Abstract:

While several devices and living skin substitutes have been developed over the past few years, none possess an integrated vasculature. Accordingly, the invention described herein provides, in part, controllable, three-dimensional human skin equivalents constructed using biodegradable microfluidic systems that encompass a variety of properties, including biodegradability, bioactivity and full functionality, and a complete microcirculation.
VASCULARIZED LIVING SKIN CONSTRUCTS AND METHODS OF USE THEREOF

FIELD OF THE INVENTION

[0001] The present invention relates generally to tissue engineering and uses of engineered tissues and tissue constructs.

CROSS REFERENCE TO RELATED APPLICATIONS

[0002] This application claims the benefit under 35 U.S.C. §119(e) of U.S. Provisional Patent Application Serial No: 61/120,323, filed on December 5, 2008, the contents of which are incorporated herein in their entirety by reference.

GOVERNMENT SUPPORT

[0003] This work was made with Government support under Grant No. EYOI5125, awarded by the National Institutes of Health. The Government has certain rights to the invention.

BACKGROUND OF THE INVENTION

[0004] In the United States each year, millions of individuals are burned with nearly 100,000 hospitalizations and several thousand thermal injury-associated deaths, both civilian and combat-derived. Systemic response to thermal injuries, such as burns, involves loss of skin barrier functionality and fluid loss, microbial infection, vascular derangements and interstitial edema, and overall organ dysfunction.

[0005] A burn is a type of injury that may be caused by heat, electricity, chemicals, light, radiation, or friction. Burns can be highly variable in terms of the tissue affected, the severity, and resultant complications. Muscle, bone, blood vessel, dermal and epidermal tissue can all be damaged with subsequent pain due to profound injury to nerves. Depending on the location affected and the degree of severity, a burn victim may experience a wide number of potentially fatal complications including shock, infection, electrolyte imbalance and respiratory distress. Beyond physical complications, burns can also result in severe psychological and emotional distress due to scarring and deformity.

[0006] A major problem facing clinicians treating severe burn injury is the limited availability of donor skin and the lack of success with currently available skin substitutes. Most of these treatments are suboptimal due to the likelihood of tissue rejection, the requirement for the donor skin or the skin substitute to be replaced numerous times during the healing process, and the disappointing functional and cosmetic outcomes generally obtained. Most of the
available treatments that do manage to heal the wound form a skin that does not have any of the
normal skin appendages such as hair or sweat or sebaceous glands, is usually discolored, and
forms unsightly and deforming scars that often result in reduced mobility of the affected body
part.

[0007] For most serious burn injuries, skin grafting is employed to replace the damaged
skin. Surgical removal (excision or debridement) of the damaged skin is followed by skin
grafting. The grafting serves two purposes: it can reduce the course of treatment needed (and
time in the hospital), and it can improve the function and appearance of the area of the body
which receives the skin graft. There are two types of skin grafts, the more common type is where
a thin layer is removed from a healthy part of the body (the donor section), or a full thickness
skin graft, which involves pitching and cutting skin away from the donor section. A full
thickness skin graft is more risky, in terms of the body accepting the skin.

[0008] While autologous tissue transfers can be very effective in securing wound
healing, such procedures are invasive, painful, and expensive, and cannot be performed by many
wound care practitioners. Bioengineered skin substitutes have emerged over the past two
decades, with the initial intention to replace autografts, allografts, and xenografts in burn
applications. While such skin substitutes have found application in the active treatment of
chronic venous and chronic diabetic ulcers, they have been shown in practice not to persist on
the wound, and so are not very effective as "skin equivalents."

SUMMARY OF THE INVENTION

[0009] An ideal skin equivalent would adhere quickly to a skin lesion such as a wound,
and once applied would persist, mimicking the physiology, such as the vasculature, and some of
the mechanics of normal skin. Furthermore, a skin equivalent would not be subject to immune
rejection by the host, and would be highly effective in accelerating tissue regeneration and
wound repair, rather than accelerating wound healing by secondary intent. In practical terms,
skin equivalents ideally represent artificial, off-the-shelf alternatives to skin grafts that avoid the
pain and potential complications of harvesting, are always available in any quantity needed, and
can be applied in an office setting.

[0010] Provided herein are compositions and medical devices, and methods of use
thereof, comprising sustainable, vascularized living skin equivalents comprising a silk fibroin
component and a biological component. The vascularized living skin equivalents or constructs
described herein are also referred to as "fabricated microfluidic scaffolds" bearing living cells.
In some embodiments, the vascularized living skin equivalents or constructs can further
comprise bioactive or therapeutic agents. Also provided herein are methods of using the vascularized living skin equivalents or constructs for treating wounds and/or promoting tissue regeneration. In some aspects, these vascularized living skin equivalents or constructs can promote tissue regeneration and/or wound healing when applied to open wounds that result from surgery or trauma. These vascularized living skin equivalents or constructs are transformative technological innovations with applications in would repair science, would care, and wound healing therapeutics, including the care of civilian wounds and combat casualties, and in personalized medicine. Advantages of these vascularized living skin constructs over existing skin substitutes include the provision of an existing and integrated vasculature in these constructs, which helps the constructs to persist, and the ability to seed these constructs with the subject's own cells, *i.e.*, autologous cells, which has benefits in maintaining and integrating the constructs as grafts without rejection.

Accordingly, in one aspect a composition comprising a vascularized living tissue construct is provided, where the construct fabricated silk microfluidic scaffold and at least one living cell. In another aspect, the use of a composition comprising a vascularized living tissue construct is provided, where the construct comprises a fabricated silk microfluidic scaffold and at least one living cell, in a subject in need thereof. In another aspect, the use of a composition comprising a vascularized living tissue construct is provided, where the construct comprises a fabricated silk microfluidic scaffold and at least one living cell, in the preparation of a medicament for treatment of a wound.

In another aspect, a method of treating a subject having a wound is provided, comprising administering to a subject having a wound, a composition comprising a vascularized living tissue construct, where the construct comprises a fabricated silk microfluidic scaffold and at least one living cell.

In some embodiments of these aspects and all such aspects described herein, the silk microfluidic scaffold further comprises a flat silk film to which a silk microchannel film is bonded. In some such embodiments, the silk microchannel film comprises a microchannel network. The design of the microchannel network can be varied to mimic physiologic properties of the microcirculation within the scaffold. In some embodiments, each microchannel network has at least one inlet channel and at least one outlet channel. In some embodiments, the silk microfluidic scaffold further comprises at least one bioerodible micropost within a lumen of the microchannel network. Such bioerodible microposts are used to increase the mechanical strength and properties of the microchannel network.

In some embodiments of these aspects, the constructs comprising a fabricated microfluidic scaffold and at least one living cell are implantable in a subject. In such
embodiments, implanting refers to an act of delivering a scaffold to a site within a subject and of affixing the scaffold to the site. Such implanting methods may also include minimally invasive methods such as by catheter-based technology or by needle injection. Such implanting methods may also include the use of surgical fasteners and biocompatible adhesives.

[0015] In some embodiments of these aspects, the at least one living cell is a eukaryotic cell, preferably a mammalian cell. In some such embodiments, the at least one cell is a progenitor cell, such as an ectodermal or vascular endothelial progenitor cell. In some such embodiments, the at least one living cell is a differentiated cell, such as a keratinocyte or a vascular endothelial cell. In some such embodiments, the at least one living cell expresses keratin-12 expressing cell. In other embodiments of these aspects, the at least one living cell is autologous to the subject into which the composition is being implanted. In other embodiments, the at least one living cell is an allogenic cell obtained from a donor.

[0016] In further embodiments of these aspects, the vascularized living tissue constructs may be used to promote healing of deep tissue wounds, such as puncture wounds, bullet wounds, or wounds that result from the surgical removal of a substantial amount of tissue, such as skin tissue. In some such embodiments of these aspects and all such aspects described herein, it may be advantageous for the compositions to further comprise one or more therapeutic agents. Such therapeutic agents can be introduced into the constructs by any useful method, including both static methods and/or dynamic methods. In other such embodiments, one or more therapeutic agents can be added to the construct before it is implanted in the patient. In some embodiments, a therapeutic agent can be released from the construct. The one or more therapeutic agents introduced to vascularized living tissue constructs for administration to a subject in need thereof include those agents useful in the treatment of the subject or those that would provide a therapeutic benefit to the subject, such as antimicrobial agents, growth factors, emollients, retinoids, and topical steroids.

[0017] Other aspects provide methods of fabricating a microfluidic scaffold. Such methods comprise the steps of (i) designing a microchannel network, (ii) fabricating a mold comprising the microchannel network, (iii) casting an aqueous solution on the mold to form a microchannel film, and (iv) bonding the microchannel film to a flat film to form a microfluidic scaffold.

[0018] In some embodiments of such aspects, the aqueous solution is an aqueous silk solution, which forms a silk microchannel film when cast on the mold. In some embodiments of the aspect, the flat film is a flat silk film. In some embodiments, increased mechanical stability can be provided to the microchannel network by the introduction of at least one bioerodible micropost inserted in a lumen of the microchannel network. In such embodiments, the at least
one bioerodible micropost can be introduced prior to the bonding of the flat film to the microchannel film. In some embodiments, a bioerodible micropost can be comprised of silk.

[0019] In other embodiments of this aspect, the method further comprises adding at least one living cell to the microfluidic scaffold. The living cell can be added to the scaffold through a variety of means, including, but not limited to, directly seeding the at least one living cell onto the microfluidic scaffold, or introducing the at least one living cell through a fluid flowing through the microchannel network. In such embodiments, the at least one living cell can be a progenitor cell, such as an ectodermal or vascular endothelial progenitor cell, or a differentiated cell, such as a keratinocyte or a vascular endothelial cell. In some embodiments, the microchannel network comprises or is lined with at least one vascular endothelial progenitor cell or vascular endothelial cell line. More than one type of cell can be simultaneously or successively introduced to the constructs described herein.

[0020] Other aspects described herein provide wound closure kits or systems comprising one or more vascularized living tissue constructs used for wound closure therapies by allowing growth of neodermal tissue around a vascularized living tissue construct. In some embodiments of such aspects, the one or more vascularized living tissue constructs comprise a silk microfluidic scaffold and one or more cells. In some such embodiments, the wound closure kits or systems include additional reagent(s), and/or ingredient(s), and/or one or more containers, for performing any methods of the invention.

[0021] Other aspects of the invention described herein provide vascularized living tissue constructs for use in high-throughput drug screening. In some such aspects, the vascularized living skin constructs are used in methods and assays to determine pharmacokinetic parameters and toxicity of drugs. In some such aspects, a multiplex array for drug screening is provided comprising at least two vascularized living tissue constructs, where each vascularized living tissue construct comprises a silk microfluidic scaffold and at least one living cell. In some embodiments, such multiplex vascularized living tissue construct arrays can be used for screening of different drugs on the same type of tissue, e.g., skin, and in other embodiments such multiplex vascularized living tissue construct arrays can be used for screening of the same drugs on the a variety of tissue types, e.g., skin, cornea, cancer tissue. In other embodiments of such aspects, the multiplex vascularized living tissue construct arrays can comprise constructs comprising cancerous or malignant cells, such as cells derived or obtained from basal cell cancers, squamous cell cancers, and melanomas, to test the effects of different drugs on, for example, the cellular proliferation of these cancers in a 3-dimensional context.

