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Electrically Stimulated Adipose Stem Cells on Polypyrrole-Coated Scaffolds for Smooth Muscle Tissue Engineering

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Abstract—We investigated the use of polypyrrole (PPy)-coated polymer scaffolds and electrical stimulation (ES) to differentiate adipose stem cells (ASCs) towards smooth muscle cells (SMCs). Since tissue engineering lacks robust and reusable 3D ES devices we developed a device that can deliver ES in a reliable, repeatable, and cost-efficient way in a 3D environment. Long pulse (1 ms) or short pulse (0.25 ms) biphasic electric current at a frequency of 10 Hz was applied to ASCs to study the effects of ES on ASC viability and differentiation towards SMCs on the PPy-coated scaffolds. PPy-coated scaffolds promoted proliferation and induced stronger calponin, myosin heavy chain (MHC) and smooth muscle actin (SMA) expression in ASCs compared to uncoated scaffolds. ES with 1 ms pulse width increased the number of viable cells by day 7 compared to controls and remained at similar levels to controls by day 14, whereas shorter pulses significantly decreased viability compared to the other groups. Both ES protocols supported smooth muscle expression markers. Our results indicate that electrical stimulation on PPy-coated scaffolds applied through the novel 3D ES device is a valid approach for vascular smooth muscle tissue engineering.

Keywords—Mesenchymal stem cells, Poly (trimethylene carbonate), Conductive polymers, Physical stimulation, Vascular tissue engineering.

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ABBREVIATIONS

ALP	Alkaline phosphatase
ASC	Adipose stem cell
CPE	Constant phase element capacitance
ES	Electrical stimulation
μCT	Micro-computed tomography
MHC	Myosin heavy chain
PPy	Polypyrrole
PTMC	Poly (trimethylene carbonate)
SEM	Scanning electron microscope
SMA	Smooth muscle actin
SMC	Smooth muscle cell
TGF-β	Transforming growth factor-β
Ti/TIN	Titanium nitride coated titanium

INTRODUCTION

Vascular tissue engineering using adult stem cells and biomaterials has raised high expectations to meet the increasing need for vascular graft substitutes. Adipose stem cells (ASCs), also known as adipose-derived stem cells, have shown potential in vascular tissue engineering as they possess differentiation ability towards vascular smooth muscle cells (SMCs), and can be harvested in large numbers with minimal risk for the patient.[9,20] To induce differentiation of

ASCs towards SMCs, transforming growth factor-β (TGF-β) has been identified as one of the most important factors.[20] However, TGF-β is involved in a great diversity of cell-specific gene responses[26] and therefore efficient and specific biochemical induction by solely using different combinations of growth factors and biomolecules remains a major challenge.

Electrical stimulation (ES) has been shown to direct the contractile phenotype of SMCs.[24] Therefore, application of ES *via* conductive 3D scaffolds in combination with growth factors could provide a valid approach to induce ASC differentiation towards SMC lineages. Although, ASCs have been shown to respond to various ES *in vitro*,[2,16,21] the effect of ES on differentiation of ASCs towards SMCs has not so far been studied.

ES in 3D configurations provides a meaningful environment for studying cell response as it better resembles the *in vivo* environment than standard 2D configurations where cell behavior can be drastically different from that *in vivo*.[12,25] Currently available 3D ES systems are usually built in single petri dishes which limits the number of specimens that can be stimulated in a single experiment.[32] Therefore, a robust, sterilizable ES device that can simultaneously stimulate several samples in parallel electrical circuits and apply a range of electrical parameters is needed.

Polypyrrole (PPy) is an interesting electroactive polymer for muscle tissue engineering applications due to its good compatibility with a range of cell types.[2,11,17,22,33] PPy can be electrochemically or chemically polymerized, the latter method enabling coating of insulating scaffold matrices.[11,22] SMC behaviour has been tested on several different electrochemically-doped PPy surfaces including hyaluronic acid (HA), heparin, paratoluene sulphonate, and nitrate as dopants,[24,31] with HA-doped PPy showing promise for supporting SMC attachment and spreading.[24] HA-doped PPy was also reported to have a crucial role in regulation of angiogenesis and hence SMC activity.[27] Extensive characterization of electrochemically polymerized PPy films and their cell responses has been performed by Gilmore *et al.*[8] The study showed that differently doped PPy films supported C2C12 and primary mouse myoblast adhesion, proliferation and differentiation to varying degrees depending on the physical properties of the films.[8] In addition, in our previous work we have demonstrated that human ASC proliferation and attachment can be induced by PPy-coating of polylactide (PLA) scaffolds.[22] However the potential of PPy to support ASC differentiation towards SMCs has not been reported yet.

Here, we first studied whether PPy coatings on porous poly (trimethylene carbonate) (PTMC) scaffolds could enhance ASC growth and differentiation towards SMC tissue. PTMC is a synthetic, *in vivo* surface eroding elastomer previously reported to be a suitable scaffold material e.g. for vascular tissue engineering.[28,29] However, as it is an insulating polymer, we hypothesized that coating it with electroactive PPy could better facilitate delivery of ES to the cells seeded within the structures. Next, the effect of biphasic electric current on ASC growth and differentiation towards smooth muscle in PPy-coated PTMC scaffolds was assessed using our novel in-house-built ES device. The novelty of this low electrical impedance ES system arises from the use of sterilizable titanium nitride (TIN) coated titanium (Ti) electrodes and a mechanically rigid and robust design, which allows repeatable ES of 24 specimens simultaneously with up to 4 different electrical parameters.

