Conductive interpenetrating networks of polypyrrole and polycaprolactone encourage electrophysiological development of cardiac cells

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Abstract

Conductive and electroactive polymers have the potential to enhance engineered cardiac tissue function. In this study, an interpenetrating network of the electrically-conductive polymer polypyrrole (PPy) was grown within a matrix of flexible polycaprolactone (PCL) and evaluated as a platform for directing the formation of

functional cardiac cell sheets. PCL films were either treated with sodium hydroxide to render them more hydrophilic and enhance cell adhesion or rendered electroactive with PPy grown via chemical polymerization yielding PPy-PCL that had a resistivity of 1.0 ± 0.4 kΩ cm, which is similar to native cardiac tissue. Both PCL and PPy-PCL films supported cardiomyocyte attachment; increasing the duration of PCL pre-treatment with NaOH resulted in higher numbers of adherent CMs per unit area, resulting in cell densities which were more similar to those on PPy-PCL films (1,568 ± 126 cells mm^{-2}, 2,880 ± 439 cells mm^{-2}, 3,623 ± 456 cells mm^{-2} for PCL with 0, 24, 48 h of NaOH pretreatment, respectively; 2,434 ± 166 cells mm^{-2} for PPy-PCL). When cardiomyocytes were cultured on the electrically-conductive PPy-PCL, more cells were observed to have peripheral localization of the gap junction protein connexin-43 (Cx43) as compared to cells on NaOH-treated PCL (60.3 ± 4.3% vs. 46.6 ± 5.7%). Cx43 gene expression remained unchanged between materials. Importantly, the velocity of calcium wave propagation was faster and calcium transient duration was shorter for cardiomyocyte monolayers on PPy-PCL (1612 ± 143 μm/s, 910 ± 63 ms) relative to cells on PCL (1129 ± 247 μm/s, 1130 ± 20 ms). In summary, PPy-PCL has demonstrated suitability as an electrically-conductive substrate for culture of cardiomyocytes, yielding enhanced functional properties; results encourage further development of conductive substrates for use in differentiation of stem cell-derived cardiomyocytes and cardiac tissue engineering applications.

1 Introduction

Heart disease is the leading cause of mortality in the United States, accounting for about 25% of all deaths in 2009 [1]. Better treatment options to enable true cardiac regeneration are needed to augment total heart transplant, for which donor organs are in chronic short supply. Engineered cardiac tissues having properties similar to native myocardium have the potential to contribute to reaching this goal, both through their use in drug-testing platforms for the identification of more effective pharmaceutical therapies and directly in future clinical treatments. However, creating tissues *in vitro* that have the electromechanical properties of adult native cardiac tissue remains an elusive goal. By replicating microenvironmental cues present in native myocardium, biomimetic materials may facilitate formation and maturation of functional engineered cardiac tissues [2,3].

Electrical signaling and conductivity are important considerations in designing a substrate that mimics the properties of native cardiac tissue. Electrical signals propagating through native myocardium trigger the organized contraction of the heart, resulting in blood being pumped throughout the body. Improper conduction of electrical signals can result in deadly arrhythmias. Electrical stimulation has been previously shown to be important in directing the structure and electrophysiological properties of cardiomyocytes and engineered cardiac tissue during *in vitro* culture. Electrical stimulation of cardiac cell monolayers has been shown to increase alignment of neonatal rat ventricular myocytes [4,5], increase cell coupling [4,5], decrease the heterogeneity of conduction velocity [6], and increase the amplitude of cardiac construct synchronous contractions [4]. Shortened action potential durations and decreased spontaneous contractile activity have also been reported in neonatal rat ventricular

myocytes undergoing electrical stimulation [7]. In aligned cardiac tissues created using human pluripotent stem cell-derived cardiomyocytes, electrical stimulation resulted in higher conduction velocities and improved electrophysiological properties [8]. Use of conductive substrates, including the electrically conductive polymer scaffold polyaniline-poly(glycerol-sebacate) [9], has also resulted in enhanced engineered cardiac tissue function.

Multiple avenues for creating conductive substrates for cell growth have been explored, including the use of carbon nanotubes [10–12], use of homogenously dispersed gold particles [13], and entirely polymer-based scaffolds incorporating electrically-conductive polymers such as polypyrrole (PPy) [14,15]. The cost of gold nanoparticles may prohibit their large-scale application as electroactive biomaterials, whereas the potential cytotoxicity of carbon nanotubes may limit their use within biodegradable implantable scaffolds [16]. PPy has been used widely as an electrically conductive polymer for neural tissue engineering applications [15,17]. The selection of the dopant counterions during the synthesis of electroactive polymers has an effect on the biocompatibility of the electroactive polymer (PPy) *in vitro*, with high molecular weight dopants (typically polyanions such as polystyrene sulfonate) being attractive because they will not readily leach from the polyelectrolyte complex formed with the (typically) polycationic PPy [18–21]. Interestingly, *in vivo* studies of electroactive scaffolds have been carried out and histological analyses of tissue surrounding polypyrrole-based tissue scaffolds implanted subcutaneously or intramuscularly in rats reveal immune cell infiltration comparable to FDA-approved poly(lactic acid-co-glycolic acid) [22] or FDA-approved poly(D,L-lactide-co-glycolide) [23]. Likewise, no significant

inflammatory response was observed with polypyrrole-based sciatic nerve guidance channels implanted in rats after 8 weeks [24], polypyrrole-coated electrodes in rat brains after 3 or 6 weeks [25], or most pertinently, polypyrrole-based tissue scaffolds implanted in the coronary artery of rats after 5 weeks [26]. Although differences in the individual studies (i.e. the chemical/mechanical/topological properties of the scaffolds, the site of implantation, and the methodology used to evaluate the immune response) make it challenging to compare the results of each study, PPy-based biomaterials exhibit levels of immunogenicity that are comparable with other FDA-approved biomaterials and have potential for clinical translation, in addition to use for cell growth *in vitro*.

