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Davalos

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(54) **IRREVERSIBLE ELECTROPORATION TO CREATE TISSUE SCAFFOLDS**

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CPC ***C12N 13/00*** (2013.01); ***A61K 35/12*** (2013.01); ***C12N 5/0068*** (2013.01); *C12N 2533/90* (2013.01)

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(57) **ABSTRACT**

The present invention provides engineered tissue scaffolds, engineered tissues, and methods of using them. The scaffolds and tissues are derived from natural tissues and are created using non-thermal irreversible electroporation (IRE). Use of IRE allows for ablation of cells of the tissue to be treated, but allows vascular and neural structures to remain essentially unharmed. Use of IRE thus permits preparation of thick tissue scaffolds and tissues due to the presence of vasculature within the scaffolds. The engineered tissues can be used in methods of treating subjects, such as those in need of tissue replacement or augmentation.

41 Claims, 7 Drawing Sheets

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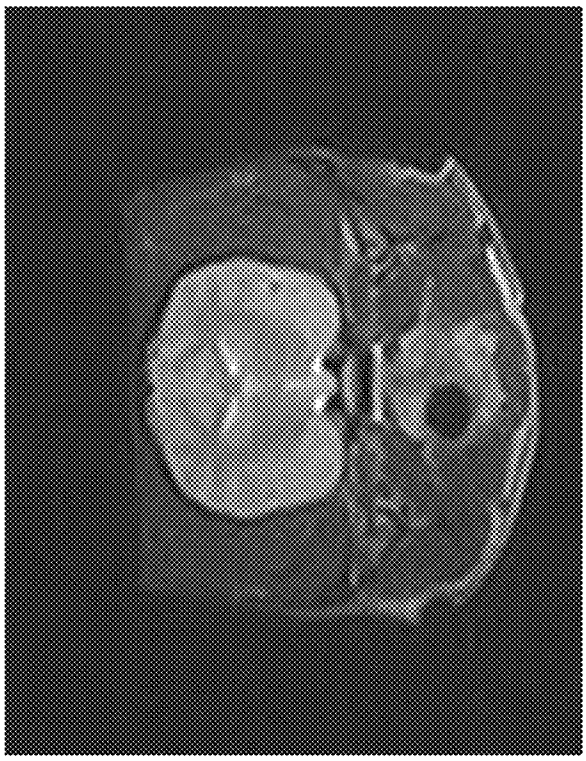

