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Electrospinning polyaniline-contained gelatin nanofibers for tissue engineering applications

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Abstract

Polyaniline (PANi), a conductive polymer, was blended with a natural protein, gelatin, and co-electrospun into nanofibers to investigate the potential application of such a blend as conductive scaffold for tissue engineering purposes. Electrospun PANi–contained gelatin fibers were characterized using scanning electron microscopy (SEM), electrical conductivity measurement, mechanical tensile testing, and differential scanning calorimetry (DSC). SEM analysis of the blend fibers containing less than 3% PANi in total weight, revealed uniform fibers with no evidence for phase segregation, as also confirmed by DSC. Our data indicate that with increasing the amount of PANi (from 0 to ~5% w/w), the average fiber size was reduced from 803 ± 121 nm to 61 ± 13 nm ($p < 0.01$) and the tensile modulus increased from 499 ± 207 MPa to 1384 ± 105 MPa ($p < 0.05$). The results of the DSC study further strengthen our notion that the doping of gelatin with a few % PANi leads to an alteration of the physicochemical properties of gelatin. To test the usefulness of PANi-gelatin blends as a fibrous matrix for supporting cell growth, H9c2 rat cardiac myoblast cells were cultured on fiber-coated glass cover slips. Cell cultures were evaluated in terms of cell proliferation and morphology. Our results indicate that all PANi-gelatin blend fibers supported H9c2 cell attachment and proliferation to a similar degree as the control tissue culture-treated plastic (TCP) and smooth glass substrates. Depending on the concentrations of PANi, the cells initially displayed different morphologies on the fibrous substrates, but after 1week all cultures reached confluence of similar densities and morphology. Taken together these results suggest that PANi-gelatin blend nanofibers might provide a novel conductive material well suited as biocompatible scaffolds for tissue engineering.

Keywords: Electrospinning; Polyaniline (PANi); Gelatin; Tissue engineering; H9c2 cardiac myoblasts

1. Introduction

Fiber-based porous scaffolds, which structurally mimic the extracellular matrix (ECM), have been generated from numerous natural or synthetic biopolymers, such as lyophilized elastin–collagen [1], accellularized aortic elastin and collagen [2], poly(ether-ether-ketone)/-hydroxyapatite biocomposites [3], and chitosan (CS)-based materials [4]. All these scaffolds are biocompatible, and enhance cell in vitro growth.

Recent studies demonstrated the usefulness of electrospinning as platform technology for generating fibrous scaffolds, for tissue engineering purposes. Various biodegradable synthetic polymers [5,6], peptide copolymers [7], and natural proteins [8–10] have been electrospun into micro/nanofibers for a multitude of biomedical applications such as scaffolds used in tissue engineering [11–14], wound dressing [15], drug delivery [16], and vascular grafts [17].

Since their discovery some 30 years ago, electrically conductive polymers, also known as "synthetic metals" are

Abbreviations: CPD, critical point drying; CPSA, camphorsulfonic acid; DMEM, Dulbecco's modified Eagle's medium; DSC, differential scanning calorimetry; EDC, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride; ECM, extracellular matrix; HFP, 1, 1, 1, 3, 3, 3-hexafluoro-2-propanol; PANi, polyaniline; PBS, phosphate buffered saline; PLGA, poly(lactide-*co*-glycolide); PPy, polypyrrole; SEM, scanning electron microscope; TCPS, tissue culture-treated polystyrene; TNBS, 2, 4, 6-trinitro-benzensulfonic acid

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finding increasing number of applications in many areas of applied chemistry and physics, such as light emitting diodes [18]. More recently, there is a growing interest in conductive polymers also for diverse biomedical applications, including for tissue engineering. We like others, have based our rationale for using conductive polymers on the hypothesis that a multitude of cell functions, such as attachment, proliferation, migration, and differentiation could be modulated through electrical stimulation [19]. Common classes of organic conductive polymers include polyacetylene, polypyrrole (PPy), polythiophene, polyaniline (PANi), and poly(para-phenylene vinylene). Some of these conductive polymers, especially PPy, have found some intriguing biomedical applications, such as for the synthesis of nanoparticles [20], immobilization of proteins [21], and coating devices with PPy materials [22]. Schmidt and her coworkers were the first ones to employ PPy for tissue engineering purposes, demonstrating that electrical stimulation enhanced NGF induced neuronal differentiation of PC 12 cells [23]. Subsequently the same group also functionalized the surface of chlorine-doped PPy to anchor peptide molecules that promote nerve regeneration, blood vessel growth or other biological processes [24]. Lakard et al. cultured olfactory cells on PPy to investigate cell adhesion and proliferation [25]. George et al. examined PPy biocompatibility and found neurons and glial cells enveloped the PPy implant [26]. Several other scaffolds containing PPy have been used for a variety of other applications of tissue engineering [27,28].

By comparison, only few groups have considered PANi, one of the otherwise most studied organic conducting polymers, as potential conductive substrates for tissue-engineering applications [29,30]. In the past two decades, the ability of varying oxidative state has allowed the conducting polymer PANi to be studied in wide range of research fields, such as corrosion protection of metals [31], as substrates for light-emitting devices [32] or as the electrode in the battery [33]. It is only quite recently that the tunable electroactivity of PANi has been explored in the realm of diverse biological application such as for biosensors [34] or as scaffolds in tissue engineering [35]. Mattioli-Belmonte et al. were the first ones to demonstrate that PANi is biocompatible in vitro and in long-term animal studies in vivo [36]. Nevertheless, most of those groups investigated the biological properties in the form of films, instead of nanofibers. Díaz. et al. reported that doped, conductive PANi blended with polystyrene (PS) and/or polyethylene oxide (PEO) could be electrospun yielding nanofibers [37]. In this paper, we describe our study on electrospinning a novel blend of conductive camphorsulfonic acid-doped emeraldine PANi (C-PANi) and gelatin and provide detailed investigations of the ensuing submicron sized fibers. Gelatin is a frequently used biomaterial for tissue engineering application, especially in cardiac tissue engineering [38,39]. Our data indicate that the addition of PANi to gelatin results in homogeneous electrospun fibers and that with increasing PANi concentration, the fiber size is reduced from approximately 800 nm to less than 100 nm concomitant with an increase in the tensile modulus of fibrous scaffolds. H9c2 rat cardiac myoblast cells were selected and cultured on these fibrous substrates, in view of our long-term goal to generate scaffolds for engineering cardiac tissues in vivo. Cell culture result demonstrates that PANi-gelatin blend fibers are biocompatible, supporting cell attachment and proliferation.

