at the distal end. In some embodiments contacting at least a portion of the elongate body with the first solution includes advancing at least the portion of the elongate body into the proximal end of the first reservoir. In some embodiments passing the portion of the elongate body that was in contact with the first solution through the aperture to remove excess alginate material includes advancing the portion of the first

elongate body through the first reservoir and out the aperture of the distal end of the first reservoir. In some embodiments a plug at a distal end of the elongate body prevents the first solution from leaving the first reservoir through the aperture prior to advancing the portion of the elongate body through the proximal end of the first reservoir. In some embodiments the first solution is added to the first reservoir prior to advancing the portion of the elongate body into the proximal end of the first reservoir.

Figures 2A-8 schematically depict a setup for performing steps 12 through 16 of the method 10, in accordance with some embodiments. One of ordinary skill in the art in view of the present disclosure will appreciate that other setups may be employed for performing steps 12 through 16 of the method 10. In Figures 2A and 2B, the elongate body 30 is inserted into a first reservoir 34 containing the first solution comprising alginate. A distal end 31b of the elongate body 30 includes the aperture 36 (*see* Figure 4B). The first reservoir 34 is positioned over a second reservoir 38 containing the second solution including $CaCl_2$. Figure 2B is a detail view of an image of the distal end of the elongate body 30 and the first reservoir 34 over the second reservoir 38. Figures 3A-5 are schematic images of various components shown in Figures 2A and 2B. Figures 3A and 3B are a side view and a top view, respectively, of a guide 40 for the elongate body. Figure 3C is a perspective image of a prototype guide. Figures 4A and 4B are a side view and a top view, respectively, of the first reservoir 34 for containing the first solution. Figure 4C is a perspective image of a prototype first reservoir. As shown Figure 4A, the first reservoir 34 has a proximal end 35a and a distal end 35b. As shown in Figure 4B, an aperture 36 is disposed in the distal end of the first reservoir 34. Figure 5A depicts the elongate body 30 having a tube forming portion 31, a proximal end 31a, and a distal end 31b. The elongate body 30 also includes a plug 33 at the distal end 31b. Figure 5B is an image of a prototype elongate body with a plug.

Figure 6A schematically depicts forming an assembly of the elongate body 30, the first reservoir 34, and the guide 40. The guide 40 is placed on the first reservoir 34. The proximal end 31a of the elongate body is inserted through the aperture 36 of the first reservoir 34 and through the guide 40 until the plug 33 is in contact with the aperture 34 thereby plugging or blocking flow out of the aperture 34. The assembly of the elongate body 30, first reservoir 34, and the guide 40 is placed over the second reservoir 38 as shown in Figures 2A and 2B with the plug 33 blocking the aperture. The first reservoir 34 is then filled with the first solution. Figure 6B is an image of a prototype of the assembly.

Figure 7 schematically depicts contacting the portion of the elongate body 30 with the first solution by advancing the portion of the elongate body 30 into the proximal end of the first reservoir 35a. Advancing the elongate body 30 as shown by arrow 42 also passes the portion of the elongate body 30 that was in contact with the first solution through the aperture 36 (*see* Figure 4B) at the distal end 35b of the first reservoir to remove excess alginate material. Advancing the elongate body 30 as shown by arrow 42 also extends the portion of the elongate body with adhered alginate 44 into the second reservoir 38 and into contact with the second solution. As shown in Figure 8, the tube forming portion 32 of the elongate body with adhered alginate may be left in the second solution until the alginate gels forming an alginate tube 46.

Turning again to Figure 1, in some embodiments, after the alginate is gelled to form an alginate tube 46, a plurality of perforations or holes are formed in the alginate tube 46 (step 22). In Figure 1 step 22 is shown in dotted lines to indicate that this step is optional. In other embodiments, a plurality of perforations or holes are not made in the alginate tube prior to proceeding to step 20. Figure 9A schematically depicts formation of a plurality of perforations or holes 50a-50e in the alginate tube 46 using a needle 54. Figure 9B is an image of an alginate tube before perforation and Figure 9C is an image of an alginate tube after perforation. As would be appreciated by one of ordinary skill in the art, any suitable tool could be employed to form the plurality of perforations or holes, such as a punch, a hollow needle, *etc*. In some embodiments, a diameter or width of the perforations or holes falls in a range of about 100 to about 200 μm, about 110 to about 200 μm, about 120 to about 200 μm, about 130 to about 200 μm, about 140 to about 200 μm, about 150 to about 200 μm, about 160 to about 200 μm, about 170 to about 200 μm, about 180 to about 200 μm, about 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, or about 180 μm in diameter. In some embodiments, the number of perforations or holes falls in a range of 0.1 to 100 per millimeter. In some embodiments, the number of perforations or holes falls in a range of 0.2 to 4 per millimeter. As shown, the perforations or holes are evenly spaced and oriented in a line; however, in other embodiments, a spacing between perforations or holes varies. In some embodiments, the perforations or holes are oriented in other patterns along a length of the tube, as would be appreciated by one of skill in the art. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

In some embodiments, for example, when the bioprotein tubes disclosed herein are for use as synthetic vasculature, the diameter of the tubes is about 1.0 to about 2.0 mm (*e.g.*, about 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, or about 2.0 mm), with perforations of about 100 μm to about 200 μm in diameter (about 110 to about 200 μm, about 120 to about 200 μm, about 130 to about 200 μm, about 140 to about 200 μm, about 150 to about 200 μm, about 160 to about 200 μm, about 170 to about 200 μm, about 180 to about 200 μm, about 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, or about 180 μm in diameter), such perforations spaced about every 1.0 to about 2.0 mm (*e.g.*, about 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, or about 2 mm) along the length of the tube. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

In some embodiments, the gelled alginate tube 46 is removed from the elongate body before the perforation or holes are formed as shown in Figure 10. In some embodiments, the gelled alginate tube is placed back on the elongate body after the perforations or holes are formed. In other embodiments, the gelled alginate tube 46 remains on the elongate body while the perforations or holes are formed.

Turning again to Figure 1, the method further includes positioning a first end 47a of the tube 46 in contact with a third solution 56 including gelatin and/or a biogenic polymer and drawing the third solution into the tube by at least partially withdrawing the elongate body 20 from a second end 47b of the tube (step 20) as shown in Figure 10. The method also includes advancing the elongate body 30 toward the first end 47a of the tube to expel excess third solution material 56 from the tube 46 (step 22) as shown in Figure 11.

In some embodiments the third solution includes gelatin. In some embodiments, the third solution includes a biogenic polymer. In some embodiments, the biogenic polymer includes one or more extracellular matrix (ECM) proteins such as collagen, fibronectin, laminin and combinations thereof. In some embodiments, the biogenic polymer includes one or more of bioproteins, peptides, nucleic acids, and other bio-active molecules. Other suitable biogenic polymers include, for example, silk, a keratin, an elastin, a fibrillin, a fibrinogen, a fibrin, a thrombin, a vimentin, a neurofilament, an integrin, a hyaluronic acid, chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, heparin sulfate, heparin, keratan sulfate, a proteoglycan, an amyloid, an actin, a myosin, a chitosan, a titin, and combinations thereof.

Suitable solutions of gelatin for use in the present invention include about 0.1% to about 10% (w/v, *e.g.,* about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.25, 1.5, 1.75, 2, 2.25, 2.5, 2.75, 3, 3.25, 3.5, 3.75, 4, 4.25, 4.5, 4.75, 5, 5.25, 5.5, 5.75, 6, 6.25, 6.5, 6.75, 7, 7.25, 7.5, 7.75, 8, 8.25, 8.5, 8.75, 9, 9.25, 9.5, 9.75, or about 10%. Suitable solutions of a biogenic polymer, *e.g.,* fibronectin, for use in the present invention include about 0.1% to about 10% (w/v, *e.g.,* about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.25, 1.5, 1.75, 2, 2.25, 2.5, 2.75, 3, 3.25, 3.5, 3.75, 4, 4.25, 4.5, 4.75, 5, 5.25, 5.5, 5.75, 6, 6.25, 6.5, 6.75, 7, 7.25, 7.5, 7.75, 8, 8.25, 8.5, 8.75, 9, 9.25, 9.5, 9.75, or about 10%. Amounts intermediate to the above recited amount (*e.g.,* about 2.3%) are also contemplated to be part of the invention.

Turning again to Figure 1, in some embodiments, the method 10 further includes positioning the first end 47a of the tube 46 in contact with a fourth solution 58 including a cross-linking agent and drawing the fourth solution 58 into the tube by at least partially withdrawing the elongate body 30 from the second end 47b of the tube (step 24) as shown by Figure 12. In some embodiments, the alginate tube 46 may then be removed from the elongate body 30 and soaked in the fourth solution as shown in Figure 13. Step 24 is shown with dotted lines indicating that need not be performed in all embodiments.

In some embodiments the cross-linking agent includes microbial transglutaminase (MTG). In some embodiments the cross-linking agent includes genipin. In some embodiments the cross-linking agent comprises riboflavin and UV light is employed to affect photocrosslinking. One of ordinary skill in the art will appreciate that other suitable cross-linking agents may also or alternatively be employed.

A suitable solution of mTG may have a concentration of about 0.01% to about 5% (w/v), *e.g.,* about 0.1%, about 0.2%, about 03%, about 0.4%, about 0.5%, about 0.6%, about 0.7%, about 0.8%, about 0.9%, about 0.1%, about 0.15% about 0.25%, about 0.35%, about 0.45%, about 0.55%, about 0.65%, about 0.75%, about 0.85%, about 0.95%, 1.0%, about 1.25%, about 1.5%, about 1.75%, about 2%, about 2.25%, about 2.5%, about 2.75%, about 3%, about 3.25%, about 3.5%, about 3.75%, about 4%, about 4.25%, about 4.5%, about 4.75%, or about 5%.

The bioprotein tubes prepared according to the methods of the invention may have an inner diameter of about 0.05 millimeters (mm) to about 3.75 mm, *e.g.,* about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3,

2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, or about 3.75 mm. The bioprotein tubes prepared according to the methods of the invention may have a tube wall thickness of about 0.05 millimeters (mm) to about 3.75 mm, *e.g.,* about 0.05, .01, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, or about 3.5 mm. Ranges, diameters, and thicknesses intermediate to the above recited amount ranges, diameters, and thicknesses are also contemplated to be part of the invention.

B. Formation of Bioprotein Tubes by Extruded Stream

In some aspects, the methods of forming a bioprotein tube include extruding a stream of a bioprotein precursor solution comprising alginate through an orifice into a gelation solution. The gelation solution comprises calcium chloride which initiates gelation at an outer surface of the extruded stream as it enters the gelation solution. The extruded stream of bioprotein precursor solution is maintained in the gelation solution while gelation proceeds from the outer surface inward to form a gelled front advancing from the gelled outer surface toward a central axis of the extruded stream. Prior to the gelled front reaching the central axis of the partially gelled extruded stream is exposed to a wash solution to cease gelation and remove the un-gelled bioprotein precursor solution from the extruded stream, resulting in the production of a bioprotein tube having an outer surface at the gelled outer surface, an inner surface at the gelled front, and a lumen.

Suitable orifices for use in the foregoing methods include, for example, an orifice of a nozzle and an orifice of a needle, *e.g.*, a 90° square tip needle. Exemplary orifice diameters that may be used in some exemplary embodiments range between about 0.05 μm and about 10000 μm, *e.g.*, between about 0.05 and about 500, between about 0.05 and 100, between about 0.1 and 1000, between about 0.1 and 500, between about 0.1 and 100, between about 1 and 1000, between about 1 and 500, between about 1 and 100, between about 10 and 1000, between about 10 and 500, between about 10 and 100, between about 50 and 1000, between about 50 and 500, between about 50 and 100, between about 100 and 1000, between about 100 and 500, between about 150 and 500, between about 200 and 500, between about 250 and 500, between about 250 and 450, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.15, 0.2, 0.225, 0.25, 0.275, 0.3, 0.325, 0.35, 0.375, 0.4, 0.425, 0.45, 0.475, 0.5, 0.525, 0.55, 0.575, 0.6, 0.625, 0.65, 0.675, 0.7, 0.725, 0.75, 0.075, 0.8, 0.825, 0.85, 0.825, 0.9,

0.925, 0.95, 0.975, 1.0, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, or about 10000 μm. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

Exemplary orifices may have any suitable cross-sectional geometry including, but not limited to, circular, oval, square, rectangular, *etc.*

Orifices may be arrayed for high-throughput production of bioprotein tubes.

In other aspect of the invention, the methods of producing a bioprotein tube include extruding an annular stream of a bioprotein precursor solution comprising alginate through a nozzle into a gelation solution. The gelation solution comprises calcium chloride which initiates gelation at an outer surface and an inner surface of the annular stream. The extruded annular stream is maintained in the gelation solution while cross-linking and gelation proceeds throughout the extruded annular stream, to produce a bioprotein tube including an outer surface, an inner surface, and a lumen.

In some embodiments, the methods of the invention include extruding an annular stream of bioprotein precursor solution through a nozzle associated with an orifice of a rotating reservoir into the cross-linking solution in a collection device. Suitable devices which include a rotating reservoir and a collection device for use in the methods of the invention are described in PCT/US2014/016197, filed February 13, 2014, and published as WO 2014/127099, the entire contents of which are incorporated herein by reference. For example, suitable devices include a reservoir for holding a bioprotein precursor solution and including a surface having one or more orifices, wherein one or more of the orifices are associated with a nozzle for extruding the bioprotein precursor solution in an annular stream, a first motion generator configured to impart rotational motion to the reservoir, the rotational motion of the reservoir causing extrusion of the solution through the one or more nozzles, and a collection device holding a gelation solution, the collection device configured and positioned to accept the annular stream extruded from the reservoir, a second motion generator couplable to the collection device, the second motion generator configured to impart rotational motion to the gelation solution in the collection device to generate a liquid vortex including an air gap, wherein the reservoir and the collection device

are positioned such that the one or more nozzles of the reservoir are positioned in the air gap of the liquid vortex in the collection device; and wherein the extrusion of the bioprotein precursor solution into the air gap and subsequently into the gelation solution of the liquid vortex in the collection device causes formation of bioprotein tubes. One embodiment of a suitable device, referred to as an "immersed rotary jet spinning" or "iRJS" device is depicted in Figure 10.

Accordingly, in one embodiment, the methods of the invention further include rotating the bioprotein precursor solution about an axis of rotation to cause extrusion of the bioprotein precursor solution in one or more annular streams, generating a liquid vortex in a collection device for collecting the one or more annular streams of the bioprotein precursor solution, the liquid vortex including a central air gap, and collecting the one or more annular streams of the bioprotein precursor solution in the collection device, wherein the one or more jets are initially ejected through the air gap of the liquid vortex and subsequently through the gelation solution in the liquid vortex of the collection device; wherein the extrusion of the bioprotein precursor solution into the air gap and subsequently into the gelation solution in the collection device causes formation of one or more bioprotein tubes.

An exemplary reservoir may have a volume ranging from about one nanoliter to about 1 milliliter, about one nanoliter to about 5 milliliters, about 1 nanoliter to about 100 milliliters, or about one microliter to about 100 milliliters, for holding the liquid material. Some exemplary volumes include, but are not limited to, about one nanoliter o about 1 milliliter, about one nanoliter to about 5 milliliters, about 1 nanoliter to about 100 milliliters, one microliter to about 100 microliters, about 1 milliliter to about 20 milliliters, about 20 milliliters to about 40 milliliters, about 40 milliliters to about 60 milliliters, about 60 milliliters to about 80 milliliters, about 80 milliliters to about 100 milliliters, but are not limited to these exemplary ranges. Exemplary volumes intermediate to the recited volumes are also part of the invention. In certain embodiment, the volume of the reservoir is less than about 5, less than about 4, less than about 3, less than about 2, or less than about 1 milliliter.

In other embodiments, larger reservoirs may be used, such as reservoirs having a volume of about 1 L to about 100 L, *e.g.,* reservoirs having a volume of about 1, about 5, about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 45, about 50, about 55, about 60, about 65, about 70, about 75, about 80, about 85, about 90, about 95,

about 100 L, or more. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

In embodiments of larger reservoirs, such reservoirs may be top loading such that a polymer solution is continuously fed into the reservoir when in operation.

In some embodiments, the reservoir may include one or more inlet ports, each coupled to one or more inlet pipes for introducing one or more material solutions and/or one or more other fluids (*e.g.,* air pressure) into the reservoir. An exemplary inlet pipe may be coupled to one or more storage devices that store a material solution or to one or more devices that produce a material solution. One or more material solutions may be fed into the reservoir through the inlet port at a constant flow rate or at variable flow rates. In an exemplary embodiment, the inlet port may be closed temporarily or permanently after the reservoir is filled. In another exemplary embodiment, the inlet port may remain open for continuous or intermittent filling of the reservoir. In an exemplary embodiment, the reservoir may be pre-filled and the filled reservoir may not include the inlet pipe and may have one or more temporarily or permanently sealed inlet ports. In another exemplary embodiment, the inlet port may remain coupled to the inlet pipe and the reservoir may be filled continuously or in one or more sessions.

Exemplary orifices may have any suitable cross-sectional geometry including, but not limited to, circular, oval, square, rectangular, *etc.* The one or more nozzles associated with an exemplary orifice controls the shape of the solution exiting the reservoir through the orifice. The locations, cross-sectional geometries and arrangements of the orifices on the reservoir, and/or the locations, cross-sectional geometries and arrangements of the nozzles on the orifices, may be configured based on the desired characteristics of the resulting fibers, *etc.*

An exemplary nozzle is provided integrally or removably on a reservoir so that the nozzle is associated with a single orifice. In another exemplary embodiment, exemplary nozzles are provided replaceably on orifices so that one nozzle provided on an orifice may be replaced by another nozzle.

Exemplary orifices may have any length. For example, orifice lengths that may be used in some exemplary embodiments range between about 0.001 m and about 0.1 m, between about 0.001 m and about 0.01 m, between about 0.001 m and about 0.005 m, between about 0.002 m and about 0.005 m, between about 0.001 m and about 0.05 m,

between about 0.0015 m and about 0.007 m, between about 0.002 m and about 0.007 m, between about 0.0025 m and about 0.0065 m, between about 0.002 m and about 0.006 m, *e.g.*, about 0.0015, 0.002, 0.0025, 0.003, 0.0035, 0.004, 0.0045, 0.005, 0.0055, 0.006, 0.0065, 0.007, 0.0075, 0.008, 0.0085, 0.009, 0.0095, 0.01, 0.015, 0.02, 0.025, 0.03, 0.035, 0.04, 0.045, 0.05, 0.055, 0.06, 0.065, 0.07, 0.075, 0.08, 0.085, 0.09, 0.095, or 0.1 m. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

Exemplary orifice diameters that may be used in some exemplary embodiments range between about 0.05 μm and about 1000 μm, *e.g.*, between about 0.05 and about 500, between about 0.05 and 100, between about 0.1 and 1000, between about 0.1 and 500, between about 0.1 and 100, between about 1 and 1000, between about 1 and 500, between about 1 and 100, between about 10 and 1000, between about 10 and 500, between about 10 and 100, between about 50 and 1000, between about 50 and 500, between about 50 and 100, between about 100 and 1000, between about 100 and 500, between about 150 and 500, between about 200 and 500, between about 250 and 500, between about 250 and 450, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.15, 0.2, 0.225, 0.25, 0.275, 0.3, 0.325, 0.35, 0.375, 0.4, 0.425, 0.45, 0.475, 0.5, 0.525, 0.55, 0.575, 0.6, 0.625, 0.65, 0.675, 0.7, 0.725, 0.75, 0.075, 0.8, 0.825, 0.85, 0.825, 0.9, 0.925, 0.95, 0.975, 1.0, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, or 1000 μm. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

The outer orifice diameter or outer nozzle diameter of exemplary coaxial extruders that may be used range between about 0.1 μm and about 1000 μm, *e.g.*, between about 0.05 and about 500, between about 0.05 and 100, between about 0.1 and 1000, between about 0.1 and 500, between about 0.1 and 100, between about 1 and 1000, between about 1 and 500, between about 1 and 100, between about 10 and 1000, between about 10 and 500, between about 10 and 100, between about 50 and 1000, between about 50 and 500, between about 50 and 100, between about 100 and 1000, between about 100 and 500, between about 150 and 500, between about 200 and 500, between about 250 and 500, between about 250 and 450, *e.g.,* about 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.15, 0.2, 0.225, 0.25, 0.275, 0.3, 0.325, 0.35, 0.375, 0.4, 0.425, 0.45, 0.475, 0.5, 0.525, 0.55, 0.575, 0.6, 0.625, 0.65, 0.675, 0.7, 0.725, 0.75, 0.075, 0.8, 0.825, 0.85, 0.825, 0.9, 0.925, 0.95, 0.975, 1.0, 1.5, 2, 2.5, 3,

3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, or 1000 μm.

The inner orifice diameter or inner nozzle diameter of exemplary coaxial extruders that may be used range between about 0.025 μm and about 500 μm, *e.g.*, between about 0.025 and about 500, between about 0.05 and 100, between about 0.1 and 1000, between about 0.1 and 500, between about 0.1 and 100, between about 1 and 1000, between about 1 and 500, between about 1 and 100, between about 10 and 1000, between about 10 and 500, between about 10 and 100, between about 50 and 1000, between about 50 and 500, between about 50 and 100, between about 100 and 1000, between about 100 and 500, between about 150 and 500, between about 200 and 500, between about 250 and 500, between about 250 and 450, *e.g.,* about 0.025, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.15, 0.2, 0.225, 0.25, 0.275, 0.3, 0.325, 0.35, 0.375, 0.4, 0.425, 0.45, 0.475, 0.5, 0.525, 0.55, 0.575, 0.6, 0.625, 0.65, 0.675, 0.7, 0.725, 0.75, 0.075, 0.8, 0.825, 0.85, 0.825, 0.9, 0.925, 0.95, 0.975, 1.0, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, or 1000 μm. *See* Figures 35A through 36B, which illustrate the outer orifice or nozzle diameter *Do* and the inner orifice or nozzle diameter *Di* for two different embodiments of reservoirs configured for coaxial extrusion.

In exemplary embodiments, the one or more nozzles (and/or orifices) are horizontally spaced from the liquid vortex by a distance of between about 0.1-8.0 cm, between about 1-8.0 cm, between about 1.5-7.5 cm, between about 2.0-7.0 cm, between about 2.5-6.5 cm, between about 2.5-6.0 cm, between about 2.5-5.5 cm, between about 2.5-5.0 cm, between about 3.0-6.0 cm, between about 3.0-5.5 cm, between about 3.0-5.0 cm, *e.g.,* about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 3.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1,3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.5, 7.5, 7.6, 7.7, 7.8, 7.9, or about 8.0 cm. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention. Extrusion of the precursor solution into the air gap extends the solution and alignment and subsequent contact with the liquid of the liquid vortex in the collection device causes gelation of the precursor solution. In an exemplary embodiment, the collection device is disposed vertically below the reservoir (see, *e.g.,*

Figure 23).

The reservoir and collection device may be constructed of any material, *e.g.,* a material that can withstand heat and/or that is not sensitive to chemical organic solvents. In one embodiment, the reservoir and the collection device are made up of glass or a plastic material, *e.g.,* polypropylene, polyethylene, or polytetrafluoroethylene. In another embodiment, the reservoir and the collection device are made up of a metal, *e.g.,* aluminum, steel, stainless steel, tungsten carbide, tungsten alloys, titanium or nickel.

Any suitable size or geometrically shaped reservoir or collector may be used in the devices of the invention. For example, the reservoir and/or collector may be round, rectangular, or oval. The reservoir and/or collector may be round, oval, rectangular, or a half-heart shape. The collector may also be shaped in the form of any living organ, such as a heart, kidney, liver lobe(s), bladder, uterus, intestine, skeletal muscle, or lung shape, or portion thereof. The collector may further be shaped as any hollow cavity, organ or tissue, such as a circular muscle structure, *e.g.,* a sphincter or iris, or, for the fabrication of protective clothing, a human head, a torso, a hand, etc.

In one embodiment, the devices of the invention further comprise a component suitable for continuously feeding the polymer into the rotating reservoir, such as a spout or syringe pump

The reservoir may also include a heating element for heating and/or melting the polymer.

In certain embodiments, the collection device is maintained at about room temperature, *e.g.,* about 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or about 30°C and ambient humidity, *e.g.,* about 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, or about 90% humidity. The devices may be maintained at and the methods may be formed at any suitable temperature and humidity.

