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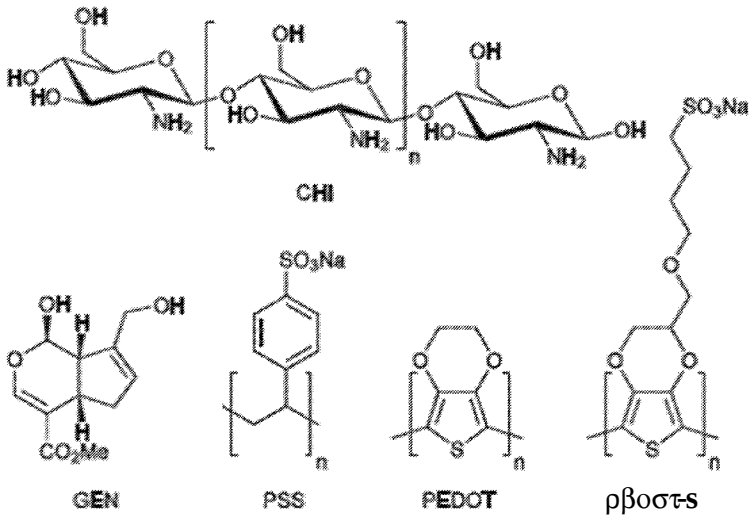

Fig. 1.1

(57) Abstract: Embodiments of the present disclosure provide for structures including a conformal conductive scaffold, methods of making the structure, method of using structure, and the like.

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CONFORMAL CONDUCTIVE SCAFFOLDS AND METHODS OF USING CONFORMAL CONDUCTIVE SCAFFOLDS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of and priority to U.S. Provisional Application Serial No. 62/192,752, having the title "CONFORMAL CONDUCTIVE SCAFFOLDS AND METHODS OF USING CONFORMAL CONDUCTIVE SCAFFOLDS," filed on July 15, 2015, the disclosure of which is incorporated herein in by reference in its entirety.

BACKGROUND

Cells inhabit environments known as the extracellular matrix (ECM) which consists of a mixture of different biomolecules, and the precise composition and topographical properties are different in different tissues *(e.g.*, bone, brain, muscle, skin). Cells interact intimately with the ECM, not only constructing the biomolecules, but assist its organization in 3D space, and its degradation (which is important for tissue remodeling); reciprocally, cells respond to the ECM *(e.g.*, by modifying their size, shape). Cellular alignment is observed in organs and tissues such as bones, muscles and skin, and this alignment is important for the healthy functioning of the organ/tissue.

SUMMARY

Embodiments of the present disclosure provide for structures including a conformal conductive scaffold, methods of making the structure, method of using structure, and the like.

An embodiment of the present disclosure provides for a structure, among others, that includes a conformal conductive scaffold including a polyelectrolyte complex, chitosan, and gelatin, which are crosslinked by genipin. In an embodiment, the polyelectrolyte complex can be a PEDOT derivative, polypyrrole-polystyrenesulfonate (PSS), polyaniline-PSS, polythiophene-PSS, or a combination thereof.

An embodiment of the present disclosure provides for a method of alignment of cells, among others, that includes: providing a structure having a conformal conductive scaffold including a polyelectrolyte complex, chitosan, and gelatin, which are crosslinked by genipin; introducing cells to the structure; periodically providing electrical stimulation to cells; and aligning the cells on the structure. In an embodiment, the cell is a human dermal fibroblast or

a human mesenchymal stem cell. In an embodiment, the electrical stimulation can be a direct current (DC) or an alternating current (AC).

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.

Figure 1.1 illustrates molecules employed in this study. Abbreviations: (CHI) Chitosan, (GEN) Genipin. (PSS) Polystyrenesulfonate, (PEDOT) Poly(3,4-ethylenedioxythiophene), and (PEDOT-S) Sulfonated PEDOT derivative.

Figures 1.2A-E show adhesion and proliferation of human dermal fibroblasts on various surfaces up to 4 days in culture. Fig. 1.2A illustrates tissue culture plate controls. Fig. 1.2B illustrates PEDOT-PSS-based multilayer films. Fig. 1.2C illustrates PEDOT-S-based multilayer films. Cells were stained with a LIVE/DEAD® Viability/Cytotoxicity Kit, live cells were green and dead cells were red. Scale bars represent 250 μm. Fig. 1.2D illustrates cell viability after 4 days in culture as determined with a LIVE/DEAD® Viability/Cytotoxicity Kit. Fig. 1.2E illustrates cell adhesion after 2 days (grey bars) or 4 days (black bars) as determined by the AlamarBlue® assay. Error bars represent standard deviations (n = 3).

Figures 1.3A-F show analysis of the morphology of human dermal fibroblasts on various surfaces with optional electrical stimulation. Figure 1.3A illustrates PEDOT-PSS-based multilayer films without electrical stimulation, PEDOT-PSS(-). Figure 1.3B illustrates PEDOT-PSS-based multilayer films with electrical stimulation, PEDOT-PSS(+). Figure 1.3C illustrates PEDOT-S-based multilayer films without electrical stimulation, PEDOT-S(-). Figure 1.3D illustrates PEDOT-S-based multilayer films with electrical stimulation PEDOT-S(+). DAPI-stained nuclei are blue and Alexa Fluor® 488-stained actin is green. Scale bars represent 200 μm. Figure 1.3E illustrates assessment of cell alignment. Figure 1.3F illustrates assessment of cell length. Error bars represent standard errors of the mean (n = 150 or more).

Figure 1.4 is an example of an experimental setup for dip coating multilayer films using a Gilson 223 Sample Changer converted for use as a dip coater.

Figure 1.5 is a schematic of an experimental setup for electrical stimulation of PEDOT-based films (Not to scale). Abbreviations: (CE) counter electrode, (CT) copper tape, (PCW) polycarbonate well, (RE) reference electrode, and (WE) working electrode.

Figure 1.6 illustrates cell alignment & length assessment.

Figures 1.7A-B show polar plots for multilayer films. Fig. 1.7A illustrates PEDOT-PSS-based films. Fig. 1.7B illustrates PEDOT-S-based films. Black circles represent cells without electrical stimulation, whereas red circles represent cells with electrical stimulation; black and red lines represent the corresponding trend lines.

Figures 1.8A-B are FDVISCs on multilayer films after 3 days in culture. Fig. 1.8A illustrates PEDOT-PSS-based multilayer films. Fig. 1.8B illustrates PEDOT-S-based multilayer films. DAPI-stained nuclei are blue and Alexa Fluor® 488-stained actin is green. Scale bars represent 150 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 structures including a conformal conductive scaffold, methods of making the structure, method of using structure, and the like. Embodiments of the present disclosure can be used to align cells *(e.g.,* fibroblasts and human mesenchymal stem cells) on the surface of the conformal conductive scaffold. In a particular embodiment, cells can be cultured on the surface of the conformal conductive scaffold in an appropriate medium for the particular type of cell and then a current *(e.g.,* DC) is applied to the conformal conductive scaffold so that the cells align on the surface of the conformal conductive scaffold, where the electrical stimulation can be periodically applied to the electroactive scaffold.

In this regard, embodiments of the disclosure provide for a structure that includes the conformal conductive scaffold that can be used to align cells. An embodiment of the present disclosure includes introducing cells to the conformal conductive scaffold, where the conformal conductive scaffold and the cells are cultured in an appropriate medium. Subsequently, electrical stimulation can be periodically applied to the cells to cause the cells to align on the conformal conductive scaffold. In particular, the cells may align along the backbone of the polymer. In an embodiment, the cells align along the direction of the dip

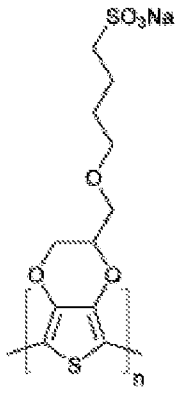
coating used to dispose the polymer film on the structure. As shown in Example 1, electrical stimulation of the conformal conductive scaffold in the presence of cells shows alignment of the cells, which is a characteristic of some cells types including bone cells, cardiac cells, and nerve cells.

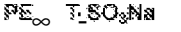
The term "conformal" as used herein refers to materials that are typically three dimensional and may or may not be inherently conductive.

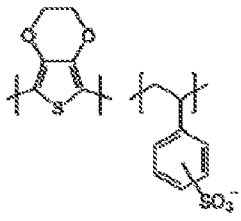
In an embodiment, the structure includes the conformal conductive scaffold that has a polyelectrolyte complex, chitosan, and gelatin film, where the chitosan and the gelatin film can crosslink with genipin. In an embodiment, the polyelectrolyte complex is selected from the group consisting of: PEDOT derivative, polypyrrole-polystyrenesulfonate (PSS), polyaniline-PSS, polythiophene-PSS, and a combination thereof. The polymer film can be coated on the structure using various techniques, including dip coating, drop coating, and spin coating. In an embodiment, the film can have a thickness of about 1 nm to 1 cm and area of about 10 mm^2 to 10 m^2.