MATERIALS AND METHODS

Scaffold Fabrication by Salt Leaching

High molecular weight PTMC ($M_w = 600\,000$ g/mol) was obtained from Medisse (The Netherlands). Manufacturing of PTMC scaffolds was based on the method used by Song *et al.*[29] PTMC was dissolved in chloroform (Merck, Germany, AR) to create a 2% wt/v solution. NaCl (Sigma-Aldrich, USA) was sieved to particle sizes of 38–250 μm using stainless steel sieves. NaCl particles were added to the PTMC solution and stirred overnight to obtain a homogenous dispersion. The NaCl/PTMC ratio was 2.3/1 g. The PTMC/NaCl dispersion was precipitated in a non-solvent (70% Ethanol, EtOH, Merck, AR), collected and left to dry overnight in the fume hood. The precipitated PTMC/NaCl was cut into $5 \times 5 \times 5$ mm^3 pieces and melt-pressed (Fontijne laboratory press THB008, The Netherlands) at 160 °C for a total of 6 min. First a force of 30 kN was applied for 4 min, then a force of 200 kN for 2 min. The membranes were stored in poly(ethylene)/poly(amide) bags, vacuum-sealed, and exposed to 25 kGy of gamma irradiation from a ^{60}Co source (Isotron, The Netherlands) for cross-linking.

Scaffolds were cut into circular shapes with diameters of 10 mm and thickness of 1 mm. The NaCl particles were leached out of the membranes by soaking in deionized water for 5 days at room temperature. The water was changed five times during the leaching process. After the salt leaching, the scaffolds were chemically coated with PPy by oxidation of pyrrole with iron (III) chloride hexahydrate ($FeCl_3$; Sigma-Aldrich) in the presence of HA from *Streptococcus equi* (Sigma-Aldrich). Polymerizations were carried out for 10 min at room temperature resulting in uniform, conductive PPy coatings (Fig. 1). The PPy coated scaffolds were rinsed with deionized water to

remove unreacted reagents and dried in air at room temperature.

Scaffold characterization

Micro-computed tomography (μCT) was conducted with a General Electric Explore Locus SP at a resolution of 15.8 μm on PPy-coated and uncoated PTMC scaffold using 3 scaffolds from each group. 3D maps were generated by GE MicroView software. Poresize, poresize distribution and porosity were calculated with Mathworks Matlab 2008 (USA).

Coated and uncoated scaffolds were imaged by scanning electron microscopy (SEM; JSM – 6360 LV, JEOL, Japan) using a low (3 kV) acceleration voltage to prevent sample damage and induce contrast between electrically conductive and insulating areas. PTMC scaffolds were coated with a 20 nm sputter-coated gold layer (SCD 050, Balzers AG, Liechtenstein) before imaging.

Through-plane DC conductivity of the dry scaffolds (surface area 1.1 cm^2, thickness 1.2 mm) was measured using a HP3442A DC micro-ohm meter (Hewlett-Packard, USA) in 4-wire configuration. The scaffolds were placed between two circular 1.1 cm^2 copper electrodes and subjected to 5 N/cm^2 compression which was optimal for obtaining good electrical contact with the samples, minimum deformation and stable measurement. The reading of the ohm meter was registered 15 s after applying the measurement current. The conductivity measurements were repeated for six parallel samples.

Electrode Characterization

Impedance of Ti/TiN electrodes was measured at the frequency range of 1 Hz to 1 kHz in phosphate-buffered saline (PBS) using a HP35670A spectrum analyser. Two electrodes (Fig. 2a) with geometric surface area of 1 cm^2 and electrode spacing of 1 mm were immersed in PBS. Sinusoidal test voltage of 10 mV (peak-to-peak) was applied to the electrodes and the current was measured across a 100 Ohm resistor. The impedance data of the single cell unit was fitted to the constant phase element capacitance (CPE) model, described in detail in supplementary data 1. A similar circuit model has been earlier utilized by Tandon *et al.* in the characterization of carbon rod stimulation electrodes.[32] Data analysis of the impedance spectra was done using the curve fitting tool provided in Origin Pro 8.5.1 software (Origin lab Corporation, USA).

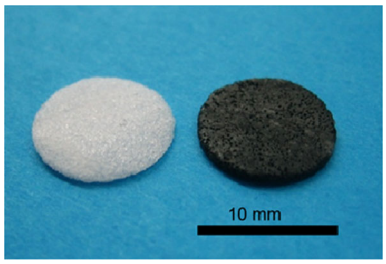

FIGURE 1. Uncoated (left) and PPy-coated (right) PTMC scaffolds used in the study.

Isolation and Culture of Adipose Stem Cells

Adipose tissue was obtained from tissue harvests from surgical procedures on four female donors with an average age of 52 ± 13 years. The operations were conducted in the Department of Plastic Surgery, Tampere University Hospital. The tissue harvesting and the use of ASCs were conducted in accordance with the Ethics Committee of the Pirkanmaa Hospital District (R03058). The ASC isolation method was presented previously by Haimi *et al.*[10] In brief, the samples from adipose tissue were digested with collagenase type I (1.5 mg/ml; Invitrogen, USA), centrifuged and filtered. Cells were expanded in maintenance medium consisting of Dulbecco's Modified Eagle Medium/Ham's Nutrient Mixture F-12 (DMEM/F-12 1:1, Invitrogen, USA), 10% human serum type AB (HS; PAA Laboratories GmbH, Pasching, Austria), 1% L-glutamine (GlutaMAX I; Invitrogen) and 1% pen-strep (100 U/mL penicillin, 0.1 mg/mL streptomycin; Lonza, Verviers, Belgium).

After primary culture in T-75 flasks, ASCs of passage 1 were harvested and analyzed by flow cytometry (FACS Aria; BD Biosciences, Belgium) as previously described.[2] Monoclonal antibodies against CD14-PE-Cy7, CD19-PE-Cy7, CD45RO-APC, CD73-PE, CD90-APC (BD Biosciences, USA); CD34-APC, HLA-DR-PE (Immunotools, Germany); and CD105-PE (R&D Systems, USA)) were used. Analysis was performed on 10,000 cells per sample, and unstained cell samples were used to compensate for the background autofluorescence levels.