Polycaprolactone (PCL) has previously demonstrated strong compatibility as an underlying base material in the formation of conductive polymer substrates. Electroactive composites of PCL and polyaniline have been previously shown to support the adhesion and proliferation of H9c2 cells (derived from rat heart tissue) [27] and the differentiation of human mesenchymal stem cells towards cardiogenic outcomes [28], whereas electroactive composites of PCL and PPy were demonstrated to support the adhesion and proliferation of primary rabbit cardiomyocytes and the expression of cardiac-specific proteins (α-actinin, troponin-T, and connexin 43 (Cx43)) [29]. Consequently, interpenetrating networks of PPy and PCL (PPy-PCL) may offer a commercially viable, electrically conductive alternative to existing materials used for culturing cardiac tissue monolayers and for cardiac differentiation of pluripotent stem cells. PCL is a biodegradable polyester that has been FDA-approved for use clinically in drug delivery devices and as a component of biomaterials used for bone regeneration [30]. Interpenetrating networks of PPy and PCL have demonstrated physiological levels

of conductivity [15], and copolymers of PPy and PCL have been shown to enhance neurite outgrowth from rat PC12 cells [24], which, like cardiomyocytes, respond to electrical stimuli. The conductivity of the PPy-PCL creates an environment conducive to electrical stimulation, thereby mimicking conditions found in the heart. Measurements of cardiac tissue resistivity vary, but are typically around 0.5 kΩ cm [31]. Based on these properties, PPy-PCL-based materials are interesting substrates for *in vitro* studies of cardiomyocytes and are of potential clinical relevance for cardiac tissue engineering.

Conductive materials that provide an electrical environment which effectively mimics the physiological conditions in the heart may enhance electrical signaling in engineered cardiac tissues, similar to changes observed in response to exogenous electrical pacing. Cx43 is a vital gap junction protein found within the myocardium responsible for establishing electrical and chemical coupling between cardiomyocytes, and is therefore a critical feature in the formation of mature engineered heart tissue. For instance, engineered heart tissue composed of anisotropically aligned cells and well-organized Cx43 demonstrated improved twitch force relative to isotropic scaffolds [32]. Application of exogenous electrical pacing to condition engineered heart tissue has been shown to improve Cx43 density and organization [33]. Exogenous electrical pacing has also been demonstrated to influence electrical and calcium wave propagation and may also be implicated in reduced heterogeneity of engineered heart tissue. Providing a more physiological electrical environment for formation of engineered cardiac tissues may contribute to enhanced Cx43 organization, improving electrical signal propagation in the engineered myocardium.

The overall objective of this study was to determine the efficacy of the conductive PPy-PCL interpenetrating networks for use as electroactive substrates for cardiomyocyte culture. We first assessed the ability of PPy-PCL to support the adhesion and growth of cardiomyocytes and thereafter the differences between cardiomyocyte monolayers cultured on either PCL or PPy-PCL. A simple three-step fabrication method was established and the surface resistivity and hydrophobicity of the materials were characterized. Cardiomyocyte cell sheets were formed on the PPy-PCL and PCL substrates and cell adhesion, cell viability, cell size, cell morphology, and Cx43 expression and organization were quantified. Propagation of calcium transients across the cardiomyocyte cell sheets was visualized through optical mapping; calcium transient velocity, duration and frequency of spontaneous propagation were quantified. Comparing cardiomyocyte phenotype and the resulting cardiac monolayer tissue properties on the non-conductive and conductive substrates allows us to assess the usefulness of mimicking the inherent conductivity of native myocardium in an *in vitro* setting.

2 Materials and Methods

2.1 Materials

All materials were purchased from Sigma-Aldrich (St. Louis, MO) unless noted otherwise.

2.2 Fabrication of PCL-Based Films

The fabrication process for NaOH-treated PCL, as well as for PPy-PCL, is represented in Fig. 1 and detailed below.

2.2.1 PCL Film Preparation

Heat-pressed PCL films were prepared by dissolving PCL beads (20 w/v%) in dichloromethane (Acros Organics, Thermo Fisher Scientific, Waltham, MA), casting on a flat surface and allowing the solvent to evaporate overnight. The solvent-cast PCL films were flattened between 1/8" mirror-finished 316 stainless steel sheets (McMaster-Carr, Atlanta, GA) on a Wabash G30H hydraulic press (Wabash MPI, Wabash, IN) at 80˚C with 1 ton of force for five min. After cooling, films were cut out of the resulting PCL sheet using a 20 mm diameter punch.

2.2.2 NaOH Treatment of PCL

To optimize PCL surfaces for cell adhesion, PCL films were subjected to NaOH treatment [34,35]. PCL films were treated with 3 N NaOH for periods of 0, 1.5, 3, 24, or 48 h, rinsed in PBS (Lonza, Walkersville, MD) and incubated in deionized water such that all samples were subjected to aqueous conditions for a duration of 48 h. Samples were then dried using a nitrogen gun and characterized or sterilized for cellular experiments. Hereafter these films are referred to as PCL-0 (for PCL that was not exposed to NaOH), or PCL-1.5, PCL-3, PCL-24 or PCL-48 respectively, depending on the duration of exposure to NaOH.