Figure 1A

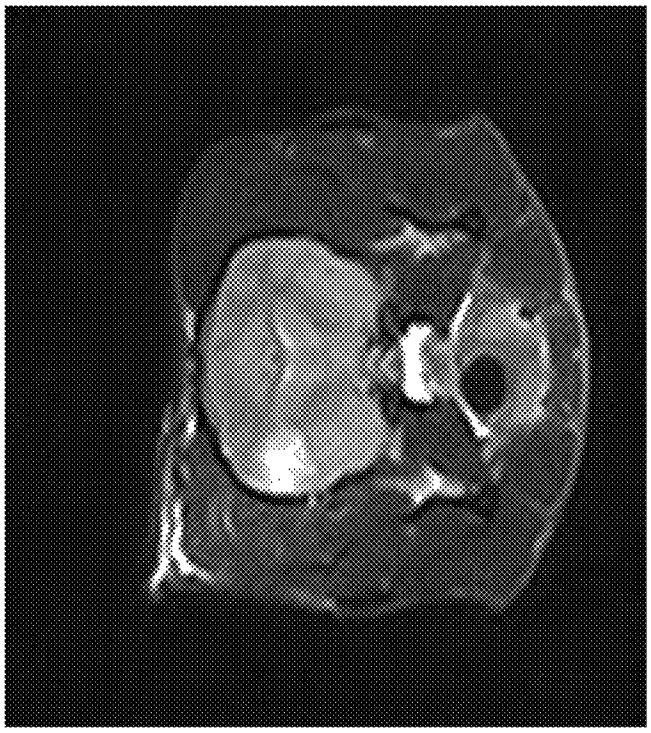

Figure 1B

Figure 1C

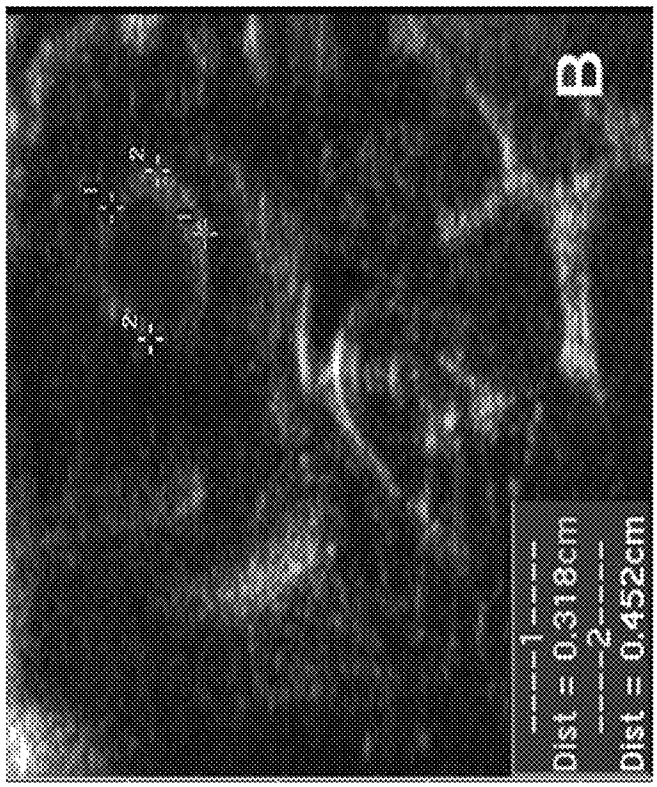

Figure 2

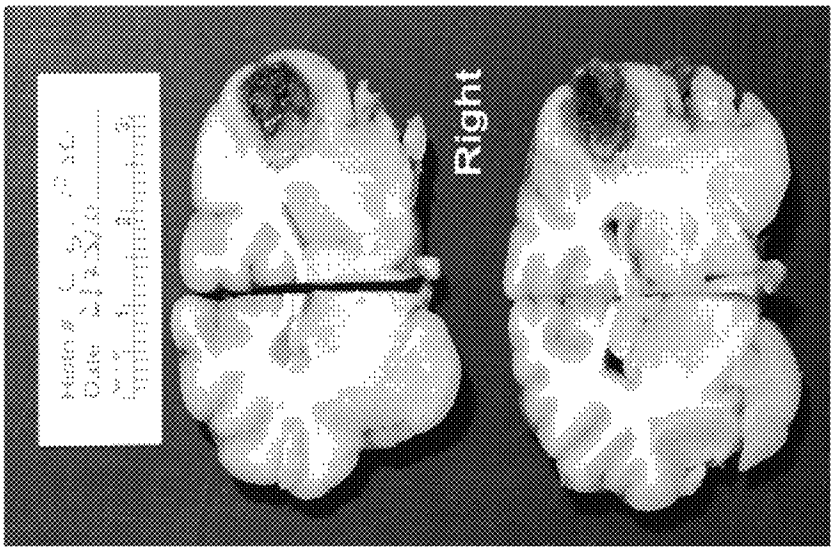

Figure 3

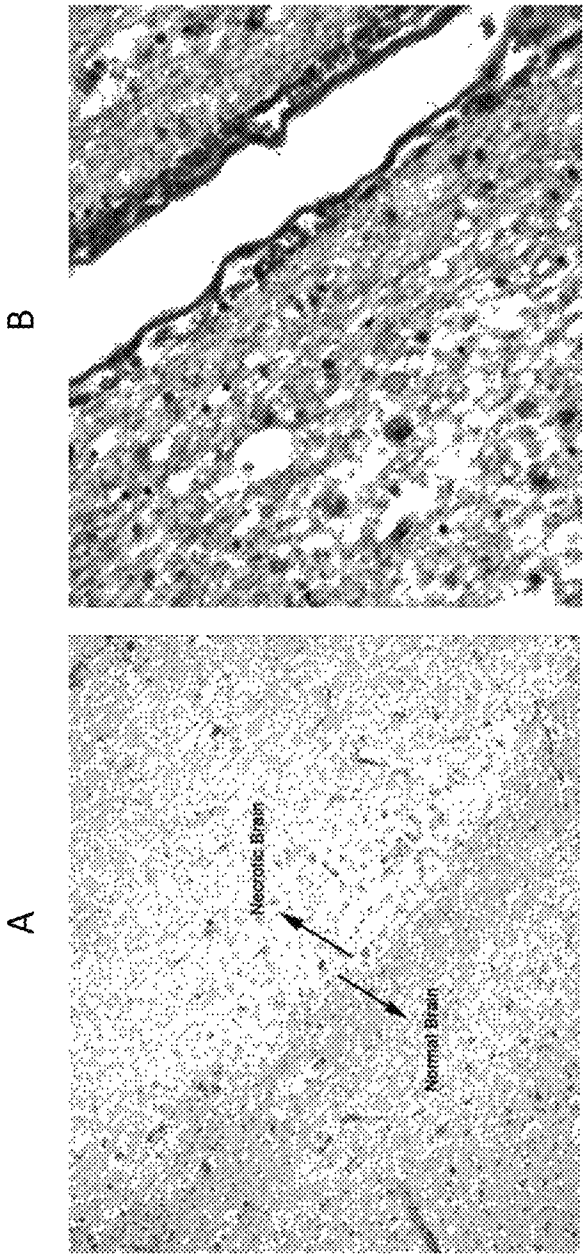

Figure 4

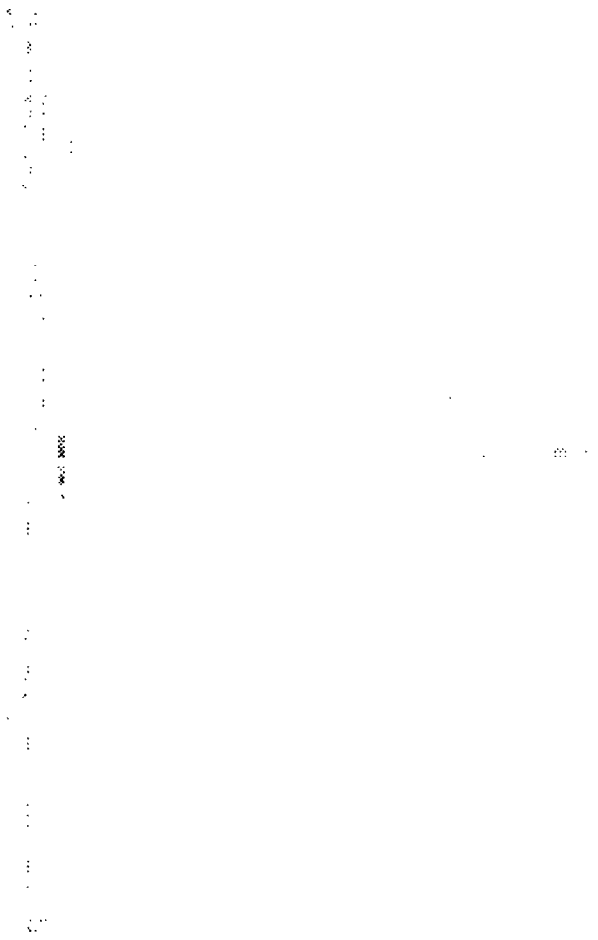

Figure 5

IRREVERSIBLE ELECTROPORATION TO CREATE TISSUE SCAFFOLDS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application relies on and claims the benefit of the filing date of U.S. provisional patent application No. 61/125,840, filed 29 Apr. 2008, the entire disclosure of which is hereby incorporated herein by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to the field of biomedical engineering. More specifically, the invention relates to methods of producing naturally-derived scaffolds for creation of tissues for medical uses, and tissues created from those scaffolds.

Description of Related Art

Tissue engineering holds great promise for treating some of the most devastating diseases of our time. Because engineered tissue and organ replacements can be developed in a laboratory, therapies can potentially be delivered on a large scale, for multiple disease states with dramatic reduction in waiting times for patients. The concept of engineering tissue using selective cell transplantation has been applied experimentally and clinically for a variety of disorders, including the successful use of engineered bladder tissue for bladder reconstruction, engineered injectable chondrocytes for the treatment of vesicoureteral reflux and urinary incontinence, and vascular grafts.

For clinical use for humans, the process involves the in vitro seeding and attachment of human cells onto a scaffold. Once seeded, the cells proliferate, migrate into the scaffold, and differentiate into the appropriate cell type for the specific tissue of interest while secreting the extracellular matrix components required to create the tissue. The three dimensional structure of the scaffold, and in particular the size of pores and density of the scaffold, is important in successful proliferation and migration of seeded cells to create the tissue of interest. Therefore, the choice of scaffold is crucial to enable the cells to behave in the required manner to produce tissues and organs of the desired shape and size.

To date, scaffolding for tissue engineering has usually consisted of natural and synthetic polymers. Methods known in the art for forming scaffolds for tissue engineering from polymers include solvent-casting, particulate-leaching, gas foaming of polymers, phase separation, and solution casting. Electrospinning is another popular method for creating scaffolds for engineered tissues and organs, but widely used techniques suffer from fundamental manufacturing limitations that have, to date, prevented its clinical translation. These limitations result from the distinct lack of processes capable of creating electrospun structures on the nano-, micro-, and millimeter scales that adequately promote cell growth and function.

Of fundamental importance to the survival of most engineered tissue scaffolds is gas and nutrient exchange. In nature, this is accomplished by virtue of microcirculation, which is the feeding of oxygen and nutrients to tissues and removing waste at the capillary level. However, gas exchange in most engineered tissue scaffolds is typically accomplished passively by diffusion (generally over distances less than 1 mm), or actively by elution of oxygen from specific types of material fibers. Microcirculation is difficult to engineer, particularly because the cross-sectional

dimension of a capillary is only about 5 to 10 micrometers (μm; microns) in diameter. As yet, the manufacturing processes for engineering tissue scaffolds have not been developed and are not capable of creating a network of blood vessels. Currently, there are no known tissue engineering scaffolds with a circulation designed into the structure for gas exchange. As a result, the scaffolds for tissues and organs are limited in size and shape.

In addition to gas exchange, engineered tissue scaffolds must exhibit mechanical properties comparable to the native tissues that they are intended to replace. This is true because the cells that populate native tissues sense physiologic strains, which can help to control tissue growth and function. Most natural hard tissues and soft tissues are elastic or viscoelastic and can, under normal operating conditions, reversibly recover the strains to which they are subjected. Accordingly, engineered tissue constructs possessing the same mechanical properties as the mature extracellular matrix of the native tissue are desirable at the time of implantation into the host, especially load bearing structures like bone, cartilage, or blood vessels.

There are numerous physical, chemical, and enzymatic ways known in the art for preparing scaffolds from natural tissues. Among the most common physical methods for preparing scaffolds are snap freezing, mechanical force (e.g., direct pressure), and mechanical agitation (e.g., sonication). Among the most common chemical methods for preparing scaffolds are alkaline or base treatment, use of non-ionic, ionic, or zwitterionic detergents, use of hypo- or hypertonic solutions, and use of chelating agents. Among the most common enzymatic methods for preparing scaffolds are use of trypsin, use of endonucleases, and use of exonucleases. Currently, it is recognized in the art that, to fully decellularize a tissue to produce a scaffold, two or more of the above-noted ways, and specifically two or more ways from different general classes (i.e., physical, chemical, enzymatic), should be used. Unfortunately, the methods used must be relatively harsh on the tissue so that complete removal of cellular material can be achieved. The harsh treatments invariable degrade the resulting scaffold, destroying vasculature and neural structures.

The most successful scaffolds used in both pre-clinical animal studies and in human clinical applications are biological (natural) and made by decellularizing organs of large animals (e.g., pigs). In general, removal of cells from a tissue or an organ for preparation of a scaffold should leave the complex mixture of structural and functional proteins that constitute the extracellular matrix (ECM). The tissues from which the ECM is harvested, the species of origin, the decellularization methods and the methods of terminal sterilization for these biologic scaffolds vary widely. However, as mentioned above, the decellularization methods are relatively harsh and result in significant destruction or degradation of the extracellular scaffold. Once the scaffold is prepared, human cells are seeded so they can proliferate, migrate, and differentiate into the specific tissue. The intent of most decellularization processes is to minimize the disruption to the underlying scaffold and thus retain native mechanical properties and biologic properties of the tissue. However, to date this intent has not been achieved. Snap freezing has been used frequently for decellularization of tendinous, ligamentous, and nerve tissue. By rapidly freezing a tissue, intracellular ice crystals form that disrupt cellular membranes and cause cell lysis. However, the rate of temperature change must be carefully controlled to prevent the ice formation from disrupting the ECM as well.