Definitions
Unless stated otherwise, the following terms and phrases have the meanings provided below:

As defined herein, a "tissue" refers to an aggregation of similarly specialized cells united in the performance of a particular function. As defined herein, "skin" refers to the outer integument or covering of the body, consisting of the dermis and the epidermis and resting upon the subcutaneous tissues.

As defined herein, a "lesion" refers to any abnormal tissue found on or in an organism, usually damaged by disease or trauma. Lesions are caused by any process that damages tissues. Trauma, including thermal burns, electrocution, and chemical burns can also cause lesions. An additional classification of lesions is based on whether or not a lesion occupies space. A "space occupying lesion," as defined herein, occupies space and may impinge on nearby structures, whereas a "non-space occupying lesion" is simply a hole in the tissue, e.g. a small area of the brain that has turned to fluid following a stroke, a gap in the skin caused by injury, such as burns. As defined herein, "wound" refers to a lesion caused by an injury or damage, usually restricted to those with disruption of the normal continuity of structures, such as the epidermal or dermal layers of the skin, any damage to the epithelial layer of a tissue, such as a corneal epithelial layer. A wound can also be referred to as an "injury" or "trauma." As defined herein, the phrase "full-thickness skin wound" refers to a skin wound with the loss or penetration of epidermis, and all of the dermis or at least the depth of dermis that includes most or all sources of epidermal cells from epidermal adnexae (glands and follicles). An "open wound", as defined herein, refers to a wound that communicates with the atmosphere by direct exposure. As defined herein, the phrase a "clean surgical skin wound" refers to a full or partial thickness skin wound that is created by surgical excision or incision and that is free of necrotic tissue, without significant bleeding, and without significant microbial contamination.

As defined herein, "wound inflammation" refers to a localized protective response elicited by injury or destruction of tissues, which serves to destroy, dilute, or wall off (sequester) both the injurious agent and the injured tissue. It is characterized in the acute form by the classical signs of pain (dolor), heat (calor) redness (rubor), swelling (tumor), and loss of function (functio laesa). Histologically, it involves a complex series of events, including dilation of arterioles, capillaries, and venules, with increased permeability and blood flow; exudation of fluids, including plasma proteins; and leukocytic migration into the inflammatory focus.

As defined herein, "wound contraction" refers to the physiological phenomenon whereby an open skin wound undergoes shrinkage and spontaneous closure. As defined herein, "wound closure" refers to the provision of an epithelial cover over a wound. It can be accomplished by approximating wound edges, performing a skin (auto)graft, or allowing...
spontaneous healing from the edges. As defined herein, a "scar" refers to the fibrous tissue replacing normal tissues destroyed by injury or disease.

[0027] As defined herein, "tissue regeneration" refers to healing of a tissue whereby lost tissue is replaced by proliferation of cells, which reconstruct the normal architecture. Tissue regeneration can be used, for example, to heal a wound caused by trauma or injury.

[0028] As defined herein, "skin replacement surgery" refers to surgery or surgical procedures that permanently replaces lost skin with healthy skin or a skin equivalent that may be natural, synthetic, or a combination thereof.

[0029] As defined herein, "graft" refers to any tissue or organ for implantation or transplantation. As defined herein, an "autograft" is a graft of tissue derived from another site in or on the body of the organism receiving it. As defined herein, a "full thickness skin autograft" refers to a skin autograft consisting of the epidermis and the full thickness of the dermis. As defined herein, a "split thickness skin autograft" refers to a skin autograft consisting of the epidermis and a portion of the dermis. As defined herein, an "epidermal autograft" refers to an autograft consisting primarily of epidermal tissue, including keratinocyte stem cells, but with little dermal tissue.

[0030] As defined herein, "engraftment" refers to incorporation of grafted tissue into the body of the host. As defined herein, "dermal tissue engraftment" refers to engraftment of dermal tissue resulting in reestablishment of vascular connections with cellular and extracellular matrix remodeling in the dermis. As defined herein, "epidermal tissue engraftment" refers to engraftment of an epidermal autograft by a process of epidermal tissue regeneration resulting in a confluent epidermis and permanent wound closure.

[0031] As used herein the term "comprising" or "comprises" is used in reference to compositions, methods, and respective component(s) thereof, that are essential to the invention, yet open to the inclusion of unspecified elements, whether essential or not. As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment of the invention. The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

[0032] As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural references unless the context clearly dictates otherwise. Thus for example, references to "the method" includes one or more methods, and/or steps of the type described herein and/or which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.
[0033] All patents and other publications identified are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the present invention. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

[0034] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as those commonly understood to one of ordinary skill in the art to which this invention pertains. Although any known methods, devices, and materials may be used in the practice or testing of the invention, the methods, devices, and materials in this regard are described herein.

**BRIEF DESCRIPTION OF THE DRAWINGS**

[0035] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate embodiments of the invention and, together with the description, serve to explain the objects, advantages, and principles of the invention.

[0036] **Figure 1** depicts a fabrication process of a silk microchannel film.

[0037] **Figure 2** depicts silk microfluidic scaffolds in an array for drug discovery and screening purposes.

[0038] **Figure 3** shows various representations of silk microfluidic networks. Figure 3A shows a diagrammatic representation of a bifurcated network showing bioerodible microposts positioned to insure patency. Figure 3B displays the visualization of the patent micromolded, bonded silk network. Figure 3C shows a scanning electron microscope image of the silk microfluidic channel and bioerodible microposts.

[0039] **Figure 4** shows the creation of ectodermally-derived progenitor populations. Figures 4A-4D show 2-D culture of human embryonic stem cells (hESC). Figure 4E show 3-D culture of epithelial/dermal construct (H/E-stained section). In brief, directed differentiation from hESC generated ectodermal subpopulations enriched in keratin 18-expressing cells (Figure 4C) underwent further differentiation and enrichment in keratin 12-expressing cells (Figure 4D). When these cells were grown at an air liquid interface on a collagen gel harboring hESC-derived mesenchymal cells, a multilayer epithelium is formed (Figure 4E), which can then be integrated into vascularized living skin equivalents.
DETAILED DESCRIPTION OF THE INVENTION

While several devices and living skin substitutes have been developed over the past few years, none possess an integrated vasculature. Ideally, a skin equivalent would have such properties as the ability to adhere quickly to a skin lesion such as a wound, and once applied, persist, and mimic the physiology, such as the vasculature, and some of the mechanics of normal skin. Furthermore, a skin equivalent would not be subject to immune rejection by the host, and would be highly effective in accelerating tissue regeneration and wound repair rather than accelerating wound healing by secondary intent. Accordingly, the invention described herein provides, in part, controllable, three-dimensional human skin equivalents constructed using biodegradable microfluidic systems that encompass a variety of properties, including biodegradability, bioactivity and full functionality, as well as a complete, pre-existing microcirculation.

The compositions described herein are capable of sustaining the living organ equivalent ex vivo and enable surgical integration in patients. In addition, this technology provides solutions for high-throughput drug discovery and screening in vitro. The ability to deliver this new class of autologous, vascularized living skin equivalents or constructs provides a long-lasting impact on regenerative medicine, such as for those subjects suffering with traumatic-injury-induced or thermally-induced wounds.

Fabrication of Vascularized Living Skin Equivalents or Constructs

Design of MicroChannel Networks. MicroChannel network designs can be designed and formulated to meet certain criteria, such as specific ranges of wall shear stress, pressure, flow, and other critical fluid mechanical properties. The design of a microchannel network for use in the vascularized living skin equivalent compositions and constructs described herein can be varied in a controlled manner, both temporally and spatially, in order to mimic physiologic properties of the microcirculation within the vascularized living skin equivalent construct. In some embodiments, each microchannel network design has at least one inlet...
channel and at least one outlet channel, where the inlet channel branches at least once to form, for example, a branching network geometry and is a channel that allows transport of fluid into the scaffold, and the outlet channel is a channel forming from the convergence of one or more microchannels, and is a channel that allows the transport of fluid away from the scaffold.

[0046] As used herein, a "microchannel" refers to a channel of a size and/or dimension that is small enough such that the properties of fluids that flow through the lumen of the channel follow "microfluidic behavior." As defined herein, the "lumen" refers to the inside space of a tubular structure, such as a microchannel. As defined herein, "microfluidics" refers to the behavior, precise control and manipulation of fluids that are geometrically constrained to a small, typically sub-millimeter, scale. Typically, micro means one of the following features: small volumes (nl, pl, fl), small size, low energy consumption, and effects of the micro domain. The behavior of fluids at the microscale can differ from 'macrofluidic' behavior in that factors such as surface tension, energy dissipation, and fluidic resistance start to dominate the system. At small scales (channel diameters of around 100 nanometers to several hundred micrometers) different properties of fluid dynamics appear. In particular, the Reynolds number (which compares the effect of momentum of a fluid to the effect of viscosity) can become very low. A key consequence of this is that fluids, when side-by-side, do not necessarily mix in the traditional sense; molecular transport between them must often be through diffusion. This property is important in many microfluidic devices. Fluid properties that can be modified when a fluid flows through a microchannel network or through a microfluidic device include, but are not limited to, laminar flow, surface tension, electrowetting, fast thermal relaxation, electrical surface charges, and diffusion properties.

[0047] A microfluidic device can be identified by the fact that it has at least one channel with at least one dimension of the lumen less than 1 mm, preferably less than 750 µm, less than 500 µm, less than 250 µm, less than 100 µm, less than 90 µm, less than 80 µm, less than 70 µm, less than 60 µm, less than 50 µm, less than 40 µm, less than 30 µm, less than 20 µm, less than 19 µm, less than 18 µm, less than 17 µm, less than 16 µm, less than 15 µm, less than 14 µm, less than 13 µm, less than 12 µm, less than 11 µm, less than 10 µm, less than 9 µm, less than 8 µm, less than 7 µm, less than 6 µm, or less than 5 µm, termed herein as a "microchannel." In some embodiments, a microchannel can be the size of a human capillary, which ranges from about 5-10 µm. Common fluids used in microfluidic devices or constructs include biological fluids, such as whole blood, serum, plasma, platelet samples, urine, cerebrospinal fluids, saliva, protein or antibody solutions and various buffers. A "microchannel network" as described herein has at least one inlet and at least one outlet microchannel permitting the flow of fluids through the network. Such a network will generally comprise a plurality of branches stemming from an inlet
and a plurality of branches or joints that coalesce to join the outlet. In this sense, the microchannel network mimics a naturally occurring capillary bed in structure and function.