To illustrate the interaction of the components, the following is an example of the interaction. In an embodiment where the polyelectrolyte complex is a PEDOT derivative *(e.g.,* PEDOT-polystyrenesulfonate (PSS)), the negatively charged sulfates on PEDOT-PSS, (or those on PEDOT-S, shown below), will non-covalently interact with the positively charged moieties on chitosan or gelatin *(e.g.,* amines), while genipin will covalently crosslink with chitosan and gelatin.

In an embodiment, the PEDOT derivative can include a sulfonated PEDOT having the

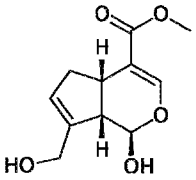


following structure:  (wherein n is 1 to infinity *(e.g.,* 10,000, 100,000, 1,000,000, or more)) or the PEDOT derivative can be PEDOT-polystyrenesulfonate (PSS)



(PEDOT-PSS) (where the unit of each be 1 to infinity *(e.g.,* 10,000, 100,000, 1,000,000, or more)). PEDOT-PSS is a mixture of two ionomers that form a macromolecular salt. In other embodiments, another negatively charged conducting polymer can be used such as polypyrrole-PSS, polyaniline-PSS, or polythiophene-PSS.

Genipin is a chemical a gardena fluid extract. Genipin has the following structure:



Chitosan is an oligosaccharide including β-linked D-glucosamine and N-actyl-D-glucosamine that are randomly distributed in the oligomer. In an embodiment, the chitosan has a molecular weight of about 1 kDa to 20 MDa.

Gelatin is an irreversibly hydrolyzed form of collagen, where the hydrolysis results in the reduction of protein fibrils into smaller peptides, which will have broad molecular weight ranges *(e.g.,* about 1 kDa to 20 MDa) associated with physical and chemical methods of denaturation, based on the process of hydrolysis.

In an embodiment, the cells can be human mesenchymal stem cells, human dermal fibroblasts, myoblasts, osteoblasts, osteoclasts, neurons, Schwann cells, pluripotent stem cells, or the like. In an embodiment where the cells are human dermal fibroblast, the medium can be HDF growth medium, 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.

Electrical stimulation can include direct contact of the material with a power source via a wire, wireless energy transfer, magnetic force, and the like. In an embodiment, the electrical stimulation can be direct current (DC) or alternating current (AC). 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 may only include a single long pulse that may last seconds to minutes, to hours, to days. 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 structure having the conformal conductive scaffold. In an embodiment, the conformal conductive scaffold can include one or more agents (*e.g.*, a chemical or biological agent), where the agent can be disposed indirectly or directly on the conformal conductive scaffold. As described herein, the agent can include a stem cell such as a human mesenchymal stem cell or human dermal fibroblast.

In addition, an additional agent that can be disposed on the conformal conductive scaffold 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 monomelic 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 cells. In an embodiment, the agent is included in an effective amount to accomplish its purpose, where such factors to accomplish the purpose are well known in the medical arts.

In general, the agent can be bound to the structure 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-electroactive scaffold interactions could be controlled through the inclusion of cell-adhesive peptides (*e.g.*, RGD, YIGSR, KQAGDV, KHIFSDDSSE, KRSR), protease-labile domains (*e.g.*, APGL, VRN, or

indeed oligoalanines that are degraded by elastase), osteoinduction can be enhanced *(e.g.,* NSPVNSKIPKACCVPTELSAI), and directing mineralization *(e.g.* FHRRIKA).

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 1:

We report the preparation of conducting polymer-based films. Composite biomaterials based on poly(3,4-ethylenedioxythiophene) derivatives, chitosan and gelatin were prepared and their physicochemical properties were characterized. Fibroblasts adhered to and proliferated on the films, and were shown to align with a DC current passed through the films.

The specific properties of bodily tissues act as cues (individually or in concert) that determine the behaviour of cells that inhabit them, and are utilised to engineer instructional tissue scaffolds to facilitate the regeneration of functional tissues.[l] The topographical properties of tissues may instruct cells to align, as is clearly observable within the anisotropically aligned pores observed in bone, cardiac, nerve and other tissues [2]. Scientists and engineers have reported novel methodologies of imparting biomimetic porous structures within biomaterials, including the removal of sacrificial templates embedded within a matrix *(e.g.,* colloidal crystals, ice crystals, electrospun fibers), and 3D printing technologies that offer methods of precisely positioning pores with specific geometries in such structures. [2]

Endogenous electric fields are another cue influencing cell behaviour (e.g. processes such as embryogenesis and wound healing). Electrical fields are also known to align cells, with endogenous electric fields playing a role in the alignment of cells,[3] and exogenous electric fields have been shown to align a variety of cell types (including astrocytes, epithelial cells, fibroblasts) in vitro,[3] motivating the development of electrically conductive materials that instruct cell alignment aided by application of an electrical field/current that mimics endogenous electrical fields/currents.[3]

Conducting biomaterials based on conducting polymers (CPs) such as derivatives of polyaniline, polypyrrole or polythiophene, have potential for both long term biomedical applications (e.g. electrodes) and short term biomedical applications (e.g. drug delivery or

tissue engineering).[4] CP-based scaffolds have been developed for the regeneration of bone and nerve tissues, and organs including the heart and skin.[4]

Layer-by-layer assembly is a method of producing thin multilayer films that was popularized by Mohwald and others.[5] Conductive multilayer films have been prepared from a variety of organic electronic components, often for applications in energy, of which a number of examples based on CPs exist.[5] Wallace and coworkers reported the preparation of bioerodible CP-based multilayer films based on an anionic sulfonate-displaying polythiophene derivative (with a M_w = 13160 Da and M_n = 6682 Da as determined by GPC) and cationic polyethyleneimine (17 kDa),[6] both of which are below the renal filtration limit of approximately 50 kDa; and the films were shown to be suitable for the growth and proliferation of cells derived from the connective and muscle tissue of mice (L929 and C2C12 cells, respectively).[6] They subsequently showed that it was possible to prepare CP-based multilayer films based solely on anionic and cationic polythiophene derivatives that could be -disassembled upon the application of an electrochemical trigger (a potential step of 650 mV for 19-42 hours).[7] Wei and coworkers reported the first example of biologically functional biodegradable CP-based multilayer films for bone tissue engineering, that were used for the electrical stimulation of osteoblast precursor MC3T3-E1 cells (derived from mice) that resulted in increased expression of osteopontin and runt-related transcription factor 2 which are markers of osteogenesis.[8]

Here we describe the preparation of CP-based films composed of poly(3,4-ethylenedioxythiophene) (PEDOT) derivatives, chitosan and gelatin, that are crosslinked by genipin (Figure 1.1). These conducting films enabled the electrical stimulation of human dermal fibroblasts cultured thereon which resulted in the preferential alignment of the cells with a DC current passed through the films.

Films were deposited on non-conductive glass slides that were previously rendered positively charged by modification with aminopropyltrimethoxysilane. The polyelectrolyte complex composed of PEDOT and polystyrenesulfonate (PSS), PEDOT-PSS, or the sulfonated PEDOT derivative PEDOT-S (Figure 1.1) were used for film preparation because they are electrochemically stable over periods long enough to facilitate short term electrical stimulation of tissues in their vicinity, and have been shown to be relatively non-immunogenic after implantation in various tissues. The polysaccharide chitosan was chosen as the cationic polymer to interact with the anionic PEDOTs (PEDOT-PSS and PEDOT-S, respectively) as it is also known to be relatively non-immunogenic (particularly by comparison with polyethyleneimine) and promotes cell adhesion. Furthermore,

electrochemically reducing the backbone of PEDOT-S makes it less positively charged which in turn reduces the number of sulfonates necessary to dope the polymer backbone rendering the surface charge of PEDOT-S films to be predominantly negative; therefore, PEDOT-S can be employed as a surface coating that enables electrochemically triggered cell desorption. Therefore we employed gelatin as a surface coating to render the films cell adhesive, and crosslinked the films with genipin (a natural crosslinker of biopolymers including chitosan, collagen and gelatin, which is markedly less toxic than the more commonly used glutaraldehyde), to ensure film stability for the duration of the experiments.

Film preparation by drop casting yielded films with μιη-scale roughnesses, water contact angles (WCAs) of approximately 50°, and conductivities of the order of 10^{-7} S cm^{-1} (Table 1). By contrast, layer-by-layer assembly via dip-coating (Figure 1.4) yielded multilayer films that were smoother (nm-scale roughnesses) and more conductive, 10^{-4} to 10^{-3} S cm^{-1} (Table 1). Film conductivities are on a similar order of magnitude to those of mammalian tissues (typically ≥ 10^{-4} S cm^{-1}).[9]

Table 1. Surface properties of the films.

