Electrospun Functionalized Polyaniline Copolymer-Based Nanofibers with Potential Application in Tissue Engineering[a]

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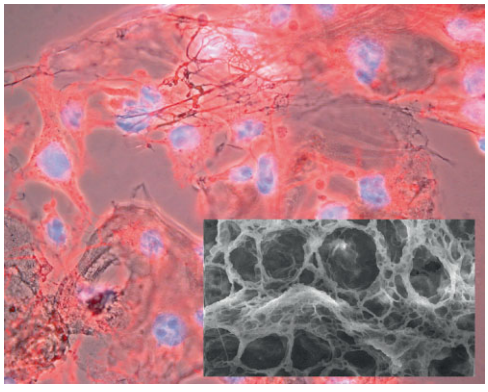
Nanofibrous blends of HCl-doped poly(aniline-*co*-3-aminobenzoic acid) (3ABAPANI) copolymer and poly(lactic acid) (PLA) were fabricated by electrospinning solutions of the polymers, in varying relative proportions, in dimethyl sulfoxide/tetrahydrofuran mixture. The morphology, mechanical and electrical properties of the nanofibers were characterized and an assessment of their bioactivity performed. To assess cell morphology and biocompatibility, pure PLA and 3ABAPANI-PLA nanofibrous mats were deposited in the form of three-dimensional networks with a high degree of connectivity, on glass substrates, and their ability to promote proliferation of COS-1 fibroblast cells was determined. The nanofibrous electrospun 3ABAPANI-PLA blends gave enhanced cell growth, potent antimicrobial capability against *Staphylococcus aureus* and electrical conductivity. This new class of nanofibrous blends can potentially be employed as tissue engineering scaffolds, and in particular have showed promise as the basis of a new generation of functional wound dressings that may eliminate deficiencies of currently available antimicrobial dressings.



Introduction

Considerable effort has been directed toward developing scaffolds for tissue engineering using biodegradable and biocompatible synthetic or natural polymers such as gelatine, collagen, poly(L-lactide-*co*-ε-caprolactone) and chitosan-based materials, which enhance in vitro cell growth.[1–4] Conducting polymers (CPs) are of interest for tissue engineering, because new technologies being developed will require biomaterials that not only physically support tissue growth, but are electrically conductive and thus able to stimulate specific cell functions or trigger cell responses. Polyaniline (PANI) and polypyrrole (PPy) are effective for carrying current in biological environments and are consequently being considered for locally delivering electrical stimuli at the site of damaged tissue to promote wound healing.[5–7] Studies with PANI have demonstrated that it supports adhesion and proliferation of H9c2 cardiac myoblasts and enhances in vitro neurite extension.[8–10] Although these studies highlight the potential use of CPs in regulating cell responses, the limited

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[a] Supporting information for this article is available at the bottom of the article's abstract page, which can be accessed from the journal's homepage at http://www.mbs-journal.de, or from the author.

DOI: 10.1002/mabi.201000237

processability of CPs is an obstacle for fabrication in a form suitable for cell culture.

Electrospinning can produce nanofibers or nanofibrous assemblies of CP/thermoplastic blends, in which the diameters of the nanofibers are typically two to three orders of magnitude smaller than the diameters of fibers prepared by conventional spinning techniques. The main limiting factor in production of CP nanofibers by electrospinning has been preparing CP solutions with sufficiently high concentration. That problem can be solved by blending CP with known fiber-forming polymers to obtain high viscosity solutions from which CP-containing blend nanofibers can be electrospun. The insolubility of CPs such as PANI, in most common solvents can be circumvented by copolymerizing aniline with substituted anilines that impart solubility to the resulting functionalized PANI copolymers (fPANIs).[11,12] Copolymerization of aniline with aminobenzoic acids (ABAs) gives copolymers that are soluble in basic aqueous media, and in polar solvents such as *N*-methyl-2-pyrrolidone (NMP) and dimethyl sulfoxide (DMSO).[11]

