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(54) **Title:** DECELLULARIZED TISSUE/NANOFIBER/HYDROGEL HYBRID MATERIAL FOR OPTIMIZED TISSUE REGENERATION

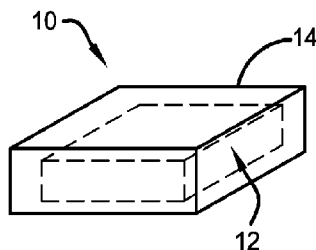


FIG. 1A

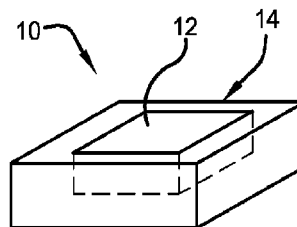


FIG. 1B

(57) **Abstract:** A wound dressing includes decellularized tissue and fibers at least partially surrounded by a PEG hydrogel, wherein the PEG hydrogel is structured by covalent bonds formed via an oxime ligation reaction of a polyethylene glycol (PEG) containing component and a cross-linking component. A method of making a hydrogel includes mixing a polyethylene glycol (PEG) component with a cross-linking component in a buffer solution, the PEG component having multiple arms with terminal functionality selected from ketone functionality and aldehyde functionality, and the cross-linking component having multiple arms with terminal aminoxy functionality, the hydrogel forming in the buffer solution by oxime ligation reaction, wherein the pH of the buffer solution used to make the hydrogel has a pH range of from 1.5 to 7.6, and the concentration of the buffer solution is from about 10 mM to about 100 mM

**DECELLULARIZED TISSUE/NANOFIBER/HYDROGEL HYBRID MATERIAL FOR OPTIMIZED TISSUE  
REGENERATION**

**CROSS-REFERENCE TO RELATED APPLICATIONS**

- 5   **[0001]**    This application claims the benefit of U.S. Provisional Application No. 62/116,600 filed on February 16, 2015, the content of which is incorporated herein by reference.

**FIELD OF THE INVENTION**

- 10   **[0002]**    The present invention generally relates to the use and method of making a hybrid material containing decellularized tissue. Specifically, the hybrid material comprises decellularized tissue that has been encased and stabilized in a PEG hydrogel matrix. In some embodiments, the PEG hydrogel matrix contains nanofibers which further assist in encasing and stabilizing the decellularized tissue.

15

**BACKGROUND OF THE INVENTION**

- [0003]**    Tissue engineering is a biomedical technique used to develop synthetic/biological scaffolds that can repair, maintain, or improve tissue function. An important feature of the scaffolds is its ability to allow cellular attachment, proliferation, and differentiation, followed by the development of healthy tissues prior to the scaffold's degradation. Human mesenchymal stem cells (hMSC) can sense their local environment and respond by altering their migration, signaling, and proliferation. Thus, the mechanical properties of scaffolds (i.e. modulus/elasticity) directly influence the maturation of hMSC's. Consequently, one key optimization for
- 20    tissue engineering is the mechanical properties of the scaffolds, which are governed by the target tissue.

- [0004]**    The most commonly used material for many applications in soft tissue repair is a polypropylene mesh. The polypropylene mesh is typically over engineered beyond native tissue parameters, primarily in strength. However, as a result of this
- 30    over engineering, the patient who uses the polypropylene mesh will have discomfort. Furthermore, the polypropylene mesh elicits a strong immunogenic response that leads to inflammation and fibrous capsule formation that result in a plethora of undesired consequences for tissue repair.

[0005] However, the large water content and favorable elastic properties of hydrogels make them promising candidates for various biomedical applications, such as scaffolds for tissue engineering. Hydrogels are materials that are primarily composed of hydrophilic cross-linked networks, containing as much as 99% water.

5 Synthetic polymers are the most preferred polymer to use in hydrogels, due to their reproducibility, high purity, and controlled molecular weight. Polyethylene glycol-based hydrogels, in particular, are most preferred because of their versatility in end group modification, hydrophilic chemical structure, and protein absorption resistance. Polyethylene glycol is also referred to herein by the well-known PEG  
10 abbreviation.

[0006] Notably, PEG-based hydrogels with terminal acrylate functionality have been found to be quite useful in tissue engineered scaffolds. Upon irradiation with ultraviolet light, they can form a three-dimensional hydrogel network with tunable mechanical properties dependent on the cross-link density, and the mass fraction of  
15 the polymer precursors. Unfortunately, free radical polymerizations have limited control, which leads to multiple cross-linking reactions across the solution, which leads to the formation of micro-clusters that are tied together by the polymer chains. Such networks are not favorable for incorporating biological cues due to their inhomogeneity and complex micro-cluster structural formations.

20 [0007] For more well defined cross-linked networks, orthogonal chemistries (click chemistries) are being used, specifically oxime "click" chemistry. "Click" chemistries in general are defined as highly efficient, robust, and orthogonal reactions that form hydrogels having superior properties in comparison to *in situ* polymerized gels. Specifically, oxime chemistry employs a kinetically dependent  
25 reaction that varies with buffer pH and ionic strength. Oxime chemistry allows for very mild reaction conditions because no metal catalyst is required, the polymerization temperature is between 20-40 °C, the pH ranges from 2-7, and the only by-product produces is water.

[0008] However, very few hydrogel systems are capable of achieving the wide  
30 range of elasticities without altering the chemical makeup, and therefore the physical properties of the scaffold, and controlling the physical properties of the scaffold is essential for the application of hydrogels in tissue engineering. Therefore, there is a need in the art for a hydrogel system that allows for the modulus to be dialed in, using

alternative means to control the mechanical properties, such as pH and buffer strength.

- [0009]** The novel hydrogels and hydrogel formation methods herein are also employed to provide novel wound dressings utilizing decellularized extracellular matrix. Decellularized extracellular matrix is generally known and forms an important component of the full hybrid wound dressings disclosed herein. Decellularized tissue has shown incredible regenerative capabilities when used in wound healing applications, and studies are currently investigating the potential of decellularization in full organ transplantation. It is believed the reason for this material's success is derived from the numerous proteins that reside in the matrix allowing for excellent wound healing performance. These proteins are cleaved by metalloproteinases within the wound, and signal to incoming cells to migrate to the wound, proliferate, and to lay down more matrix. The removal of cellular DNA from the material eliminates the immunogenic response seen in allograft and xenograft tissue transplantations, allowing for full clinical translation of this material. However, it is so quickly degraded in a wound site, such that in many cases the material disappears before full regeneration is complete. In these cases, wound recurrence occurs, leading to a second surgery using synthetic materials. This can be seen for example in the use of decellularized extracellular matrix in hernia repair.
- [0010]** The present invention provides further advances in the art by providing wound dressing better able to support and deliver the decellularized extracellular matrix to the wound site.

#### **SUMMARY OF THE INVENTION**

- [0011]** **[TO BE COMPLETED BY RENNER KENNER]**
- [0012]** In a first embodiment, the present invention provides a wound dressing comprising: decellularized tissue and fibers at least partially surrounded by a PEG hydrogel, wherein the PEG hydrogel is structured by covalent bonds formed via an oxime ligation reaction of a polyethylene glycol (PEG) containing component and a cross-linking component.
- [0013]** In a second embodiment, the present invention provides a wound dressing as in the first embodiment, wherein the fibers are formed from polymers selected from the group consisting of poly(ester urea) (PEU), polycaprolactone (PCL), poly-L-

lactide (PLLA), and poly(lactic-co-glycolic acid) (PLGA), functionalized PEU, functionalized PCL, functionalized PLLA, and functionalized PLGA.

[0014] In a third embodiment, the present invention provides a wound dressing as in either the first or second embodiment, wherein the PEG-containing component is tri-  
5 functional or greater containing ketone functionality, aldehyde functionality, or a combination thereof on the terminal end of the chains and having a molecular weight ranging from about 1,000 Da to about 50,000 Da.

[0015] In a fourth embodiment, the present invention provides a wound dressing as in any of the first through third embodiments, wherein the cross-linking component is  
10 selected from the group consisting of a 2-arm cross-linking component, a 4-arm cross-linking component, or a mixture thereof.

[0016] In a fifth embodiment, the present invention provides a wound dressing as in any of the first through fourth embodiments, wherein the PEG hydrogel is formed using a buffer solution, wherein the buffer solution the buffer solution is selected from the  
15 group consisting of citric acid, disodium phosphate, and combinations thereof.

[0017] In a sixth embodiment, the present invention provides a wound dressing as in any of the first through fifth embodiments, wherein the pH of the buffer solution is from about 1.5 to about 7.5.

[0018] In a seventh embodiment, the present invention provides a wound dressing as  
20 in any of the first through sixth embodiments, wherein the pH of the buffer solution is from about 2.0 to about 7.6.

[0019] In an eighth embodiment, the present invention provides a wound dressing as in any of the first through seventh embodiments, wherein the concentration of the buffer solution is from about 10 mM to about 100 mM

25 [0020] In a ninth embodiment, the present invention provides a wound dressing as in any of the first through eighth embodiments, wherein the concentration of the buffer solution is from about 20 mM to about 50 mM.

[0021] In a tenth embodiment, the present invention provides a method of making a wound dressing comprising the steps of laying down decellularized tissue into a bath  
30 of a buffer solution and PEG-containing component, adding fibers to the top of the decellularized, and then adding a cross-linking component to the bath of buffer solution and the PEG-containing component to create a PEG hydrogel to at least partially surround the decellularized tissue and fibers.

[0022] In an eleventh embodiment, the present invention provides a method of making a wound dressing as in the tenth embodiment, wherein the fibers are formed from polymers selected from the group consisting of poly(ester urea) (PEU), polycaprolactone (PCL), poly-L-lactide (PLLA), and poly(lactic-co-glycolic acid) (PLGA), functionalized PEU, functionalized PCL, functionalized PLLA, and functionalized PLGA.

[0023] In a twelfth embodiment, the present invention provides a method of making a wound dressing as in any of the tenth through eleventh embodiments, wherein the fibers are electrospun onto the decellularized tissue.

10 [0024] In a thirteenth embodiment, the present invention provides a method of making a wound dressing as in any of the tenth through twelfth embodiments, wherein the PEG-containing component is tri-functional or greater containing ketone functionality, aldehyde functionality, or a combination thereof on the terminal end of the chains and having a molecular weight ranging from about 1,000 Da to about 15 50,000 Da.

[0025] In a fourteenth embodiment, the present invention provides a method of making a wound dressing as in any of the tenth through thirteenth embodiments, wherein the cross-linking component is selected from the group consisting of a 2-arm cross-linking component, a 4-arm cross-linking component, or a mixture thereof.

20 [0026] In a fifteenth embodiment, the present invention provides a method of making a wound dressing as in any of the tenth through fourteenth embodiments, wherein the buffer solution is selected from the group consisting of citric acid, disodium phosphate, and combinations thereof.

[0027] In a sixteenth embodiment, the present invention provides a method of making a wound dressing as in any of the tenth through fifteenth embodiments, wherein the pH of the buffer solution is from about 1.5 to about 7.5 and the concentration of the buffer solution is from about 10 mM to about 100 mM.

[0028] In a seventeenth embodiment, the present invention provides a method of making a wound dressing as in any of the tenth through sixteenth embodiments, wherein the pH of the buffer solution is from about 2 to about 7.6 and the concentration of the buffer solution is from about 20 mM to about 50 mM.

30 [0029] In an eighteenth embodiment, the present invention provides a method of making a hydrogel comprising the steps of mixing a polyethylene glycol (PEG)

component with a cross-linking component in a buffer solution, the PEG component having multiple arms with terminal functionality selected from ketone functionality and aldehyde functionality, and the cross-linking component having multiple arms with terminal aminooxy functionality, the hydrogel forming in the buffer solution by  
5 oxime ligation reaction, wherein the pH of the buffer solution used to make the hydrogel has a pH range of from 1.5 to 7.6, and the concentration of the buffer solution is from about 10 mM to about 100 mM.

[0030] In a nineteenth embodiment, the present invention provides a method of making a hydrogel as in the eighteenth embodiment, wherein the buffer solution is a  
10 phosphate-citrate buffer solution.

[0031] In a twentieth embodiment, the present invention provides a wound dressing comprising: decellularized tissue at least partially surrounded by a PEG hydrogel, wherein the PEG hydrogel is structured by covalent bonds formed via an oxime ligation reaction of a polyethylene glycol (PEG) containing component and a cross-linking component.

15 [0032] In a twenty-first embodiment, the present invention provides a wound dressing comprising: fibers at least partially surrounded by a PEG hydrogel, wherein the PEG hydrogel is structured by covalent bonds formed via an oxime ligation reaction of a polyethylene glycol (PEG) containing component and a cross-linking component.