Sample	Ra (μm)	Rq (μm)	Water Contact Angle (°)	Conductivity, σ (S cm^{-1})
PEDOT-PSS drop cast	0.67 ± 0.23	0.99 ± 0.35	51.6 ± 20.3	8.9 x 10^{-7} ± 1.1 %
PEDOT-S drop cast	1.27 ± 0.42	1.760 ± 0.58	54.7 ± 5.1	1.8 x 10^{-7} ± 8.1 %
PEDOT-PSS dip-coated	0.25 ± 0.04	0.36 ± 0.07	53.1 ± 9.6	3.0 x 10^{-4} ± 13.8 %
PEDOT-S dip-coated	0.05 ± 0.02	0.08 ± 0.04	46.6 ± 1.5	3.0 x 10^{-3} ± 19.7 %

Ra) average roughness. Rq) root-mean-square roughness. Error bars represent standard deviations. (the two highlighted numbers were different than in the provisional, please confirm this are correct)

With a view to the application of the multilayer films as coatings for biomaterials we cultured human dermal fibroblasts (FIDFs) on their surfaces and compared them to commercially available tissue-culture treated Corning® Costar® tissue culture plate (TCP)

controls. HDFs cultured on TCP controls adhered to and spread on the TCP as expected (Figure 1.2A), FIDFs had somewhat more rounded morphologies when cultured on the PEDOT-PSS-based or PEDOT-S-based multilayer films (Figure 1.2B and 1.2C) which is indicative of slightly poorer cell adhesion to the conducting substrates despite the gelatin coating. Interestingly, we found that cell viability for the HDFs cultured on the PEDOT-based films was comparable, and indeed somewhat better than for HDFs cultured on TCP controls (Figure 1.2D). We found that HDFs adhered to and proliferated on the surface of the films over the period of 4 days, although HDFs cultured on the TCP control substrates proliferated somewhat faster than on the PEDOT-PSS-based or PEDOT-S-based films (Figure 1.2E), similar to the findings of Wallace and coworkers with mouse derived L929 and C2C12 cells on polythiophene-based films.[6]

To assess the potential of the PEDOT-based films to act as instructive coatings for biomaterials, we investigated four different systems: 1) cells seeded on PEDOT-PSS-based multilayer films without electrical stimulation, 2) cells seeded on PEDOT-PSS-based multilayer films with electrical stimulation, 3) cells seeded on PEDOT-S-based multilayer films without electrical stimulation, and 4) cells seeded on PEDOT-S-based multilayer films with electrical stimulation using a custom built setup (Figure 1.5). Those samples without electrical stimulation were cultured for 3 days, whereas those that were electrically stimulated were cultured for 1 day without stimulation, followed by one period of stimulation at 10 mV mm^{-1} for 4 hours, then 44 hours without stimulation.

We found that both the PEDOT-PSS-based and PEDOT-S-based substrates were stable to electrical stimulation at 10 mV mm^{-1} for 4 hours, and moreover that HDFs maintained adherence to the substrates after electrical stimulation. We observed that HDFs displayed a moderate preference for aligning with the direction in which the slides were dipped during the dip coating process (Figures 1.3A-E). Cells without any preferential alignment would be expected to have an average orientation of 45° (Figures 1.3A-F, 1.6 and 1.7A-B), however, we saw that HDFs on PEDOT-PSS-based multilayer films had an average orientation of 38.6° ± 1.5° (Figures 1.3A and 1.3E), whereas HDFs on PEDOT-S-based multilayer films had an average orientation of 35.3° ± 1.8° (Figure 1.3B and 1.3E), which is likely to be because the polymer chains were aligned during the dip coating process as is commonly observed for multilayer films prepared in this fashion. Interestingly, electrical stimulation of HDFs on the conductive substrates led to an increase in their propensity to align with the direction of the DC current passed through the substrate, and we found that HDFs on PEDOT-PSS-based multilayer films had an average orientation of 27.7° ± 1.7°

(Figures 1.3C and 1.3E), whereas FIDFs on PEDOT-S-based multilayer films had an average orientation of 32. 1° ± 1.1° (Figures 1.3C and 1.3E). The fact that the increase in cell alignment was somewhat lower for the cells on the PEDOT-S-based multilayer films than the PEDOT-PSS-based multilayer films is probably because of minor changes in the surface chemistry of the films that alter cell-substrate interactions as reported by Berggren and coworkers for PEDOT-S-based films. Further evidence in support of this hypothesis can be found in changes in the length of the cells on the PEDOT-S-based films after electrical stimulation, with their average lengths reduced from approximately 135 μm to 80 μm (Figures 1.3C, 1.3D, 1.3F, 1.6 and 1.7A-B). Nevertheless, the increased cell alignment on both PEDOT-PSS-based and PEDOT-S-based multilayer films suggests that they may find application as instructive biomaterial coatings, potentially for tissue engineering of skin or a variety of other niches in which cell alignment is an important feature. [2]

Cell alignment within specific tissues is clearly observable within the anisotropically aligned pores observed in bone, cardiac, nerve and other tissues. This has motivated the development of novel materials that instruct the cells thereon/therein to align, most commonly through topological cues engineered into the materials.

In this example we report the preparation of CP-based multilayer films. Films based on poly(3,4-ethylenedioxythiophene) derivatives, chitosan and gelatin were prepared by dip coating and their physicochemical properties characterized. Fibroblasts cultured on their surfaces of such films were shown to adhere and proliferate in vitro, and moreover, to respond to a DC current passed through the films by aligning with the current. Such conductive films have prospects for the development of thin conformal bioactive coatings that induce cell alignment upon the surface of implantable biomaterials. Furthermore, the fact that human mesenchymal stem cells adhere to the films (Figure 1.8A-B) highlights their potential for patient specific applications as personalized medical devices.

Manufacturing multilayer films by dip coating is very attractive as it is simple and industrially scalable. Moreover, the properties of the films could be easily tuned by altering the contents of the dipping baths. For example, using water soluble CPs with molecular weights below the renal filtration limit of approximately 50 kDa allows the preparation of bioerodible conductive films, and it is likely that adhesion could be improved by coating with mixtures of extracellular matrix-derived proteins (e.g. collagen, fibronectin, laminin). We believe the simplicity of our approach enables us to tailor the properties of the films to specific niche applications (and potentially specific patients).

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Example 1 Experimental Details

Materials and methods

Unless otherwise stated, all chemicals were of ACS grade, purchased from Sigma-Aldrich and used as received without further purification, e.g., Poly(3,4-ethylenedioxythiophene)-poly(styrenesulfonate) 1.1% in H_20, neutral pH, high-conductivity grade (PEDOT-PSS), chitosan (70 kDa), phosphate buffered saline (PBS) tablets etc. Hydroxymethyl EDOT was purchased from Sarchem Laboratories, Inc., and ITO slides were purchased from Ted Pella, Inc. For cell culture experiments, all reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise stated. Human dermal fibroblasts (hDFs) were purchased from Lonza (Gaithersburg, MD).

Film Preparation:

Aminopropyltrimethoxysilane-functionalized glass microscope slides were used as positively charged substrates for the deposition of conducting polymer-based films. Negatively charged polymers were either poly(3,4-ethylenedioxythiophene)-poly(styrenesulfonate) 1.1% in H_2O, neutral pH, high-conductivity grade (PEDOT-PSS), or PEDOT-S; whereas the positively charged polymer was chitosan (70 kDa) that was dissolved in aqueous acetic acid (1 % v/v).

Drop cast films were prepared by briefly vortexing aqueous solutions of the polymers (typically 1 mg mL^{-1}, adjusted to pH 7 and buffered with 10 mmol PBS), casting on glass slides, air drying for 24 hours and then drying under vacuum for 48 hours.

Multilayer films were prepared from aqueous solutions of the polymers (typically 1 mg mL^{-1}, adjusted to pH 7 and buffered with 10 mmol PBS) using a Gilson 223 Sample Changer (Gilson, Inc. Middleton, WI, USA) converted for use as a dip coater controlled by a script written in LabVIEW (National Instruments, Inc., Austin, TX, USA). Films were deposited by repetitive sequences of: dipping in negatively charged polymer solution (dip time 15 minutes), air drying 1 minute, rinsing in water (dip time 30 seconds), air drying 1

minute, dipping in chitosan (70 kDa, dip time 15 minutes), air drying 1 minute, rinsing in water (dip time 30 seconds), air drying 1 minute. The films were air dried for 24 hours and then dipped in a solution of gelatin (1 mg/mL, dip time 15 min), air dried for 1 minute, rinsed in water (dip time 30 seconds), air dried 1 minute, rinsed in water (dip time 30 seconds), air dried for 24 hours and vacuum dried for 48 hours.

Profilometry:

Profilometry was carried out using a Veeco Dektak 6M Stylus Profilometer (Veeco Instruments Inc., NY) fitted with a 12.5 μm stylus tip. The profilometer was isolated on an air table to reduce ambient vibrations. The profilometer was operated at 10 mg of stylus force, and used to record profiles of distances of ca. 1 cm, recording data points every 555 nm. Data analysis was carried out with the software provided by the manufacturer, which allowed the determination of the thickness and roughness of the films. The surface roughness parameters are analyzed and reported in accordance with the ISO 25178 series. The average roughness (Ra) is the arithmetic average of the deviation from the mean line, and is the most used international parameter of roughness, and the root-mean-square roughness (Rq) is based upon this.