Films containing PANI have been found to have antibacterial activity against the growth of non-antibiotic resistant *Escherichia coli* and *Staphylococcal* organisms.[13] These films contained 1–10 wt.% of PANI relative to the amount of polyvinyl alcohol or polyethylene used because of the processability problems that would result if higher amounts of PANI were used. Gizdavic-Nikolaidis et al.[14] recent discovered potent antimicrobial effects of fPANIs on a broad range of antibiotic-sensitive and resistant Gram-negative and Gram-positive pathogenic bacteria including *Staphylococcus aureus*, *E. coli*, *Pseudomonas aeruginosa*, *Salmonella enteric*, *Enterococcus*, *S. sciuri*, *Enterobacter* sp., *Campylobacter jejuni*, and others. The active materials, used in small proportions (0.03–1 wt.%), show very high and rapid killing effects for the tested microorganisms (10^{12} CFU $\cdot$ mL^{-1}). The materials are used in such small proportion that their cost is low, and they can be incorporated in or coated on conventional polymers. They are mostly insoluble in water, stable to sterilization at 121 °C, and their antimicrobial activity has been shown in preliminary testing to permit multiple usage.

The aim of the present work was to prepare nanofibrous blends of the emeraldine salt (ES) form of poly(aniline-*co*-3-aminobenzoic acid) (3ABAPANI) with poly(lactic acid) by electrospinning, to obtain enhanced cell growth coupled with potent antimicrobial capability.

Experimental Part

Solution Preparation

Poly(aniline-*co*-3-aminobenzoic acid) (3ABAPANI) as the emeraldine base (EB) form was synthesized from a comonomer mixture with equimolar proportions of aniline and 3ABA, using potassium iodate (KIO_3) as oxidizing agent and hydrochloric acid. The synthesis gave a 50–60% yield of polymers with enhanced solubility.[11,15,16] The ES form was prepared by adding 0.1 g of synthesized 3ABAPANI (EB) to HCl solution (100 mL, 1 mol $\cdot$ L^{-1}), and allowing the mixture to stand for 24 h. The sample was then filtered, washed repeatedly with distilled water, and vacuum dried at 40 °C overnight.

Nanofibers were electrospun from pure PLA (4 wt.%) and a series of mixtures of PLA and 3ABAPANI, with 3ABAPANI (ES):PLA mass ratios 5:95, 15:85, 30:70, and 45:55, dissolved in DMSO/THF (50:50) mixture. The total polymer concentration in each electrospinning solution was maintained at 4 wt.%. The choice of solvent was dictated primarily by the finding that all of the PLA/3ABAPANI mixtures dissolved in the DMSO/THF mixture to give solutions with sufficiently high solute concentration to make production of electrospun, bead-free nanofibers possible. In analogous work on PLA/PANI mixtures Patra et al.[17] utilized a similar combination of volatile ($CHCl_3$) and relatively non-volatile dimethylformamide (bp 153 °C) solvents.

Electrospinning Parameters

Nanofibrous mats were obtained with solution delivery rate 2 mL $\cdot$ h^{-1}, and 10–15 kV applied to the metallic needle. Smaller voltage was insufficient to overcome the surface tension and viscoelastic forces of the polymer solution. The distance between the metallic needle and collector was in the range 6–8 cm.

Complete removal of the relatively non-volatile DMSO (bp 189 °C) from the electrospun nanofibers was accomplished by using a heated collector to facilitate evaporation of DMSO, and by thoroughly rinsing the nanofiber deposits with phosphate buffered saline (PBS). The ability of the nanofibers to support cell growth was regarded as confirmation of the absence of residual DMSO.

Contact Angle Measurements

Static contact angles of distilled water on the surface of the electrospun nanofibers were measured with a Cam 100 optical contact angle meter (KSV Instruments, Monroe, CT, USA), equipped with a CCD camera. This measurement was used to evaluate the hydrophilicity/hydrophobicity of the electrospun nanofibers with varying 3ABAPANI:PLA weight ratios. The electrospun nanofibers were deposited on cellophane film to cover an area $\approx 10 \times 10$ mm^2. About 5×10^{-6} L of distilled water was pipetted onto the electrospun nanofiber surface. Temporal images of the droplet were taken, and the contact angle was calculated by computer analysis of the acquired images.