Rotational speeds of the reservoir in exemplary embodiments may range from about 1,000 rpm-400,000 rpm, for example, about 1,000 rpm to about 40,000 rpm, about 1,000 rpm to about 20,000 rpm, about 3,000 rpm-85,000 rpm, about 3,000 rpm-50,000 rpm, about 3,000 rpm-25,000 rpm, about 5,000 rpm-20,000 rpm, about 5,000 rpm to about 15,000 rpm, or about 50,000 rpm to about 400,000 rpm, *e.g.*, about 1,000, 1,500, 2,000, 2,500, 3,000,

3,500, 4,000, 4,500, 5,000, 5,500, 6,000, 6,500, 7,000, 7,500, 8,000, 8,500, 9,000, 9,500,10,000, 10,500, 11,000, 11,500, 12,000, 12,500, 13,000, 13,500, 14,000, 14,500, 15,000, 15,500, 16,000, 16,500, 17,000, 17,500, 18,000, 18,500, 19,000, 19,500, 20,000, 20,500, 21,000, 21,500, 22,000, 22,500, 23,000, 23,500, 24,000, 32,000, 50,000, 55,000, 60,000, 65,000, 70,000, 75,000, 80,000, 85,000, 90,000, 95,000, 100,000, 105,000, 110,000, 115,000, 120,000, 125,000, 130,000, 135,000, 140,000, 145,000, 150,000 rpm, about 200,000 rpm, 250,000 rpm, 300,000 rpm, 350,000 rpm, or 400,000 rpm. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

In certain embodiments, rotating speeds of about 50,000 rpm-100,000 rpm or about 50,000 rpm-400,000 rpm are intended to be encompassed by the methods of the invention. In one embodiment, devices employing rotational motion may be rotated at a speed greater than about 50, 000 rpm, greater than about 55,000 rpm, greater than about 60,000 rpm, greater than about 65,000 rpm, greater than about 70,000 rpm, greater than about 75,000 rpm, greater than about 80,000 rpm, greater than about 85,000 rpm, greater than about 90,000 rpm, greater than about 95,000 rpm, greater than about 100,000 rpm, greater than about 105,000 rpm, greater than about 110,000 rpm, greater than about 115,000 rpm, greater than about 120,000 rpm, greater than about 125,000 rpm, greater than about 130,000 rpm, greater than about 135,000 rpm, greater than about 140,000 rpm, greater than about 145,000 rpm, greater than about 150,000 rpm, greater than about 160,000 rpm, greater than about 165,000 rpm, greater than about 170,000 rpm, greater than about 175,000 rpm, greater than about 180,000 rpm, greater than about 185,000 rpm, greater than about 190,000 rpm, greater than about 195,000 rpm, greater than about 200,000 rpm, greater than about 250,000 rpm, greater than about 300,000 rpm, greater than about 350,000 rpm, or greater than about 400,000 rpm.

Exemplary devices employing rotational motion may be rotated for any desired period of time, such as a time sufficient to form a desired length of bioprotein tubes (or desired collection of nanometer dimension polymeric fibers.

In some embodiments, a suitable bioprotein precursor solution further comprises gelatin and/or a biogenic polymer. For example, bioprotein tubes prepared using a bioprotein precursor solution that includes primarily or only alginate have minimal cell adhesiveness. However, a gelatin and/or a biogenic polymer may be mixed with alginate in

the bioprotein precursor solution in order to promote cell adhesion on and/or in the bioprotein tubes.

Suitable solutions of alginate for use in the present invention include about 1% (w/v) to about 8% (w/v) solutions of alginate, *e.g.,* about 1.0, about 1.25, about 1.5, about 1.75, about 2.0, about 2.25, about 2.5, about 2.75, about 3.0, about 3.25, about 3.5, about 3.75, about 4.0, about 4.25, about 4.5, about 4.75, about 5.0, about 5.25, about 5.5, about 5.75, about 6.0, about 6.25, about 6.5, about 6.75, about 7.0, about 7.25, about 7.5, about 7.75, about 8.0%. Suitable solutions of gelatin for use in the present invention include about 0.1% to about 10% (w/v, *e.g.,* about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.25, 1.5, 1.75, 2, 2.25, 2.5, 2.75, 3, 3.25, 3.5, 3.75, 4, 4.25, 4.5, 4.75, 5, 5.25, 5.5, 5.75, 6, 6.25, 6.5, 6.75, 7, 7.25, 7.5, 7.75, 8, 8.25, 8.5, 8.75, 9, 9.25, 9.5, 9.75, or about 10%. Suitable solutions of a biogenic polymer, *e.g.,* fibronectin, for use in the present invention include about 0.1% to about 10% (w/v, *e.g.,* about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.25, 1.5, 1.75, 2, 2.25, 2.5, 2.75, 3, 3.25, 3.5, 3.75, 4, 4.25, 4.5, 4.75, 5, 5.25, 5.5, 5.75, 6, 6.25, 6.5, 6.75, 7, 7.25, 7.5, 7.75, 8, 8.25, 8.5, 8.75, 9, 9.25, 9.5, 9.75, or about 10%. Amounts intermediate to the above recited amount (*e.g.,* about 2.3%) are also contemplated to be part of the invention.

Suitable ratios of alginate to gelatin and/or biogenic polymer in a bioprotein precursor solution comprising the foregoing solutions for use in the methods of the invention and which are suitable to maintain the stability of the bioprotein tubes in a liquid (*e.g.,* water or tissue culture media) include about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1 about 4:1, about 3:1, about 2:1, about 1:1, or about 0.5:1. Ratios intermediate to the above recited ratios (*e.g.,* about 5.5:1, about 4.9:1, about 6:1.2) are also contemplated to be part of the invention.

Suitable biogenic polymers for use in bioprotein precursor solution include, for example, silk, a keratin, an elastin, a fibrillin, a fibrinogen, a fibrin, a thrombin, a fibronectin, a laminin, a collagen, a vimentin, a neurofilament, an integrin, a hyaluronic acid, chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, heparin sulfate, heparin, keratan sulfate, a proteoglycan, an amyloid, an actin, a myosin, a chitosan, and a titin.

The gelation solution may include about 1%, about 1.25%, about 1.5%, about 1.75%, about 2%, about 2.25%, about 2.5%, about 2.75%, about 3%, about 3.25%, about 3.5%, about 3.75%, about 4%, about 4.25%, about 4.5%, about 4.75%, or about 5% calcium

chloride. Amounts intermediate to the above recited amount (*e.g.,* about 2.3%) are also contemplated to be part of the invention.

In some embodiments, a cross-linking agent is added to the gelation solution to, *e.g.,* enhance the long-term stability of the bioprotein tubes. For example, in embodiments in which the bioprotein precursor solution comprises gelatin, a microbial transglutaminase ("mTG" or "meat glue") may be included. In some embodiments the cross-linking agent may genipin. In some embodiments the cross-linking agent comprises riboflavin. For example, in some embodiments, the bioprotein precursor solution comprises mTG at a concentration of about 0.01% to about 5% (w/v), *e.g.,* about 0.1%, about 0.2%, about 03%, about 0.4%, about 0.5%, about 0.6%, about 0.7%, about 0.8%, about 0.9%, about 0.1%, about 0.15% about 0.25%, about 0.35%, about 0.45%, about 0.55%, about 0.65%, about 0.75%, about 0.85%, about 0.95%, 1.0%, about 1.25%, about 1.5%, about 1.75%, about 2%, about 2.25%, about 2.5%, about 2.75%, about 3%, about 3.25%, about 3.5%, about 3.75%, about 4%, about 4.25%, about 4.5%, about 4.75%, or about 5%. In other embodiments, a bioprotein tube is contacted with a cross-linking agent, *e.g.,* mTG, after the tube is gelled, *e.g.,* a gelation solution comprising mTG at a concentration of about 0.01% to about 5% (w/v), *e.g.,* about 0.1%, about 0.2%, about 03%, about 0.4%, about 0.5%, about 0.6%, about 0.7%, about 0.8%, about 0.9%, about 0.1%, about 0.15% about 0.25%, about 0.35%, about 0.45%, about 0.55%, about 0.65%, about 0.75%, about 0.85%, about 0.95%, 1.0%, about 1.25%, about 1.5%, about 1.75%, about 2%, about 2.25%, about 2.5%, about 2.75%, about 3%, about 3.25%, about 3.5%, about 3.75%, about 4%, about 4.25%, about 4.5%, about 4.75%, or about 5%. A wash solution suitable to cease gelation and remove any ungelled precursor solution may comprise water (*e.g.,* distilled water or CaCl2, *e.g.,* CaCl2 at a concentration of about 1%, about 1.25%, about 1.5%, about 1.75%, about 2%, about 2.25%, about 2.5%, about 2.75%, about 3%, about 3.25%, about 3.5%, about 3.75%, about 4%, about 4.25%, about 4.5%, about 4.75%, or about 5% calcium chloride.) at a temperature of about 25°C.

The bioprotein tubes prepared according to the methods of the invention may have an outer diameter of about 0.1 millimeters (mm) to about 4.0 mm, *e.g.,* about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, or about 4.0 mm. The bioprotein tubes prepared according to the methods of the invention may have an inner diameter of about 0.05 millimeters (mm) to about 3.75 mm, *e.g.,* about 0.1, 0.2, 0.3, 0.4,

0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, or about 3.75 mm. The bioprotein tubes prepared according to the methods of the invention may have a tube wall thickness of about 0.05 millimeters (mm) to about 3.75 mm, *e.g.,* about 0.05, .01, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, or about 3.5 mm. Ranges, diameters, and thicknesses intermediate to the above recited amount ranges, diameters, and thicknesses are also contemplated to be part of the invention.

The bioprotein tubes formed using the methods of the invention may be of any length. For example, the bioprotein tubes may be about 1 nanometer, about 10 feet, or about 500 yards. Additionally, the bioprotein tubes may be cut to a desired length using any suitable instrument.

The bioprotein tubes prepared according to the methods of the invention may be unbranched and have an essentially constant diameter, unbranched with an undulating or varying diameter, branched, or a combination of both branched and unbranched.

The rate of extrusion of the stream of bioprotein precursor solution may be constant, *e.g.,* to produce an unbranched bioprotein tube having an essentially constant diameter, or pulsatile, *e.g.,* to produce a bioprotein tube having varying diameters, or may be varied periodically during extrusion of the stream. In exemplary embodiments the rate of extrusion is about 1 ml of bioprotein precursor solution per minute to about 60 ml/minute, about 1 ml/minute to about 50 ml/minute, about 1 ml/minute to about 40 ml/minute, about 1 ml/minute to about 30 ml/minute, about 1 ml/minute to about 20 ml/minute, or about 1 ml/minute to about 10 ml/minute, *e.g.,* about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, or about 60 ml/minute. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

By varying the rate of extrusion, the concentration of alginate (and the concentrations of gelatin and/or biogenic polymer), the concentration of gelation solution, the amount of time that the stream is present in the gelation solution, and/or the orifice or nozzle diameter, the dimensions of the bioprotein tubes can be customized.

For example, a bioprotein tube having an outer diameter of about 3 mm and an inner diameter of about 2.75 mm may be prepared by extruding a bioprotein precursor solution comprising 50 mg/ml of alginate through an orifice having a diameter of about 3 mm into a

solution of about 20 mg/ml calcium chloride and exposing the tube to a wash solution within about 5 seconds. A bioprotein tube having an outer diameter of about 1 mm and an inner diameter of about 0.80 may be prepared by extruding a bioprotein precursor solution comprising 50 mg/ml of alginate and gelatin at a ratio of 3:1 through an orifice into a solution of about 5 mg/ml calcium chloride and exposing the tube to a wash solution within about 10 sec.

C. Characteristics and Further Processing of Bioprotein Tubes Formed by Casting on an Elongate Body or Formed by Extrusion

The bioprotein tubes prepared according to the methods of the invention may have an elastic modulus (*e.g.*, tensile (Young's) modulus or biaxial modulus) of between about 1 kiloPascal (kPa) and 1000 kPa, *e.g,* about 100, 200, 300, 400, 500, 600, 700, 800, 900, or about 1000 kPa. In some embodiments, the bioprotein tubes prepared according to the methods of the invention may have an elastic modulus of between about 100 kPa and about 600 kPa, *e.g,* about 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, or 600 kPa. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

The crosslinked bioprotein tubes prepared according to the methods described herein are hydrogels that can take up a significant amount of water. The inventors demonstrated that the hydrogels have sufficient structural integrity to be dehydrated (e.g., for storage or shipment) and rehydrated for use without degradation of mechanical properties of the bioprotein tubes.

The bioprotein tubes formed according to the methods of the invention may be contacted with additional agents and optionally cultured in an appropriate medium, such as a tissue culture medium. Contacting the bioprotein tubes with the additional agents will allow the agents to, for example, coat the inside and/or outside (fully or partially) of the tubes, or in the case of, for example, cells (*e.g.,* endothelium and/or smooth muscle cells), to mimic natural vasculature. Contacting the bioprotein precursor solution with additional agents during the fabrication of the bioprotein tubes also allows the agents to be incorporated into the bioprotein tubes themselves.

In one embodiment, a bioprotein tube may be contacted, *e.g.,* seeded, with a plurality of living cells, *e.g.,* vascular smooth muscle cells, myocytes (*e.g.*, cardiac

myocytes), skeletal muscle, myofibroblasts, airway smooth muscle cells, osteoblasts, myoblasts, neuroblasts, fibroblasts, glioblasts, germ cells, hepatocytes, chondrocytes, keratinocytes, connective tissue cells, glial cells, epithelial cells, endothelial cells, vascular endothelial cells, hormone-secreting cells, cells of the immune system, neural cells, and cells that will differentiate into contractile cells (*e.g.*, stem cells, *e.g.,* embryonic stem cells or adult stem cells, progenitor cells or satellite cells). In one embodiment, the cells are vascular endothelial cells. In one embodiment, bioprotein tubes treated with a plurality of living cells may be cultured in an appropriate medium *in vitro*. Such cultured cells exhibit characteristics and functions typical of such cells *in vivo*. The plurality of living cells may comprise one or more types of cells.

The cells may be normal cells, abnormal cells (*e.g.*, those derived from a diseased tissue, or those that are physically or genetically altered to achieve an abnormal or pathological phenotype or function), normal or diseased vasculature cells derived from embryonic stem cells or induced pluripotent stem cells.

The term "progenitor cell" is used herein to refer to cells that have a cellular phenotype that is more primitive (*e.g.*, is at an earlier step along a developmental pathway or progression than is a fully differentiated cell) relative to a cell which it can give rise to by differentiation. Often, progenitor cells also have significant or very high proliferative potential. Progenitor cells can give rise to multiple distinct differentiated cell types or to a single differentiated cell type, depending on the developmental pathway and on the environment in which the cells develop and differentiate.

The term "progenitor cell" is used herein synonymously with "stem cell."

The term "stem cell" as used herein, refers to an undifferentiated cell which is capable of proliferation and giving rise to more progenitor cells having the ability to generate a large number of mother cells that can in turn give rise to differentiated, or differentiable daughter cells. The daughter cells themselves can be induced to proliferate and produce progeny that subsequently differentiate into one or more mature cell types, while also retaining one or more cells with parental developmental potential. The term "stem cell" refers to a subset of progenitors that have the capacity or potential, under particular circumstances, to differentiate to a more specialized or differentiated phenotype, and which retains the capacity, under certain circumstances, to proliferate without substantially differentiating. In one embodiment, the term stem cell refers generally to a

naturally occurring mother cell whose descendants (progeny) specialize, often in different directions, by differentiation, *e.g.*, by acquiring completely individual characters, as occurs in progressive diversification of embryonic cells and tissues. Cellular differentiation is a complex process typically occurring through many cell divisions. A differentiated cell may derive from a multipotent cell which itself is derived from a multipotent cell, and so on. While each of these multipotent cells may be considered stem cells, the range of cell types each can give rise to may vary considerably. Some differentiated cells also have the capacity to give rise to cells of greater developmental potential. Such capacity may be natural or may be induced artificially upon treatment with various factors. In many biological instances, stem cells are also "multipotent" because they can produce progeny of more than one distinct cell type, but this is not required for "stem-ness." Self-renewal is the other classical part of the stem cell definition. In theory, self-renewal can occur by either of two major mechanisms. Stem cells may divide asymmetrically, with one daughter retaining the stem state and the other daughter expressing some distinct other specific function and phenotype. Alternatively, some of the stem cells in a population can divide symmetrically into two stems, thus maintaining some stem cells in the population as a whole, while other cells in the population give rise to differentiated progeny only. Formally, it is possible that cells that begin as stem cells might proceed toward a differentiated phenotype, but then "reverse" and re-express the stem cell phenotype, a term often referred to as "dedifferentiation" or "reprogramming" or "retrodifferentiation".

The term "embryonic stem cell" is used to refer to the pluripotent stem cells of the inner cell mass of the embryonic blastocyst (see U.S. Patent Nos. 5,843,780, 6,200,806, the contents of which are incorporated herein by reference). Such cells can similarly be obtained from the inner cell mass of blastocysts derived from somatic cell nuclear transfer (see, for example, U.S. Patent Nos. 5,945,577, 5,994,619, 6,235,970, which are incorporated herein by reference). The distinguishing characteristics of an embryonic stem cell define an embryonic stem cell phenotype. Accordingly, a cell has the phenotype of an embryonic stem cell if it possesses one or more of the unique characteristics of an embryonic stem cell such that that cell can be distinguished from other cells. Exemplary distinguishing embryonic stem cell characteristics include, without limitation, gene expression profile, proliferative capacity, differentiation capacity, karyotype, responsiveness to particular culture conditions, and the like.

The term "adult stem cell" or "ASC" is used to refer to any multipotent stem cell derived from non- embryonic tissue, including fetal, juvenile, and adult tissue. Stem cells have been isolated from a wide variety of adult tissues including blood, bone marrow, brain, olfactory epithelium, skin, pancreas, skeletal muscle, and cardiac muscle. Each of these stem cells can be characterized based on gene expression, factor responsiveness, and morphology in culture. Exemplary adult stem cells include neural stem cells, neural crest stem cells, mesenchymal stem cells, hematopoietic stem cells, and pancreatic stem cells.

Cells for seeding can be cultured *in vitro*, derived from a natural source, genetically engineered, or produced by any other means. Any natural source of prokaryotic or eukaryotic cells may be used. Embodiments in which the bioprotein tubes contacted with a plurality of living cells are implanted in an organism can use cells from the recipient, cells from a conspecific donor or a donor from a different species, or bacteria or microbial cells.

In one embodiment of the invention, a bioprotein tube is contacted with a plurality of endothelial cells and cultured such that a living tissue is produced. In another embodiment of the invention, a bioprotein tube is contacted with a plurality of smooth muscle cells and cultured such that a living tissue is produced. In one particular embodiment, the living tissue is a vascular tissue.

In other embodiments of the invention, a bioprotein tube is contacted with a biologically active polypeptide or protein, such as, collagen, fibrin, elastin, laminin, fibronectin, integrin, hyaluronic acid, chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, heparin sulfate, heparin, and keratan sulfate, and proteoglycans. In one embodiment, the polypeptide or protein is lipophilic.

In still other embodiments, a bioprotein tube is contacted with nucleic acid molecules and/or nucleotides, or lipids.

A bioprotein tube may also be contacted with a pharmaceutically active agent. Suitable pharmaceutically active agents include, for example, anesthetics, hypnotics, sedatives and sleep inducers, antipsychotics, antidepressants, antiallergics, antianginals, antiarthritics, antiasthmatics, antidiabetics, antidiarrheal drugs, anticonvulsants, antigout drugs, antihistamines, antipruritics, emetics, antiemetics, antispasmodics, appetite suppressants, neuroactive substances, neurotransmitter agonists, antagonists, receptor blockers and reuptake modulators, beta-adrenergic blockers, calcium channel blockers, disulfiram and disulfiram-like drugs, muscle relaxants, analgesics, antipyretics, stimulants,

anticholinesterase agents, parasympathomimetic agents, hormones, anticoagulants, antithrombotics, thrombolytics, immunoglobulins, immunosuppressants, hormone agonists/antagonists, vitamins, antimicrobial agents, antineoplastics, antacids, digestants, laxatives, cathartics, antiseptics, diuretics, disinfectants, fungicides, ectoparasiticides, antiparasitics, heavy metals, heavy metal antagonists, chelating agents, gases and vapors, alkaloids, salts, ions, autacoids, digitalis, cardiac glycosides, antiarrhythmics, antihypertensives, vasodilators, vasoconstrictors, antimuscarinics, ganglionic stimulating agents, ganglionic blocking agents, neuromuscular blocking agents, adrenergic nerve inhibitors, anti-oxidants, vitamins, cosmetics, anti-inflammatories, wound care products, antithrombogenic agents, antitumoral agents, antiangiogenic agents, anesthetics, antigenic agents, wound healing agents, plant extracts, growth factors, emollients, humectants, rejection/anti-rejection drugs, spermicides, conditioners, antibacterial agents, antifungal agents, antiviral agents, antibiotics, biocidal agents, anti-biofouling agents, tranquilizers, cholesterol-reducing drugs, antitussives, histamine-blocking drugs, or monoamine oxidase inhibitors.

Other suitable pharmaceutically active agents include growth factors and cytokines. Growth factors useful in the present invention include, but are not limited to, transforming growth factor-α ("TGF-α"), transforming growth factor-β ("TGF-β"), platelet-derived growth factors including the AA, AB and BB isoforms ("PDGF"), fibroblast growth factors ("FGF"), including FGF acidic isoforms 1 and 2, FGF basic form 2, and FGF 4, 8, 9 and 10, nerve growth factors ("NGF") including NGF 2.5s, NGF 7.0s and beta NGF and neurotrophins, brain derived neurotrophic factor, cartilage derived factor, bone growth factors (BGF), basic fibroblast growth factor, insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), granulocyte colony stimulating factor (G-CSF), insulin like growth factor (IGF) I and II, hepatocyte growth factor, glial neurotrophic growth factor (GDNF), stem cell factor (SCF), keratinocyte growth factor (KGF), transforming growth factors (TGF), including TGFs alpha, beta, beta1, beta2, and beta3, skeletal growth factor, bone matrix derived growth factors, and bone derived growth factors and mixtures thereof. Cytokines useful in the present invention include, but are not limited to, cardiotrophin, stromal cell derived factor, macrophage derived chemokine (MDC), melanoma growth stimulatory activity (MGSA), macrophage inflammatory proteins 1 alpha (MIP-1alpha), 2, 3 alpha, 3 beta, 4 and 5, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, TNF-α, and TNF-β. Immunoglobulins useful in the present invention include,

but are not limited to, IgG, IgA, IgM, IgD, IgE, and mixtures thereof.

Other agents that may be used to contact the bioprotein tubes of the invention, include, but are not limited to, growth hormones, leptin, leukemia inhibitory factor (LIF), tumor necrosis factor alpha and beta, endostatin, angiostatin, thrombospondin, osteogenic protein-1, bone morphogenetic proteins 2 and 7, osteonectin, somatomedin-like peptide, osteocalcin, interferon alpha, interferon alpha A, interferon beta, interferon gamma, interferon 1 alpha, amino acids, peptides, polypeptides, and proteins, *e.g.*, structural proteins, enzymes, and peptide hormones.

For agents such as nucleic acids, any nucleic acid can be used to contact the bioprotein tubes. Examples include, but are not limited to deoxyribonucleic acid (DNA), ent-DNA, and ribonucleic acid (RNA). Embodiments involving DNA include, but are not limited to, cDNA sequences, natural DNA sequences from any source, and sense or anti-sense oligonucleotides. For example, DNA can be naked (*e.g.*, U.S. Pat. Nos. 5,580,859; 5,910,488) or complexed or encapsulated (*e.g.*, U.S. Pat. Nos. 5,908,777; 5,787,567). DNA can be present in vectors of any kind, for example in a viral or plasmid vector. In some embodiments, nucleic acids used will serve to promote or to inhibit the expression of genes in cells inside and/or outside the bioprotein tubes. The nucleic acids can be in any form that is effective to enhance uptake into cells.

Agents used to treat the bioprotein tubes of the invention may also be cell fragments, cell debris, organelles and other cell components, tablets, and viruses as well as vesicles, liposomes, capsules, nanoparticles, and other agents that serve as an enclosure for molecules. In some embodiments, the agents constitute vesicles, liposomes, capsules, or other enclosures that contain agents that are released at a time after contacting, such as at the time of implantation or upon later stimulation or interaction. In one illustrative embodiment, transfection agents such as liposomes contain desired nucleotide sequences to be incorporated into cells that are located in or on the bioprotein tubes.

Magnetically or electrically reactive materials are examples of other agents that are optionally used to contact the bioprotein tubes of the present invention. Examples of magnetically active materials include but are not limited to ferrofluids (colloidal suspensions of magnetic particles), and various dispersions of electrically conducting polymers. Ferrofluids containing particles approximately 10 nanometers in diameter, polymer-encapsulated magnetic particles about 1-2 microns in diameter, and polymers with

a glass transition temperature below room temperature are particularly useful. Examples of electrically active materials are polymers including, but not limited to, electrically conducting polymers such as polyanilines and polypyrroles, ionically conducting polymers such as sulfonated polyacrylamides are related materials, and electrical conductors such as carbon black, graphite, carbon nanotubes, metal particles, and metal-coated plastic or ceramic materials.

Suitable biocides for contacting the bioprotein tubes of the invention, include, but are not limited to, organotins, brominated salicylanilides, mercaptans, quaternary ammonium compounds, mercury compounds, and compounds of copper and arsenic.

Antimicrobial agents, which include antibacterial agents, antiviral agents, antifungal agents, and anti-parasitic agents, may also be used to contact the bioprotein tubes of the invention.

The present invention is also directed to the bioprotein tubes produced using the methods of the invention, as well as, tissues and edible food products, *e.g.,* tubular food products, *e.g.,* sausage casing and candy, prepared using the bioprotein tubes of the invention.

II. Uses of the Bioprotein Tubes of the Invention

The bioprotein tubes of the invention support liquid perfusion and resist compression (*e.g.,* during muscle contraction) as well as cell infiltration, and are therefore suitable for use as, among other things, synthetic vasculature and for the culture of three-dimensional tissues in tissue engineering and drug screening applications.