While freezing can be an effective method of cell lysis, it must be followed by processes to remove the cellular material from the tissue.

Cells can be lysed by applying direct pressure to tissue, but this method is only effective for tissues or organs that are not characterized by densely organized ECM (e.g., liver, lung). Mechanical force has also been used to delaminate layers of tissue from organs that are characterized by natural planes of dissection, such as the small intestine and the urinary bladder. These methods are effective, and cause minimal disruption to the three-dimensional architecture of the ECM within these tissues. Furthermore, mechanical agitation and sonication have been utilized simultaneously with chemical treatment to assist in cell lysis and removal of cellular debris. Mechanical agitation can be applied by using a magnetic stir plate, an orbital shaker, or a low profile roller. There have been no studies performed to determine the optimal magnitude or frequency of sonication for disruption of cells, but a standard ultrasonic cleaner appears to be effective. As noted above, currently used physical treatments are generally insufficient to achieve complete decellularization, and must be combined with a secondary treatment, typically a chemical treatment. Enzymatic treatments, such as trypsin, and chemical treatment, such as ionic solutions and detergents, disrupt cell membranes and the bonds responsible for intercellular and extracellular connections. Therefore, they are often used as a second step in decellularization, after gross disruption by mechanical means.

It is also recognized in the art that any processing step currently known that is used to remove cells will alter the native three-dimensional architecture of the ECM. This is an undesirable side-effect of the treatment, and attempts have been made to minimize the amount of disruption of the ECM.

SUMMARY OF THE INVENTION

The present invention provides an advancement over tissue engineering techniques known in the art. Specifically, the present invention provides a method of making engineered tissue scaffolds using irreversible electroporation (IRE) to decellularize natural tissue. Use of IRE to decellularize tissue provides a controlled, precise way to destroy cells of a tissue or organ, while leaving the underlying ECM, including vascularization, neural tubes, and other gross morphological features of the original tissue intact. The decellularized scaffolds are then suitable for seeding with cells of the appropriate organism. Where the process is performed in vitro, the seeded tissue is suitable for implantation into the organism as replacement tissue. In addition to methods of producing scaffolds, the invention also provides the decellularized scaffolds themselves, as well as methods of fabrication of engineered tissues and organs built from such scaffolds. Furthermore, the invention provides for use of the engineered scaffolds and the engineered tissues and organs built from such scaffolds.

Non-thermal IRE is a method to kill undesirable cells using electric fields in tissue while preserving the ECM, blood vessels, and nerves. Certain electrical fields, when applied across a cell, have the ability to permeabilize the cell membrane through a process that has come to be called "electroporation". When electrical fields permeabilize the cell membrane temporarily, after which the cells survive, the process is known as "reversible electroporation". Reversible electroporation has become an important tool in biotechnology and medicine. Other electrical fields can cause the cell membrane to become permeabilized, after which the cells die. This deadly process is known as "irreversible electroporation". Non-thermal irreversible electroporation is a new, minimally invasive surgical technique to ablate undesirable tissue, for example, tumor tissue. The technique is easy to apply, can be monitored and controlled, is not affected by local blood flow, and does not require the use of adjuvant drugs. The minimally invasive procedure involves placing needle-like electrodes into or around the targeted area to deliver a series of short and intense electric pulses that induce structural changes in the cell membranes that promote cell death. The voltages are applied in order to electroporate tissue without inducing significant joule heating that would significantly damage major blood vessels and the ECM. For a specific tissue type and set of pulse conditions, the primary parameter determining the volume irreversibly electroporated is the electric field distribution within the tissue. Recent IRE animal experiments have verified the many beneficial effects resulting from this special mode of non-thermal cell ablation, such as preservation of major structures including the extracellular matrix, major blood vessels, and myelin sheaths, no scar formation, as well as its promotion of a beneficial immune response.

However, the usefulness of IRE in generating tissue scaffolds for tissue engineering has not been recognized. The present invention, for the first time, discloses implementation of non-thermal IRE in the widely divergent field of tissue engineering. Use of non-thermal IRE in preparing tissue scaffolds not only provides a novel means for achieving that goal, but addresses long felt needs in the tissue engineering field. In various embodiments, the needs that are addressed are: preparation of tissue scaffolds with the underlying matrix essentially intact; preparation of tissue scaffolds having the ability to provide circulation, and preferably including microcirculation; preparation of tissue scaffolds having the ability to provide spaces for neural infiltration; and preparation of relatively thick (e.g., greater than 100 μm in thickness) engineered tissues.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate one (several) embodiment(s) of the invention, and together with the written description, serve to explain certain principles of the invention.

FIG. **1** shows magnetic resonance imaging (MRI) images of tissue after non-thermal IRE on canine tissue. The images show that non-thermal IRE decellularization zones were sharply demarcated T1 iso- to hypo-intense, T2 hyperintense and mild and peripherally contrast enhancing following intravenous administration of gadolinium, consistent with fluid accumulation within decellularization sites and a focal disruption of the blood-brain-barrier. FIG. **1**A shows an MRI before IRE, T2 weighted; FIG. **1**B shows superficial non-thermal IRE decellularization site, T2 weighted; and FIG. **1**C shows post-contrast T1 weighted; the dog's right is conventionally projected on the left.

FIG. **2** shows an ultrasound image of tissue 24 hour post-IRE treatment. The IRE decelluarization zone is clearly visible as a well demarcated, hypoechoic circular lesion with a hyperechoic rim.

FIG. **3** shows photographs of fixed brain sections to show position and character of decellularized volume.

FIG. **4** depicts images of brain tissue after non-thermal IRE treatment. FIG. **4**A shows a sharp delineation of brain tissue showing the regions of normal and necrotic canine

brain tissue after IRE. FIG. **4**B shows IRE treated brain tissue showing sparing of major blood vessels.

FIG. **5** shows a three-dimensional MRI source reconstruction of a superficial lesion site.

DETAILED DESCRIPTION OF VARIOUS EMBODIMENTS OF THE INVENTION

Reference will now be made in detail to various exemplary embodiments of the invention. It is to be understood that the following discussion of exemplary embodiments is not intended as a limitation on the invention, as broadly disclosed above. Rather, the following discussion is provided to give the reader a more detailed understanding of certain aspects and features of the invention.

Before embodiments of the present invention are described in detail, it is to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting. Further, where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit, unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither, or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the term belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. The present disclosure is controlling to the extent it conflicts with any incorporated publication.

As used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a pulse" includes a plurality of such pulses and reference to "the sample" includes reference to one or more samples and equivalents thereof known to those skilled in the art, and so forth.

Tissue engineering of tissue and organ replacements generally involves in vitro seeding and attachment of human cells onto a scaffold. To date, the most successful scaffolds for tissue engineering have been natural and made by chemically and/or mechanically decellularizing organs of large animals (e.g., pigs). Such techniques have been successful in making scaffolds to build thin organs, such as bladders, which have been successfully implanted in humans. Nevertheless, the field of tissue engineering is currently limited to organs that are less than about 1 mm thick because the process to decellularize the scaffold destroys vital blood vessels (as well as nerves and other architecture.

The present invention provides decellularize scaffolds, which are created at least in part using non-thermal irreversible electroporation (IRE). IRE is a method to kill undesirable cells using electric fields, which is known in the field of medical devices and tumor treatment. The procedure involves delivering a series of low energy (intense but short) electric pulses to the targeted tissue. These pulses irrecoverably destabilize the cell membranes of the targeted tissue, thereby killing the cells affected by the electrical field. The treatment is non-thermal, essentially only affects the cell membranes of the targeted tissue, and does not affect the nerves or blood vessels within the treated tissue. The organ may need to be perfused during the procedure, which is a routine technique in the medical arts.