Mechanical Property and Conductivity Measurements

Apparent compressive strength (CS) of electrospun PLA and 3ABAPANI-PLA nanofibrous mats were determined with a Rheometric Scientific Mark IV instrument. It was difficult to form dogbone specimens that were sufficiently robust to enable handling for tensile testing, so an alternative procedure was adopted that allowed compression testing to be done. The

electrospun nanofibrous mats were deposited directly on the metal disk sample holder designed for the DMTA instrument.

Conductivity was measured using the 4-probe technique (Jandal Multi Height Four-Point Probe with DC current source), by means of which two pairs of contacts are used to measure the conductivity at ambient temperature.

Cell Culture

African Green Monkey fibroblast COS-1 cells (ATCC CRL-1650) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% foetal calf serum (Invitrogen) with 1% penicillin and streptomycin (Invitrogen) and incubated at 37 °C/5% CO_2. Cell proliferation was measured in a continuous fluorescence assay using Resazurin (Sigma–Aldrich). The nanofiber samples (3ABAPANI-PLA blends, PLA control and glass coverslip control) were autoclave sterilized and placed in a 24-well tissue culture plate (TCP). COS-1 cells were suspended in 1% w/v Resazurin (in complete DMEM) at 10^5 cells · mL^{-1} and 1 mL of suspended cells was aliquoted onto the nanofiber blends, glass control, and TCP control. The samples were incubated for 24 h and the Resazurin-containing media collected. The samples were washed with 1 mL complete DMEM and fresh complete DMEM containing 1% Resazurin was added to each sample. The collected sample was centrifuged at 1600 rpm for 5 min and duplicate 100×10^{-6} L aliquots were dispensed into a 96-well black microtitre plate. The fluorescence was read at emission and excitation wavelengths 530 and 590 nm, respectively, using a Synergy HT Multi-Mode Microplate Reader (BioTek). This procedure was repeated over the course of 4 days. For each well the data were normalized to the initial fluorescence readings at day 0.

Microscopy

To examine the biocompatibility of the nanofibers, African Green Monkey fibroblast COS-1 cells (ATCC CRL-1650) fibroblasts grown on PLA and 3ABAPANI-PLA blend nanofibers were examined using scanning electron microscopy (SEM). COS-1 cells were seeded onto glass coverslips coated in nanofibers at 10 000 cells per slide and grown for 3 days. The slides were washed with PBS and fixed with 2.5% glutadialdehyde for 1 h, then dehydrated in a series of 15, 30, 50, 80, and 100% ethanol washes. The samples were dried in a critical point dryer, coated with gold/palladium mixture, and examined by SEM.

To examine the biocompatibility of the nanofibers, the glass microscope slides upon which polymers had been deposited were autoclave sterilized and aseptically placed in sterile Petri dishes. African Green Monkey fibroblast COS-1 cells (ATCC CRL-1650) were seeded at 10^6 cells · mL^{-1} in plastic Petri dishes. Cells were incubated at 37 °C/5% CO_2 in air for 2 days. After incubation the slide was washed twice in PBS to remove media components. The cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. After fixation the slides were washed with PBS twice. The cells were incubated cells in Phalloidin-Texas red (Molecular Probes; Invitrogen) diluted 1:100 in PBS for 15 min. After the incubation period the slides were washed twice with PBS and allowed to air-dry for 15 min or until completely dry. 20×10^{-6} L of DAPI containing Prolong Gold (Molecular Probes; Invitrogen) was deposited on top of the cells then mounted with a coverslip and examined using epifluorescence microscopy. The same procedure was followed to stain cells with 5×10^{-6} M SYTO-9 and 30×10^{-6} M propidium iodide (PI, Baclight Invitrogen; 15 min, 37 °C, in PBS) which is followed by visualization by epifluorescence microscopy.