2. Materials and methods

2.1. Materials

Polyaniline (PANi, emeraldine base, Mw 100,000), gelatin (bovine skin, type B powder, Bloom number 225), and camphorsulfonic acid (CPSA) were purchased from Sigma-Aldrich. For electrospinning, both PANi and gelatin were dissolved in 1,1,1,3,3,3-hexafluoro-2-Propanol (HFP, Sigma-Aldrich). The non-zero cross-linker 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide Hydrochloride (EDC) was purchased from PIERCE. All cell culture media and supplements were from Hyclone, disposable tissue culture supplies were from Fisher.

2.2. Electrospinning

Immediately before electrospinning, 15 mg emeraldine base PANi and 15 mg CPSA were dissolved in 5 ml HFP with stirring at room temperature for 1 h, followed by filtering through a regular qualitative filter paper (Whatman). Similarly, 800 mg gelatin powder was dissolved in 10 ml HFP with stirring and heating at 50 °C for 3 h. Thus, the concentration of the pure CPSA-PANi solution was 3% (w/v) and pure gelatin solution 8% (w/v). Samples for electrospinning were then prepared by mixing two solutions at volume ratios of PANi to gelatin at 15:85, 30:70, 45:55, and 60:40, respectively. The calculated concentrations for each of the components in the various samples are listed in Table 1. For convenience, all descriptions are expressed in volume ratio in the following sections. Electrospinning was carried out with the high voltage power supply (ES30-0.1P, Gamma High Voltage Research, Inc.) set at 10 kV with an air gap distance of 10 cm as previously described [9]. Depending upon the intended use, PANi-gelatin blend fibers were collected as ~0.2–~0.5 mm thick mats either on microscope glass slides (Fisher) for fiber morphological characterization or on 15 mm diameter circular glass covers (Fisher) for cell culture assessment; those fibers were electrospun into ~0.5–~1 mm thick mats for mechanical testing.

2.3. Crosslinking

For cell culture, electrospun PANi-gelatin blend fibers were crosslinked with 0.2% EDC in 90% ethanol for up to 2 h at room temperature. Upon

Table 1
Concentration of PANi and gelatin in mixed solution

PANi: gelatin ratio (v/v)	Mass in 100 ml HFP		PANi mass ratio (w/w) (%)	Total solute concentration (w/v) (%)
	PANi (mg)	Gelatin (mg)		
Pure gelatin	0	8000	0	8.00
15:85	45	6800	0.66	6.85
30:70	90	5600	1.58	5.69
45:55	135	4400	2.98	4.54
60:40	180	3200	5.33	3.38

crosslinking, the fibrous scaffolds were sterilized in 70% ethanol for 1 hour followed by 3 rinses with distilled water (dd H_2O) and soaked overnight in 1 × phosphate buffered saline (PBS). The degree of crosslinking was determined using 2,4,6-trinitro-benzensulfonic acid (TNBS, Sigma) as described by Sheu et al. [40] and Simmons et al. [41]. In brief: to a sample of 2–5 mg of the cross-linked PANi-gelatin scaffold, 1 ml of 4% (w/v) $NaHCO_3$ solution (pH 8.5) and 1 ml of freshly prepared 0.5% (w/v) TNBS solution in distilled water were added. After allowing the reaction to take place for 2 h at 40 °C, 2 ml of 6 N HCl was added, and the temperature was raised to 60 °C. Solubilization of PANi-gelatin was achieved within 90 min. The resulting solution was diluted with 4 ml of dd H_2O, and the absorbance was measured with a Spectrophotometer (BioMate, Thermo Electron Corporation). The degree of croslsinking was expressed as percent of loss in free primary amino-group after crosslinking. The degree of crosslinking was calculated as follows:

$$\text{Degree of crosslinking } (\%) = 1 - (\text{absorbance}_s/\text{mass}_s)/(\text{absorbance}_n/\text{mass}_n) \quad (1)$$

where the subscripts s and n denote the sample and non-crosslinked gelatin, respectively.

2.4. Cell culture

In view of our long-term goal of generating scaffolds for cardiac tissue engineering, we used H9c2 cardiac rat myoblast cells (ATCC CRL-1446; American Type Culture Collection, Rockville, MD, USA) for studying cell attachment to and proliferation on PANi-gelatin blend fibers. H9c2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose supplemented 4 mM L-glutamine, 25 units/ml penicillin/streptomycin (Cellgro, Herndon VA), and 10% fetal bovine serum (Hyclone, Logan UT) under standard culture conditions (37 °C, 5% CO_2). Mats (~0.2 mm in thickness) of blend fibers with different ratios of PANi and gelatin were electrospun on circular 15 mm glass coverslips. Then fibers were crosslinked and the substrates were secured in 24-well culture plates using Viton O-rings (Cole-Parmer) [42]. Before seeding cells, fiber scaffolds were soaked in complete DMEM medium for another 3 h.

Cell attachment and proliferation were measured with a continual fluorescence assay, using AlamarBlue™ (AB, Biosource, Alameda CA) [43]. H9c2 cells were seeded onto the various fibrous PANi-gelatin scaffolds, control glass cover slips, and TCP surfaces at a density of 10,000 cells/well. Following overnight attachment in complete DMEM in a tissue culture incubator, supernatants were removed and 1.5 ml fresh complete DMEM containing 5% (v/v) AB was added into each well. After another 4 h incubation, triplicate 100 μl aliquots of the AB containing medium was removed from each well for fluorescence measurement. Subsequently the cells were re-fed with fresh medium without AB. For continual assessment of cell proliferation, the AB assay was performed every other day on the same cell population for up to 6 days until the cells reached confluence. For each well the data were normalized to the initial AB fluorescence readings at day 0 (taken 12 h post plating).