In a first aspect, the invention provides a method of making a decellularized tissue scaffold. In general, the method comprises treating, in vitro or in vivo, a tissue comprising cells and an underlying scaffold with an electrical field of sufficient power and duration to kill cells of the tissue, but not to disrupt to a significant extent the underlying scaffold. The method is suitable for producing a tissue scaffold for use in tissue engineering. Although the source of the tissue is not limited, in exemplary embodiments, the tissue is from a relatively large animal or an animal recognized as having a similar anatomy (with regard to the tissue of interest) as a human, such as a pig, a cow, a horse, a monkey, or an ape. In embodiments, the source of the tissue is human, use of which can reduce the possibility of rejection of engineered tissues based on the scaffold. In preferred embodiments, the method leaves intact vascular structures of the tissue, such as capillaries. In embodiments, the method leaves intact neural tubes present in the tissue before treatment with the electrical field. As used herein, the term "intact" refers to a state of being whereby an element is capable of performing its original function to a substantial extent. Thus, for example, an intact capillary is a capillary that is capable of carrying blood and an intact neural tube is a tube that can accommodate a neuron. In embodiments, cells of the vascular system and neural system remain intact. In such embodiments, the cells can remain as part of the scaffold and engineered tissue, or may be removed by suitable treatment techniques or by cells that are seeded onto a scaffold or by cells of a body that receives the engineered tissue.

According to the method, a tissue is exposed to an electrical field that is adequate in time and power to cause killing of cells of the tissue, but not adequate to significantly destroy the scaffolding upon and within which the cells exist. Furthermore, the electrical field does not cause irreversible tissue damage as a result of heating of the tissue. Various ways of providing such an electrical field are possible. General parameters follow; however, those of skill in the art are fully capable of devising alternative combinations to achieve the same end result without undue experimentation. In typical embodiments, one or more electrical pulses are applied to the tissue to cause cell membrane disruption as a result of the electricity and not substantially as a result of heat. Where two or more pulses are used, the pulses are separated by a period of time that allows, among other things, the tissue to cool so that thermal damage does not occur to a significant extent. For example, one or more electrical pulses can be applied to the tissue of interest for a duration in a range of from about 5 microseconds (μs) to about 62 seconds. For convenience, a short period of treatment might be desired. As such, in preferred embodiments, electrical pulses are applied for a period of about 1-10000 μs. Further, although there is no limit on the number of

pulses to be delivered to the tissues, in preferred embodiments, from about 1 to about 100 pulses are applied to the tissue. For example, in an exemplary embodiment, about 10-1000 pulses of about 100 μs each in duration are applied to the tissue to cause cellular ablation.

There are several parameters that can be monitored and adjusted in using non-thermal IRE for preparation of tissue scaffolds. One such parameter is voltage gradient. In some embodiments, the pulses produce a voltage gradient in a range of from about 10 volt/cm to about 10,000 volt/cm. Voltage gradient (electric field) is a function of the distance between electrodes and electrode geometry, which will vary depending on the size of the tissue sample, tissue properties, and other factors. In some embodiments, two electrodes are used, and they are placed about 5 mm to 10 cm apart. Typical electrode diameters range from 0.25-1.5 mm and typically 2 or 4 electrodes are used. In embodiments, one bipolar electrode is used. Also, the "electrode" can have parts of it insulating (including using a non-conductive sheath) and parts of it conductive (e.g., at the tip) to ensure proper application of the electrical current and to minimize production of excessive heat in parts of the tissue.

Appropriate electrical fields and durations of exposure are those that have been reported in the literature as being suitable for medical treatment of tissues for tumor ablation. Exemplary exposure parameters include: ninety 90 microsecond (μs) pulses at 1.5 kV/cm at a frequency of 1 Hz; eighty 100 μs pulses at 2.5 kV/cm at a frequency of 1 Hz; one 20 millisecond pulse at 400 V/cm; ten 100 μs pulses at 3800 V/cm at a frequency of 10 pulses per second; ninety 100 μs pulses ranging from 1000 to 1667 V/cm at a frequency of about 1 Hz; and eighty pulses of 100 μs ranging from 1000 to 3000 V/cm at about 1 Hz. In general, the frequency of pulsing can be as low as twice the pulse width and can be quite a bit farther apart. Any suitable frequency that allows for electroporation without significant thermal damage to the tissue is acceptable. Furthermore, electrical current can be supplied as either DC or AC.

The shape and size of the electrodes are not critical to practice of the invention. Those of skill in the art may choose any shape and size that is suitable for transmitting the desired electrical field into the tissue. For example, the electrodes may be circular in shape, ovoid, square, rectangular, diamond-shaped, hexagonal, octagonal, etc. Likewise, the surface area of the electrodes is not critical to practice of the invention. Thus, for example, the surface area may be about 0.5 square centimeter, about 1 square centimeter, or greater.

Exposing the tissue to the electrical field generates heat. To ensure that tissue damage due to heat is avoided, the amount of energy transmitted to the tissue is set below a threshold level per unit time. Time and temperature parameters are known in the art for IRE, and any suitable combination may be used. For example, the temperature of the tissue can be monitored during treatment for ablation, and the electrical pulses adjusted to maintain the temperature at 100° C. or less, such as 60° C. or less. Preferably, the temperature is maintained at 50° C. or less.

In some embodiments, the method includes adjusting the applied voltage, length of the pulses, and/or number of pulses to obtain irreversible electroporation averaged over the biological cells of the tissue, thereby achieving irreversible electroporation of the biological cells in the tissue at a level that minimizes damage to non-target tissue. Likewise, in some embodiments, the duration of the applied voltage is adjusted in accordance with the current-to-voltage ratio to achieve irreversible electroporation of identified tissue cells, whereby cell membranes are disrupted in a manner resulting in cell death. Additional exemplary parameters are disclosed below.

The present invention thus comprises a method for the creation of scaffolds, involving the placement of electrodes into or near the vicinity of the original tissue with the application of electrical pulses causing irreversible electroporation of the cells throughout the entire treated region. It is to be noted that placement of the electrodes defines the treated region; thus, the treated region may be only a portion of an entire tissue or organ that is used as the starting material. The electric pulses irreversibly permeate the membranes of treated cells, thereby invoking cell death. The length of time of the electrical pulses, the voltage applied, and the resulting membrane permeability are all controlled within defined ranges. Application of electric pulses results in cell death, but preserves some or all of the vascular and neural structures, preferably including those involved in microcirculation. Thus, in some embodiments, microcirculation structures may be partially or totally damaged, but larger structures maintained.

For in vitro practice of this aspect of the invention, secondary techniques for removing cellular material can be used. For example, any of the known physical, chemical, or enzymatic techniques can be used to remove cellular debris from the irreversibly permeabilized cells. Likewise, the treated tissue can be attached to an artificial perfusion pump, which can pump a liquid composition (e.g., a detergent-containing aqueous composition) through the treated tissue, resulting in removal of cell debris from the scaffold. Importantly, such secondary treatments, where applied, can be applied under relatively gentle conditions, which allow for removal of cellular debris but also retention of the scaffolding structure (including vascular and neural structures). The use of non-thermal IRE allows for such gentle procedures, and improves the scaffold that is ultimately produced, as compared to procedures non relying on non-thermal IRE.

For in vivo practice of the method, the debris remaining from the irreversibly permeabilized cells may be left in situ and may be removed by natural processes, such as the body's own circulation and immune system.

The amount of tissue ablation achievable through the use of irreversible electroporation without inducing thermal damage is considerable, as disclosed and described herein.

The concept of irreversible electroporation to decellularize tissues is different from other forms decellularization used in the art. Irreversible electroporation is different from chemical and physical methods or cell lysis via osmotic imbalance because it uses electricity to kill the cells. Irreversible electroporation is a more benign method because it destroys only the cell membrane of cells in the targeted tissue and does no damage to the underlying ECM. Chemical and physical methods can damage vital structures, such as the ECM, blood vessels, and nerves. In contrast, IRE of the type described here, solely uses electrical pulses to serve as the active means for inducing cell death by a specific means, i.e., by fatally disrupting the cell membrane.

Irreversible electroporation may be used for the decellularizing tissue in a minimally invasive procedure that does not or does not substantially affect the ECM. Its non-selective mode of decellularization is acceptable in the field of tissue engineering and provides results that in some ways are comparable to sonication, inducing an osmotic imbalance, freezing, or chemical decellularization.