The antimicrobial activity of nanofiber samples (PLA control, 45:55 3ABAPANI-PLA) and a glass control was assessed as follows. The nanofiber blends were autoclave sterilized, then 1 mL of PBS containing 10^6 CFU · mL^{-1} *S. aureus* 6838 was deposited onto the surface of each sample. The slides were incubated for 24 h at 37 °C and >85% humidity. After incubation the slide was washed twice in PBS and the Baclight stain mixture was applied to the slides. The samples were covered and incubated at room temperature for 20 min. The samples were then covered with a coverslip and examined using epifluorescence microscopy.

Results and Discussion

Characterization of Electrospun Nanofibers

Nanofibrous blends of 3ABAPANI [a random copolymer formed from comonomer mixtures with equimolar propor-

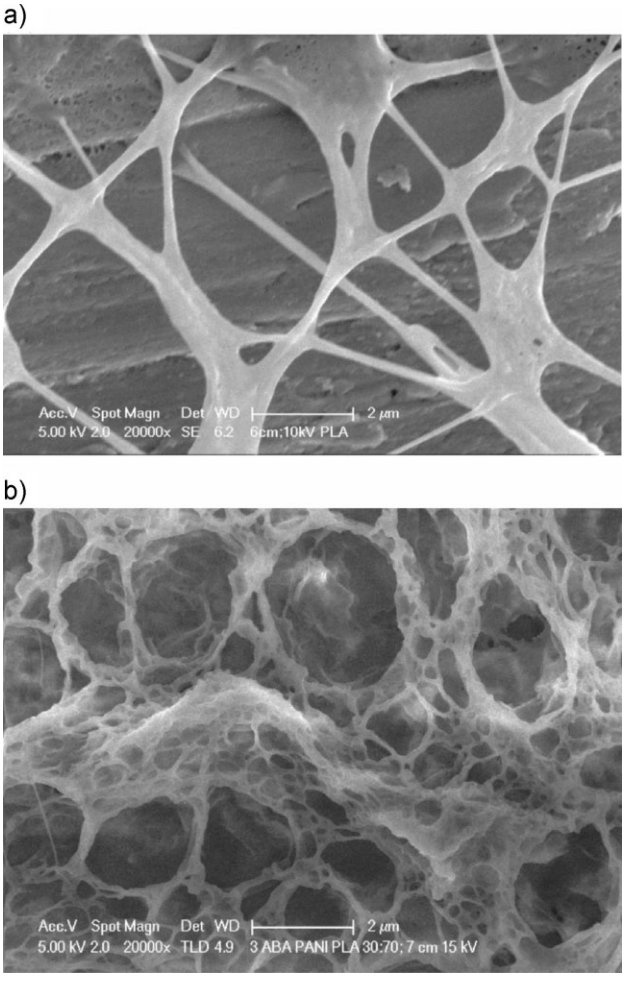

Figure 1. Scanning electron microscopy (SEM) micrographs of (a) pure PLA, (b) 30:70 3ABAPANI-PLA blend.

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DOI: 10.1002/mabi.201000237

Table 1. Mean nanofiber diameter range (d), apparent CS, conductivity (σ), and contact angle (θ) of PLA and 3ABAPANI-PLA nanofibrous mats.

Sample	Weight ratio[a)]	d[b)]	CS[b)]	σ	θ
		nm	MPa	mS · cm^{-1}	°
PLA	0:100	170 ± 50	12.0 ± 1.0	0.9	72.8
3ABAPANI-PLA	5:95	110 ± 35	15.5 ± 1.0	2.4	74.5
	15:85	80 ± 20	18.1 ± 1.0	3.9	78.6
	30:70	35 ± 8	28.8 ± 1.0	6.9	84.6
	45:55	25 ± 7	30.0 ± 1.0	8.1	93.5

[a)]3ABAPANI:PLA; [b)]mean ± std. deviation.

tions of aniline and 3-aminobenzoic acid (3ABA)], and PLA were prepared by electrospinning solutions of the polymers in a DMSO/THF mixture. The experimental variables were optimized by varying the voltage applied to the metallic needle, solution delivery rate and distance between the metallic needle and collector.