2.5. Microscopy

For cytological assessment, samples were fixed with 10% buffered formalin for 1 h at room temperature and then left overnight in PBS at 4 °C. The samples were washed once with 1 × PBS, and the cells permeablized for 15 min in 0.2% Triton-X 100 (Sigma) in PBS (Cellgro). Following a gentle wash in PBS, the samples were incubated for 15 min in PBS containing 2 μg/ml Hoechst 33258 (*bis*-benzimide, Sigma), a nuclear stain, and 1 μg/ml rhodamine-phalloidin (Phalloidin TRITC-labeled, Sigma), a specific stain for microfilaments. Samples were visualized on a Leica DMRX microscope equipped with the appropriate fluorescence filters. Digital images were acquired using a Leica 300F camera. To assess the ultrastructure of H9c2 cells cultured on various PANi-gelatin blend fibers, samples were fixed with 2.5% glutaraldehyde for 1 h at room temperature and then left overnight in PBS at 4 °C. The samples were washed with PBS, and dehydrated in 15%, 30%, 50%, 70%, 85%, 95%, and 100% (twice) graded ethanol for 10 min each followed by drying in a critical point dryer (CPD, SPI CPD 7501, West Chester PA). The dry samples were sputter coated with platinum, and visualized with a scanning electron microscope (SEM, XL-30 Environmental SEM-FEG). For each digital electron micrograph, the average fiber diameters at different ratios of PANi to gelatin were calculated using UTHSCSA ImageTool 3.0 imaging software, measuring approximate 10 fibers per image. A total of 3–4 images from independent preparations were evaluated.

2.6. Microtensile test

The tensile properties of electrospun PANi-gelatin composite fibrous scaffolds were characterized by a Kawabata Evaluation System (KES-G1, Kato Tech Co., Japan) using routine mechanical testing methods for fabric materials, as previously described [9].

2.7. Conductivity analysis

Conductivity of gelatin fiber and PANi-gelatin blend fiber covered substrates were measured by using 4-probe technique (Model 173 Potentiostat/Galvanostat), in which two pairs of contacts are used to measure the conductivity.

2.8. Differential scanning calorimetry (DSC)

The thermal properties of the pure gelatin and PANi-contained gelatin were studied by DSC, (TA Instruments DSC Q100), using 10 mg of various electrospun materials. All measurements were performed under nitrogen atmosphere at a heating rate of 10 °C/min. All experiments were performed twice, yielding similar results.

2.9. Statistics

The number of independent replica is listed individually for each experiment. Where applicable, all data are expressed as mean ± standard deviation. Student's *t*-test and single factor ANOVA were used for parameter estimation and hypothesis testing, with $P<0.05$ and $P<0.01$ considered as being statistically significant.

3. Results and discussion

3.1. Morphology of PANi-gelatin blend fibers

Fibers were electrospun from pure gelatin and the other four sets of PANi-gelatin blend fibers with volume ratios of PANi to gelatin solution of, respectively, 15:85, 30:70, 45:55, and 60:40. Fig. 1 shows SEM micrographs of gelatin fibers electrospun from pure gelatin solution and the various PANi-gelatin blends. With increasing the concentration of PANi in the solution, the fiber sizes decreased. As seen in Fig. 2, a quantitative analysis of at least 30 fibers from 2–3 independent samples each indicates that the mean fiber size decreased from 803 ± 121 nm for pure gelatin fibers to 61 ± 13 nm for 60:40 PANi-gelatin blend fibers. Statistical analysis revealed that the fiber sizes with different volume ratios of PANi and gelatin are significantly different from each other ($P<0.01$). As also seen in Fig. 2, the biggest decrease in the fiber size occurs when the PANi to gelatin ratio is increased from 30:70 to 45:55.

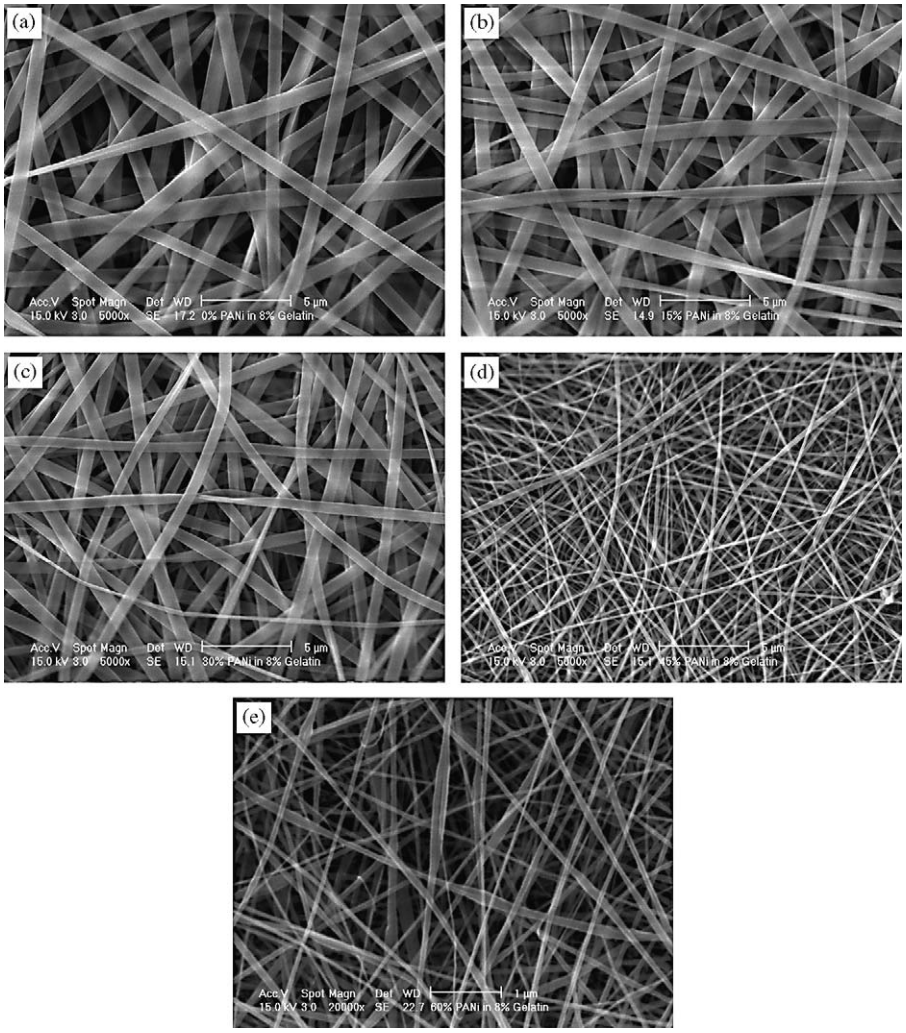

Fig. 1. SEM micrographs of gelatin fibers (a) and PANi-gelatin blend fibers with ratios of (b) 15:85; (c) 30:70; (d) 45:55; and (e) 60:40. Original magnifications are 5000 × for (a–d) and 20000 × for (e). Figure shows the electrospun fibers were homogeneous while 60:40 fibers were electrospun with beads.

