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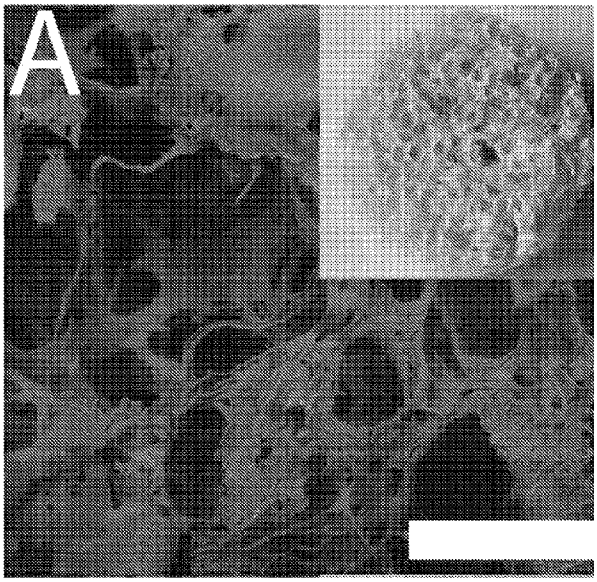

Fig. 1A

(57) Abstract: Embodiments of the present disclosure provide for conductive foams (e.g., silk foams, polymer foams) methods of making the conductive foam, method of using the conductive foam, and the like.

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ELECTROACTIVE SCAFFOLDS AND METHODS OF USING ELECTROACTIVE SCAFFOLDS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of and priority to U.S. Provisional Application Serial No. 62/192,686, having the title "ELECTROACTIVE SCAFFOLDS AND METHODS OF USING ELECTROACTIVE SCAFFOLDS," filed on July 15, 2015, the disclosure of which is incorporated herein in by reference in its entirety.

BACKGROUND

Bone tissues are hierarchically structured composite materials composed of both soft and hard matter (i.e. cell-rich vascularized soft tissue, and collagen-/hydroxyapatite-rich hard tissue). Bone conditions and disorders that require surgical intervention motivate development of novel biomaterials that facilitate bone tissue regeneration. Engineered bone tissue scaffolds to control cell outcomes in a rational fashion are of particular interest for such applications. Thus, there is a need for engineered bone tissue scaffolds.

SUMMARY

Embodiments of the present disclosure provide for conductive foams *(e.g.,* silk foams, polymer foams) methods of making the conductive foam, method of using the conductive foam, and the like.

An embodiment of the present disclosure includes a method of differentiation of human mesenchymal stem cells, among others, that includes: providing a conductive foam with an interpenetrating polymer network on the surface of the foam, wherein the interpenetrating polymer network is made from a doped conductive polymer that is either a self-doped conducting polymer or a conducting polymer and a dopant; introducing human mesenchymal stem cells to the conductive foam, wherein the conductive foam and the human mesenchymal stem cells are cultured in a osteogenic medium; and periodically providing electrical stimulation to the human mesenchymal stem cells to cause differentiation of human mesenchymal stem cells towards osteogenic outcomes. In an embodiment, the method further includes providing increased ALP activity, increased collagen deposition, increased Ca^{2+} deposition, or a combination thereof, on the foams relative to not periodically providing electrical stimulation.

An embodiment of the present disclosure includes a structure, among others, that includes: a conductive foam having an interpenetrating polymer network on the surface of the foam, wherein the interpenetrating polymer network is made from a doped conductive polymer that is either a self-doped conducting polymer or a conducting polymer and a dopant, wherein human mesenchymal stem cells are disposed within the conductive foam.

Other compositions, structures, methods, features, and advantages will be or become apparent to one with skill in the art upon examination of the following drawings and detailed description. It is intended that all such additional compositions, apparatus, methods, features and advantages be included within this description, be within the scope of the present disclosure, and be protected by the accompanying claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Further aspects of the present disclosure will be more readily appreciated upon review of the detailed description of its various embodiments, described below, when taken in conjunction with the accompanying drawings.

Figures 1A-B demonstrate physicochemical analysis of the tissue scaffolds. Fig. 1A is an SEM image of non-conductive silk foam with inset photograph of the bulk foam. Fig. IB is an SEM image of conductive silk foam, with inset photograph of the bulk foam, and the structure of the self-doped CP composed of pyrrole and 2-hydroxy-5-sulfonic aniline overlaid. Scale bars represent 400 μπι, and the bulk foams were 4 mm in diameter and height. Figs. 1C and ID are XPS and FTIR spectra, respectively; grey lines represent spectra of non-conductive silk foams and black lines represent spectra of conductive silk foams. The appearance of peaks in the XPS spectrum of the conductive silk foam at 168 and 400 eV, and in the FTIR spectrum of the conductive silk foam at 1203, 1033, 927 and 895 cm-1 confirm that the surface chemistry of the silk foams changed after growth of an interpenetrating network of CPs.

Figures 2A-F are biochemical analyses. Figure 2A illustrates *in vitro* degradation assay: white bars, silk foam without enzyme; light grey bars, silk foam with enzyme; dark grey bars, conductive silk foam without enzyme; black bars, conductive silk foam with enzyme. Figure 2B illustrates HMSC viability after incubation with ethanol (15% v/v, toxic control) or different concentrations of CP. Figures 2C-F illustrate quantitative studies of cell culture experiments: light grey bars, silk foam; dark grey bars, conductive silk foam without electrical stimulation; black bars, conductive silk foam with electrical stimulation.

Figures 3A-R show histological analysis of the scaffolds at various points in time. Hematoxylin and eosin (H&E) staining of sections of non-conductive scaffolds results in characteristic blue staining of cell nuclei, and characteristic pink staining of intracellular and extracellular proteins *(e.g.*, actin or silk, respectively); Alizarin staining results in characteristic orange-red staining of calcium deposits that are early stage markers of matrix mineralization; the CP is black. Figures 3A to 3F are non-conductive silk foams: Fig. 3A illustrates 10 days, H&E, Fig. 3B illustrates 10 days, Alizarin, Fig. 3C illustrates 20 days, H&E, Fig. 3D illustrates 20 days, Alizarin, Fig. 3E illustrates 30 days, H&E, Fig. 3F illustrates 30 days, Alizarin. Figures 3G to 3L are conductive silk foams without electrical stimulation: Fig. 3G illustrates 10 days, H&E, Fig. 3H illustrates 10 days, Alizarin, Fig. 31 illustrates 20 days, H&E, Fig. 3J illustrates 20 days, Alizarin, Fig. 3K illustrates 30 days, H&E, Fig. 3L illustrates 30 days, Alizarin. Figures 3M to Fig. 3R are conductive silk foams with electrical stimulation: Fig. 3M illustrates 10 days, H&E, Fig. 3N illustrates 10 days, Alizarin, Fig. 30 illustrates 20 days, H&E, Fig. 3P illustrates 20 days, Alizarin, Fig. 3Q illustrates 30 days, H&E, and Fig. 3R illustrates 30 days, Alizarin. Scale bars represent 100 μm.

Figure 4 shows high magnification SEM image of the smooth underside of the foams in contact with the petri dish during foam preparation (foams are subsequently removed from the petri dish template during salt leaching). The left side of the image is of non-conductive silk foam. The right side of the image is of conductive silk foam clearly shows the presence of CP-based nanoparticles on the surface of the silk. Scale bar represents 1 μm.

DETAILED DESCRIPTION

This disclosure is not limited to particular embodiments described, and as such may, of course, vary. The terminology used herein serves the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present disclosure will be limited only by the appended claims.

Where a range of values is provided, each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the disclosure. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges and are also encompassed within the disclosure, 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 disclosure.

As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present disclosure. Any recited method may be carried out in the order of events recited or in any other order that is logically possible.

Embodiments of the present disclosure will employ, unless otherwise indicated, techniques of organic chemistry, biochemistry, microbiology, molecular biology, pharmacology, medicine, and the like, which are within the skill of the art. Such techniques are explained fully in the literature.

Prior to describing the various embodiments, the following definitions are provided and should be used unless otherwise indicated.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art of microbiology, molecular biology, medicinal chemistry, and/or organic chemistry. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described herein.

As used in the specification and the appended claims, the singular forms "a," "an," and "the" may include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a support" includes a plurality of supports. In this specification and in the claims that follow, reference will be made to a number of terms that shall be defined to have the following meanings unless a contrary intention is apparent.

Discussion :

Embodiments of the present disclosure provide for conductive foams *(e.g.,* silk foams, polymer foams), methods of making the conductive foam, methods of using the conductive foam, and the like. In a particular embodiment, a cell such as a human mesenchymal stem cell can be incubated with the conductive silk foam and cultured in an osteogenic medium so that the stem cells differentiate towards osteogenic outcomes.

In this regard, embodiments of the disclosure provide for methods of differentiation of human mesenchymal stem cells. An embodiment of the present disclosure includes introducing human mesenchymal stem cells to the conductive foam *(e.g.,* conductive silk

foam), where the conductive foam and the human mesenchymal stem cells are cultured in an osteogenic medium. In an embodiment, the conductive foam has electroactive characteristics so that an electrical stimulation can be periodically applied to the conductive foam. Subsequently, electrical stimulation can be periodically applied to the human mesenchymal stem cells to cause differentiation of human mesenchymal stem cells towards osteogenic outcomes.

In an embodiment, application of electrical stimulation to the conductive silk foam increases ALP activity, increased collagen formation, and/or increased Ca^{2+} deposition on the foams of the nonwoven mat, which can lead to formation of calcified bone-like extracellular matrix. As shown in Example 1, electrical stimulation of the conductive silk foam in the presence of human mesenchymal stem cells in the osteogenic medium shows increased differentiation towards osteogenic outcomes as compared to conductive silk foam without electrical stimulation and other types of materials.

In an embodiment, the interpenetrating polymer network is formed from a conducting polymer *(e.g.,* polypyrrole) and a dopant *(e.g.,* 2-hydroxy-5-sulfonic aniline). In an embodiment, the conducting polymer can include either a self-doped conducting polymer or a conducting polymer and a dopant. The interpenetrating polymer network is disposed on the surface of the conductive foams. In an embodiment, the amount of the conducting polymer and dopant disposed on the conductive foams can be about 1 to 100% by mass, where the range includes each 1% increment *(e.g.,* 1 to 10%, about 50 to 80%, about 20 to 60%, and the like).

In an embodiment, the conductive foam is a conductive silk foam that has a porous scaffold of silk protein having interpenetrating polymer networks. In an embodiment, the silk foams can include fibroin or related proteins. In an embodiment, the silk can be natural silks from any species, including but not limited to: silk worms, spiders, lacewings, caddisfly larvae, bees; or recombinantly/genetically engineered proteins inspired by silks; or chemically produced peptides/proteins/polymers inspired by silks. As used herein, the term "fibroin" includes fibroin obtained from a solution containing a dissolved silk (typically silkworm silk). The silkworm silk protein may be obtained, for example, from *B.mori,* and the spider silk may be obtained from *N. clavipes.* In an alternative embodiment, the silk can be obtained from a solution containing a genetically engineered silk, produced by bacteria, yeast, mammalian cells, transgenic animals or transgenic plants. Silk solutions can be prepared by any conventional method known to one skilled in the art, and one approach is described in detail in the Example.

In an embodiment, the conductive foam has usable mechanical properties as well as pore structure, where the one or more of the pores can be interconnected with or among one another. In an embodiment, the conductive foam can have a length of about 1 to 1000 millimeters and a width of about 1 micrometers to 1000 millimeters. In an embodiment, the density of the conductive foam can be about 0.01 and 10 g per cm^3. In an embodiment, the porosity of the conductive foam can be about 3 and 99%, and the pores are typically interconnected and have pore sizes of 10 to 1000 μm, where the diameter may be the same throughout the pore or vary in diameter.