20 **BRIEF DESCRIPTION OF THE DRAWINGS**

[0033] Fig. 1A is a schematic perspective view of a generic embodiment of the present invention, showing regenerative material completely encased in hydrogel;

[0034] Fig. 1B is a schematic perspective view of a generic embodiment of the present invention, showing regenerative material that is only partially encased or  
25 surrounded by the hydrogel such that the regenerative material is at least partially exposed;

[0035] Fig. 2 is a schematic side elevational view of a first embodiment of a wound dressing having only decellularized tissue as the regenerative material;

[0036] Fig. 3 is a schematic side elevational view of a second embodiment of a wound dressing having nanofibers as the regenerative material;

30 [0037] Fig. 4 is a schematic side elevational view of a third embodiment of a wound dressing having decellularized material supported by nanofibers as the regenerative material;

[0038] Fig. 5 is an exemplary reaction scheme for the synthesizing of a particular PEG-containing component with ketone functionality;

- [0039] Fig. 6 is an exemplary reaction scheme for the synthesizing of a particular four-arm cross-linking component;
- [0040] Fig. 7 provides a general schematic for manufacturing a wound dressing such as that of Fig. 2;
- 5 [0041] Fig. 8 provides a general schematic for manufacturing a wound dressing such as that of Fig. 3;
- [0042] Fig. 9 provides a general schematic for manufacturing a wound dressing such as that of Fig. 4;
- [0043] Fig. 10 shows hydrogel storage modulus and loss modulus as a function of  
10 buffer strength;
- [0044] Fig. 11 shows hydrogel storage modulus and loss modulus as a function of pH;
- [0045] Fig. 12 shows hydrogel storage modulus and loss modulus as a function of the weight percentage of PEG-containing component;
- [0046] Fig. 13 is a graph showing a strain sweep performed to determine the linear  
15 viscoelastic regime;
- [0047] Fig. 14 is a graph showing a frequency sweep performed to determine the linear viscoelastic regime;
- [0048] Fig. 15 is a general schematic of a specific hydrogel formation in accordance with this invention, the hydrogel formation notably using a cross-linking reaction  
20 involving oxime formation, which produces water as a by-product and does not require a metal catalyst,
- [0049] Fig. 16 is an exemplary reaction scheme for the synthesizing of a particular 4-arm PEG-containing component;
- [0050] Fig. 17 is an exemplary reaction scheme for the synthesizing of a particular  
25 arm PEG-containing component;
- [0051] Fig. 18 is an exemplary reaction scheme for the synthesizing of a particular 2-arm PEG-containing component;
- [0052] Fig. 19 is an exemplary reaction scheme for the synthesizing of a particular four-arm cross-linking component;
- 30 [0053] Fig. 20 is an exemplary reaction scheme for the synthesizing of a particular four-arm cross-linking component;
- [0054] Fig. 21 is an exemplary reaction scheme for the synthesizing of a particular three-arm cross-linking component; and

[0055] Fig. 22 shows data for a hydrogel system of 4-arm aminooxy cross-linker made from 10k MW PEG and equimolar amounts of 10k 4-arm PEG-ketone.

#### **DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS**

5 [0056] With reference to Figs. 1A and 1B, a broad characterization of a wound dressing of this invention is shown and designated by the numeral 10. The wound dressing 10 includes regenerative material 12 supported and at least partially surrounded by a polyethylene glycol (PEG) hydrogel 14. Through methods disclosed herein, the PEG hydrogel 14 is made to have mechanical properties making it suitable  
10 for placement at an intended wound site. The regenerative material 12 can be provided through different components.

[0057] The description “at least partially surrounded” can be understood by comparison of Figs. 1A and 1B, showing a wound dressing 10 and wound dressing 10'. In Fig. 1A, the regenerative material 12 is completely surrounded or encased in  
15 PEG hydrogel 14. In Fig. 1B, the regenerative material 12 is flush with the top surface of the PEG hydrogel 14 such that it is not fully surrounded or encased. In Fig. 1A, the regenerative material 12 is not immediately exposed, but will become so as the hydrogel biodegrades. In Fig. 1B, the regenerative material 12 is exposed at least partially. Each embodiment might have preferred applications with different types of  
20 wounds. These general schematics of Figs. 1A and 1B should be appreciated as being applicable to the embodiments of Figs. 2-4, with the understanding that Figs. 2-4 provide side view schematics for simplicity.

[0058] In a first embodiment shown in Fig. 2, a wound dressing 110 includes decellularized tissue 116 (also known as “decellularized extracellular matrix  
25 material”) as the regenerative material 112.

[0059] In a second embodiment shown in Fig. 3, a wound dressing 210 includes fibers 218 as the regenerative material 212, wherein the fibers are functionalized to provide regenerative properties.

[0060] In a third embodiment shown in Fig. 4, a wound dressing 310 the  
30 regenerative material 312 includes decellularized tissue 316 reinforced with fibers 318 as the regenerative material 112. In this embodiment, the regenerative properties can be provided by the decellularized tissue, alone, or both the decellularized tissue and the fibers. In some embodiments, the fibers will simply be

biodegradable and will serve to provide mechanical support for the decellularized tissue for the time period prior to their biodegradation. It is known that decellularized tissue can degrade too quickly at a wound site, thus disappearing before full regeneration is achieved (i.e., before an acceptable level of healing of the wound), thus allowing for recurrence of the wound. This is noted particularly in applications such as hernia repair. Thus, the fibers are provided to improve the performance of the wound dressing by degrading slower than the decellularized tissue and providing a structural integrity to the overall wound dressing suitable to prohibit recurrence.

5 [0061] In each embodiment, the PEG hydrogel 14 at least partially surrounds the regenerative material 112, 212, or 312. By at least partially surround, it is meant that when the PEG hydrogel 14 is formed, the PEG hydrogel 14 either completely encapsulates the regenerative material 112, 212, or 313, or a portion of the regenerative material 112, 212, or 313 is exposed wherein the rest of the regenerative material 112, 212, or 313 is encapsulated by the PEG hydrogel 14.

15 [0062] By varying the kinetics of cross-linking within the PEG hydrogel 14, small changes in the structural features of the PEG hydrogel 14 can be achieved. The kinetics of the network formation of the PEG hydrogel 14 are influenced by pH and buffer strength, and are utilized to intrinsically control the degree of heterogeneity within the microstructure of the PEG hydrogel 14. By varying the pH and buffer strength used in the formation of the PEG hydrogel 14, the properties of the PEG hydrogel can be controlled, but yet the chemical identity, concentration, and stoichiometry of the PEG hydrogel 14 is maintained. When the PEG hydrogel 14 is formed in accordance with the method provided herein below, the gelation is rapid but takes enough time to allow for manipulation and placement of the wound dressing (110, 210, or 310) at a desired wound site. When properly implanted, the PEG hydrogel 14 will degrade into readily metabolized and safe byproducts in a matter of days, leaving the regenerative material 112, 212, or 312 exposed for optimal wound healing performance. This PEG hydrogel 14 therefore acts mostly as a delivery vehicle and laminating agent for the regenerative material 112, 212, or 312.

25 [0063] The decellularized tissue (or decellularized extracellular matrix material) is selected to be non-toxic and biocompatible in its intended end application. The decellularized extracellular matrix contains structural proteins such as: elastin, laminin; functional proteins such as growth factors and cytokines, polysaccharides or

mineral phases. This material is commercially available in different forms known to those of skill in the art to be useful for different applications. In some embodiments, any tissue that is void of cellular DNA or remnant components that illicit an immunogenic reaction would be acceptable in this application, and are to be understood as “decellularized tissue.”

**[0064]** In embodiments where fibers are used to support decellularized tissue, the fibers can be formed from any fiber material that does not induce a prominent inflammatory reaction when used for the intended application at a wound site. In some such embodiments, the fibers can be formed of polymers selected from the group consisting of poly(ester urea) (PEU), polycaprolactone (PCL), poly-L-lactide (PLLA), and poly(lactic-co-glycolic acid) (PLGA), functionalized PEU, functionalized PCL, functionalized PLLA, and functionalized PLGA, wherein the functionalization serves to induce regeneration of tissue at the wound site, i.e., the polymer is functionalized with functional groups that provide regenerative properties. In embodiments where they are used alone, the fibers are formed of polymers having functional groups that provide regenerative properties, in some such embodiments, the fibers can be formed of polymers selected from the group consisting of functionalized PEU, functionalized PCL, functionalized PLLA, and functionalized PLGA.

**[0065]** In embodiments where the polymers forming the fibers include functional groups, those functional groups can be selected from the group consisting of peptides, carbohydrates, proteins, oligonucleotides, small molecule drugs, an oxygen atom connected to a alkyl or aryl group containing an alkyne group, an alkene group, an azide group, a benzyl protected phenol group, a ketone group or a strained cyclooctyne. In some embodiments, the polymers include peptides that signal to cells to regenerate tissue. In some embodiments, the functional groups are peptides selected from the group consisting of RGD, BMP 2, BMP 7, BMP 9, and OGP. In some embodiments, the peptide is RGD, a component of fibronectin that signals for cell attachment.

**[0066]** In some embodiments, the fibers are first formed by electrospinning and then functionalized. In other embodiments, the fibers are first functionalized and then formed into fibers by electrospinning. In some embodiments, the fibers are functionalized post-formation as disclosed in *Post-Electrospinning “Triclick”*

*Functionalization of Degradable Polymer Nanofibers*, by Becker M. L, et al., ACS Macro Lett., 2015, 4 (2), pp 207–213; and *Postelectrospinning “Click” Modification of Degradable Amino Acid-Based Poly(ester urea) Nanofibers*, by Becker M. L, et al, Macromolecules, 2013, 46 (24), pp 9515–9525, which are incorporated herein by  
5 reference.

**[0067]** Guidance on other useful fibers that can be used in some embodiments can be found in *“Click” reactions: a versatile toolbox for the synthesis of peptide-conjugates*, by Becker M. L, et al, Chemical Society Reviews, Volume 43, issue 20; and *Branched Amino Acid Based Poly(ester urea)s with Tunable Thermal and Water Uptake*  
10 *Properties*, by Becker M. L, et al, Macromolecules, 2015, 48 (9), pp 2916–2924.

**[0068]** In some embodiments, the fibers are biodegradable. The above mentioned polymers are biodegradable. In some embodiments, the fibers are biodegradable at a rate that matches the rate of tissue regeneration at the intended wound site.

**[0069]** In some embodiments the fibers are electrospun fibers. In some  
15 embodiments, the fibers are nanofibers. In some embodiments, the fibers are nanofibers that have been formed by electrospinning such that the polymer forming the nanofibers is chosen so as to be electrospinnable. The polymer materials above are suitable for electrospinning. For purposes herein, a nanofiber is to be understood as a fiber having a diameter of less than 2 microns. In particular embodiments, the  
20 nanofibers have a diameter of less than 1.5 microns, in other embodiments, less than 1.0 microns, in other embodiments, less than 750 nm, in other embodiments, less than 500 nm, in other embodiments, less than 400 nm, and, in other embodiments, less than 350 nm.

**[0070]** In some embodiments, the fibers comprise hyperbranched amino acid-based poly(ester urea), such as those disclosed in U.S. Patent Application No. 14/939,216, which is herein incorporated by reference.  
25

**[0071]** In some embodiments, the fibers have the ability to have their surfaces derivatized with peptides and fluorescent probes using bio-orthogonal reaction strategies, such as those fibers disclosed in International Patent Application No. PCT/US14/58264, which is herein incorporated by reference.  
30

**[0072]** In some embodiments, the fibers comprise an amino acid-based poly(ester urea) with amino acid residues selected from L-leucine, L-isoleucine, L-

valine or combinations thereof, such as those disclosed in International Patent Application No. PCT/US14/62888, which is herein incorporated by reference.

**[0073]** In some embodiments, the fibers have a round, smooth, and consistent morphology. It has been found that electrospinning can provide such morphologies.

5 **[0074]** In some embodiments, the fibers have a diameter greater than about 150 nm, in other embodiments the diameter is greater than about 400 nm, and in yet other embodiments the diameter is greater than about 700 nm. In some  
10 embodiments, the fibers have a diameter less than about 1500 nm, in other embodiments the diameter is less than about 1100 nm, and in yet other  
embodiments the diameter is less than about 900 nm. In some embodiments, the  
fibers have a diameter of from about 150 nm to about 1500 nm, in other  
embodiments the diameter is from about 400 nm to about 1100 nm, and in yet other  
embodiments the diameter is from about 700 nm to about 900 nm.