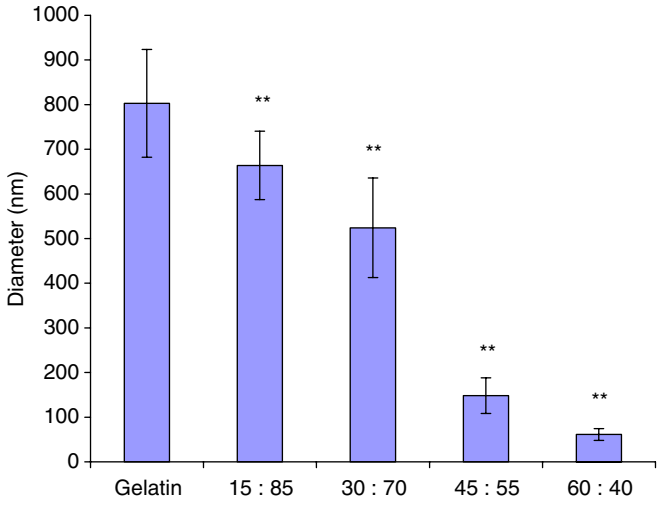

Fig. 2. Sizes of electrospun gelatin fibers and PANi-gelatin blend fibers at different volume ratios. Electrospun fibers were examined by SEM and data are expressed as means ± SD, $n = 10-20$, **: $P < 0.01$, values are significantly different from the previous group compared.

In our previous studies on electrospun gelatin fibers, we found that reducing the concentration of gelatin solution significantly decreased fiber sizes. This conclusion was also validated here because increasing the volume ratio of PANi solution and decreasing the volume ratio of gelatin solution did reduce the total concentrations of PANi-gelatin from 8%, to 6.8%, 5.7%, 4.5%, and 3.4%. However, for pure gelatin fibers, the reduction of the gelatin concentration to less than 5% was accompanied by a significant formation of beads among the fibers, so the minimum size of continuous, bead-free gelatin fibers was around 200 nm [9]. For PANi-gelatin blend fibers the ensuing fibers were still continuous at the gelatin concentration of 3.4% (volume ratio of PANi to gelatin is 60:40) and fiber size was <100 nm, with occasional appearance of beads. Any further reduction in gelatin concentration induced significant bead formation. We therefore conclude, that when blended with PANi, the limit for the reproducible formation of continuous, bead-free fibers is at ~4% (around v/v ratio of 50:50).

3.2. Determination of the degree of crosslinking

As detailed in the Materials and methods section, the extent of crosslinking of PANi-gelatin fibers, was expressed as the percent loss in free primary amino-groups after crosslinking. In order to determine the optimal crosslinking time for all fiber scaffolds, we measured the time course of crosslinking for a single volume ratio of 30:70 as representative for all our samples. As seen in Fig. 3, the crosslinking degree increased sharply within first 30 min to gradually reach saturation after 1 h. While the maximum degree of crosslinking still might have increased with longer crosslinking times, we deemed 60 min as sufficient for subsequent cell culture experiments.

3.3. Mechanical and electrical properties of PANi-gelatin blend fiber sheets

Gelatin and PANi-gelatin blend fibers were electrospun into ~1 mm thick fiber mats to measure their mechanical and electrical properties. Based on the strain–stress measurements of those fibrous scaffolds, tensile strength, elongation, and moduli were calculated, as seen in Table 2. The tensile strength of the scaffolds increased with increasing PANi content, e.g from 5.77 ± 0.96 MPa for pure gelatin fibers to 10.49 ± 0.96 MPa for 45:55 ratio of PANi to gelatin. At the same time, the maximum deformation, elongation, of the scaffolds dropped from 0.69 ± 0.33 for pure gelatin fibers to 0.09 ± 0.03 for PANi-gelatin blend fibers with ratio of 45:55. The tensile modulus of a gelatin fibrous sheet is 499 ± 207 MPa and then increased to 1384 ± 105 MPa for 45:55 PANi-gelatin fibers. It can be concluded that increasing the concentration of PANi in the blend solution strengthens the electrospun blend fibers. This finding is yet another evidence for the integration of PANi into the fibers. In testing the elasticity of the PANi-gelatin blend fibrous scaffolds, we observed that with addition of more PANi, the fibrous mats became less elastic.

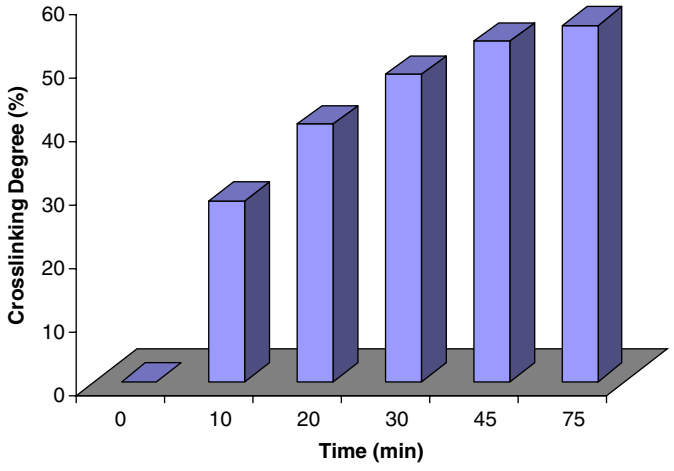

Fig. 3. Crosslinking degree measurement for PANi-gelatin blend fibers with volume ratio of 30:70.