In an embodiment, the conductive foam can be a conductive polymer foam such as a synthetic polymer, a natural polymer, or a combination thereof. In an embodiment, the synthetic polymer can include polycaprolactone, polyesters, polyamides, polycaprolactone (PCL), poly-L-lactic acid (PLLA), poly lactic-co-glycolic acid (PLGA), and combinations thereof. In an embodiment, the natural polymer can be proteins, polysaccharides, lignins, polyalanine, oligoalanine, collagen, cellulose, chitin, chitosan, and a combination thereof. In an embodiment, the conductive foam can include a mixture of different types of polymers and/or silks *(e.g.,* a portion of proteins such as silks and polysaccharides such as hyaluronic acid).

In an embodiment, the interpenetrating polymer network can be formed from the conducting polymer and the dopant. In an embodiment, the conducting polymers include polypyrrole, polyaniline, polythiophene, poly(3,4-ethylenedioxythiophene), poly fluorenes, polyphenylenes, polypyrenes, polyazulenes, polynapthalenes, polyindoles, polyazepines, poly(p-phenylene sulfide)s, poly(p-phenylene vinylene)s, and polyfurans. In an embodiment, there are biodegradable versions, in which there are block of conducting units within a polymer chain containing biodegradable bonds *(e.g.* esters and amides), that can also be used as the conducting polymer.

In an embodiment, the dopant can be a polymer that has the opposite charge to the conducting polymer, and can have a low molecular weight *(e.g.,* chlorine ions, tosylate ions, and the like) or high molecular weight *(e.g.,* collagen, hyaluronic acid, and the like). In an embodiment, the dopant can be chemically linked to the backbone of the polymer *(i.e.,* self-doped) potentially as a monomer *(e.g.* 2-hydroxy-5-sulfonic aniline).

In an embodiment, the conducting polymer and the dopant can be disposed on the silk foams by incubating the foams with conducting polymer, dopant, and an agent capable of polymerizing the monomer(s) *(e.g.,* an oxidizing agent such as ferric chloride, a light source or suitable electrochemistry apparatus) for an appropriate time period *(e.g.,* about 1 to 36

hours or about 24 hours). After incubation, the residual materials can be washed away and the foams having interpenetrating polymer networks are formed. Additional details are provided in Example 1.

In an embodiment the osteogenic medium is based on standard cell culture medium with the optional addition of other components such as serum, non-essential amino acids, bone morphogenetic protein 2 (BMP-2), dexamethasone, β-glycerophosphate, ascorbic acid, ascorbic acid-2-phosphate, heparin, retinoic acid, and 1,25-dihydroxycholecalciferol (for example: high glucose Dulbecco's Modified Eagle Medium (DMEM, 425 mL); fetal bovine serum (50 mL); antibiotic-antimycotic (5 mL); non-essential amino acids (5 mL), dexamethasone (100 nM), β-glycerol phosphate (10 mM) and ascorbic acid (50 μM)). The volume of medium used should be in line with the recommended guidelines of the manufacturer of the cell culture dishes.

Electrical stimulation can include direct contact of the material with a power source via a wire, wireless energy transfer, magnetic force, and the like. The term "periodically" refers to applying the electrical stimulation at established time frames that may be at regular or irregular time intervals on the time frames of seconds, hours, days, weeks, or months *(e.g.,* about 1 s to 2 months, about 1 hour to 1 day, about 1 day to 1 month, or other the like) depending upon the specific circumstances. In an embodiment, the impulses of the electrical stimulation can last on the time frame of seconds, hours, or days *(e.g.,* about 1 second to 1 day, about 10 seconds to 1 hour, about 1 minute to 12 hours, about 1 hour to 1 day, or the like) depending upon the specific circumstances. In an embodiment, the electrical stimulation can be in the range of millivolts to volts *(e.g.,* about 10 mV to 10 volts, about 1 mV to 100 mV, or the like). The time frame, duration of electrical stimulation, and intensity of the electrical stimulation can be designed based on particular circumstances and requirements of a specific situation.

As stated above, embodiments of the present disclosure provide for a conductive foam, where the foam includes interpenetrating polymer networks. In addition, human mesenchymal stem cells *(e.g.,* on collagen-1 coated substrates) and the differentiated products of the stem cells are disposed within the conductive foam. Furthermore, the conductive foam includes ALP, collagen, and/or Ca^{2+}, which can be deposited on the foams. In an embodiment, the conductive foam can include one or more agents *(e.g.,* a chemical or biological agent), where the agent can be disposed indirectly or directly on the conductive foam. As described herein, the agent can include a stem cell such as a human mesenchymal stem cell.

In addition, an additional agent that can be disposed on the conductive foam can include, but is not limited to, a drug, a therapeutic agent, a radiological agent, a small molecule drug, a biological agent *(e.g.*, polypeptides *(e.g.*, proteins such as, but not limited to, antibodies (monoclonal or polyclonal)), antigens, nucleic acids (both monomeric and oligomeric), polysaccharides, haptens, sugars, fatty acids, steroids, purines, pyrimidines, ligands, and aptamers) and combinations thereof, that can be used to image, detect, study, monitor, evaluate, and the like, the differentiation of the stem cells. In an embodiment, the agent is included in an effective amount to accomplish its purpose *(e.g.*, ALP production and/or Ca^{2+} production), where such factors to accomplish the purpose are well known in the medical arts.

In general, the agent can be bound to the conductive silk foam by a physical, biological, biochemical, and/or chemical association directly or indirectly by a suitable means. The term "bound" can include, but is not limited to, chemically bonded *(e.g.*, covalently or ionically), biologically bonded, biochemically bonded, and/or otherwise associated with the electroactive supramolecular polymeric assembly. In an embodiment, being bound can include, but is not limited to, a covalent bond, a non-covalent bond, an ionic bond, a chelated bond, as well as being bound through interactions such as, but not limited to, hydrophobic interactions, hydrophilic interactions, charge-charge interactions, π-π stacking interactions, combinations thereof, and like interactions. In an embodiment, cell-conductive silk foam interactions could be controlled through the inclusion of cell-adhesive peptides *(e.g.*, RGD, YIGSR, KQAGDV, KHIFSDDSSE, KRSR), and protease-labile domains *(e.g.*, APGL, VRN, or indeed oligoalanines that are degraded by elastase).

While embodiments of the present disclosure are described in connection with the Examples and the corresponding text and figures, there is no intent to limit the disclosure to the embodiments in these descriptions. On the contrary, the intent is to cover all alternatives, modifications, and equivalents included within the spirit and scope of embodiments of the present disclosure.

EXAMPLE:

Stimuli-responsive materials enabling the behaviour of the cells that reside within them to be controlled are vital for the development of instructive tissue scaffolds for tissue engineering. This example describes the preparation of conductive silk foam-based bone tissue scaffolds that enable the electrical stimulation of human mesenchymal stem cells to enhance their differentiation towards osteogenic outcomes.

Introduction:

Bone tissues are hierarchically structured composite materials composed of both soft and hard matter *(i.e.,* cell-rich vascularized soft tissue, and collagen-/hydroxyapatite-rich hard tissue). Bone conditions and disorders that require surgical intervention motivate development of novel biomaterials that facilitate bone tissue regeneration.[1] Engineered bone tissue scaffolds to control cell outcomes in a rational fashion are of particular interest for such applications.

An incredibly diverse variety of materials have been investigated for their potential application in bone repair and regeneration,[2] including non-biodegradable materials (such as ceramics, glasses, polymethylmethacrylate and titanium)[2] or biodegradable materials (such as autografts, allografts and polycaprolactone),[2] and moreover multifunctional materials capable of drug delivery.[3] Biopolymer-based tissue scaffolds represent a particularly interesting class of biomaterials because of the versatile materials morphologies accessible via aqueous processing, and a variety of both polysaccharides and proteins have been investigated for their application as bone tissue scaffolds.[4] Natural silk proteins and recombinant silk-inspired proteins are frequently used as base materials for both drug delivery devices and tissue scaffolds with encouraging results both *in vitro* and in preclinical studies.[5]

Electromagnetic fields may be employed for the non-invasive stimulation of bone growth, or as invasive implantable biointerfaces such as cardiac pacemakers and neural electrodes. Biointerfaces based on conductive polymers (CPs), such as derivatives of polyaniline, polypyrrole or polythiophene, are of interest for both long term applications as low impedance coatings for electrodes with biomimetic mechanical properties and potentially for short term applications as drug delivery devices or tissue scaffolds for tissue engineering.[6]

Pro-regenerative CP-based tissue scaffolds have been developed for various tissues.[6,7] Electrical stimulation of C2C12 mouse myoblasts (a common model for muscle cells) *in vitro* results in increased contractile activity and maturation relative to non-stimulated controls,[8] and therefore, C2C12-adhesive polythiophene-based hydrogels with biomimetic mechanical properties represent promising muscle tissue scaffolds.[9] Likewise, electrical stimulation of peripheral nerve gaps *in vivo* improves the rate of recovery, thus, polypyrrole-based materials with biomimetic topographies have promise as nerve tissue scaffolds.™

The concept of using CP-based materials as bone tissue scaffolds was first reported by Langer and coworkers,[11] who found that applying a potential step of 20 mV/mm across 2-dimensional polypyrrole films enhanced the differentiation of bone marrow-derived stromal cells towards osteogenic outcomes, as confirmed by an increase in alkaline phosphatase (ALP) activity per cell relative to non-stimulated control substrates,[11] and further developed by others.[12] Oligoaniline-based CPs are increasingly popular biomaterials,[6i,6j] and such polymers have been shown to promote osteogenic differentiation.[12c]

A variety of conductive protein-based materials have been prepared previously.[6g] Some examples include those based on individual components of the extracellular matrix (e.g. collagen),[13] and decellularized tissues containing a variety of extracellular matrix proteins. Additionally, functionalization of spider[14] and silkworm[15] silks with polypyrrole yields anti-static silk textiles, or novel stimuli-responsive actuators. Here we describe the preparation of conductive 3D silk foams and their use as instructive bone tissue scaffolds that enable electrical stimulation of human mesenchymal stem cells (HMSCs), thereby enhancing osteogenic differentiation (and this is to the best of our knowledge the first report of electrical stimulation on such a large scaffold).

Experimental Section

Materials

Unless otherwise stated, all chemicals for synthesis and physicochemical analysis were of ACS grade, purchased from Sigma-Aldrich and used as received without further purification. Phosphate buffered saline (PBS) was at pH 7.4. Reagents for cell culture were purchased from Invitrogen (Carlsbad, CA) unless otherwise noted. Human mesenchymal stem cells (HMSCs) from a 24 year old drug- and disease-free male were purchased from Lonza (Gaithersburg, MD).

Preparation of silkfoams

Aqueous solutions of silk fibroin were prepared in accordance with the literature.[17] Briefly, silk cocoons of B. mori silkworms were degummed by boiling in an aqueous solution of Na_2C0_3 (0.02 M) for 20 min, followed by rinsing thoroughly with distilled water. The extracted silk fibroin was then dissolved in aqueous LiBr (9.3 M) at 60 °C for 4 h, and thereafter dialyzed against ultrapure water using a Slide-a-Lyzer dialysis cassette (MWCO 3,500, Life Technologies, Carlsbad, CA, USA) for 2 days. The solution was centrifuged at 9000 rpm *(ca.* 12,700 g) at 4 °C for 20 min, transferred to a fresh centrifuge tube, and

centrifuged at 9000 rpm *{ca.* 12,700 g) at 4 °C for a further 20 min to remove any solids. The final concentration of silk fibroin aqueous solution was 8 w/v%, as determined by dry weight analysis. If necessary, solutions could be stored for a few weeks at 4 °C without the onset of premature precipitation. Glass petri dishes were almost filled with NaCl (particle size of 425-500 μm) and aqueous solutions of silk fibroin (8 wt%) were poured over the salt. The petri dish was covered and left at room temperature for 48 h, after which the petri dish was immersed in water (2L) and the NaCl extracted for 2 days changing the water at least three times a day (minimum 6 washes). Samples were cut to lengths appropriate for the various subsequent experiments using a disposable biopsy punch and a razor blade. The porous silk scaffolds were either stored in ultrapure water at 4 °C or lyophilized and stored at room temperature.