The expression levels of the ASCs used in the experiments are presented in Table 1. Positive expression (≥75%) was found in CD73, CD90, and CD105, lack of expression (≤2%) was found in CD14, CD19, CD45, and HLA-DR, and moderate expression (2–50%) was found in CD34. Apart from highly varying expression profile of CD34, this expression profile is in accordance with that defined by the Mesenchymal and Tissue Stem Cell Committee of the ISCT.[4] However, varying expression of CD34 has been shown to be typical for ASCs when cultured in medium supplemented with human serum.[14]

The cell response to PPy-coated and uncoated scaffolds was studied with ASCs cultured in smooth

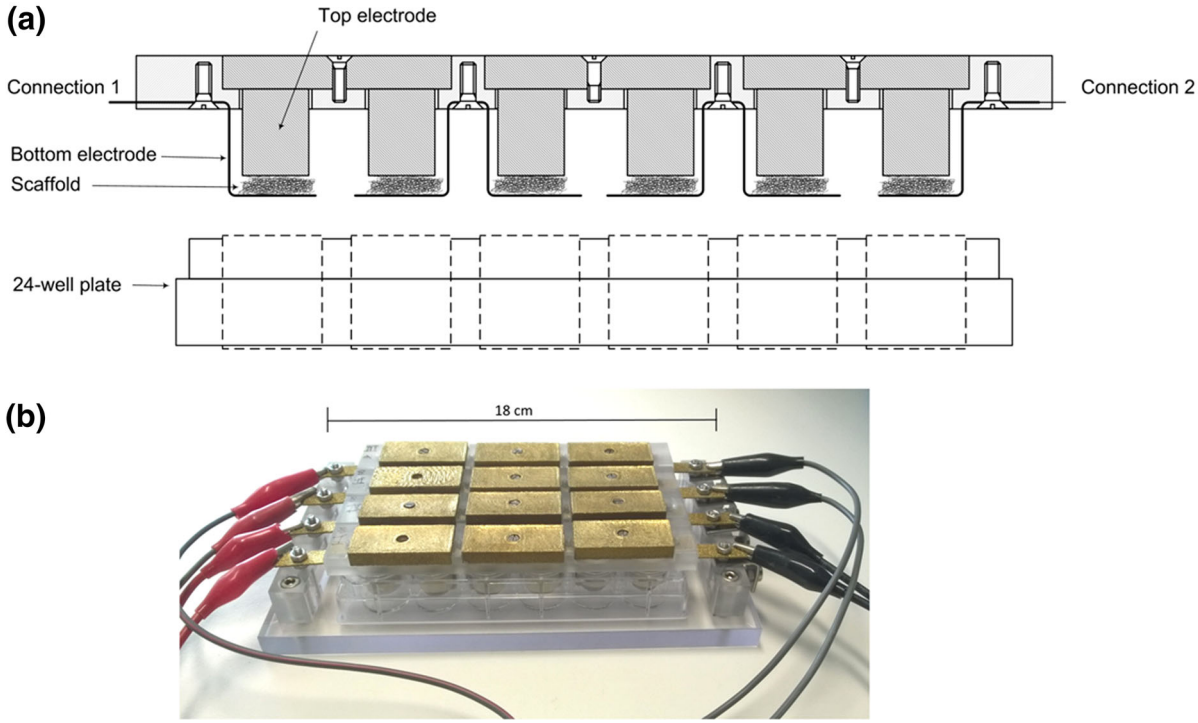

FIGURE 2. (a) Side view of a 6-well stimulation channel. The six wells were connected in series and the 3D scaffolds fitted between the top and bottom electrodes. (b) Assembly for 3D electrical stimulation device on 24-well plate.

TABLE 1. The surface protein expression of ASCs from the four female donors.

Antigen	Surface protein	Mean	SD
CD14	Serum lipopolysaccharide binding protein	0.9	0.7
CD19	B lymphocyte-lineage differentiation antigen	0.4	0.4
CD34	Sialomucin-like adhesion molecule	26.5	34.2
CD45	Leukocyte common antigen	1.4	0.6
CD73	Ecto-50-nucleotidase	78.4	16.7
CD90	Thy-1 (T cell surface glycoprotein)	99.0	0.9
CD105	SH-2, endoglin	94.3	6.7
HLA-DR	Major histocompatibility class II antigens	0.8	0.5

muscle differentiation medium for 11 days before seeding to better observe late differentiation of ASCs after 14 days of culture on the scaffolds. The differentiation medium consisted of maintenance medium supplemented with 5 ng/mL of TGF-β1 (Santa Cruz Biotechnology, USA). For the ES studies, only PPy-coated scaffolds were used and ASCs were expanded in maintenance medium and cultured in differentiation medium during the experiments. Scaffold comparison experiments were repeated twice and electrical stimulation experiments three times. ASCs from different patients were necessarily used in each experiment.

Cell Seeding

Scaffolds were disinfected by soaking in 70% ethanol for 30 min and air dried. Scaffolds were then rinsed five times with PBS and incubated in DMEM for 48 h. The scaffolds used for ES experiments, including the unstimulated control scaffolds, were soaked in 0.1% fibronectin (FN; Sanquin, The Netherlands) in PBS for 2 h before cell seeding to further induce differentiation.[5] Each scaffold was seeded with 120,000 cells in 50 μL of medium on top of the scaffolds and cells allowed to attach for 3 h before transferring the scaffolds to the 24 well plates and covering with medium.

Electrical Stimulation of Adipose Stem Cells

The 3D stimulation electrodes depicted in Fig. 2 utilized TiN coating on metallic Ti. The 24-well stimulation system comprised of top and bottom electrodes attached to a plexiglass support lid and assembled into 4 ES channels, each channel containing 6 individual wells connected electrically in series. Within a single well, the two Ti/TiN electrodes were in contact with the cell culture medium and the scaffolds were placed into the gap between the Ti/TiN electrodes. Electrical connections (connections 1 and 2 in Fig. 2a) were made to both ends of each channel, hence the stimulation current was identical through the six wells connected in series. Because the impedance of the individual wells and the samples connected in series cannot be matched exactly, the potential difference across each well could be slightly different. Given the variation of the through-plane conductivity of the scaffolds was in the order of 20%, it was estimated that the variation of the cell potential difference was a maximum of 5 mV per electrode pair.