For example, a bioprotein tube prepared according to the methods of the invention may be implanted or grafted in a subject, using standard surgical techniques, for the treatment of a vascular disease (*e.g.,* cardiovascular, cerebrovascular, and peripheral vascular diseases).

The bioprotein tubes prepared according to the methods of the invention may be used to produce an engineered tissue by providing a bioprotein tube prepared according to the methods of the invention and embedding the tube within two stacked tissue scaffolds comprising a plurality of nanometer dimension polymeric fibers comprising alginate to form a tissue scaffold assembly, contacting the assembly with a plurality of cells, such as

muscle cells (*e.g.,* skeletal muscle cells), and culturing the cells under appropriate conditions to form a three-dimensional tissue.

Suitable tissue scaffolds comprising a plurality of nanometer dimension polymeric fibers comprising alginate may be prepared as described in PCT/US2014/016197, filed February 13, 2014, and published as WO 2014/127099, the entire contents of which are incorporated herein by reference. As described *supra*, suitable devices include a reservoir for holding a bioprotein precursor solution and including a surface having one or more orifices for extruding the bioprotein precursor solution, a first motion generator configured to impart rotational motion to the reservoir, the rotational motion of the reservoir causing ejection of the solution through the one or more orifices, and a collection device holding a gelation solution, the collection device configured and positioned to accept the ejected solution from the reservoir, a second motion generator couplable to the collection device, the second motion generator configured to impart rotational motion to the gelation solution in the collection device to generate a liquid vortex including an air gap, wherein the reservoir and the collection device are positioned such that the one or more orifices of the reservoir are positioned in the air gap of the liquid vortex in the collection device; and wherein the extrusion of the bioprotein precursor solution into the air gap and subsequently into the gelation solution of the liquid vortex in the collection device causes formation of nanometer dimension polymeric fibers comprising alginate.

The nanometer dimension polymeric fibers may have a diameter of about 15, 20, 25, 30, 35,40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 33, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690, 700, 710, 720, 730, 740, 750, 760, 770, 780, 790, 800, 810, 820, 830, 840, 850, 860, 870, 880, 890, 900, 910, 920, 930, 940, 950, 960, 970, 980, 990, 1000 nanometers. Sizes and ranges intermediate to the recited diameters are also part of the invention.

The polymeric fibers for use in the methods of the invention may be of any length. In one embodiment, the length of the polymeric fibers is dependent on the length of time the device is in motion and/or the amount of polymer fed into the system. For example, the polymeric fibers may be about 1 nanometer, about 1 meter, or about 500 meters in length. Additionally, the polymeric fibers may be cut to a desired length using any suitable instrument.

For the preparation of 3-D tissue, the bioprotein tubes and the tissue scaffold preferably comprise alginate, gelatin, water, and a cross-linking agent.

As alginate and gelatin are edible and approved for human consumption in the United States and Europe, in one embodiment, the 3-D tissue is cultured meat comprising skeletal muscle tissue. For example, one bioprotein tube having an inner diameter of about 1 mm and a length of about 10 cm positioned between two polymeric scaffolds, each having a thickness of about 0.5 mm and a width of about 1 mm, is sufficient to remove waste and provide nutrients to thereby permit culture of a three-dimensional tissue, *e.g.,* edible tissue, which is about 1 mm wide, about 1.5 mm thick, and about 10 cm long,

In other embodiments, bioprotein tubes comprising alginate and/or gelatin are suitable as casings for edible tubular food products. For example, flavoring and/or coloring may be added to the bioprotein precursor solution during production of the bioprotein tubes which may be subsequently filled with, for example, sweetened jelly and/or cream, for the preparation of a confection.

In some embodiments, the bioprotein tubes and the 3-D tissue prepared according to the methods of the invention is suitable for use in *in vitro* drug screening assays to determine the effects of a test compound on living tissue by examining the effect of the test compound on various biological responses, such as for example, cell viability, cell growth, migration, differentiation and maintenance of cell phenotype, electrophysiology, metabolic activity, muscle cell contraction, osmotic swelling, structural remodeling and tissue level pre-stress.

Numerous physiologically relevant parameters, *e.g.,* muscle activities, *e.g.,* biomechanical and electrophysiological activities, can be evaluated using the methods and devices of the invention. For example, in one embodiment, the bioprotein tubes and the 3-D tissues prepared according to the methods of the present invention can be used in contractility assays for contractile cells, such as muscular cells or tissues, such as chemically and/or electrically stimulated contraction of vascular, airway or gut smooth muscle, cardiac muscle, vascular endothelial tissue, or skeletal muscle. In addition, the differential contractility of different muscle cell types to the same stimulus (*e.g.*, pharmacological and/or electrical) can be studied.

In another embodiment, the bioprotein tubes and the 3-D tissues prepared according to the methods of the present invention can be used for measurements of solid stress due to

osmotic swelling of cells. For example, as the cells swell the muscle tissue will bend and as a result, volume changes, force and points of rupture due to cell swelling can be measured.

In another embodiment, the bioprotein tubes and the 3-D tissues prepared according to the methods of the present invention can be used for pre-stress or residual stress measurements in cells. For example, vascular smooth muscle cell remodeling due to long term contraction in the presence of endothelin-1 can be studied.

Further still, the bioprotein tubes and the 3-D tissues prepared according to the methods of the present invention can be used to study the loss of rigidity in tissue structure after traumatic injury, *e.g.,* traumatic brain injury. Traumatic stress can be applied to vascular smooth muscle 3-D tissues as a model of vasospasm. These tissues can be used to determine what forces are necessary to cause vascular smooth muscle to enter a hyper-contracted state. These tissues can also be used to test drugs suitable for minimizing vasospasm response or improving post-injury response and returning vascular smooth muscle contractility to normal levels more rapidly.

In other embodiments, the bioprotein tubes and the 3-D tissues prepared according to the methods of the present invention can be used to study biomechanical responses to paracrine released factors (*e.g.*, vascular smooth muscle dilation due to release of nitric oxide from vascular endothelial cells, or cardiac myocyte dilation due to release of nitric oxide).

In other embodiments, the bioprotein tubes and the 3-D tissues prepared according to the methods of the present invention can be used to evaluate the effects of a test compound on an electrophysiological parameter, *e.g.,* an electrophysiological profile comprising a voltage parameter selected from the group consisting of action potential, action potential morphology, action potential duration (APD), conduction velocity (CV), refractory period, wavelength, restitution, bradycardia, tachycardia, reentrant arrhythmia, and/or a calcium flux parameter, *e.g.,* intracellular calcium transient, transient amplitude, rise time (contraction), decay time (relaxation), total area under the transient (force), restitution, focal and spontaneous calcium release, and wave propagation velocity. For example, a decrease in a voltage or calcium flux parameter of a muscle tissue comprising cardiomyocytes upon contacting the tissue with a test compound, would be an indication that the test compound is cardiotoxic.

In yet another embodiment, the bioprotein tubes and the 3-D tissues prepared according to the methods of the present invention can be used in pharmacological assays for measuring the effect of a test compound on the stress state of a tissue. For example, the assays may involve determining the effect of a drug on tissue stress and structural remodeling of the muscle tissue. In addition, the assays may involve determining the effect of a drug on cytoskeletal structure (*e.g.,* sarcomere alignment) and, thus, the contractility of the muscle tissue.

In still other embodiments, the bioprotein tubes and the 3-D tissues prepared according to the methods of the present invention can be used to measure the influence of biomaterials on a biomechanical response. For example, differential contraction of vascular smooth muscle remodeling due to variation in material properties (*e.g.*, stiffness, surface topography, surface chemistry or geometric patterning) of polymeric thin films can be studied.

In further embodiments, the bioprotein tubes and the 3-D tissues prepared according to the methods of the present invention can be used to study functional differentiation of stem cells (*e.g.*, pluripotent stem cells, multipotent stem cells, induced pluripotent stem cells, and progenitor cells of embryonic, fetal, neonatal, juvenile and adult origin) into contractile phenotypes. For example, undifferentiated cells, *e.g.,* stem cells, are coated on the scaffolds and differentiation into a contractile phenotype is observed by thin film bending. Differentiation into an anisotropic tissue may also be observed by quantifying the degree of alignment of sarcomeres and/or quantifying the orientational order parameter (OOP). Differentiation can be observed as a function of: co-culture (*e.g.*, co-culture with differentiated cells), paracrine signaling, pharmacology, electrical stimulation, magnetic stimulation, thermal fluctuation, transfection with specific genes, chemical and/or biomechanical perturbation (*e.g.*, cyclic and/or static strains).

In another embodiment, the bioprotein tubes and the 3-D tissues prepared according to the methods of the present invention may be used to determine the toxicity of a test compound by evaluating, *e.g.,* the effect of the compound on an electrophysiological response of a muscle tissue. For example, opening of calcium channels results in influx of calcium ions into the cell, which plays an important role in excitation-contraction coupling in cardiac and skeletal muscle fibers. The reversal potential for calcium is positive, so calcium current is almost always inward, resulting in an action potential plateau in many excitable cells. These channels are the target of therapeutic intervention, *e.g.*, calcium

channel blocker sub-type of anti-hypertensive drugs. Candidate drugs may be tested in the electrophysiological characterization assays described herein to identify those compounds that may potentially cause adverse clinical effects, *e.g.*, unacceptable changes in cardiac excitation, that may lead to arrhythmia.

For example, unacceptable changes in cardiac excitation that may lead to arrhythmia include, *e.g.,* blockage of ion channel requisite for normal action potential conduction, *e.g.,* a drug that blocks Na^+ channel would block the action potential and no upstroke would be visible; a drug that blocks Ca^{2+} channels would prolong repolarization and increase the refractory period; blockage of K^+ channels would block rapid repolarization, and, thus, would be dominated by slower Ca^{2+} channel mediated repolarization.

In addition, metabolic changes may be assessed to determine whether a test compound is toxic by determining, *e.g.,* whether contacting with a test compound results in a decrease in metabolic activity and/or cell death. For example, detection of metabolic changes may be measured using a variety of detectable label systems such as fluormetric/chromogenic detection or detection of bioluminescence using, *e.g.,* AlamarBlue fluorescent/chromogenic determination of REDOX activity (Invitrogen), REDOX indicator changes from oxidized (non-fluorescent, blue) state to reduced state(fluorescent, red) in metabolically active cells; Vybrant MTT chromogenic determination of metabolic activity (Invitrogen), water soluble MTT reduced to insoluble formazan in metabolically active cells; and Cyquant NF fluorescent measurement of cellular DNA content (Invitrogen), fluorescent DNA dye enters cell with assistance from permeation agent and binds nuclear chromatin. For bioluminescent assays, the following exemplary reagents may be used: Cell-Titer Glo luciferase-based ATP measurement (Promega), a thermally stable firefly luciferase glows in the presence of soluble ATP released from metabolically active cells.

The bioprotein tubes and the 3-D tissues prepared according to the methods of the present invention are also useful for evaluating the effects of particular delivery vehicles for therapeutic agents *e.g.,* to compare the effects of the same agent administered *via* different delivery systems, or simply to assess whether a delivery vehicle itself (*e.g.*, a viral vector or a liposome) is capable of affecting the biological activity of the muscle tissue. These delivery vehicles may be of any form, from conventional pharmaceutical formulations, to gene delivery vehicles. For example, the devices of the invention may be used to compare the therapeutic effect of the same agent administered by two or more different delivery systems (*e.g.*, a depot formulation and a controlled release formulation). The bioprotein

tubes and the 3-D tissues prepared according to the methods of the present invention may also be used to investigate whether a particular vehicle may have effects of itself on the tissue. As the use of gene-based therapeutics increases, the safety issues associated with the various possible delivery systems become increasingly important. Thus, the bioprotein tubes and the 3-D tissues prepared according to the methods of the present invention may be used to investigate the properties of delivery systems for nucleic acid therapeutics, such as naked DNA or RNA, viral vectors (*e.g.*, retroviral or adenoviral vectors), liposomes and the like. Thus, the test compound may be a delivery vehicle of any appropriate type with or without any associated therapeutic agent.

Furthermore, the bioprotein tubes and the 3-D tissues prepared according to the methods of the present invention are a suitable *in vitro* model for evaluation of test compounds for therapeutic activity with respect to, *e.g.,* a muscular and/or neuromuscular disease or disorder. For example, the bioprotein tubes and the 3-D tissues prepared according to the methods of the present invention (*e.g.*, comprising muscle cells) may be contacted with a candidate compound by, *e.g.,* diffusion of the test compound added drop-wise on the surface of a muscle tissue, diffusion of a test compound through the culture medium, or immersion in a bath of media containing the test compound, and the effect of the test compound on muscle activity (*e.g.,* a biomechanical and/or electrophysiological activity) may be measured as described herein, as compared to an appropriate control, *e.g.,* an untreated muscle tissue. Alternatively, the bioprotein tubes and the 3-D tissues prepared according to the methods of the present invention may be bathed in a medium containing a candidate compound, and then the cells are washed, prior to measuring a muscle activity (*e.g.,* a biomechanical and/or electrophysiological activity) as described herein. Any alteration to an activity determined using the device in the presence of the test agent (as compared to the same activity using the device in the absence of the test compound) is an indication that the test compound may be useful for treating or preventing a muscle disease.

Evaluation of muscle activity includes determining the degree of contraction and the rate or frequency of contraction/rate of relaxation compared to a normal control in the absence of the test compound. An increase in the degree of contraction or rate of contraction indicates that the compound is useful in treatment or amelioration of pathologies associated with myopathies such as muscle weakness or muscular wasting. Such a profile also indicates that the test compound is useful as a vasocontractor. A decrease in the degree of contraction or rate of contraction is an indication that the

compound is useful as a vasodilator and as a therapeutic agent for muscle or neuromuscular disorders characterized by excessive contraction or muscle thickening that impairs contractile function.

Compounds evaluated in this manner are useful in treatment or amelioration of the symptoms of muscular and neuromuscular pathologies such as those described below. Muscular Dystrophies include Duchenne Muscular Dystrophy (DMD) (also known as Pseudohypertrophic), Becker Muscular Dystrophy (BMD), Emery-Dreifuss Muscular Dystrophy (EDMD), Limb-Girdle Muscular Dystrophy (LGMD), Facioscapulohumeral Muscular Dystrophy (FSH or FSHD) (Also known as Landouzy-Dejerine), Myotonic Dystrophy (MMD) (Also known as Steinert's Disease), Oculopharyngeal Muscular Dystrophy (OPMD), Distal Muscular Dystrophy (DD), and Congenital Muscular Dystrophy (CMD). Motor Neuron Diseases include Amyotrophic Lateral Sclerosis (ALS) (Also known as Lou Gehrig's Disease), Infantile Progressive Spinal Muscular Atrophy (SMA, SMA1 or WH) (also known as SMA Type 1, Werdnig-Hoffman), Intermediate Spinal Muscular Atrophy (SMA or SMA2) (also known as SMA Type 2), Juvenile Spinal Muscular Atrophy (SMA, SMA3 or KW) (also known as SMA Type 3, Kugelberg-Welander), Spinal Bulbar Muscular Atrophy (SBMA) (also known as Kennedy's Disease and X-Linked SBMA), Adult Spinal Muscular Atrophy (SMA). Inflammatory Myopathies include Dermatomyositis (PM/DM), Polymyositis (PM/DM), Inclusion Body Myositis (IBM). Neuromuscular junction pathologies include Myasthenia Gravis (MG), Lambert-Eaton Syndrome (LES), and Congenital Myasthenic Syndrome (CMS). Myopathies due to endocrine abnormalities include Hyperthyroid Myopathy (HYPTM), and Hypothyroid Myopathy (HYPOTM). Diseases of peripheral nerves include Charcot-Marie-Tooth Disease (CMT) (Also known as Hereditary Motor and Sensory Neuropathy (HMSN) or Peroneal Muscular Atrophy (PMA)), Dejerine-Sottas Disease (DS) (Also known as CMT Type 3 or Progressive Hypertrophic Interstitial Neuropathy), and Friedreich's Ataxia (FA). Other Myopathies include Myotonia Congenita (MC) (Two forms: Thomsen's and Becker's Disease), Paramyotonia Congenita (PC), Central Core Disease (CCD), Nemaline Myopathy (NM), Myotubular Myopathy (MTM or MM), Periodic Paralysis (PP) (Two forms: Hypokalemic - HYPOP - and Hyperkalemic - HYPP) as well as myopathies associated with HIV/AIDS.

The bioprotein tubes and the 3-D tissues prepared according to the methods of the present invention are also useful for identifying therapeutic agents suitable for treating or

ameliorating the symptoms of metabolic muscle disorders such as Phosphorylase Deficiency (MPD or PYGM) (Also known as McArdle's Disease), Acid Maltase Deficiency (AMD) (Also known as Pompe's Disease), Phosphofructokinase Deficiency (PFKM) (Also known as Tarui's Disease), Debrancher Enzyme Deficiency (DBD) (Also known as Cori's or Forbes' Disease), Mitochondrial Myopathy (MITO), Carnitine Deficiency (CD), Carnitine Palmityl Transferase Deficiency (CPT), Phosphoglycerate Kinase Deficiency (PGK), Phosphoglycerate Mutase Deficiency (PGAM or PGAMM), Lactate Dehydrogenase Deficiency (LDHA), and Myoadenylate Deaminase Deficiency (MAD).

In addition to the disorders listed above, the screening methods described herein are useful for identifying agents suitable for reducing vasospasms, heart arrhythmias, and cardiomyopathies.

Vasodilators identified as described above are used to reduce hypertension and compromised muscular function associated with atherosclerotic plaques. Smooth muscle cells associated with atherosclerotic plaques are characterized by an altered cell shape and aberrant contractile function. Such cells are used to populate a thin film, exposed to candidate compounds as described above, and muscular function evaluated as described above. Those agents that improve cell shape and function are useful for treating or reducing the symptoms of such disorders.

Smooth muscle cells and/or striated muscle cells line a number of lumen structures in the body, such as uterine tissues, airways, gastrointestinal tissues (*e.g.*, esophagus, intestines) and urinary tissues, *e.g.*, bladder. The function of smooth muscle cells in the presence and absence of a candidate compound may be evaluated as described above to identify agents that increase or decrease the degree or rate of muscle contraction to treat or reduce the symptoms associated with a pathological degree or rate of contraction. For example, such agents are used to treat gastrointestinal motility disorders, *e.g.*, irritable bowel syndrome, esophageal spasms, achalasia, Hirschsprung's disease, or chronic intestinal pseudo-obstruction.

The present invention is next described by means of the following examples. However, the use of these and other examples anywhere in the specification is illustrative only, and in no way limits the scope and meaning of the invention or of any exemplified form. The invention is not limited to any particular preferred embodiments described

herein. Many modifications and variations of the invention may be apparent to those skilled in the art and can be made without departing from its spirit and scope. The contents of all references, patents and published patent applications cited throughout this application, including the figures, are incorporated herein by reference.

EXAMPLES

Example 1. Production of Bioprotein Tubes by Extrusion

One or more bioprotein solutions are required to produce bioprotein tubes. At least one of the bioprotein precursors gels with a sufficiently high gelation rate to form structurally stable tubes during bioprotein solution extrusion into a gelation bath.

Alginate bioproteins are suitable for tube production because they gel rapidly in $CaCl_2$ solutions. Alginate bioproteins are also biocompatible and optically transparent.

Additional bioproteins are mixed with the alginate precursors without impeding tube formation. For example, gelatin was mixed with alginate to produce tubes that promote biological cell adhesion. These can be used to culture vascular cells in the tubes, for example endothelial and/or smooth muscle cells. Furthermore, addition of gelatin and subsequent action of crosslinking agents such as microbial transglutaminase (mTG or "meat glue") can enhance the long-term structural stability of the bioprotein tubes.

The composition of the gelation solution and the addition of any further crosslinking agents are selected based on optimal gelation and crosslinking of bioprotein precursors.

Gelating solution concentration is adjusted to initiate sufficiently fast gelation to impart structurally stable tubular geometry to extruded bioproteins. Subsequent bathing time in the gelation solution determines tube wall thickness. The addition of further crosslinking agents can be used to enhance the tubes' long-term structural stability.

Application-dependent cell adhesion, proliferation, and organization either inside the tubes, on the tube's inner walls, within the tube walls, or on the outer wall surface can be controlled by suitable selection of production process conditions. Cell:tube adhesion probability can be adjusted by varying the concentration of cell-adhesive or non-adhesive bioprotein precursors. Extrusion nozzle geometry can be engineered to define tube microarchtecture, for example to produce grooves on the surface of the tubes to promote cell alignment.

Pure alginate tubing having minimal cell adhesiveness can be used to produce acellular synthetic vasculature. This is useful for many food applications in which cell adhesion should be minimized. For cultured meat production in particular, cell-free synthetic vasculature is ideal to supply thick edible tissues with serum-free culture media.

Endothelium and/or smooth muscle cells can be cultured in and on bioprotein tubes to mimic natural vasculature. This is useful when tissue engineering application require vasculature that mimics native vasculature.

By adjusting bioprotein composition and crosslinking agent concentration, bioprotein tube dissolution rate and lifetime in culture can be precisely controlled. This can be used to produce tubes that are gradually replaced with natural vasculature.

In other aspects, the methods include formulating a bioprotein precursor and gelation bath solutions; and extrude soluble bioproteins into the gelation bath and allow tubes to partially gel. Gelation occurs from the outside-in, forming tube walls; and remove partially gelled bioprotein tubes to a heated bath and perfuse with warm water to wash away ungelled precursors from the tube interior. In this method's simplest form, bioproteins are extruded through cylindrical metallic tubes (*e.g.*, needle tips). Extrusion tube inner diameter determines extruded tube outer diameter. Arrayed extruders can be used to scale up tube production. In another form, bioproteins are extruded through a device having multiple joined orifices to produce joined branching tubes that resemble natural vasculature.

Example 2. Production of Bioprotein Tubes by Casting on an Elongate Body

In one aspect, the methods for the fabrication of bioprotein tubes include use of an elongate body which is generally contacted with a first solution comprising alginate, a second solution comprising CaCl2, and a third solution comprising gelatin and/or a biogenic polymer.

Bioprotein tubes were produced by the method 10 described above with respect to Figures 1 through 13. Figures 2B, 3C, 4C, 5B, 9B, and 9C are images of the prototype system used in Example 2. The elongate body was a metal stick with a diameter of about 1500 micrometers. The plug was formed by wrapping paraffin film around the distal end of the metal stick and using a wound suture to affix the paraffin film to the metal stick. The

first solution was 3% alginate in water. The second solution was 2.5% $CaCl_2$ in water. The stick with adhered alginate was left in the second solution for about 15 minute to gel the alginate forming the alginate tube. The alginate tube was removed from the metal stick for perforation with a 23G hollow needle having an outer diameter of about 0.6 mm and an inner diameter of about 0.33 mm, which formed circular perforations in the tube each having a diameter of about 100 micrometers. The alginate tube was placed over the metal stick after perforation for further processing. In other embodiments, the alginate tube may not be perforated prior to further processing. A third solution of 10% gelatin in water was drawn into the alginate tube and excess gelatin was released. A fourth solution of 0 0.2 % MTG (w/v) in water was drawn into the alginate-gelatin tube and the tube was soaked in a bath of the fourth solution for cross-linking.

Tubes were formed using apertures for removing excess alginate material having different aperture diameters, which resulted in tubes with similar inner diameters and different outer diameters and wall thicknesses. Figures 24A through 24C are images of a resulting tube illustrating measurement of the inner tube diameter and of the tube wall thickness.

Figure 25 is a graph of inner tube diameter for extruded bioprotein tubes and cast bioprotein tubes with aperture diameters of 2500, 2750 and 3000 micrometers. Aperture diameter is referred to as polymer outlet diameter in the graph. As shown, the cast tubes had smaller error bars corresponding to more reliable inner tube diameter measurements. The standard error from the mean was 16, 9 and 14 micrometers for the cast tubes as compared with 43 micrometers for the extruded tubes.

Figure 26 is a graph of tube wall thicknesses for extruded bioprotein tubes and cast bioprotein tubes with aperture diameters of 2500, 2750 and 3000 micrometers. As shown, the extruded tubes and the cast tubes had about the same standard error from the mean for wall thickness.

As shown in Figure 27, a bioprotein tube may be used in a microfluidic platform that employs a tissue culture chamber and permits imaging of the tube and attached cells. Figure 28 depicts perforation of the gel tube while in the tissue culture chamber. Figure 29 is an image of an alginate tube within the tissue culture chamber. Figure 30 is an image of the microfluidic platform showing the pump and the flow sensor as well as the tissue culture chamber. Experiments performed using alginate bioprotein tubes and alginate and

gelatin bioprotein tubes in the microfluidic platform demonstrated that the tubes supported continuous perfusion for at least 40 hours.

In order to demonstrate whether alginate tubes and gelatin coated alginate tubes prepared by casting on an elongate body permit cell attachment, 100 µl of a stock of $2.5x10^4$ human-umbilical-vein-endothelial-cells (HUVEC cells) per ml was added to the tubes in a petri dish with Lonza EGM-2 media and cultured at 37°C, 5% CO_2 for at least one day. Cells were visualized by optical microscopy. Figure 31A is a cross-sectional view of an alginate tube. Figures 31B and 31C are images of the alginate tube interior after culturing with HUVEC for 1 day in a petri dish. As shown, the HUVEC cells did not adhere to the alginate tube. Figure 32A is a cross-sectional view of a gelatin-coated alginate tube stained with eosin, which reveals gelatin. Figures 32B and 32C are images of the gelatin-coated tube interior after culturing with HUVEC for one day in a petri dish. As shown, the HUVEC cells adhered to the gelatin-coated alginate.