One exemplary embodiment of the invention includes a method whereby cells of tissue are irreversibly electroporated by applying pulses of very precisely determined length

and voltage. This may be done while measuring and/or observing changes in electrical impedance in real time and noting decreases at the onset of electroporation and adjusting the current in real time to obtain irreversible cellular damage without thermal damage. The method thus may include use of a computing device and sensors to monitor the effects of the electrical treatment. In embodiments where voltage is applied, the monitoring of the impedance affords the user knowledge of the presence or absence of pores. This measurement shows the progress of the pore formation and indicates whether irreversible pore formation, leading to cell death, has occurred.

Yet another embodiment includes a method whereby the onset and extent of electroporation of cells in tissue can be correlated to changes in the electrical impedance (which term is used herein to mean the voltage over current) of the tissue. At a given point, the electroporation becomes irreversible. A decrease in the resistivity of a group of biological cells occurs when membranes of the cells become permeable due to pore formation. By monitoring the impedance of the biological cells in a tissue, one can detect the average point in time in which pore formation of the cells occurs, as well as the relative degree of cell membrane permeability due to the pore formation. By gradually increasing voltage and testing cells in a given tissue, one can determine a point where irreversible electroporation occurs. This information can then be used to establish that, on average, the cells of the tissue have, in fact, undergone irreversible electroporation. This information can also be used to control the electroporation process by governing the selection of the voltage magnitude. Other imaging techniques can be employed to monitor how much area has been treated (e.g., ultrasound, MRI).

The invention provides the simultaneous irreversible electroporation of multitudes of cells providing a direct indication of the actual occurrence of electroporation and an indication of the degree of electroporation averaged over the multitude. The discovery is likewise useful in the irreversible electroporation of biological tissue (masses of biological cells with contiguous membranes) for the same reasons. The benefits of this process include a high level of control over the beginning point of irreversible electroporation.

One feature of embodiments of the invention is that the magnitude of electrical current during electroporation of the tissue becomes dependent on the degree of electroporation so that current and pulse length are adjusted within a range predetermined to obtain irreversible electroporation of targeted cells of the tissue while minimizing cellular damage to surrounding cells and tissue. Yet another feature of embodiments of the invention is that pulse length and current are precisely adjusted within ranges to provide more than mere intracellular elecctro-manipulation which results in cell death and less than that which would cause thermal damages to the surrounding tissues. Another feature of embodiments is that measuring current (in real time) through a circuit gives a measurement of the average overall degree of electroporation that the cells in the tissue achieve.

Yet other features of embodiments include: the precise electrical resistance of the tissue can be calculated from cross-time voltage measurement with probe electrodes and cross-current measurement with the circuit attached to electroporation electrodes; the precise electrical resistance of the tissue is calculated from cross-time voltage measurement with probe electrodes and cross-current measurement with the circuit attached to electroporation electrodes; and electrical measurements of the tissue can be used to map the electroporation distribution of the tissue. It is noted that, in irreversible electroporation it is possible and perhaps even preferred to perform the current or EIT measurements a substantial time (several minutes or more) after the electroporation to verify that it is indeed irreversible.

In embodiments of the method, it is preferred to remove cellular debris from the decellularized scaffolding after primary cell destruction with non-thermal IRE. In such embodiments, any known technique for doing so may be used, including any of the known physical, chemical, and/or enzymatic methods. In one exemplary embodiment, removal of cellular material is accomplished, at least in part, through perfusion of the tissue scaffolding with an appropriate agent (e.g., water, pH-adjusted water, an aqueous solution of one or more chelating agents, etc.), using general diffusion, transmittal via remaining intact vasculature, or a mixture of the two.

For in vitro methods, it is preferred that the scaffold be sterilized, especially where the scaffold is to be used to prepare engineered tissues and organs for implantation into a host. Sterilization and/or removal of debris after decellularization is usually conducted for scaffolds that will be used as implants to reduce the risk of patient rejection (for example, due to DNA fragments). When a scaffold requires some type of sterilization, methods published in the literature for sterilization of scaffolds can be employed.

For in vitro methods, the method of making a decellularized tissue scaffold results in a decellularized tissue scaffold that is isolated from its natural environment. For in vivo methods, the method of making a decellularized tissue scaffold results in a tissue scaffold that is devoid of normal cellular material. Thus, in an aspect of the invention, an engineered tissue scaffold is provided. The engineered tissue scaffold comprises a natural scaffold that is removed from its natural environment and/or from which cellular material has been removed. The engineered tissue scaffold of the invention contains at least some, preferably most, and more preferably substantially all or all, of the vascular structures (i.e., arteries, veins, capillaries) present in the tissue in its natural state. In embodiments, the tissue scaffold comprises at least some, preferably most, and more preferably substantially all or all of the neural structures present in the tissue in its natural state. In embodiments, the scaffold further comprises the cells that constitute these vascular structures and/or these neural structures. Thus, preferably, the engineered tissue scaffold contains a reduced number of the cells naturally populating the scaffold. In preferred embodiments, a majority of the original cells, more preferably substantially all of the original cells, and most preferably all of the original cells, are absent from the engineered scaffold. In embodiments, the remaining cells are cells that comprise vascular or neural structures. Furthermore, in preferred embodiments, some, most, or all of the cellular debris from the original cells is absent from the engineered scaffold. Likewise, in embodiments, the tissue scaffold contains some or all of the neurons originally present in the tissue. However, in embodiments, the neurons are destroyed but the neural tubes in which the neurons existed remain intact.

In some embodiments, the engineered scaffold comprises cell debris from cells originally (i.e., naturally) populating the scaffold. As discussed above, in such embodiments, the cell debris can be removed using known means. Alternatively, some or all of the cell debris may be left in and on the scaffold. In embodiments where cell debris is left on the scaffold, it can be later removed by the action of new cells seeded onto the scaffold and/or during the process of seeding, infiltration, and growth of new cells. For example,

where new cells are seeded onto a scaffold comprising cell debris, the action of the new cells infiltrating and growing, alone or in combination with a perfusion means for feeding and supporting the new cells, can result in removal of the cell debris.

The present invention provides, for the first time, engineered tissue scaffolds that comprise vascular structures that can function in providing nutrients and gases to cells growing on and in the scaffolds. The use of non-thermal IRE to create the engineered scaffolds permits retention of these important structures, and thus provides for improved scaffolds for use in medical, veterinary, and research activities. The invention thus provides engineered scaffolds capable of having relatively large dimensions. That is, because reseeded cells growing within the inventive scaffolds need not be close (i.e., within 1 mm) to an external surface in order to obtain nutrients and gas, the engineered scaffolds may be thicker than scaffolds previously known in the art. Engineered scaffolds may have thicknesses of any desirable range, the only limitation being the ability to generate the appropriate electrical field to cause decellularization. However, such a limitation is not a significant physical constraint, as placement of electrodes to effect IRE is easily adjusted and manipulated according to the desires of the practitioners of the invention.

Engineered scaffolds of the invention can have thicknesses that approach or mimic the thicknesses of the tissues and organs from which they are derived. Exemplary thicknesses range from relatively thin (i.e., 1 mm or less) to moderately thick (i.e., about 5 mm to 1 cm) to relatively thick (i.e., 5 cm or more).

The disclosure, above, has focused on engineered tissue scaffolds and methods of making them. The invention also encompasses engineered tissues and methods of making them. In general, the methods of making engineered tissues comprises: seeding an engineered scaffolding according to the invention with a cell of interest, and exposing the seeded scaffold to conditions whereby the seeded cells can infiltrate the scaffold matrix and grow. Seeding of the scaffold can be by any technique known in the art. Likewise, exposing the seeded scaffold to conditions whereby the cells can infiltrate the scaffold and grow can be any technique known in the art for achieving the result. For example, it can comprise incubating the seeded scaffold in vitro in a commercial incubator at about 37° C. in commercial growth media, which can be supplemented with appropriate growth factors, antibiotics, etc., if desired. Those of skill in the art are fully capable of selecting appropriate seeding and proliferation techniques and parameters without a detailed description herein. In other words, with respect to seeding and growth of cells, the scaffolds of the present invention generally behave in a similar manner to other natural scaffolds known in the art. Although the present scaffolds have beneficial properties not possessed by other scaffolds, these properties do not significantly affect seeding and growth of cells.