3D stimulation electrodes were sterilized by autoclaving at 120 °C. ES was initiated on the day after cell seeding. The full capability of this ES screening system was utilized to determine suitable parameters of ES for stimulation of adipose stem cells in preliminary experiments (data not shown). The resulting 2 variations of biphasic electric current, presented in Table 2 were chosen for closer examination due to the observed enhancement of cell differentiation. Both variations consisted of biphasic pulses at frequencies of 10 Hz, with current densities of 50 $\mu A/cm^2$, but varied in pulse width of either 0.25 or 1 ms. Cells were stimulated for 4 h per day over 14 days and characterized for both viability and SMC protein expression. Stimulation waveforms were generated by DS8000 digital stimulator (World Precision Instruments, USA) and converted to current output by A365 Stimulus isolators. The potential developed over each electrode assembly was monitored using an E-corder 401 and Chart software (eDAQ Pty Ltd, Australia).

Cell Attachment and Viability

Viability was assessed using calcein AM (Life Technologies, Australia) to visualize viable cells and propidium iodide (Sigma-Aldrich) to visualize dead cells according to manufacturers' protocols. Samples were examined with a fluorescence microscope (Axioimager, Zeiss, Germany). Green fluorescence of stimulated samples was compared with those of controls using ImageJ. Briefly, the brightness threshold for detecting exposed pixels was adjusted to a level where only cells and no background noise was present in the images. The total number of cell-specific pixels in each image was counted. Sample sizes of 4–5 images at ×100 magnification were used for analysis at day 7 and sample sizes of ten images at day 14.

TABLE 2. ES paradigms used in the study.

Group	Electrical stimulation parameters
Control	No stimulation
ES-1 ms	10 Hz, 50 μA, 1 ms pulse width
ES-0.25 ms	10 Hz, 50 μA, 0.25 ms pulse width

Cell proliferation

Cell proliferation was studied on PPy-coated and uncoated scaffolds using CyQuant® Cell Proliferation Assay Kit (Molecular Probes®, Life Technologies, Australia). The experiment was performed according to the manufacturer's protocol. In brief, on the day of the analysis, samples were carefully washed with PBS and cells within the scaffolds were lysed in 0.1% Triton-X 100 (Sigma-Aldrich) in PBS and stored at −80 °C until analysis. After thawing and thorough mixing of the lysate, 20 μl of three replicates of each sample were mixed with CyQuant® GR dye and lysis buffer. The fluorescence was measured with a microplate reader (TECAN Safire, Switzerland) at 480/520 nm.

Immunofluorescence Staining

Cells were washed with PBS and fixed with 3.7% paraformaldehyde (Sigma-Aldrich) in PBS for 10 min. All washing steps were performed with PBS and incubation steps were performed at room temperature, unless otherwise stated. After fixation, cells were washed twice and permeabilized with PBS containing 0.1% Triton X-100 for 10 min.

Cells were washed and incubated with blocking-buffer containing 10% (v/v) normal goat serum (Gibco, Life Technologies) in PBS for 1 h. Subsequently, cells were incubated with the antibodies: anti-human calponin (Abcam, USA), anti-human myosin heavy chain (MHC; Abcam), and anti-human smooth muscle actin (SMA; Abcam), diluted in blocking buffer, for 2 h at room temperature or overnight at 4 °C. Cells were washed twice and incubated with secondary AlexaFluor 488-conjugated goat anti-mouse IgG or AlexaFluor 594-conjugated goat anti-rabbit IgG (Molecular Probes, Life Technologies)) diluted in blocking buffer for 1 h in the dark at room temperature. After three washing steps, nuclei were counterstained with 4′6-diamidino-2-phenylindole (DAPI;

Molecular Probes) in PBS and samples transferred to fresh PBS. Immunofluorescence was assessed with a fluorescence microscope (scaffold comparison imaged with EVOS, Advanced Microscopy Group, USA; ES experiments imaged with Axioimager, Carl Zeiss, Germany using 100 W mercury lamp) and five random images were taken for each condition using the same exposure time. Immunofluorescence was quantified using ImageJ from images of ×100 magnification in each group. Images were processed as described for Cell attachment and Viability analysis (Sect. 2.7). DAPI stained nuclei were counted and the total number of pixels for immuno-stained fluorescent images was normalized to the number of DAPI-stained nuclei in each image.

Statistical Analysis

ASC proliferation was compared at three different time points (1, 7 and 14 days) and analyzed using two-way ANOVA. Tukey–Kramer corrections were used for post hoc analysis. Triplicate technical replicates for each sample were used. Semi-quantitative analysis of immunomarker expression of ASCs under ES was performed using one-way ANOVA. Microsoft Excel Professional Plus 2013 was used for analyzing data from both experiments. Semi quantitative analysis of cell viability during ES was analyzed using one-way ANOVA and IBM SPSS statistics version 21. All data was presented as mean ± standard deviation. Results were considered statistically significant when $p < 0.05$.

RESULTS

Scaffold Characterization

According to μCT, the pore size of the PTMC scaffolds ranged from 38 to 250 μm. Pore size distribution is presented in Fig. 3. PPy coating of the PTMC scaffolds did not notably change the porosity. For PTMC scaffolds, a porosity of 54 ± 2.1% was achieved, while for the PPy-coated scaffolds, porosity was 56 ± 4.2%.

According to SEM images (Fig. 4), the pores were evenly distributed and were inter-connected through the scaffolds. PPy coating formed characteristic nano- and microscale topography[22] clearly roughening the surface of the PTMC scaffolds. From the cross-sections, the thickness of the PPy coating was estimated to vary between 2 and 5 μm. The through plane conductivity of the PPy-coated scaffolds was 5.84 ± 1.3 S/m.

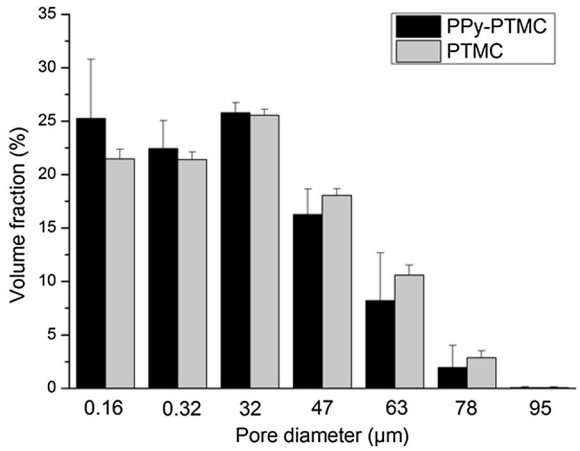

FIGURE 3. Pore size distribution of the scaffolds.