[0048] The flow of fluids, such as biological fluids, through a microchannel or microchannel network can be actuated by external pressure sources, external mechanical pumps, integrated mechanical micropumps, or by combinations of capillary forces and electrokinetic mechanisms.

[0049] Mold Fabrication. In some embodiments of the constructs described herein, standard photolithography is used to create a "master mold," such as an SU-8/silicon silicon master mold. As defined herein, "optical lithography" or "photolithography," is a process used in the fabrication of miniature structures, termed herein as "microfabrication," to selectively remove parts of a thin film or the bulk of a substrate, such as silicon, quartz, glass, or other such substrates. Photolithography uses light to transfer a geometric pattern from a photo mask to a light-sensitive chemical photo resist, or termed simply "resist," onto the substrate, such as a silicon wafer. A series of chemical treatments is used to engrave the exposure pattern, such as a microchannel network, into the material underneath the photo resist. Main advantages of using photolithography in fabricating the molds include the ability to have exact control over the shape and size of the objects created, and because photolithography can create patterns over an entire surface simultaneously. Typically, photolithography utilizes ultraviolet light generated by gas-discharge lamps using mercury, sometimes in combination with noble gases such as xenon. Other light sources for use in photolithography include "deep ultraviolet", produced by excimer lasers, using, for example, krypton fluoride and argon fluoride.

[0050] Having made a master mold for use in fabricating the vascularized living skin equivalents or constructs described herein, techniques such as "soft lithography" are then used to create an inverse replica, or "negative" mold, for example, using Polydimethyl Siloxane (PDMS). As defined herein, "soft lithography" includes the technologies of micro-contact printing (µCP), replica molding (REM), microtransfer molding (µTM), micromolding in capillaries (MIMIC), solvent-assisted micromolding (SAMIM), and patterning by etching at the nanoscale (PENs). Advantages of soft lithography for use in the various embodiments of the invention include lower cost than traditional photolithography for mass production of the negative molds to be used herein, suitability for applications in biotechnology, greater pattern-transferring methods than traditional lithography techniques, and the lack of requirement for a photo-reactive surface.

[0051] In some embodiments of the aspects described herein, a degassed resin, such as polydimethyl piloxane (PDMS) or fluorosilicone, is poured atop the silicon master mold, creating a "negative mold" or "PDMS stamp." In some embodiments, the negative mold can
also be attached to an external source by tubing, so that a liquid, such as an aqueous silk fibroin solution, may be passed through the channels on its surface. In some embodiments, the negative mold is laminated to a separate surface.

Silk Casting. Methods for preparation of concentrated aqueous silk fibroin solutions for use in fabricating silk microchannel films of the vascularized living skin equivalents constructs described herein are discussed below.

As used herein, the term "fibroin" includes silkworm fibroin and insect or spider silk protein (Lucas et al., Adv. Protein Chem 13: 107-242 (1958)). Preferably, fibroin is obtained from a solution containing a dissolved silkworm silk or spider silk. The silkworm silk protein can be obtained, for example, from Bombyx mori, and the spider silk can be obtained from Nephila clavipes. In other embodiments, silk proteins suitable for use in the vascularized living skin equivalents of the present invention can be obtained from a solution containing a genetically engineered silk, such as from bacteria, yeast, mammalian cells, transgenic animals or transgenic plants. See, for example,WO 97/08315 and US Patent 5,245,012.

The silk fibroin solution to be concentrated can be prepared by any conventional method known to one skilled in the art, for example, any of the methods described in WO 2004/000915, WO 2004/062697, WO 2005/012606, WO 2005/000483, WO 2005/123114,WO 2006/042287, WO 2006/076711, US 2007/0212730, WO 2007/016524, WO 2008/118133, WO 2008/127404, WO 2008/118211, WO 2008/127401, WO 2008/127402, WO 2008/127403, WO 2008/127405, WO 2008/140562, WO 2008/106485, WO 2008/150861, WO 2009/023615, and WO 2009/061823. For example, in some embodiments, B. mori cocoons are boiled for about 20-30 minutes in an aqueous solution. In some embodiments, the aqueous solution is about 0.02M Na₂CO₃. The cocoons are rinsed, for example, with water, to extract the sericin proteins and wax. The extracted silk fibroin is then dissolved in an aqueous salt solution. Salts useful for this purpose include lithium bromide, lithium thiocyanate, calcium nitrate or other chemicals capable of solubilizing silk. In some embodiments, the extracted silk is dissolved in about 9-12 M LiBr solution. In some embodiments, the solution is about 9.3M LiBr solution. The salt is consequently removed using, for example, dialysis. In some embodiments, the silk fibroin solution is dialyzed against distilled water, using, for example, a dialysis cassette. In some embodiments, the aqueous silk fibroin solution is prepared in the absence of organic solvents or harsh chemicals.

In some embodiments, the aqueous silk fibroin solution is then concentrated using, for example, dialysis against a hygroscopic polymer, for example, PEG, a polyethylene oxide, amylose or sericin. In some embodiments, the PEG is of a molecular weight of 8,000-10,000 g/mol and has a concentration of 25 - 50%. In some embodiments, a slide-a-lyzer
dialysis cassette (Pierce, MW CO 3500) can be used for the dialysis. However, any dialysis system can be used. The dialysis is for a time period sufficient to result in a final concentration of aqueous silk solution between 10 - 30%. In most cases, dialysis for 2 - 12 hours is sufficient. In some embodiments, the final concentration of aqueous silk solution is 10-13%.

As an example, in some embodiments of the invention, silkworm cocoons are processed to aqueous silk fibroin solutions by sericin protein extraction in Na₂CO₃, dissolved in LiBr, and dialyzed against, for example, H₂O. The aqueous silk fibroin solutions are then concentrated to a 10-13% solution by reverse dialysis.

The concentrated aqueous silk solution can be processed into hydrogels, foams, films, threads, fibers, meshes, and scaffolds using processes known in the art. See, e.g., Altman, et al., *Biomaterials* 24:401, 2003.

Aqueous silk fibroin solutions can be formed easily into mechanically robust films of thermodynamically-stable beta-sheets, with control of thicknesses from a few nanometers to hundreds of micrometers or more. Such films, termed herein as "silk fibroin films" or "silk films," can be formed by casting of purified aqueous silk fibroin solution which crystallizes upon exposure to air, humidity or dry nitrogen gas, as some examples, without the need for exogenous crosslinking reactions or post processing crosslinking for stabilization. A silk film that is cast in the absence of a mold, such that the silk film has no functional or embedded attributes on either face of the silk film is termed herein as a "flat silk film."

In some embodiments, in order to provide functional features to the silk film, the silk film is formed by casting an aqueous silk fibroin solution in a mold. For example, in some embodiments, an aqueous silk fibroin solution is cast onto a mold, such as a "negative mold," having a desired design or pattern, and dried. In some embodiments, the negative mold is a PDMS mold. In some embodiments, the desired design is a microchannel network design. In some embodiments, after casting the aqueous silk fibroin solution is then allowed to crystallize in free air at ambient temperature and pressure. In most cases, under these settings, dry silk films are produced after approximately 16 hours. In other embodiments, alternative post-processing techniques can be used to shorten the time necessary for beta-sheet film formation. Such alternative post-processing techniques include water vapor annealing or exposure to methanol. For example, in some embodiments, the cast silk is delaminated and treated with a 50% methanol solution for 4 hours to produce water-stable "silk microchannel films. A silk film that has been cast on a mold having a microchannel network design is termed herein as a "silk microchannel film."

In some embodiments, one or more biocompatible polymers are added to the aqueous silk fibroin solution to generate composite matrices. Non-limiting examples of
biocompatible polymers useful in the present invention include, for example, polyethylene oxide (PEO) (US 6,302,848), polyethylene glycol (PEG) (US 6,395,734), collagen (US 6,127,143), fibronectin (US 5,263,992), keratin (US 6,379,690), polyaspartic acid (US 5,015,476), polylysine (US 4,806,355), alginate (US 6,372,244), chitosan (US 6,310,188), chitin (US 5,093,489), hyaluronic acid (US 387,413), pectin (US 6,325,810), polycaprolactone (US 6,337,198), polylactic acid (US 6,267,776), polyglycolic acid (US 5,576,881), polyhydroxyalkanoates (US 6,245,537), dextrans (US 5,902,800), and polyanhydrides (US 5,270,419). In some embodiments, two or more biocompatible polymers can be used. In some embodiments, the silk film comprises from about 50 to about 99.99 part by volume aqueous silk protein solution, and from about 0.01 to about 50 part by volume of a biocompatible polymer, e.g., polyethylene oxide (PEO). Preferably, the resulting silk blend film is from about 60 to about 240 µm thick, however, thicker samples can easily be formed by using larger volumes or by depositing multiple layers.

[0061] As used herein, the term "porous" or "porosity" refers to the property of a silk film, such as a silk microchannel film described herein, to permit the passage of materials, for example, from the lumen of a microchannel, into and through the silk film, and vice versa. The silk films described herein can be designed to encompass a range of porosities, from those that do not substantially permit the passage of cells or proteins, to those that substantially permit the passage of proteins, but not cells, to those that permit the passage of both. The porosity of a silk microchannel film or a silk microfluidic scaffold as described herein can be expressed in terms of a measured/calculated permeability coefficient.