Water contact angle measurements:

Measurements were carried out with a high-speed contact angle measurement device (FTA200 video-based semi-automatic contact angle goniometer supplied by First Ten Angstroms, Inc., Portsmouth, VA, USA). Images of a drop of deionized water (2 μL) laid on the surface of the samples were recorded at a frame rate of 360 frames per second, and the contact angles for the droplets were recorded after 3 seconds of contact with the film. The reported values are the average of at least 3 measurements at different positions on a film.

Conductivity Determination:

Resistance (*R* in Ω) was measured between two silver electrodes using a digital multimeter (DM-8A, Sperry Instrument, Milwaulkee, WI). The resistivity, p (Ω/cm), of the films was determined in accordance with equation 1:

$$p = Rwt/L \qquad (1)$$

The resistance, *R*, was recorded in at least ten different positions on the materials, *Wis* the width of the film, *t* corresponds to the thickness of the film (as determined via profilometry), and *L* is the distance between the two silver electrodes. The conductivity (S/cm) of the films was determined in accordance with equation 2:

$$\sigma = 1/p \qquad (2)$$

In Vitro Cell Culture Studies

Sample preparation and conditions for in vitro cell culture:

Pristine indium tin oxide (ITO) slides (Ted Pella, Inc.) or glass slides coated with electroactive multilayer films (of ca. 1 cm^2) were inserted in tissue culture plates and sterilized by incubation in 70 % ethanol solution, followed by exposure to UV for 30 minutes. After sterilization, films were incubated for 30 minutes under 3 mm of HDF growth medium 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 HDF seeding. Cell viability before starting the experiment was determined by the Trypan Blue (Sigma, USA) exclusion method, and the measured viability exceeded 95 % in all cases. Cells were seeded at 5,000 cells per cm^2 under 3 mm of medium, and incubated at 37 °C, 95% humidity, and a $C0_2$ content of 5%.

Cell proliferation studies:

After 2 days the cells were washed gently with PBS, followed by the addition of fresh medium containing 10% v/v of the AlamarBlue® reagent. After 2.5 hours of culture, the medium was aspirated and replaced with fresh medium, and 100 μ of the aspirated medium containing the AlamarBlue® reagent was placed in a 96 well plate, and the fluorescence was measured with a fluorimeter (Synergy HT Multi-Mode Microplate Reader, Biotek US, Winooski, VT). Two controls were considered during the measurement of the fluorescence: the first was wells containing medium alone (i.e. no cells or AlamarBlue® reagent), which was not fluorescent; and the second was wells that contained the AlamarBlue® reagent but no cells (used for baseline correction). Numbers of cell adhered to the various surfaces studied herein are reported relative to their initial seeding density of 5,000 cells per cm^2, which was assigned an arbitrary value of *100%*. After another 2 days (i.e. at 4 days after initial seeding) this process was repeated. The medium was aspirated and replaced once more at 6 days after initial seeding, and finally after a total of 8 days in culture the viability of the cells was evaluated using a LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells (Molecular Probes, Eugene, OR). The medium was removed and cells on the surfaces were incubated with 4 μM ethidium and 2 μM calcein AM in PBS for 15 min at 37 °C in the dark. Live cells were stained green because of the cytoplasmic esterase activity, which results in reduction of calcein AM into fluorescent calcein, and dead cells were stained red by ethidium, which enters the cells via damaged cell membranes and becomes integrated into the DNA strands. Fluorescence images of cells were captured using a color CCD camera (Optronics® MagnaFire, Goleta, CA, USA) attached to a fluorescence microscope (IX-70;

Olympus America Inc.). Cells were counted with the cell counter tool (plugin) in the open source program ImageJ, all cells on all images were counted. Results of AlamarBlue® assays presented are the average of four samples and ethidium/calcein stained images are representative of 3 samples (typically 3 images per sample).

Cell orientation studies in the absence or presence of electrical stimulation:

Cell orientation studies employed a custom built setup (see Figure 1.6). Electroactive multilayer films supported on glass slides were (width of 2.5 cm, length of 7.5 cm) were sterilized by incubation in 70 % ethanol solution, followed by exposure to UV for 30 minutes. Polycarbonate wells (square polycarbonate blocks, thickness of 1 cm, sides of 2.5 cm, with square holes with sides of 0.9 cm cut out), Dow Corning® high vacuum grease, and medium binder clips (Staples®, Framingham, MA) were sterilized by autoclaving. Holes were drilled into the sides of 10 cm polystyrene Petri dishes using a Dremel saw (Lowes, Mooresfield, NC, USA), and the plates were sterilized by exposure to UV for 60 min. Adhesive-backed copper tape (5 mm width, Ted Pella, Inc.), waterproof Kapton® tape (1 cm width, Fisher Scientific, Waltham, MA, USA), wires and alligator clips were sterilized by exposure to UV for 60 min.

Electroactive multilayer films supported on glass slides and secured in position with two thin strips of adhesive-backed copper tape that were attached to the films, parallel to one another and separated by a distance of ca. 4 cm. One face of the polycarbonate wells was coated with vacuum grease and placed on the electroactive tissue scaffolds, greased side down, in contact with the glass slide. A binder clip on either side of the well was used to secure this in position and render it water tight. A strip of copper tape was run between the parallel copper strips attached to the scaffolds and the ends of the slides as points of contact for the alligator clip-terminated wires attached to the multipotentiostat (CH Instruments, Austin, TX, USA). The counter and reference electrodes were connected together and clipped to copper tape on one side of the slide, and the working electrode was clipped to copper tape on the other side of the slide. HDFs were plated and cultured for 1 day as described above. A potential step of +10 mV mm^{-1} was placed across the substrate for the duration of 4 hours, after which the wires were disconnected and the substrates cultured as normal for a further 40 hours. The medium was aspirated and the samples were washed gently with PBS. Cells were fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.1% Triton X-100 (Fluka) and 2% bovine serum albumin (BSA) in PBS buffer for 5 min, followed by blocking with 2% BSA in PBS buffer for 30 min at room temperature. Actin filaments and cell nuclei within cells were stained with Alexa Fluor 488® Phalloidin (Life Technologies, USA) for 30

min and 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, USA) for 5 min. The cells were then washed three times with PBS buffer and stored at 4 °C until images were acquired. Fluorescence images of cells were captured using a colour CCD camera (Optronics® MagnaFire, Goleta, CA, USA) attached to a fluorescence microscope (IX-70; Olympus America Inc.). Images are representative of 3 samples. Images were analysed using ImageJ. A line was drawn across cells for measurements, and lengths in pixels were converted to lengths in μιη using the measure bar in ImageJ. Angles were determined measuring from the left side of the image to the right side of the image to ensure that all angles measured would be within the 1st and 2nd quadrants. In Excel the absolute value of the negative angles were taken so all angles measured would be converted to the 1st quadrant, and these data were converted to cartesian coordinates. Polar plots were generated automatically using a polar plot add-in (http://www.andypope.info/charts/polarplot.htm) and the data transferred to Excel for further calculations. A minimum of 150 cells were counted per experiment, and error bars represent standard errors.

Human Mesenchymal Stem Cell Adhesion Studies:

HMSCs were supplied by Lonza (Walkersville, MD). Samples were prepared as described above (in "film preparation"). 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 5,000 cells cm-2, and incubated at 37 °C, 95 % humidity, and a C02 content of 5 %. Samples were fixed and stained as described above (n = 3).

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

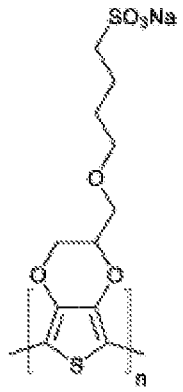
We claim:

1. A structure, comprising:

a conformal conductive scaffold including a polyelectrolyte complex, chitosan, and gelatin, which are crosslinked by genipin.

2. The structure of claim 1, wherein the polyelectrolyte complex is selected from the group consisting of: PEDOT derivative, polypyrrole-polystyrenesulfonate (PSS), polyaniline-PSS, polythiophene-PSS, and a combination thereof.

3. The structure of claim 2, wherein the PEDOT derivative is a sulfonated PEDOT



having the following structure: PEDOT-S¾ Na or the PEDOT derivative is PEDOT-polystyrenesulfonate (PSS) (PEDOT-PSS).

4. The structure of claims 1, 2 or 3, wherein the conformal conductive scaffold is a polymer film.

5. The structure of claim 4, wherein the polymer film is a multi-layer film assembled by dip-coating.

6. The structure of claim 5, wherein the multi-layer film has a conductivity similar to that of mammalian tissues.

7. The structure of claim 6, wherein the conductivity is about 10^{-4} S cm^{-1} to 10^{-3} S cm^{-1}.

8. A method of alignment of cells, comprising:

providing a structure having a conformal conductive scaffold including a polyelectrolyte complex, chitosan, and gelatin, which are crosslinked by genipin;

introducing cells to the structure;

periodically providing electrical stimulation to cells; and

aligning the cells on the structure.