Table 2
Mechanical tensile properties of gelatin and PANi-gelatin fiber scaffolds ($n = 4$)

		Modulus (MPa)	Strength (MPa)	Elongation
Gelatin fibers		499 ± 207	5.77 ± 0.96	0.96 ± 0.33
PANi-gelatin fibers	15:85	614 ± 75	6.56 ± 1.06	0.49 ± 0.10
	30:70	$1043 \pm 184^{**}$	$9.28 \pm 1.97^{*}$	$0.12 \pm 0.03^{**}$
	45:55	$1384 \pm 105^{*}$	10.49 ± 0.96	0.09 ± 0.03

Note: Mechanical tensile data are expressed as means $\pm$ SD, $n = 4$, $^{**}P < 0.01$; $^{*}P < 0.05$, values are significantly different from the previous group compared.

Table 3
Conductivities of gelatin and PANi-gelatin blend fiber covered substrates

	Gelatin fibers	PANi-gelatin blend fibers			
		15:85	30:70	45:55	60:40
Conductivity (S/cm)	0.005	0.01	0.015	0.017	0.021

In addition to the mechanical properties, we also measured the conductivity of electrospun PANi-gelatin blends. As shown in Table 3, the conductivity of a fibrous sheet made of pure gelatin is small (0.005 S/cm). With increasing the amount of PANi in the blend the conductivity of the sheets increased about four-fold. The modest, yet significant increase in conductivity with increasing PANi concentration is further evidence for the homogeneous incorporation of the conductive polymer into the electrospun blend fibers. As an organic acid, CPSA can protonate the emeraldine base of PANi to make it a polyelectrolyte and therefore provide optimal solubility necessary for electrospinning. The solvent used for CPSA-doped PANi emeraldine salt is the main factor to affect conductivity, which ranges from 0.1 S/cm in chloroform to 200–400 S/cm in *m*-cresol; *m*-cresol is been regarded to act as 'secondary dopant' to facilitate expanding the coil-like chains [44,45]. Although the effects of HFP on PANi emeraldine salt have not yet been studied, we surmise that this alcoholic solvent may work similarly to phenols, and thus optimize the conformation along the PANi chain. Although the electrical conductivity of the PANi-gelatin composite is much lower in comparison to the parent CPSA-doped PANi films cast from precursor solutions, these levels of conductivity are still adequate for studying the effects of electrical stimulation on cell growth and differentiation in vitro [29].

3.4. DSC analysis of PANi-gelatin fibers

DSC, a widely used thermoanalytical technique, was employed to assess some of the physicochemical properties, such as endothermic or exothermic processes, characteristic of the PANi-gelatin blend materials. The results of the

DSC study further strengthen our notion that the doping of gelatin with a few % PANi leads to an alteration of the physicochemical properties of gelatin. The differential heat flow curves for gelatin and PANi-gelatin blends are shown in Fig. 4(a). As seen in Fig. 4(b), the melting temperature T_m of the main endothermic transition of pure gelatin fibers was at 93.8 °C. Doping the gelatin fibers with PANi resulted in a small but significant shift in T_m (to ~97.6 °C). Importantly no phase segregation or secondary peaks are visible, suggesting that the resulting fibers are homogenous albeit with slightly altered melting properties. The "protein stabilization" (as inferred from the shift in T_m) by PANi is very similar for all the gelatin and PANi-gelatin fibers with ratios of 15:85, 30:70, and 45:55 investigated. As seen in Fig. 4(c), incorporation of PANi had more profound effect on the broad glass transition of gelatin between −50 and 10 °C. A small endothermic glass transition temperature (T_g) of pure gelatin fibers is found at ~−35 °C. Addition of small amounts of PANi changed this transition both qualitatively (becoming exothermic) and quantitatively (the temperature shifted to ~−30 °C for 45:55 PANi-gelatin

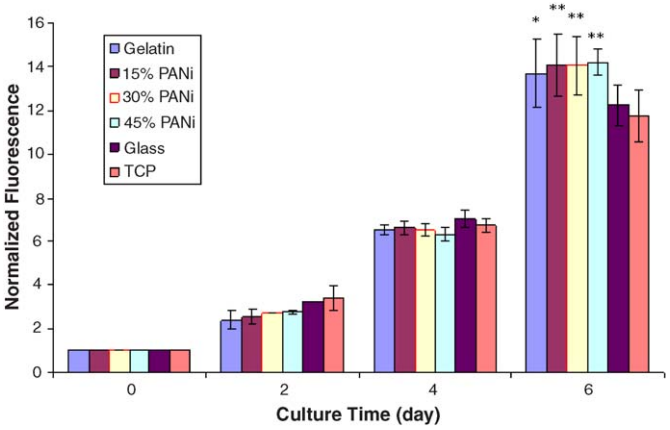

Fig. 5. H9c2 myoblase cell proliferation on various substrates. H9c2 cells were cultured on electrospun gelatin and PANi-gelatin blend fibers with volume ratio of 15:85, 30:70, 45:55, and control glass and TCP substrates over a 6 days time course. Cell proliferation/metabolic activity was evaluated using the Alamar Blue (AB) Assay. The data were normalized to the AB fluorescence reading at day 0. Data are expressed as means ± SD, $n = 6$. *: $P < 0.05$; **: $P < 0.01$, values are significantly different from those on glass and TCP substrates at day 6.