2.2.3 Preparation of conductive PPy-PCL

To generate conductive PCL-based materials, an interpenetrating network of PPy was grown within PCL films by adaptation of a previously described polymerization technique [15,17]. Briefly, a solution of polystyrene sulfonic acid (70,000 MW, 15.98 g L^{-1}) and pyrrole (5.99 g L^{-1}, 0.084 M) was prepared in DI water and mixed well. PCL films were added and the solution was incubated at room temperature for 1 h. Subsequently,

ferric chloride (37.0 g L^{-1}, 0.228 M) was added to the mixture. This final mixture was incubated at 4º C for 24 h, at which point the PPy-PCL samples were removed, rinsed with DI water, and air-dried prior to testing.

2.3 Material Characterization

PCL and PPy-PCL material characterization included assessment of: water contact angle (WCA), electrical resistivity, material hardness and elasticity, surface chemistry, and imaging using scanning electron microscopy (SEM) as described below.

2.3.1 Water Contact Angle (WCA)

To assess the hydrophobicity of the treated films, the WCA of the PCL was measured. The WCA of the NaOH-treated PCL and the PPy-PCL was quantified using a ramé-hart contact angle goniometer and DROPimage software (ramé-hart, Succasunna, NJ). Three separately prepared samples were tested for each treatment condition.

2.3.2 Nanoindentation

To determine the elastic modulus and stiffness for PCL and PPy-PCL films, material mechanical analysis was carried out using nanoindentation. Samples were loaded into an MTS Nanoindenter XP with a Berkovich diamond tip under continuous stiffness operation. Parameters were set to include a harmonic displacement target of 2 nm and a minimum thermal drift rate of 0.05 nm/s. The samples were indented to a depth of at least 2 μm and the values of elastic modulus and hardness were continually recorded throughout displacement. Arrays of at least five indents were performed for each experimental condition to obtain a statistical response.

2.3.3 Quantification of Surface Chemistry

To confirm surface modification of PCL films via NaOH treatment or PPy synthesis, samples were evaluated using X-ray photoelectric spectroscopy (XPS). XPS analyses were performed using AXIS Ultra DLD with monochromatic Al Kα radiation as the X-ray source. Survey and high resolution spectra were recorded with a pass energy of 160 eV and 20 eV, respectively, at constant analyzer transmission energy mode. The binding energy of the system was referenced to Ag $3d_{5/2}$ at 368.3 eV.

2.3.4 Scanning Electron Microscopy (SEM)

PCL and PPy-PCL samples, both with and without cells, were imaged using SEM to observe the surface properties of the material. Briefly, samples with cells were fixed in a solution of 3% paraformaldehyde for 2 h at room temperature, followed by incubation in 2% osmium tetroxide for 2 h. Material samples were dried using serial dilutions of ethanol followed by a chemical drying step in hexamethyldisilazane for 30 min. All samples were allowed to dry completely in air prior to mounting on aluminum stubs and sputter-coating using gold (EMS 550X Auto Sputter Coating Device, Electron Microscope Services, Hatfield, PA). Gold-coated samples imaged using SEM (Zeiss EVO 50 SEM, Carl Zeiss Microscopy, Jena, Germany).

2.3.5 Surface resistivity

The surface resistivity of PCL and PPy-PCL films was determined with a Keithley 4200-SCS Parameter Analyzer (Keithley, Cleveland, OH) and the four-point probe method; this approach is optimal for flat materials such as those described herein and helps to eliminate the influence of instrumentation internal resistance on the recorded resistance. Four probes, spaced equidistantly from one another, were applied to the surface of a

sample. A known current was applied to the outer two probes while the inner two probes were used to measure the voltage on the sample surface. Ohm's Law was then used to solve for the surface resistivity of each sample. The resistivity of each sample was confirmed prior to sterilization and culture of cardiomyocytes.

2.4 Cell Culture

2.4.1 HL-1 Maintenance

HL-1 atrial myocytes, a cell line derived from adult mouse atria [36] were obtained from Dr. William Claycomb (Louisiana State University Health Sciences Center, New Orleans, LA) and cultured *in vitro* using Claycomb media supplemented with 10% fetal bovine serum (FBS), 0.1 mM norepinephrine, 2 mM L-glutamine (Lonza), and 100 μg mL^{-1} penicillin-streptomycin (Lonza). HL-1 cells were expanded and passed according to standard protocols [36] prior to use in all experiments.

2.4.2 HL-1 Cell Response to PCL-Based Films

PCL-based films were placed in a 12-well plate and sterilized by incubation in 70% ethanol (Pharmco-AAPER, Brookfield, CT) solution and exposure to UV light for 1 h. The ethanol solution was aspirated and the films were washed with PBS and dried. Films were incubated in a solution of (0.02 w/v%) gelatin-(5×10^{-6} g/mL) fibronectin for at least 1 hr at 37°C to better promote cellular attachment to each surface. HL-1 cells were then seeded on the film surface at a density of 1,300 cells mm^{-2}. Cell culture medium was exchanged every 24 h.

2.4.3 LIVE/DEAD Assay

A LIVE/DEAD assay (Invitrogen, Life Technologies, Grand Island, NY) was used to quantify the viability of the cells on each surface 6 days post-seeding. Briefly, cells were incubated at room temperature for 20 min in a solution of calcein-AM (2 mM) and ethidium homodimer-1 (4 mM) in PBS. The solution was aspirated, cells were rinsed with PBS and PBS was added prior to imaging using fluorescence microscopy. Live cells were characterized by intracellular esterase activity which cleaves the calcein-AM to form fluorescent green calcein. Dead cells were reported by the presence of red fluorescent ethidium homodimer-1, which enters through the ruptured cell membrane and binds to nucleic acids in the nucleus. HL-1 cells were imaged using a Nikon Eclipse-Ti inverted fluorescence microscope (Nikon Instruments Inc., Melville, NY) and an Andor Luca S camera (Andor, Belfast, UK). Viability of cells on three separate films per condition was quantified using the cell counter plugin for ImageJ (NIH).