Electrode Characterization

The measured data and the fit to CPE model of the electrode impedance is depicted in Fig. 5. The best reduced Chi squared fit parameters to the data were: a series resistance R_S of 8.5 Ohm cm^2, a polarization resistance R_P of 1×10^{15} Ohm cm^2, and a cell double layer capacitance of 2×10^{-5} F, along with ideality exponent of 0.83. Characteristics of some TiN recording and stimulation electrodes have been earlier reviewed.[3] According to the analysis, the current Ti/TiN electrode was electrochemically stable under the test conditions (10 mV peak-to-peak). Furthermore, the charging of the electrode was purely capacitive, and no evidence of electrochemical corrosion was found. As expected, polarization of the electrodes was relatively low, which was also evidenced by the low potential build-up during biphasic electric current stimulation, in the range of 10-60 mV ruling out the possibility of any significant electric field effects in our stimulation setup. The performance of the ES set up was monitored daily during ES experiments by measuring potential changes across the system. Samples of the potential curves for each experiment are presented in Supplementary data 2. Both the total and polarization voltages were reproducible between experiments and no build-up of potential was observed during any of the experiments. This indicated that there were no changes in electrode impedance as a result of autoclaving or ES applied over 14 day time periods.

Comparison of Cell Proliferation and SMC Protein Expression on PPy-Coated and Uncoated PTMC Scaffolds

The increase in cell number, as reflected by the increase in DNA content of cell lysates, was statistically significant only on PPy-coated scaffolds over the time

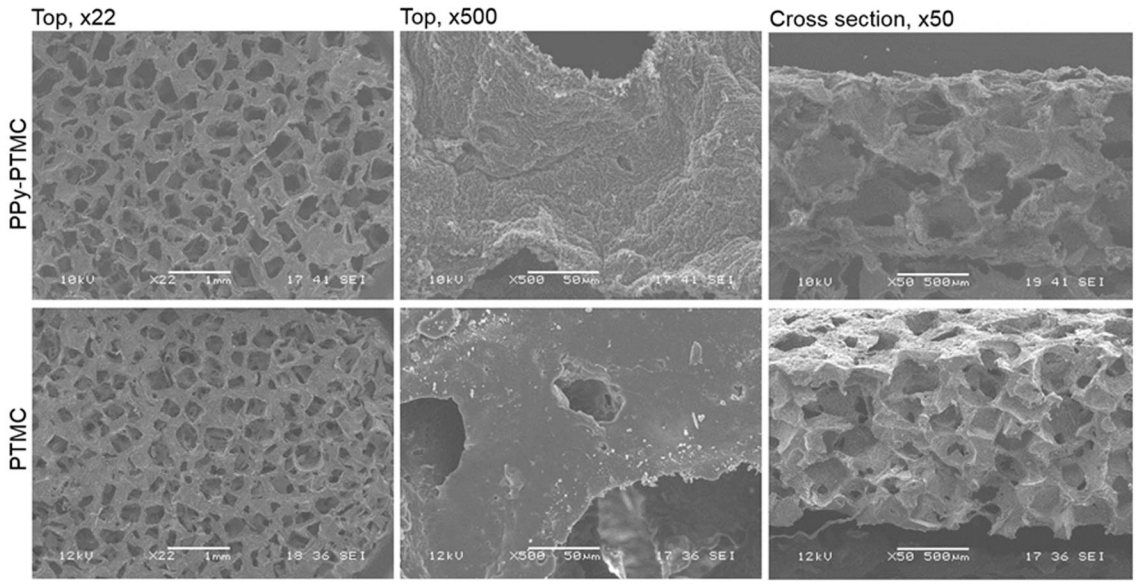

FIGURE 4. SEM images from PTMC and PPy-PTMC scaffolds at different magnifications. PTMC scaffolds were sputtered with gold to increase conductivity and prevent charging.

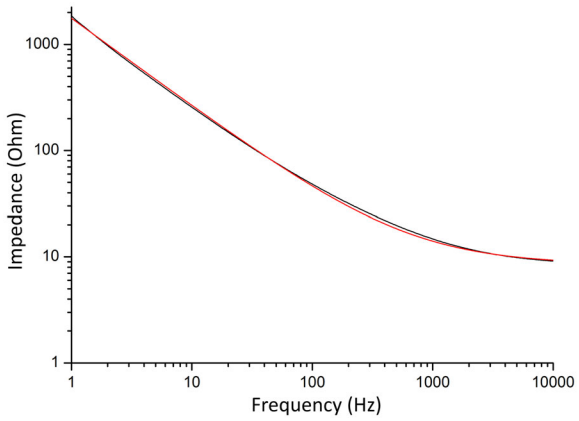

FIGURE 5. The measured impedance data (black line) and the data fitted to CPE model (red line) for Ti/TiN stimulation electrodes.

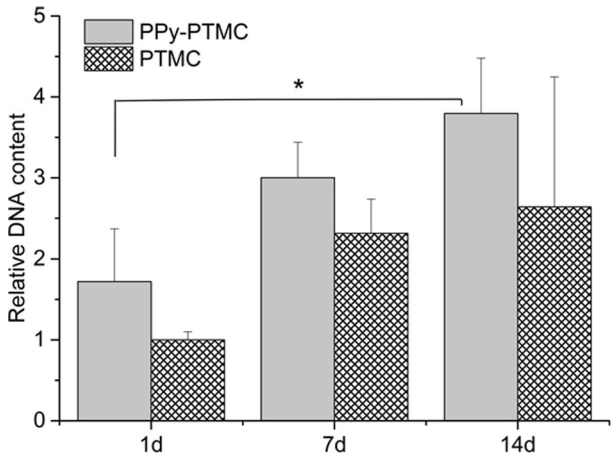

FIGURE 6. DNA content in ASC-seeded PPy-coated PTMC and PTMC scaffolds relative to 1d PTMC samples. The increase in cell number between 1 and 14 day time points was statistically significant on PPy-PTMC scaffolds. *$p < 0.05$.

period 1–14 days. While cells were viable in both scaffold types, as evidenced by live/dead (calcein/PI staining, data not shown) no statistically significant differences in DNA content were found between the PPy-coated and uncoated groups (Fig. 6).