Engineered tissues have been developed as replacements for injured, diseased, or otherwise defective tissues. An important goal in the field of tissue engineering is to develop tissues for medical/therapeutic use in human health. In view of the difficulty and ethical issues surrounding use of human tissues as a source of scaffolds, tissues from large animals are typically used for the source material for natural scaffolds. The xenotypic scaffolds are then seeded with human cells for use in vivo in humans. While the presently disclosed engineered tissues are not limited to human tissues based on animal scaffolds, it is envisioned that a primary configuration of the engineered tissues will have that make-up. Thus, in embodiments, the engineered tissues of the invention are tissues comprising human cells on and within a scaffold derived from an animal tissue other than human tissue.

For certain in vivo uses, animal tissue is subjected in vivo to non-thermal IRE, and the treated tissue cleared of cell debris by the host animal's body. Thus, in certain in vivo embodiments, no secondary cell debris removal step is required, as the host animal's body itself is capable of such removal (this concept applies to in vivo creation of scaffolds in humans as well). The treated tissue is then seeded in vivo, for example with human cells, and the seeded cells allowed to infiltrate the scaffold and grow. Upon suitable infiltration and growth, the regenerated tissue is removed, preferably cleaned of possible contaminating host cells, and implanted into a recipient animal, for example a human. In such a situation, it is preferred that the host animal is one that has an impaired immune system that is incapable or poorly capable of recognizing the seeded cells as foreign cells. For example, a genetically modified animal having an impaired immune system can be used as the host animal. Alternatively, for example, the host animal can be given immune-suppressing agents to reduce or eliminate the animal's immune response to the seeded cells.

It is important to note at this point that the recipient can be any animal. It thus can be a human or another animal, such as a companion animal (i.e., a pet, such as a dog or cat), a farm animal (e.g., a bovine, porcine, ovine), or a sporting animal (e.g., a horse). The invention thus has applicability to both human and veterinarian health care and research fields.

Whether in vivo or in vitro, the choice of cells to be seeded will be left to the practitioner. Many cell types can be obtained, and those of skill in the tissue engineering field can easily determine which type of cell to use for seeding of tissues. For example, one may elect to use fibroblasts, chondrocytes, or hepatocytes. In embodiments, embryonic or adult stem cells, such as mesenchymal stem cells, are used to seed the scaffolds. The source of seeded cells is not particularly limited. Thus, the seeded cells may be autologous, syngenic or isogenic, allogenic, or xenogenic. However, because a feature of the present invention is the production of scaffolds and tissues that have reduced immunogenicity (as compared to scaffolds and tissues currently known in the art), it is preferred that the seeded cells be autologous (with relation to the recipient of the engineered tissue). In certain embodiments, it is preferred that the seeded cells be stem cells or other cells that are able to differentiate into the proper cell type for the tissue of interest.

Alternatively or additionally, the in vivo method of creating a scaffold and the in vivo method of creating an engineered tissue can include treating tissue near the non-thermal IRE treated cells with reversible electroporation. As part of the reversible electroporation, one or more genetic elements, proteins, or other substances (e.g., drugs) may be inserted into the treated cells. The genetic elements can include coding regions or other information that, when expressed, reduces interaction of the cells with the seeded cells, or otherwise produces anti-inflammatory or other anti-immunity substances. Short-term expression of such genetic elements can enhance the ability to grow engineered tissues in vivo without damage or rejection. Proteins and other substances can have an effect directly, either within the reversibly electroporated cells or as products released from the cells after electroporation.

Certain embodiments of the invention relate to use of human scaffolds for use in preparation of engineered human

tissues. As with other engineered tissues, such engineered tissues can be created in vitro, in vivo, or partially in vitro and partially in vivo. For example, tissue donors may have part or all of a tissue subjected to non-thermal IRE to produce a scaffold for tissue engineering for implantation of a recipient's cells, then growth of those cells. Upon infiltration and growth of the implanted cells, the tissue can be removed and implanted into the recipient in need of it. Of course, due to ethical concerns, the donor tissue should be tissue that is not critical for the life and health of the donor. For example, the tissue can be a portion of a liver. The engineered tissue, upon removal from the host and implanted in the recipient, can regenerate an entire functional liver, while the remaining portion of the host's liver can regenerate the portion removed.

Up to this point, the invention has been described in terms of engineered tissue scaffolds, engineered tissues, and methods of making them. It is important to note that the invention includes engineered organs and methods of making them as well. It is generally regarded that organs are defined portions of a multicellular animal that perform a discrete function or set of functions for the animal. It is further generally regarded that organs are made from tissues, and can be made from multiple types of tissues. Because the present invention is generally applicable to both tissues and organs, and the distinction between the two is not critical for understanding or practice of the invention, the terms "tissue" and "organ" are used herein interchangeably and with no intent to differentiate between the two.

Among the many concepts encompassed by the present invention, mention may be made of several exemplary concepts relating to engineered tissues. For example, in creating engineered organs, the initial organ can be completely removed of cells using irreversible electroporation prior to reseeding (this is especially relevant for organs having at least one dimension that is less than 1 mm); the organ can be irreversibly electroporated in sections and reseeded to allow the human cells to infiltrate small sections at a time; the organ can be irreversibly electroporated in incremental slices introducing the cells in stages, so that no human viable cells are in contact with the viable animal cells they are replacing; the organ can be irreversibly electroporated entirely or in sections and the human cells can be injected into targeted locations in the organ; the entire organ can be irreversibly electroporated to kill the animal cells, then human cells can be replanted on top of the organ to infiltrate the scaffold and replace the other cells (as the animal cells die, the human cells will fill in and substitute, thereby creating a new organ.)

Having provided isolated engineered tissues and organs, it is possible to provide methods of using them. The invention contemplates use of the engineered tissues in both in vitro and in vivo settings. Thus, the invention provides for use of the engineered tissues for research purposes and for therapeutic or medical/veterinary purposes. In research settings, an enormous number of practical applications exist for the technology. One example of such applications is use of the engineered tissues in an ex vivo cancer model, such as one to test the effectiveness of various ablation techniques (including, for example, radiation treatment, chemotherapy treatment, or a combination) in a lab, thus avoiding use of ill patients to optimize a treatment method. For example, one can attach a recently removed liver (e.g., pig liver) to a bioreactor or scaffold and treat the liver to ablate tissue. Another example of an in vivo use is for tissue engineering.

The engineered tissues of the present invention have use in vivo. Among the various uses, mention can be made of methods of in vivo treatment of subjects (used interchangeably herein with "patients", and meant to encompass both human and animals). In general for certain embodiments, methods of treating subjects comprise implanting an engineered tissue according to the invention into or on the surface of a subject, where implanting of the tissue results in a detectable change in the subject. The detectable change can be any change that can be detected using the natural senses or using man-made devices. While any type of treatment is envisioned by the present invention (e.g., therapeutic treatment of a disease or disorder, cosmetic treatment of skin blemishes, etc.), in many embodiments, the treatment will be therapeutic treatment of a disease, disorder, or other affliction of a subject. As such, a detectable change may be detection of a change, preferably an improvement, in at least one clinical symptom of a disease or disorder affecting the subject. Exemplary in vivo therapeutic methods include regeneration of organs after treatment for a tumor, preparation of a surgical site for implantation of a medical device, skin grafting, and replacement of part or all of a tissue or organ, such as one damaged or destroyed by a disease or disorder (e.g., the liver). Exemplary organs or tissues include: heart, lung, liver, kidney, urinary bladder, brain, ear, eye, or skin. In view of the fact that a subject may be a human or animal, the present invention has both medical and veterinary applications.

For example, the method of treating may be a method of regenerating a diseased or dysfunctional tissue in a subject. The method can comprise exposing a tissue to non-thermal IRE to kill cells of the treated tissue and create a tissue scaffold. The method can further comprise seeding the tissue scaffold with cells from outside of the subject, and allowing the seeded cells to proliferate in and on the tissue scaffold. Proliferation produces a regenerated tissue that contains healthy and functional cells. Such a method does not require removal of the tissue scaffold from the subject. Rather, the scaffold is created from the original tissue, then is re-seeded with healthy, functional cells. The entire process of scaffold creation, engineered tissue creation, and treatment of the subject is performed in vivo, with the possible exception of expansion of the cells to be seeded, which can be performed, if desired, in vitro.