In order to demonstrate that the gelatin-coated alginate tubes prepared by casting on an elongate body permit cell proliferation, 100 µl of a stock of $2.5x10^4$ GFP-HUVEC cells per ml were added to the tubes in a microfluidic device (see, *e.g.,* Figures 27-30) with Lonza EGM-2 media and cultured with continuous perfusion at 37°C, 5% CO_2 for at least one day. Cells were visualized by confocal fluorescent microscopy using an excitation wavelength of 488 nm.

The side cross-sectional view of Figure 33A and the detail view of Figure 33B show that the GFP-HUVEC cells attached to the gelatin-coated alginate tube and proliferated on the tube interior. Furthermore, these data demonstrate the mechanical strength and resistance to blockage of the tubes as they were continuously perfused for about 2 days without collapse and without blockage. The line in Figure 33A marks the cross-section used to produce the image in Figure 33C. The end cross-sectional view of Figure 33C shows that the cells attached and proliferated throughout the cylindrical tube interior.

Figures 34A and 34B show proliferation of the cells through a perforation in the gelatin-coated alginate tube demonstrating that perforations in the tubes can be used to deliver cells to surrounding matrix or fibrous scaffold. Thus, the cells proliferating through the perforations can sprout forming a branched structure extending into surrounding matrix, fibrous scaffold or tissue. For example, the surrounding tissue could be skeletal muscular tissue enabling production of thick (*e.g.,* greater than about 200 µm, *e.g.,* the maximum

distance that a cell can be positioned from a blood supply which permits cell survival) continuous tissue.

Example 3: Elastic Moduli of Bioprotein Tubes Produced by Extrusion

Various bioprotein tubes produced by extrusion, using methods as described above, were assessed for tensile strength.

Bioprotein tubes used for these analyses included (1) alginate and gelatin bioprotein tubes prepared from an 8% solution of alginate and an 8% solution of gelatin, mixed at a ratio of 3:1 (alginate to gelatin) which were crosslinked by immersing the tubes in 5% mTG (5 grams mTG per 10 mL water) for 30 minutes, and, for comparison, (2) alginate and gelatin bioprotein tubes prepared from an 8% solution of alginate and an 8% solution of gelatin, mixed at a ratio of 3:1 (alginate to gelatin) without later crosslinking, and (3) bioprotein tubes comprising alginate prepared from an 8% w/v solution of alginate without gelatin and without later crosslinking. Four tubes of each type were used in the testing. The inner and outer diameters of the different types tubes are provided in Table 1 below. The ratio of the inner diameter to the outer diameter for each tube was about 0.7.

Table 1 - Inner and Outer Diameters for Tube Types

	Alg-Ge-MTG		***Alg-Gel***		***Alg***	
	Outer Diameter (mm)	**Inner Diameter (mm)**	**Outer Diameter (mm)**	**Inner Diameter (mm)**	**Outer Diameter (mm)**	**Inner Diameter (mm)**
Mean	1.328	0.924	1.1895	0.829	1.545	1.077
Standard Deviation	0.176	0.213	0.0353	0.115	0.203	0.231

The tensile strength of four tubes for teach type of tube (*i.e.*, alginate-gelatin tubes crosslinked with mTG, alginate-gelatin tubes without crosslinking, and alginate tubes without crosslinking) was measured to determine the tensile modulus (Young's modulus) of each tube type as shown below in Table 2.

Table 2 - Young's Modulus for Tube Types

Tube Type	Tensile Modulus ± Standard Deviation in kPa
Alg-Ge-MTG	379.8±28.29
Alg-Gel	165.02±7.238
Alg	498.6±6.49

The extruded and crosslinked alginate-gelatin biotubes exhibited a substantial tensile modulus of about 380 kPa. It is expected that the value of the biaxial modulus of the tubes would be similar. The cross-linking of the alginate-gelatin biotubes significantly increased the tensile modulus of the tubes.

Example 4: Preparation of Bioprotein Scaffolds and Cultured Meat Production

Skeletal muscle cells must be provided a scaffold in order to direct the location and directional alignment of individual cells. For cultured meat, the scaffold must be edible and cost effective. Using immersion Rotary Jet Spinning (iRJS; see, *e.g.,* PCT/US2014/016197, filed February 13, 2014, and published as WO 2014/127099), fibrous three-dimensional scaffolds were produced from water-soluble bioprotein precursors.

Alginate and gelatin are both edible and approved for human consumption in the United States and Europe and are, thus, suitable for use in the scaffolds. In addition, materials can be combined to form composite scaffolds that leverage the respective benefits of each material component. For example, cell adhesion to alginate fibers can be enhanced by gelatin inclusion. Fiber morphology and cell adhesion can both be controlled by adjusting process parameters.

Virtually any cell type can be cultured in the scaffold. For cultured meat production, skeletal muscle progenitors from various animal sources can be expanded and cultured within the scaffold.

Production of large three-dimensional muscle tissue requires effective nutrient supply to cells that are cultured within the tissue. Extruded alginate tubes that are embedded in the scaffold can accomplish this task. Alginate tubes are preferable to alternative pore-based vasculature strategies because they are structurally stable and can be biochemically tailored to limit cell adhesion and therefore avoid clogging by proliferating cells. Alginate

tubes are edible. They can also be biochemically tailored for controlled rates of dissolution within the scaffold.

Fibrous scaffolds support directed three-dimensional cell proliferation and differentiation. They are a fundamental component of cultured (*i.e.,* engineered) tissues, including skeletal muscle tissues.

EQUIVALENTS

In describing embodiments of the invention, specific terminology is used for the sake of clarity. For purposes of description, each specific term is intended to at least include all technical and functional equivalents that operate in a similar manner to accomplish a similar purpose. Additionally, in some instances where a particular embodiment of the invention includes a plurality of system elements or method steps, those elements or steps may be replaced with a single element or step; likewise, a single element or step may be replaced with a plurality of elements or steps that serve the same purpose. Further, where parameters for various properties are specified herein for embodiments of the invention, those parameters can be adjusted up or down by 1/20th, 1/10th, 1/5th, 1/3rd, ½, etc., or by rounded-off approximations thereof, unless otherwise specified. Moreover, while this invention has been shown and described with references to particular embodiments thereof, those skilled in the art will understand that various substitutions and alterations in form and details may be made therein without departing from the scope of the invention; further still, other aspects, functions and advantages are also within the scope of the invention. The contents of all references, including patents and patent applications, cited throughout this application are hereby incorporated by reference in their entirety. The appropriate components and methods of those references may be selected for the invention and embodiments thereof. Still further, the components and methods identified in the Background section are integral to this disclosure and can be used in conjunction with or substituted for components and methods described elsewhere in the disclosure within the scope of the invention.

We claim:

1. A method of producing a bioprotein tube comprising:

contacting at least a portion of an elongate body with a first solution comprising alginate;

passing the at least a portion of the elongate body that was in contact with the first solution through an aperture to remove excess alginate adhered to the elongate body;

exposing the at least a portion of the elongate body with adhered alginate material to a second solution comprising $CaCl_2$ to gel the adhered alginate, thereby forming a tube on the at least a portion of the elongate body;

positioning a first end of the tube in contact with a third solution comprising gelatin and/or a biogenic polymer and drawing the third solution into the tube by at least partially withdrawing the elongate body from a second end of the tube; and

advancing the elongate body toward the first end of the tube to expel excess third solution material.

2. The method of claim 1, further comprising, after expelling excess third solution material, positioning the first end of the tube in contact with a fourth solution comprising a cross-linking agent and drawing the fourth solution into the tube by at least partially withdrawing the elongate body from the second end of the tube.

3. The method of claim 2, further comprising, positioning the tube in a bath of the fourth solution.

4. The method of claim 2, wherein the cross-linking agent of the fourth solution comprises microbial transglutaminase (MTG).

5. The method of claim 2, wherein the cross-linking agent comprises genipin.

6. The method of claim 2, wherein the cross-linking agent comprises riboflavin.

7. The method of claim 1, further comprising forming a plurality of perforations or holes in the tube.

8. The method of claim 7, wherein the plurality of perforations or holes in the tube are formed prior to positioning the first end of the tube in contact with the third solution.

9. The method of claim 8, wherein the tube is at least partially removed from the elongate body prior to forming the plurality of perforations or holes in the tube and the tube is positioned back on the elongate body prior to drawing the third solution into the tube.

10. The method of claim 8, wherein a diameter or width of each of the plurality of perforations or holes falls in a range of 1 micrometer to half an inner diameter of the tube.

11. The method of claim 8, wherein the number of perforations or holes in the plurality of perforations or holes falls in a range of 0 to 500 per millimeter.

12. The method of claim 1, wherein contacting the at least a portion of the elongate body with the first solution comprises advancing the at least a portion of the elongate body into a first reservoir holding the first solution.

13. The method of claim 12, wherein exposing the at least a portion of the elongate body with adhered alginate material to the second solution comprising $CaCl_2$ to gel the adhered alginate comprises extending the at least a portion of the elongate body with adhered alginate material into a second reservoir containing the second solution.

14. The method of claim 12, wherein the first reservoir comprises a proximal end and a distal end with the aperture disposed at the distal end;

wherein contacting the at least a portion of the elongate body with the first solution comprises advancing the at least a portion of the elongate body into the proximal end of the first reservoir; and

wherein passing the at least a portion of the elongate body that was in contact with the first solution through the aperture to remove excess alginate material comprises advancing the at least a portion of the elongate body through the first reservoir and out the aperture of the distal end of the first reservoir.

15. The method of claim 14, wherein a plug at a distal end of the elongate body prevents the first solution from leaving the first reservoir through the aperture prior to advancing the at least a portion of the elongate body through the proximal end of the first reservoir.

16. The method of claim 14, wherein the method further comprises:

positioning the elongate body extending through the first reservoir with the plug at the distal end of the elongate body blocking the reservoir aperture; and

adding the first solution to the first reservoir prior to advancing the at least a portion of the elongate body into the proximal end of the first reservoir.

17. The method of claim 1, wherein the portion of the elongate body on which the tube is formed is the tube forming portion of the elongate body, and wherein a diameter or width of the elongate body throughout the tube-forming portion falls in a range of 10 micrometers to 5 centimeters

18. The method of claim 1, wherein the portion of the elongate body on which the tube is formed is the tube forming portion of the elongate body, and wherein a diameter or width of the elongate body throughout the tube-forming portion falls in a range of 500 micrometers to 5 millimeters.

19. The method of claim 1, wherein the portion of the elongate body on which the tube is formed is the tube forming portion of the elongate body, and wherein a diameter or width of the aperture is in a range of 10 micrometers to 5 millimeters larger than a diameter or width of the tube forming portion of the elongate body.

20. The method of claim 1, wherein the third solution comprises gelatin.

21. The method of claim 1, wherein the third solution comprises extracellular matrix proteins.

22. The method of claim 21, wherein the third solution comprises one or more of collagen, fibronectin, laminin, and combinations thereof.

23. The method of claim 1, wherein the third solution comprises one or more of bioproteins, peptides, nucleic acids, and combinations thereof.

24. A method of producing a bioprotein tube comprising:

extruding a stream of a bioprotein precursor solution comprising alginate through an orifice into a gelation solution comprising calcium chloride to initiate gelation at an outer surface of the extruded stream, thereby forming a gelled outer surface of the extruded stream;

maintaining the extruded stream in the gelation solution while gelation proceeds forming a gelled front advancing from the gelled outer surface toward a central axis of the extruded stream; and

exposing the extruded stream to a wash solution to cease gelation and remove the un-gelled bioprotein precursor solution from the extruded stream prior to the gelled front reaching the central axis of the extruded stream, thereby producing a bioprotein tube having an outer surface at the gelled outer surface, an inner surface at the gelled front, and a lumen.

25. The method of claim 24, wherein the orifice is an orifice of a needle.

26. A method of producing a bioprotein tube comprising:

extruding an annular stream of a bioprotein precursor solution comprising alginate through a nozzle into a gelation solution comprising calcium chloride to initiate cross-linking and gelation at an outer surface of the extruded annular stream and at an inner surface of the extruded annular stream; and

maintaining the extruded annular stream in the gelation solution while cross-linking and gelation proceeds throughout the extruded annular stream, thereby producing a bioprotein tube including an outer surface, an inner surface, and a lumen.

27. The method of claim 26, wherein the bioprotein precursor solution is in a reservoir and the nozzle is associated with an orifice.

28. The method of claim 24 or 26, wherein an extrusion rate is constant during extrusion of the stream.

29. The method of claim 24 or 26, wherein the extrusion rate varies during extrusion of the stream.

30. The method of claim 24 or 26, wherein the extrusion rate is periodically varying during extrusion of the stream.

31. A method of producing one or more bioprotein tubes comprising:

rotating a bioprotein precursor solution about an axis of rotation to cause extrusion of the solution in one or more annular streams;

generating a gelation solution vortex in a collection device for collecting the one or more streams of the solution, the liquid vortex including a central air gap; and

collecting the one or more streams of the solution in the collection device, wherein the one or more streams are initially extruded through the air gap of the gelation solution vortex and subsequently through the gelation solution in the gelation solution vortex of the collection device;

wherein the extrusion of the solution into the air gap and subsequently into the gelation solution in the collection device causes formation of one or more bioprotein tubes.

32. The method of any one of claims 24-27 and 31, wherein the gelation solution comprises about 1% to about 5% calcium chloride.

33. The method of any one of claims 24-27 and 31, wherein the gelation solution further comprises a microbial transglutaminase.

34. The method of any one of claims 24-27 and 31, wherein the bioprotein precursor solution further comprises gelatin.

35. The method of claim 34, wherein the ratio of alginate to gelatin is about 10:1.

36. The method of claim 34, wherein the ratio of alginate to gelatin is about 5:1.

37. The method of claim 34, wherein the ratio of alginate to gelatin is about 1:2.

38. The method of any one of claims 24-27 and 31, wherein the bioprotein precursor solution further comprises a biogenic polymer.

39. The method of claim 38, wherein the biogenic polymer is selected from the group consisting of silk, a keratin, an elastin, a fibrillin, a fibrinogen, a fibrin, a thrombin, a fibronectin, a laminin, a collagen, a vimentin, a neurofilament, an amyloid, an actin, a myosin, and a titin.

40. A bioprotein tube prepared according to any one of claims 1-27 and 31.

41. The method of any one of claims 1-27 and 31, further comprising seeding cells on the bioprotein tube.

42. The method of claim 41, wherein the cells are endothelial cells or smooth muscle cells.

43. The method of claim 42, wherein the cells are cultured under appropriate conditions to produce a vascular tissue.

44. A vascular tissue prepared according to the method of claim 43.

45. A method for treating a subject having a vascular disease, comprising providing the vascular tissue of claim 44, and implanting the tissue in the subject, thereby treating the subject having a vascular disease.

46. A method for preparing a three-dimensional tissue, comprising

embedding a bioprotein tube prepared according to the method of any one of claims 1-27 and 31 between two stacked polymeric tissue scaffolds comprising a plurality of nanometer dimension polymeric fibers comprising alginate to form a tissue scaffold assembly,

contacting the assembly with a plurality of cells, and

culturing the cells under appropriate conditions, thereby preparing a three-dimensional tissue.

47. A three-dimensional tissue prepared according to the method of claim 46.

48. The method of claim 46, wherein the cells are myocytes.

49. An edible three-dimensional muscle tissue prepared according to the method of claim 48.

50. The method of claim 46, wherein the plurality of nanometer dimension polymeric fibers further comprise gelatin.

51. The method of claim 50, wherein the ratio of alginate to gelatin is about 10:1.

52. The method of claim 50, wherein the ratio of alginate to gelatin is about 5:1.

53. The method of claim 50, wherein the ratio of alginate to gelatin is about 1:2.

54. The method of claim 50, wherein the plurality of nanometer dimension polymeric fibers further comprise a biogenic polymer.

55. The method of claim 54, wherein the biogenic polymer is selected from the group consisting of silk, a keratin, an elastin, a fibrillin, a fibrinogen, a fibrin, a thrombin, a fibronectin, a laminin, a collagen, a vimentin, a neurofilament, an amyloid, an actin, a myosin, and a titin. In one embodiment, the polymeric fiber is a biohybrid fiber.

56. An edible tubular food product comprising the bioprotein tubes prepared according to the methods of any one of claims 1-27 and 31.

57. A method for identifying a compound that modulates a tissue function, the method comprising

providing a vascular tissue prepared according to the method of claim 43 or a three-dimensional tissue prepared according to the method of claim 46;

contacting said tissue with a test compound; and

determining the effect of the test compound on a tissue function in the presence and absence of the test compound, wherein a modulation of the tissue function in the presence of said test compound as compared to the tissue function in the absence of said test compound indicates that said test compound modulates a tissue function, thereby identifying a compound that modulates a tissue function.

58. A method for identifying a compound useful for treating or preventing a tissue disease, the method comprising

providing a vascular tissue prepared according to the method of claim 43 or a three-dimensional tissue prepared according to the method of claim 46;

contacting said tissue with a test compound; and

determining the effect of the test compound on a tissue function in the presence and absence of the test compound, wherein a modulation of the tissue function in the presence of said test compound as compared to the tissue function in the absence of said test compound indicates that said test compound modulates a tissue function, thereby identifying a compound useful for treating or preventing a tissue disease.

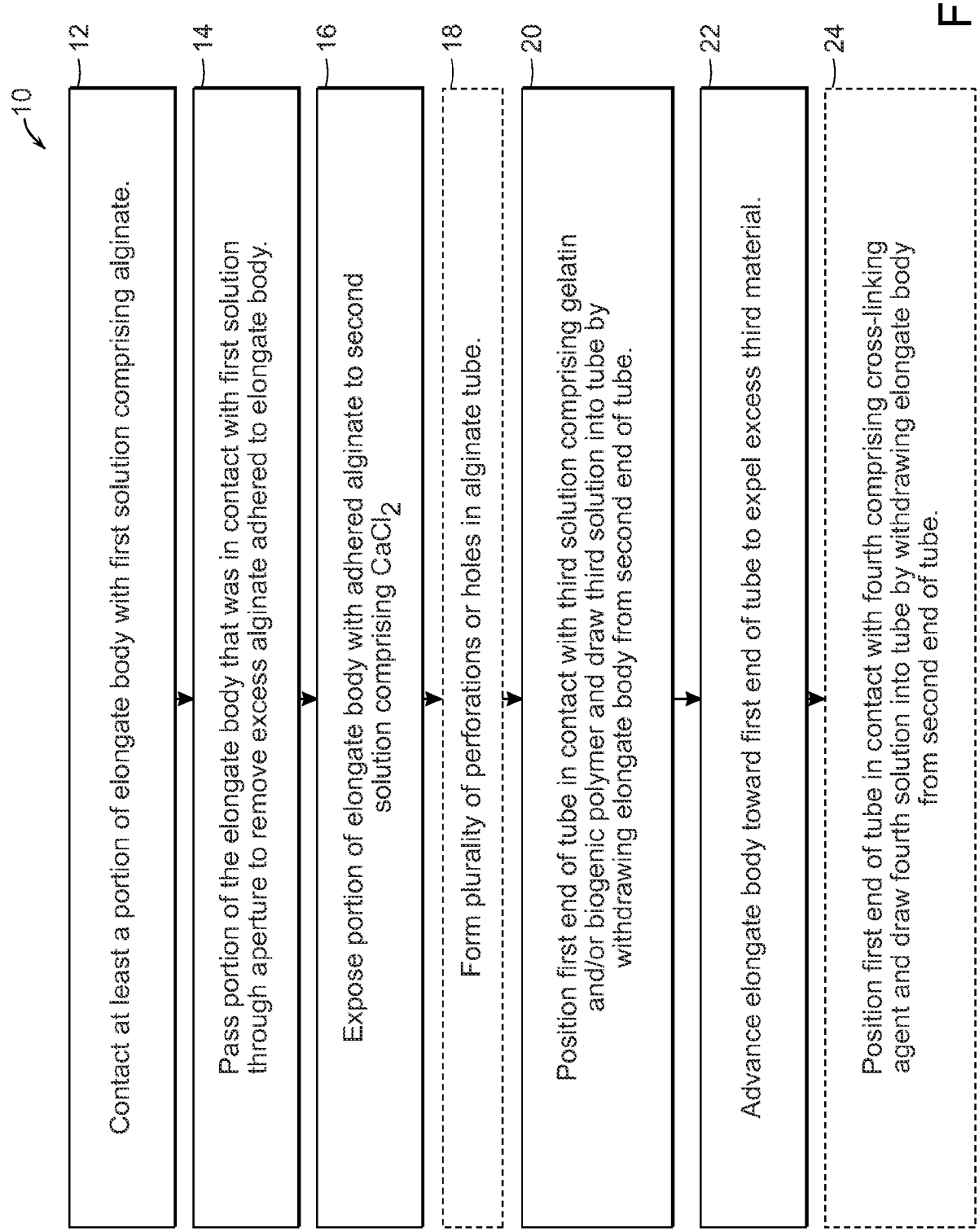

10
12
Contact at least a portion of elongate body with first solution comprising alginate.
14
Pass portion of the elongate body that was in contact with first solution through aperture to remove excess alginate adhered to elongate body.
16
Expose portion of elongate body with adhered alginate to second solution comprising $CaCl_2$
18
Form plurality of perforations or holes in alginate tube.
20
Position first end of tube in contact with third solution comprising gelatin and/or biogenic polymer and draw third solution into tube by withdrawing elongate body from second end of tube.
22
Advance elongate body toward first end of tube to expel excess third material.
24
Position first end of tube in contact with fourth comprising cross-linking agent and draw fourth solution into tube by withdrawing elongate body from second end of tube.