Preparation of conductive silkfoams with interpenetrating networks of poly(pyrrole-co-(2-hydroxy-5-sulfonic aniline))

Interpenetrating networks of conductive polymers and silk fibroin were prepared by adaptation of the literature.[10d, 18] Briefly, pyrrole was purified by passage over basic alumina. 2-hydroxy-5-sulfonic aniline (0.473 g, 2.5 mmol) was dissolved in HC1 solution (1 M, 50 mL) in disposable 50 mL centrifuge tubes. Pyrrole (0.175 mL, 2.5 mmol) was added and the sample cooled to 0 °C. 60 silk foams (4 mm in height and diameter) were added and incubated at 0 °C for 1 hour. $(NH_4)_2S_2O_8$ (1.142 g, 5 mmol) and $FeCl_3$ (0.005 g, catalytic)[19] were added, and the samples were shaken to assure dissolution of the ammonium persulfate and ferric chloride and then incubated for a further 24 h at 4 °C. The reaction mixture was decanted and the foams were washed with water, however, the interpenetrating network was inhomogeneous after this time. Therefore, the process was repeated, and a solution of 2-hydroxy-5-sulfonic aniline (0.473 g, 2.5 mmol) in HC1 (1 M, 50 mL), pyrrole (0.175 mL, 2.5 mmol) was added to the foams, followed by $(NH_4)_2S_2O_8$ (1.142 g, 5 mmol) and $FeCl_3$ (0.005 g, catalytic).[19] The samples were shaken and then incubated for a further 24 h at 4 °C. Homogeneously black conductive foams were removed from the reaction mixture, placed in fresh distilled water, sonicated for 5 min, and then exhaustively washed (to remove monomers, oligomers and initiators) with deionized water until the water used to wash the materials was clear, colourless and the pH was neutral *{ca.* 3 days), after which they were washed with ethanol (1 day at 21 °C with two changes of ethanol). Conductive silk foams with an interpenetrating network of poly(pyrrole-co-(2-hydroxy-5-sulfonic aniline)) were

dried under high vacuum at 21 °C (mass differences in the non-conductive and conductive foams were within experimental error negligible).

Porosity determination

The porosity of the samples (n = 5) was measured by liquid displacement.[16c] Hexane was used as the liquid as it does not swell or shrink the sample. The sample was immersed in a known volume *(V\)* of hexane in a graduated cylinder for 5 min. The total volume of hexane and the hexane-impregnated sample was recorded as V_2. The hexane-impregnated sample was then removed from the cylinder and the residual hexane volume was recorded as $V3$. The total volume V of the sample was:

$$V = (V_2 - V_1) + (V_1 - V_3) = V_2 - V_3 \quad (1)$$

$$V_2 - V_1 \quad (2)$$

$$V_1 - V_3 \quad (3)$$

V2-V1 is the volume of the polymer scaffold. V_1-V_3 is the volume of hexane within the scaffold. The porosity of the scaffold (ε, %) was calculated from:

$$\varepsilon\ (\%) = ((V_1 - V_3)/(V_2 - V_1)) \times 100 \quad (4)$$

Swelling properties

Samples of dry weight (W_d) were immersed in PBS at room temperature for 24 h.[16c] After excess water was removed, the wet weight of the scaffold (W_s) was determined (n = 5). The swelling ratio *(SR)* and equilibrium water content *(EWC)* of the scaffold was calculated from:

$$SR = (W_s - W_d)/W_d \quad (5)$$

$$EWC\ (\%) = \left(\frac{W_s - W_d}{W_s}\right) \times 100 \quad (6)$$

Mechanical properties

Compressive tests were performed using an Instron Materials Testing Machine 5543 Series Single Column System (Instron, Norwood, MA) with Bluehill 2 software. Samples (diameter 4 mm, height 4 mm) were stored in PBS (1 mL) for 24 hours prior to testing. Each sample was compressed to 90 % of its original height at a rate of 0.05 mm s^{-1} using a 50 N

load cell (n = 16). The compressive stress and strain were plotted and the average compressive strength as well as the compressive modulus and standard error of the mean determined. The elastic modulus was defined by the slope of the initial linear section of the stress-strain curve. The compressive strength was determined by drawing a line parallel starting at 1% strain. The point at which this line crossed the stress-strain curve was defined as the compressive strength of the scaffold.[22c]

Scanning electron microscopy (SEM)

Samples were mounted on a Scanning Electron Microscopy (SEM) stub and sputter coated with Pt/Pd (15 nm) using a Cressington 208 Benchtop Sputter Coater. All samples were imaged using a Zeiss Supra 40 VP field emission scanning electron microscope.

X-ray photoelectron spectroscopy (XPS)

XPS was carried out on the samples to confirm that the surface chemistry of the scaffolds had changed after the growth of an interpenetrating network of the CP within the silk matrix. XPS was performed on a Kratos Axis X-ray photoelectron spectrometer (Kratos Analytical Ltd., Manchester, UK). The binding energy was calibrated using the C 1s photoelectron peak at 284.6 eV as a reference. The CasaXPS computer program was used for peak fitting of the C 1s and O 1s peaks in the XPS spectra. The reported spectra are representative of two measurements at different positions on a sample.

Fourier Transform infrared spectroscopy (FTIR)

A Thermo Scientific Nicolet 380 FTIR Spectrometer (Thermo Fisher Scientific Inc., USA) was used to record spectra in attenuated total reflectance (ATR) mode at 21 °C with a 1 cm^{-1} resolution and 128 scans (corrected for background and atmosphere using the software provided with the spectrometer). Samples were secured in position on the ATR crystal using the built-in clamp.

In vitro enzymatic degradation assay

The degradation of the samples was evaluated using protease XIV (EC 3.4.24.31). Samples (4 mm diameter, 4 mm height) were immersed in PBS (1 mL) and incubated for 24h at 37 °C. Samples were washed with PBS and ultrapure water, dried on Kimwipes and their initial mass determined. Samples were incubated at 37 °C in PBS optionally containing the enzyme protease XIV (1U mL^{-1}). At specific times, samples were washed with PBS and

ultrapure water, dried on Kimwipes and weighed. The enzyme solution was replaced with freshly prepared solution every 24 h (n = 5, error bars represent standard deviations).

Cell source & preparation

HMSCs were isolated from bone marrow aspirate (Lonza, Walkersville, MD) as described previously.[28] Briefly, aspirate from a male donor under 25 years old was combined with HMSC proliferation medium (MEM alpha with 10% fetal bovine serum (FBS), 1% antibiotic/antimycotic, 1% nonessential amino acids (NEAA)) and cultured at 37 C with 5% $C0_2$ in a humidified environment. Flasks were rocked every day to allow HMSCs to adhere and medium was added every 3-4 days until HMSCs reached 80% confluence. Non-adherent cells were removed via PBS washes and the HMSCs were cultured in proliferation medium until either passaged or frozen.

Toxicity of the CPs as assessed via Cell Titer-Glo® luminescent cell viability assay kit

HMSC viability was assessed using a Cell Titer-Glo® luminescent cell viability assay kit (Promega, USA) in accordance with the with the manufacturer's protocol, using a Synergy HT Multi-Mode Microplate Reader (Biotek, USA) to analyze the luminescence of the samples. As controls, cells seeded on tissue-culture treated Corning® Costar® tissue culture plates in pristine cell culture medium or cell culture medium containing 15% v/v ethanol were considered (n = 3, error bars represent standard deviations).

In vitro culture of human Mesenchymal stem cells without electrical stimulation

Foams were sterilized by electron beam sterilization (Nutek Corporation, Hayward, CA, USA). After sterilization, the samples were incubated for 30 minutes in 24 well plates containing HMSC growth medium that was composed of: high glucose Dulbecco's Modified Eagle Medium (DMEM, 440 mL); fetal bovine serum (50 mL); antibiotic-antimycotic (5 mL); non-essential amino acids (5 mL), and 2 ng mL^{-1} basic fibroblast growth factor. Medium was aspirated and replaced prior to HMSC seeding. Cell viability before starting the experiment was determined by the Trypan Blue exclusion method, and the measured viability exceeded 95 % in all cases. HMSCs were seeded at *ca.* 0.5 x 10^6 cells per foam, and incubated at 37 °C, 95 % humidity, and a $C0_2$ content of 5 %. After 3 days the medium was aspirated, the materials were washed gently with PBS and transferred to a fresh 24 well plate containing osteogenic medium that was composed of: high glucose Dulbecco's Modified Eagle Medium (DMEM, 425 mL); fetal bovine serum (50 mL); antibiotic-antimycotic (5

mL); non-essential amino acids (5 mL), dexamethasone (100 nM), β-glycerol phosphate (10 mM) and ascorbic acid (50 μM). Thereafter the osteogenic medium was aspirated and replaced every 2 days until the samples were analyzed.

In vitro culture of human mesenchymal stem cells with electrical stimulation

Electrical stimulation of human mesenchymal stem cells was achieved employing a custom built setup. A conductive composite composed of a dispersion of multiwalled carbon nanotubes (0.1 g) and Pellethane® 2363-80A TPU (Lubrizol, Wickliffe, OH, USA) (1 g) in chloroform (10 mL) was painted on the tip of copper wires and carefully embedded in the top and bottom of the conductive foams. The tip of the wire was bent to act as a hook inside the foam and secured in position by the application of more nanotube-loaded Pellethane®. The chloroform was allowed to evaporate and the samples were dried under high vacuum, yielding waterproof and conductive electrical contacts enabling the passage of an electrical current through the samples.

Foams were sterilized by electron beam sterilization (Nutek Corporation, Hayward, CA, USA). After sterilization, the samples were incubated for 30 minutes in 24 well plates containing HMSC growth medium that was composed of: high glucose Dulbecco's Modified Eagle Medium (DMEM, 440 mL); fetal bovine serum (50 mL); antibiotic-antimycotic (5 mL); non-essential amino acids (5 mL), and 2 ng mL^{-1} basic fibroblast growth factor. Medium was aspirated and replaced prior to HMSC seeding. Cell viability before starting the experiment was determined by the Trypan Blue exclusion method, and the measured viability exceeded 95 % in all cases. HMSCs were seeded at *ca.* 0.5 x 10^6 cells per foam, and incubated at 37 °C, 95 % humidity, and a $C0_2$ content of 5 %. After 3 days the medium was aspirated, the materials were washed gently with PBS and transferred to a fresh 24 well plate containing osteogenic medium that was composed of: high glucose Dulbecco's Modified Eagle Medium (DMEM, 425 mL); fetal bovine serum (50 mL); antibiotic-antimycotic (5 mL); non-essential amino acids (5 mL), dexamethasone (100 nM), β-glycerol phosphate (10 mM) and ascorbic acid (50 μM).

The tips of the copper wires attached to the foams were wound around alligator clip-terminated wires attached to the multipotentiostat (CH Instruments, Austin, TX, USA). The counter and reference electrodes were connected together and clipped to the copper wire protruding from one end of the sample, and the working electrode was clipped to copper wire protruding from the other side of the sample. Wires and alligator clips were secured in position with adhesive copper tape (Ted Pella, Inc., Reading, CA, USA) and wrapped in

Parafilm® to render them electrically insulating and waterproof (i.e. suitable for use inside an incubator). A potential step of 100 mV/mm was placed across the samples for the duration of 4 h per day for 6 days, after which the wires were disconnected and the substrates cultured as normal. Throughout the electrical stimulation experiments the osteogenic medium was aspirated and replaced every 2 days. Thereafter the osteogenic medium was aspirated and replaced every 2 days until the samples were analyzed.