In yet another exemplary embodiment, a tissue scaffold is created using non-thermal IRE to ablate a tissue in a donor animal. The treated tissue is allowed to remain in the donor's body to allow the body to clear cellular debris from the tissue scaffold. After an adequate amount of time, the treated tissue is removed from the donor's body and implanted into the recipient's body. The transplanted scaffold is not reseeded with external cells. Rather, the scaffold is naturally reseeded by the recipient's body to produce a functional tissue.

The present invention eliminates some of the major problems currently encountered with transplants. The methods described herein reduce the risk of rejection (as compared to traditional organ transplants) because the only cells remaining from the donor organ, if any, are cells involved in forming blood vessels and nerves. Yet at the same time, vascular and neural structures are maintained. As a result, the present invention provides a relatively rapid, effective, and straightforward way to produce engineered tissues having substantially natural structure and function, and having reduced immunogenicity. As such, the engineered tissues of the present invention are highly suitable for therapeutic and cosmetic use, while having a relatively low probability of rejection. In embodiments where human organs are used as the source of the scaffold (e.g., from live organ donors or cadavers), the risk of rejection is very small.

EXAMPLES

The invention will be further explained by the following Examples, which are intended to be purely exemplary of the invention, and should not be considered as limiting the invention in any way.

As a general background to the Examples, it is noted that the inventor and his colleagues have successfully demonstrated decellularization using IRE 1) in vivo and ex vivo, 2) to show that different tissues can be utilized, 3) to show that the area affected can be predicted using numerical modeling, 4) to show how numerical modeling can be used to ensure the ECM, blood vessels, and nerves are not thermally damaged, 5) while the organ was attached to a perfusion system, 6) while demonstrating preservation of major vasculature and ECM, and 7) with verification through imaging.

Ideally IRE performed ex vivo should be done as the tissue is perfused in a bioreactor. Perfusion of tissue in a bioreactor has been published in the literature, and the parameters disclosed therein can be generally applied within the present context. IRE is a special mode for cell ablation perfectly suitable to creating scaffolds because it kills the cells in the targeted area while sparing major blood vessels, connective tissue, nerves, and the surrounding tissue. Typically, mild enzymes or chemicals (non-ionic detergents, zwitterionic detergents, chelating agents, enzymatic methods) are used to facilitate removal of DNA fragments after decellularization. (It should be noted that for IRE in vivo, the removal of cells can be accomplished by the body's natural system.

The following is an "ideal" approach to implementing IRE ex vivo with a bioreactor perfusion system:

a) attach freshly excised organ to bioreactor perfusion system to maintain physiological environment (e.g., 37° C.);

b) perfuse organ with saline;

c) insert electrodes into targeted area;

d) apply IRE pulses;

e) optional: use gentle chemical (e.g., non-ionic detergent) or physical technique to remove cellular content/debris;

f) seed cells into the targeted/treated area;

g) perfuse organ with nutrients/growth media (demonstration of perfusion during IRE in Edd et al., 2006);

h) maintain bioreactor perfusion system at optimal conditions for cell growth (37° C.)

i) optional: repeat steps b-h until entire desired volume of tissue has been treated (e.g., to treat the entire organ).

It is to be noted that the order can be switched in many of these items.

Example 1

IRE on Freshly Excised Mouse Tissue to Create a Scaffold: IRE Scaffold Test Protocol

A single mouse was sacrificed via CO_2 asphyxiation and the liver was surgically removed. Two round sections (1 experimental, 1 control) were removed from the liver using a 5 mm core borer. A straight edge was then cut into each section to facilitate orientation and imaging. The first section was subjected to eighty 100 μs 2500 V/cm pulses at 4 Hz. The second section was not treated and left as control. Each section was then divided into 5 samples using a scalpel. The outer samples were discarded, leaving three samples from the experimental section and three samples from the control section.

One sample from the experimental section and one sample from the control section were subjected to sonication for 2 hours at 37° C. One sample from the experimental section and one sample from the control section were subjected to agitation via stir bar for 2 hours at 37° C. with a rotational rate of 60-300 rpm. One sample from the experimental section and one sample from the control section were placed in a water bath for 2 hours at 37° C.

Each of the experimental samples was then cut in half. Each section was then rinsed twice in DI water. Half of the experimental samples were fixed in formaldehyde for histology. The remaining experimental samples and all of the control samples were placed in individual 1.5 mL microvials of DI water and flash frozen in liquid nitrogen. The samples were freeze dried for 24-48 hours prior to imaging.

Results indicated that the experimental parameters were adequate for cell ablation of the tissue. Furthermore, no thermal damage to tissue was observed, and ECM, blood vessels, and nerves were preserved. Using this protocol and other parameters disclosed herein, various different IRE protocols can be designed to ensure no thermal damage to ECM, blood vessels, and nerves. Furthermore, highly customizable field distributions can be attained using different electrode geometries. Also, as shown in Edd and Davalos, 2007, tissue heterogeneity can be accounted for using numerical models.

Example 2

IRE Performance Indicia

To illustrate 1) the possibility to monitor creation of the scaffold in real-time using imaging techniques, 2) the variety of tissues that can be used, and 3) how to preserve vasculature, a healthy female purpose bred beagle was used. Nine sets of ten pulses were delivered with alternating polarity between the sets to prevent charge build-up on the electrode surfaces. The maximum voltage-to-distance ratio used was 2000 V/cm because the resulting current did not exceed 2 amps. The charge that was delivered to the brain during the IRE procedure was 22.5 mC, assuming ninety pulses (50 μs pulse durations) that would result from a maximum hypothetical current of 5 amps.

TABLE 1

IRE pulse parameters						
ELECTRODES	EXPOSURE LENGTH [mm]	GAP DISTANCE [mm]	VOLTAGE [V]	VOLTAGE TO DISTANCE RATIO [V/cm]	PULSES	PULSE DURATION [μs]
1 mm Monopolar	5	5	500	1000	90	50
Bipolar	Standard	7	1600	2000	90	50

Method: After induction of general anesthesia, a routine parietotemporal craniectomy defect was created to expose the right temporal lobe of the brain. Two decelluarization sites were performed: 1) a deep lesion within the caudal aspect of the temporal lobe using a monopolar electrode configuration (6 mm electrode insertion depth perpendicular to the surface of the target gyrus, with 5 mm interelectrode

distance), and 2) a superficial lesion in the cranial aspect of the temporal lobe using a bipolar electrode (inserted 2 cm parallel to the rostrocaudal length of the target gyrus, and 2 mm below the external surface of the gyrus). Intraoperative adverse effects that were encountered included gross microhemorrhages around the sharp monopolar electrode needles following insertion into the gyrus. This hemorrhage was controlled with topical application of hemostatic foam. Subject motion was completely obliterated prior to ablating the superficial site by escalating the dose of atracurium to 0.4 mg/kg. Grossly visible brain edema and surface blanching of the gyrus overlying the bipolar electrode decelluarization site was apparent within 2 minutes of completion of IRE at this site. This edema resolved completely following intravenous administration of 1.0 g/kg of 20% mannitol. No adverse clinically apparent effects attributable to the IRE procedure, or significant deterioration in neurologic disability or coma scale scores from baseline evaluations were observed.

Methods to monitor creation of scaffold: A unique advantage of IRE to create scaffolds is its ability to be monitored in real-time using imaging techniques, such as electrical impedance tomography, MRI, and ultrasound. Below, this Example shows MRI examinations performed immediate post-operatively, which demonstrate that IRE decelluarization zones were sharply demarcated (FIGS. **1**A-C).

As shown in FIG. **1**, neurosonography performed intraoperatively and at 1 hour and 24 hours post-procedure demonstrated clearly demarcated decellularization zones and visible needle tracts within the targeted brain parenchyma. Intraoperatively and immediately postoperatively, the decellularization zones appeared as hypoechoic foci with needle tracts appearing as distinct hyperechoic regions (FIG. **2**). Neurosonographically, at the 24 hour examination the IRE decellularization zone was hypoechoic with a hyperechoic rim (FIG. **2**). Compared to the 1 hour post-operative sonogram, the IRE decelluarization zone appeared slightly larger (1-2 mm increase in maximal, two dimensional diameter). EEG performed in the post-operative period revealed focal slowing of the background rhythm over the right temporal region in association with the decelluarization zones.