2.4.4 Analysis of Adherent Cell Density and Cellular Size

HL-1 cells were fluorescently labeled to quantify the density of adherent cells and the average surface area of individual cells on the films 10 days post-seeding; PPy-PCL is opaque, meaning cells are unable to be visualized under bright field microscopy. After fixation with a 50:50 acetone (Macron Chemicals, Center Valley, PA)/ethanol solution, HL-1 cells were permeabilized using a 0.1% Triton X-100 solution in PBS, washed with PBS and incubated in a 2.5 (v/v)% solution of Alexa Fluor® 488 Phalloidin (Invitrogen) in PBS for 20 min at room temperature. HL-1 cells were then counterstained with DAPI (Invitrogen, 1:36000 from stock) and samples were dehydrated and mounted in Prolong

Gold (Life Technologies). Images of HL-1 cells on the film surfaces were acquired using a Nikon confocal microscope (Nikon Instruments Inc).

Morphological analysis was performed using ImageJ by outlining five to ten individual cells in each of 3-4 images per film. The average cell size was determined for each film and used for statistical analysis (n = 3 to 4). Analysis of adherent cell density was carried out using ImageJ. All nuclei within each image were counted and then divided by the unit area of each image to find the cell density (n = 3 films per condition).

2.4.5 Immunocytochemistry

Qualitative assessment of the expression and localization of the gap junction protein Cx43 in HL-1 cells adhered on PCL-based films was achieved with immunocytochemistry at 10 days post-seeding. HL-1 cells were fixed using a 50:50 acetone/ethanol solution for 10 min at -20°C. The HL-1 cells were then permeabilized using a 0.1% Triton X-100 solution in PBS and blocked using a 3% solution of FBS in PBS. Following blocking, samples were incubated in rabbit-anti-Cx43 antibody (diluted 1:200 in blocking buffer) for 2 h at room temperature. After rinsing, the secondary antibody Alexa Fluor 488 goat-anti-rabbit (Invitrogen, diluted 1:200 in blocking buffer) was added and incubated for 2 h at room temperature. To quantify the number of cells per area, HL-1 cells were counterstained with DAPI (Invitrogen, 1:36000 from stock) and samples were dehydrated and mounted in Prolong Gold (Life Technologies). Images of HL-1 cells on the film surfaces were acquired using a Nikon confocal microscope.

To eliminate the potential for bias, this analysis was carried out by a blinded observer and automated image re-naming and order randomization was employed.

Image analysis was performed using the cell counter plugin for ImageJ. For each image, the number of cells with positive peripheral staining for Cx43 was counted. Then the number of remaining cells, i.e. those without peripheral Cx43 expression, was counted. For each individual film, three images were quantified; the percentage of cells with peripheral Cx43 expression was then calculated and the average value used for statistical analysis (n = 4 films per condition).

2.4.6 Analysis of Connexin-43 expression by qPCR

To quantify the effect of film type on gap junction gene expression, HL-1 cells were evaluated using qPCR. Cells were seeded on PCL and PPy-PCL films. After 7 days of culture, RNA was isolated using a MicroElute Total RNA Kit (Omega Bio-Tek, Norcross, GA). Cells were physically disrupted in lysis buffer prior to homogenization (Homogenizer Spin Columns, Omega Bio-Tek). The solution containing nucleic acid was transferred to collection columns and treated according to the manufacturer's instructions; the concentration of diluted RNA was determined via spectroscopy (NanoDrop, Thermo Fisher) prior to generation of a cDNA library (qScript cDNA SuperMix, Quanta Biosciences, Gaithersburg, MD). Quantification of Cx43 (Forward: GGTGGACTGCTTCCTCTCAC, Reverse: ATCGCTTCTTCCCTTCACG) was carried out via qPCR using a SYBR green amplification kit (PerfeCTa SYBR Green SuperMix, Quanta Biosciences) in an iCycler iQ5 (Bio-Rad, Hercules, CA). PCR conditions were 95°C for 5 min, followed by 40 cycles of amplification at 95℃ for 10s, and 55℃ for 30s. GAPDH (Forward: GAAGGGCATCTTGGGCTAC, Reverse: GCCTCTCTTGCTCAGTGTCC) was used as a housekeeping gene for all trials.

Relative quantification of Cx43 was determined using the ΔΔCt method; a total of n = 5 samples were analyzed from each treatment group.

2.5 Optical Mapping

Optical mapping was performed on confluent HL-1 cell monolayers 10 to 12 days post-seeding on PCL-based films. Tyrode's solution was prepared as previously described [37]. Rhod 2 (Invitrogen) calcium indicator was prepared by first creating a 1 mM stock solution through addition of anhydrous dimethylsulfoxide followed by sonication at 50º C for 30 min. To prepare the working calcium indicator solution, 5 µl of 1 mM Rhod-2 stock solution and 5 µl of Pluronic F-127 were added per 1 ml of Tyrode's solution. Following removal of cell culture media, 1 ml of calcium indicator solution was added to each well and allowed to incubate at room temperature for 20 min. After the incubation period, the wells were rinsed three times with Tyrode's solution prior to imaging.