Biochemical assays

Cell viability on the scaffolds at various time points was assessed with an AlamarBlue® assay kit and a Synergy HT Multi-Mode Microplate Reader (Bio-tek US, Winooski, VT) in accordance with the manufacturer's protocol, and numbers of cells adhered to the samples studied herein are reported relative to their initial seeding density, which was assigned an arbitrary value of 100%. The DNA content and Alkaline Phosphatase (ALP) activity of samples that were broken up in a buffer of 0.2% Triton X-100 were quantified concurrently, using the PicoGreen® assay (Life Technologies, Thermo Fisher Scientific Inc., USA) for DNA quantitation in accordance with the manufacturer's protocol, a SensoLyte® *p*NPP Alkaline Phosphatase Assay Kit (AnaSpec, Inc., Freemont, CA, USA) for ALP quantitation in accordance with the manufacturer's protocol, and a Synergy HT Multi-Mode Microplate Reader (Bio-tek US, Winooski, VT). The calcium content of samples was quantified using a Calcium Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA) in accordance with the manufacturer's protocol, and the collagen content of samples was quantified using a Sircol™ Assay Kit (Biocolor Ltd., Carrickfergus, UK) in accordance with the manufacturer's protocol, and a Synergy HT Multi-Mode Microplate Reader (Bio-tek US, Winooski, VT). Data were normalized to DNA quantity, ($n = 13$, error bars represent standard errors of the mean).

Histology

Slices of the samples (thickness of 10 μm) were prepared on a Leica CM-1950 UV cryostat (Leica Microsystems Inc., Buffalo Grove, IL, USA). Hematoxylin & Eosin (H&E) staining: samples were dehydrated in xylene and ethanol, rehydrated in water, dipped in Harris Hematoxylin solution to stain nuclei, rinsed in water and placed in Scott's solution until they turned blue, rinsed again in water, dipped in Eosin solution to stain the ECM, then dehydrated prior to being covered with a coverslip in xylene-based mounting media. Alizarin red staining: samples were dipped in 1% Alizarin red stain with a pH of 4.1-4.3 for 2 minutes,

followed by 20 dips in acetone, followed by 20 dips in 50/50 Acetone/Xylene, then Histomount was used to cover each slide and the samples were left to dry overnight before imaging (images are representative of 3 samples).

Results and Discussion

Preparation and characterization of scaffolds

The porosity of bones varies widely; cortical canals have porosities of approximately 3.5%, whereas trabecular bones have porosities of approximately 80%, and bone tissue scaffolds typically require networks of interconnected pores with sizes of approximately 100 μm to allow for ingrowth of cells and vascularization of the scaffold.[16] Silk foams with interconnected pores with sizes greater than 100 μιη (Figure 1A) were prepared by salt leaching (using salt particles of 425-500 μιη).[17] Sacrificial templates are commonly used to impart porosity to biomaterials, and while it is possible to generate porous materials with well-defined pore interconnectivity by the removal of colloidal crystals (yielding inverse opals),[18] or 3D printed porogens,[19] our all-aqueous approach is appealing because it is cheap and scalable.[17] We rendered the scaffolds conductive by generation of an interpenetrating network of a self-doped CP within the silk foam matrix. The self-doped CPs were composed of pyrrole and 2-hydroxy-5-sulfonic aniline (Figure 1B),[20] and their polymerization within the silk foams was initiated by ammonium persulfate and ferric chloride.[21] When the scaffolds were homogeneously coloured, they were washed thoroughly with water and ethanol to remove the by-products that were not within or attached to the silk matrix (e.g. initiators, monomers, oligomers and polymers). The resulting conductive foams had the same pore size distributions, swell ratio and equilibrium water content as non-conductive foams (Figure IB, Table 1), however, the porosity of the foams (as determined by hexane displacement) was moderately reduced because of the presence of an interpenetrating network of the CPs within the hydrogel-like matrix of inter-/intra-molecularly crosslinked silk proteins that constitute the foam (Table 1). To within experimental error there are no differences in the mechanical properties of the materials before or after the reaction to render the scaffolds conductive; and the compressive moduli (approximately 80 kPa) and strengths (approximately 8 kPa) of the non-conductive foams and conductive foams (Table 1) would be acceptable for non-load-bearing bone tissues, and could be reinforced as necessary for load bearing tissues.[22] The conductivity of the self-doped CPs[20] was 6.1 x 10^{-4} S cm^{-1}, which is on a similar order of magnitude to those of mammalian tissues (typically ≥ 10^{-4} S cm^{-1}).[231]

Sample	Non-conductive silk foam	Conductive silk foam
Porosity, ε (%)	89.6 ± 0.6	70.8 ± 4.2
Swell ratio	14.0 ± 1.4	12.5 ± 1.4
Equilibrium water content (%)	93.3 ± 0.7	92.5 ± 0.8
Compressive modulus (kPa)	99.3 ± 30.4	74.7 ± 31.9
Compressive strength (kPa)	9.2 ± 4.2	7.4 ± 3.9

Table 1. Physicochemical properties of the foams. Errors quoted are standard deviations.

SEM showed that the surface of the non-conductive foams are relatively smooth on the nanometer scale, whereas the conductive foams have aggregates of CP nanoparticles (composed of individual nanoparticles typically of 30-60 nm) on their surface (Figure 4).[20] X-ray photoelectron spectra of the non-conductive and conductive foams (Figure 1C) confirm that the surface chemistry of the foams has changed, with the appearance of peaks in the spectra of the conductive scaffolds at 168 eV (S 2p) and 400 eV (N Is) resulting from the CP. Infrared spectra (Figure ID) exhibit peaks at 1620 cm^{-1} and 1520 cm^{-1} corresponding to the amide I and amide II peaks, respectively, indicating the silk foam is β-sheet rich. Shoulders at 1541 cm^{-1} and 1496 cm^{-1} are characteristic of oligoanilines, and peaks at 1203 cm^{-1} (asymmetric S=0 stretching), 1033 cm^{-1} (C-H in-plane deformation and/or symmetric S=0 stretching), 927 cm^{-1} and a shoulder at 895 cm^{-1} (C-H out-of-plane deformation of aromatic rings and/or bipolaron bands)[20] confirm that the conductivity of the scaffolds is due to the presence of the CPs depicted in Figure IB.

While *in vitro* degradation assays do not accurately reproduce patient-specific immune responses or tissue-specific enzyme distributions, they are useful to confirm the potential of materials to degrade and their relative propensities to do so. Silk proteins are well-known to degrade *in vivo,* and protease XIV is the most commonly used enzyme to mimic their degradation *in vitro;*[24] therefore, we incubated the non-conductive and conductive foams in phosphate buffered saline (PBS) at 37 °C in the absence or presence of protease XIV (1 U/mL) and measured their mass at specific time points (Figure 2A). We observed no significant mass loss for foams in the absence of the enzyme, whereas both non-

conductive and conductive foams were observed to decrease in mass over the course of the experiment due to enzyme-mediated proteolysis; *in vivo* the silk component of the scaffolds is likely to degrade over the period of months to years.[24] Mass loss for the non-conductive foams was faster than for the conductive foams, which is potentially because the interpenetrating network of non-degradable CPs hinder the enzymes access to the backbone of the protein. The gradual degradation of the silk protein would leave behind a small residue of CPs. Consequently, we assessed the toxicity of the CPs using a Cell Titer-Glo® luminescent cell viability assay (Figure 2B). We found cell viability to be high when exposed to low concentrations (0.2 mg mL^{-1}) of CPs and to decrease above 1.5 mg mL^{-1} (a concentration greater than the mass in the individual foams), yet the CPs are markedly less toxic than nanoparticles composed of polypyrrole alone,[25] or indeed poly(3-thiophene acetic acid).[9] Silk-based materials are relatively non-immunogenic *in vivo*, with inflammatory responses in rats typically lower than collagen or polylactic acid,[26] as is also true of polyaniline [27] and polypyrrole [27] derivatives. Hence, we conclude that, while imperfect, such CPs represent valuable lead structures for the future development of conductive biomaterials.

In vitro cell culture

With a view to the application of the foams as bone tissue scaffolds, we seeded bone marrow-derived HMSCs [28] in the scaffolds and cultured them in osteogenic medium for up to 30 days. Three conditions were considered: 1) cells seeded on non-conductive silk foams, 2) cells seeded on conductive silk foams without electrical stimulation, and 3) cells seeded on conductive silk foams with electrical stimulation (3 days without stimulation, 6 days with stimulation at 100 mV/mm for 4 hours per day, no stimulation thereafter). In all cases, cells adhered to the substrates and remained active for the duration of the experiments as confirmed by an AlamarBlue® assay (Figure 2C). As controls we seeded FDVISCs on non-conductive silk foams in osteogenic medium and observed their differentiation towards osteogenic fates using biochemical assays for alkaline phosphatase (ALP) activity, Ca^{2+} deposition, collagen production (Figure 2D to 2F) and histology (Figure 3A to 3F).[29] Relative to the non-conductive silk foams ALP expression was increased on both the conductive foams with or without electrical stimulation (Figure 2D), which is likely to be a result of differences in the surface chemistry altering protein deposition from the medium onto the scaffolds.[30] Calcium deposition was increased on both the conductive foams with or without electrical stimulation, with approximately double the mass of calcium present in samples exposed to electrical stimulation after 30 days (Figure 2E). Likewise, collagen production was notably higher on the conductive scaffolds, and electrical stimulation

markedly increased collagen production (Figure 2F). Thus, quantitative biochemical analyses of the scaffolds reveal that, while the non-conductive silk scaffolds support differentiation of HMSCs towards osteogenic outcomes, the application of an electrical stimulus to HMSCs residing in an conductive scaffold enhances their differentiation towards osteogenic fates, and the increased quantities of calcium and collagen are an important step towards the formation of calcified extracellular matrix associated with bone.

Histological analysis of the scaffolds (Figures 3A-3R) confirmed that the FDVISCs differentiated towards osteogenic outcomes in all cases based on hematoxylin and eosin (H&E) staining and Alizarin staining. H&E staining of sections of non-conductive scaffolds (Figure 3A, 3C and 3E) resulted in characteristic blue staining of cell nuclei, and characteristic pink staining of intracellular and extracellular proteins (e.g. actin or silk, respectively). [29] Alizarin staining (Figure 3B, 3D and 3F) resulted in characteristic orange-red staining of calcium deposits that are early stage markers of matrix mineralization. [29c, 31] H&E staining of sections of conductive scaffolds without electrical stimulation (Figure 3G, 31 and 3K) or with electrical stimulation (Figure 3M, 30 and 3Q) was a darker pink than the non-conductive equivalents, resulting from increased collagen production by the HMSCs on the scaffolds (Figure 2F). Likewise, Alizarin staining of sections of conductive scaffolds without electrical stimulation (Figure 3H, 3J and 3L) or with electrical stimulation (Figure 3N, 3P, 3R) was a darker red than the non-conductive equivalents, because of the increased calcium deposition in the scaffolds (Figure 2E). Qualitative analysis of the scaffolds via histology supports our quantitative biochemical analyses of the scaffolds: while the non-stimulated scaffolds support differentiation of HMSCs towards osteogenic outcomes, electrical stimulation of HMSCs enhances their biochemical phenotype.