[0062] Methods to modulate the porosity of a silk film are known to those of skill in the art, and can comprise the use of the addition of granular salts to silk solutions to vary the degree of porosity in a silk film. As one non-limiting example, granular sodium chloride (for example, crystal sizes from 150 to 700 µm) can be used to increase porosity of a silk film. For example, an aqueous silk solution can be lyophilized and dissolved in hexafluoro-2-propanol to generate a non-aqueous silk solution. Such a non-aqueous silk solution can be about 15%. This non-aqueous silk solution can be poured into a mold containing leveled granular sodium chloride, and then placed covered at 4°C for the amount of time necessary for the fibroin solution to diffuse around the salt crystals, which can be about 30 minutes. The weight ratio of salt to fibroin used can be about 2 to 1. The hexafluoro-2-propanol can be allowed to evaporate for about for 24 hours, and the salt/silk composite is immersed in methanol. This immersion induces the structural transition to a β-sheet, ensuring the films are insoluble in aqueous solutions. After removing the methanol, the films can be immersed in water several times for at least a day to extract residual salt. Such treatments can be used to vary the porosity of the silk
In some embodiments, the silk films or aqueous silk solutions can further comprise one or more therapeutic or bioactive agents. In some embodiments, the silk solution is mixed with one or more therapeutic agents prior to forming the material, such as a silk film. In other embodiments, one or more therapeutic agents are loaded into the silk material after it is formed into a specific structure, for example, a silk film for use in a vascularized living skin equivalent. The variety of different therapeutic agents that can be used in conjunction with the silk films or aqueous silk solutions is vast, and includes small molecules, proteins, peptides and nucleic acids. In general, therapeutic agents that may be administered via the silk films or aqueous silk solutions of the invention include, without limitation: anti-infectives, such as antibiotics and antiviral agents; chemotherapeutic agents (i.e. anticancer agents); anti-rejection agents; analgesics and analgesic combinations; anti-inflammatory agents; hormones such as steroids; growth factors (bone morphogenetic proteins (i.e. BMP's 1-7), bone morphogenic-like proteins (i.e. GFD-5, GFD-7 and GFD-8), epidermal growth factor (EGF), fibroblast growth factor (i.e. FGF 1-9), platelet derived growth factor (PDGF), insulin like growth factor (IGF-I and IGF-II), transforming growth factors (i.e. TGF-β-III), vascular endothelial growth factor (VEGF)); anti-angiogenic proteins such as endostatin, and other naturally derived or genetically engineered proteins, polysaccharides, glycoproteins, or lipoproteins. Additionally, the silk films or aqueous silk solutions of the present invention can be used as a delivery system to deliver any type of molecular compound, including, but not limited, pharmacological materials, vitamins, sedatives, steroids, hypnotics, antibiotics, chemotherapeutic agents, prostaglandins, radiopharmaceuticals, proteins, peptides, nucleotides, carbohydrates, simple sugars, cells, genes, anti-thrombotics, anti-metabolics, growth factor inhibitor, growth promoters, anti-coagulants, anti-mitotics, fibrinolytics, anti-inflammatory steroids, and monoclonal antibodies.

Silk films or aqueous silk solutions containing therapeutic or bioactive agents can be formulated by mixing one or more therapeutic agents with the silk films or aqueous silk solutions. Alternatively, in some embodiments one or more therapeutic agents could be coated onto the material, such as a silk film, preferably with a pharmaceutically acceptable carrier. Any pharmaceutical carrier can be used that does not dissolve the silk films or aqueous silk solutions. The therapeutic agents, may be present as a liquid, a finely divided solid, or any other appropriate physical form.
The silk materials described herein can be further modified after fabrication of the silk molds. For example, after casting the silk fibroin solution in the negative PDMS molds, silk films, such as silk microchannel films, can be coated with additives, such as bioactive substances that function as receptors or chemoattractors for a desired population of cells, such as the cells to be integrated into the silk microchannel films to form vascularized living skin equivalents or constructs. Such coatings can be applied through, for example, absorption or chemical bonding.

Additives suitable for use with the constructs described herein include biologically or pharmacologically active compounds. Examples of biologically active compounds include, but are not limited to: cell attachment mediators, such as collagen, elastin, fibronectin, vitronectin, laminin, proteoglycans, or peptides containing known integrin binding domains e.g. "RGD" integrin binding sequence, or variations thereof, that are known to affect cellular attachment (Schaffner P & Dard 2003 Cell Mol Life Sci. Jan;60(1): 119-32; Hersel U. et al. 2003 Biomaterials. Nov;24(24):4385-415); biologically active ligands; and substances that enhance or exclude particular varieties of cellular or tissue ingrowth. For example, the steps of cellular population of a 3-dimensional scaffold matrix preferably are conducted in the presence of growth factors effective to promote proliferation of the cultured cells employed to populate the matrix. Agents that promote proliferation will be dependent on the cell type employed for use in the living skin equivalents or constructs. For example, when fibroblast cells are employed, a growth factor for use herein may be fibroblast growth factor (FGF), most preferably basic fibroblast growth factor (bFGF) (Human Recombinant bFGF, UPSTATE Biotechnology, Inc.). Other examples of additive agents that enhance proliferation or differentiation include, but are not limited to, osteoinductive substances, such as bone morphogenetic proteins (BMP); cytokines, growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF-I and II) TGF-β, and the like. As used herein, the term additive also encompasses antibodies, DNA, RNA, modified RNA/protein composites, glycogens or other sugars, and alcohols.

Silk Bonding. The silk microchannel films described herein can be combined with other silk-based structures to form various 3-dimensional structures, such as silk microfluidic scaffolds, silk scaffolds, silk sponges, or other silk composite structures, for applications such as living skin equivalents or other biomedical devices. In some embodiments, glycerol can be easily blended with any silk composite to alter the mechanical properties of the silk-based structure.

For example, in some embodiments, microchannel silk films are bonded to flat silk films to form a "silk microfluidic scaffold," such that the face of the microchannel silk film
on which the microchannel network is present is bonded to the flat silk film to form functional and defined microchannels through which fluids, such as biological fluids, can flow. In some embodiments, flexible tip needles are inserted into the microchannel inlet and outlet to provide a means of introducing fluids into the microchannel network of a silk microfluidic scaffold. In some embodiments, a silk microchannel film and a flat silk film are bonded together using an aqueous silk solution. In some embodiments, the aqueous silk solution used for bonding is about 8%. In some embodiments, a silk microchannel film and flat silk film are first visually aligned prior to the bonding step. For example, in some embodiments, a silk microchannel film and flat silk film are visually aligned and bonded together using an 8% aqueous solution. Usually, such bonding can occur at 70°C, though any temperature suitable for bonding the two films can be used. In some embodiments, mechanical pressure is also applied to enhance the bonding of the two silk films.

In some embodiments, the microchannel network of the silk microfluidic scaffold further comprises one or more bioerodible posts. The term "bioerodible" as used herein is synonymous with the term "biodegradable." These terms denote the property of an object, such as a post, to undergo degradation, erosion and solubilization as a result of hydrolysis of labile linkages at the physiologic conditions of use. Accordingly, "bioerodible posts," as used herein refer to any structure that are placed within the microchannel network of silk microfluidic scaffold to provide additional mechanical support. A post can, for example, be of a cylindrical, polygonal, or columnar shape. The shape per se of the bioerodible posts is not as important as the ability of such a bioerodible post to provide the necessary increased mechanical stability within the microchannel network to permit fluids to flow through, without substantially impeding the flow of such fluids through the microchannel network.

Such posts can be placed in the microchannel network to provide increased mechanical stability within the network. In some embodiments, one or more bioerodible posts are placed in the lumen of a microchannel. In some embodiments, the increase in mechanical stability is at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 100% greater than the mechanical stability of the microchannel network in the absence of such bioerodible posts. The suitability of the placement of such bioerodible posts in the microchannel network can be determined by techniques known to one of skill in the art. For example, the placement of the bioerodible posts can be based on the substantial absence of any effects, such as increased eddying effects, on fluid flowing through the microchannel network.

Cellularization of Silk Microfluidic Scaffolds
The silk microfluidic scaffolds described herein can be used to create compositions comprising organized vascularized living tissue equivalents with a predetermined form and structure, either in vitro, ex vivo, or in vivo. For example, in some embodiments, a vascularized living tissue equivalent or construct is produced from a silk microfluidic scaffold ex vivo that is functional from the start and can be used as an in vivo implant. Alternatively, the silk microfluidic scaffold can be seeded with cells capable of forming a desired tissue, or organ, such as the skin, and then implanted as a vascularized living tissue equivalent or construct to promote growth in vivo. Thus, the scaffolds can be designed to form tissue with a "customized fit" that is specifically designed for implantation in a particular patient, i.e., through the use of autologous cells. For example, the vascularized living skin equivalents or constructs described herein can be used as skin grafts in subjects in need, such as burn victims. In some embodiments, the silk mirofluidic scaffolds can be cellularized with dissociated cells, e.g., keratinocytes, mesenchymal cells, chondrocytes, or hepatocytes, to create a three-dimensional tissue or organ. It should be understood that in the context of the constructs described herein, a fabricated microfluidic scaffold "comprising" a living cell has the cell attached to or resident on the scaffold including, but not limited to, attachment or residence within the lumen of a microchannel. Thus, to the extent that blood cells or other cells in the circulation pass through the microfluidic scaffold those cells are not "comprised" by the tissue constructs described herein.

Further, it is to be understood that as used herein, "vascularized" is meant that a tissue construct comprises a microfluidic network of passages or channels. The passages or channels preferably comprise, are lined with, or otherwise include vascular endothelial cells or cells capable of giving rise through a proliferation and differentiation program to such vascular endothelial cells.

In some embodiments, the silk microfluidic scaffolds are cellularized with multilayer populations of cells, such as, for example, a multilayer epithelium. In some embodiments, the microchannel network of the silk microfluidic scaffold is cellularized with endothelial cells, to form "endothelialized microchannels." Vascularized living tissue equivalents or constructs provided by the present invention can be used in any species.

As defined herein, the term "vascularized living tissue equivalent" or "vascularized living tissue construct" refers to a composition or device comprising a silk microfluidic scaffold and at least one living cell, preferably a eukaryotic cell, more preferably a mammalian cell. The living cell of such compositions and devices can be found within the microchannel network of the silk microfluidic scaffold, such as an endothelial cell that lines a microchannel, or around, above, within, or below, the silk microfluidic scaffold, such as cells of
a differentiated, stratified epithelial multilayer that are formed above the silk microfruidic scaffold as a consequence of epithelial progenitor cells proliferating within the scaffold.

[0075] A number of different cell types or combinations thereof may be employed in the present invention, depending upon the intended function of the vascularized living tissue equivalent being produced. These cell types include, but are not limited to: keratinocytes, fibroblasts, mesenchymal cells, smooth muscle cells, skeletal muscle cells, cardiac muscle cells, epithelial cells, endothelial cells, urothelial cells, myoblasts, chondrocytes, chondroblasts, osteoblasts, osteoclasts, hepatocytes, bile duct cells, pancreatic islet cells, thyroid, parathyroid, adrenal, hypothalamic, pituitary, ovarian, testicular, salivary gland cells, adipocytes, and precursor cells. For example, mesenchymal cells may be employed to form vascularized living skin equivalents; smooth muscle cells and endothelial cells may be employed for muscular, tubular vascularized living tissue equivalents, e.g., constructs intended as vascular, esophageal, intestinal, rectal, or ureteral constructs; chondrocytes may be employed in cartilaginous vascularized living tissue equivalents; cardiac muscle cells may be employed in heart vascularized living tissue equivalents; hepatocytes and bile duct cells may be employed in liver vascularized living tissue equivalents; epithelial, endothelial, fibroblast, and nerve cells may be employed in constructs intended to function as replacements or enhancements for any of the wide variety of tissue types that contain these cells. In general, any cells may be employed that are found in the natural tissue to which the vascularized living tissue equivalent or construct is intended to correspond. In addition, progenitor cells, such as human embryonic stem cells or myoblasts, may be employed to produce their corresponding differentiated cell types. In some instances it may be preferred to use neonatal cells or tumor cells.