Figure 1

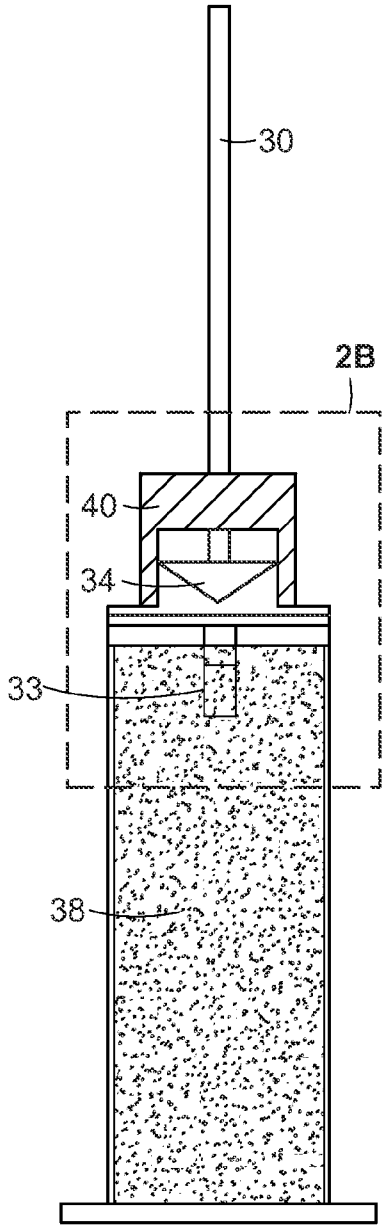


Figure 2A

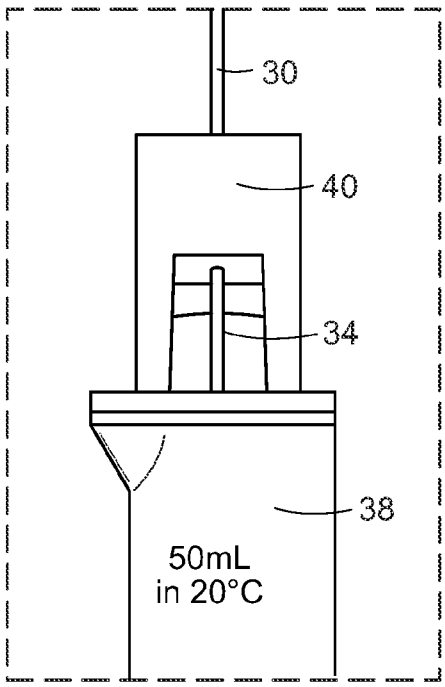


Figure 2B

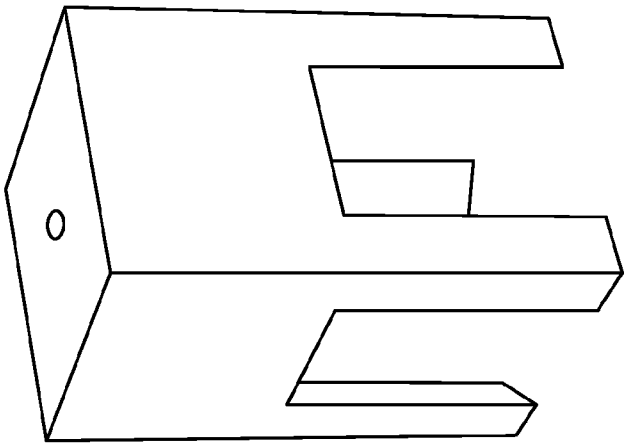


Figure 3C

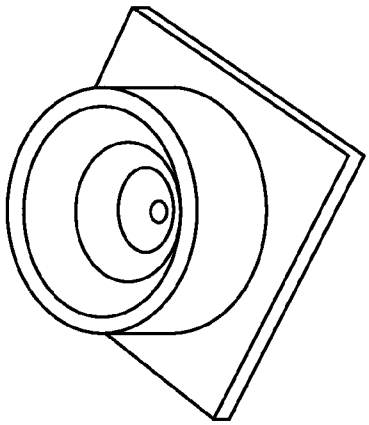


Figure 4C

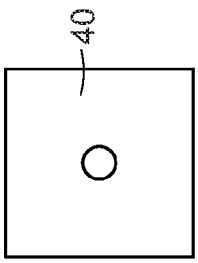


Figure 3B

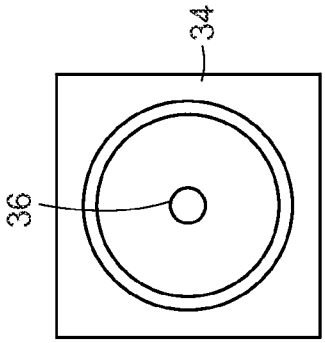


Figure 4B

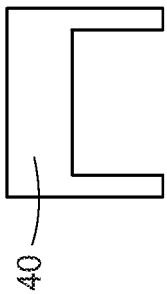


Figure 3A

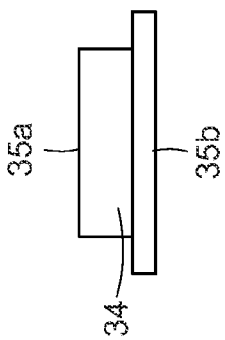


Figure 4A

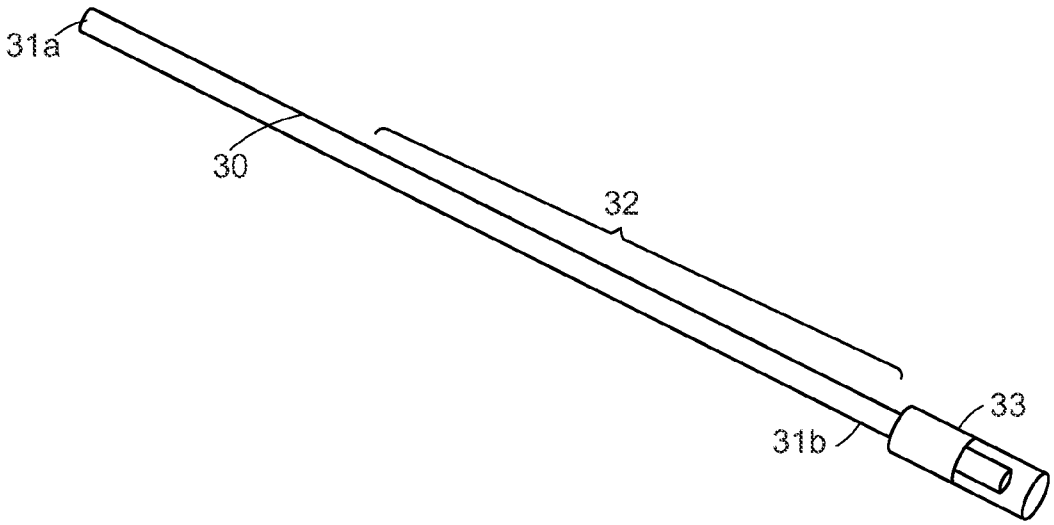


Figure 5A

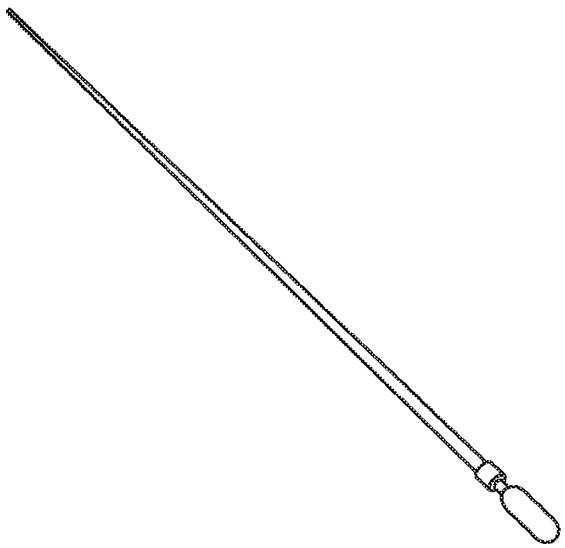


Figure 5B

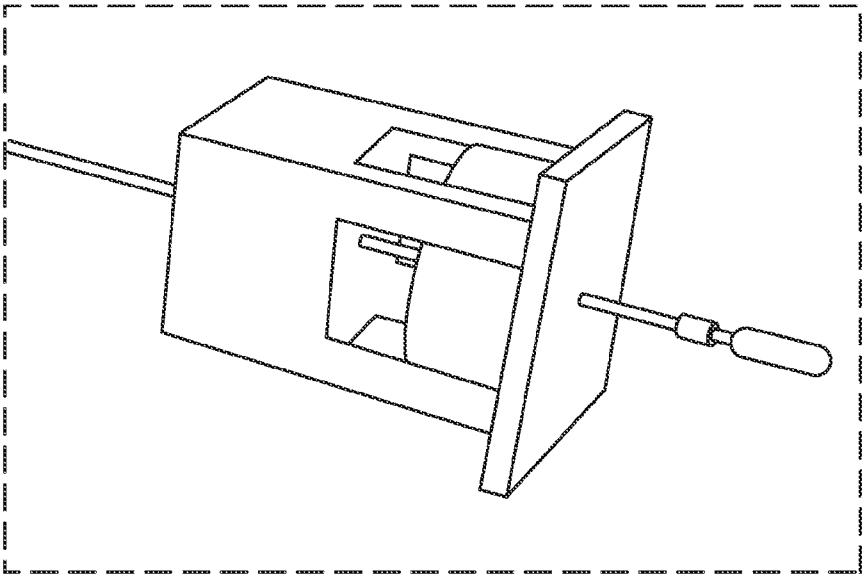


Figure 6B

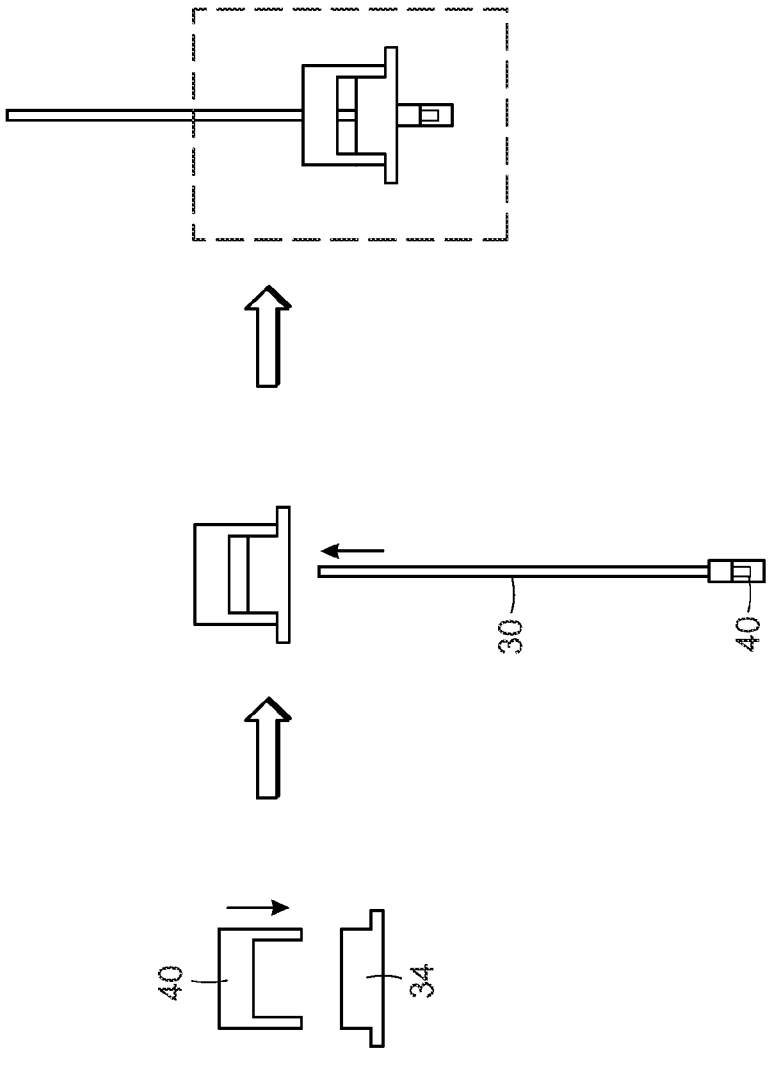


Figure 6A

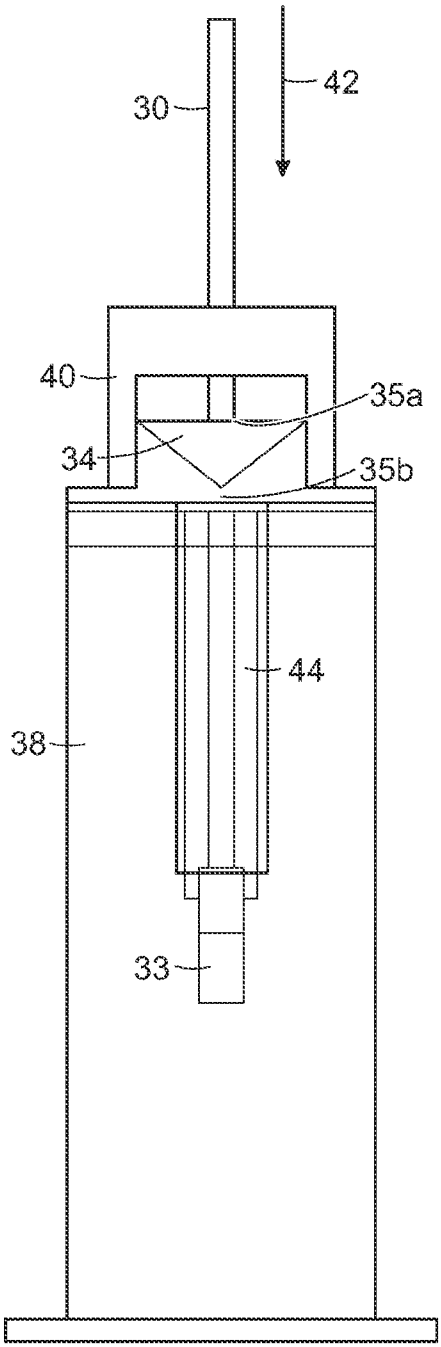


Figure 7

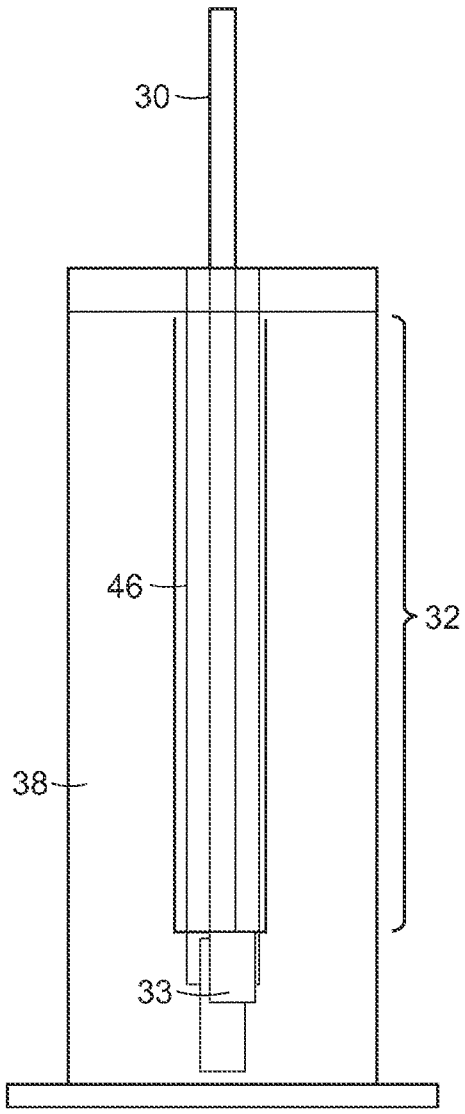


Figure 8

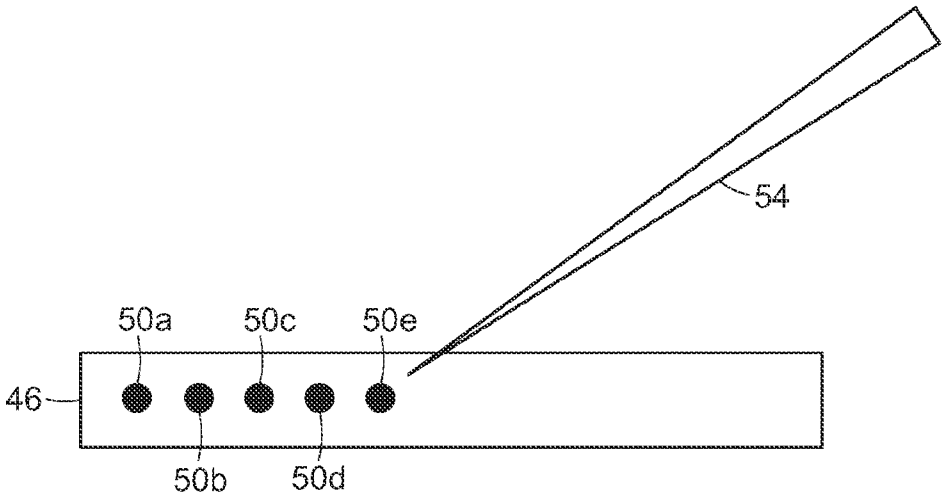


Figure 9A

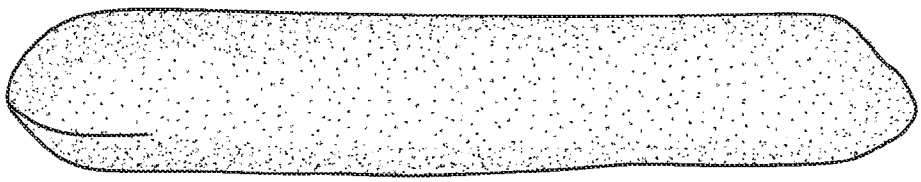


Figure 9B

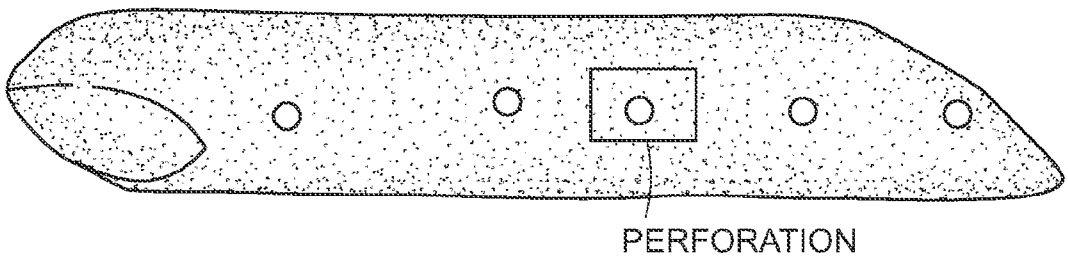


Figure 9C

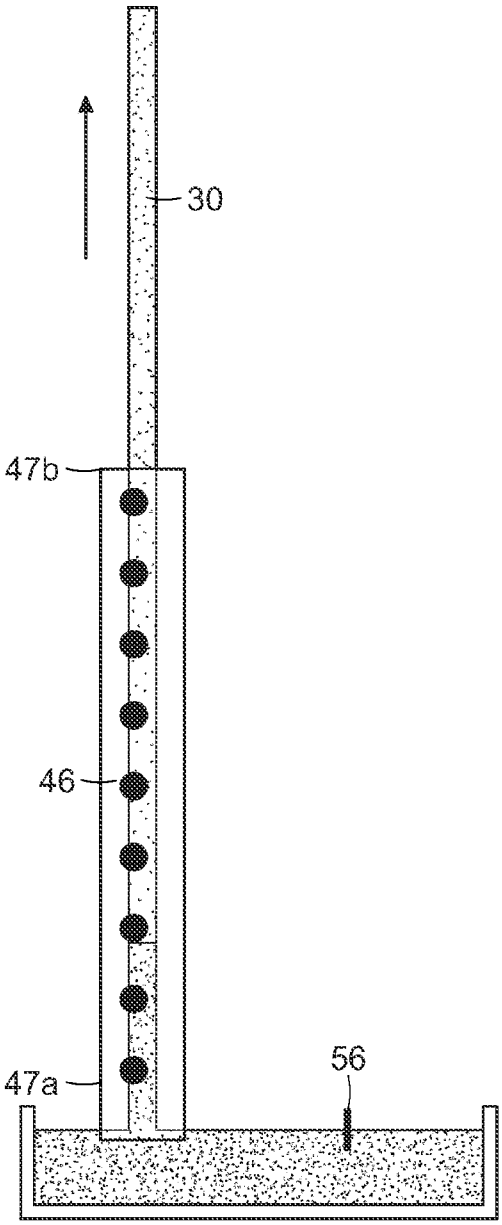


Figure 10

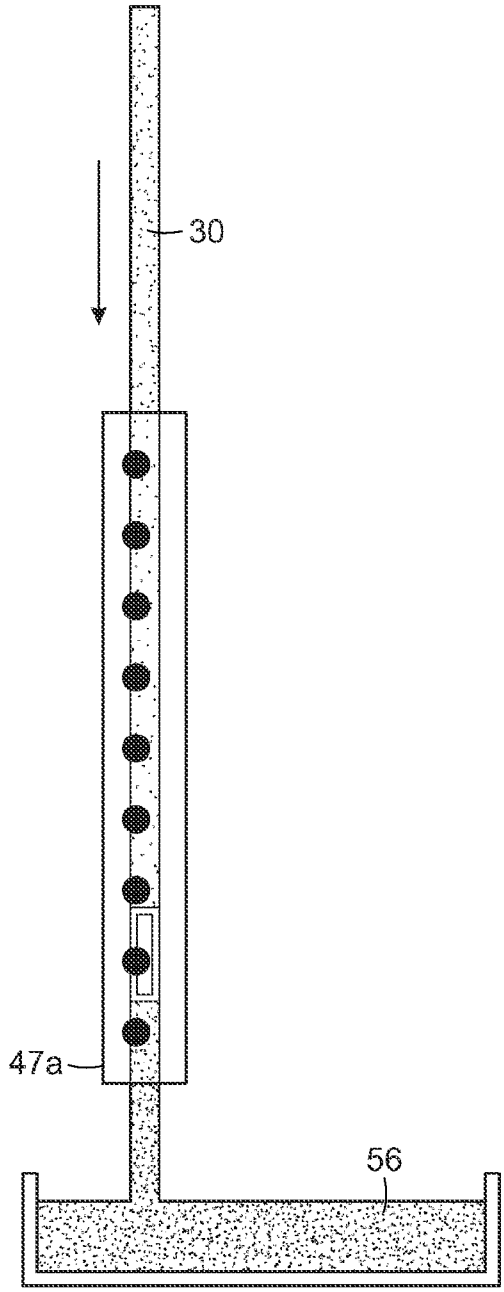


Figure 11

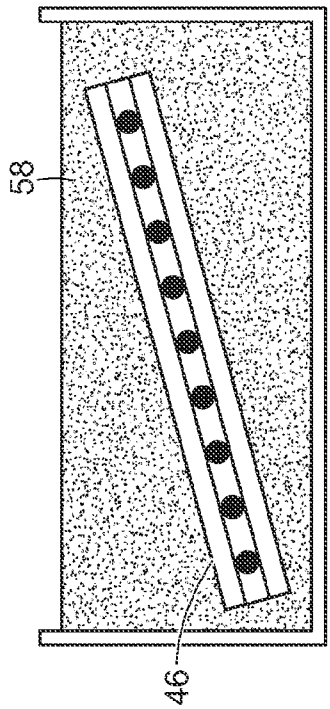


Figure 13

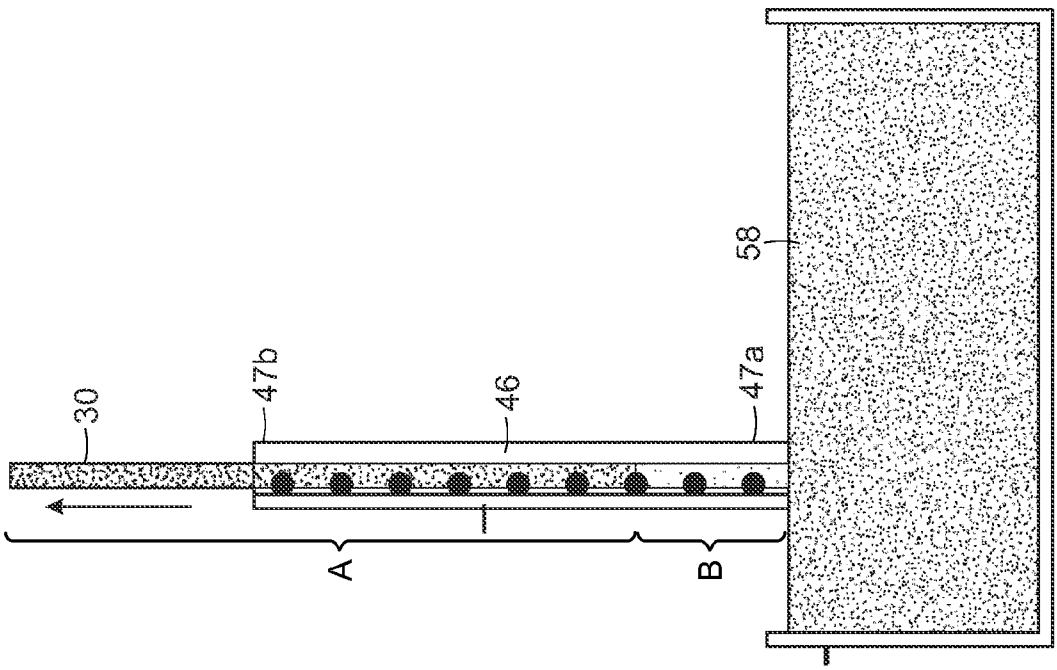


Figure 12

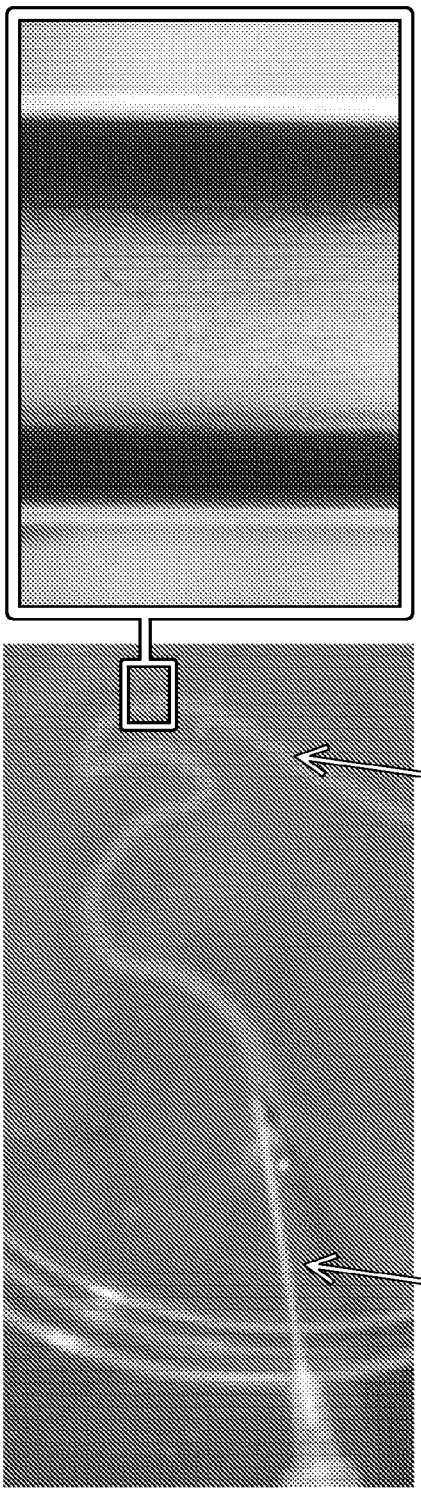


Figure 14A

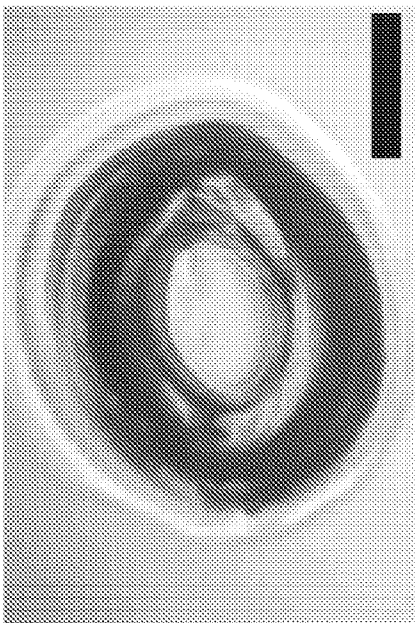