Bone tissue engineering is a vibrant field of research, and as noted above, an incredible variety of materials have been investigated for bone tissue engineering, and silk proteins are a class of materials that has shown great promise both *in vitro* and *in vivo* in preclinical trials. [5] Herein we report the first study of a conducting silk derivative and its application as a bone tissue scaffold that facilitates electrical stimulation of HMSCs and promotes their differentiation towards osteogenic outcomes. We observe that levels of ALP expression relative to the smooth non-conductive silk foams was increased on the rougher conductive scaffolds both without and with electrical stimulation (Figure 2D). We believe that this is a result of differences in the surface chemistry altering protein deposition from the medium onto the scaffolds, [30] which Bose and coworkers show to modify cell-material interactions *in vitro*,[32] yet Epinette and Manley conclude that microstructure and surface

charge are not the sole factors at play in osteoinduction in the clinic.[33] Indeed, our experimental data for non-conductive silk foams, and conductive silk foams without/with electrical stimulation enabled us to observe that electrical stimulation markedly enhanced calcium deposition and collagen production (Figure 2E, 2F and 3).

Conclusions

In summary, there is a need for pro-regenerative biomaterials for the treatment of bone conditions and disorders requiring surgical intervention. Engineered bone tissue scaffolds with properties that enable the behaviour of residing cells to be rationally controlled are of particular interest. We report herein the first examples of conductive 3D silk foam-based bone tissue scaffolds via a simple process under aqueous conditions and characterized their physicochemical properties. The CPs are only significantly toxic above 1.5 mg mL^{-1}, which is markedly less toxic than nanoparticles composed of polypyrrole[23] or indeed poly(3-thiophene acetic acid).[9] Moreover, the CPs reported here display markedly better electrochemical stability than poly(3-thiophene acetic acid)[9] which enabled us to electrically stimulate cells residing in the scaffolds, and we believe that the CPs represent valuable lead structures for the future development of conductive biomaterials. While there are reports of electrical stimulation of cells on 3D scaffolds composed of electrospun fibers,[34] these mats tend not to be more than 1 millimeter in thickness, and our scaffolds were 4 millimeters in height and diameter, and this is to the best of our knowledge the first report of electrical stimulation on such a large scaffold. The conductive scaffolds enable electrical stimulation of HMSCs residing therein and enhance their differentiation towards osteogenic outcomes as confirmed by both quantitative biochemical assays and qualitative histological analysis. Importantly, electrical stimulation increased quantities of calcium and collagen deposited in the scaffolds, which is an important step towards the formation of calcified extracellular matrix associated with bone.

References:

[1] a) A. R. Amini, C. T. Laurencin, S. P. Nukavarapu, *Crit. Rev. Biomed. Eng.* 2012, *40,* 363; b) J. F. Mano, G. A. Silva, H. S. Azevedo, P. B. Malafaya, R. A. Sousa, S. S. Silva, L. F. Boesel, J. M. Oliveira, T. C. Santos, A. P. Marques, N. M. Neves and R. L. Reis, *J. R. Soc. Interface,* 2007, *4,* 999; c) A. J. Salgado, O. P. Coutinho, R. L. Reis, *Macromol. Biosci.* 2004, *4,* 743; d) J. Ma, S. K. Both, F. Yang, F. Z. Cui, J. Pan, G. J. Meijer, J. A. Jansen, J. J. van den Beucken. *Stem Cells Transl. Med.* 2014, *3,* 98; e) J. I. Dawson, J. Kanczler, R. Tare, M.

Kassem, R. O. Oreffo., *Stem Cells.* 2014, *32,* 35; f) Y. Liu, J. Lim, S. H. Teoh, *Biotechnol. Adv.* 2013, *31,* 688; g) D. Marolt, M. Knezevic and G. Vunjak-Novakovic, *Stem Cell Res. Ther.* 2010, *1,* 10; h) M. Frohlich, W. L. Grayson, L. Q. Wan, D. Marolt, M. Drobnic, G. Vunjak-Novakovic, *Curr. Stem Cell Res. Ther.* 2008, *3,* 254; i) W. L. Grayson, T. P. Martens, G. M. Eng, M. Radisic, G. Vunjak-Novakovic, ***Sent.*** *Cell Dev. Biol,* 2009, *20,* 665; j) C. A. Lyssiotis, L. L. Lairson, A. E. Boitano, H. Wurdak, S. Zhu, P. G. Schultz, *Angew. Chem. Int. Ed.* 201 1, *50,* 200.

[2] U. G. K. Wegst, H. Bai, E. Saiz, A. P. Tomsia, R. O. Ritchie, *Nat. Mater.,* 2015, *14,* 23.

[3] J. R. Porter, T. T. Ruckh, K. C. Popat, *Biotechnol. Prog.* 2009, *25,* 1539.

[4] a) C. Vepari, D. L. Kaplan, *Prog. Polym. Sci.* 2007, *32,* 991; b) S. Gomes, I. B. Leonor, J. F. Mano, R. L. Reis, D. L. Kaplan, *Prog. Polym. Sci.* 2012, *37,* 1; c) S. Pina, J. M. Oliveira, R. L. Reis, *Adv. Mater.* 2015, *27,* 1143; d) J. G. Hardy, T. R. Scheibel, *J. Polym. Sci. Part A: Polym. Chem.,* 2009, *47,* 3957; e) K. Schacht, T. Scheibel, *Curr. Opin. Biotechnol.* 2014, *29,* 62.

[5] a) S. Saha, B. Kundu, J. Kirkham, D. Wood, S. C. Kundu, X. B. Yang, *PLoS One,* 2013, *8,* e80004; b) Z. Zhao, Y. Li, M.-B. Xie, *Int. J. Mol. Sci.,* 2015, *16,* 4880; c) F. Vollrath, D. Porter, C. Holland, *Soft Matter,* 201 1, 7, 9595; d) M. Widhe, J. Johansson, M. Hedhammar, A. Rising, *Biopolymers,* 2012, *97,* 468; e) D. Pra, G. Freddi, J. Minic, A. Chiarini, U. Armato, *Biomaterials,* 2005, *26,* 1987; f) J. G. Hardy, L. M. Romer, T. R. Scheibel, *Polymer,* 2008, *49,* 4309; g) J. G. Hardy, A. Leal-Egana, T. R. Scheibel, *Macromol. Biosci.,* 2013, *13,* 1431; h) J. G. Hardy, A. Pfaff, A. Leal-Egana, A. H. E. Muller, T. R. Scheibel, *Macromol. Biosci.,* 2014, *14,* 936; i) L. Meinel, S. Hofmann, V. Karageorgiou, C. Kirker-Head, J. McCool, G. Gronowicz, L. Zichner, R. Langer, G. Vunjak-Novakovic, D. L. Kaplan, *Biomaterials,* 2005, *26,* 147; j) Y. Liu, R. You, G. Liu, X. Li, W. Sheng, J. Yang, M. Li, *Int. J. Mol. Sci.,* 2014, *15,* 7049; k) S. K. Nitta, K. Numata, *Int. J. Mol. Sci.,* 2013, *14,* 1629; 1) M. Yang, Y. Shuai, W. He, S. Min, L. Zhu, *Int. J. Mol. Sci.,* 2012, *13,* 7762; m) K.-H. Zhang, Q. Ye, Z.-Y. Yan, *Int. J. Mol. Sci.,* 2012, *13,* 2036.

[6] a) M. Berggren, A. Richter-Dahlfors, *Adv. Mater.,* 2007, *19,* 3201; b) J. Rivnay, R. M. Owens, G. G. Malliaras, *Chem. Mater.* 2014, *26,* 679-685; c) T. H. Qazi, R. Rai, A. R. Boccaccini, *Biomaterials,* 2014, *35,* 9068; d) R. Balint, N. J. Cassidy, S. H. Cartmell, *Acta Biomaterialia,* 2014, *10,* 2341; e) P. J. Molino, G. G. Wallace, *APLMaterials,* 2015 3, 014913; f) M. Muskovich, C. J. Bettinger, *Adv. Healthcare Mater.,* 2012, *1,* 248; g) J. G. Hardy, J. Y. Lee, C. E. Schmidt, *Curr. Opin. Biotech.* 2013, *24,* 847; h) D. C. Martin, J. Wu,

C. M. Shaw, Z. King, S. A. Spanninga, S. Richardson-Burns, J. Hendricks, Junyan Yang, *Pofym. Rev.,* 2010, *50,* 340; i) B. Guo, L. Glavas, A. C. Albertsson, *Prog. Polym. Sci.,* 2013, *38,* 1263; j) D. M. Thompson, A. N. Koppes, J. G Hardy, C. E. Schmidt, *Annu. Rev. Biomed. Eng.,* 2014, *16,* 397; k) Anthony Guiseppi-Elie, *Biomaterials,* 2010, *31,* 2701; 1) S. C. Luo, *Pofym. Rev.,* 2013, *53,* 303; m) M. A. Fernandez- Yague, S. A. Abbah, L. McNamara, D. I. Zeugolis, A. Pandit, M. J. Biggs, *Adv. Drug Deliver. Rev.,* 2015, DOI: doi:10.1016/j.addr.2014.09.005; n) J. G. Hardy, D. J. Mouser, N. Arroyo-Curras, S. Geissler, J. K. Chow, L. Nguy, J. M. Kim, C. E. Schmidt, *J. Mater. Chem. B,* 2014, *2,* 6809; o) D. Svirskis, J. Travas-Sejdic, A. Rodgers, S. Garg, *J. Control. Release,* 2010, *146,* 6.

[7] a) N. K. Guimard, N. Gomez, C. E. Schmidt, *Prog. Pofym. Sci.,* 2007, *32,* 876; b) A. D. Bendrea, L. Cianga, I. Cianga, *J. Biomater. Appl.,* 201 1, *26,* 3.

[8] S. Ahadian, J. Ramon- Azcon, S. Ostrovidov, G. Camci-Unal, H. Kaji, K. Ino, H. Shiku, A. Khademhosseini, T. Matsue, *Biomed. Microdevices,* 2013, *15,* 109.

[9] D. Mawad, E. Stewart, D. L. Officer, T. Romeo, P. Wagner, K. Wagner, G. G. Wallace, *Adv. Funct. Mater.,* 2012, *22,* 2692.

[10] a) M. B. Runge, M. Dadsetan, J. Baltrusaitis, A. M. Knight, T. Ruesink, E. A. Lazcano, L. Lu, A. J. Windebank, M. J. Yaszemski, *Biomaterials,* 2010, *31,* 5916; b) X. Liu, J. Chen, K. J. Gilmore, M. J. Higgins, Y. Liu, G. G. Wallace, *J. Biomed. Mater. Res. A.,* 2010, *94,* 1004; c) A. F. Quigley, K. J. Bulluss, I. L. Kyratzis, K. Gilmore, T. Mysore, K. S. Schirmer, E. L. Kennedy, M. O'Shea, Y. B. Truong, S. L. Edwards, G. Peeters, P. Herwig, J. M. Razal, T. E. Campbell, K. N. Lowes, M. J. Higgins, S. E. Moulton, M. A. Murphy, M. J. Cook, G. M. Clark, G. G. Wallace, R. M. Kapsa, *J. Neural Eng.,* 2013, *10,* 016008; d) J.G. Hardy, R. C. Cornelison, R. C. Sukhavasi, R. J. Saballos, P. Vu, D. L. Kaplan, C. E. Schmidt, *Bioengineering,* 2015, *2,* 15; e) C. Martin, T. Dejardin, A. Hart, M. O. Riehle, D. R. Cumming, *Adv. Healthc. Mater.,* 2014, *3,* 1001.

[11] V. P. Shastri, N. Rahman, I. Martin, R. Langer, *Mat. Res. Soc. Symp. Proc,* 1999, *550,* 215.