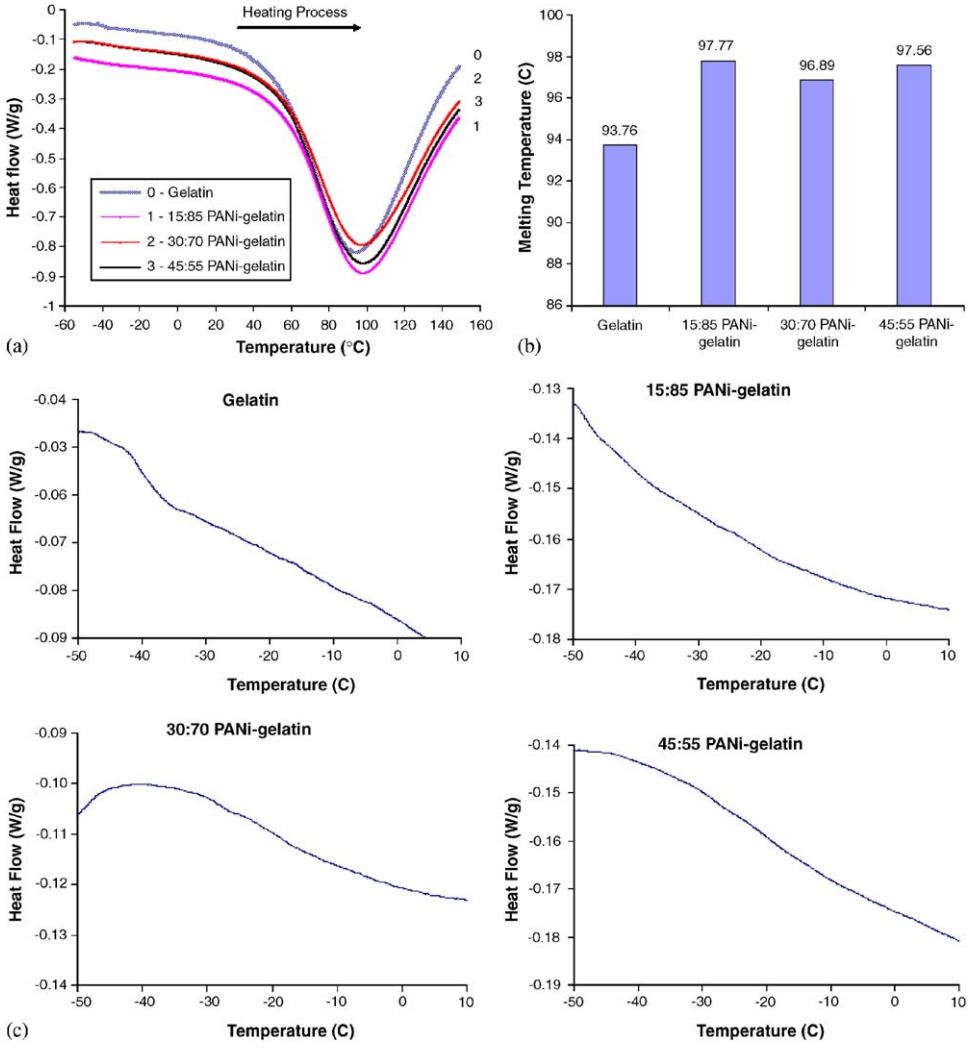

Fig. 4. DSC measurement of electrospun fibers composed of pure gelatin and of PANi-gelatin blends with ratios of 15:85, 30:70, and 45:55: (a) Heat flow of the fibers during heating process; (b) comparative analysis of the melting temperatures (T_m) of the fibers; and (c) glass-transition curves of the fibers.

fibers). These data indicate that the crystalline domain of gelatin may have been altered during the synthesis of the blend nanofibers.

3.5. Cell proliferation

For each of the experimental conditions (different blends, gelatin, glass, and TCP as controls), H9c2 rat cardiac myoblasts were seeded at a density of 10,000 cells/well in triplicate. The cells were allowed to proliferate for up to 6 days. Over the 6 days time course, relative cell numbers were assessed continually every other day using the Alamar blue assay, as described in Materials and methods. Fig. 5 shows the normalized AB fluorescence measurements of cells on electrospun gelatin and PANi-gelatin blend fibers with volume ratio of 15:85, 30:70, 45:55, and control glass and TCP substrates. Cardiac myoblast cells proliferated similarly on all substrates during the 6 days culture period, albeit with some slight differences. At day 2, the cell numbers on the fibrous substrates were slightly lower on the fibrous mats than on the control smooth glass and TCP surfaces. Conversely, at day 6, there were more cells on the fibrous gelatin ($P<0.05$) and PANi-gelatin ($P<0.01$) substrates indicating higher degree of proliferation. A possible reason is that fibrous substrates, being rougher, provided more surfaces for the cells to grow on than the smooth glass and TCP surfaces. We conclude that all our fibrous substrates are equally biocompatible for H9c2 cells and support cell proliferation as well as conventional "gold-standards", such as tissue culture-treated polystyrene.

3.6. Cell morphology

In order to assess the morphology and cytoskeletal architecture of H9c2 cells on the different substrates, the