Recordings of calcium wave propagation across the HL-1 cell sheets were acquired using an iXon Ultra DU897 EMCCD camera (Andor) coupled to an Optomask Adjustable Field Mask (Andor), which was utilized to enhance frame rate in cropped sensor mode, and Nikon Elements software. Propagating calcium waves were recorded in Tyrode's solution at room temperature (22 °C) in the absence of exogenous stimulation (i.e., spontaneous wave propagation). For each film, a minimum of three recordings were taken with an average length of 10 sec at a rate of approximately 500 frames per second. Data was analyzed using a modified version of a previously established MATLAB (MathWorks, Natick, MA) script [37–39]. Using this script, calcium transient velocity, calcium transient duration (time to 50% recovery), and calcium wave frequency were quantified for each condition (n = 3 to 4).

2.6 Image and Statistical Analysis

As detailed above, quantification of cell viability, cell density, cell size, and Cx43 location was performed using ImageJ. Non-treated PCL was used as an initial control for viability and cell density experiments comparing the effect of PCL NaOH pre-treatment duration; NaOH-treated PCL films shown to support comparable cellular viability and density to PPy-PCL were used as a control for gap junction and calcium handling experiments. After quantification, the data was imported into MATLAB (MathWorks) where statistical analysis was performed and plots were generated. Images from three to four films were quantified for each treatment condition and averaged (n = 3 to 4). For statistical analysis involving two treatment conditions, a two-tailed, two-sample t-test was performed assuming equal variances. For statistical analysis involving more than two conditions, one-way ANOVA was performed and significant differences between groups were identified using the Tukey-Kramer method. A p-value of less than 0.05 was considered statistically significant. All error bars represent standard deviations.

3 Results

3.1 NaOH Treatment of PCL Decreases WCA of Films

Based on previous studies, treatment with NaOH was anticipated to affect the surface chemistry of PCL by hydrolysis of the ester group, yielding materials displaying carboxylic acid and hydroxyl groups on their surfaces [30,40]. NaOH treatment of PCL resulted in an exposure time-dependent decrease in hydrophobicity, as demonstrated by the change in WCA measurements (Fig. 2A). PCL films that were treated with water

alone (PCL-0) had an average contact angle of 78 ± 3°; values for WCA decreased incrementally as the time of NaOH treatment was increased (denoted by PCL-hours of NaOH treatment: PCL-1.5: 64 ± 5°, PCL-3: 58 ± 8°, PCL-24: 35 ± 5°, PCL-48: 24 ± 5°). PPy-PCL showed super-hydrophilic characteristics, consistent with previous studies [41]; the water droplet spread across the surface of the material upon contact giving an effective WCA of 0°.

3.2 PCL and PPy-PCL Material Properties

XPS analysis confirmed the surface modification of NaOH-treated PCL and polymerization of PPy-PCL (Fig. 2B, Supplemental Figure 1). Treatment of the PCL with NaOH over 24 h period resulted in a shift of the carbon 1s peak, which is indicative of formation of carbonyl and alcohol groups (via hydrolysis of the ester bond). Successful polymerization of PPy within the PCL backbone was confirmed through identification of a nitrogen 1s peak (at ~400 eV) which was not present in the PCL films.

Because material stiffness is known to affect phenotype of developing cardiomyocytes, nanoindentation was used to evaluate the mechanical properties of PCL after NaOH treatment and PPy polymerization. Both the PCL-24 and the PPy-PCL films exhibited similar elastic modulus (1.07 ± 0.05 GPa, 0.93 ± 0.19 GPa) and hardness (0.073 ± 0.008 GPa, 0.071 ± 0.02 GPa)(Supplemental Figure 2).

3.3 Conductive PPy-PCL Films Have Biomimetic Resistivity

Generation of an interpenetrating network of PPy within the non-conductive PCL films produced materials with electrical resistivities that closely mimicked the resistivity of native cardiac muscle. PCL and NaOH-treated films had infinite resistivity indicating that they did not conduct electricity, while the interpenetrating networks of PPy-PCL had an

average resistivity of 1.0 ± 0.4 kΩ cm (Fig. 2C). In comparison, native cardiac muscle has approximate resistivity of 0.5 kΩ cm [31], which is a factor of two lower than the PPy-PCL, and adipose tissue has approximate resistivity of 2.6 kΩ cm [31], which is a factor of two higher than the PPy-PCL films (Supplemental Table 1). We therefore conclude that generation of an interpenetrating network of PPy within PCL generates films with resistivities that closely mimic that of native cardiac muscle.

3.4 High Cell Viability Observed on all Films

HL-1 cells adhered and remained viable when seeded on the surface of PCL-based films. PPy-PCL and PCL-24 surfaces were visualized both with and without cells using SEM imaging (Fig. 3). PCL-based films were mostly smooth with the presence of nano-scale cracks with respective thickness of 100-200 nm. The presence of an interpenetrating network of PPy increased the surface roughness somewhat (Fig. 3) with the thickness of the films increasing by less than 500 nm after generation of the PPy network. PCL-24 supported the attachment of HL-1 cells with a combination of elongated and rounded morphologies; in contrast, HL-1 cells on PPy-PCL films appeared more elongated with fewer rounded cells adherent to the underlying HL-1 cell monolayer. The surface chemistry of the films was found to have no effect on the viability of the cells (Fig. 4). Based on quantification following LIVE/DEAD staining, cell viability was found to be approximately 90% on all films.