SEM micrographs of fabricated nanofibrous material showed neuron-like structures for pure PLA (Figure 1a) and three-dimensional honeycomb-like (Figure 1b and Supporting Information Figure S3) network structures for 3ABAPANI-PLA blends. The charge retained on the nanofibers and the characteristics of the collector screen influence the self-assembly of the fibers.[18,19] The presence of tetrahydrofuran (THF) as a solvent component may have had an influence on the nanostructured morphology: a similar morphology has been observed for a thermoplastic polyurethane electrospun from a solution in THF/DMF (60:40).[18] The conductivity of the polymer and the short throw distance may be contributing factors, because the nanofibrous mats were formed very rapidly compared to nanofibers of PLA alone. More detailed study of the factors responsible for the unusual morphology was outside the scope of the present study, which was focused on the potential of electrospun 3ABAPANI-PLA nanofibrous material to be utilized in tissue engineering. In addition, as noted elsewhere the extended network structure was thought to be advantageous, in terms of connectivity, to the more usual assemblies of single nanofibers that are produced by electrospinning. The 3ABAPANI-PLA honeycomb structures showed (Figure 1b) very high porosity, which was expected to facilitate cell growth.

The diameter range for PLA nanofibers was 170 ± 50 nm (see Supporting Information Figure S1 and Table 1). The biggest decrease in fiber diameter occurred when the 3ABAPANI-PLA ratio was changed from 15:85 to 30:70 (Figure 2). Nanofiber diameter tended to decrease (to a minimum of 15 nm) with increasing (a) electrospinning voltage, and (b) concentration of 3ABAPANI in solution (see Supporting Information Figure S2).

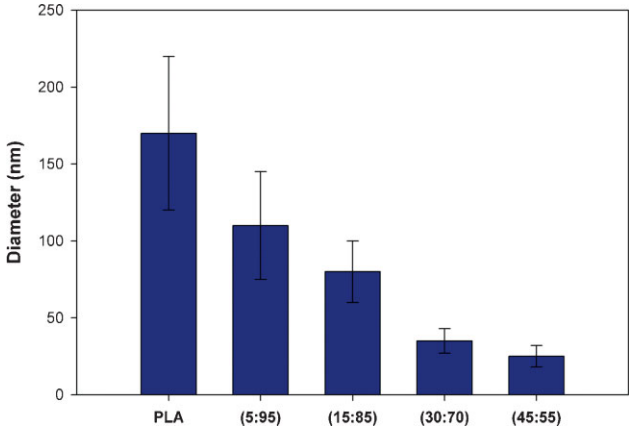

Figure 2. Average diameter of electrospun PLA and 3ABAPANI-PLA blend nanofibers.

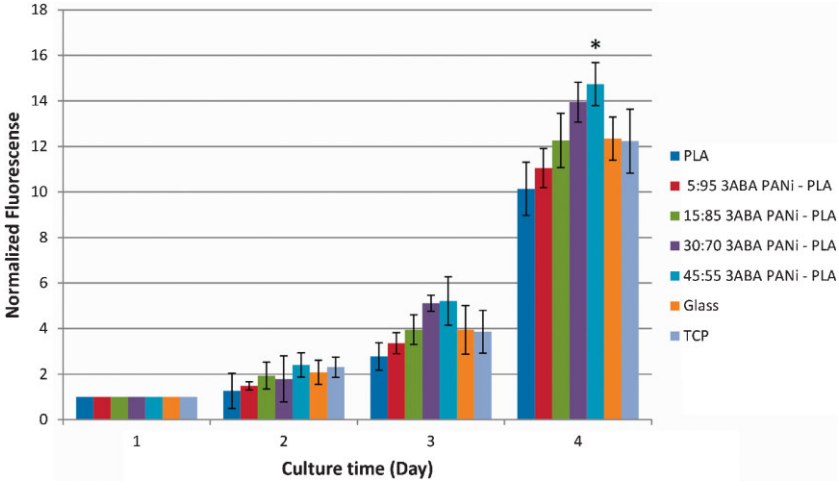

Figure 3. COS-1 fibroblast proliferation on 3ABAPANI-PLA substrates.

The conductivity (σ) of the blends (Table 1) increased linearly with CP content

$$\sigma/S\left[\mathrm{cm}^{-1}\right] = 1.806 \times 10^{-3} + 0.148w; R^2 = 0.97 \quad (1)$$

where w is the wt.% CP.