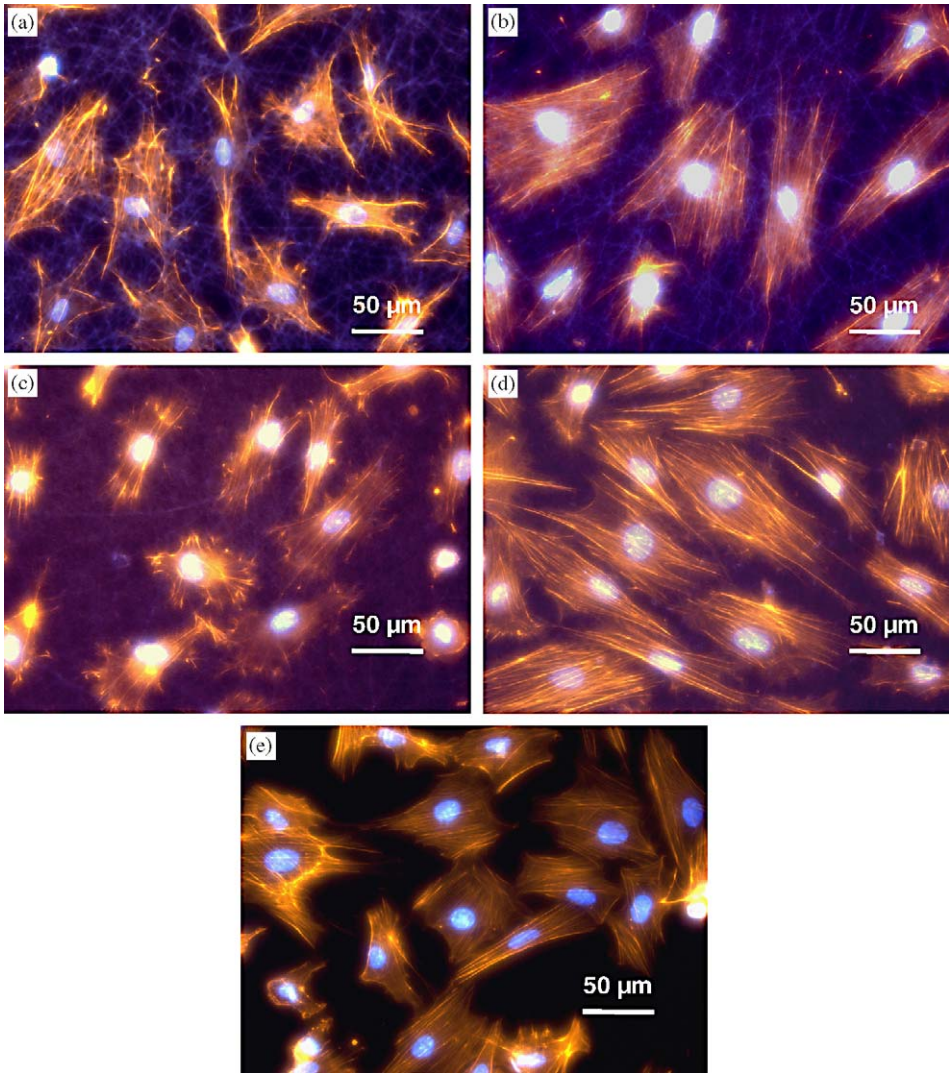

Fig. 6. Morphology of H9c2 myoblast cells at 20 h of post-seeding on: (a) gelatin fiber; (b) 15:85 PANi-gelatin blend fiber; (c) 30:70 PANi-gelatin blend fiber; (d) 45:55 PANi-gelatin blend fibers; and (e) glass matrices. Staining for nuclei-bisbenzimide and actin cytoskeleton-phalloidin, fibers-autofluorescence, original magnification 400 × .

cells were fixed at 20 h of post-seeding and at day 6 for monolayer confluence assay. The cells were permeabilized and stained with bis-benzimide (Hoechst 33258) and rhodamine-phalloidin for nuclei and cytoskeleton, respectively, and then visualized using fluorescence microscope as detailed in Materials and methods. Gelatin fibers are autofluorescent [9], however, with addition of more and more PANi, the fluorescence of blend fibers decreases because (a) PANi is not autofluorescent and (b) the fibers become smaller and smaller. As see in Fig. 6, pure gelatin fibers can be seen very clearly (Panel a); similarly the 15:85 (Panel b) and 30:70 (Panel c) PANi-gelatin blend fibers are still visible, albeit rather faintly. By contrast, 45:55 PANi-gelatin blend fibers are almost not visible (panel d). Cells growing at low density on the bigger-fiber substrates show a large number of prominent microfilament-rich pseudopodia attaching to individual fibers (see Panels a–c). By contrast, cells growing on the smaller fibers are more spread (Panel d) and exhibit a smooth muscle-like morphology and cytoskeletal architecture (prominent stress fibers) more similar to the cells growing on the smooth glass surface (Panel e). These results are reminiscent of our recent study, in which we studied the attachment and alignment of bovine aortic endothelial cells to micropatterned surfaces. In that study we found that the cells will align with micropatterend groves as long as their width exceeds 500 nm. Below that cells will align randomly and spread just as if plated on smooth surfaces [46]. Similarly, Min et al. also reported their investigation on the cytocompatibility and cell behavior onto the electrospun fabricate silk fibroin (SF) nanofibers; human keratinocyte and fibroblast cells attached and spread on the SF nanofibers [47]. Under our conditions, H9c2 cells attached, spread, migrated, and proliferated to confluence within 6 days and formed nearly identical monolayers on all substrates investigated, as shown in Fig. 7.

In addition to visualization by light microscopy, we used scanning electron microscopy for close inspection of the cell morphology. As highlighted in Fig. 8, H9c2 cultured on gelatin fibers extended pseudopodia which attached onto the large gelatin fibers (Panel a). Fig. 8(b) shows a large pseudopodium (or a bundle of pseudopodia) growing along the gelatin fiber. Similarly, the pseudopodia grew and extended along PANi-gelatin fibers (see panel c, panel d, and panel e). Interestingly, with decreasing fiber size the pseudopodia were getting smaller on smaller fibers. This observation was also confirmed by fluorescence images from cell F-actin stained cells in Fig. 6. As a control, pseudopodia extended by cells growing on glass were small, around 100–200 nm in diameter, as shown in Fig. 8(f). These SEM micrographs also reveal another important finding, namely that cells preferentially grow and spread out on smaller fibers, which more closely resemble their in vivo ECM.

Taken together our results open the opportunities for further investigations into the use of electrospun fibrous matrices containing conductive emeraldine base PANi for tissue engineering applications, such as for the building of functional cardiac, cardiovascular and neuronal tissue constructs. These data confirm and extend our previous observations on the usefulness of PANi as substrates for cardiomyblasts and cells of neuronal origin [29]. Together these results with previous reports by Schmidt and her colleagues [23,24] on the use of polypyrrole, our results strengthen the notion of the feasibility of using inherently electrical conductive scaffolds for modulating growth and differentiation of various cells for tissue engineering and regenerative purposes.