**[0075]** In some embodiments, the fibers form a fabric structure having a pore  
15 size greater than about 1.0  $\mu\text{m}$ , in other embodiments the pore size is greater than  
about 20  $\mu\text{m}$ , and in yet other embodiments the pore size is greater than about 50  
 $\mu\text{m}$ . In some embodiments, the fibers form a fabric structure having a pore size less  
than about 125  $\mu\text{m}$ , in other embodiments the pore size is less than about 100  $\mu\text{m}$ ,  
and in yet other embodiments the pore size is less than about 75  $\mu\text{m}$ . In some  
20 embodiments, the fibers have form a fabric structure having a pore size of from about  
1.0  $\mu\text{m}$  to about 125  $\mu\text{m}$ , in other embodiments the a pore size is from about 20  $\mu\text{m}$  to  
about 100  $\mu\text{m}$ , and in yet other embodiments the a pore size is from about 50  $\mu\text{m}$  to  
about 75  $\mu\text{m}$ .

**[0076]** In some embodiments, the fibers have a rate of degradation of from 3  
25 months to 2 years depending on application and need.

**[0077]** In some embodiments, the fibers have a cell viability of greater than 80%,  
in other embodiments greater than 90%, and in yet other embodiments greater than  
95%. In some embodiments, the fibers have a cell viability of between about 80% and  
about 100%, in other embodiments the fibers have a cell viability of between about  
30 90% and about 100%, and in yet other embodiments the fibers have a cell viability of  
between about 95% and about 100%.

**[0078]** Depending upon the embodiment being made, the decellularized tissue  
(Fig. 2), the fibers (Fig. 3), or the decellularized tissue reinforced with fibers (Fig. 4)

is at least partially surrounded and supported by a polyethylene glycol (PEG) hydrogel specifically taught herein.

5     **[0079]**     The PEG hydrogel 14 component is formed via an oxime ligation reaction of a polyethylene glycol (PEG) containing component and a cross-linking component in a buffer solution. The oxime ligation reaction produces water as a by-product, and is herein demonstrated to provide hydrogels with variable mechanical properties depending of the pH of the buffer.

10    **[0080]**     The PEG component and the cross-linking component are chosen so as to form crosslinked networks wherein distal functional ends of the PEG component and cross-linking component react through the oxime ligation reaction to form the hydrogel system. An example is shown in Fig. 15.

15    **[0081]**     To form suitable networks, a significant portion of the components must be tri-functional or tetra-functional or even penta-functional, etc. This can also be considered in relation to the chain arms or “arms” of a given component. Tri-functional components will be seen to have three arms terminating in the appropriate functional group as taught below. Similarly, tetra-functional components will be seen to have four arms terminated appropriately. A suitable number of the component must have tri-functionality and higher to create the covalently bonded network necessary for the formation of a suitably stable hydrogel. The ratios of each type of functional component necessary to form a suitable hydrogel will be appreciated to change with respect to the molecular weight of the components and the length of the arms serving to form the covalent network.

20    **[0082]**     In some embodiments, the PEG-containing component is selected to be tri-functional or greater, i.e., having three arms or four arms providing reactive terminal groups. In some embodiments, the cross-linker component has at least three functional sites for cross-linking, i.e., at least three arms providing reactive terminal groups, and the PEG component is di-functional, i.e., having only two arms. In embodiments currently practiced, the PEG-containing component is tetra-functional, and the cross-linking component is tetra-functional, and they are mixed in equimolar ratios (Fig. 15). It is found that a good homogenous network is formed from this combination, and slight alterations of this system could be used to tailor the properties around a useful range such as those found in particular studies experimentally presented herein below.

30    **[0083]**     In some embodiments, the PEG-containing component is tri-functional (3 arms) or tetra-functional (4-arms; e.g. Fig. 15). In some embodiments, the PEG-containing component has a molecular weight (MW) greater than about 1,000 Dalton

(Da), in other embodiments greater than about 2,000 Da, and in yet other embodiments greater than about 6,000 Da. In some embodiments, the PEG-containing component has a MW less than about 50,000 Da, in other embodiments less than about 25,000 Da, and in yet other embodiments less than about 10,000 Da.

5 In some embodiments, the PEG-containing component has with a MW ranging from about 1,000 Da to about 50,000 Da (1 kDa to 50 kDa), in other embodiments from about 2 kDa to 25 kDa, and in yet other embodiments, from about 6 kDa to about 10 kDa.

**[0084]** A sufficient number of the terminal ends of the arms of the components  
10 include certain functionality to participate in the oxime ligation reaction. Thus, in some embodiments, the PEG-containing component includes ketone functionality or aldehyde functionality or a mixture thereof to participate in the oxime reaction disclosed herein for forming the hydrogel. In some embodiments, the ketone functionality is afforded via coupling with levulinic acid.

15 **[0085]** In some embodiments, the PEG-containing component is selected from the group consisting of 10 kDa 4-arm PEG containing ketone functionality on the terminal end of the arms, In some embodiment the PEG containing component is a 4-arm PEG containing ketone functionality at the terminal end of each arm, and is synthesized via DIC-coupling of levulinic acid as in Fig. 5. For more examples of  
20 possible PEG-containing components see Fig. 16, which shows a 4-arm PEG-containing component, Fig. 17, which shows a 3-arm PEG-containing component, and Fig. 18, which shows a 2-arm PEG-containing component.

**[0086]** In some embodiments, the cross-linking component is selected to be tri-functional or greater, i.e., having three arms or four arms providing reactive terminal  
25 groups. In some embodiments, additional two-arm (di-functional) cross-linker is included in the creation of the hydrogel. In some embodiments, the cross-linking component contains either 4 arms or 2 arms. The cross-linking components are functionalized with aminoxy groups to enable oxime crosslinking with the ketone or aldehyde functionality of the PEG-containing component. In some embodiments, the two arm cross-linking  
30 component contains an allyl group for peptide or other conjugations. This is particularly suitable for functionalization by a thiol-ene “click” reaction post gel formation. Tuning the amount of 2-arm cross-linker that is incorporated also provides another handle for tuning the modulus of the gel. Aside from this, the post-gelation functionalization through the

allyl group is the primary desire for the 2-arm cross-linker. It is possible to “click” peptides to the surface of the gel that would provide favorable signals for cell migration and proliferation.

5     **[0087]**     In some embodiments, the 2-arm cross-linker is made by coupling 3-allyloxy-1,2-propanediol and boc-protected aminooxy acetic acid followed by deprotection to afford the aminooxy functional group. The allyl group (alkene) can be functionalized through a thiol-ene “click” reaction post gelation.

10     **[0088]**     In some embodiments, the cross-linking component is a 4 arm PEG molecule with MW from 2,000 – 10,000 containing aminooxy functional groups. In some embodiments, the cross-linking component is a 4-arm aminooxy cross-linking component synthesized from pentaerythritol and aminooxy acetic acid.

15     **[0089]**     In some embodiments, the cross-linking component is a 4-arm cross-linking component synthesized from tetra-PEG of various MW (2,000-10,000 Da) and aminooxy acetic acid. For example of 4-arm cross-linking components, see Figs. 6, 19, and 20, and for an example of a 3-arm cross-linking component, see Fig. 21.

20     **[0090]**     The purpose of making the cross-linker larger is to make the hydrogel softer. By using a larger cross-linker (10kDa) gels with as low of modulus as ~5kPa and as high as ~30kPa have been achieved in practicing this invention. This large of a range could not be achieved with small cross-linker and a 10 kDa ketone-PEG. In some embodiments, the cross-linker is made identical in size to the main PEG-containing component to provide a structurally homogeneous network

25     **[0091]**     In some embodiments, when the PEG-containing component contains aldehyde functionality, the buffer solution has a pH range of from 1.5 to 7.5, in other embodiments the pH has a range of from 2.0 to 7.6, in other embodiments the pH has a range of from 5.0 to 7.5, and in yet other embodiments the pH has a range of from 5.0 to 7.6.

30     **[0092]**     In some embodiments, the buffer solution is water or a phosphate-citrate buffer solution. In some embodiments, the buffer solution is water or a phosphate-citrate buffer solution. The use of this buffer solution allows for more acidic pH levels to be attained than phosphate buffer alone. The buffer solution has a pH range of 2.6-7.6. The buffer solution is prepared by mixing various amounts (depending on desired pH and buffer strength) of citric acid monohydrate ( $C_6H_8O_7 \cdot H_2O$ ) and sodium phosphate dibasic ( $Na_2HPO_4$ ) in water.

**[0093]** In some embodiments, the concentration of the buffer solution is selected from about 10 mM to about 100 mM. In other embodiments the concentration of the buffer solution used was selected from about 20 mM to about 50 mM.

**[0094]** The hydrogel is formed upon introducing both the PEG containing component and a 2-arm cross-linking component or a 4-arm cross-linking component or a mixture of 2-arm and 4-arm cross-linking components to the buffer solution.

**[0095]** In some embodiments, the PEG-containing component and the cross-linking component are mixed at equimolar ratios. In some embodiments, particularly when the hydrogel is made using a mixture of different cross-linking components (i.e. some 4-arm and some 2-arm with allyl group), the final ratio of PEG-containing component: cross-linking component was still equimolar, but the ration of 4-arm crosslinking component: 2-arm cross-linking component is from 95:5, 90:10, and/or 85:15.

**[0096]** Buffer concentration plays a crucial role in this oxime ligation hydrogel system. A notable rise in the storage modulus is observed when the buffer concentration was elevated from 10 mM to 20 mM, and a large drop appeared when the concentration was raised to 50 mM and greater. The overall trend suggests that an increase in buffer strength may lead to an increase in the heterogeneity of the network, which will produce a mechanically weaker scaffold. The ionization degree in the polymer gels plays a crucial role in determining the state of spatial inhomogeneity. The inversion phenomenon of a charged hydrogel network is expected when the degree of ionization exceeds a certain level. Inversion here stands for the anomalous cross-linking dependence that takes place due to the competition between two effects of the cross-linking: one is the random distribution of cross-links in the network, and the other one is the suppressed tendency of a phase-segregated structure. An increase of inversion may directly induce a weakness in strength of the formed hydrogels.

**[0097]** Since oxime ligation is a pH sensitive reaction, a change in pH values of the buffer should also influence the final strength of gels. A drop in storage moduli is observed with a decrease in the pH. In an oxime reaction, a lower pH value accelerates the ligation rate. If the movement of the PEG molecules is limited within a confined space, the cross-linking between the ketone and the aminooxy groups may be inhibited or allow for more dangling ends and loops (network defects). The

inhomogeneity for this system under pH = 5.7 is expected to be higher than the one under pH = 7.1.

**[0098]** A trend of increasing mesh size was also observed with an increase in buffer strength. The variation in buffer strength has a more prominent effect on the kinetic dependence of network formation for mesh size control. Experiments were run to determine the function of buffer concentration and pH as they relate to mesh size and it was concluded that the mesh size clearly increases in size with increasing buffer strength, suggesting the development of heterogeneities within the structure at higher buffer concentrations. Overall, the trends suggested that small scale heterogeneities are responsible for the weakening/loosening of the network with increasing buffer strength.

**[0099]** In one embodiment, as shown in Fig. 7, the wound dressing 110 is formed by first introducing decellularized tissue 116 into a bath 114 of the buffer solution used for the manufacture of the PEG hydrogel 14. Then, the PEG containing component and the cross-linking component used to create the PEG hydrogel 14 is added to the bath 114 to create the PEG hydrogel 14. In some embodiments, the cross-linking component is added after the PEG containing component. Depending upon where the decellularized tissue is held in the buffer solution, the formed PEG hydrogel 14 will either partially or fully surround the decellularized tissue 116 (e.g., Figs. 1A, 1B). Network formation of the PEG hydrogel 14 occurs quickly; depending of the pH of the buffer solution, the gelation time may occur between 5-30 minutes.

**[00100]** In one embodiment, as shown in Fig. 8, the wound dressing 210 is formed by electrospinning nanofibers 218 into a bath 114 of the buffer solution used for the manufacture of the PEG hydrogel 14. The electrospinning solution used to create the nanofibers 218 is loaded into a reservoir 220 that is mounted with a needle. High voltage is applied to the reservoir 220 so that the electrospinning solution experiences instability, and spins into the bath 114 of the buffer solution. Then, the PEG- containing component and the cross-linking component used to create the PEG hydrogel 14 are added to the bath 114 to create the PEG hydrogel 14, and the formed PEG hydrogel 14 at least partially surrounds the electrospun nanofibers 218. In some embodiments, the cross-linking component is added after the PEG containing component. Network formation of the PEG hydrogel 14 occurs quickly; depending of the pH of the buffer solution, the gelation time may occur between 5-30 minutes.