[12] a) S. Meng, Z. Zhang, M. Rouabhia, *J. Bone Miner. Metab.,* 201 1, *29,* 535; b) J. Pelto, M. Bjorninen, A. Palli, E. Talvitie, J. Hyttinen, B. Mannerstrom, R. Suuronen Seppanen, M. Kellomaki, S. Miettinen, S. Haimi, *Tissue Eng. Part A.,* 2013, *19,* 882; c) J. Cao, Y. Man, L. Li, *Biomed. Rep.,* 2013, 7, 428.

[13] A. Blau, C. Weinl, J. Mack, S. Kienle, G. Jung, C. Ziegler, *J. Neurosci. Methods,* 2001, 772, 65.

[14] E. L. Mayes, F. Vollrath, S. Mann, *Adv. Mater.,* 1998, *10,* 801.

[15] a) I. Cucchi, A. Boschi, C. Arosio, F. Bertini, G. Freddi, M. Catellani, *Synthetic Metals,* 2009, *159,* 246; b) I. S. Romero, N. P. Bradshaw, J. D. Larson, S. Y. Severt, S. J. Roberts, M. L. Schiller, J. M. Leger, A. R. Murphy, *Adv. Funct. Mater.,* 2014, *24,* 3866.

[16] a) G. A. P. Renders, L. Mulder, L. J. Van Ruijven, T. M. G. J. Van Eijden, *J. Anat.* 2007, *210,* 239; b) L. Cardoso, S. P. Fritton, G. Gailani, M. Benalla, S. C. Cowin, *J. Biomech.* 2013, *46,* 253; c) V. Karageorgiou, D. Kaplan, *Biomaterials,* 2005, *26,* 5474.

[17] D. N. Rockwood, R. C. Preda, T. Yucel, X. Wang, M. L. Lovett, D. L. Kaplan, *Nature Protocols,* 201 1, *6,* 1612.

[18] a) V. M. Swinerd, A. M. Collins, N. J. V. Skaer, T. Gheysens, S. Mann, *Soft Matter,* 2007, *3,* 1377; b) Y. Y. Diao, X. Y. Liu, G. W. Toh, L. Shi, J. Zi, *Adv. Funct. Mater.,* 2013, *23,* 5373; c) S. Kim, A. N. Mitropoulos, J. D. Spitzberg, H. Tao, D. L. Kaplan, F. G. Omenetto, *Nature Photon.,* 2012, *6,* 818.

[19] J. G. Torres-Rendon, T. Femmer, L. De Laporte, T. Tigges, K. Rahimi, F. Gremse, S. Zafarnia, W. Lederle, S. Ifuku, M. Wessling, J. G. Hardy, A. Walther, *Adv. Mater.,* 2015, DOI: 10.1002/adma.201405873.

[20] X. G. Li, Z. Z. Hou, M. R. Huang, M. G. Moloney, *J. Phys. Chem. C,* 2009, *113,* 21586.

[21] R. H. Karlsson, A. Herland, M. Hamedi, J. A. Wigenius, A. Aslund, X. Liu, M. Fahlman, O. Inganas, P. Konradsson, *Chem. Mater.,* 2009, *21,* 1815.

[22] B. B. Mandal, A. Grinberg, E. S. Gil, B. Panilaitis, D. L. Kaplan, *Proc. Natl. Acad. Sci. USA,* 2012, *109,* 7699.

[23] a) C. Gabriel, S. Gabriel and E. Corthout, *Phys. Med. Biol,* 1996, *41,* 223 1; b) 107 S. Gabriel, R. W. Lau and C. Gabriel, *Phys. Med. Biol,* 1996, *41,* 2251; c) S. Gabriel, R. W. Lau and C. Gabriel, *Phys. Med. Biol,* 1996, *41,* 2271.

[24] a) Y. Cao, B. Wang, *Int. J. MolSci.,* 2009, *10,* 1514; b) K. Shang, J. Rnjak-Kovacina, Y. Lin, R. S. Hayden, H. Tao, D. L. Kaplan, *Transl. Vis. Sci. Technol,* 2013, *2,* 2; c) U. J. Kim, J. Park, H. J. Kim, M. Wada, D. L. Kaplan, *Biomaterials,* 2005, *26,* 2775.

[25] A. Vaitkuviene, V. Kaseta, J. Voronovic, G. Ramanauskaite, G. Biziuleviciene, A. Ramanaviciene, A. Ramanavicius, *J. Hazard. Mater.,* 2013, *250–251,* 167.

[26] L. Meinel, S. Hofmann, V. Karageorgiou, C. Kirker-Head, J. McCool, G. Gronowicz, L. Zichner, R. Langer, G. Vunjak-Novakovic, D. L. Kaplan, *Biomaterials,* 2005, *26,* 147.

[27] M. Mattioli-Belmonte, G. Giavaresi, G. Biagini, L. Virgili, M. Giacomini, M. Fini, F. Giantomassi, D. Natali, P. Torricelli, R. Giardino, *Int. J. Artif. Organs.,* 2003, *26,* 1077.

[28] a) G. H. Altman, R. L. Horan, I. Martin, J. Farhadi, P. R. Stark, V. Volloch, J. C. Richmond, G. Vunjak-Novakovic, D. L. Kaplan, *FASEB J* , 2002, *16,* 270; b) J. J. Li, E. S. Gil, R. S. Hayden, C. Li, S. I. Roohani-Esfahani, D. L. Kaplan, H. Zreiqat, *Biomacromolecules,* 2013, *14,* 2179.

[29] a) S.H. Park, E. S. Gil, H. Shi, H. J. Kim, K. Lee, D. L. Kaplan, *Biomaterials,* 2010, *31,* 6162; b) A. Augst, D. Marolt, L. E. Freed, C. Vepari, L. Meinel, M. Farley, R. Fajardo, N. Patel, M. Gray, D. L. Kaplan, G. Vunjak-Novakovic, *J. Roy. Soc. Interface,* 2008, *5, 929;* c) C. Correia, S. Bhumiratana, L. P. Yan, A. L. Oliveira, J. M. Gimble, D. Rockwood, D. L. Kaplan, R. A. Sousa, R. L. Reis, G. Vunjak-Novakovic, *Acta Biomaterialia,* 2012, *8,* 2483.

[30] H. M. Rawel, K. Meidtner, J. Kroll, *J. Agric. Food Chem.,* 2005, *53,* 4228.

[31] P. C. Bessa, E. R. Balmayor, J. Hartinger, G. Zanoni, D. Dopier, A. Meinl, A. Banerjee, M. Casal, H. Redl, R. L. Reis, M. van Griensven, *Tissue Eng. Part C Methods,* 2010, *16,* 937.

[32] S. Tarafder, S. Bodhak, A. Bandyopadhyay, S. Bose, *J. Biomed. Mater. Res. Part B Appl. Biomater.,* 201 1, *97B,* 306.

[33] *Fifteen years of clinical experience with hydroxyapatite coatings injoint arthroplasty,* (Eds: J.-A. Epinette, M. T. Thomas), Springer-Verlag, Paris, France 2004.

[34] a) L. Ghasemi-Mobarakeh, M. P. Prabhakaran, M. Morshed, M. H. Nasr-Esfahani, H. Baharvand, S. Kiani, S. S. Al-Deyab, S. Ramakrishna, *J. Tissue Eng. Regen. Med.,* 201 1, 5, el7; b) J. Y. Lee, *Polymer Reviews,* 2013, *53,* 443.

It should be noted that ratios, concentrations, amounts, and other numerical data may be expressed herein in a range format. It is to be understood that such a range format is used for convenience and brevity, and thus, should be interpreted in a flexible manner to include not only the numerical values explicitly recited as the limits of the range, but also to include all the individual numerical values or sub-ranges encompassed within that range as if each numerical value and sub-range is explicitly recited. To illustrate, a concentration range of "about 0.1% to about 5%" should be interpreted to include not only the explicitly recited concentration of about 0.1 wt% to about 5 wt%, but also include individual concentrations *(e.g.,* 1%, 2%, 3%, and 4%) and the sub-ranges *(e.g.,* 0.5%, 1.1%, 2.2%, 3.3%, and 4.4%) within the indicated range. In an embodiment, the term "about" can include traditional

rounding according to significant figures of the numerical value. In addition, the phrase "about 'x' to 'y'" includes "about 'x' to about 'y'"\

Many variations and modifications may be made to the above-described embodiments. All such modifications and variations are intended to be included herein within the scope of this disclosure and protected by the following claims.

CLAIMS

We claim:

1. A method of differentiation of human mesenchymal stem cells, comprising:

providing a conductive foam with an interpenetrating polymer network on the surface of the foam, wherein the interpenetrating polymer network is made from a doped conductive polymer that is either a self-doped conducting polymer or a conducting polymer and a dopant;

introducing human mesenchymal stem cells to the conductive foam, wherein the conductive foam and the human mesenchymal stem cells are cultured in a osteogenic medium; and

periodically providing electrical stimulation to the human mesenchymal stem cells to cause differentiation of human mesenchymal stem cells towards osteogenic outcomes.

2. The method of claim 1, wherein the conducting polymer is a self-doped conducting polymer formed from pyrrole and 2-hydroxy-5-sulfonic aniline

3. The method of claim 2, wherein the interpenetrating polymer network is poly(pyrrole-co-(2-hydroxy-5-sulfonic aniline).

4. The method of claims 1 or 2, further providing increased ALP activity, increased collagen deposition, increased Ca^{2+} deposition, or a combination thereof, on the foams relative to not periodically providing electrical stimulation.

5. The method of claims 1 or 2, wherein the conductive foam has a height of about 1 to 1000 millimeters and a width of about 1 millimeter to 1000 millimeters.

6. The method of claim 1, wherein the conductive foam is a conductive silk foam or a conductive polymer foam.

7. The method of claim 6, wherein the conductive silk foam has a porosity of about 3 to 99 %.

8. The method of claim 6, wherein the conductive polymer foam is selected from the group consisting of: polycaprolactone, polyesters, polyamides, PCL, PLLA, PLGA, proteins, polysaccharides, lignins, polyalanine, oligoalanine, collagen, cellulose, chitin, chitosan, and a combination thereof.

9. A structure, comprising:

a conductive foam having an interpenetrating polymer network on the surface of the foam, wherein the interpenetrating polymer network is made from a doped conductive polymer that is either a self-doped conducting polymer or a conducting polymer and a dopant, wherein human mesenchymal stem cells are disposed within the conductive foam.

10. The structure of claim 9, wherein the conducting polymer is a self-doped conducting polymer composed of pyrrole and 2-hydroxy-5-sulfonic aniline.

11. The structure of claim 9, wherein the interpenetrating polymer network is poly(pyrrole-co-(2-hydroxy-5-sulfonic aniline).

12. The structure of claim 9, wherein conductive foam has a height of about 1 to 1000 millimeters and a width of about 1 millimeters to 1000 millimeters.

13. The structure of claim 9, wherein the conductive foam includes ALP, collagen, and Ca^{2+} deposition on the foams.

14. The structure of claim 9, wherein the conductive foam has interconnected pores.

15. The structure of claim 14, wherein the interconnected pores have a diameter of about 10 μιη ïo 1000 μιη.

16. The structure of claim 9, wherein the conductive foam has a density of about 0.01 to 10 grams per cm^3.

17. The structure of claim 9, wherein the conductive foam is a conductive silk foam or a conductive polymer foam.

18. The structure of claim 17, wherein the conductive polymer foam is selected from the group consisting of: polycaprolactone, polyesters, polyamides, PCL, PLLA, PLGA, proteins, polysaccharides, lignins, polyalanine, oligoalanine, collagen, cellulose, chitin, chitosan, and a combination thereof.