[0076] The cells that are used for in the various aspects of the present invention should be derived from a source that is compatible with the intended recipient or subject. Cells can be obtained from donors (allogenic) or from recipients (autologous). Cells can also be of established cell culture lines, or even cells that have undergone genetic engineering. Pieces of tissue can also be used, which may provide a number of different cell types in the same structure, such as cells obtained from biopsies, or microdissection techniques. The cells are obtained from any suitable donor, either human or animal, or from the subject into which they are to be implanted. As used herein, the term "host" or "subject" includes mammalian species, including, but not limited to, humans, monkeys, dogs, cows, horses, pigs, sheep, goats, cats, mice, rabbits, rats.

[0077] The cells can be dissociated using standard techniques and seeded onto and into the microfruidic silk scaffold. In vitro culturing optionally may be performed prior to implantation. Alternatively, the microfruidic silk scaffold is implanted into the subject and
allowed to vascularize, via endothelialization of the scaffold. One or more cells can be injected into the silk microfluidic scaffold after implantation if so desired. Methods and reagents for culturing cells in vitro and implantation of a tissue scaffold are known to those skilled in the art. [0078] In other aspects, various means are provided to introduce cells into the silk microfluidic scaffolds to form the vascularized living tissue equivalents or constructs described herein. In some embodiments, cells are seeded directly onto the silk microfluidic scaffolds. For example, cells can be differentiated into an appropriate cell type in vitro or ex vivo and the differentiated cells integrated into a silk microfluidic scaffold to form a vascularized living tissue equivalent or construct. In other embodiments, cells can be introduced through the microchannel network of the silk microfluidic scaffold, for example, through an inlet channel. [0079] For example, in some embodiments, multipotent cells such as human embryonic stem cells (hESC) can be used as a source of epithelial and mesenchymal tissues for the construction of vascularized living tissue, e.g., skin, equivalents or constructs. Directed differentiation of ectodermal subpopulations enriched in keratin 18-expressing cells can be generated from hESC. Such cells can then undergo further differentiation and enrichment into keratin 12-expressing cells. When such cells are grown at an air liquid interface on a collagen gel harboring hESC-derived mesenchymal cells, a multilayer epithelium is formed, which can then be integrated into the silk microfluidic scaffolds to form a vascularized living skin equivalent.

[0080] In some embodiments, the microfluidic silk scaffold described herein can itself be implanted in vivo and serve as tissue substitute (e.g. to substitute for skin). Such implants, would require no seeding of cells, but contain an addition e.g., RGD, that attracts cells.

[0081] As used herein, the term "totipotent cells" refers to cells have the potential to become any cell type in an adult body; any cell type(s) of the extraembryonic membranes (e.g., placenta). Totipotent cells include the fertilized egg and approximately the first 4 cells produced by its cleavage.

[0082] As used herein, the term "pluripotent stem cells" refer to stem cells with the potential to make any differentiated cell in the body, but which cannot contribute to making the components of the extraembryonic membranes which are derived from the trophoblast. Different types of pluripotent stem cells include Embryonic Stem Cells (ESCs) (may also be totipotent in primates), Embryonic Germ Cells (EGCs), and Embryonic Carcinoma Cells (ECCs). In some embodiments, human ESCs are used. The term "pluripotent" as used herein refers to a cell with the capacity, under different conditions, to differentiate to more than one differentiated cell type, and preferably to differentiate to cell types characteristic of all three germ cell layers. Pluripotent cells are characterized primarily by their ability to differentiate to more than one cell type,
preferably to all three germ layers, using, for example, a nude mouse teratoma formation assay. Pluripotency is also evidenced by the expression of embryonic stem (ES) cell markers, although the preferred test for pluripotency is the demonstration of the capacity to differentiate into cells of each of the three germ layers.

[0083] 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.

[0084] 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, and it is essential as used in this document. 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" by persons of ordinary skill in the art.
In the context of cell ontogeny, the adjective "differentiated ", or "differentiating" is a relative term meaning a "differentiated cell" is a cell that has progressed further down the developmental pathway than the cell it is being compared with. Thus, a stemcell, as this term is defined herein can differentiate to lineage-restricted precursor cells (such as a mesodermal stem cell), which in turn can differentiate into other types of precursor cells further down the pathway (such as a tissue specific precursor, for example, a cardiomyocyte precursor), and then to an end-stage differentiated cell, which plays a characteristic role in a certain tissue type, and may or may not retain the capacity to proliferate further.

The term "embryonic stem cell " is used to refer to the pluripotent stem cells of the inner cell mass of the embryonic blastocyst (see US Patent Nos. 5,843,780, 6,200,806, 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, US 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.

The term "progenitor cell " is used herein to refer to cells that have a cellular phenotype that is more primitive (i.e., is at an earlier step along a developmental pathway or progression than is a fully differentiated cell) relative to a cell to which it can give rise by differentiation. Typically, 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 "differentiated cell" refers to a primary cell that is not pluripotent as that term is defined herein. Stated another way, the term "differentiated cell" refers to a cell of a more specialized cell type derived from a cell of a less specialized cell type (e.g., a stem cell such as an induced pluripotent stem cell) in a cellular differentiation process.

As used herein, the term "somatic cell" refers to a cell forming the body of an organism, as opposed to germline cells. In mammals, germline cells (also known as "gametes") are the spermatozoa and ova which fuse during fertilization to produce a cell called a zygote, from which the entire mammalian embryo develops. Every other cell type in the mammalian body—apart from the sperm and ova, the cells from which they are made (gametocytes) and undifferentiated stem cells—is a somatic cell: internal organs, skin, bones, blood, and connective tissue are all made up of somatic cells. In some embodiments the somatic cell is a "non-embryonic somatic cell", by which is meant a somatic cell that is not present in or obtained from an embryo and does not result from proliferation of such a cell in vitro. In some embodiments the somatic cell is an "adult somatic cell", by which is meant a cell that is present in or obtained from an organism other than an embryo or a fetus or results from proliferation of such a cell in vitro. In some embodiments, where a differentiated cell or population of differentiated cells are cultured in vitro, the differentiated cell can be cultured in an organotypic slice culture, such as described in, e.g., meneghel-Rozzo et al., (2004), Cell Tissue Res, 316(3):295-303. As used herein, the term "adult cell" refers to a cell found throughout the body after embryonic development.

As used herein, the term "multipotent stem cells" refers to stem cells that can only differentiate into a limited number of types. For example, the bone marrow contains multipotent stem cells that give rise to all the cells of the blood but may not be able to differentiate into other cell types. Such "multipotent" cells have the ability to differentiate into more than one cell type in response to distinct differentiation signals. Examples of multipotent cells include, but are not limited to, bone marrow stromal cells (BMSC), and adult or embryonic stem cells.

Appropriate growth conditions for mammalian cells are well known in the art (Freshney, R.I. (2000) Culture of Animal Cells, a Manual of Basic Technique. Hoboken NJ, John Wiley & Sons; Lanza et al. Principles of Tissue Engineering, Academic Press; 2nd edition May 15, 2000; and Lanza & Atala, Methods of Tissue Engineering Academic Press; 1st edition October 2001). Cell culture media generally include essential nutrients and, optionally, additional elements such as growth factors, salts, minerals, vitamins, etc., that may be selected according to the cell type(s) being cultured. Particular ingredients may be selected to enhance cell growth, differentiation, secretion of specific proteins, etc. In general, standard growth media
include, for example, Dulbecco's Modified Eagle Medium, low glucose (DMEM), with 110 mg/L pyruvate and glutamine, supplemented with 10-20% fetal bovine serum (FBS) or calf serum and 100 U/ml penicillin or various other standard media well known to those in the art. Growth conditions will vary dependent on the type of mammalian cells in use and the tissue desired.

[0092] In general, the length of the growth period of a cell for use in the vascularized living tissue equivalents or constructs will depend on the particular vascularized living tissue equivalent or construct being produced. The growth period can be continued until the vascularized living tissue equivalent or construct has attained certain desired properties, e.g., until the vascularized living tissue equivalent or construct has reached a particular thickness, size, strength, composition of proteinaceous components, and/or a particular cell density. Methods for assessing these parameters are known to those skilled in the art.

[0093] In some embodiments, following a first growth period the vascularized living tissue equivalent or construct can be seeded with a second population of cells, which may comprise cells of the same type as used in the first seeding, or cells of a different type. The vascularized living tissue equivalent can then be maintained for a second growth period which may be different in length from the first growth period and may employ different growth conditions. Multiple rounds of cell seeding with intervening growth periods may be employed.

[0094] In some embodiments, vascularized living tissue and organ equivalents are generated for humans. In other embodiments, tissues and organs are generated for animals such as, dogs, cats, horses, monkeys, or any other mammal.

**Pharmaceutical Compositions of Vascularized Living Tissue Equivalents or Constructs**

[0095] Provided herein, in some aspects, are pharmaceutical compositions comprising one or more vascularized living tissue equivalents or constructs together with a pharmaceutically acceptable carrier diluent or excipient. The pharmaceutical compositions described herein can be used to promote or otherwise facilitate cell migration, tissue regeneration, wound healing, and other treatments.

[0096] As used herein, the phrase "pharmaceutically acceptable", and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a mammal without the production of undesirable physiological effects such as nausea, dizziness, gastric upset and the like. Each carrier must also be "acceptable" in the sense of being compatible with the other ingredients of the formulation. A pharmaceutically acceptable carrier typically will not promote the raising of an immune response to an agent with which it is
admixed, unless so desired. Carriers for the tissue constructs described herein include, as non-limiting examples, saline, plasma, glycerol, isotonic polymer solutions etc.

[0097] In addition, if desired, the tissue constructs can contain or be in contact with minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which stabilize or preserve the viability of the constructs. Exemplary liquid carriers further include sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, polyethylene glycol and other solutes.

**Administration of Vascularized Living Tissue Equivalents or Constructs**

[0098] In some aspects, vascularized living tissue equivalents or constructs are provided for use in subjects in need thereof. For example, in some aspects, vascularized living skin equivalents or constructs are provided for use in subjects having wounds, such as burn injuries.

[0099] The term "skin" is commonly used to describe the body covering of any animal, but technically it refers only to the body covering of vertebrates, who all have the same basic skin structure. An organism's skin is essential to its survival. It forms a physical barrier that helps prevent harmful microorganisms and chemicals from entering the body, it prevents the loss of body fluids, protects internal body structures from injury, and protects them from the potentially damaging ultraviolet rays of the sun. In addition, the skin helps regulate body temperature, excretes certain waste products, and is an important sensory organ. Skin contains various types of specialized nerve cells responsible for the sense of touch, pain, pressure, etc.