**[00101]** In one embodiment, as shown in Fig. 9, the wound dressing 310 is formed by first introducing decellularized tissue 316 into a bath 114 of the buffer solution and the used for the manufacture of the PEG hydrogel 14. Then nanofibers 318 are electrospun into the bath 114 of buffer solution and onto the decellularized tissue  
5 316. The nanofibers tend to lie on the decellularized tissue and while there is some static interaction, the two components tend to delaminate from each other, particularly when submerged in fluid. This is the reason for including the hydrogel component. The electrospinning solution used to create the nanofibers 318 is loaded into a reservoir 320 that is mounted with a needle. High voltage is applied to the  
10 reservoir 320 so that the electrospinning solution experiences instability, and spins onto the decellularized tissue 316, which is laying in the bath 114 of the buffer solution. Then, the PEG containing component and the cross-linking component used to create the PEG hydrogel 14 are added to the bath 114 to create the PEG hydrogel 14, and the formed PEG hydrogel 14 at least partially surrounds the regenerative  
15 material 312 which includes the decellularized tissue 316 reinforced with nanofibers 318. In some embodiments, the cross-linking component is added after the PEG containing component. Network formation of the PEG hydrogel 14 occurs quickly; depending of the pH of the buffer solution, the gelation time may occur between 5-30 minutes.

**[00102]** The strength of the buffer solution selected changes the cross-linking kinetics of the reaction between the PEG-containing component and the cross-linking component. It is also theorized that the strength of the buffer solution affects the modulus of the hydrogel formed. The overall trend suggests that an increase in buffer strength may lead to an increase in the heterogeneity of the network, which  
20 will produce a mechanically weaker scaffold.

**[00103]** The inhomogeneity that results from cross-linking kinetics is a direct result of the pH of the buffer solution. The oxime cross-linking reaction between the PEG-containing component and the cross-linking component is catalyzed in mildly acidic conditions, thus, the cross-linking is faster at lower pH and results in more  
25 defects/inhomogeneity. Therefore, gels formed under lower pH conditions have lower modulus than those formed at higher pH. The optimum pH is dependent on the target tissue. If the target tissue is more rigid, then you would use a higher pH and form a more rigid hydrogel (and vice versa for a softer tissue)

30

5 [00104] By varying the buffer pH and the ionic strength and molecular weights of the components, it is possible to obtain hydrogels ranging from 80-90% water content with a storage modulus of 1.0-30 kPa using conditions that will not harm cells. In some embodiments, the present invention provided hydrogels with storage modulus of from 5.0 to 30.0 kPa.

10 [00105] These properties allow tuning the modulus of the gels without changing the actual chemistry. The hydrogel is produced by simply mixing equimolar ratios of the two precursor components into the buffer of choice. The precursors consist of a PEG containing component and 2-arm and 4-arm cross-linking components as taught herein.

15 [00106] The entire composite material can be easily implanted to the site of injury during laparoscopic surgery (in the case of hernia repair) similar to current techniques for hernia mesh placement. The hydrogel will degrade in a matter of days, leaving the electrospun nanofiber encapsulated tissue exposed for optimal wound healing performance. This hydrogel therefore acts mostly as a delivery vehicle and laminating agent for the nanofiber/ECM complex.

20 [00107] After the hydrogel has degraded, polymer nanofibers containing bioactive peptides will stimulate wound healing as they slowly degrade over time, allowing for tissue integration into the porous network. The polymer nanofibers degrade into amino acids and diols, where the diols neutralize the acidic components resulting in less inflammation, and the byproducts are easily metabolized and eliminated from the body.

25 [00108] Beneath the fibers, extracellular matrix will be exposed at a slower rate than if it were implanted without nanofibers. This slower rate of exposure allows for slower degradation, but enhances the healing response by releasing native tissue proteins.

30 [00109] The full construct will degrade at a slower rate than conventionally used decellularized tissue, while maintaining the highly bioactive regenerative response seen when using decellularized tissue to heal wounds.

#### **EXPERIMENTAL**

[00110] The following experimental section will illustrate how to fabricate the PEG hydrogel component of the hybrid material, and provides additional background

on hydrogels and the novelty of the present PEG hydrogel and the ability to tailor properties thereof.

**[00111]** Hydrogels are materials that are primarily composed of hydrophilic cross-linked networks, containing as much as 99% water. Synthetic polymers are arguably more favorable in hydrogel systems due to their reproducibility, high purity, and controlled molecular weight. Polyethylene glycol (PEG)-based hydrogels, in particular, have become one of the most extensively studied systems because of their versatility in end group modification, hydrophilic chemical structure, and protein absorption resistance. In several studies, such as those done by *Nanostructured PEG-Based Hydrogels with Tunable Physical Properties for Gene Delivery to Human Mesenchymal Stem Cells*, Biomaterials, 2012, 33 (27), 6533-6541 by Li et al. and *Human Chondrocyte Extracellular Matrix Formation and Phenotype Maintenance using RGD-Derivatized PEGDM Hydrogels Possessing a Continuous Young's Modulus Gradient*. Acta Biomaterialia. 2013, 9 (4), 6095-6104 by Smith Callahan, et al., which are herein incorporated by reference, PEG hydrogels were fabricated to mechanically mimic extracellular matrix (ECM) for various types of cell cultures.

**[00112]** Notably, PEG-based hydrogels with terminal acrylate functionality have been examined widely as tissue engineered scaffolds. Upon irradiation with ultraviolet light, they can form a three dimensional hydrogel network with tunable mechanical properties dependent on the cross-link density, and the mass fraction of the polymer precursors. Unfortunately, free radical polymerizations have limited control, which leads to multiple cross-linking reactions across the solution. The heterogeneous cross-linking leads to the formation of micro-clusters that are eventually tied together by the polymer chains. Such networks are not favorable for incorporating biological cues due to their inhomogeneity and complex micro-cluster structural formation. For better defined cross-linked networks, orthogonal chemistries ("click" chemistries) are currently being explored.

**[00113]** "Click" chemistries are generally defined as highly efficient, robust and orthogonal reactions. With regards to hydrogel formation, some of the extensively investigated "click" reactions include the Michael addition, thiol-ene addition, tetrazine-norbornene addition, copper(I) catalyzed alkyne-azide cycloaddition, and strain-promoted alkyne-azide cycloaddition. In many instances, the hydrogels produced from "click" reactions show superior properties in comparison to in situ

polymerized gels. As a result of their orthogonal nature, multiple "click" reactions can be carried out in one system, which allows for post-functionalization of the hydrogel. The PEG based hydrogels that will be discussed herein involves oxime "click" chemistry.

- 5 **[00114]** Oxime chemistry employs a kinetically dependent reaction that varies with buffer pH and ionic strength. Several studies, have utilized oxime reactions for hydrogel fabrication. One such study, *Biocompatible Hydrogels by Oxime Click Chemistry*, *Biomacromolecules*. 2012, 13 (10), 3013-3017, by Maynard, et al, which is herein incorporated by reference, developed an 8-aml aminooxy PEG cross-linked
- 10 with glutaraldehyde to form hydrogels for supporting cell adhesion. They were able to tune the mechanical properties of their hydrogel based on the mass fraction of PEG, effectively altering the cross-linking ratio. Another study, *Oxime Cross-Linked Injectable Hydrogels for Catheter Delivery*, *Advanced Materials*. 2013, 25 (21), 2937-2942, by Christman et al., which is herein incorporated by reference, applied oxime
- 15 hydrogels to catheter delivery systems, in which a range of gelation times were achieved by controlling the buffer pH. These injectable hydrogels exhibited rapid gelation upon injection into tissue, demonstrating practicality for use in treatment of arterial diseases. *Peptide-Functionalized Oxime Hydrogels with Tunable Mechanical Properties and Gelation Behavior*, *Biomacromolecules*. 2013, 14 (10), 3749-3758, by
- 20 Becker, M.L., et al., which is herein incorporated by reference, showed that the storage modulus for oxime gels changed significantly as the pH was decreased from 7.6 to 4.5. They were also able to photo-pattern peptide functionalities post-gelation via thiol-ene "click" chemistry. Oxime chemistry has proven to be a truly robust method for hydrogel formation, and it allows for very mild conditions: no metal
- 25 catalyst is required, 20-40 °C with pH ranges from 2-7 is suitable, and the by-product of the reaction is water.

- [00115]** The Oxime, PEG-based hydrogel system possesses widely tunable mechanical properties, without altering the chemistry of the network. Previous research has incorporated multiple chemistries into their hydrogel systems, or
- 30 utilized chemically different substrates altogether, to study the cell's response with varying mechanical properties. However, very few hydrogel systems are capable of achieving a wide range of elasticities without altering the chemical makeup of the scaffold. The advantage of such a system is to show, definitively, the effect of

substrate elasticity on cell response. Therefore, a hydrogel system is needed that would allow for the modulus to be dialed in, using alternative means to control the mechanical properties, such as the pH and strength of the buffer. To better understand how pH and buffer strength influence the elasticity of an oxime hydrogel, an investigation of the detailed microstructure was performed. Controlling the physical properties is essential for the application of hydrogels, and it is important to understand how the microstructure impacts those properties.

### Materials

[00116] The 4-arm, 10K polyethylene glycol was purchased from Creative PEGWorks. All other commercial reagents and solvents were purchased from Sigma-Aldrich or Fisher Scientific and used as received unless noted otherwise. All synthesis reactions were performed under nitrogen unless noted otherwise.

### Instrumentation

[00117] The <sup>1</sup>H-NMR spectra were obtained using a Varian NMRS 300 MHz spectrometer. All chemical shifts are reported in ppm (δ), and referenced to the chemical shifts of residual solvent resonances (<sup>1</sup>H-NMR CDCl<sub>3</sub> 7.26 ppm, D<sub>2</sub>O 4.79 ppm). Abbreviations for multiplicities are listed as follows: s = singlet, d = doublet, t = triplet, br = broad singlet, m = multiplet. FT-IR spectra were recorded by a DIGILAB EXCALIBUR Series FTS3000, with a scanned wavenumber range from 400 to 4000 cm<sup>-1</sup>. Samples were prepared by grinding KBr powder with dried sample powder into pellets and spectra were recorded after 64 scans. Baseline was deducted and normalized to the same reference peak intensity.

[00118] Mass spectrometry for the 4-arm keto-PEG was performed using a Bruker UltraFlex III MALDI tandem time-of-flight (TOF/TOF) mass spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with a Nd:YAG laser emitting at 355 nm. The matrix and cationization salt were DCTB (2-[(2E)-3-(4-tert-butylphenyl)-2-methylprop-2-enylidene]malonitrile) and sodium trifluoroacetate, respectively. Solutions of the matrix (20 mg/mL) and cationizing salt (10 mg/mL) were prepared in THF, and the polymer sample (10 mg/mL) was prepared in water. The matrix and cationizing agent solutions were mixed in 10:1 (v/v) ratio and applied to the target. After drying, a spot of the sample was applied, followed by an additional drop of the matrix/cationizing agent. For the 4-arm aminoxy cross-linking component, the spectrum was collected using a Bruker HCTultra

II quadrupole ion trap (QIT) mass spectrometer (Billerica, MA) equipped with an ESI source.

5 **[00119]** The pH values of buffers were tested using an Orion® 350 PerpHecT® benchtop pH meter with an Orion® ROSS® Sure-Flow pH electrode at room temperature. The PEG hydrogels were cast to be 2 mm thick in 25 mm diameter, and soaked in buffer overnight to reach swelling equilibrium. The equilibrium moduli were determined using an ARES G2 (TA Instruments, New Castle, DE) with 25 mm serrated parallel plate geometry and a 6 N axial force. Hydrogels were immersed in buffer during testing to keep the gel from drying up.

10 **[00120]** Initially, a strain sweep was performed to determine the viscoelastic region, and 1% strain was selected from frequency sweeps. The frequency sweep ramped from  $0.1 \text{ rad}\cdot\text{s}^{-1}$  to  $100 \text{ rad}\cdot\text{s}^{-1}$ , all frequencies were within the viscoelastic region, and  $100 \text{ rad}\cdot\text{s}^{-1}$  was selected for reporting. Temperature was controlled at  $37^\circ\text{C}$  by a forced convection oven to mimic physiological conditions. Small angle neutron scattering was used to identify the chain and gel structure. All measurements were taken at the National Institute of Standards and Technology Center for Neutron Research (NCNR)

15 **[00121]** Using instruments NG7 and NGB30, the scattering “wavevector”  $Q$  was measured. Where  $Q = (4\pi/\lambda) \cdot \sin(\theta/2)$ ,  $\lambda$  is a neutron beam wavelength and  $\theta$  is the scattering angle. Using three detector distances of 12.5 m, 4 m, and 1.3 m, a  $Q$  range of  $0.0035 \text{ \AA}^{-1}$  to  $0.6 \text{ \AA}^{-1}$  was measured. The scattering results were circularly averaged over the 2D detector to attain the 1D scattering of  $Q$  versus intensity. The data was fit with a Zimm model in the  $Q$  range of  $0.032$  to  $0.077 \text{ \AA}^{-1}$ , and the correlation length of the gel nanostructure was determined. In addition, the Correlation length model for cross-linked gels was used to fit the entire  $Q$  range, from which the Porod exponent and correlation length were determined.