19. The structure of claim 9, wherein the conductive foam is a conductive silk foam.

20. The structure of claim 19, wherein the conductive foam has a porosity of about 3 to 99 %.

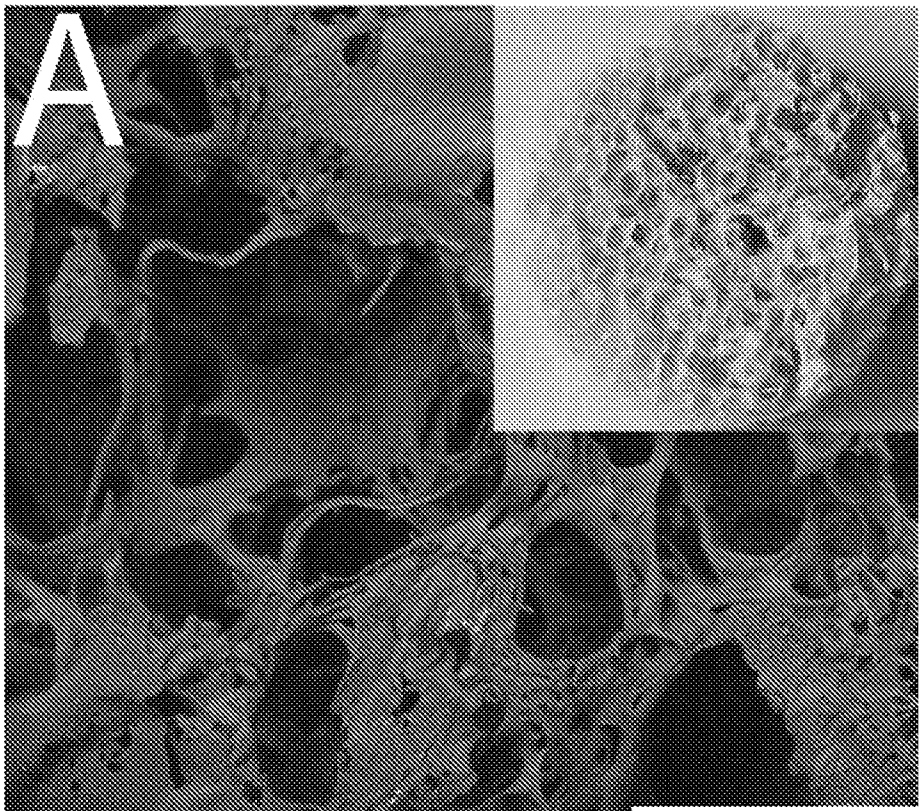

Fig. 1A

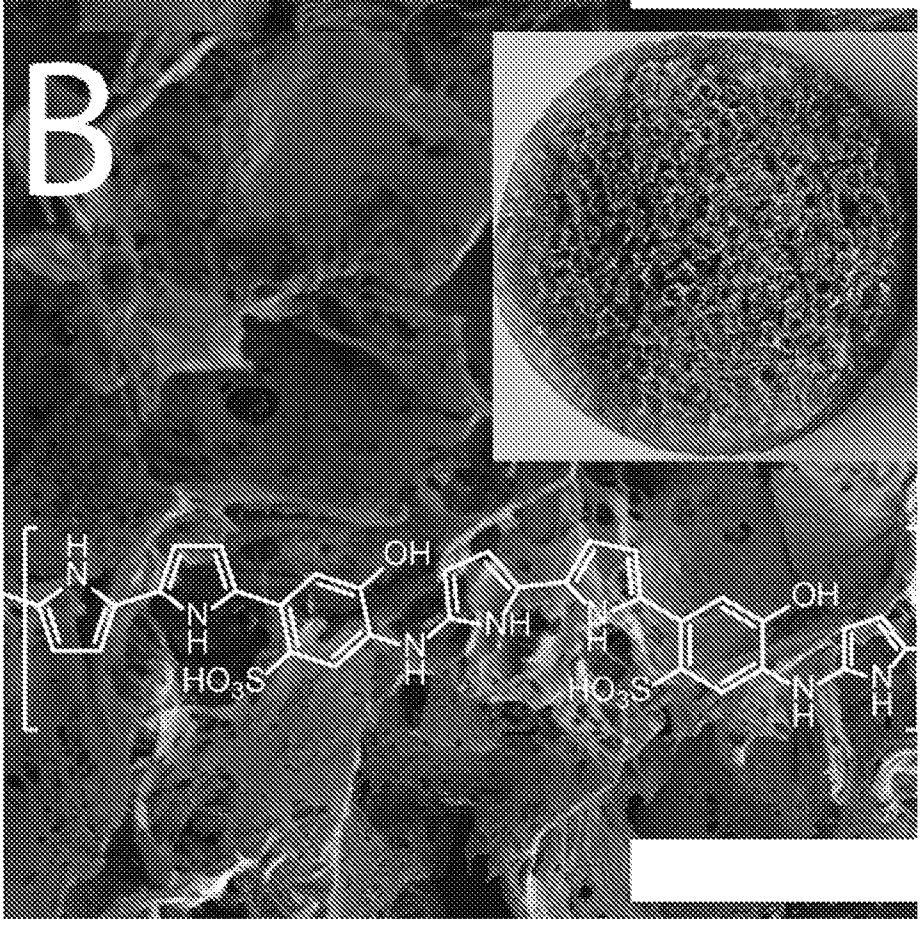

Fig. 1B

C

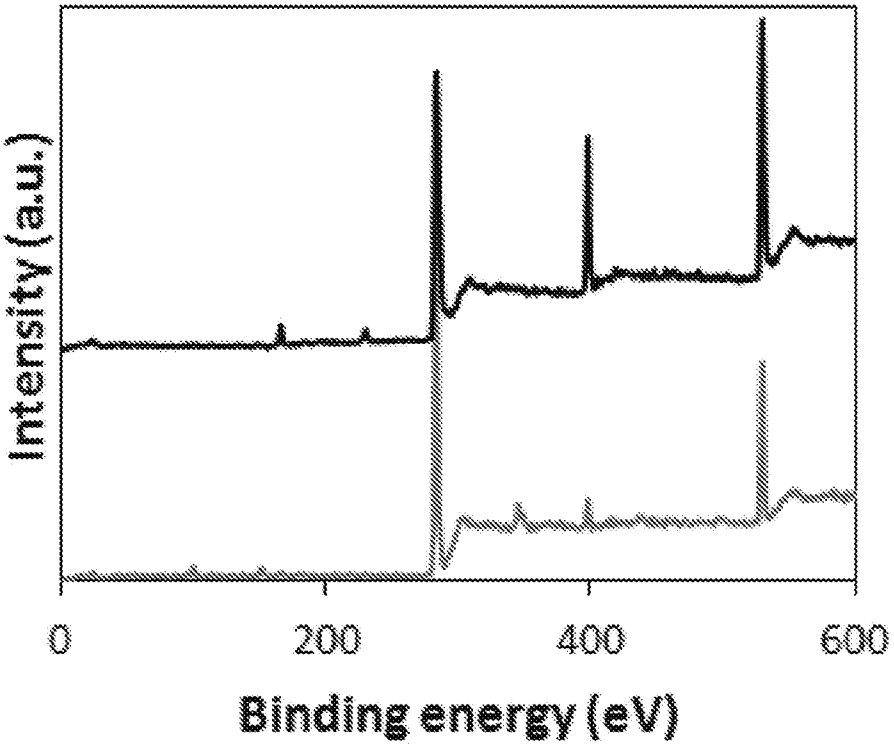

Fig. 1C

D

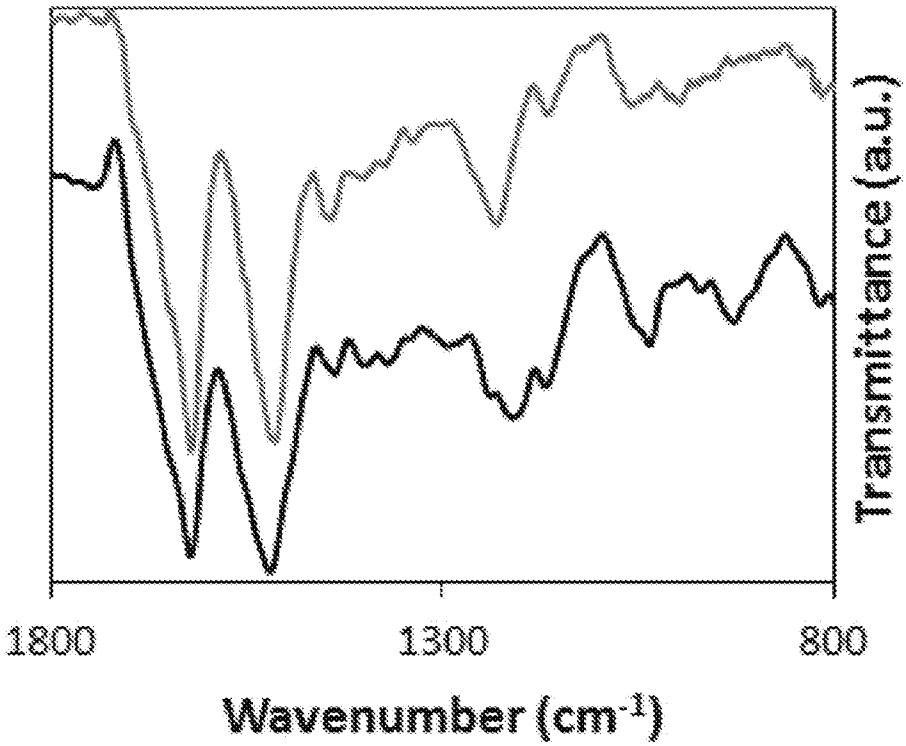

Fig. 1D

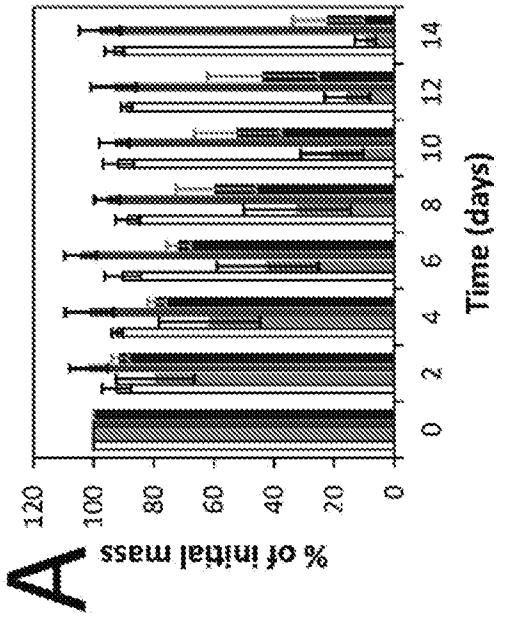

Fig. 2A

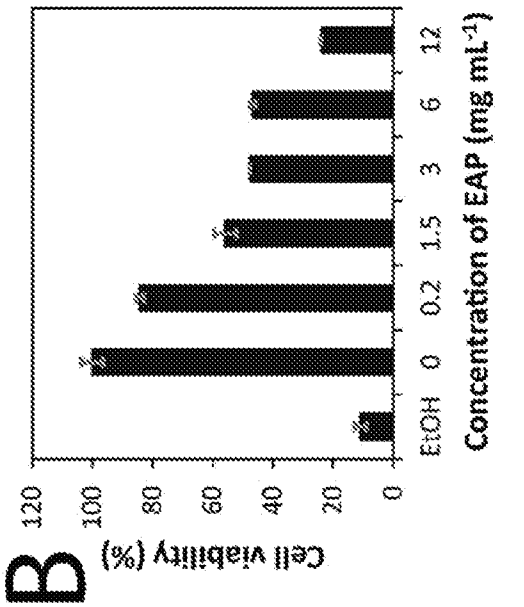

Fig. 2B

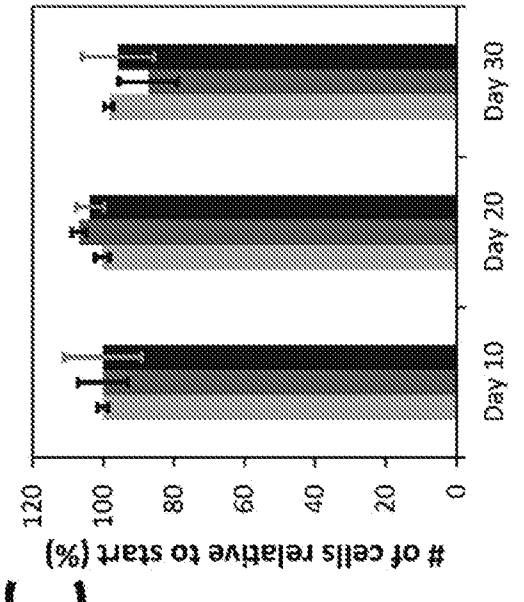

Fig. 2C

Fig. 2D

Fig. 2E

Fig. 2F

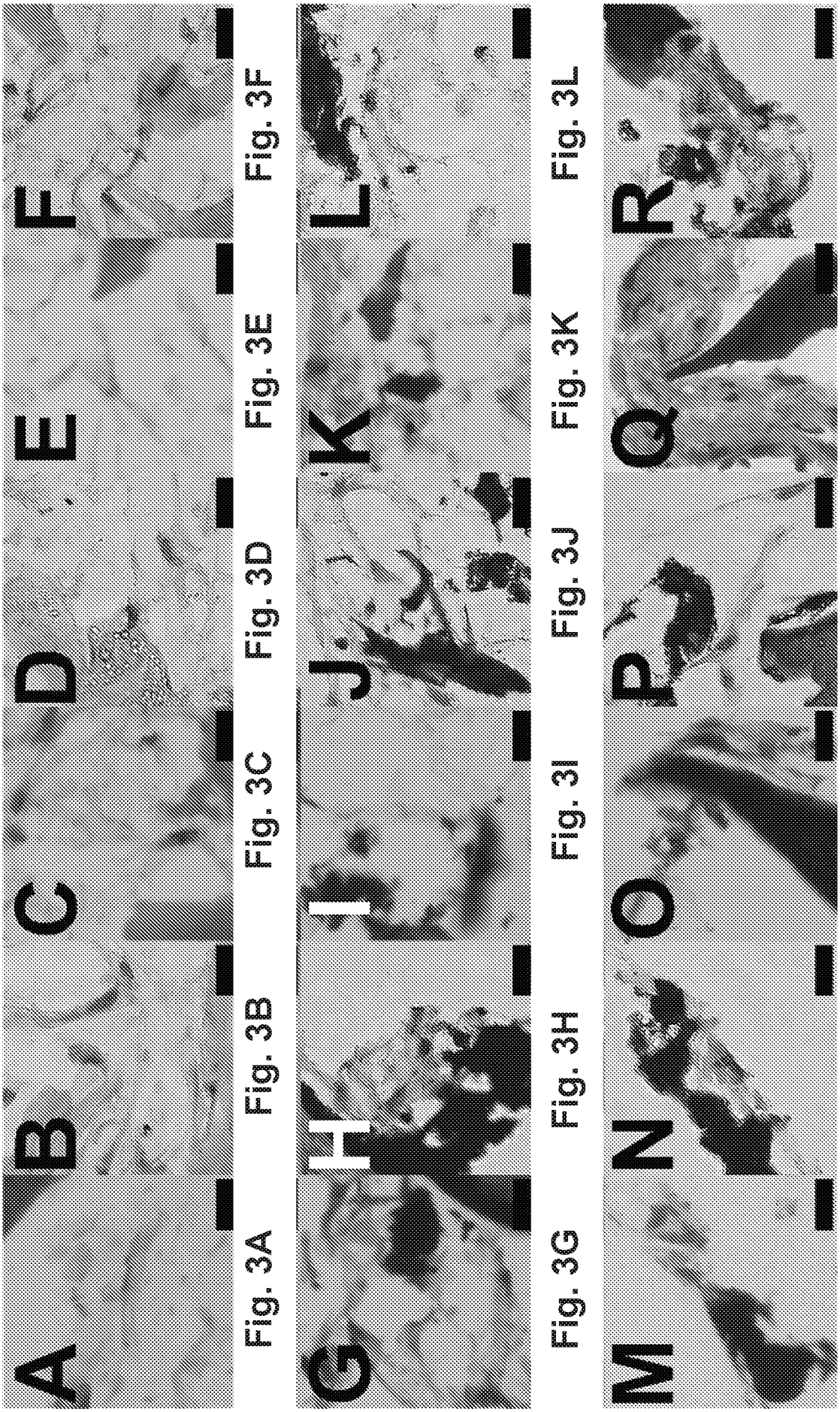

Fig. 3A Fig. 3B Fig. 3C Fig. 3D Fig. 3E Fig. 3F

Fig. 3G Fig. 3H Fig. 3I Fig. 3J Fig. 3K Fig. 3L

Fig. 3M Fig. 3N Fig. 3O Fig. 3P Fig. 3Q Fig. 3R

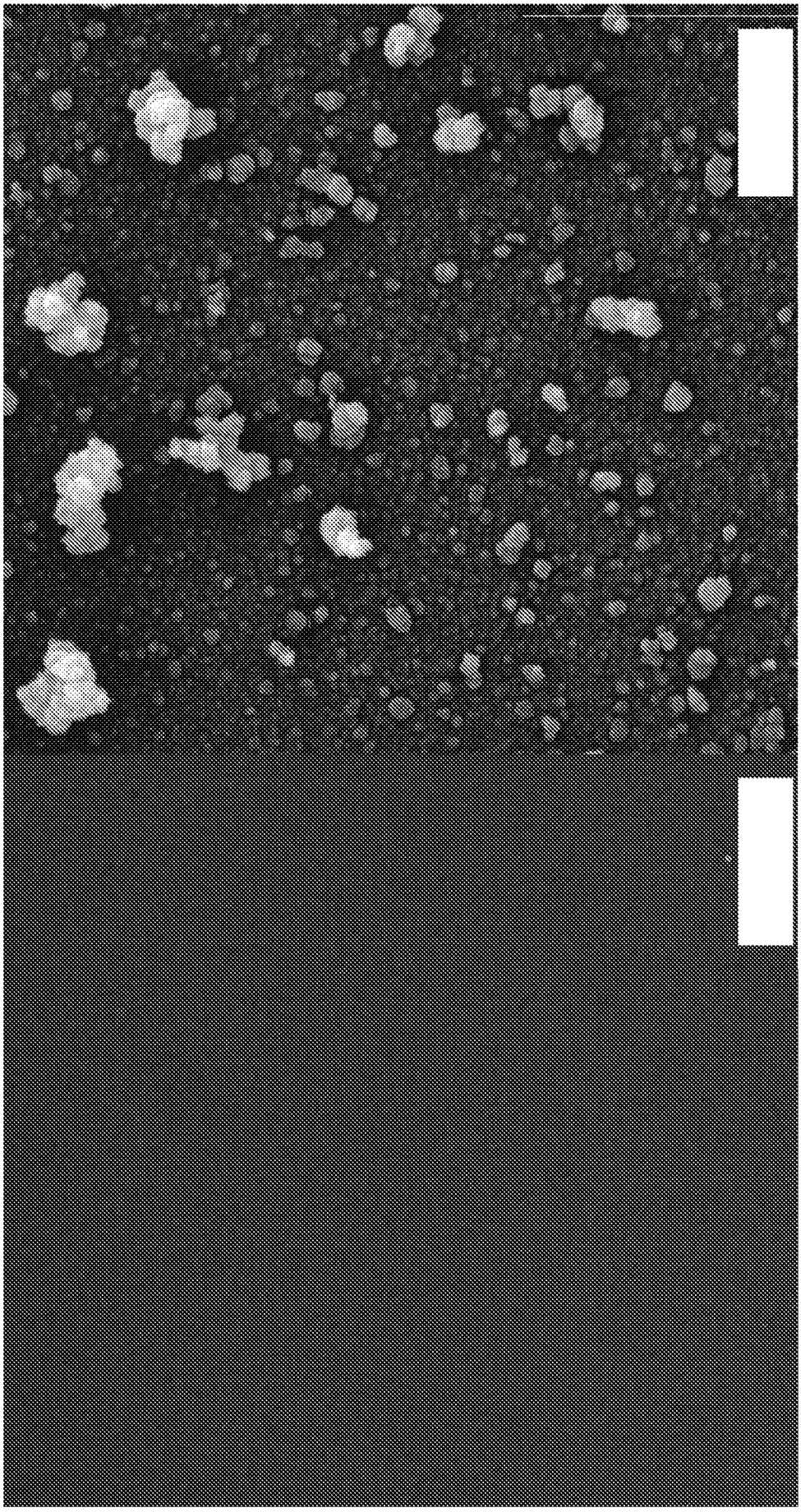

Fig. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/41889

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A01N 63/00, C12N 5/00, C12N 5/077 (2016.01)
CPC - C12N 5/0657
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) - A01N 63/00, C12N 5/00, C12N 5/077 (2016.01)
CPC - C12N 5/0657

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/377

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, Google Scholar, Google Web
Terms searched: differentiation human mesenchymal stem cells conductive foam interpenetrating polymer network coating foam aniline pyrrole silk polypyrrole-co-(2-hydroxy-5-sulfonic aniline) electroactive electric stimulation collagen calcium deposit density porous scaffold

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Pelto et al., 'Novel Polypyrrole-Coated Polylactide Scaffolds Enhance Adipose Stem Cell Proliferation and Early Osteogenic Differentiation', Tissue Engineering Part A. March 2013, 19(7-8): 882-892, abstract; pg 883, para 3; pg 884, para 3; pg 885, para 3;	1-20