With increase in the proportion of 3ABAPANI in the blend the conductivity increased to 8.1 $\mathrm{mS \cdot cm^{-1}}$ which is in agreement with the previously published value for PANI-gelatine blend.[10] The apparent CS of the fibrous scaffolds also increased with increase in 3ABAPANI content in the blend from 12 MPa for pure PLA fibers to 30 MPa for 45:55 weight ratio of 3ABAPANI to PLA (Table 1).

The contact angle measurements showed that nanofibrous mats of both PLA and 3ABAPANI blend, deposited on cellophane film, were substantially more hydrophobic than the cellophane substrate, for which the water contact angle was measured as 42.8°. Furthermore, the contact angle, hence hydrophobicity increased markedly with increase in proportion of 3ABAPANI. Variation of the proportion of 3ABAPANI in the 3ABAPANI-PLA blend thus provides control of the hydrophilicity/hydrophobicity of the nanofibrous mat.

The hypothesis that a multitude of cell functions (including proliferation) can be modulated through electrical stimulation has been extensively tested experimentally, and a variety of conductive polymers has been utilized to provide electrical stimulation, including PANI as an electrospun blend with gelatine.[10] The honeycomb structure provides a continuous conducting path through the whole network and is virtually free from resistive contacts. By contrast, conduction in the usual form of nanofiber assembly is through essentially point contacts between nanofibers. The conductivity of the nanofibrous honeycomb networks (of the order $10^{-2}\,\mathrm{S \cdot cm^{-1}}$) should facilitate electrical stimulation of damaged tissue due to the continuous conducting network.

Cell Proliferation and Biocompatibility

To assess cell morphology and biocompatibility, 3ABAPANI-PLA nanofibrous mat was deposited on glass substrates (Figure 3), and proliferation of COS-1 fibroblast cells was measured using Resazurin assay. COS-1 fibroblasts were seeded at $10^5\,\mathrm{cells \cdot mL^{-1}}$ on each 3ABAPANI-PLA nanofibrous substrate and AB fluorescence measured over a period of 4 days. COS-1 fibroblasts proliferated similarly on each of the experimental substrates, although a significant increase in proliferation ($p<0.05$) was observed on the 3ABAPANI-PLA nanofibrous mat compared to tissue culture-treated polystyrene (TCP) and glass substrates. The fibrous substrates, being rougher, provided more surface sites for cell growth than did the smooth glass and TCP surfaces.

To examine the biocompatibility of 3ABAPANI-PLA nanofibers, COS-1 fibroblasts grown on the nanofibers were examined using SEM. The SEM images show interesting effects of the nanofibers on fibroblast growth. Fibroblasts have a planar morphology and project a dendritic network of pseudopodia as they grow. Figure 4c shows a fibroblast with normal morphology on 30:70