[00100] The skin is the human body's largest organ. The skin is very resilient, constantly regenerating itself and exhibiting a strong ability to repair itself after injury. The skin is made up of two distinct layers, the epidermis and the dermis. The epidermis is the outer layer of the skin and is a tough, waterproof, protective layer. The dermis, or inner layer, is thicker than the epidermis and gives the skin its strength and elasticity. The two layers of the skin are anchored to one another by a thin but complex layer of tissue known as the basement membrane which is composed of a series of elaborately interconnecting molecules that serve to hold the skin together. Below the dermis is the subcutaneous layer, the hypodermis, which is a layer of tissue composed of protein fibers and adipose tissue. Although not technically part of the skin itself, the subcutaneous layer contains glands and other skin structures, as well as sensory receptors involved in the sense of touch.

[00101] A burn is a type of injury that may be caused by heat, electricity, chemicals, light, radiation, or friction. Burns can be highly variable in terms of the tissue affected, the severity,
and resultant complications. Skin (both dermal and epidermal tissue), muscle, bone, and blood vessels can all be damaged by burn injuries. Depending on the location affected and the degree of severity, a burn victim may experience a wide number of potentially fatal complications including shock, infection, electrolyte imbalance and respiratory distress.

Treatment of severe burns often requires skin grafting, where skin, either epidermal, dermal, or both, are taken from unburned sites on a body, i.e. donor sites, and grafting that skin onto the burn wound. Ideally, the grafted skin attaches to the underlying tissue and effectively closes the wound.

A skin graft "takes" or is successful when new blood vessels and tissue form in the injured area and cells of the graft remain viable and proliferate to maintain or expand cell number. Sometimes, skin grafts do not take because of complications such as infection (the most common cause of graft failure) or shearing (pressure causing a graft to detach from the skin). A skin graft can be "autologous," where the donor and recipient are the same (also known as an autograft); "isogeneic," where the donor and recipient are genetically identical (i.e., an isograft or syngraft); "allogeneic," where the donor and recipient are of the same species; and "xenogeneic," where the donor and recipient are of different species (e.g., xenograft or heterograft).

By using a patient's own skin to cover a burn wound, as in an autograft, the risk of tissue rejection is eliminated. However, skin grafts are often a challenge for patients with severe burns across large portions of their body. In these instances there may not be sufficient donor site skin to immediately cover all of the individual's wounds, and thus alternative sources for the skin graft must be used. Skin flaps are a complex type of skin graft that attach donor skin and underlying tissue by surgically connecting blood supply from the wound to the transferred skin. Skin flaps and other skin replacement methods are sometimes used in situations where standard skin grafts are not possible or where alternative methods are preferred.

In addition to providing vascularized living tissue equivalents or constructs as described above, methods of using such vascularized living tissue equivalents or constructs are encompassed herein. Generally, in some aspects a vascularized living tissue equivalent or construct can be implanted by using any suitable medical procedure that facilitates use of the vascularized living tissue equivalent or construct to provide a therapeutic benefit to a subject in need thereof.

As used herein, the terms "implanted" and "implantation" and like terms refer to an act of delivering a vascularized living tissue equivalent or construct, such as a vascularized living skin equivalent or construct, to a site within a subject and of affixing the vascularized living tissue equivalent or construct to the site. The site of implantation in a patient typically is
"at or near a site for wound healing or tissue generation or regeneration in the subject," meaning the vascularized living tissue equivalent or construct is implanted in, on, onto, adjacent to or in proximity to a desired site of delivery to facilitate healing and/or tissue generation and/or regeneration to repair an injury or defect in the patient and/or to achieve a desired effect in the patient, such as wound drainage.

The delivery method may also include minimally invasive methods such as by catheter-based technology or by needle injection. The subject may be human or animal. The vascularized living tissue equivalent or construct may be delivered by any surgical procedure, including minimally invasive techniques, such as laparoscopic surgery, as well as invasive techniques such as thoracic surgery and fasciotomy. In certain non-limiting embodiments, the vascularized living tissue equivalents or constructs are used as surgical fabrics. For example and without limitation, in some embodiments, the vascularized living tissue equivalents can be implanted in a patient during laparoscopic procedures to repair or to reinforce fasciae that have been damaged or weakened. In some embodiments, the vascularized living tissue equivalents can also be used to re-join organs that have been separated as a result of surgery, to treat hernias, and to promote the healing of surgical incisions.

The vascularized living tissue equivalents or constructs may be implanted alone or implanted in conjunction with surgical fasteners, such as sutures, staples, adhesives, and the like. Additionally, biocompatible adhesives, such as, without limitation, fibrin-based or silk-based glues, may be used to fasten the vascularized living tissue equivalents or constructs as well. In other non-limiting embodiments, the vascularized living tissue equivalents or constructs may be used to promote healing of deep tissue wounds, such as puncture wounds, bullet wounds, or wounds that result from the surgical removal of a substantial amount of tissue, such as in debridement procedures or removal of tumors. In some embodiments of these aspects, it may be advantageous for the vascularized living tissue equivalents or constructs to further comprise therapeutic agents, such as antibiotics or growth factors, prior to insertion into the wound.

One or more therapeutic agents can be introduced into the vascularized living tissue equivalents or constructs by any useful method, such as, without limitation absorption, adsorption, deposition, admixture with a polymer composition used to manufacture the vascularized living tissue equivalents or constructs and linkage of the agent to a component of the vascularized living tissue equivalents or constructs. Generally, the therapeutic agents include any substance that can be coated on, embedded into, absorbed into, adsorbed to, or otherwise attached to or incorporated onto or into the vascularized living tissue equivalents or constructs that would provide a therapeutic benefit to a subject in need thereof. In some embodiments, the
vascularized living tissue equivalents or constructs may be "loaded" with therapeutic agent(s) by using static methods. For instance, a vascularized living tissue equivalent or construct can be immersed into a solution containing the therapeutic agent permitting the agent to absorb into and/or adsorb onto the vascularized living tissue equivalent or construct. The vascularized living tissue equivalents or constructs may also be loaded by using dynamic methods. For instance, a solution containing the therapeutic agent can be perfused or electrodeposited into the vascularized living tissue equivalents or constructs. If a solution containing a therapeutic agent is perfused into the vascularized living tissue equivalents or constructs, the microchannel network may be used to facilitate the perfusion. In other embodiments, a therapeutic agent can be added to the vascularized living tissue equivalent or construct before it is implanted in the patient. In some embodiments, a therapeutic agent is released from the vascularized living tissue equivalent or construct. For example, the vascularized living tissue equivalents can be designed so that anti-inflammatory drugs are released from the vascularized living tissue equivalent or constructs to decrease an immune response. In other embodiments, a therapeutic agent is intended to substantially remain within the vascularized living tissue equivalents or constructs. For example and without limitation, chemoattractants are maintained within the vascularized living tissue equivalents or constructs to promote cellular migration and/or cellular infiltration into the scaffold upon implantation into a subject. In some embodiments, at least one therapeutic agent is added to the scaffold before it is implanted in the patient.

[0108] The one or more therapeutic agents introduced to vascularized living tissue equivalents or constructs for administration to a subject in need thereof include those agents useful in the treatment of the subject or those that would provide a therapeutic benefit to the subject. For example, a vascularized living tissue equivalent or construct for implantation into a patient may further comprise as a therapeutic agent an anti-microbial agent to prevent growth of any bacteria that were introduced during the implantation, such as during a surgery. It is to also be understood that such therapeutic agents may be different from or partially overlap with the one or more therapeutic agents introduced to a vascularized living tissue equivalent or construct during the cellularization of such a construct. Non-limiting examples of such therapeutic agents include antimicrobial agents, growth factors, emollients, retinoids, and topical steroids. Each therapeutic agent may be used alone or in combination with other therapeutic agents. For example and without limitation, a vascularized living tissue equivalent or construct comprising neurotrophic agents or cells that express neurotrophic agents may be applied to a wound that is near a critical region of the central nervous system, such as the spine. Alternatively, the therapeutic agent may be blended with the silk fibroin while the silk fibroin is being processed.
In another embodiment, the therapeutic agent is mixed with a carrier polymer (e.g., polylactic-glycolic acid microparticles) which is subsequently processed with the silk fibroin.

[0109] In certain non-limiting embodiments, the therapeutic agent is a growth factor, such as a neurotrophic or angiogenic factor, which optionally may be prepared using recombinant techniques. Non-limiting examples of growth factors include basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factors 1 and 2 (IGF-I and IGF-2), platelet derived growth factor (PDGF), stromal derived factor 1 alpha (SDF-I alpha), nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), neurotrophin-3, neurotrophin-4, neurotrophin-5, pleiotrophin protein (neurite growth-promoting factor 1), midkine protein (neurite growth-promoting factor 2), brain-derived neurotrophic factor (BDNF), tumor angiogenesis factor (TAF), corticotropic releasing factor (CRF), transforming growth factors cc and β (TGF-α and TGF-β), interleukin-8 (IL-8), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukins, and interferons. Commercial preparations of various growth factors, including neurotrophic and angiogenic factors, are available from R & D Systems, Minneapolis, Minnesota; Biovision, Inc, Mountain View, California; ProSpec- Tany TechnoGene Ltd., Rehovot, Israel; and Cell Sciences®, Canton, Massachusetts.

[0110] In certain non-limiting embodiments, the therapeutic agent is an antimicrobial agent, such as, without limitation, isoniazid, ethambutol, pyrazinamide, streptomycin, clofazimine, rifabutin, fluoroquinolones, ofloxacin, sparfloxacin, rifampin, azithromycin, clarithromycin, dapsone, tetracycline, erythromycin, ciprofloxacin, doxycycline, ampicillin, amphotericin B, ketoconazole, fluconazole, pyrimethamine, sulfadiazine, clindamycin, lincomycin, pentamidine, atovaquone, paromomycin, diclofazin, acyclovir, trifluorouridine, fosfocarnet, penicillin, gentamicin, ganciclovir, iatroconazole, miconazole, Zn-pyrithione, and silver salts such as chloride, bromide, iodide and periodate.

[0111] In certain non-limiting embodiments, the therapeutic agent is an anti-inflammatory agent, such as, without limitation, an NSAID, such as salicylic acid, indomethacin, sodium indomethacin trihydrate, salicylamide, naproxen, colchicine, fenoprofen, sulindac, diflunisal, diclofenac, indoprofen, sodium salicylamide; an anti-inflammatory cytokine; an anti-inflammatory protein; a steroidal anti-inflammatory agent; or an anti-clotting agents, such as heparin. Other drugs that may promote wound healing and/or tissue regeneration may also be included.

[0112] In certain embodiments, the vascularized living tissue equivalents or constructs comprise genetically modified cells that are capable of expressing a therapeutic substance, such as a growth factor. Cells can be modified by any useful method in the art. For example and
without limitation, the therapeutic agent is a growth factor that is released by cells transfected with cDNA encoding the growth factor. Therapeutic agents that can be released from cells include, without limitation, a neurotrophic factor, such as nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3, neurotrophin-4, neurotrophin-5, and ciliary neurotrophic factor; a growth factor, such as basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factors (IGF), platelet derived growth factor (PDGF), transforming growth factor-beta (TGF-β), pleiotrophin protein (neurite growth-promoting factor 1), and midkine protein (neurite growth-promoting factor 2); an anti-inflammatory cytokine; and an anti-inflammatory protein. The cells may be autologous, allogeneic, etc., as described in the preceding sections.