Y	US 2008/0038236 A1 (Gimble et al.) 14 February 2008 (14.02.2008) para [0017]; [0066]; [0095]; [0100]; [0102]	1-20
Y	Li et al., 'Efficient Synthesis of Intrinsically Conducting Polypyrrole Nanoparticles Containing Hydroxy Sulfoaniline as Key Self-Stabilized Units', J. Phys. Chem. C 2009, 113, 21586-21595, abstract; pg 21587, para 1;	2-3, 10-11
Y	Rockwood et al., 'Ingrowth of Human Mesenchymal Stem Cells into Porous Silk Particle Reinforced Silk Composite Scaffolds: An In Vitro Study', Acta Biomater. 201 1 January ; 7(1): 144-151 , pg 150 para 2	13
Y	US 2014/0094410 A1 (Kaplan et al.) 03 April 2014 (03.04.2014) Table 1	16
A	McCullen et al., 'Application of Low-Frequency Alternating Current Electric Fields Via Interdigitated Electrodes: Effects on Cellular Viability, Cytoplasmic Calcium, and Osteogenic Differentiation of Human Adipose-Derived Stem Cells', Tissue Engineering Part C: Methods. November 2010, 16(6): 1377-1386, abstract	1-20
A	US 5,431,998 A (Wettermark et al.) 11 July 1995 (11.07.1995) col 4, ln 7-9; ln 45-47; ln 49-51; col 5, ln 1-3; col 6, ln 12-14; col 7, ln 22-24; col 9 ln 32	1-20
A	US 6,214,260 B1 (Bessette et al.) 10 April 2001 (10.04.2001) col 3, ln 44-55; col 4 ln 28-44	1-20

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
30 August 2016	11 OCT 2016

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/41889

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2002/0034796 A1 (Shastri et al.) 21 March 2002 (21.03.2002) para [0024]-[0043]	1-20
A	US 2009/0171406 A1 (Foley et al.) 02 July 2009 (02.07.2009) para [0010]-[0014]; [0058]-[0059]; [0073]-[0080];	1-20