3.5 NaOH Treatment of PCL Increases Cell Adhesion

Achieving high densities of adherent cardiomyocytes on film materials is necessary in creating engineered cardiac tissues and can be challenging depending on the material. As NaOH treatment time of the PCL films was increased and hydrophobicity decreased,

there was a significant increase in the number of cells on the film surface per unit area (PCL-0: 1,568 ± 126 cells mm^{-2}, PCL-24: 2,880 ± 439 cells mm^{-2}, PCL-48: 3,623 ± 456 cells mm^{-2}, Fig. 5A). Furthermore, increasing the NaOH treatment times led to an overall decrease in the area of individual adhered HL-1 cells (PCL-0: 419 ± 120 μm^2, PCL-24: 270 ± 48 μm^2, PCL-48: 270 ± 60 μm^2, Fig. 5B). HL-1 adhesion and cell size on PPy-PCL was most similar to PCL-24, with PPy-PCL films having 2,434 ± 166 cells mm^{-2} and the average area of HL-1 cells on PPy-PCL being 279 ± 15 μm^2; PCL-24 was selected, therefore, for comparison with PPy-PCL in functional and gene expression experiments.

3.6 Conductive PPy-PCL Films Encourage Peripheral Localization of Cx43

Cardiomyocyte growth on conductive PPy-PCL films resulted in a change in intracellular location of the gap junction Cx43, but did not significantly alter Cx43 gene expression. In HL-1 cells grown on the electrically-conductive PPy-PCL, the gap junction protein Cx43 was frequently observed to be located around the cell periphery, whereas in HL-1 cells grown on PCL this was less frequently the case (Fig. 6). Based on analysis of immunofluorescence imaging, when cultured on PPy-PCL films, 60 ± 4% of HL-1 cells expressed peripheral Cx43, whereas 47 ± 6% of HL-1 cells expressed peripheral Cx43 when cultures on PCL-24 films ($p<0.05$, Fig. 6B). No significant difference in Cx43 gene expression was observed between HL-1 cells cultured on PPy-PCL versus PCL-24 films based on qPCR analysis (Fig. 6C).

3.7 Calcium Transient Wave Propagation Improves on Conductive PPy-PCL Films

Growth of cardiac cell monolayers on conductive PPy-PCL films positively influenced functional properties. HL-1 cell monolayers grown on PPy-PCL demonstrated an overall

increase in the average velocity of calcium transient wave propagation in comparison to cells grown on PCL-24 (Fig. 7A, B). Following 10-12 days of culture on the films, the average calcium transient wave velocity across HL-1 cell monolayers on PPy-PCL (1612 ± 143 μm/s, Supplemental Video 1) was significantly higher than that across HL-1 cell monolayers on PCL-24 (1,129 ± 247 μm/s, $p<0.05$, Supplemental Video 2). Moreover, HL-1 cells cultured on PPy-PCL also demonstrated improved calcium transient recovery relative to cells cultured on PCL-24; calcium transient durations, specifically the time to reach 50% recovery, were significantly shorter for HL-1 cells on PPy-PCL (910 ± 60 ms) relative to HL-1 cells on PCL-24 (1,130 ± 20 ms, $p<0.01$, Fig. 7C). Additionally, the frequency of spontaneous calcium transient propagation was higher for HL-1 cell monolayers on PPy-PCL as compared to on PCL-24 (0.48 ± 0.03 Hz and 0.31 ± 0.02 Hz, respectively, $p<0.01$ Fig. 7D).

4 Discussion

The overall goal of this study was to determine the efficacy of conductive PPy-PCL interpenetrating networks for use as electroactive substrates for cardiomyocyte culture. To accomplish this goal, HL-1, a murine cardiomyocyte cell line, was employed in this first study examining the potential of PPy-PCL films for use in cardiac tissue engineering applications. Unlike primary cardiomyocytes, such as neonatal rat cardiomyocytes, HL-1 cells are capable of being expanded *in vitro*. HL-1 cells have previously been used in studies aiming to increase homogeneity of cardiomyocyte contraction [42,43]. Additionally, HL-1 cells, in contrast to other contractile cell types, are often limited to spontaneous contraction among isolated, independent clusters of cells. This provided a greater opportunity to evaluate changes in response to culture on

the electrically-conductive PPy-PCL films, including changes in gene expression and functional properties, i.e. calcium transient propagation and duration, related to calcium and electrical signal handling. Future examination of other types of cardiomyocytes cultured on PPy-PCL will provide additional insights into the usefulness of this material as a conductive cardiac tissue engineering platform.

The resistivity of PPy-PCL films used in this study was 1.0 ± 0.4 kΩ cm, which is similar to the reported resistivity of cardiac tissue (approximately 0.5 kΩ cm) [31]. A variety of methods for preparing PPy-PCL films were tested including employing only solvent casting or heat pressing to prepare the PCL; however, the most consistent method was found to be the one utilizing both solvent casting and a heat press as described above. This method resulted in the creation of films with consistent topography and shape, as well as conductive substrates with reproducible resistivities. Furthermore, the PPy-PCL films exhibit elastic moduli and hardness similar to NaOH-treated PCL films (Supplemental Figure 1), as well as resistivities which are comparable to those of other conductive polymer systems that have been synthesized for use in other tissue engineering platforms (Supplemental Table 1).

Changes in PCL surface chemistry as a result of NaOH treatment and PPy polymerization may be important factors contributing to the significant increase in numbers of cells (and concomitant decrease in the average size of cells) on each material relative to the PCL-0. NaOH treatment of PCL was found to result in increased cell adhesion to the PCL films, consistent with studies using a multitude of different cell sources [34,35,44,45]. NaOH treatment causes hydrolysis of the ester bonds within the PCL films, thereby exposing carboxylic acid and hydroxyl groups on the surface which

renders the surfaces more hydrophilic [46]. Under physiologically-mimetic conditions (i.e. PBS, pH 7.4), the carboxyl groups exposed after hydrolysis of the PCL will be deprotonated and therefore negatively charged, as indeed would the sulfonate groups displayed on the polystyrene sulfonate dopant for PPy. Although all films were coated with a gelatin-fibronectin solution prior to HL-1 seeding, differing electrostatic interactions could result in differences in the relative amount and distribution of proteins adsorbed, particularly following one week in culture. Cell adhesion, which is typically mediated by the cell-matrix interactions, is heavily dependent on the distribution of different proteins that adsorb on the surface of a biomaterial [19]. While surface chemistry strongly influences cellular attachment, modifications to surface structure or topography via chemical polymerization or hydrolization may also contribute to greater adherent cell numbers observed on PCL-24 and PPy-PCL films.