#### **Synthesis of 4-arm aminooxy cross-linker**

20 **[00122]** In a 250 mL round bottom flask, 0.712 g (5.23 mmol, 1 eq) of pentaerythritol was dissolved in 20 mL of DMF, then 5.0 g (26.15 mmol, 5 eq) of (boc-aminooxy) acetic acid and 0.770 g (2.62 mmol, 0.5 eq) of DPTS were added. The reaction flask was purged with  $\text{N}_2$  and stirred vigorously until all reagents were dissolved. The reaction flask was cooled to  $0^\circ\text{C}$  in an ice bath for 15 min, followed by the injection of 4.05 mL (26.15 mmol, 5 eq) of DIC. The reaction was allowed to gradually warm to room temperature with stirring for 24 hrs. The reaction mixture was filtered to remove the urea by-product, and rotary evaporated to remove

solvent. The crude product was re-dissolved in ethyl acetate, cooled in liquid nitrogen and centrifuged to further remove urea by-product. The solution was concentrated and purified using silica column chromatography with a mobile phase of 5:3 (ethyl acetate : hexane). After rotary evaporation, a white solid intermediate  
5 was obtained. <sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>): δ = 7.93 (s, 4H, -NHCO-), 4.47 (s, 8H, -NHOCH<sub>2</sub>CO-), 4.26 (s, 8H, -COOCH<sub>2</sub>C-), 1.49 (42H, (CH<sub>3</sub>)<sub>3</sub>CO). The intermediate product was dissolved in approximately 25 mL of 4M HCl/Dioxane to remove the protecting group, and stirred for 24 hours at room temperature under an N<sub>2</sub> purge. The resulting reaction solution was precipitated in cold diethyl ether, centrifuged,  
10 and vacuum dried. A fluffy white solid was obtained (1.75 g, 79.6% yield). <sup>1</sup>H-NMR (300 MHz, D<sub>2</sub>O): δ = 4.85 (s, 2H, +NH<sub>3</sub>OCH<sub>2</sub>CO-), 4.45 (s, 2H, -COOCH<sub>2</sub>C-).

#### Synthesis of 2-allyl cross-linker vinyl extender

**[00123]** In a 250 mL round bottom flask, 0.540 uL (4.36 mmol, 1 eq) of 3-allyloxy-1,2-propanediol was dissolved in 20 mL of DMF, then 2.5 g (13.08 mmol, 3 eq) of  
15 (boc-aminooxy)acetic acid and 0.642 g (2.18 mmol, 0.5 eq) of DPTS were added. The reaction flask was purged with N<sub>2</sub> and stirred vigorously until all reagents were dissolved. The reaction flask was cooled to 0 °C in an ice bath for 15 min, followed by the injection of 2.03 mL (13.08 mmol, 3 eq) of DIC. The reaction was allowed to gradually warm to room temperature with stirring for 24 hrs. The reaction mixture  
20 was filtered to remove the urea by-product, and rotary evaporated to remove solvent. The crude product was re-dissolved in ethyl acetate, cooled in liquid nitrogen and centrifuged to further remove urea by-product. The solution was concentrated and purified using silica column chromatography with a mobile phase of 1:2 (ethyl acetate: hexane). After rotary evaporation, the white solid intermediate  
25 was obtained. <sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>): δ = 7.87 (s, 2H, -NH-), 5.75-6.00 (m, 1H, -CH=CH<sub>2</sub>), 5.15-5.40 (m, 3H, -COOCH<sub>2</sub>CHOOC-, -CH=CH<sub>2</sub>), 4.47-4.55 (m, 1H, -COOCH<sub>2</sub>CHOOC-), 4.45 (d, 4H, -NHOCH<sub>2</sub>COO-), 4.28-4.38 (m, 1H, -COOCH<sub>2</sub>CHOOC-), 4.00 (d, 2H, -OCH<sub>2</sub>CH=CH<sub>2</sub>), 3.60 (d, 2H, -CHCH<sub>2</sub>OCH<sub>2</sub>CH=CH<sub>2</sub>), 1.48 (s, 18H, (CH<sub>3</sub>)<sub>3</sub>CCO-). The intermediate product was dissolved in 25 mL of 4M HCl/Dioxane  
30 to remove the protecting group, and stirred for 24 hours at room temperature under N<sub>2</sub> purge. The resulting reaction solution was precipitated in cold diethyl ether, centrifuged, and vacuum dried. A fluffy white solid was obtained (0.95 g, 78.6% yield).

**Synthesis of keto-PEG**

**[00124]** In a 250 mL round bottom flask, 8 g (0.8 mmol, 1 eq) of 4-arm PEG (10K) was dissolved in 20 mL of  $\text{CH}_2\text{Cl}_2$  with stirring and  $\text{N}_2$  purge. Then, 0.4645 g (4.0 mmol, 5 eq) of levulinic acid was added to the flask, followed by 0.118 g (0.4 mmol, 5 eq) of DPTS. The reaction flask was cooled in an ice bath for 15 minutes, and then injected with 0.620 mL (4.0 mmol, 5 eq) of DIC using a micropipette. The reaction flask was allowed to gradually come to room temperature with stirring under nitrogen for 24 hours. Using a Buchner funnel, the reaction mixture was filtered to remove solid DIC-urea, and washed with  $\text{CH}_2\text{Cl}_2$ . The filtrate was concentrated via rotary evaporation and re-dissolved in a minimal amount of  $\text{CH}_2\text{Cl}_2$  for precipitation into cold methanol, followed by centrifugation. The precipitate was then re-dissolved in a minimal amount of solvent again, and precipitated into cold diethyl ether, followed by centrifugation at 5000 RPM for 2 minutes. The precipitate was then vacuum dried, and a white solid was obtained (7.10 g, 85.0% yield).  $^1\text{H-NMR}$  (300 MHz,  $\text{CDCl}_3$ ): 4.20-4.17 (m,  $-\text{CH}_2\text{OC}=\text{O}-\text{CH}_2-$ ), 3.79-3.76 (m,  $\text{CCH}_2\text{OCH}_2$ ), 3.67-3.46 (m,  $-\text{OCH}_2\text{CH}_2-$ ), 3.37 (br,  $\text{CCH}_2\text{OCH}_2-$ ), 2.73-2.70 (m,  $-\text{OC}=\text{OCH}_2-$ ), 2.58-2.55 (m,  $-\text{CH}_2\text{CH}_2\text{C}=\text{O}-\text{CH}_3$ ), 2.15 (br,  $-\text{CH}_2\text{C}=\text{OCH}_3$ ).

**Citric acid - disodium phosphate buffer preparation**

**[00125]** Stock solutions of 0.1 M citric acid and 0.1 M disodium phosphate were prepared with ultrapure water. The pH values and buffer concentrations for different gel systems were adjusted by mixing different portions of the two stock solutions with additional water. Precise pH values of the buffer solutions were tested with a pH meter

**Hydrogel Fabrication**

**[00126]** In general, hydrogels were fabricated using a precursor mixing method. Solutions of the 4-arm keto-PEG were prepared by dissolving a pre-weighed mass in 800  $\mu\text{L}$  of the desired buffer. Solutions of 4-arm cross-linker and 2-arm cross-linker vinyl extender were prepared by dissolving the cross-linker precursors in 200  $\mu\text{L}$  of the desired buffer. For the 4+4 hydrogel system, the 4-arm keto-PEG solution was mixed with the 4-arm aminooxy cross-linker solution under stoichiometric balance (1:1 aminooxy: ketone) with sufficient initial shaking to ensure thorough mixing of the precursors. For 4+2+4 hydrogel system, keto-PEG solution was mixed with 2-arm aminooxy alkene extender with sufficient shaking, and allowed to react for 30

minutes. Then, the remainder of the 4-ann aminooxy cross-linker was then added with additional shaking. For oscillatory shear measurement, the hydrogel precursor mixtures were cast in silicone molds for at least 8 hours to ensure complete gelation. The hydrogels were then taken out of molds, soaked in the target buffer overnight to reach swelling equilibrium. The mass fraction of total precursors was calculated using the following equation:

$$wt.\% = \frac{m(PEG) + m(cross - linker)}{m(4PEG) + m(cross - linker) + m(buffer)}$$

### Buffer Strength Effects on the Hybrid Material

**[00127]** Buffer concentration plays a crucial role in this oxime ligation hydrogel system. Fig. 10 shows the differences in the hydrogel storage modulus with different buffer concentrations (10, 20, 50, 100 mM). Interestingly, a notable rise in the storage modulus is observed when the buffer concentration was elevated from 10 mM to 20 mM, and a large drop appeared when the concentration was raised to 50 mM and greater. The buffer used in this experiment was citric acid/disodium phosphate with different concentrations. The overall trend suggests that an increase in buffer strength may lead to an increase in the heterogeneity of the network, which will produce a mechanically weaker scaffold. Alternatively, the existence of phosphate in the buffer may lead to a three component Kabachnik-Fields reaction. This reaction occurs between the phosphate and C=N bonds. It is possible that the resulting oxime linked network reacts with the phosphate ions in the solution to complete the Kabachnik-Fields reaction, causing the hydrogel network to become ionized. The ionization degree in the polymer gels plays a crucial role in determining the state of spatial inhomogeneity. The inversion phenomenon of a charged hydrogel network is expected when the degree of ionization exceeds a certain level. Inversion here stands for the anomalous cross-linking dependence that takes place due to the competition between two effects of the cross-linking: one is the random distribution of cross-links in the network, and the other one is the suppressed tendency of a phase-segregated structure. An increase of inversion may directly induce a weakness in strength of the formed hydrogels, as observed in our experiment.

### 30 Influence of pH

**[00128]** Since oxime ligation is a pH sensitive reaction, a change in pH values of the buffer should also influence the final strength of gels. Fig. 11 shows the

differences between gel moduli with the corresponding buffer pH value of the buffer used; a drop in storage moduli is observed with a decrease in the pH. In an oxime reaction, a lower pH value would accelerate the ligation rate. If the movement of the PEG molecules is limited within a confined space, the cross-linking between the ketone and the aminoxy groups may be inhibited or allow for more dangling ends and loops (network defects). The inhomogeneity for this system under pH = 5.7 is expected to be higher than the one under pH = 7.1, however a series of pH values need to be explored before drawing further conclusions.

**[00129]** Fig. 22 shows similar data for a hydrogel system of 4-arm aminoxy cross-linker made from 10k MW PEG and equimolar amounts of 10k 4-arm PEG-ketone.

#### **Impact of PEG wt.%**

**[00130]** The influence of the mass fraction of PEG in the overall hydrogel system was observed for weight percentages of 12%, 14% and 18%. As shown in Fig. 12, no significant change in storage modulus was indicated when the mass fraction changed. The hydrogels cannot be formed when the mass fraction is below 8%; only a viscous liquid was obtained. When the fraction went up to 12% and above, which in this case gels reach their peak of strength, the differences in the storage modulus were within error. An increase in storage modulus is observed with increasing mass fraction of PEG, however, some other hydrogel systems have also shown independence from the mass fraction of PEG precursor, which would be ideal for the use of a biologically relevant scaffold (12 wt.%), since large mass fractions of PEG are not necessary to increase the strength of the gel.

#### **Ratio of 4-arm: 2-arm cross-linking component**

**[00131]** The ratio of 4-arm: 2-arm cross-linking component shows a clear trend in terms of the resulting storage moduli. Testing showed that the storage modulus decreased when the ratio of 2-arm cross-linking component was increased. The highest storage modulus scaffold appears to be the system containing only 4-arm cross-linking component. This suggests that the incorporation of 2-arm cross-linking component effectively decreases the cross-linking density, and possibly introduces more sites for ineffective/incomplete cross-linking between the 4-arm aminoxy cross-linking component and the keto-PEG.

#### **Structural Properties of the Hydrogel**

**[00132]** The small angle neutron scattering was performed in order to probe the microstructure of the hydrogel scaffolds. From the SANS data, it was possible to obtain a correlation length (mesh size) data to examine the homogeneity of the network. The results exhibited a clear trend of increasing mesh size with increase  
 5 buffer strength. Although the trend with pH is not clearly distinguishable, it may be possible that the variation in buffer strength has a more prominent effect on the kinetic dependence of network formation for mesh size control. The correlation length and Porod exponent were plotted as a function of buffer concentration and pH and it was concluded that the mesh size clearly increases in size with increasing  
 10 buffer strength, suggesting the development of heterogeneities within the structure at higher buffer concentrations. The decrease in the Porod exponent with increasing buffer strength also indicates a mass fractal change from a dense three-dimensional network to a more open and loose network. Overall, the trends suggested that small scale heterogeneities are responsible for the weakening/loosening of the network  
 15 with increasing buffer strength.