Macrolevel and histologic verification of treating cells: The brain was collected within 2 hours of the time of death and removed from the cranium. Care was taken to inspect soft tissues and areas of closure created at the time of surgery. The brain was placed in 10% neutral buffered formalin solution for a minimum of 48 hours. Then, the brain was sectioned at 3 mm intervals across the short axis of the brain, in order to preserve symmetry and to compare lesions. Following gross dissection of fixed tissues, photographs were taken of brain sections in order to document the position and character of lesions as shown in FIG. **3**. Readily apparent in gross photographs of the sectioned brain are lesions created either by the physical penetration of brain substance with electrodes or created by the application of pulse through the electrodes. There are relatively well-demarcated zones of hemorrhage and malacia at the sites of pulse delivery.

Microscopic lesions correlated well with macroscale appearance. Areas of treatment are represented by foci of malacia and dissociation of white and grey matter. Small perivascular hemorrhages are present and there is sparing of major blood vessels (see FIG. **4**B). Notable in multiple sections is a relatively sharp line of demarcation (approximately 20-30 μm) between areas of frank malacia and more normal, organized brain substance (see FIG. **4**A).

Analysis to determine IRE threshold: To determine the electric field needed to irreversibly electroporate tissue, one can correlate the lesion size that was observed in the ultrasound and MRI images with that in the histopathological analysis to determine the percentage of lesion growth. Decellularized site volumes can be determined after identification and demarcation of IRE decellularization zones from surrounding brain tissue using hand-drawn regions of interest (ROI). A representative source sample image is provided in FIG. **5**.

It will be apparent to those skilled in the art that various modifications and variations can be made in the practice of the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

The invention claimed is:

1. A method of making an engineered tissue by electrical ablation comprising:

positioning at least one electrode in a target region containing tissue cells;

applying a plurality of electrical pulses through the electrode in an amount sufficient to induce cell death by electrical ablation of the tissue cells in the target region in vivo to create an engineered tissue scaffold, wherein the step of applying leaves intact vascular structures present in the target region; and

seeding the ablated target region in vivo or in vitro and allowing seeded cells to proliferate to create the engineered tissue.

2. The method of claim **1**, further comprising removing cellular debris from the ablated target region by one or more of physical, chemical and enzymatic techniques.

3. The method of claim **2**, wherein the step of removing includes introducing a detergent material to the engineered tissue scaffold after the applying step.

4. The method of claim **2**, wherein the step of removing includes pumping a material to the engineered tissue scaffold using a perfusion pump.

5. The method of claim **4**, wherein the step of pumping a material includes pumping a detergent material to the engineered tissue scaffold.

6. The method of claim **1**, where the step of seeding includes seeding the ablated target region, in vitro, the method further comprising exposing the seeded scaffold to conditions that permit growth of the seeded cells.

7. The method of claim **6**, wherein the step of exposing includes incubating the seeded target region in an incubator at a predetermined temperature.

8. The method of claim **6**, wherein:

the step of applying includes applying the electrical pulses to the target region of an animal, in vivo; and

the step of seeding includes seeding the ablated target region with cells from a human.

9. The method of claim **8**, wherein the electrical ablation is used on the target region of an animal, or of a human, and the target region seeded with cells is removed from the animal for implantation of the target region into a human.

10. The method of claim **1**, further comprising implanting the engineered tissue into a subject in need of tissue replacement, the engineered tissue replacing at least a portion of an organ that has diminished or no function wherein the organ is a heart, a lung, a liver, a kidney, a urinary bladder, a brain, an ear, an eye, or skin.

11. The method of claim 1, wherein the step of applying includes applying the electrical pulses in a manner not to cause irreversible damage to major blood vessels present in the target region.

12. The method of claim 1, further comprising attaching the target region to a bioreactor perfusion system in vitro.

13. The method of claim 12, wherein the step of attaching is performed prior to the step of seeding.

14. The method of claim 12, after the step of attaching, further comprising perfusing the target region with nutrients.

15. The method of claim 14, wherein the step of perfusing occurs after the step of seeding.

16. The method of claim 14, after the step of attaching, further comprising perfusing the target region with saline.

17. The method of claim 12, after the step of applying the electrical pulses, further comprising perfusing the target region with detergent.

18. A method of making an engineered tissue by irreversible electroporation comprising:

positioning at least one electrode in a target region containing tissue cells;

applying a plurality of electrical pulses through the electrode in an amount sufficient to induce cell death by irreversible electroporation of the tissue cells in the target region in vivo, to create an engineered tissue scaffold;

wherein the step of applying leaves intact vascular structures present in the target region; and

seeding the engineered tissue scaffold in vivo or in vitro and allowing seeded cells to proliferate to create the engineered tissue.

19. The method of claim 18, wherein:

the step of applying includes applying the electrical pulses to the target region, in vivo; and

the step of seeding includes seeding the ablated target region, in vivo.

20. The method of claim 18, wherein:

the step of applying includes applying the electrical pulses to the target region, in vivo; and

the step of seeding includes seeding the ablated target region, in vitro.

21. The method of claim 18, further comprising removing cellular debris from the ablated target region by introducing a detergent material to the engineered tissue scaffold.

22. The method of claim 18, where the step of seeding includes seeding the ablated target region, in vitro, the method further comprising incubating the seeded target region in an incubator at a predetermined temperature to permit growth of the seeded cells in the tissue scaffold.

23. The method of claim 18, further comprising implanting the engineered tissue into a subject in need of tissue replacement, the engineered tissue replacing at least a portion of an organ that has diminished or no function wherein the organ is a heart, a lung, a liver, a kidney, a urinary bladder, a brain, an ear, an eye, or skin.

24. The method of claim 18, further comprising attaching the target region to a bioreactor perfusion system in vitro.

25. The method of claim 24, after the step of attaching, further comprising perfusing the target region with nutrients.

26. The method of claim 24, after the step of applying the electrical pulses, further comprising perfusing the target region with detergent.

27. A method of making an engineered tissue scaffold by electrical ablation comprising:

positioning at least one electrode in a target region containing tissue cells;

applying a plurality of electrical pulses through the electrode in an amount sufficient to induce cell death by electrical ablation of the tissue cells in the target region;

wherein the step of applying leaves intact vascular structures present in the target region in vivo; and

removing the ablated tissue region from a body.

28. The method of claim 27, further comprising seeding the ablated tissue region with cells.

29. The method of claim 28, wherein the step of seeding includes seeding the ablated tissue region in vitro.

30. The method of claim 27, further comprising implanting the engineered tissue scaffold into a subject in need of tissue replacement, the engineered tissue scaffold replacing at least a portion of an organ that has diminished or no function wherein the organ is a heart, a lung, a liver, a kidney, a urinary bladder, a brain, an ear, an eye, or skin.

31. The method of claim 27, further comprising removing cellular debris from the ablated target region by introducing a detergent material in vitro.

32. The method of claim 27, further comprising attaching the target region to a bioreactor perfusion system in vitro.

33. The method of claim 32, after the step of attaching, further comprising perfusing the target region with nutrients.

34. The method of claim 32, after the step of applying the electrical pulses, further comprising perfusing the target region with a detergent.

35. A method of making an engineered tissue by electrical ablation comprising:

positioning at least one electrode in a target region containing tissue cells;

applying, in vivo, a plurality of electrical pulses through the electrode in an amount sufficient to induce cell death by electrical ablation of the tissue cells in the target region to create an engineered tissue scaffold, wherein the step of applying is based on a protocol that leaves intact vascular structures for microcirculation in the target region;

seeding the ablated target region and allowing seeded cells to proliferate to create the engineered tissue.

36. The method of claim 35, further comprising removing the ablated target region from a body to create the engineered tissue scaffold.

37. The method of claim 35, wherein the vascular structures for microcirculation are left intact by limiting an amount of current delivered to the target region to a maximum of 5 amps.

38. The method of claim 35, wherein the vascular structures for microcirculation are left intact by limiting a voltage gradient delivered to the target region to 10 to 10,000 V/cm.

39. The method of claim 35, wherein the vascular structures for microcirculation are left intact by limiting a charge delivered to the target region to 22.5 mC.

40. The method of claim 27, wherein the engineered tissue scaffold has a thickness that approaches or mimics the thickness of the type of tissue or organ from which the engineered tissue scaffold is derived.

41. The method of claim 27, wherein the engineered tissue scaffold has a thickness of about 5 mm or more.

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