All of the studied SMC markers were expressed more strongly when ASCs were cultured on PPy-coated scaffolds, whereas the expression of calponin, MHC and especially SMA was weak on uncoated scaffolds (Fig. 7). The expression difference for MHC was visually the most evident. MHC and SMA staining on PPy-coated scaffolds revealed the elongated and spindle-shaped morphology characteristic of mature contractile SMCs.[23] Secondary antibodies did not show strong nonspecific binding on either uncoated or PPy-coated PTMC, as shown by the negative controls for PPy-coated samples (Supplementary Fig. 2).

Cell Viability under Electrical Stimulation

Cells were viable in both stimulation groups and unstimulated controls, as shown in Fig. 8a. According to the quantitative analysis (Fig. 8b), ES-1 ms had significantly higher numbers of viable cells compared to controls on day 7. Numbers of viable cells in controls and ES-1 ms were at similar levels by day 14 while ES-0.25 ms showed significantly lower numbers of viable cells compared to the other groups. Imaging of the

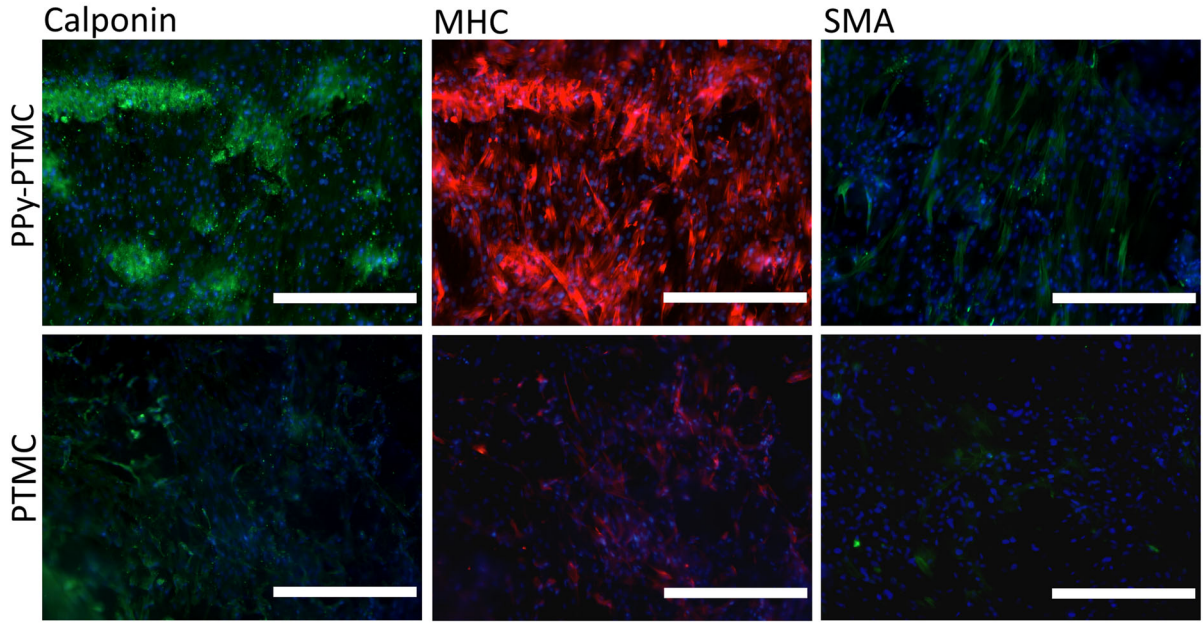

FIGURE 7. SMC immunomarker expression of ASCs in PPy-coated PTMC and uncoated PTMC scaffolds at day 14. Scale bars represent 400 μm.

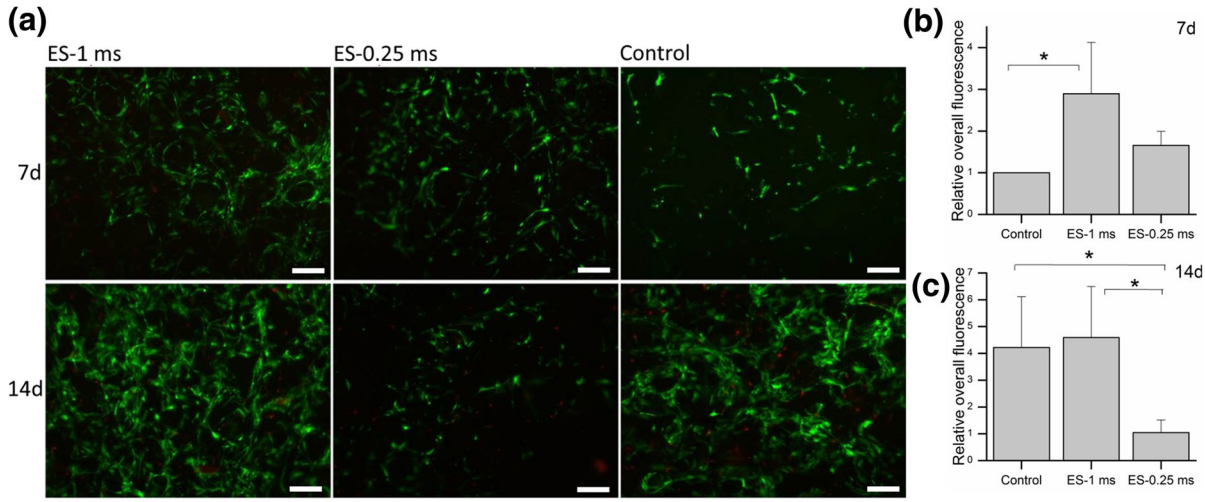

FIGURE 8. (a) Live/dead images of ASCs on PPy-coated PTMC scaffolds under ES on days 7 and 14. Control samples were not stimulated. Scale bars represent 200 μm. (b) Numbers of viable ASCs relative to controls were calculated from 1 patient line. Results were considered significant when $p < 0.05$.

scaffolds from both top and bottom revealed that cells had migrated easily through the scaffolds by day 7 (Supplementary Fig. 1).