Inability to form a syncytium through poor re-formation of gap junctions is a serious impediment to creation of engineered cardiac tissue from dissociated cardiomyocytes. One potential reason for this lack of cellular interconnectivity is the inability of the cells to sense each other electrically on the substrate. For instance, junctional conductance is lower in developing neonatal rat cardiomyocytes compared to adult rat cardiomyocytes, which may result from decreased expression or poor organization of Cx43 [47]. Cell adhesion to conductive polymers is typically mediated via both integrin and non-integrin interactions with surface-adsorbed proteins [19]. Although the underlying material is likely separated from the adherent cells by a layer of adsorbed and secreted proteins, conductive polymers influence the electrical charge within a local cellular environment, which in turn can result in changes in cell phenotype.

Another issue that hinders the creation of engineered cardiac tissue is the inability to induce synchronous contraction of cardiac cells during *in vitro* culture. Induction of synchronous contraction of cardiac cells used to create engineered cardiac tissue, either through electrical pacing or by mechanical stimulation, has led to reduced heterogeneity [6] and improved maturation [4], including maintenance of differentiated cell phenotypes [4], elongation and alignment of cells [4], and increased gap junction formation among cells [4,7].

Some studies employing conductive substrates for cardiomyocyte culture have demonstrated increased Cx43 gene expression, including neonatal rat cardiomyocytes cultured in hydrogels containing gold nanoparticles [13] or gold nanowires [5], whereas others have seen functional improvements in engineered cardiac tissues without changes in Cx43 expression [48]. In this study, analysis of Cx43 gene expression via qPCR did not reveal significant differences between HL-1 cells cultured on PPy-PCL versus on PCL. This finding suggests that the functional differences observed between cells cultured on each film may be due to improved organization and formation of gap junctions in HL-1 cells on the PPy-PCL, which has been demonstrated through fluorescence image analysis of HL-1 cells stained for Cx43 (Fig. 6). Other studies support this observed correlation between changes in cellular function and altered localization of Cx43. For instance, exogenous electrical pacing, which has shown improvement in functional maturation of cardiomyocytes, has resulted in increased presence of Cx43 structures within engineered rat myocardium [49] as well as increased Cx43 along the membranes of individual neonatal rat cardiomyocytes relative to non-stimulated myocardium [50]. Similarly, cyclical mechanical stretching of

cardiomyocytes has been shown to enhance overall Cx43 presence [51], which can include increased gap junction expression at the poles of each cell [52]. While the exact mechanism for cellular communication on conductive surfaces hasn't been elucidated, It has been postulated by others that enhanced localization of connexins and formation of gap junctions is a consequence of increased spontaneous depolarization which may be a result of cells detecting electrical signals from the local environment [13]. Enhancing Cx43 expression and organization is also of particular importance for differentiation of stem cell-derived cardiomyocytes from pluripotent stem cells and results suggest employing a conductive polymer film may be an effective tool to aid in this process.

Electrophysiological maturation, as evidenced through changes in ion channel currents, electrical propagation, and calcium handling, is a fundamental characteristic for assessing the efficacy of cardiac tissue models. In this study, optical mapping of calcium waves demonstrated increased calcium transient velocities and decreased calcium transient durations among HL-1 cells cultured on PPy-PCL in comparison to cells on PCL alone (Fig. 7). These results are consistent with observations of maturation in neonatal cardiomyocytes cultured *in vitro* for extended periods, resulting in reduction in action potential duration [53]. While fundamental knowledge elucidating the mechanism by which conductive materials improve electrophysiology is incomplete, parallels may be drawn between systems which subject *in vitro* cardiomyocytes to exogenous electrical stimulation or mechanical stretch. In such cases, improvements in conduction velocity and action potential duration are associated with transient activity of specific ion channel currents, including I_{t0}, I_{NaCa}, and $I_{Ca,L}$ [7,54]. However, improvement in conduction velocity, or calcium transient wave propagation, is also mediated by

changes in cell-cell coupling which may be influenced by exogenous electrical or mechanical pacing. This idea is consistent with the increased peripheral Cx43 presence observed among HL-1 cells cultured on PPy-PCL films (Fig. 6). Together, these data demonstrate the efficacy of conductive substrates to aid electrophysiological maturation of cells cultured on their surfaces, which may be of clinical relevance for the future design and manufacture of engineered heart tissues.

5 Conclusions

This study demonstrated that conductive PPy-PCL films effectively support cardiomyocyte culture and could be useful in enhancing the functional properties and maturation of cardiomyocyte cell sheets. Treatment of PCL films with NaOH resulted in decreased surface hydrophobicity, increased cardiomyocyte attachment and decreased cell size. PPy-PCL substrates promoted cellular attachment at comparable densities and cell areas relative to NaOH-treated PCL films and without a change in cellular viability. Cx43 gene expression was similar for HL-1 cardiomyocytes grown on PPy-PCL and PCL films. However, localization of Cx43 protein differed; when HL-1 cells were cultured on PPy-PCL, Cx43 was more frequently observed to be along the cell periphery. Additionally, HL-1 cells grown on conductive PPy-PCL films supported significantly faster calcium transient velocities and significantly lower calcium transient durations relative to HL-1 cells grown on control PCL films. These data encourage the use of conductive materials as substrates for cardiac tissue engineering; providing enhanced substrate conductivity, similar to that of native cardiac tissue, may constitute an important component in achieving proper electrophysiological development of

cardiac cell types, and may have clinical relevance for cardiac tissue engineering applications.