Figure 14B
Cross-section

Figure 14C

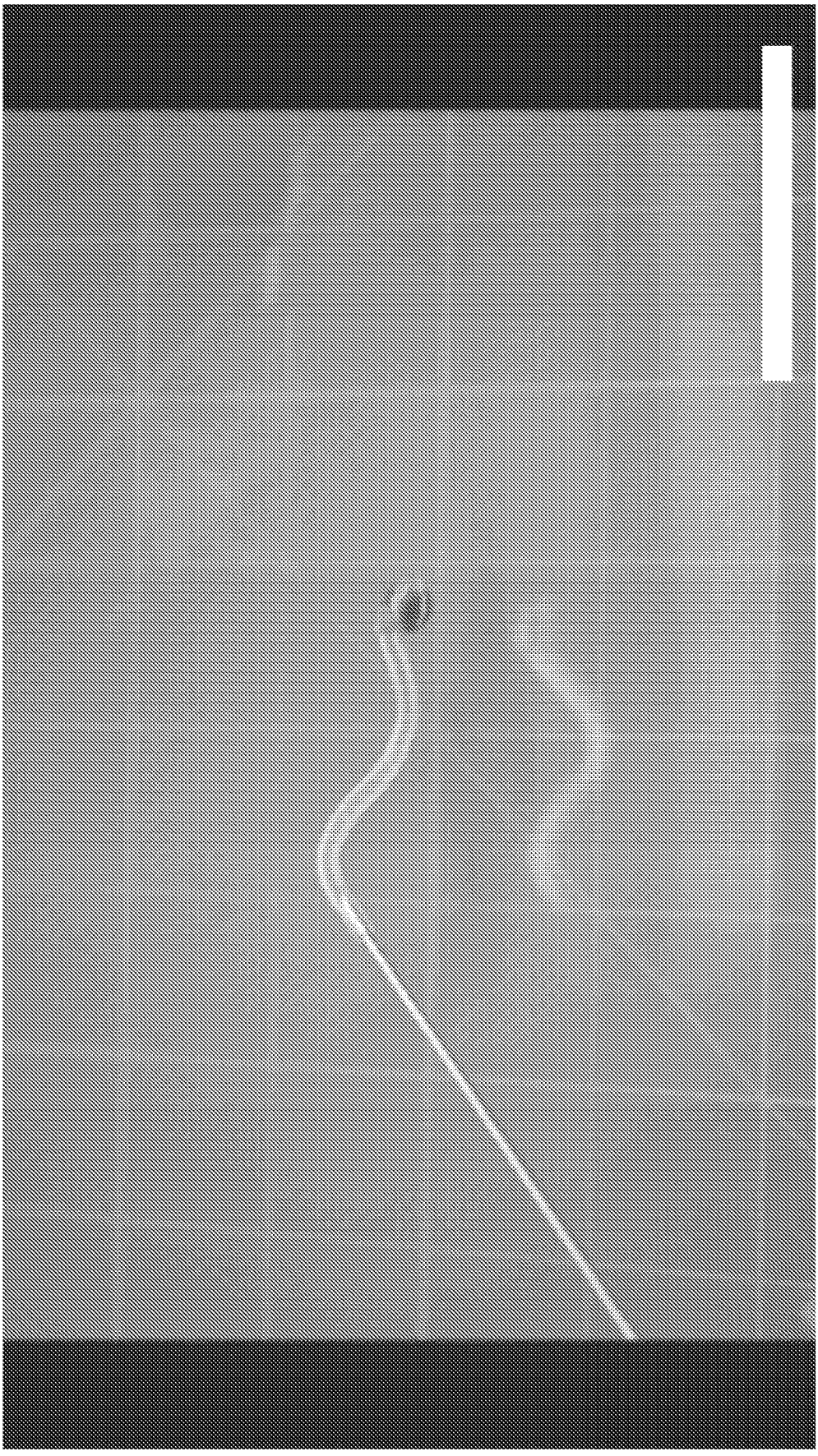
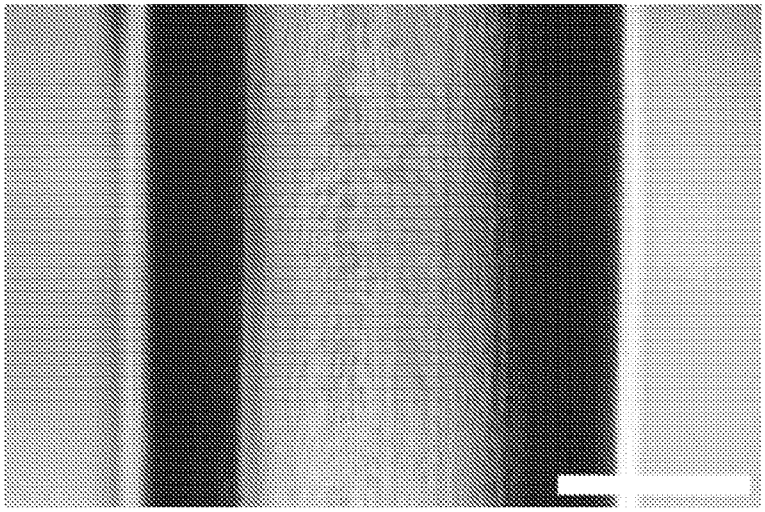


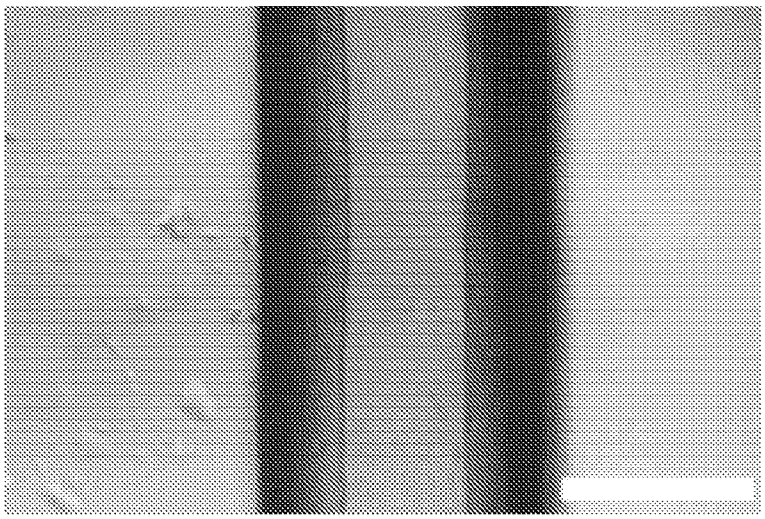
Figure 15

Figure 16A



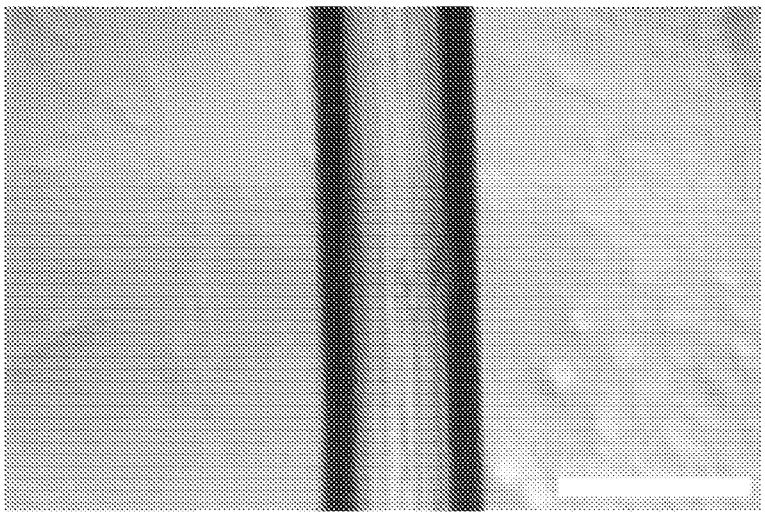
Scale: 0.5 mm

Figure 16B



Scale: 0.5 mm

Figure 16C



Scale: 0.5 mm

Figure 17A

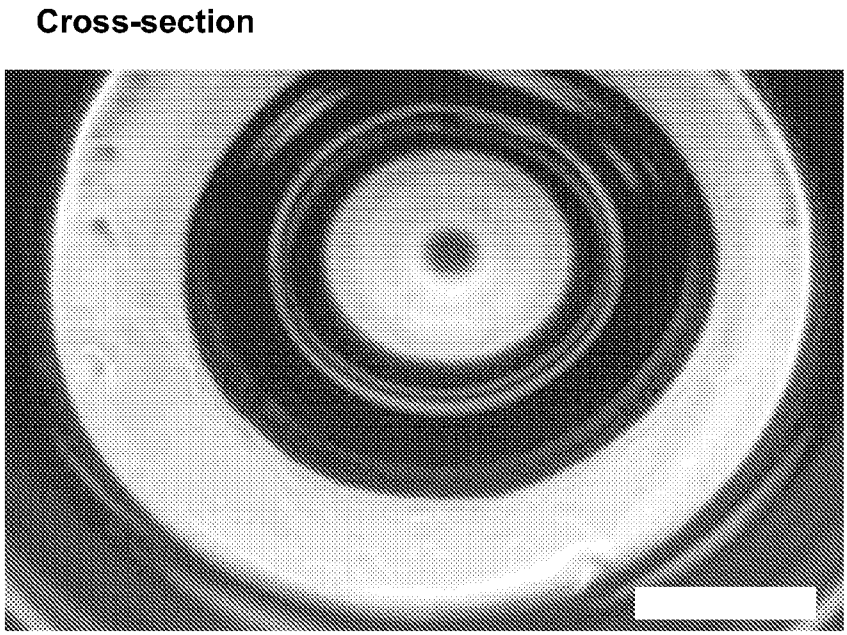


Figure 17B

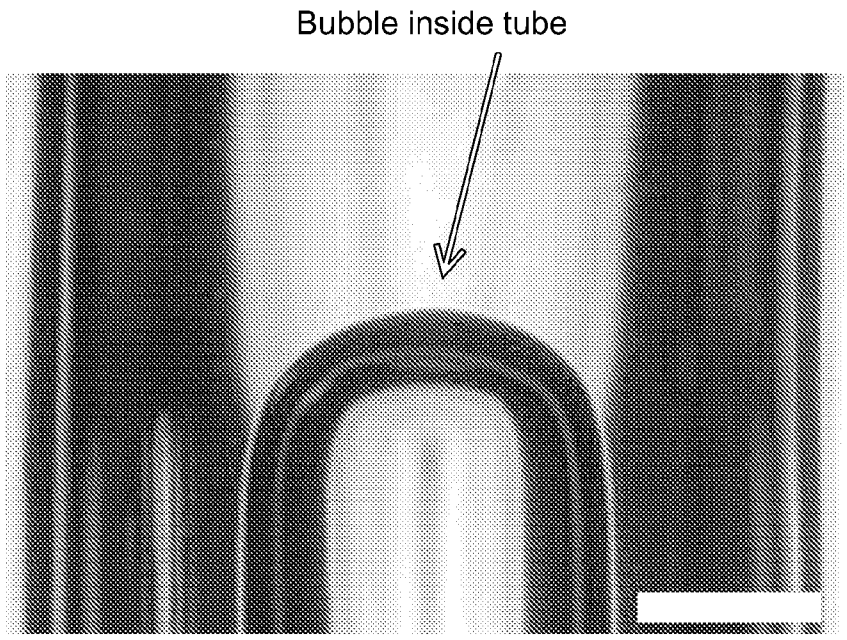
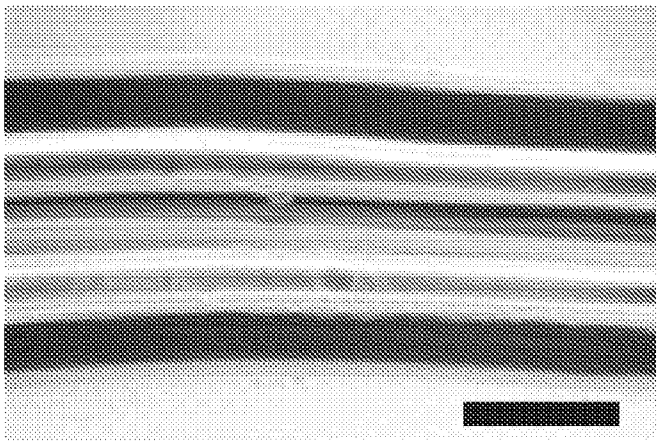
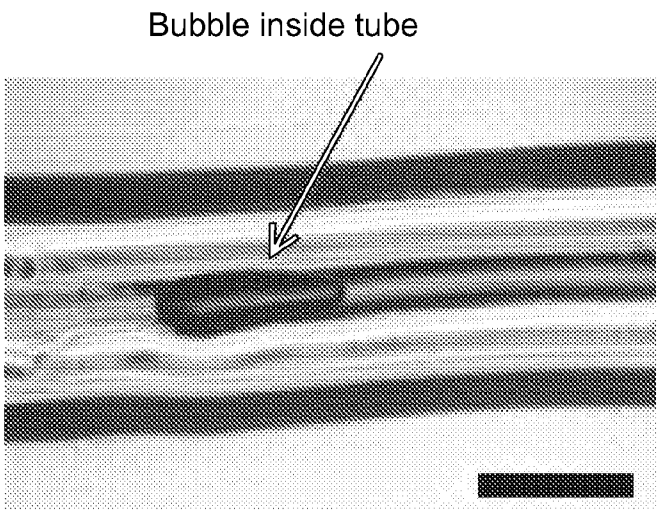


Figure 18A



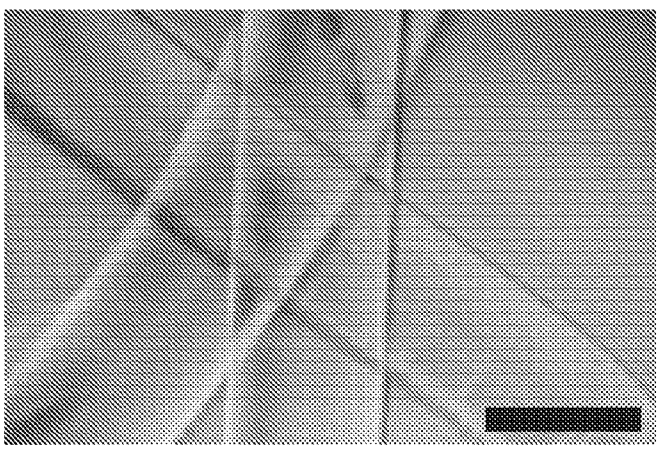
Scale: 0.5 mm

Figure 18B



Scale: 0.5 mm

Figure 18C



Scale: 0.5 mm

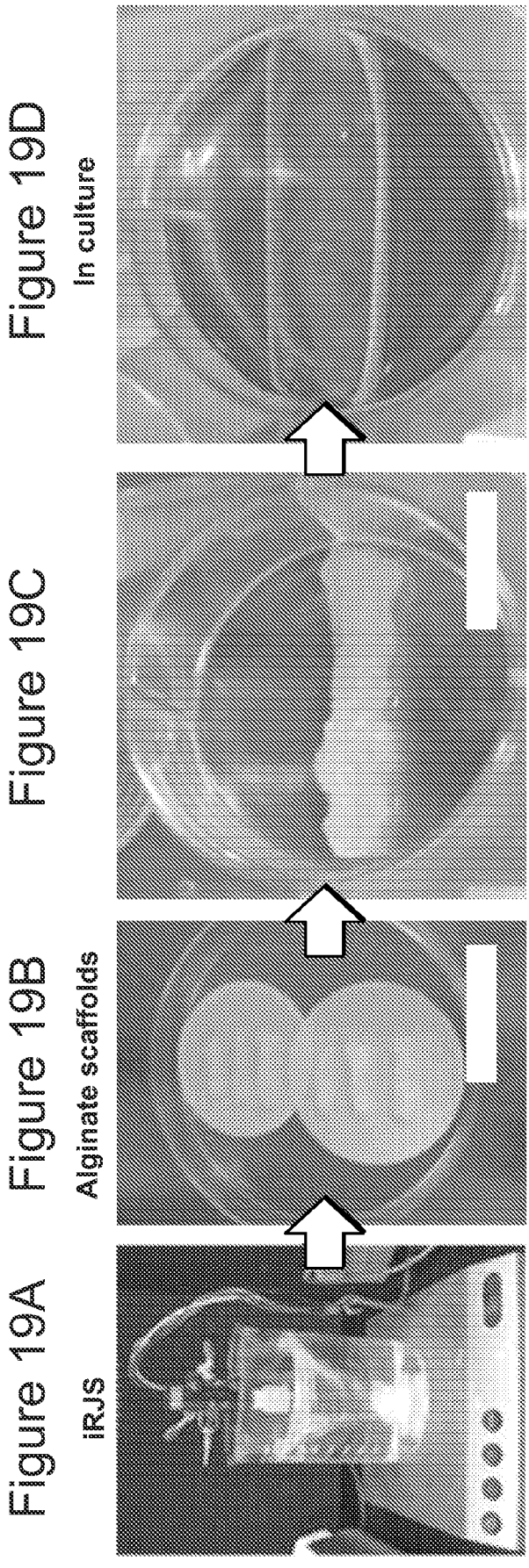


Figure 19A
iRJS
Figure 19B
Alginate scaffolds
Figure 19C
Figure 19D
In culture
Scale: 4 cm
Scale: 1 cm

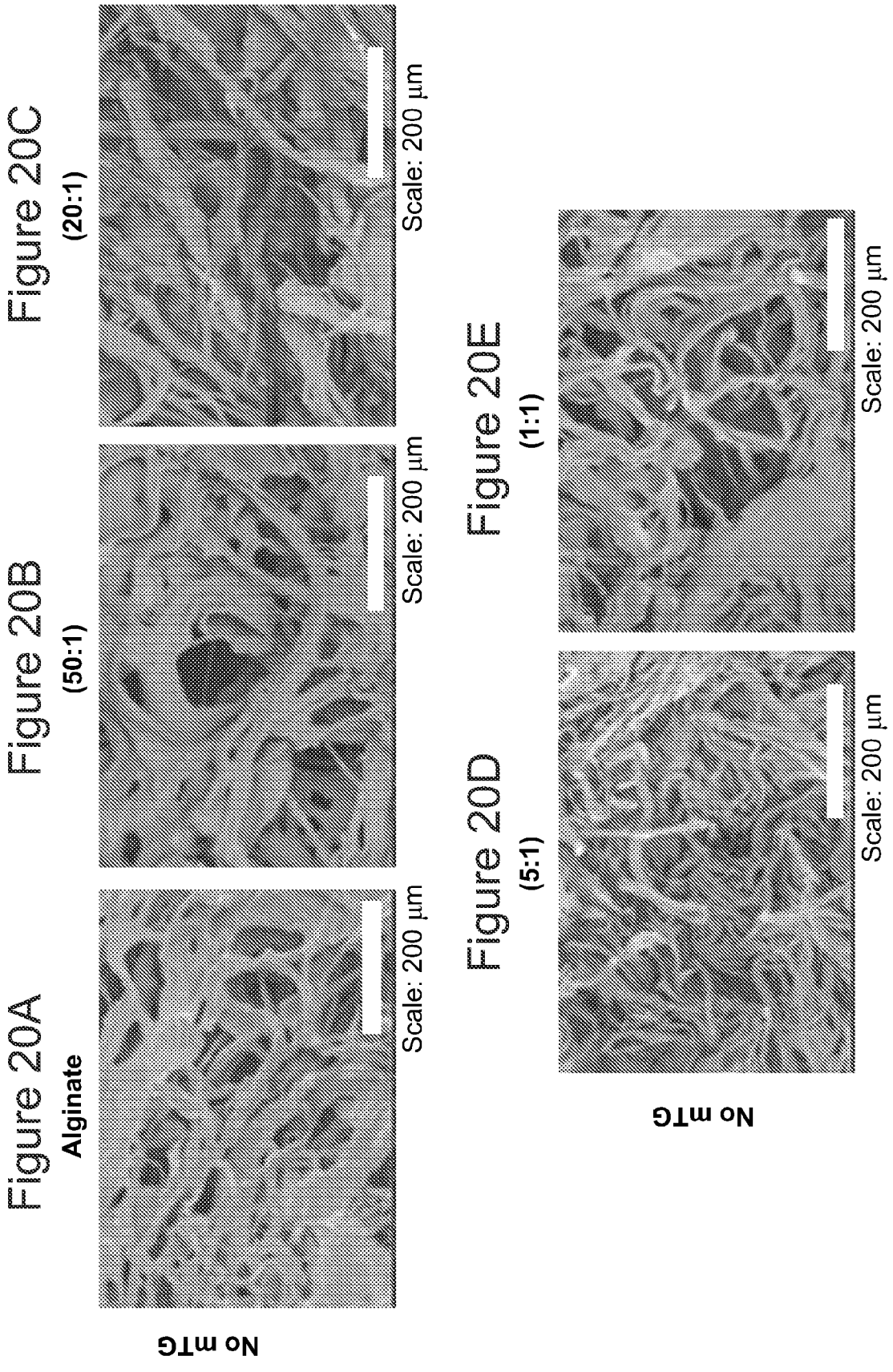


Figure 20A
Alginate
Figure 20B
(50:1)
Figure 20C
(20:1)
Figure 20D
(5:1)
Figure 20E
(1:1)
Scale: 200 µm
Scale: 200 µm
Scale: 200 µm
Scale: 200 µm
Scale: 200 µm
No mTG
No mTG

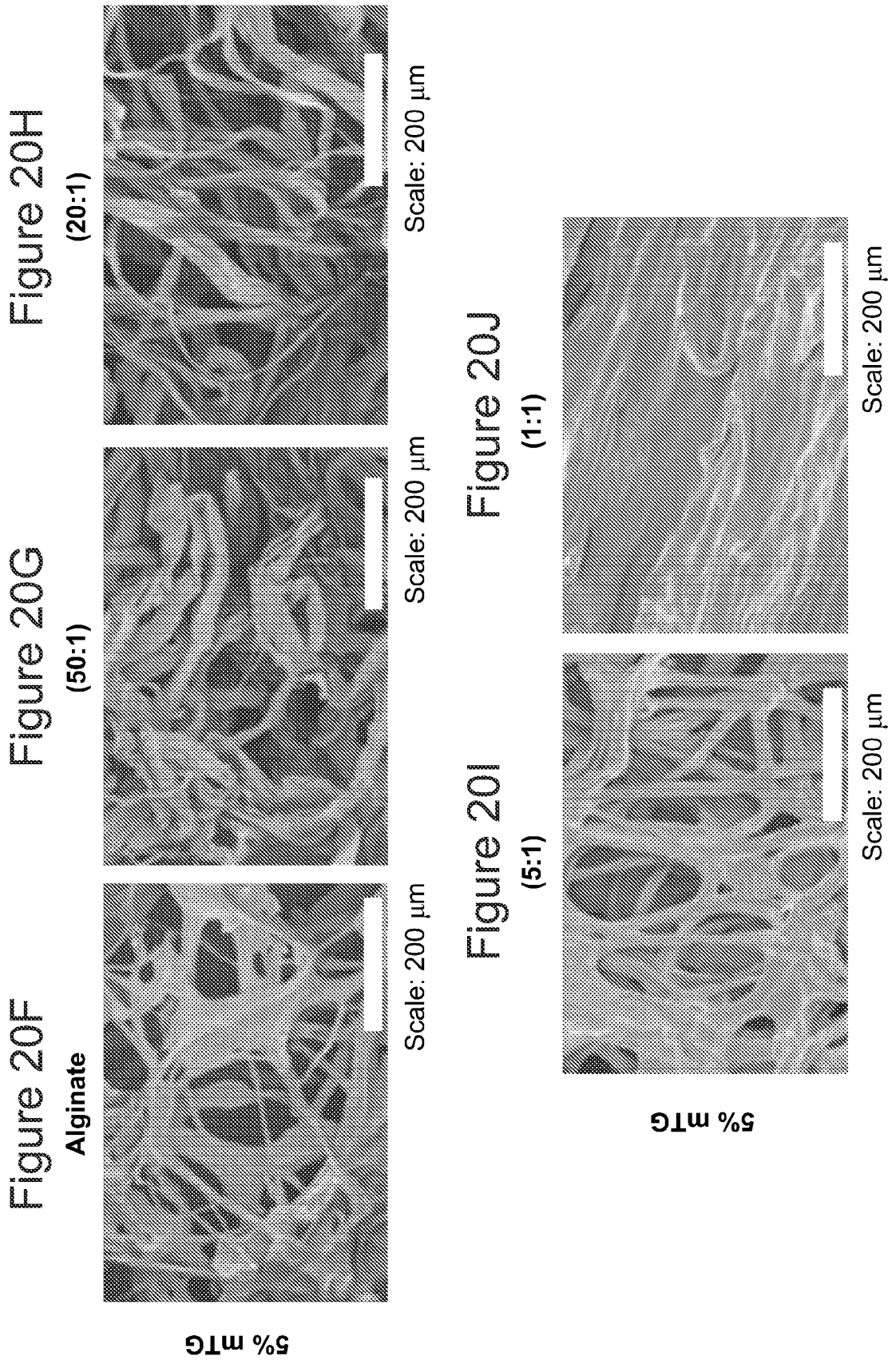


Figure 20F
Alginate
Figure 20G
(50:1)
Figure 20H
(20:1)
Figure 20I
(5:1)
Figure 20J
(1:1)
Scale: 200 µm
Scale: 200 µm
Scale: 200 µm
Scale: 200 µm
Scale: 200 µm
5% mTG
5% mTG