### Swelling Studies

**[00133]** The hydrogels were cast in a silicon mold and punched to 8 mm diameter (~2 mm thick) using a biopsy punch, then swollen in a glass vial with deionized water (2.5 mL) for 48 hours. The excess water was gently removed by blotting with a  
 20 soft tissue, and the samples were weighed using an analytical balance ( $\pm 0.01$  mg) to obtain the swollen mass ( $M_s$ ). The hydrogel samples were then lyophilized for 48 hours and weighed again to obtain the dry mass ( $M_d$ ). After determining the swollen ratio ( $Q$ ), the Flory-Rehner equations were utilized to calculate the average mesh size ( $\xi_m$ ) for hydrogels formed under each pH and buffer concentration condition ( $n = 5$ ).  
 25 The Flory-Rehner equations:

$$\frac{1}{v_2} = Q = \frac{M_s - M_d}{M_d} ;$$

$$\frac{1}{M_c} = \frac{2}{M_n} - \frac{\bar{v}/V_1 [\ln(1 - v_2) + v_2 + \chi_1 v_2^2]}{v_2^{1/3} - \frac{v_2}{2}} ;$$

$$(\bar{r}_o^2)^{1/2} = l \cdot C_n^{1/2} \cdot \left( \frac{2M_c}{M_r} \right)^{1/2} ; \text{ and}$$

$$\xi_m = v_2^{-1/3} \cdot (\bar{r}_o^2)^{1/2}$$

[00134] For this system of equations,  $V_2$  is the swollen polymer volume fraction,  $\bar{v}$  is the specific volume of PEG (0.893 cm<sup>3</sup> g<sup>-1</sup>),  $V_1$  is the molar volume of water (18 cm<sup>3</sup> mol<sup>-1</sup>),  $\bar{M}_n$  is the number-average molecular weight (10,330 g mol<sup>-1</sup>),  $\bar{M}_c$  is the average molecular weight between crosslinks,  $\chi_1$  is the polymer-solvent interaction parameter (0.426 for PEG in water),  $\bar{r}_{ee}^2$ <sup>1/2</sup> is the root-mean-square end to end distance,  $l$  is the bond length (1.46 Å), and  $C_n$  is the characteristic ratio for PEG (4.0).

### Rheology

[00135] The moduli of the hydrogels were determined using an ARES G2 Rheometer (TA Instruments, New Castle, DE) equipped with 8 mm parallel plate geometry. The hydrogel samples were centered on the test geometry, and the gap height was set to ~1.6 mm with a constant normal force of ~2 N. Initially, a strain sweep was performed (Fig. 13) to determine the linear viscoelastic regime (LVR), and 1% strain was selected for frequency sweeps. The frequency sweeps were conducted from 100 rad s<sup>-1</sup> to 0.1 rad s<sup>-1</sup>; all frequencies between 1-100 rad s<sup>-1</sup> demonstrated linear behavior (Fig. 14), and 10 rad s<sup>-1</sup> was selected for reporting ( $n = 3$ ). It should be noted that no notable drying of the hydrogels was observed during testing. Time sweeps were performed at 1% strain and 1 rad s<sup>-1</sup> to determine the gelation time. The precursor solutions were mixed and shaken for 5 seconds before injecting 400 uL of the hydrogel solution into the rheometer equipped with 25 mm parallel plates and a set gap height of 0.80 mm, and the response was measured

### Influence of Microstructure on Hydrogel Modulus

[00136] SANS experiments were performed to further explore the structural properties of the hydrogels, and to examine how the detailed microstructure may influence the mechanical properties. The SANS data showed that the correlation length increased with increasing buffer strength, which corresponds to the decline in the mechanical properties of the network. The increase in correlation length suggests there is an introduction of heterogeneities in the hydrogel network; the Porod exponent at low  $Q$  also supports this trend as it generally decreases from 3.05 to 2.75. The introduction of inhomogeneities, or network defects, could be the result of incomplete cross-linking at each of the four branches (i.e. the keto-PEG cross-linked at 3 loci rather than all 4), or dangling ends and loops. Therefore, the tunability of the mechanical properties seen for this hydrogel system is the result of controlling the degree of small-scale heterogeneities by altering the buffer strength and pH. These

results reveal the potential for using this system as a platform for various cell culture scaffolds.

**[00137]** This experiment provides necessary guidance for the creation of covalently cross-linked systems that employ a kinetically controlled oxime ligation, and demonstrate variable mechanical properties by altering the pH and buffer strength. Using SANS and rheology, it has been demonstrated that the kinetics of the ligation reaction influence the development of the microstructure, which ultimately leads to changes in the rigidity of the gel. This system holds promise to definitively demonstrate the effect of scaffold modulus on stem cell differentiation, because it does not rely on changing the precursor chemistry to change the scaffold elasticity.

**CLAIMS**

What is claimed is:

- 5           1. A wound dressing comprising:  
              decellularized tissue and fibers at least partially surrounded by a PEG  
              hydrogel, wherein the PEG hydrogel is structured by covalent bonds formed  
              via an oxime ligation reaction of a polyethylene glycol (PEG) containing  
              component and a cross-linking component.
- 10           2. The wound dressing of claim 1, wherein the fibers are formed from polymers  
              selected from the group consisting of poly(ester urea) (PEU),  
              polycaprolactone (PCL), poly-L-lactide (PLLA), and poly(lactic-co-glycolic  
              acid) (PLGA), functionalized PEU, functionalized PCL, functionalized PLLA,  
15           and functionalized PLGA.
3. The wound dressing of claim 1, wherein the PEG-containing component is tri-  
              functional or greater containing ketone functionality, aldehyde functionality, or  
              a combination thereof on the terminal end of the chains and having a  
20           molecular weight ranging from about 1,000 Da to about 50,000 Da.
4. The wound dressing of claim 1, wherein the cross-linking component is  
              selected from the group consisting of a 2-arm cross-linking component, a 4-  
              arm cross-linking component, or a mixture thereof.
- 25           5. The wound dressing of claim 1, wherein the PEG hydrogel is formed using a  
              buffer solution, wherein the buffer solution the buffer solution is selected  
              from the group consisting of citric acid, disodium phosphate, and  
              combinations thereof.
- 30           6. The wound dressing of claim 5, wherein the pH of the buffer solution is from  
              about 1.5 to about 7.5.
7. The wound dressing of claim 5, wherein the concentration of the buffer  
35           solution is from about 10 mM to about 100 mM.

8. A method of making a wound dressing comprising the steps of laying down decellularized tissue into a bath of a buffer solution and PEG-containing component, adding fibers to the top of the decellularized, and then adding a cross-linking component to the bath of buffer solution and the PEG-containing component to create a PEG hydrogel to at least partially surround the decellularized tissue and fibers.
9. The method of claim 8, wherein the fibers are formed from polymers selected from the group consisting of poly(ester urea) (PEU), polycaprolactone (PCL), poly-L-lactide (PLLA), and poly(lactic-co-glycolic acid) (PLGA), functionalized PEU, functionalized PCL, functionalized PLLA, and functionalized PLGA.
10. The method of claim 8, wherein the fibers are electrospun onto the decellularized tissue.
11. The method of claim 8, wherein the PEG-containing component is tri-functional or greater containing ketone functionality, aldehyde functionality, or a combination thereof on the terminal end of the chains and having a molecular weight ranging from about 1,000 Da to about 50,000 Da.
12. The method of claim 8, wherein the cross-linking component is selected from the group consisting of a 2-arm cross-linking component, a 4-arm cross-linking component, or a mixture thereof.
13. The method of claim 8, wherein the buffer solution is selected from the group consisting of citric acid, disodium phosphate, and combinations thereof.
14. The method of claim 8, wherein the pH of the buffer solution is from about 1.5 to about 7.5 and the concentration of the buffer solution is from about 10 mM to about 100 mM.
15. A method of making a hydrogel comprising the steps of:  
mixing a polyethylene glycol (PEG) component with a cross-linking component in a buffer solution, the PEG component having multiple arms with

terminal functionality selected from ketone functionality and aldehyde functionality, and the cross-linking component having multiple arms with terminal aminoxy functionality, the hydrogel forming in the buffer solution by oxime ligation reaction, wherein the pH of the buffer solution used to make the hydrogel has a pH range of from 1.5 to 7.6, and the concentration of the buffer solution is from about 10 mM to about 100 mM.

16. The method of claim 15 wherein the buffer solution is a phosphate-citrate buffer solution.

10

17. A wound dressing comprising:

decellularized tissue at least partially surrounded by a PEG hydrogel, wherein the PEG hydrogel is structured by covalent bonds formed via an oxime ligation reaction of a polyethylene glycol (PEG) containing component and a cross-linking component.

15

18. A wound dressing comprising:

fibers at least partially surrounded by a PEG hydrogel, wherein the PEG hydrogel is structured by covalent bonds formed via an oxime ligation reaction of a polyethylene glycol (PEG) containing component and a cross-linking component.