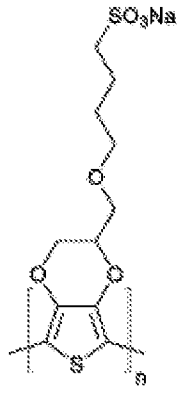
9. The method of claim 8, wherein the cell is a human dermal fibroblast or a human mesenchymal stem cell.

10. The method of claim 8 or 9, wherein the electrical stimulation is direct current (DC).

11. The method of claim 8 or 9, wherein the electrical stimulation is alternating current (AC).

12. The method of claim 8, wherein the alignment is in line with the dip-coating direction.

13. The method of claim 8, wherein the polyelectrolyte complex is selected from the group consisting of: PEDOT derivative, polypyrrole-polystyrenesulfonate (PSS), polyaniline-PSS, polythiophene-PSS, and a combination thereof.

14. The method of claim 13, wherein the PEDOT derivative is a sulfonated PEDOT having the following structure: 

PEDOT-SO3Na or the PEDOT derivative is PEDOT-polystyrenesulfonate (PSS) (PEDOT-PSS).

15. The method of claim 8, wherein the conformal conductive scaffold is a polymer film.

16. The method of claim 15, wherein the polymer film is a multi-layer film assembled by dip-coating.

17. The method of claim 16, wherein the multi-layer film has a conductivity similar to that of mammalian tissues.

18. The method of claim 17, wherein the conductivity is about 10^{-4} S cm^{-1} to 10^{-3} S cm^{-1}.

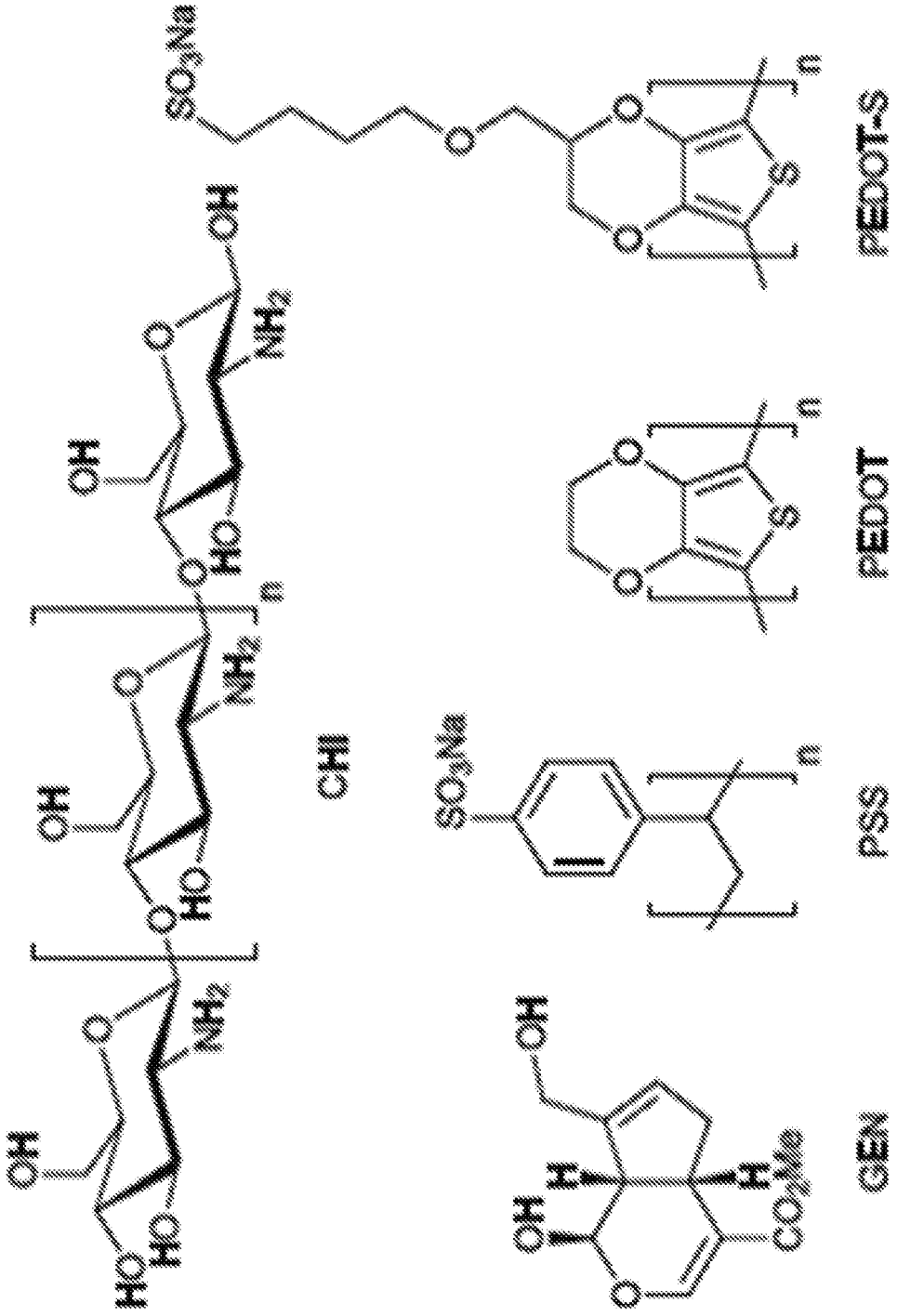


Fig. 1.1

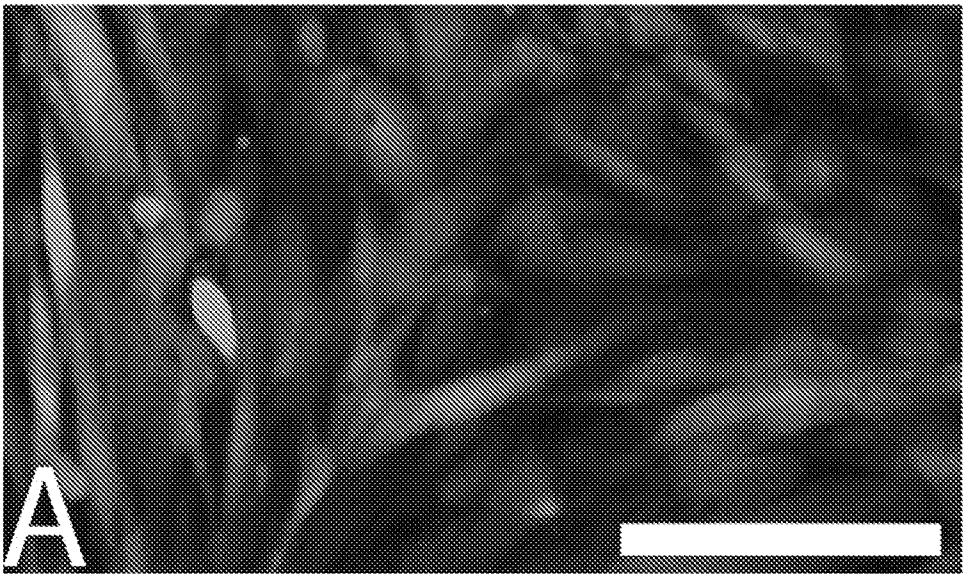


Fig. 1.2A

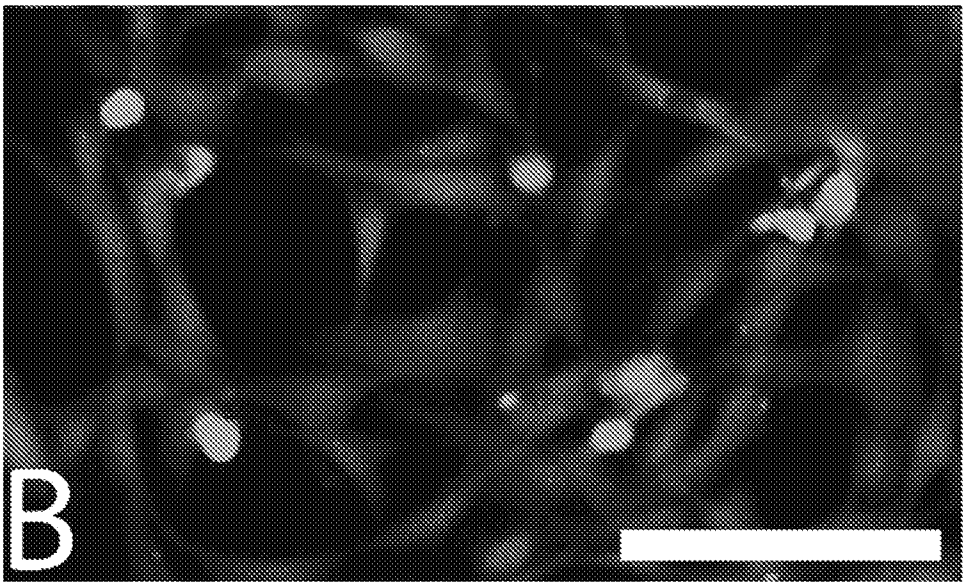


Fig. 1.2B

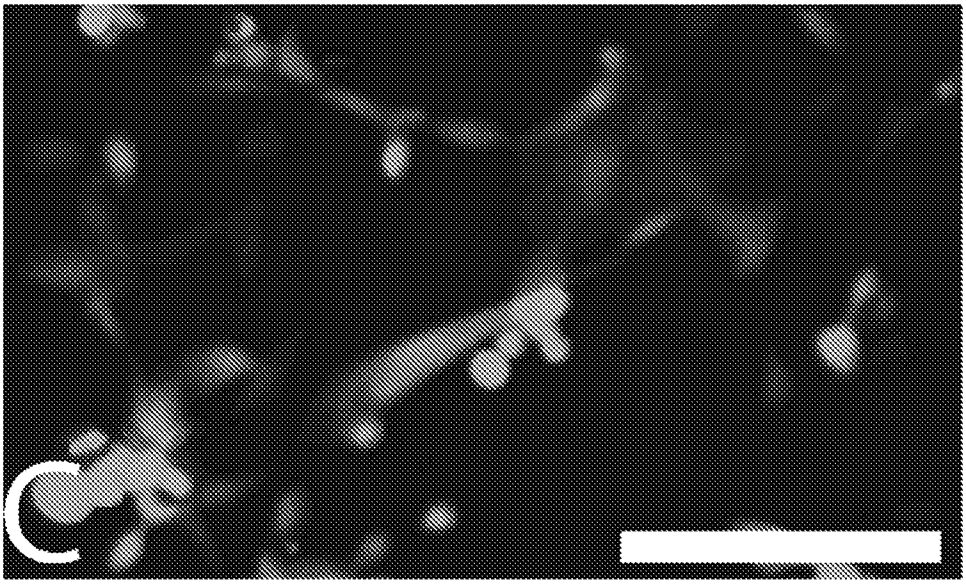


Fig. 1.2C

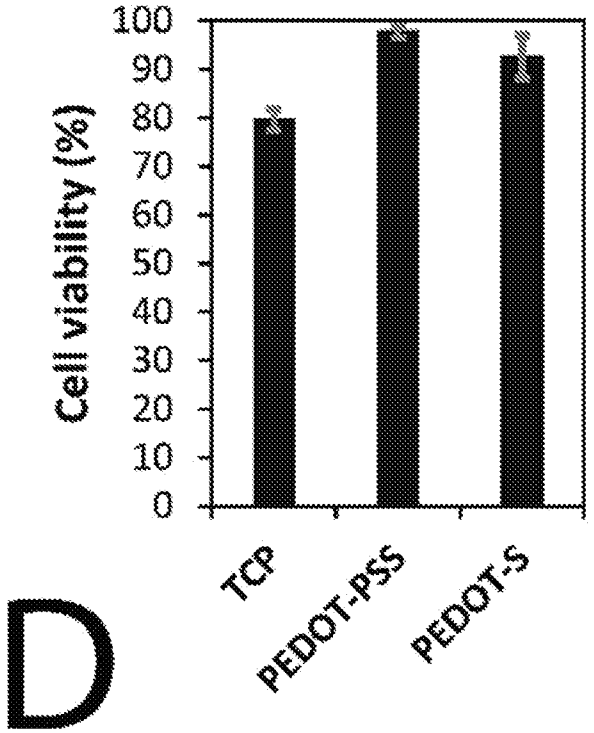


Fig. 1.2D

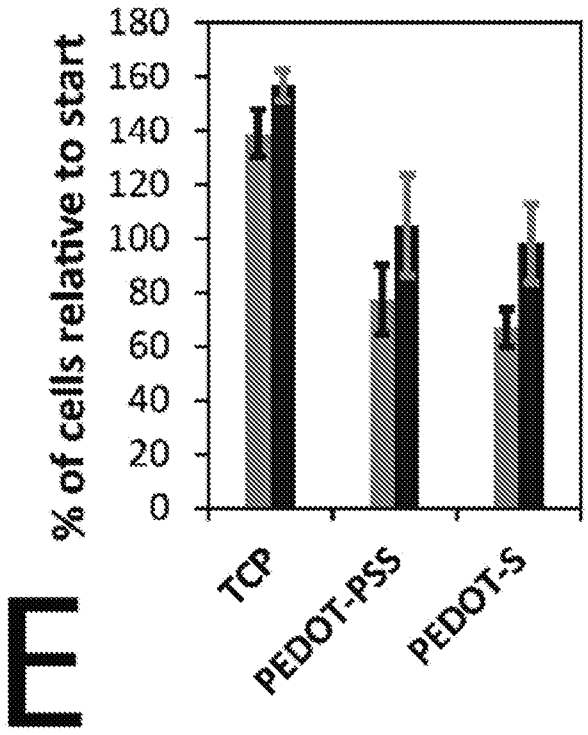


Fig. 1.2E

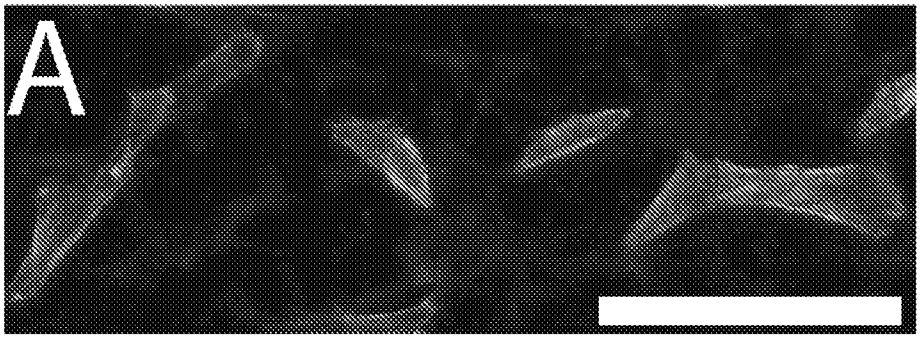


Fig. 1.3A

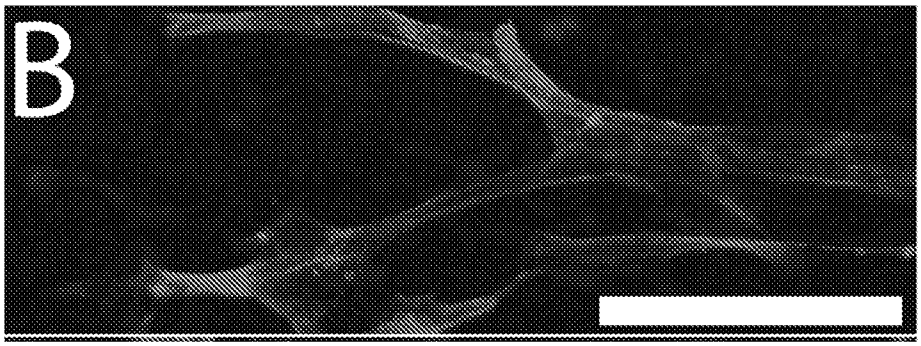


Fig. 1.3B

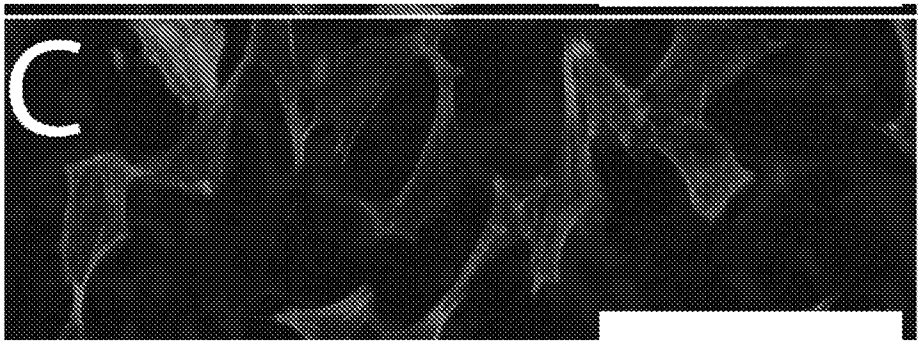


Fig. 1.3C

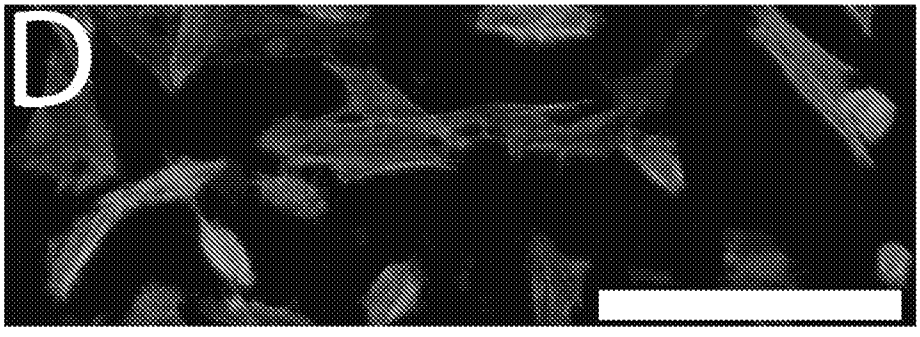


Fig. 1.3D

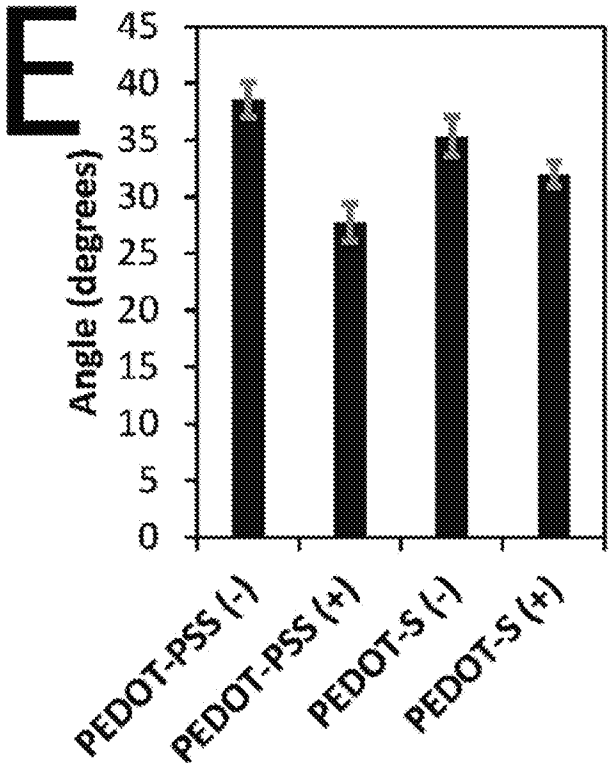


Fig. 1.3E

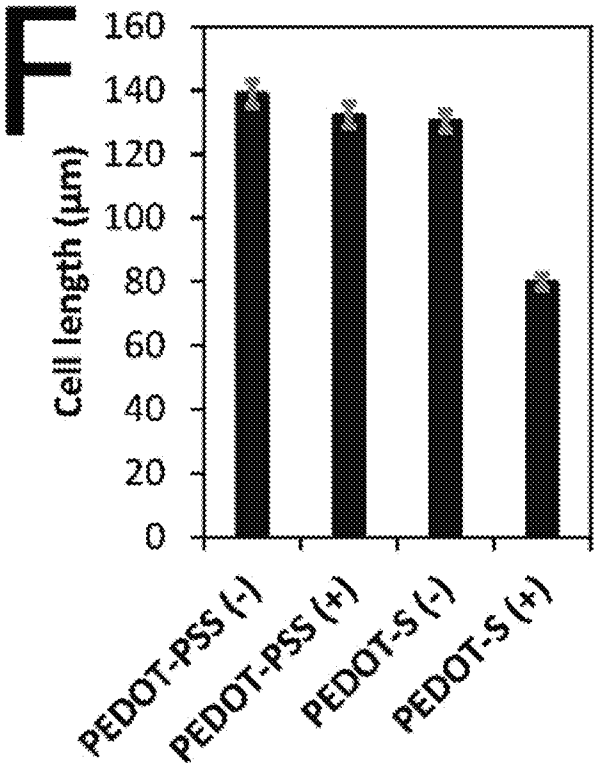


Fig. 1.3F

PC running LabVIEW script to control the Gilson 223 Sample Changer
Gilson 223 Sample Changer converted for use as a dip coater
24 place vertical plastic slide staining rack
Dipping baths:
1. Anionic polymer (PEDOT-PSS or PEDOT-S)
2. Water
3. Cationic polymer (Chitosan)
4. Water
1
2
3
4