SMC Differentiation Under Electrical Stimulation

The images show typical calponin and MHC expression in each group (Fig. 9a). Semi quantitative analysis of the immunofluorescent images (Figs. 9b) revealed no statistically significant differences between the groups when data was normalized to cell number.

DISCUSSION

An important goal of this study was to establish a novel reusable multichannel electrical stimulation device for the *in vitro* ES of tissue engineering scaffolds. In this work we showed that the same electrodes could be used reproducibly in successive experiments and sterilization cycles without any significant changes to the electrode performance and without toxicity to the cells. Mild color changes, attributable to the thin layer of oxidized Ti/TiN[18] and observed after multiple uses,

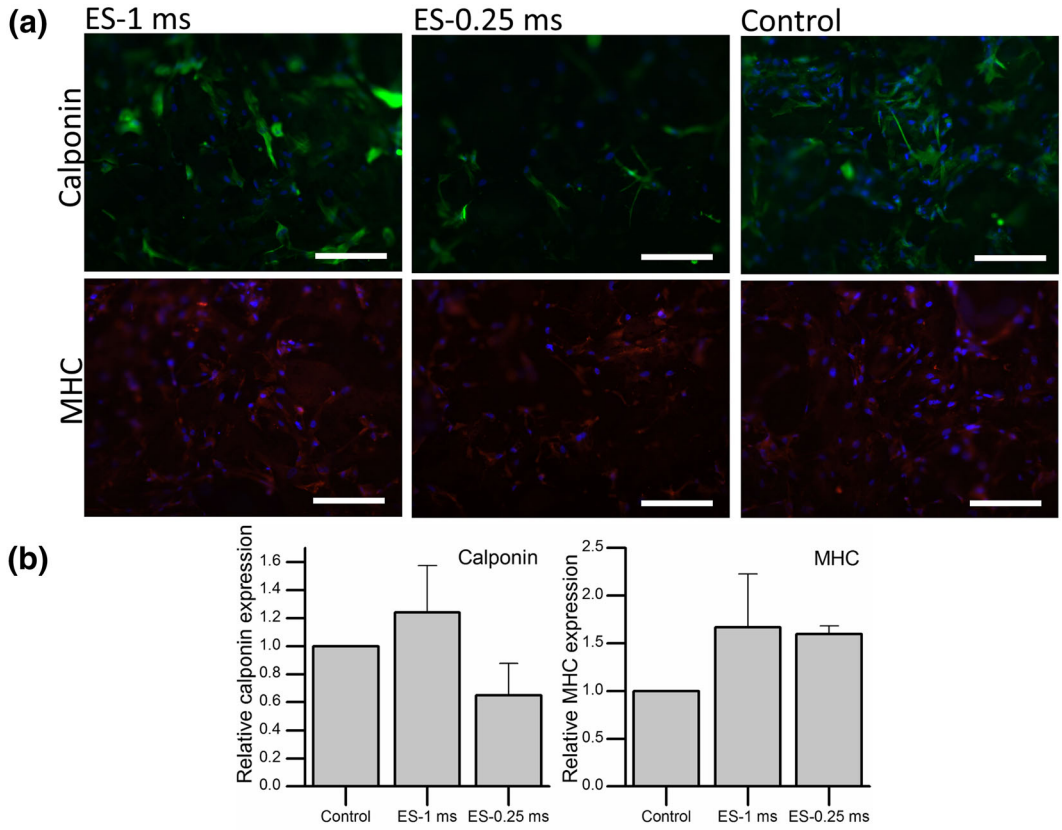

FIGURE 9. (a) Comparison of SMC immunomarker expression in unstimulated controls and two ES paradigms at day 14. Scale bars represent 200 μm. (b) Expression of smooth muscle proteins relative to controls. Data was collected from two patient lines.

did not compromise the performance of the device. Easy handling of the device, especially during media changes helped with maintaining sterility throughout the 14-day studies. This ES device provides researchers an easier, more reliable and cost-effective tool to perform repetitive ES experiments on multiple 3D samples and to systematically compare different electric current-based parameters compared to earlier systems reported in the literature.[12,13,30]

In comparison with previous studies in our group[2,22] that utilized gold (Au) electrodes in a 24 cell culture well plates, the system utilized for the present study has marked improvements. For example, the Ti/TiN electrodes were significantly less polarizable than Au electrodes, and therefore more electrochemically stable under the relevant stimulation conditions. Furthermore, the electrical connections in the current system were designed to deliver an identical pulsed bipolar stimulation current into six wells, connected in series, thus forming a single electronic channel, over long periods of time (14 days). This has not been possible in the earlier stimulator systems utilizing Au-electrodes[2,22] where the wells were electrically connected in parallel, and hence the stimulation current was less evenly distributed due to the (inevitable) small variations in the impedances over the single wells. These variations arise from the small differences in the electrode impedances, electrode geometries and properties of the scaffolds and the cells. These effects are minimized in the current setup. The drawback in choosing the current setup was that the voltage drop over each well was not exactly controlled. Hence, variations originating from the changing electrode-scaffold contact resistances, electrical properties of the porous scaffolds (about 10% variation observed), and the interactions of medium components, such as salts and proteins, with the electrode coating, were to be expected. However, the low electrode potential build-up, that is due to low polarization of the electrodes over 14 days, indicated that the electrical field stimulation of the cells was negligible and the stimulation was solely due to the current injected into the medium or transferred to the scaffold and through the PPy coating of the scaffold. In this sense, the present system is suitable for direct electric current stimulation.