20

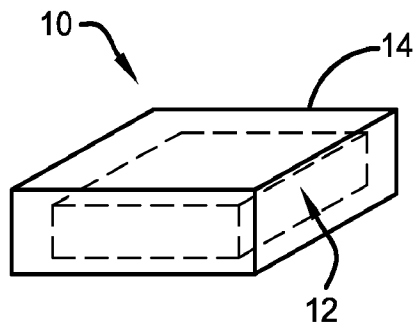


FIG. 1A

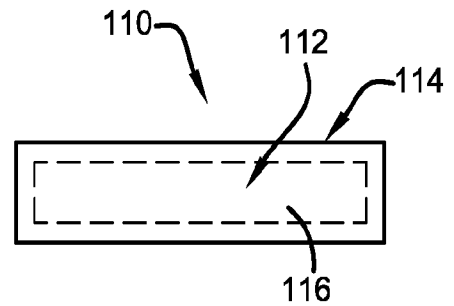


FIG. 2

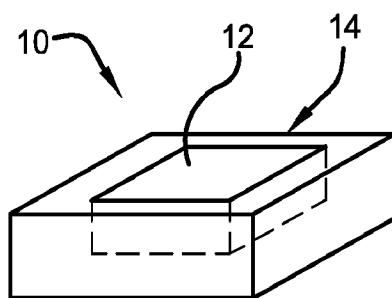


FIG. 1B

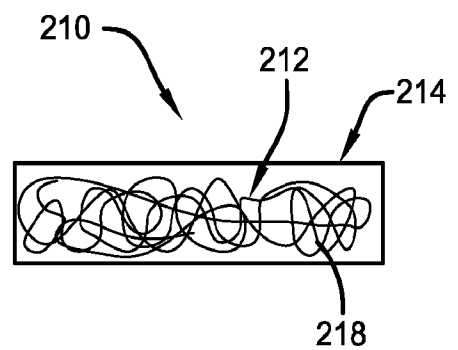


FIG. 3

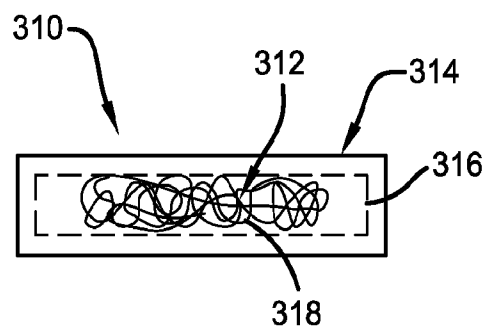


FIG. 4

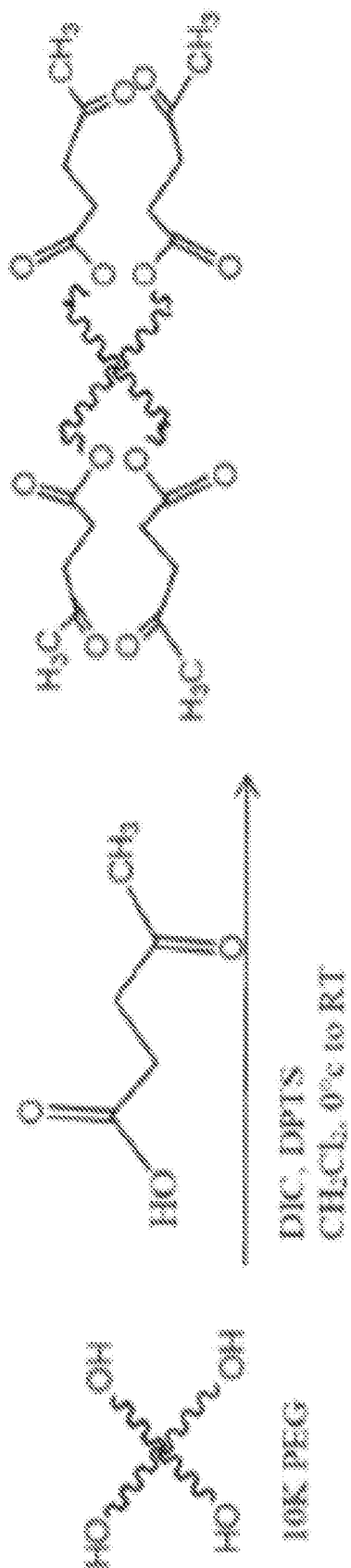


FIG. 5

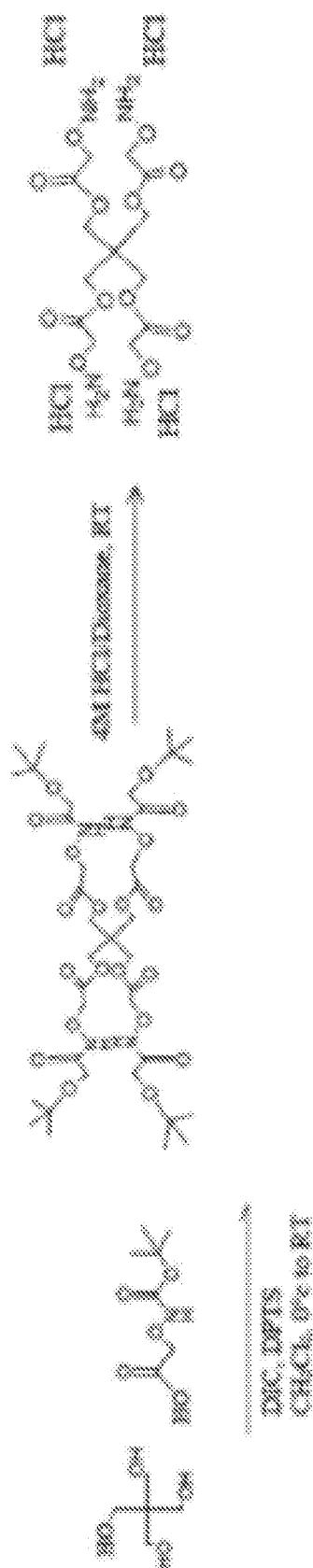


FIG. 6

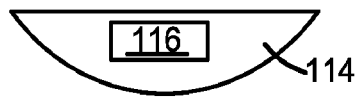


FIG. 7

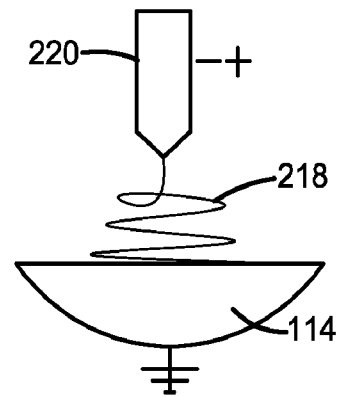


FIG. 8

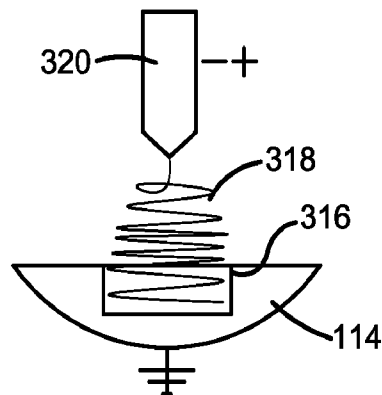


FIG. 9

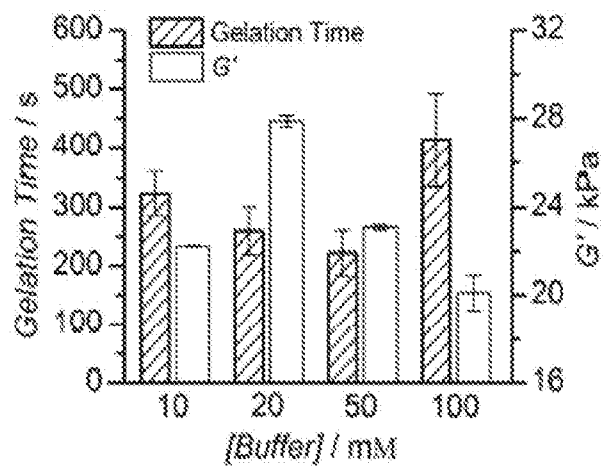


FIG. 10

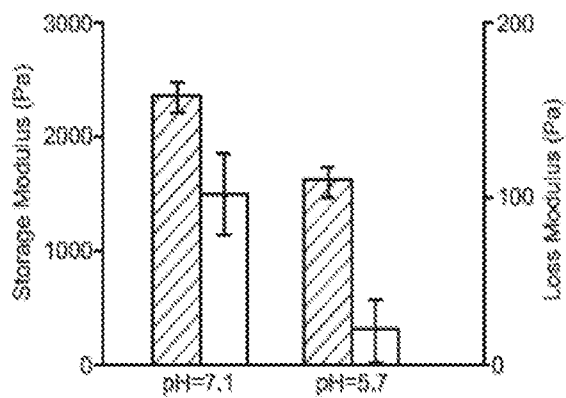


FIG. 11

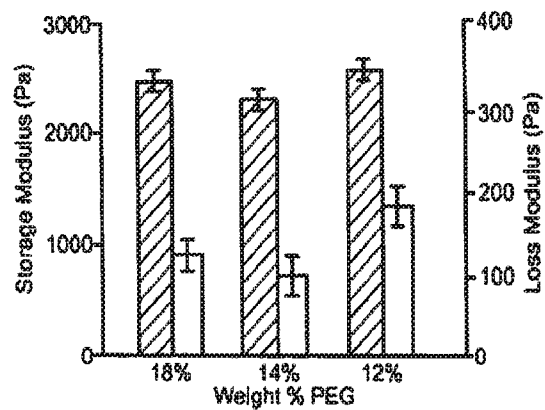


FIG. 12

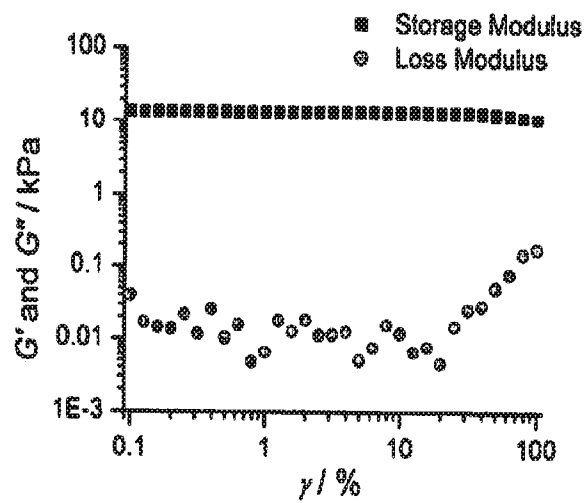


FIG. 13

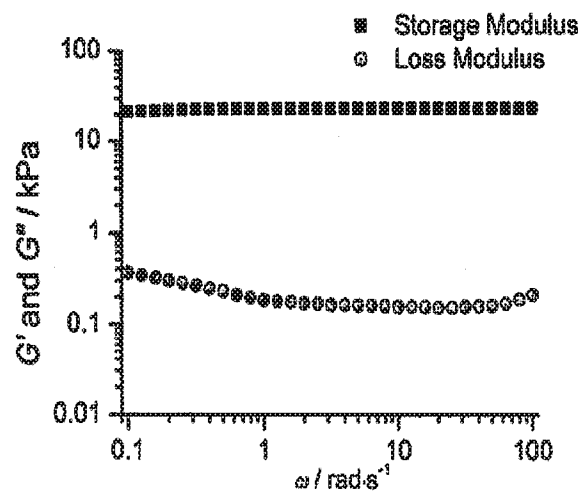


FIG. 14

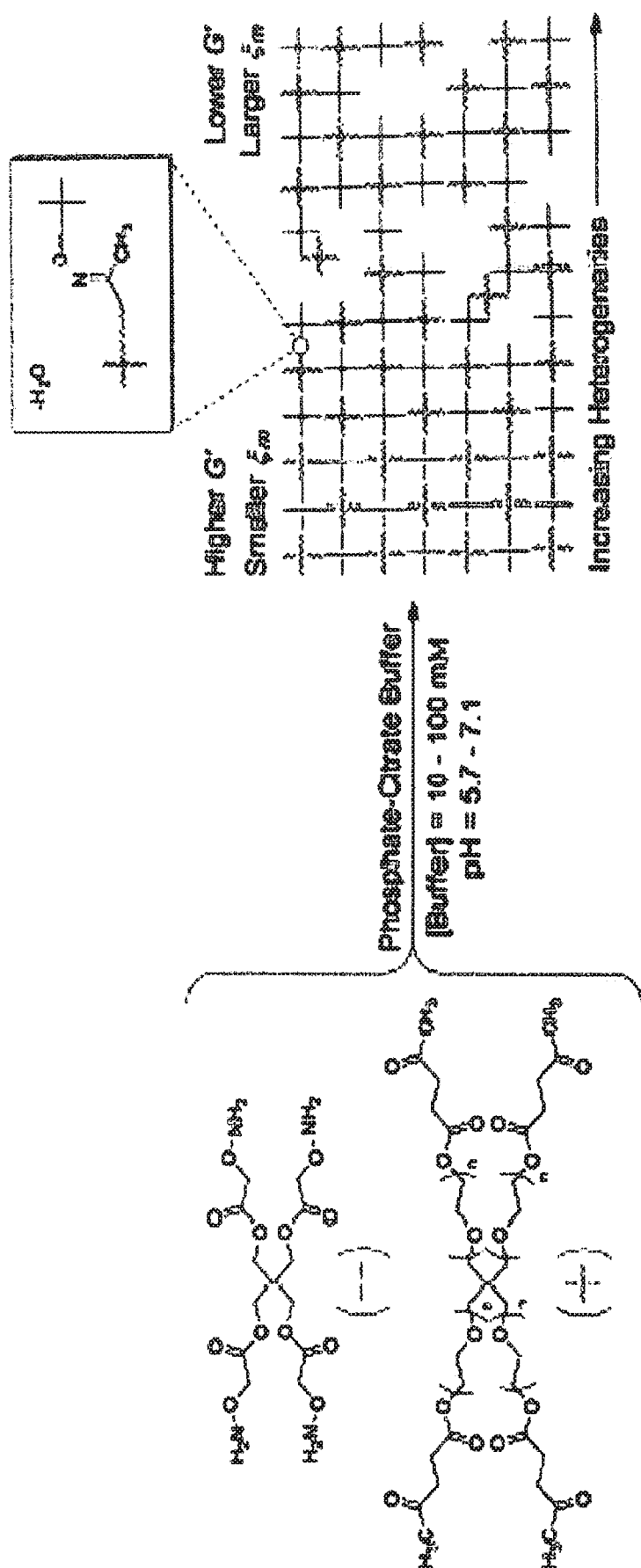
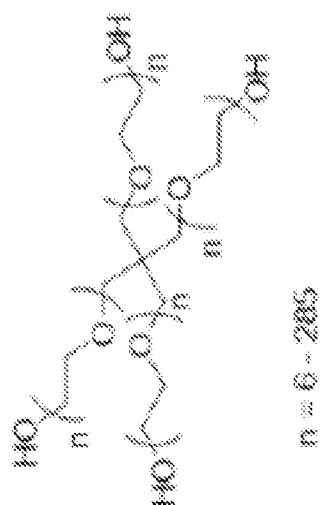
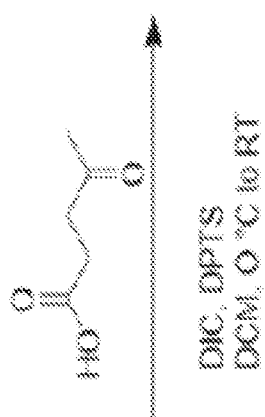
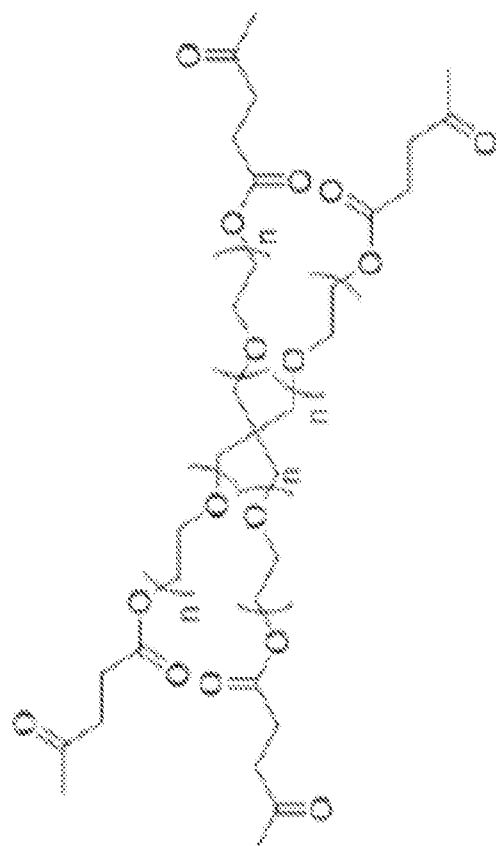
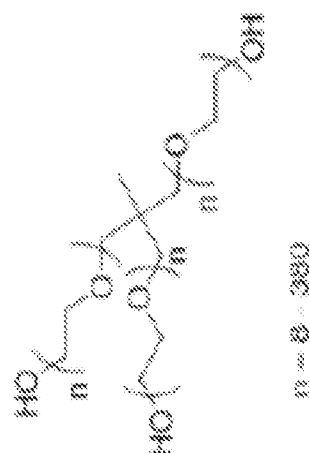
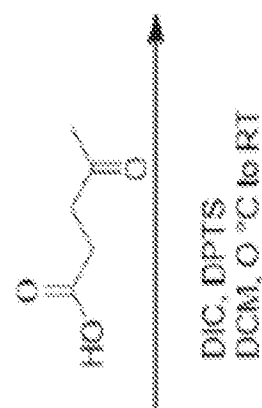
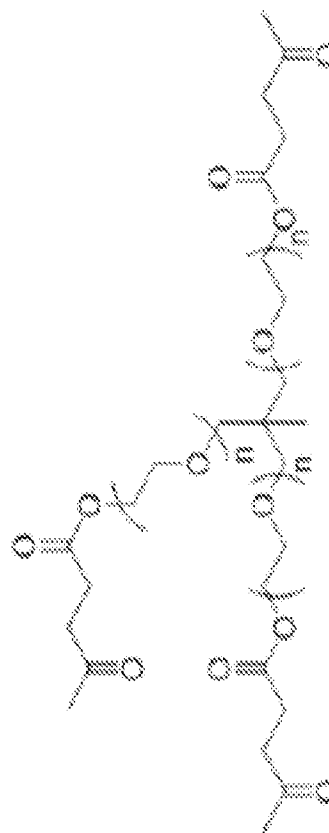