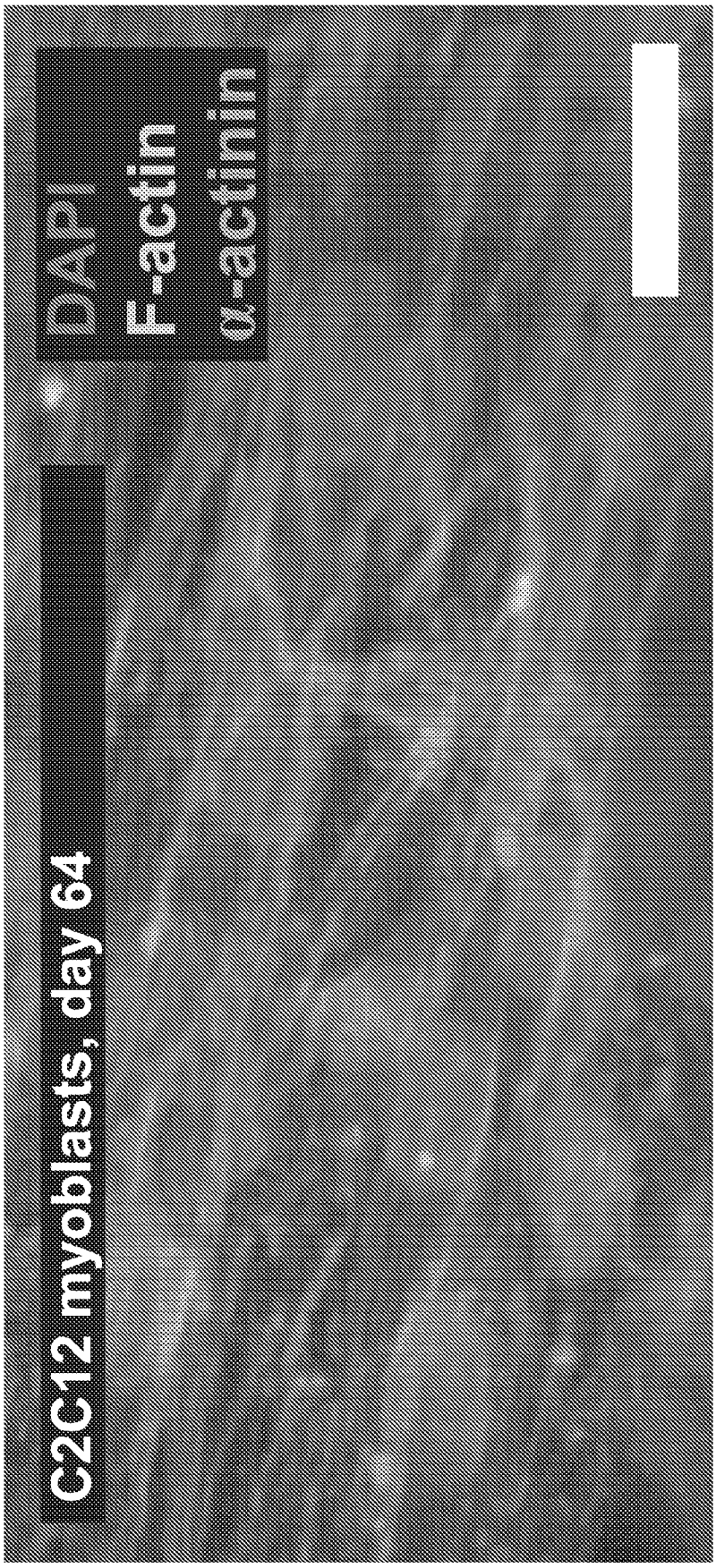


Figure 21

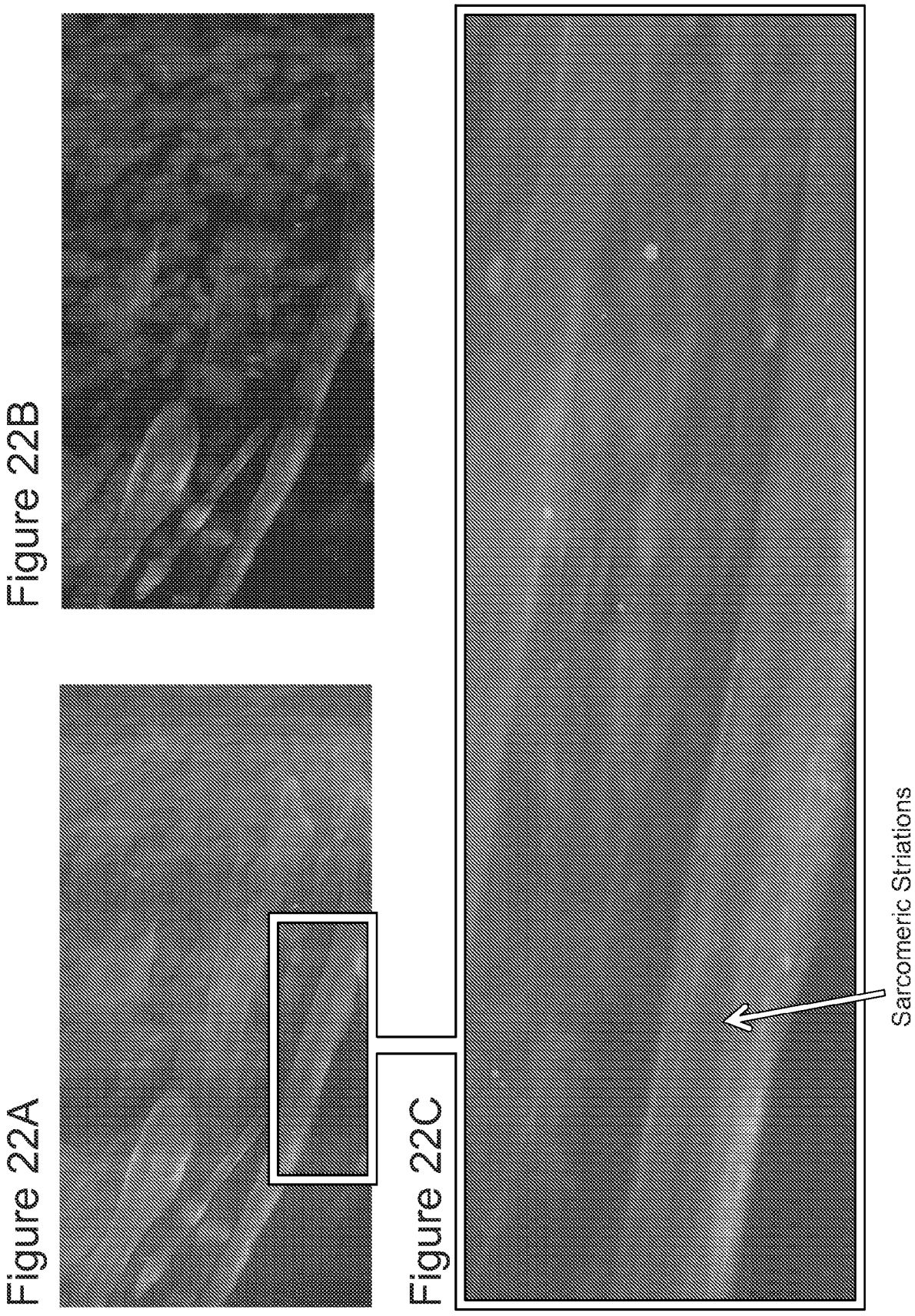


Figure 22B
Figure 22A
Figure 22C
Sarcomeric Striations

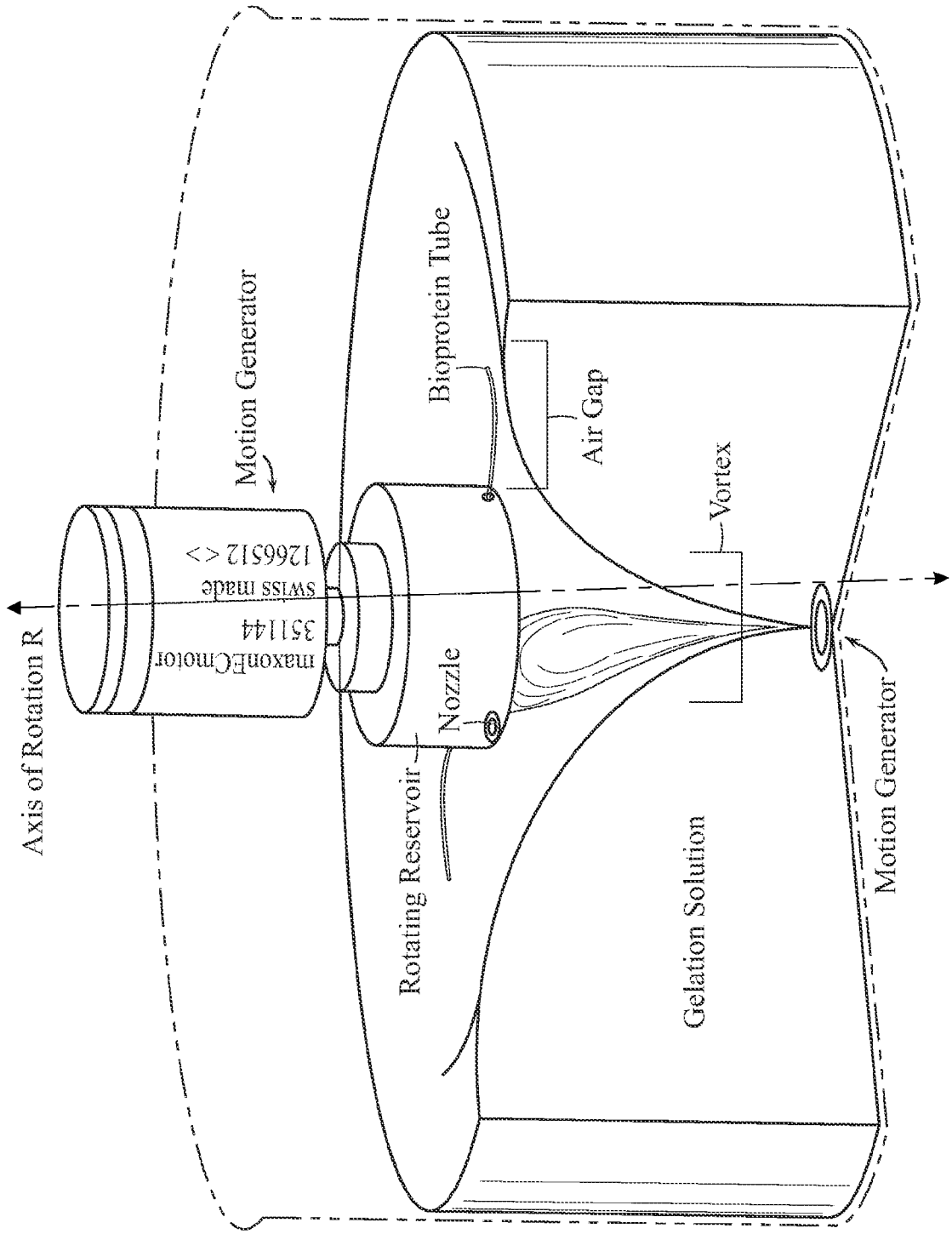

Axis of Rotation R
maxonECmotor
351144
swiss made
1266512 < >
Motion Generator
Bioprotein Tube
Air Gap
Vortex
Nozzle
Rotating Reservoir
Gelation Solution
Motion Generator