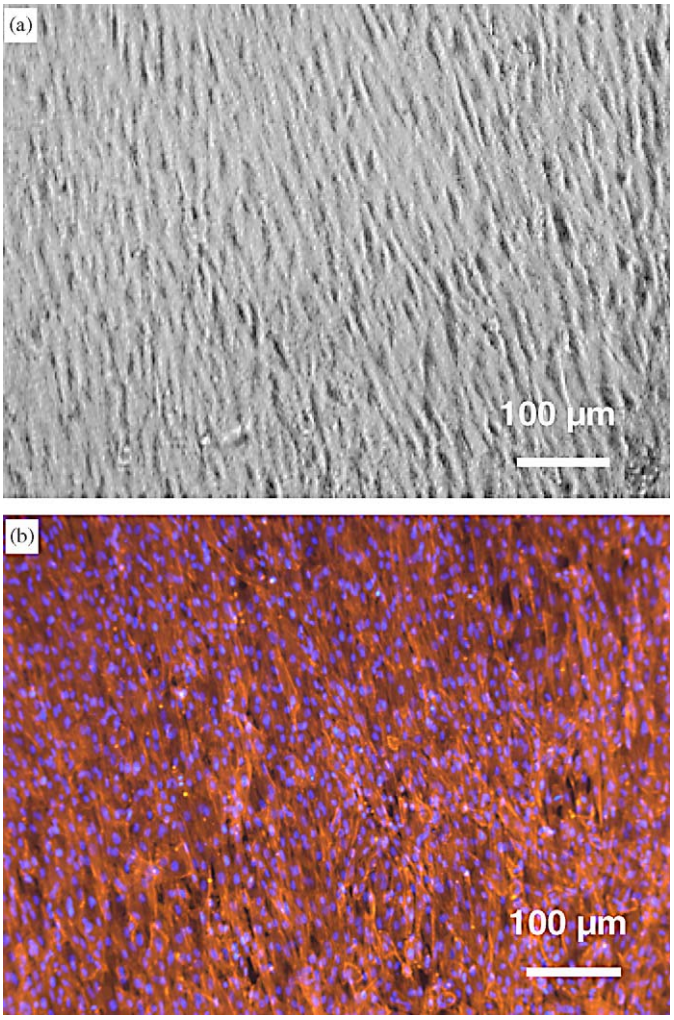

Fig. 7. H9c2 myoblast cells confluency on 30:70 PANi-gelatin blend fiber substrates: (a) phase contrast; (b) fluorescence. Staining for nuclei-bisbenzimide and actin cytoskeleton-phalloidin, original magnification 100 × .

4. Conclusions

Interactions between cells and engineered ECM are crucial for modulating or redirecting cell functions in an in vitro environment. Biocompatibility of tissue-engineered scaffolds is of primary concern since it affects cell attachment, proliferation, and further growth. In our search for novel ‘‘intelligent’’ biomaterials for cardiac

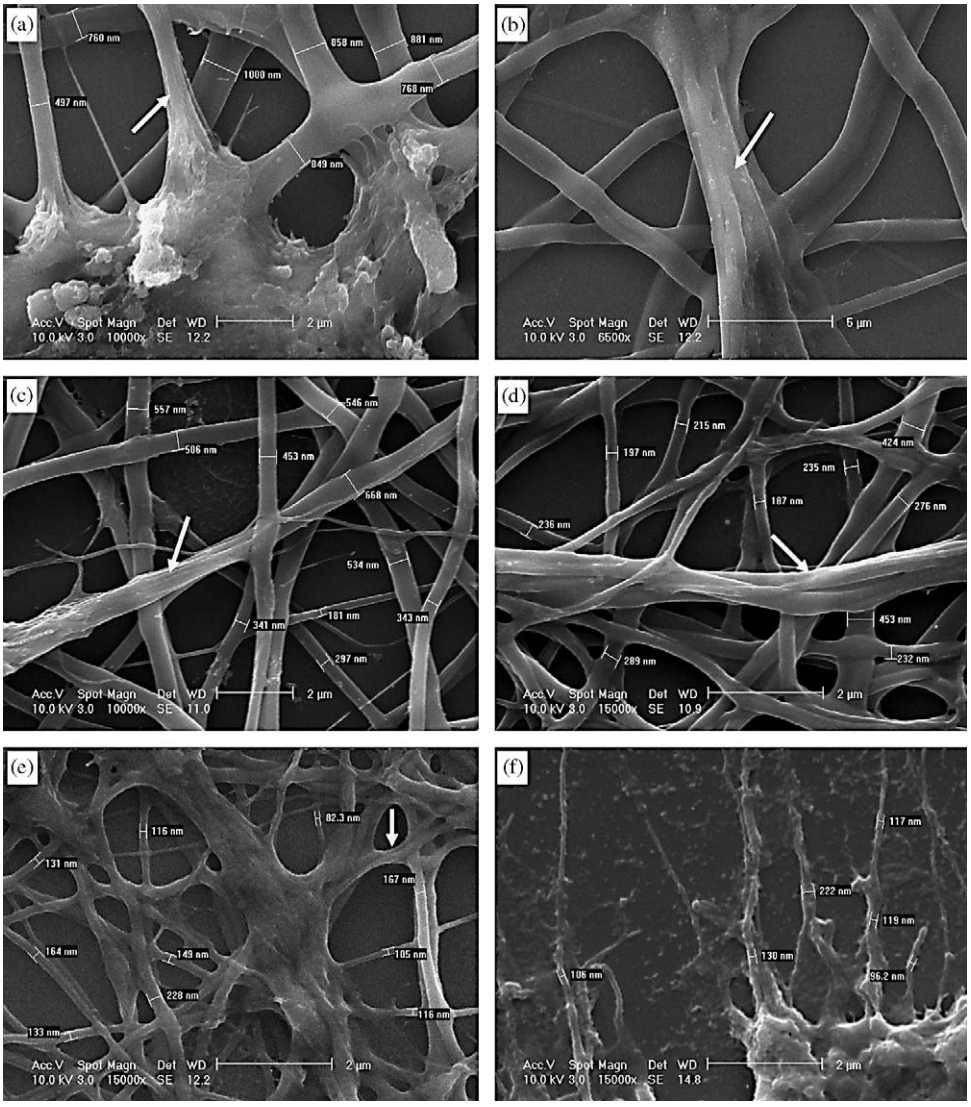

Fig. 8. SEM micrographs of H9c2 cells cultured on fibrous substrates: (a) cell pseudopodia on gelatin fibers; cell pseudopodia grew along; (b) gelatin fibers; (c) 15:85; (d)30:70; (e) 45:55 PANi-gelatin blend fibers; and (f) cell pseudopodia on glass flat surface.

and neuronal tissue engineering, we have further explored the use of electroactive polymers. In this paper we demonstrate for the first time the feasibility to generate a novel type of biocompatible fibrous scaffolds by blending an inherently conductive form of plyaniline (C-PANi) with biopolymeric macromolecules, such as gelatin. The fiber size is reduced by approx. 1 order of magnitude to <100 nm by increasing the concentration of C-PANi in the blend. Physicochemical measurements of the tensile strength, elasticity and conductivity, in concert with inspection by light and electron microscopy, confirm the uniformity of the ensuing fibers. Our experimental data demonstrate that electrospun C-PANi-gelatin blend fibers are biocompatible, supporting attachment, migration, and proliferation of H9c2 rat cardiac myoblasts. This report represents the first stage in our long-term strategy of culturing cells on "intelligent" nano-fibrous scaffolds containing electroactive polymers. In later stages, we will focus on studying the effects of electrical stimulation on the functionality of H9c2 myoblast cells for cardiac tissue engineering.

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