[0113] In some aspects, the invention provides a vascularized living skin equivalent or construct for use as a skin graft. In some embodiments, one or more vascularized living skin equivalents or constructs comprising a silk microfluidic scaffold and at least one living cell are administered to a subject having a wound. The wound may have been caused by any type of trauma or injury. In some embodiments, one or more vascularized living skin equivalents or constructs comprising a silk microfluidic scaffold and at least one living cell are administered to a subject having a surgical procedure. In some embodiments, a vascularized living skin equivalent or construct comprising a silk microfluidic scaffold and at least one living cell is administered to a subject having a skin lesion. In some embodiments, one or more vascularized living skin equivalents or constructs are administered to a subject in need of skin replacement.

[0114] In such embodiments, the number of vascularized living skin equivalents or constructs administered to the patient is dependent on the size of the area in need in the subject. In one instance, a large sheet can be prepared by scaling up the procedures described in the Examples herein. Alternatively, a number of smaller constructs can be applied to affect coverage of a larger area. The size or area will be dependent on a number of factors including the nature (cause, depth) of the wound, injury, or trauma, the age of the subject, and the size of the subject. The number of vascularized living skin equivalents necessary for administration to subject can be determined by one of skill in the art. The number of vascularized living skin equivalents administered to the subject can be at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 20, at least 30, at least 40, at least 50, at least 75, at least 100, at least 200, at least 500, at least 750, at least 1000 or more.

[0115] The terms "subject" and "individual" are used interchangeably herein, and refer to an animal, for example, a human. For treatment of conditions or disease states which are specific for a specific animal such as a human subject, the term subject refers to that specific
animal. The term "mammal" is intended to encompass a singular "mammal" and plural "mammals," and includes, but is not limited to humans; primates such as apes, monkeys, orangutans, and chimpanzees; canids such as dogs and wolves; felids such as cats, lions, and tigers; equids such as horses, donkeys, and zebras; food animals such as cows, pigs, and sheep; ungulates such as deer and giraffes; rodents such as mice, rats, hamsters and guinea pigs; and bears. In some preferred embodiments, a mammal is a human. The "non-human animals" and "non-human mammals" as used interchangeably herein, includes mammals such as rats, mice, rabbits, sheep, cats, dogs, cows, pigs, and non-human primates. The term "subject" also encompasses any vertebrate including but not limited to mammals, reptiles, amphibians and fish. However, advantageously, the subject is a mammal such as a human, or other mammals such as a domesticated mammal, e.g. dog, cat, horse, and the like, or production mammal, e.g. cow, sheep, pig, and the like are also encompassed in the term subject.

**Wound Closure Kits and Systems**

[0116] In some aspects, a wound closure kit is provided, comprising at least one vascularized living skin equivalent or construct and providing wound closure by allowing growth of neodermal tissue around a vascularized living skin equivalent or construct. In some embodiments, the wound closure system comprises one or more vascularized living skin equivalents or constructs, wherein the vascularized living skin equivalent or construct comprises a silk microfluidic scaffold and one or more cells. In various embodiments, the kit includes one or more containers, as well as additional reagent(s) and/or ingredient(s) for performing any methods of the invention. The kit can also include instructions for using the wound closure system.

**Systems for Drug Discovery and Development**

[0117] In the field of pharmaceutical research, the use of high-throughput screening has been an essential tool used for drug discovery. However, a limitation in such screening methods is the necessity for large number of cells. Furthermore, such screens are usually performed on isolated, in vitro-derived cells, versus natural tissues or tissue-like structures, in the absence of the 3-dimensional structure and vasculature that is found in the natural tissue. In addition, such isolated cells cultured in monolayer conditions, often do not posses the functional and structural properties of natural three-dimensional tissue or organ structures. Accordingly, the vascularized living skin equivalent or construct described herein provide a renewable source of skin tissue-like components that can be used for drug screening which mimics the natural structure found in living skin tissue and can be developed into a 3-dimensional structure.

[0118] In some aspects, the vascularized living tissue equivalents or constructs can be used as models for high-throughput drug screening purposes. In some such aspects, vascularized
living skin equivalents or constructs are used as a skin model for drug screening purposes, such as determination of pharmacokinetics and toxicity of drugs during drug discovery and development. Assays and criteria that can be used and adapted to measure the pharmacokinetic properties and toxicity of the drugs being tested using the vascularized living tissue equivalents or constructs described herein are well-known to the skilled artisan.

[0119] In some aspects, a method for high-throughput screening of agents using vascularized living tissue equivalents or constructs is provided. The method comprises screening for and identifying agents, and testing the effect of such agents on the vascularized living tissue equivalents or constructs of the invention, such as vascularized living skin equivalents or constructs. In some such aspects, a multiplex array for drug screening is provided comprising at least two vascularized living tissue equivalents or constructs, where each vascularized living tissue equivalent or construct comprises a silk microfluidic scaffold and at least one living cell. In some embodiments of such aspects, the multiplex array can be designed so that each construct comprises the same living cell, and different fluids comprising test drugs or compounds are passed through the microfluidic network of each construct to compare the effects of different drugs on the same tissue or cell type. In such embodiments, the inlet and outlet microchannel of each silk microfluidic scaffold of each construct can be connected to a different fluid source comprising a different drug being tested. In other embodiments, each inlet and outlet channel can be connected to a same fluid source comprising a drug being tested, while the living cell of each construct is different, i.e., the effects of the same drugs on different tissues can be tested.

For example, in such embodiments, the multiplex array comprises one vascularized living skin equivalent or construct and one vascularized living corneal equivalent or construct. In other embodiments, the multiplex array can comprise constructs comprising cancerous or malignant cells, such as cells derived or obtained from basal cell cancers, squamous cell cancers, and melanomas, to test the effects of different drugs on, for example, the cellular proliferation of these cancers in a 3-dimensional context.

[0120] For example, in some embodiments of such aspects, a combinatorial library containing a large number of potential therapeutic compounds (potential modulator compounds) is provided. Such "combinatorial chemical libraries" are then screened in one or more assays to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity, when administered to the vascularized living skin equivalents or constructs. The compounds thus identified can serve as conventional "lead compounds" or "candidate therapeutic agents," and can be tested in animal models and/or derivatized for further testing to identify additional agents with the desired characteristics or effects. For example, drugs being screened using multiplex arrays comprising vascularized living skin equivalents or
constructs can be tested for effects on cell proliferation, differentiation, survival, wound healing, malignancy inhibition, or malignancy acceleration.

[0121] As used herein, the term "drug" or "compound" or "agent" refers to a chemical entity or biological product, or combination of chemical entities or biological products, including nucleic acids, administered to a subject to treat or prevent or control a disease or condition. The chemical entity or biological product is preferably, but not necessarily a low molecular weight compound, such as a small molecule compound, but may also be a larger compound, for example, an oligomer of nucleic acids, amino acids, or carbohydrates including, without limitation, proteins, oligonucleotides, ribozymes, DNAzymes, glycoproteins, siRNAs, lipoproteins, aptamers, and modifications and combinations thereof.

[0122] As used herein, the term "small molecule" refers to a chemical agent including, but not limited to, peptides, peptidomimetics, amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, aptamers, nucleotides, nucleotide analogs, organic or inorganic compounds (i.e., including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds.

[0124] The terms "effective" and "effectiveness", as used herein, includes both pharmacological effectiveness and physiological safety. Pharmacological effectiveness refers to the ability of the treatment to result in a desired biological effect in the patient. Physiological safety refers to the level of toxicity, or other adverse physiological effects at the cellular, organ and/or organism level (often referred to as side-effects) resulting from administration of the treatment. "Less effective" means that the treatment results in a therapeutically statistically significant lower level of pharmacological effectiveness and/or a therapeutically statistically greater level of adverse physiological effects.

[0125] The present invention can further be defined in any of the following numbered paragraphs:

1. A composition comprising a fabricated silk microfluidic scaffold, said composition comprising a living cell.
2. The composition of paragraph 2, wherein said silk microfruidic scaffold comprises a microchannel network.

3. The composition of paragraph 1, wherein said cell is a eukaryotic cell.

4. The composition of paragraph 1, wherein said cell is an ectodermal progenitor cell.

5. The composition of paragraph 1, wherein the at least one living cell is a differentiated cell.

6. The composition of paragraph 1, wherein the at least one living cell is a keratin-12 expressing cell.

7. The composition of paragraph 1, wherein the at least one living cell is a keratinocyte.

8. The composition of paragraph 1, wherein the at least one living cell is a vascular endothelial cell precursor.

9. The composition of paragraph 1, wherein the at least one living cell is a vascular endothelial cell.

10. The composition of paragraph 1, wherein said fabricated microfruidic scaffold is implantable in a subject.

11. The composition of any of paragraphs 3-10, wherein said living cell is autologous to the subject into which the fabricated microfruidic scaffold is being implanted.

12. The composition of paragraph 1, wherein the silk microfruidic scaffold further comprises a flat silk film to which a silk microchannel film is bonded.

13. The composition of paragraph 1, wherein the silk microfruidic scaffold further comprises at least one bioerodible micropost within a lumen of a microchannel.

14. The use of a composition comprising a fabricated silk microfruidic scaffold and at least one living cell in a subject in need thereof.

15. The use of a composition comprising a fabricated silk microfruidic scaffold and at least one living cell in the preparation of a medicament for treatment of a wound.

16. The use of paragraphs 14 or 15, wherein the silk microfruidic scaffold comprises a microchannel network.

17. The use of paragraphs 14 or 15, wherein the silk microfruidic scaffold comprises a flat silk film to which a silk microchannel film is bonded.