Compared to published data, Ti/TiN electrode impedance in our study had a clear frequency dependence, typical of capacitive charge injection electrodes,

e.g. smooth TiN. The CPE capacitance and the polarization resistance of the cell at 10 mV (peak-to-peak) excitation were comparable to the system described by Tandon *et al.*[32] utilizing carbon rods. However, their stimulation conditions (for 2D cell monolayers and 3D scaffolds) were significantly harsher compared to our system, they applied up to 5 V (peak-to-peak) over a distance of approximately 1 cm. These conditions were clearly outside of the electrochemical stability window of both the medium and the electrodes, as evidenced by the drastic effects on the electrode impedance characteristics, especially the drop in the R_P and ideality exponent η. By contrast, our system utilized shorter electrode distances (2 mm) and much lower potentials (10–60 mV), which were inside the stability window of the TiN coated Ti electrodes and the cell culture medium. This was particularly evidenced by the high polarization resistance of the electrode.

Application of the longer pulse width of 1 ms significantly increased the number of viable cells by 7 days of stimulation but was at similar levels to controls by day 14, whereas applying shorter pulsed (0.25 ms) stimulation drastically decreased the number of viable cells by day 14. This suggested that 1 ms pulsed ES supported cell attachment and proliferation and is a valid ES protocol for promoting increases in ASC numbers.

The expression of the SMC markers calponin and MHC were maintained under both ES paradigms, there being no statistically significant differences between controls and both ES groups. Calponin and MHC are regarded as two of the essential SMC markers,[1] however the further optimization of parameters for ES of these cells in order to enhance differentiation towards the smooth muscle phenotype is warranted.

Our study is the first to compare two different pulse widths used in biphasic electric current stimulation at the frequency of 10 Hz. The two different pulse widths of biphasic electric current clearly generated different responses in ASCs as ES with shorter pulse width significantly compromised the number of viable cells in comparison with both the control and 1 ms pulses after 14 days of ES. As studies on the effect of ES on differentiation of adult stem cells towards SMCs had not been reported at the start of this work, we chose a set of parameters which were most likely to trigger ASC response without compromising viability. Pulsed biphasic electric current has commonly been exploited in ES studies for mesenchymal stem cells as it prevents accumulation of charged species and balances pH changes occurring at the electrodes.[2,12,13,22] The low frequency (10 Hz) was chosen for its common use in muscle stimulation[15] falling within the range (5–500 Hz) reported by Rowlands *et al.* They reported increases in proliferation and upregulation of contractile protein expression of SMCs under sinusoidal alternating currents with amplitude of 50 μA. The current density in our study was adjusted to be in a similar range to the earlier 3D ES studies of adult stem cells.[12,22,24]

Regarding pulse widths in biphasic current stimulation of stem cells, both 1 ms[19,21,30] and 0.25 ms[12,13,30] pulse widths have been reported in the literature with positive outcomes in cell response. In our work, cells were exposed to four times longer direct currents under ES-1 ms compared to ES-0.25 ms. Direct current or biphasic electric current stimulation (i.e. pulsed direct current) with 1 ms or longer pulses has been reported to promote differentiation pathways where cell attachment to substrate is crucial for differentiation.[2,13,19,21,34] While the reason for the difference in the number of viable cells between ES-1 and ES-0.25 ms is unclear, the increase in cell number for ES-1 ms compared to controls by day 7 may reflect better attachment of ASCs to the scaffolds.

The 2–5 μm thick PPy coatings, measured from the SEM images, were visually and microscopically intact after 14 days of ES. The through-plane conductivity of the PPy-coated scaffolds was 5.84 ± 1.3 S/m. This is higher than the conductivity of muscle, which has been reported to be 0.15–0.19 S/m when measured at the frequencies of 40 and 70 Hz.[6] The conductivity of the coatings should therefore be sufficient for muscle tissue engineering purposes. PPy has previously been studied for smooth muscle or skeletal tissue engineering only in an electrochemically polymerized form,[8,24] which limits applications mainly to 2D environments. The chemical polymerization used in the present study allows versatile coating of PPy in 3D scaffold structures. Coating PTMC with PPy provides interesting opportunities for using PTMC-based scaffolds as conductive highly elastic substrates for soft tissue engineering.

For the first time, PPy-coated PTMC scaffolds were demonstrated to be a suitable substrate for attachment, proliferation, and differentiation of ASCs towards SMCs. Cell numbers significantly increased in PPy-coated scaffolds over time and showed consistently higher cell number in comparison with uncoated PTMC scaffolds, which was in agreement with our earlier study conducted on non-woven PLA scaffolds coated with chondroitin sulphate-doped PPy.[22] PPy coatings also triggered stronger smooth muscle protein expression. The scaffold morphology, pore size and interconnectivity facilitated the attachment and migration of the cells through the scaffolds. The better differentiation of ASCs on PPy-coated scaffolds may

be explained by the better attachment of ASCs on this substrate. This may facilitate formation of focal adhesions which play a major role in adult stem cell differentiation towards anchorage-dependent lineages, such as SMCs.[7]

CONCLUSIONS

The presented novel ES device for 3D scaffolds was shown to allow stimulation of 24 scaffolds simultaneously over long periods of time without compromising the performance of the electrodes. The system should be highly valuable in experiments where large quantities of cell characterization data are required. In this study we showed that PPy-coated scaffolds increased ASC number and smooth muscle protein expression when compared with the uncoated PTMC scaffolds, and provided a suitable substrate for cells to be electrically stimulated. Biphasic electric current with 1 ms pulse width applied *via* the novel electrodes increased the numbers of viable ASCs relative to non-stimulated controls by day 7, while the pulse width of 0.25 ms decreased viable cell numbers by 14 days of ES. Both ES protocols supported smooth muscle expression markers. The presented ES stimulation system provides a feasible means for further exploring the enhanced differentiation of stem cells for tissue engineering applications.

ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (doi: 10.1007/s10439-016-1755-7) contains supplementary material, which is available to authorized users.

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DISCLOSURE

No competing financial interests exist.

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