Fig. 1.4

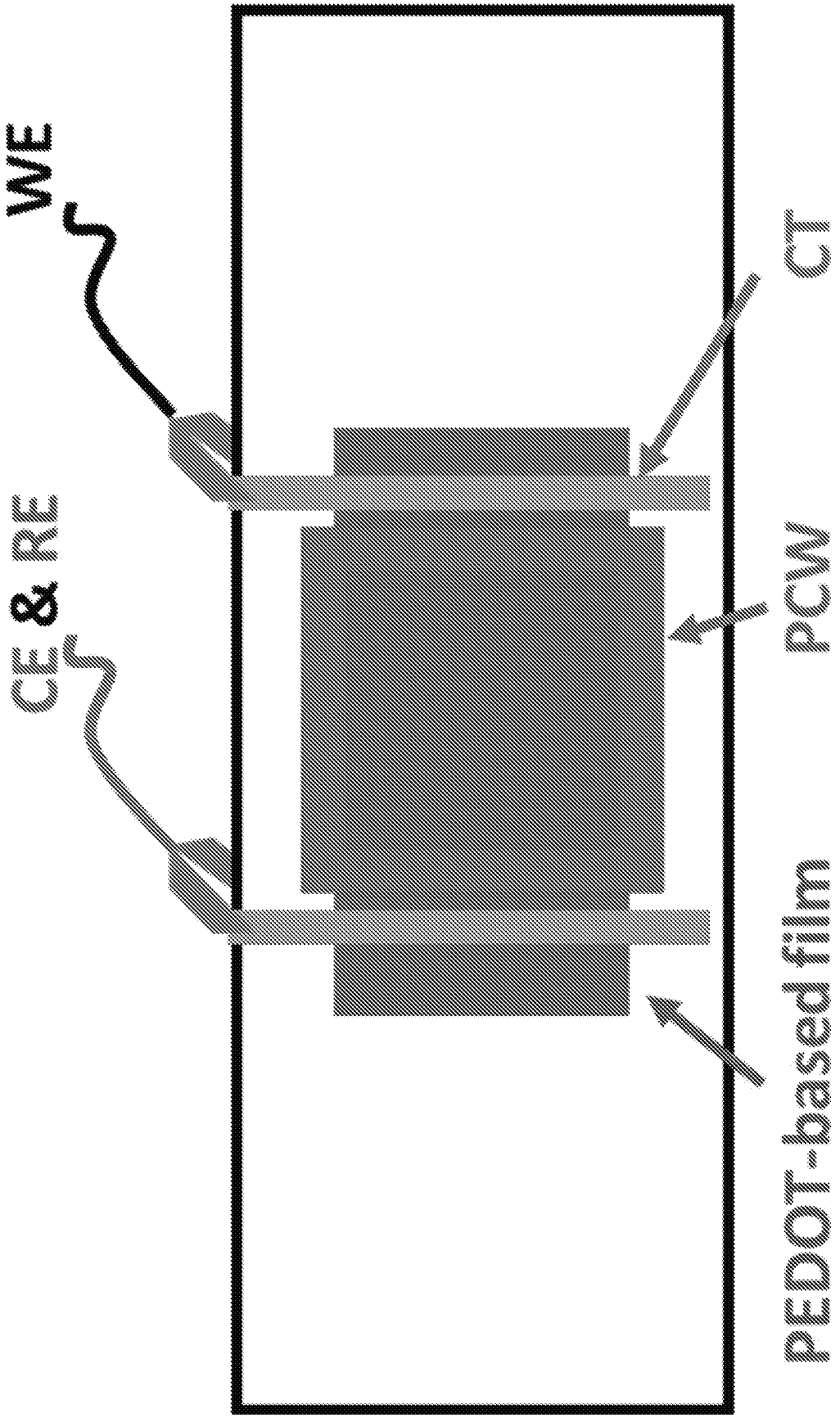


Fig. 1.5

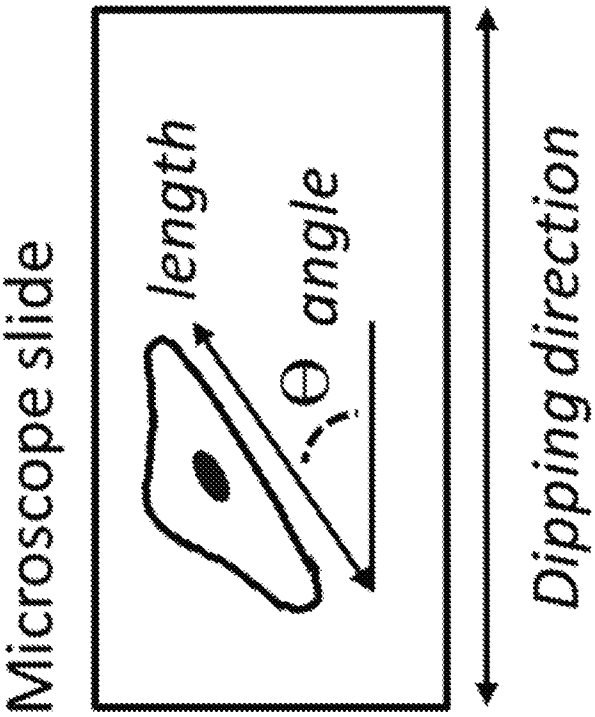


Fig. 1.6

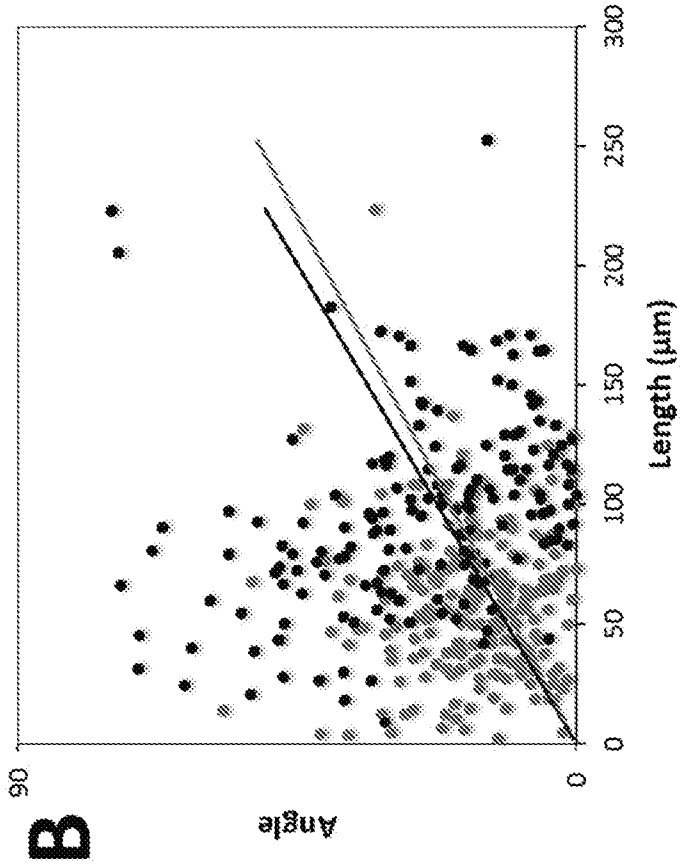


Fig. 1.7B

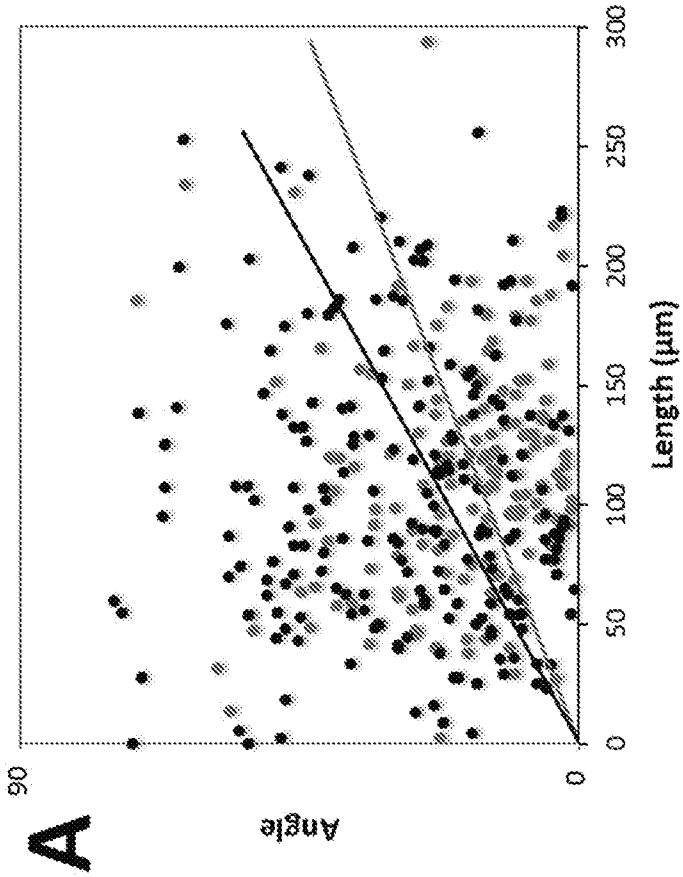


Fig. 1.7A

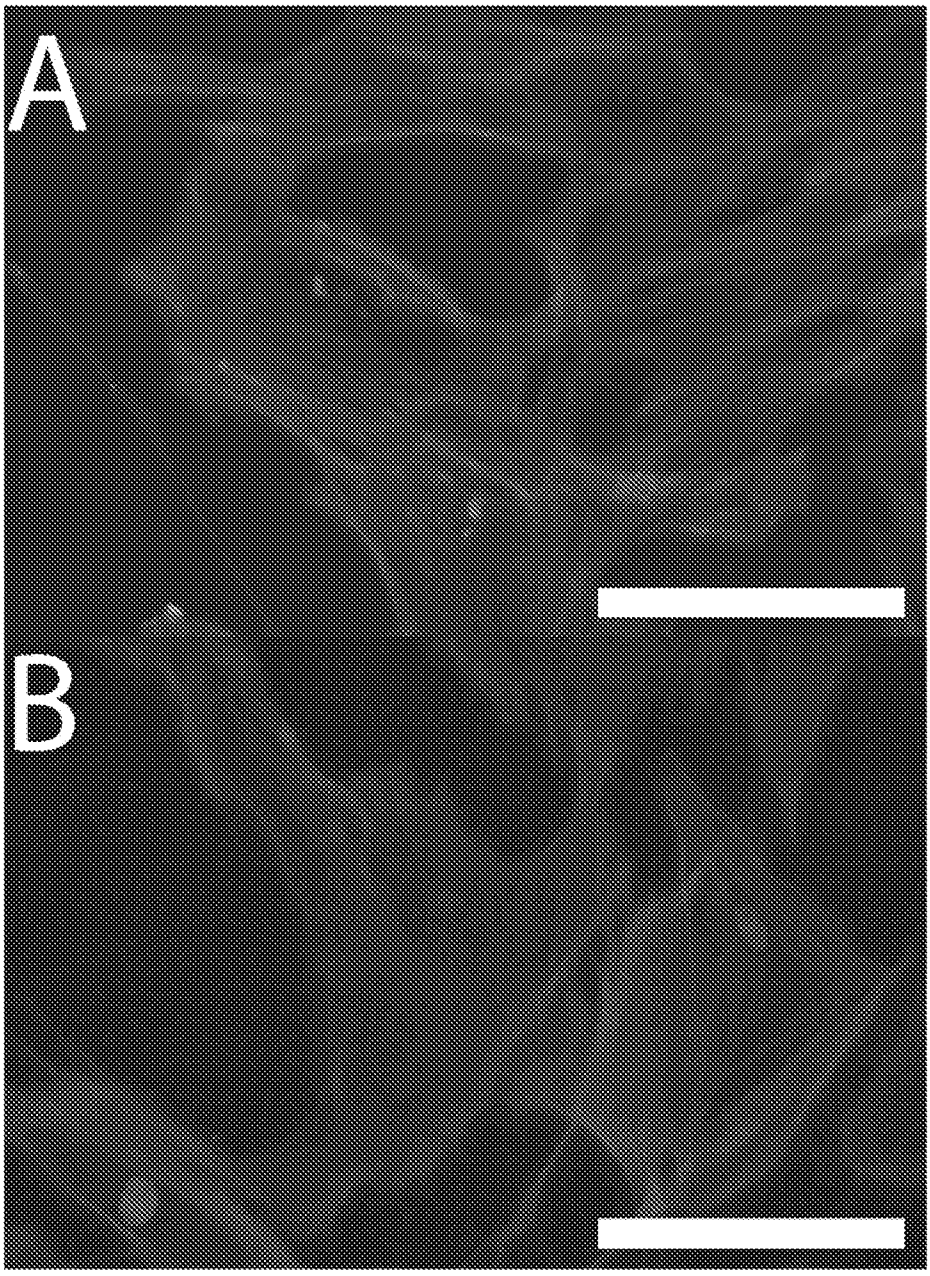


Fig. 1.8A

Fig. 1.8B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/41885

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61N 1/05; H01B 1/12 (2016.01)
CPC - A61N1/0565; A61N1/0568; A61N1/05; H01B1/124
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) - A61N 1/05; H01B 1/12 (2016.01)
CPC - A61N1/0565; A61N1/0568; A61N1/05; H01B1/124

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Patents and NPL (classification, keyword; search terms below)
USPC - 252/500; 607/122; 525/328.5

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase (AU BE BR CA CH CN DE DK EP ES FI FR GB IN JP KR SE TH TW US WO), PubWest, Google Web
search terms: conductive scaffold, polyelectrolyte complex, chitosan, gelatin, genipin, PEDOT, sulfonated, PEDOT/S03Na/PEDOT-PSS, polymer film, aligning cells, electrical stimulation

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2013/0183352 A1 (Xie) 18 July 2013 (13.07.2013), para [0008]-[0009], [0022], [0043], [0051], [0071], [0073]; Fig 7A-B, 8, 10-11.	1-18
Y	US 2012/0149111 A1 (Wegst et al.) 14 June 2012 (14.06.2012), para [0108], [0114], [0148]	1-18
Y	US 2009/0226757 A1 (Song et al.) 10 September 2009 (10.09.2009), para [0014]	3, (4-7)/3, 14
Y	US 2002/0034796 A1 (Shastri et al.) 21 March 2002 (21.03.2002), para [0025], [0029]-[0030], [0041]; Fig 7	6-7, 17-18

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

* Special categories of cited documents:
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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
15 September 2016 (15.09.2016)	17 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**

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