18. The use of paragraphs 14 or 15, wherein the living cell is a differentiated cell.

19. The use of paragraphs 14 or 15, wherein the living cell is a keratin-12 expressing cell.

20. The use of paragraphs 14 or 15, wherein the living cell is a keratinocyte.

21. The use of paragraphs 14 or 15, wherein the at least one living cell is a vascular endothelial cell precursor.
22. The use of paragraphs 14 or 15, wherein the at least one living cell is a vascular endothelial cell.
23. The use of paragraphs 14 or 15, wherein the silk microfluidic scaffold further comprises at least one bioerodible micropost within a lumen of a microchannel.
24. A method of treating a subject having a wound, comprising administering to a subject having a wound a composition comprising a fabricated silk microfluidic scaffold and at least one living cell.
25. The method of paragraph 24, wherein the fabricated microfluidic scaffold is a silk microfluidic scaffold.
26. The method of paragraph 24, wherein the silk microfluidic scaffold comprises a microchannel network.
27. The method of paragraph 24, wherein the silk microfluidic scaffold further comprises a flat silk film to which the silk microchannel film is bonded.
28. The method of paragraph 24, wherein the at least one living cell is a differentiated cell.
29. The method of paragraph 24, wherein the at least one living cell is a keratin-12 expressing cell.
30. The method of paragraph 24, wherein the at least one living cell is a keratinocyte.
31. The method of paragraph 24, wherein the at least one living cell is a vascular endothelial cell precursor.
32. The method of paragraph 24, wherein the at least one living cell is a vascular endothelial cell.
33. The method of any of paragraphs 24-31, wherein the at least one living cell is autologous to the subject having a wound.
34. The method of paragraph 24, wherein the fabricated microfluidic scaffold further comprises at least one bioerodible micropost in a lumen of a microchannel.
35. The method of paragraph 24, wherein the wound is a skin injury.
36. The method of paragraph 24, wherein the administering of the composition comprises implanting the composition in the subject.
37. A method of fabricating a microfluidic scaffold, the method comprising the steps of (i) designing a microchannel network, (ii) fabricating a mold comprising the microchannel network, (iii) casting an aqueous solution on the mold to form a microchannel film, and (iv) bonding the microchannel film to a flat film to form a microfluidic scaffold.
38. The method of paragraph 37, wherein the aqueous solution is an aqueous silk solution.
39. The method of paragraph 37, wherein the flat film is a flat silk film.
40. The method of paragraph 37, further comprising adding at least one living cell to the microfluidic scaffold.

41. The method of paragraph 40, wherein the adding at least one living cell comprises direct seeding of the at least one living cell onto the microfluidic scaffold or introducing the at least one living cell through the microchannel network.

42. The method of paragraph 37, wherein the microfluidic scaffold further comprises at least one bioerodible micropost inserted in the lumen of the microchannel network.

[0126] It should be understood that this invention is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such may vary. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims.

[0127] The present invention is further illustrated by the following Examples. These Examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

EXAMPLES

The Challenge

[0128] In the United States each year, millions of civilians are burned with nearly 100,000 hospitalizations and several thousand thermal injury-associated deaths, both civilian and combat-derived. Systemic response to thermal injury involves loss of skin barrier functionality and fluid loss, microbial infection, vascular derangements and interstitial edema, and overall organ dysfunction.

[0129] These data reflect the need for transformative technological innovations in wound repair science, wound care, wound healing therapeutics, and in the creation of sustainable, vascularized living skin equivalents.

The Approach

[0130] While several devices and living skin substitutes have been developed over the past few years, none possess an integrated vasculature. Controllable 3-dimensional human skin equivalents or constructs constructed using biodegradable microfluidics can be: biodegradable, bioactive and fully functionalized, and complete with a microcirculation. Such a pre-vascularized compartment is capable of sustaining the living organ equivalent or construct ex vivo and enable surgical integration in patients. The technology described provides solutions for high throughput drug discovery and screening in vitro, and delivers a new class of autologous, vascularized living skin equivalents or constructs that provide long-lasting impact on
regenerative medicine, especially for those suffering with traumatic-injury-induced or thermally-induced wounds.

**Fabrication Methods**

[0131] *Design of MicroChannel Network.* MicroChannel network designs are tuned to specific ranges of wall shear stress, pressure, flow and other critical fluid mechanical properties, and can be varied in a controlled manner both temporally and spatially in order to mimic physiologic properties of the microcirculation within the tissue construct.

[0132] *Mold Fabrication.* Standard photolithography was used to create a SU-8/silicon master mold. Soft lithography was then used to create an inverse replica, or "negative" mold, in Polydimethyl Siloxane (PDMS).

[0133] *Silk Casting.* Silkworm cocoons were processed to aqueous silk fibroin by sericin protein extraction in Na₂CO₃, dissolution in LiBr, and dialysis against H₂O. The silk was then concentrated to a 10-13% solution by reverse dialysis. Aqueous silk solution was cast on the PDMS mold and dried. The cast silk was then delaminated and treated with 50% methanol solution for 4 hr to produce water-stable microchannel films.

[0134] *Silk Bonding.* MicroChannel silk films were bonded to flat silk films to form a patent microfluidic network. Flexible tip needles were inserted into the microchannel inlet and outlet. A thin layer of 8% aqueous silk was applied to the bonding area. The two layers were visually aligned and bonded together at 70°C with applied mechanical pressure for 18 h.

**Silk Fibroin Microfluidic Networks:**

[0135] The branching microchannel network geometry required that is capable of providing shear forces at the channel walls comparable to those found in normal dermal microvasculature has been fabricated. In order to address the possibility of channel collapse, which might be observed for wider and shallower microchannels, the layout of the microvascular network was modified to increase mechanical stability by inserting bioerodible microposts within the channels.

[0136] A diagrammatic representation of a bifurcated network showing bioerodible microposts positioned to insure patency is shown. Visualization of the patent micromolded, bonded silk network is provided. A scanning electron microscope image of the silk microfluidic channel and bioerodible microposts was also obtained.

**Creation of Ectodermally-derived Progenitor Populations: Integration into Vascularized Living Skin Equivalents or Constructs**

[0137] Human embryonic stem cells (hESC) can be used as a source of epithelial and mesenchymal tissues for 3-D tissue construction, as shown here. Longstanding expertise in epithelial stem cell biology, 3-D tissue biology and extracellular matrix biology was used to
generate in vivo-like tissues that can be incorporated into living skin equivalents with an integrated microvasculature. Precursor cells from hESC were derived that demonstrate epithelial and mesenchymal cell differentiation. In brief, directed differentiation from hESC generated ectodermal subpopulations enriched in keratin 18-expressing cells that underwent further differentiation and enrichment for keratin 12-expressing cells. When these cells were grown at an air liquid interface on a collagen gel harboring hESC-derived mesenchymal cells, a multilayer epithelium was formed, which was then integrated into vascularized living skin equivalents or constructs.

*Cellularization of Silk Scaffold*

[0138] Cells can be seeded directly onto the silk scaffolds or can be introduced through the microfluidic network. This interface allows for a sufficient means of exchange between surface cells and the flow network during perfusion.

[0139] A patent closed network fabricated from silk with seeded human dermal microvascular endothelial cells, 24 hrs post seeding (100 and 50 μm channel diameters) was produced. Further, confluent, differentiated human dermal microvascular endothelial cells cultured on untreated silk fibroin 3 days post seeding was also obtained.

*Summary*

[0140] Successfully colonized biodegradable, branched bifurcated networks with human capillary-derived endothelial cells to be used in vascularized living skin equivalents for civilian and combat casualty care have been created and demonstrated. It has been shown that patent, branched bifurcated networks are sustained with living endothelial cells that maintain a differentiated, non-overlapping phenotype. In-channel features such as micromolded posts that can enhance capillary patency without detriment to cell seeding or imposing eddying effects were also obtained.

[0141] Such vascularized living skin equivalents are also useful for multiplex arrays of vascularized organ equivalents or constructs for high throughput drug discovery and development.

*References:*


CLAIMS

We claim:
1. A composition comprising a fabricated silk microfluidic scaffold, said composition comprising a living cell.
2. The composition of claim 1, wherein said silk microfluidic scaffold comprises a microchannel network.
3. The composition of claim 1, wherein said cell is a eukaryotic cell.
4. The composition of claim 1, wherein said cell is an ectodermal progenitor cell.
5. The composition of claim 1, wherein the at least one living cell is a differentiated cell.
6. The composition of claim 1, wherein the at least one living cell is a keratin-12 expressing cell.
7. The composition of claim 1, wherein the at least one living cell is a keratinocyte.
8. The composition of claim 1, wherein the at least one living cell is a vascular endothelial cell precursor.
9. The composition of claim 1, wherein the at least one living cell is a vascular endothelial cell.
10. The composition of claim 1, wherein said fabricated microfluidic scaffold is implantable in a subject.
11. The composition of any of claims 3-10, wherein said living cell is autologous to the subject into which the fabricated microfluidic scaffold is being implanted.
12. The composition of claim 1, wherein the silk microfluidic scaffold further comprises a flat silk film to which a silk microchannel film is bonded.
13. The composition of claim 1, wherein the silk microfluidic scaffold further comprises at least one bioerodible micropost within a lumen of a microchannel.
14. The use of a composition comprising a fabricated silk microfluidic scaffold and at least one living cell in a subject in need thereof.
15. The use of a composition comprising a fabricated silk microfluidic scaffold and at least one living cell in the preparation of a medicament for treatment of a wound.
16. The use of claims 14 or 15, wherein the silk microfluidic scaffold comprises a microchannel network.
17. The use of claims 14 or 15, wherein the silk microfluidic scaffold comprises a flat silk film to which a silk microchannel film is bonded.
18. The use of claims 14 or 15, wherein the living cell is a differentiated cell.
19. The use of claims 14 or 15, wherein the living cell is a keratin-12 expressing cell.
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23. The use of claims 14 or 15, wherein the silk microfluidic scaffold further comprises at least one bioerodible micropost within a lumen of a microchannel.
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25. The method of claim 24, wherein the fabricated microfluidic scaffold is a silk microfluidic scaffold.
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31. The method of claim 24, wherein the at least one living cell is a vascular endothelial cell precursor.
32. The method of claim 24, wherein the at least one living cell is a vascular endothelial cell.
33. The method of any of claims 24-31, wherein the at least one living cell is autologous to the subject having a wound.
34. The method of claim 24, wherein the fabricated microfluidic scaffold further comprises at least one bioerodible micropost in a lumen of a microchannel.
35. The method of claim 24, wherein the wound is a skin injury.
36. The method of claim 24, wherein the administering of the composition comprises implanting the composition in the subject.
37. A method of fabricating a microfluidic scaffold, the method comprising the steps of (i) designing a microchannel network, (ii) fabricating a mold comprising the microchannel network, (iii) casting an aqueous solution on the mold to form a microchannel film, and (iv) bonding the microchannel film to a flat film to form a microfluidic scaffold.
38. The method of claim 37, wherein the aqueous solution is an aqueous silk solution.
39. The method of claim 37, wherein the flat film is a flat silk film.
40. The method of claim 37, further comprising adding at least one living cell to the microfluidic scaffold.
41. The method of claim 40, wherein the adding at least one living cell comprises direct seeding of the at least one living cell onto the microfluidic scaffold or introducing the at least one living cell through the microchannel network.
42. The method of claim 37, wherein the microfluidic scaffold further comprises at least one bioerodible micropost inserted in the lumen of the microchannel network.
Figure 6

Differentiated, stratified epithelial layer

Epithelial progenitors proliferating within functionalized scaffold

Dermal scaffold functionalized surfaces for fibroblast and endothelialized microchannels

Endothelialized microchannel

Microchannel prior to endothelial seeding

Functionalyzed scaffold prior to dermal cell or epithelial progenitor cell inoculation