FIG. 15



$n = 6 - 285$

FIG. 16



$n = 8 - 380$

FIG. 17

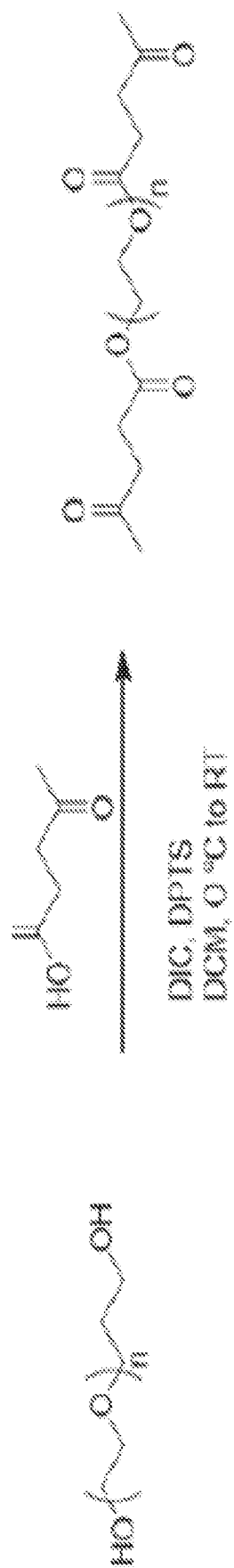


FIG. 18

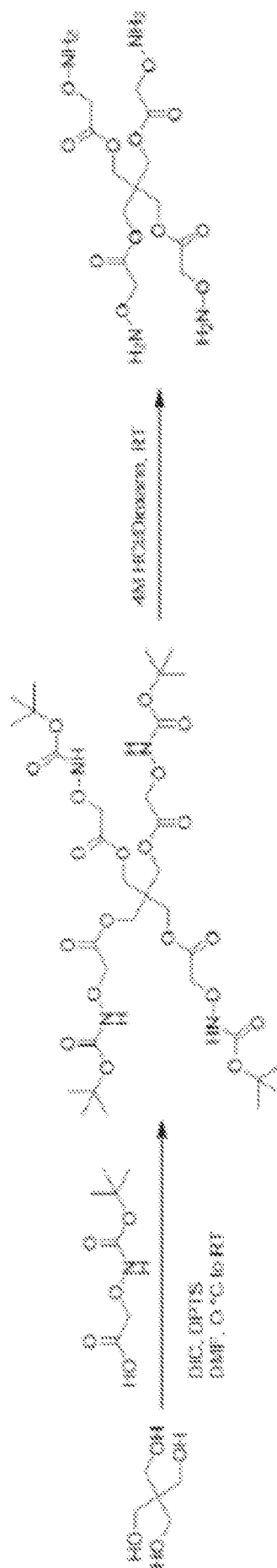
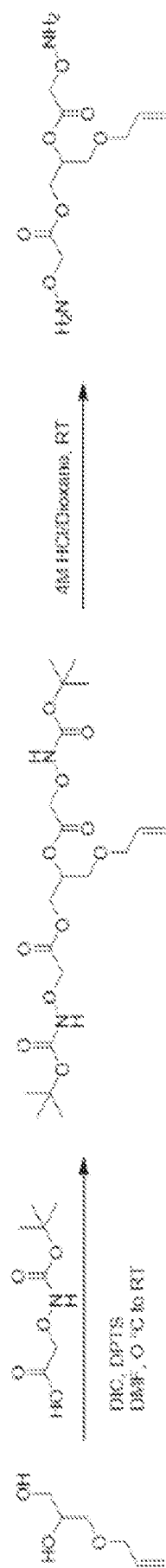
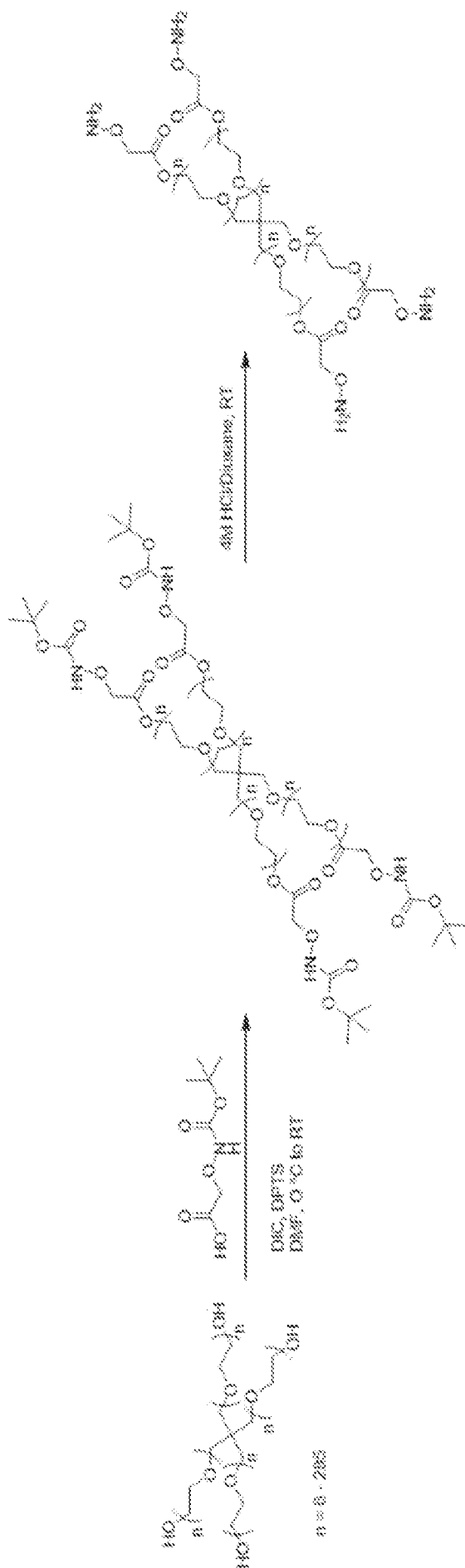


FIG. 19



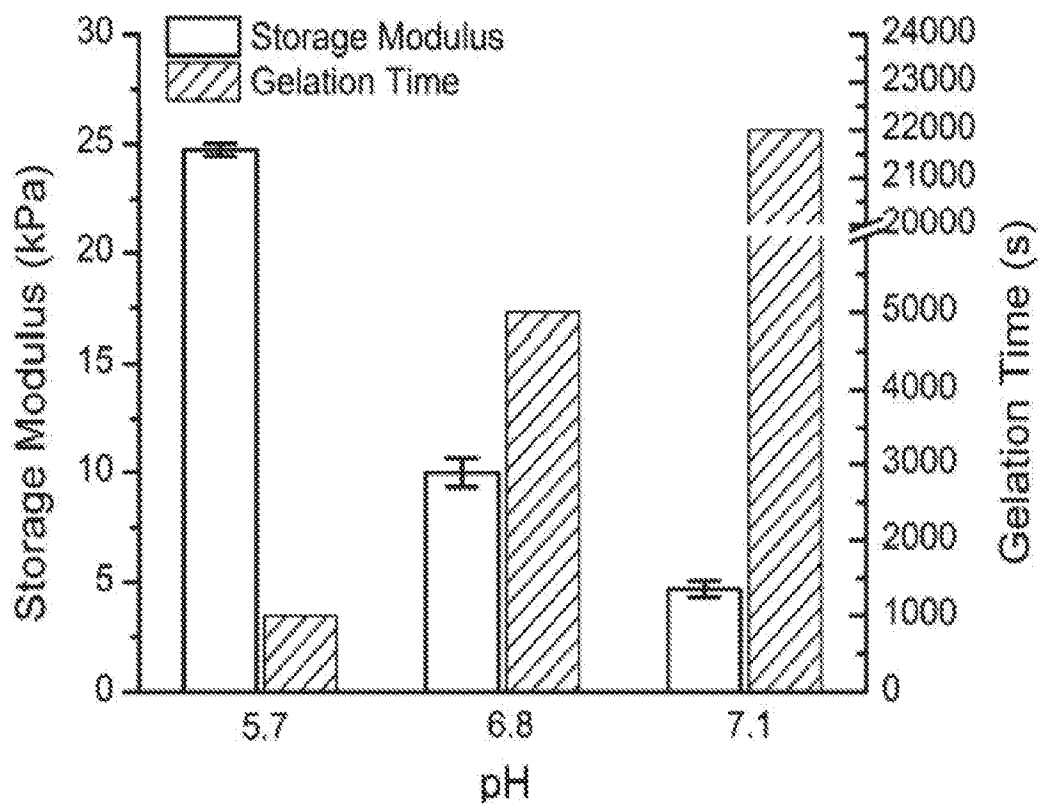


FIG. 22

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US16/18043

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 9/52, 47/42 (2016.01)

CPC - A61K 9/06, 47/42

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): A61K 9/52, 47/42 (2016.01)

CPC: A61K 9/06, 47/42

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); ProQuest; Scifinder; Google/Google Scholar;  
KEYWORDS: decellularized, tissue, fiber, hydrogel, wound, dressing, PEG, oxime, ketone, aldehyde, functionality, buffer, crosslinking

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                       | Relevant to claim No. |
|-----------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Y         | US 2014/0248328 A1 (WEHMEYER, JL et al.) 04 September 2014; abstract; paragraph [0063]; claim 39                                                                                                                                                                                                                         | 8-14                  |
| Y         | WO 2014/040026 A2 (WAKE FOREST UNIVERSITY HEALTH SCIENCES) 13 March 2014; page 3, lines 7-13, 24-26; page 4, lines 7-13, 21-22; page 18, lines 7-8; page 23, lines 14-20; page 24, lines 10-25; page 25, lines 1-4; page 28, lines 28-30; page 29, line 1; page 31, lines 5-7; page 32, lines 9-10; page 41, lines 29-31 | 1-14, 17              |
| Y         | WO 2014/039245 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 13 March 2014; paragraphs [0008]-[0009], [0015], [0065], [00157]; figure 1                                                                                                                                                                               | 1-2, 4-7, 15-18       |
| Y         | US 2012/0156164 A1 (PARK, KD et al.) 21 June 2012; paragraphs [0024], [0057], [0060], [0067]-[0068], [0073]-[0074], [0116]                                                                                                                                                                                               | 1-7, 9, 11-12, 18     |
| Y         | US 2014/0371692 A1 (EMANUEL, ON et al.) 18 December 2014; paragraphs [0081], [0315]                                                                                                                                                                                                                                      | 3, 5-7, 13, 15-16     |
| Y         | US 2010/0144902 A1 (SHU, X) 10 June 2010; paragraphs [0027]-[0029], [0059]-[0060]                                                                                                                                                                                                                                        | 15-16                 |
| Y         | US 2006/0127873 A1 (HOEMANN, C et al.) 15 June 2006; paragraph [0011]                                                                                                                                                                                                                                                    | 3, 11                 |



Further documents are listed in the continuation of Box C.



See patent family annex.

## \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

30 March 2016 (30.03.2016)

Date of mailing of the international search report

29 APR 2016

Name and mailing address of the ISA/

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