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Disclosure Statement

The authors have nothing to disclose.

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Figure Captions

Fig. 1: Fabrication of NaOH-treated PCL and PPy-PCL films. PCL was initially prepared using solvent casting and flattened with a melt press. Punched films were either treated in a solution of NaOH or rendered conductive with an interpenetrating network of PPy.

Fig. 2: Modifying PCL-based films via NaOH treatment and PPy polymerization altered their hydrophobicity and surface chemistry. (A) Water contact angle of PCL scaffolds decreased with increased exposure to NaOH. PPy-PCL was shown to have an effective contact angle of 0°. Mean values not sharing the same letter are significantly different ($p < 0.05$, $n = 3$ samples per condition). All contact angle measurements shown are significantly different from PPy-PCL. (B) Surface chemistries of PCL-based films were changed after exposure to NaOH or formation of an interpenetrating network of PPy. XPS analysis reveals shift in carbon 1s peak after NaOH treatment. Formation of a PPy network was confirmed by the presence of a nitrogen 1s peak. (C) PCL rendered conductive with PPy resulted in significantly lower resistivity. Electrical resistivity of PCL, NaOH-treated PCL and PPy-PCL films as determined with a four-point probe; PPy-PCL resistivity was shown to be on the same order of magnitude as body tissues including the cardiac muscle, skeletal muscle, and adipose tissue.

Fig. 3: Scanning electron microscopy images showing the surfaces of PCL-based films: Top left) flat PCL-24 films; Top right) PCL-24 films with HL-1 cells; Bottom left) PPy-PCL films; Bottom right) PPy-PCL films with HL-1 cells. Scale bar = 10 μm. High

magnification images (10,000x) show changes in surface roughness in response to PPy polymerization. Insert scale bar = 1 μm.

Fig. 4: Viability of HL-1 cells remained high on all tested films. (A) Representative images of HL-1 cells on PCL-24 and PPy-PCL; live cells appear green and dead cells appear red. Scale bar = 40 μm. (B) Percentage of viable HL-1 cells was quantified following culture on each type of film (n = 3 films per condition).

Fig. 5: HL-1 cells adhered and spread on PCL-based films. (A) The density of HL-1 cells on PCL-based films increased with longer NaOH pretreatment (i.e., hydrophilicity); HL-1 cell density on PPy-PCL was comparable to PCL-1.5 to PCL-24. Means not sharing the same letter are significantly different ($p < 0.05$, n = 3 films per condition). (B) HL-1 cell size trended lower with increasing time of NaOH pretreatment (i.e., increased hydrophilicity), although no statistically significant difference was observed (n = 3 films per condition). (C) Representative images of HL-1 cells on PCL-24 and PPy-PCL stained with DAPI (nuclear, blue) and Alexa Fluor® 488 Phalloidin (cytoskeletal actin, red). Scale bar = 40 μm.

Fig. 6: HL-1 cells cultured on PCL and PPy-PCL films showed changes in Cx43 localization. (A) Representative images of HL-1 cells on PCL-24 and PPy-PCL immunostained for Cx43. Scale bar = 40 μm. (B) Cx43 localization was quantified using representative images of HL-1 cells on PCL-24 and PPy-PCL. Number of HL-1 cells with peripheral expression of Cx43 was significantly higher on on the conductive

PPy-PCL versus PCL-24 ($p < 0.05$, $n = 4$ films per condition). (C) Gene expression of Cx43 was quantified for HL-1 cells cultured on PCL-24 and PPy-PCL using qPCR and trended towards increased expression on the conductive substrates ($n = 6$ films per condition).

Fig. 7: Increased calcium transient wave velocity, decreased calcium transient duration observed among HL-1 cells cultured on PPy-PCL. A) Representative maps of calcium wave propagation across spontaneously contracting HL-1 cell monolayers cultured on either PCL-24 or PPy-PCL. Scale bar = 100 μm. B) Average velocity of calcium wave propagation across HL-1 cell monolayers on PPy-PCL was significantly greater than for HL-1 cell monolayers cultured on PCL-24 ($p < 0.05$, $n = 4$ films per condition). C) Average calcium transient duration at 50% repolarization for HL-1 cells on PPy-PCL was significantly less than for HL-1 cells cultured on PCL-24 ($p < 0.05$, $n = 4$ films per condition). (D) Representative single pixel traces of calcium transients for HL-1 cells on PCL-24 and PPy-PCL.

Supplemental Table 1: Resistivities of PCL films, body tissues, and comparable conductive polymer-based tissue engineering platforms.

Supplemental Figure 1: XPS graphs showing carbon 1s peaks on films of A) PCL-0, B) PCL-24, and C) PPy-PCL. D) Nitrogen 1s peaks were present on PPy-PCL films. No nitrogen peaks were observed on PCL-0 or PCL-24.

Supplemental Figure 2: Elastic modulus and hardness vs. displacement curves for PCL-24 (A and C) and PPy-PCL (B and D) films.

Supplemental Video 1: Representative video of calcium wave propagation across spontaneously contracting HL-1 cells on PCL-24. Time bar represents 1000 ms.

Supplemental Video 2: Representative video of calcium wave propagation across spontaneously contracting HL-1 cells on PPy-PCL. Time bar represents 1000 ms.

Supplemental References

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