Figure 23

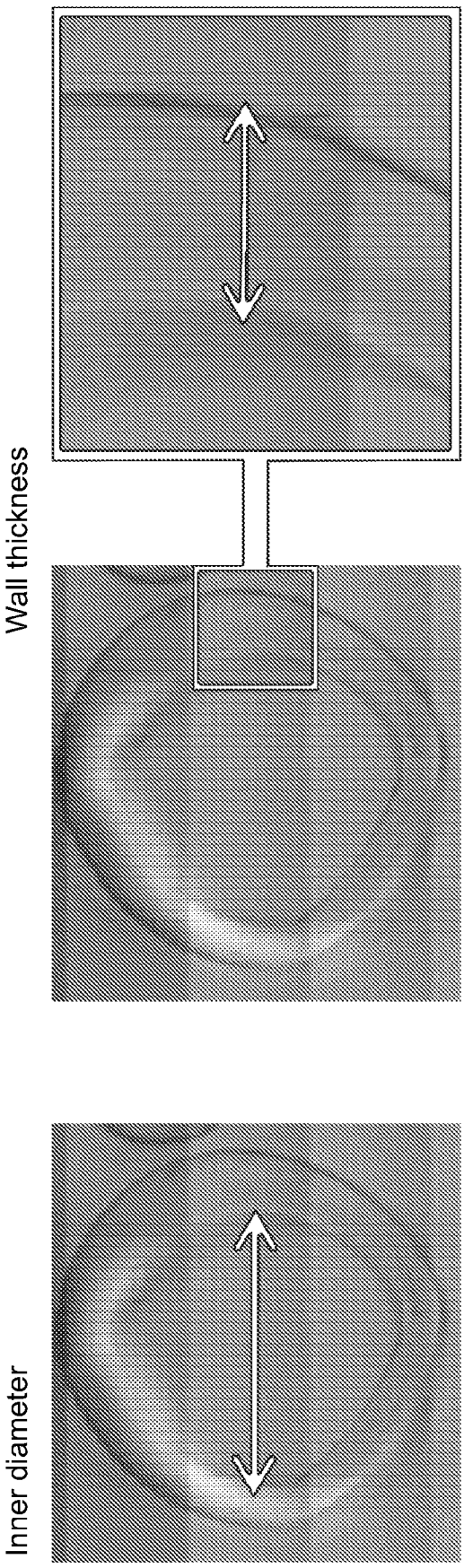


Figure 24C

Figure 24B

Figure 24A

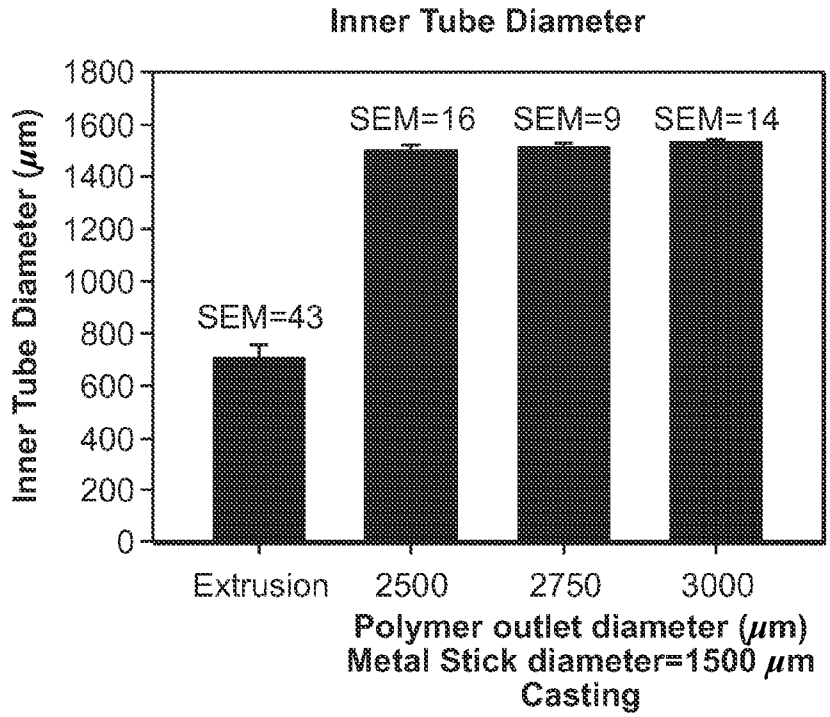


Figure 25

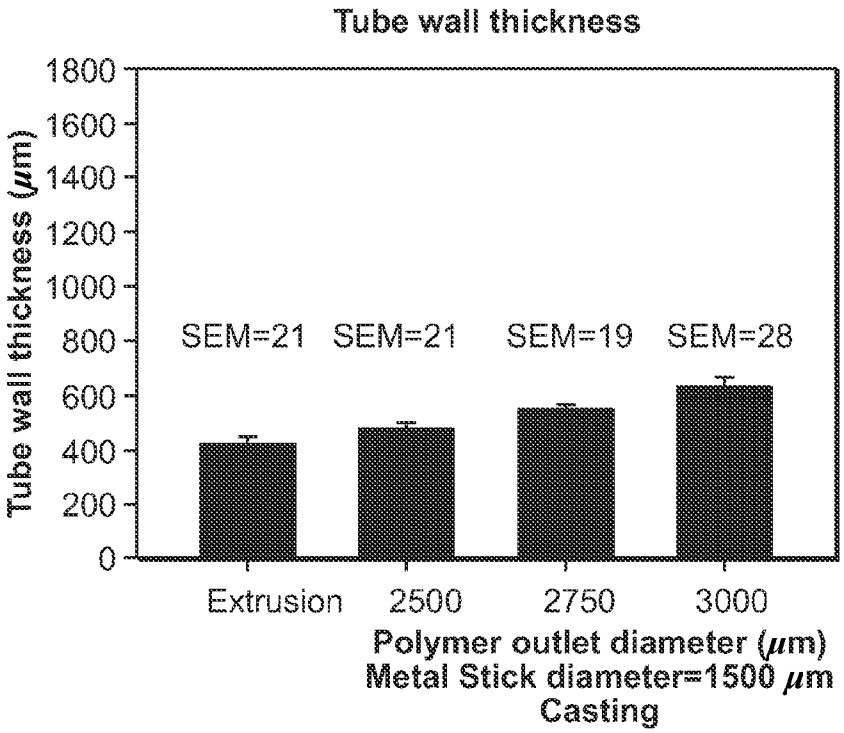


Figure 26

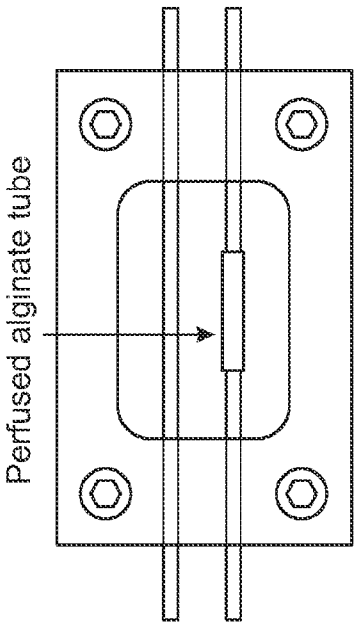


Figure 29

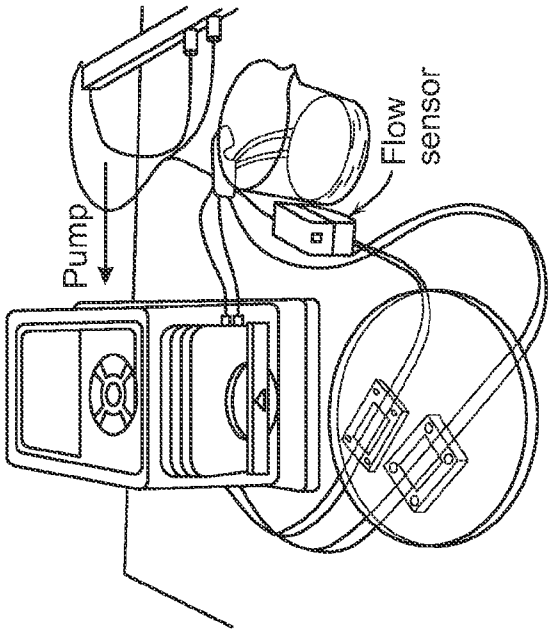


Figure 30

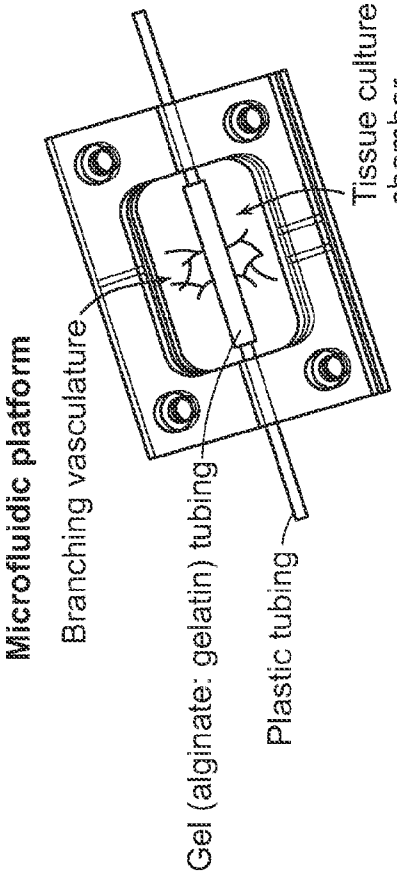


Figure 27

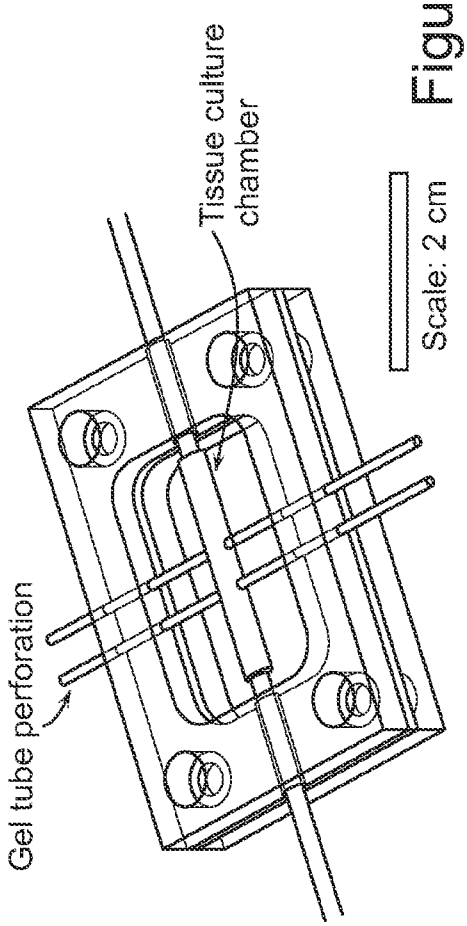


Figure 28

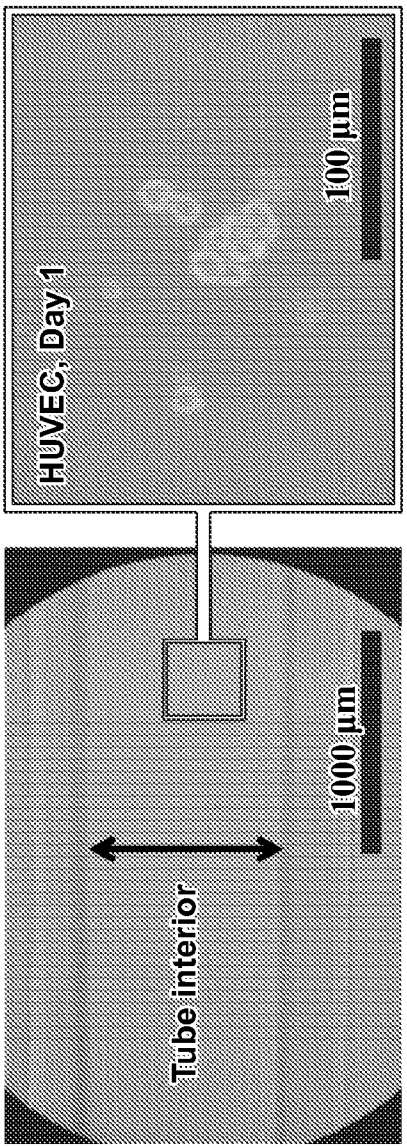


Figure 31A

Figure 31B

Figure 31C

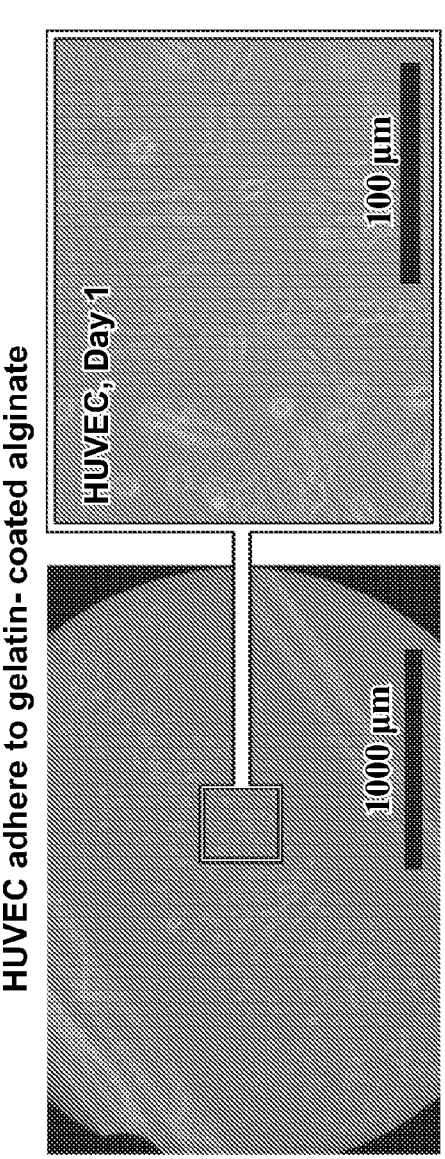


Figure 32A

Figure 32B

Figure 32C

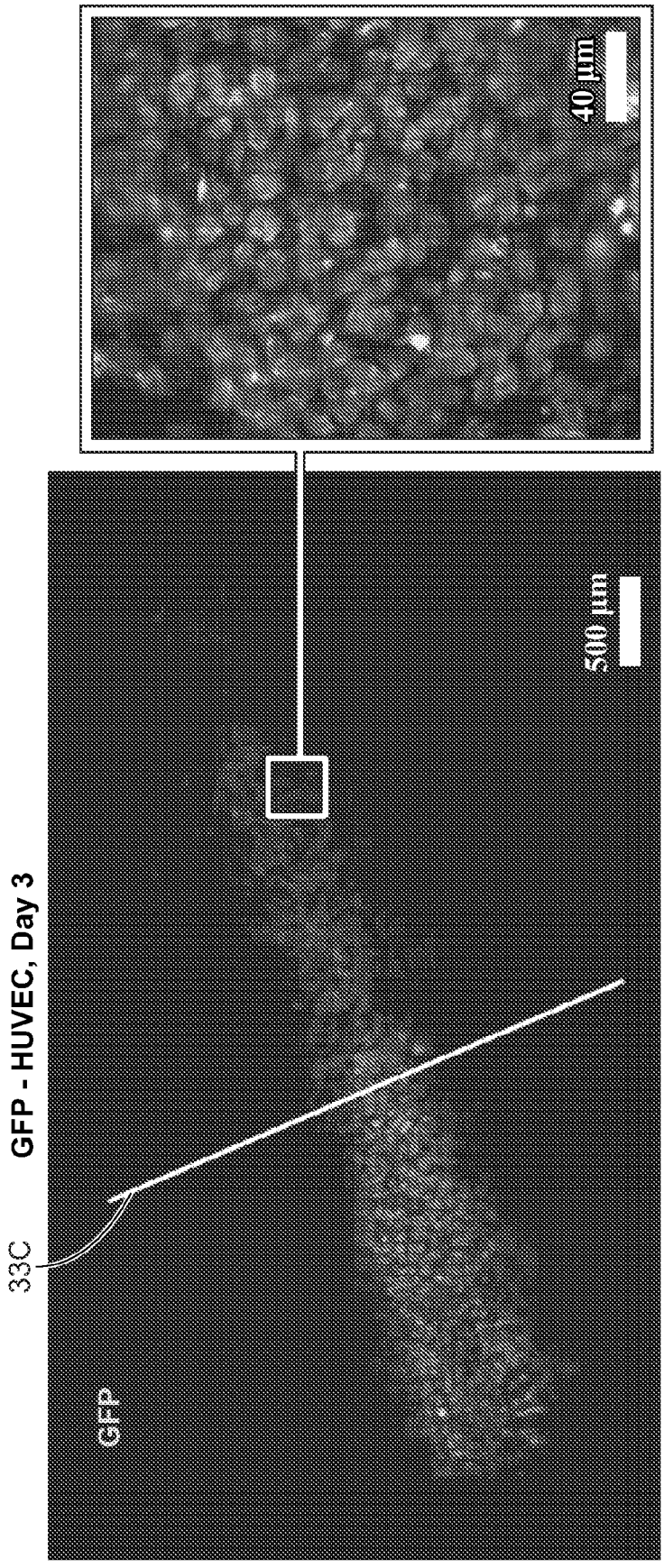


Figure 33A

Figure 33B

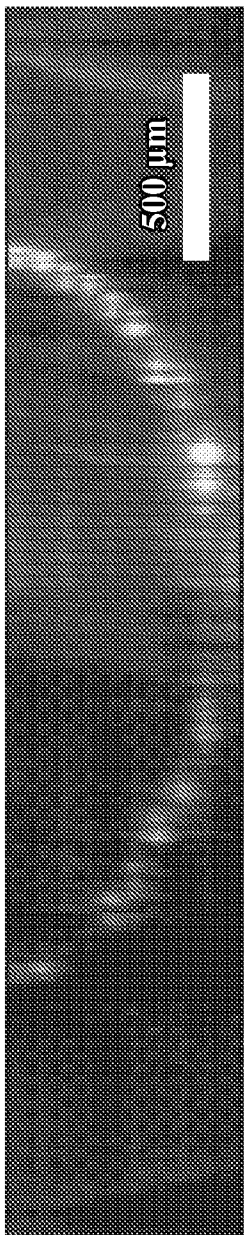


Figure 33C

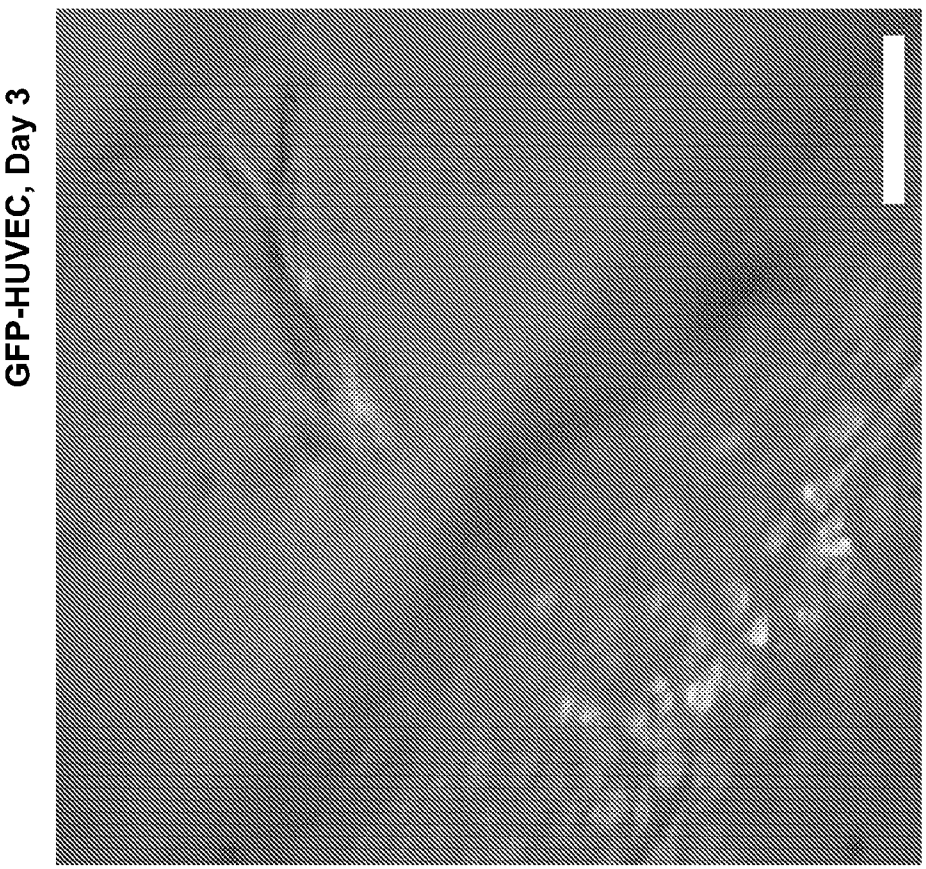


Figure 34B

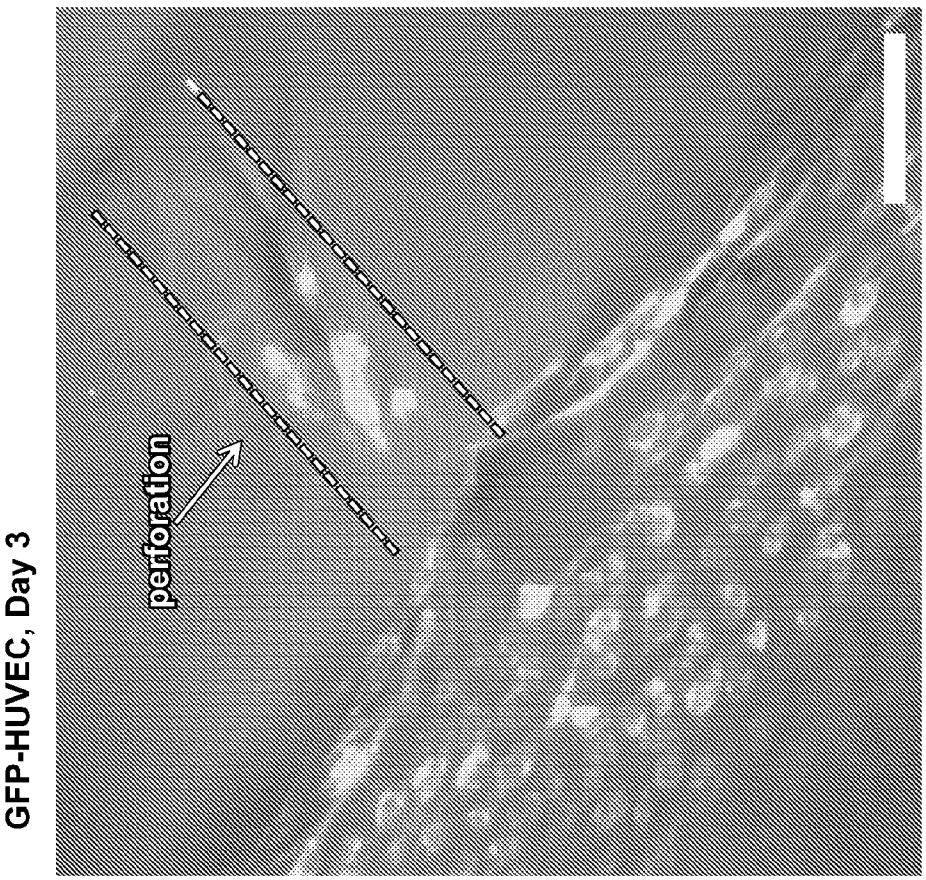


Figure 34A

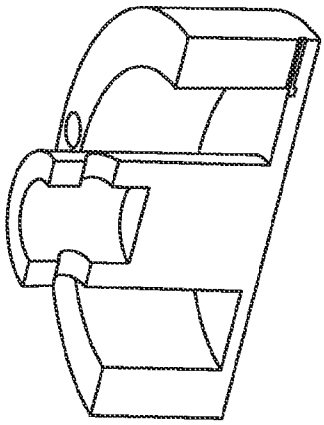


Figure 35B

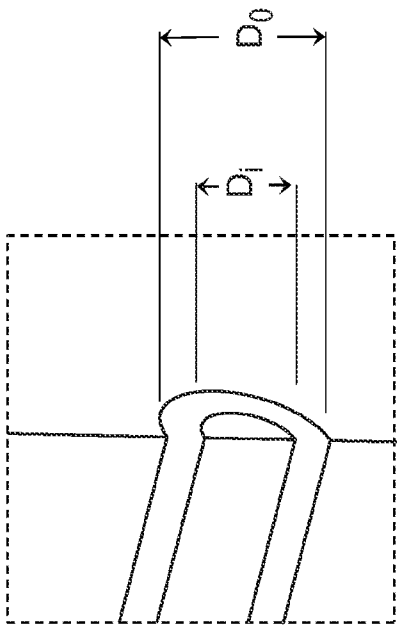


Figure 35D

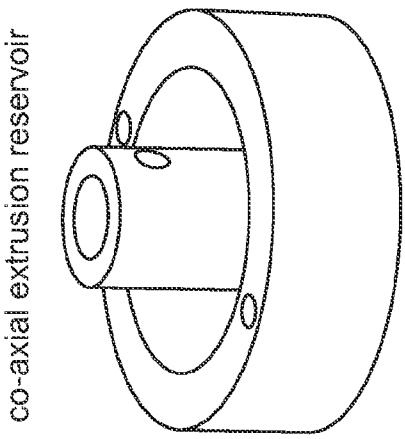


Figure 35A

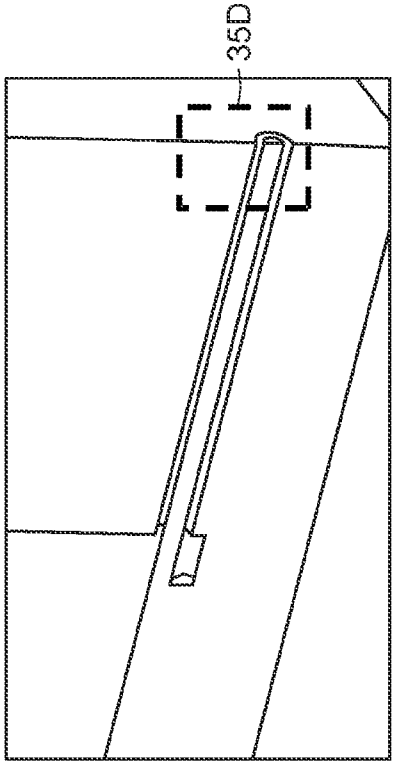


Figure 35C

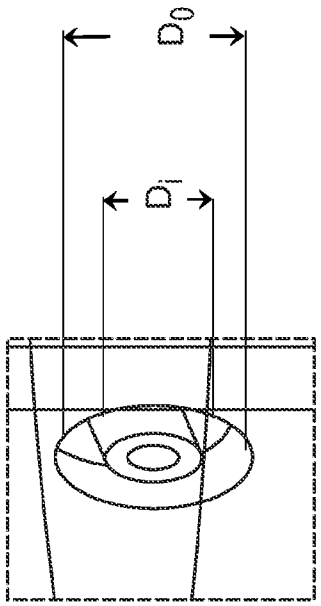


Figure 36B

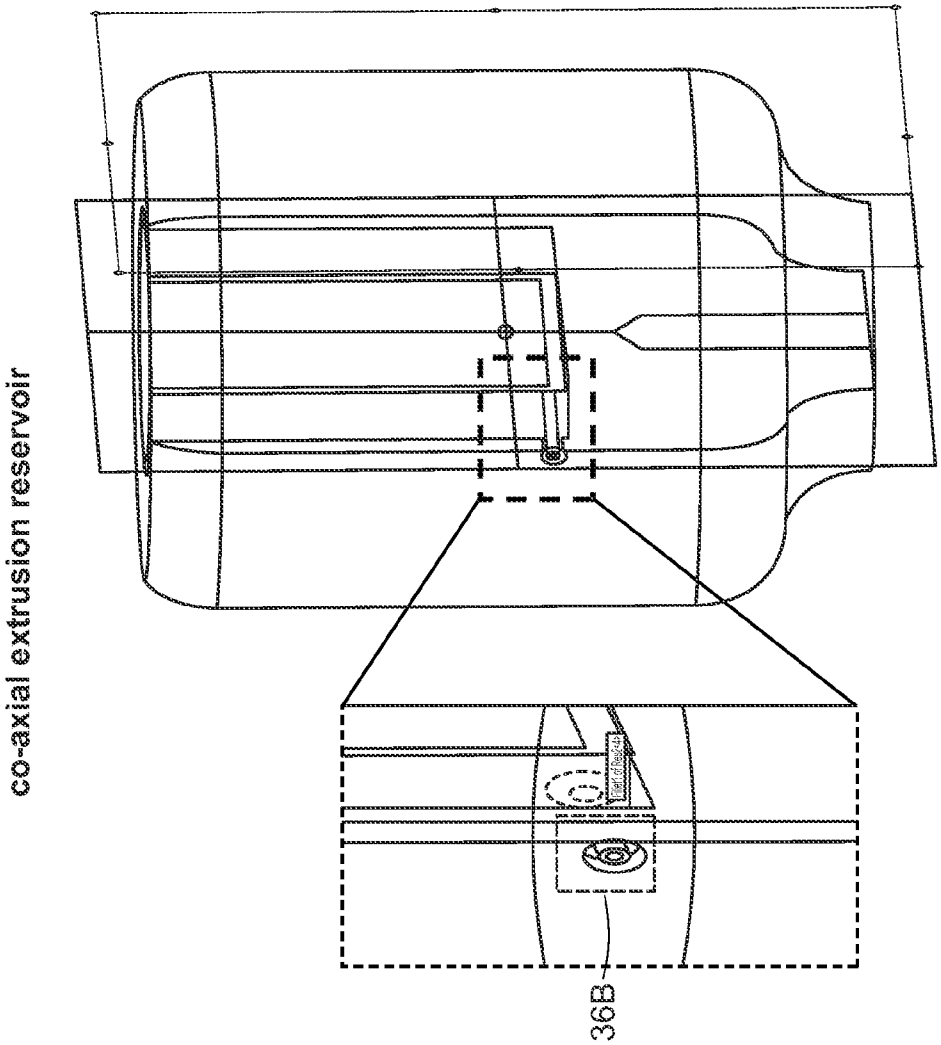


Figure 36A

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/39983

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61L 27/36, A61M 37/00, A61F 2/06 (2015.01)
CPC - A61L 2300/412, A61L 27/58, A61L 27/3808, C12N 5/0658
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): A61L 27/36, A61M 37/00, A61F 2/06 (2015.01)
CPC: A61L 2300/412, A61L 27/58, A61L 27/3808, C12N 5/0658

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 623/23.72, 424/423, 264/41, 424/93.7, 435/1.1

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, Google Scholar, Google Web, search terms: ex vivo bioprotein, hollow cylinder, tube, waste removal, vasculature, edible, alginate, biogenic polymer, elongate body, mold or form, cross-linking agent, microbial transglutaminase, a three-dimensional tissue, gelatin, genipin, reservoir, distal end, aperture, polymeric fibers,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2002/0151968 A1 (ZILLA et al.) 17 October 2002 (17.10.2002) [0009], [0010], [0020], [0022]-[0024], [0027], [0031], [0035]-[0037], [0042], [0043], Figs 2-4	1-23, (41-43, 46, 48, 50-55)/(1-23)
Y	US 2004/0126405 A1 (SAHATJIAN et al.) 01 July 2004 (01.07.2004)abstract, [0001], [0029], [0031], [0041], [0042], [0048], [0057]-[0060], [0062], [0078], [0080], [0083]	1-23, (41-43, 46, 48, 50-55)/(1-23)
Y	US 2011/0151011 A1 (FLYNN) 23 June 2011 (23.06.2011) [0030], [0031], [0079]	4-6, (41-43, 46, 48, 50-55)/(4-6)
Y	US 2004/0248722 A1 (MASON et al.) 9 December (2004 09.12.2004) para [0007], [0037], [0056], [0058], Figs 15-18	15, 16 (41-43, 46, 48, 50-55)/(15-16)

☐ Further documents are listed in the continuation of Box C. ☐

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

Date of the actual completion of the international search
04 November 2015 (04.11.2015)

Date of mailing of the international search report
08 DEC 2015

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-8300

Authorized officer:
Lee W. Young
PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/39983

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 57-58
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-23, 41-43 (in part), 46 (in part), 48 (in part), 50-55 (in part), drawn to a method of producing a bioprotein tube by forming a tube of bioprotein precursor on an elongate body solid support

Group II: Claims 24-30, 32-39 (in part), 41-43 (in part), 46 (in part), 48 (in part), 50-55 (in part), drawn to a method of producing a bioprotein tube by extruding a stream of bioprotein precursor through an orifice or nozzle into a gelation solution

Group III: Claims 31, 32-39 (in part), 41-43 (in part), 46 (in part), 48 (in part), 50-55 (in part), drawn to a method of producing a bioprotein tube by rotating a bioprotein precursor about an axis of rotation through a gelation solution vortex having a central air gap

-----please see continuation on extra sheet-----

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-23, 41-43 (in part), 46 (in part), 48 (in part), 50-55 (in part)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Continuation of: Box No. III Observations where unity of invention is lacking

Group IV: Claims 40, 44-45, 47, 49, 56, drawn to compositions comprising a bioprotein tube (i.e. bioprotein tubes, vascular tissue, three-dimensional tissue, edible food products) and methods of using said bioprotein tube compositions. [Please note that claims 40, 44, 47, 49, and 56 are product by process claims. The claim are construed as a claim to the product per se that possesses the characteristics derived from the manufacturing process stated in the claim. Therefore, the patentability of products defined by the product-by-process claims do not depend on its method of production. A product is not rendered novel merely by the fact that it is produced by means of a new process.]

The inventions listed as Groups I-IV do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

Group I requires the use of an elongate body as a solid support for forming a bioprotein tube, and deposition of a third solution comprising gelatin upon the tube, not required by Groups II-IV

Group II requires an orifice or nozzle for extruding a bioprotein precursor into a gelation solution, not required by Groups I and III-IV.

Group III requires rotating a bioprotein precursor about an axis of rotation through a gelation solution vortex having a central air gap, not required by Groups I-II and IV.

Group IV requires compositions of matter comprising a bioprotein tube, not required by the methods of Groups I-III.

Common Technical Features

The feature shared by Groups I-IV is a bioprotein tube comprising alginate that is gelled using calcium chloride.

The feature shared by Groups I-III is a method of producing a bioprotein tube comprising shaping alginate into a tubular form and gelling the alginate by contacting the alignate with a calcium chloride gelation solution. Another feature shared by Groups II and III is forming alginate into a tubular form by extrusion of alignate as an annular stream.

However, these shared technical features do not represent a contribution over prior art, because the shared technical features are taught by GB 784235 A to (Fox).

Fox discloses a bioprotein tube comprising alginate that is gelled using calcium chloride (p 1, ln 63-86).

Fox further discloses a method of producing a bioprotein tube (p 1, ln 63-64 "a process for the production of improved alginate sausage casings") comprising
shaping alginate into a tubular form by extruding the alignate as an annular stream (p 1, ln 64-68 "forming a viscous aqueous solution of a water-soluble alginate, passing said solution through an annular nozzle into a coagulating bath, whereby a tubular structure is formed"; p 3, ln 16-28 "sodium alginate was passed under pressure to an extrusion machine, where it was passed through a metering pump before being extruded through an annular orifice ... The solution extruded through the annular orifice was coagulated on entering the bath, whereby a continuous solidified tubular structure was formed"), and
gelling the alginate by contacting the alignate with a calcium chloride gelation solution (p 1, ln 68-72 "allowing the formed tubular structure to set in said coagulating bath, in which process the coagulating bath is constituted by an aqueous solution comprising a water-soluble calcium salt"; p 1, ln 85-86 "The preferred water-soluble calcium salt is calcium chloride"; p 3, ln 19- "coagulating bath having a temperature of 20 C and constituted by an aqueous solution containing 0.3% calcium chloride by weight").

Another feature shared by Groups I-III is [claim 46] a method for preparing a three-dimensional tissue, comprising embedding a bioprotein tube between two stacked polymeric tissue scaffolds comprising a plurality of nanometer dimension polymeric fibers comprising alginate to form a tissue scaffold assembly, contacting the assembly with a plurality of cells, and culturing the cells under appropriate conditions, thereby preparing a three-dimensional tissue.

-----please see next extra sheet-----

Continuation of: Box No. III Observations where unity of invention is lacking

However, this shared technical feature does not represent a contribution over prior art, because the shared technical feature is taught by US 2004/0126405 A1 to Sahatjian et al. (hereinafter 'Sahatjian').

Sahatjian discloses a method for preparing a three-dimensional tissue (para [0001] "The present invention relates generally to tissue engineering, specifically to three-dimensional scaffolding for cell and tissue culture"), comprising
embedding a bioprotein tube between two stacked polymeric tissue scaffolds comprising a plurality of nanometer dimension polymeric fibers comprising alginate to form a tissue scaffold assembly (para [0078] "a blood vessel prosthesis is made by first forming a non-woven polymer of alginate into a tube"; para [0048] "In a desirable aspect of the invention a first biodegradable polymer can be formed into a partial scaffold design followed by a second more biostable polymer to form the complete scaffold. Particularly desirable is to form a scaffold having a biostable polymer portion of the scaffold sandwiched inside two biodegradable polymer portions"; para [0057] "Non-limiting examples of tissues which can be repaired and/or reconstructed using the scaffolding described herein include ... vascular tissue"),
contacting the assembly with a plurality of cells (para [0058] "The scaffold may be used as a substrate for the growth of cells appropriate for the particular application. For example, scaffolding may be seeded with osteoblasts to repair bone defects, mesothelial cells to repair a pericardial membrane, mesothelial cells to repair the abdomen, epithelial cells to repair skin, epithelial cells to repair esophagus, and so on"; para [0078] "Endothelial cells are seeded onto the interior of the tube and smooth muscle cells are seeded onto the exterior of the tube"), and
culturing the cells under appropriate conditions, thereby preparing a three-dimensional tissue (para [0080] "a tissue modeling kit is provided, including a cell scaffold according to the invention and a plurality of viable cells from a tissue to be modeled. The viable cells are cultured in the cell scaffold"; para [0083] "forming a cell scaffold according to the invention, wherein the shape of the scaffold resembles at least a portion of a tissue to be tested, culturing cells derived from the tissue in the cell scaffold").

Groups I-IV therefore lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Item 4 (continued)

Claims 57-58 are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Note: Claim 55 contains two sentences "55. The method of claim 54, wherein the biogenic polymer is selected from the group consisting of silk, a keratin, an elastin, a fibrillin, a fibrinogen, a fibrin, a thrombin, a fibronectin, a laminin, a collagen, a vimentin, a neurofilament, an amyloid, an actin, a myosin, and a titin. In one embodiment, the polymeric fiber is a biohybrid fiber.". For purposes of this search and opinion, the biohybrid fiber is considered to be an alternative in the group of biogenic fibers.