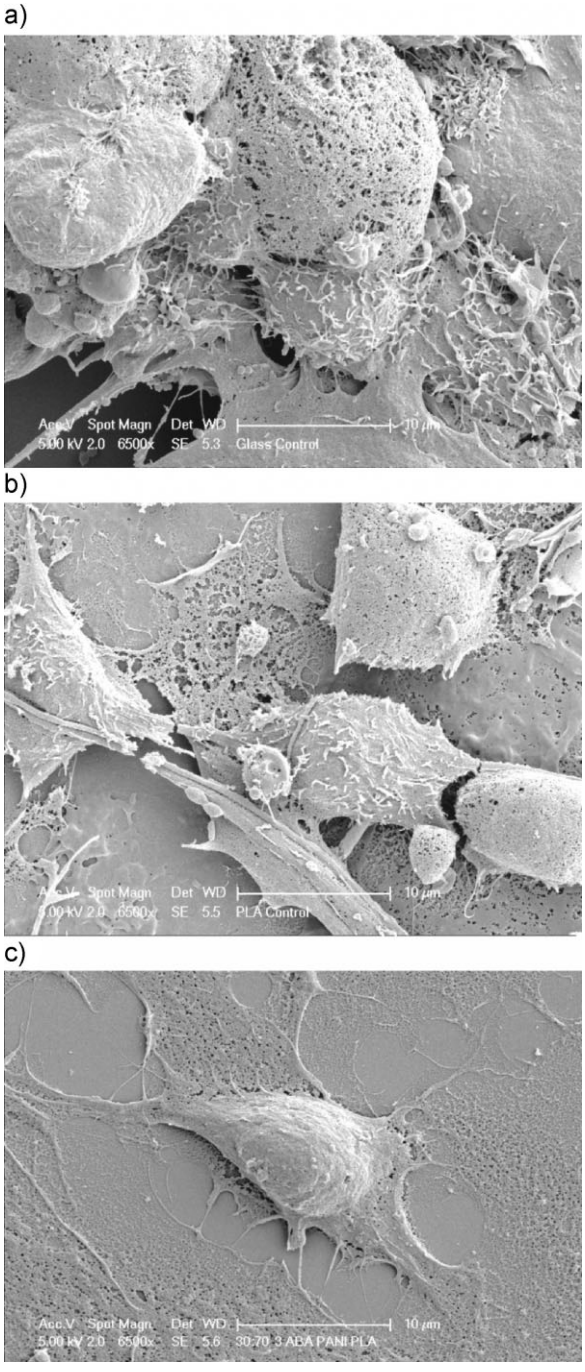

Figure 4. Cell morphology and biocompatibility. SEM micrographs of COS-1 cells growing on (a) glass, (b) PLA, and (c) 3ABAPANI-PLA substrates.

Macromol. Biosci. **2010**, *10*, 1424–1431

Macromolecular Journals

DOI: 10.1002/mabi.201000237

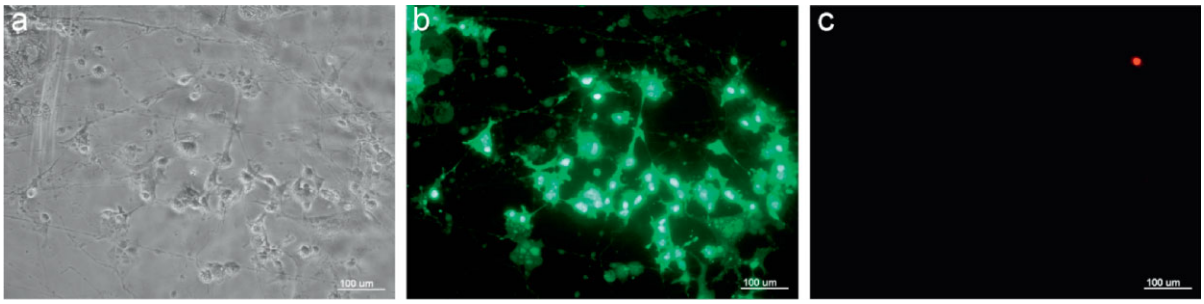

Figure 5. COS-1 fibroblast cells growth on 3ABAPANI-PLA 30:70. (a) Phase contrast, (b) SYTO-9, and (c) PI.

3ABAPANI-PLA substrate. However, on the glass and PLA substrates (Figure 4a and b) the fibroblasts were more spherical and in some cases smaller than the average fibroblast size (≈20 μm). As this is not normal behavior for fibroblasts it may indicate that these substrates (glass and PLA) had an adverse effect on their growth. This observation also correlates with the proliferation assay which showed that fibroblast proliferation was lower on glass and PLA compared to the 3ABAPANI-PLA substrates.

Cell viability was determined using BacLight to visualize viable cells by epifluorescence microscopy. The BacLight kit utilizes the nucleic acid stains SYTO-9 and PI. Five separate fields of view were photographed for each sample and the total amount of live and dead cells counted to calculate the total viable COS-1 fibroblasts on each sample. The majority of COS-1 fibroblasts (>99%) were viable and had proliferated on 3ABAPANI-PLA nanofibrous mat, confirming biocompatibility of the electrospun materials. There was no significant difference between cell viability with each treatment. As an example, the COS-1 fibroblasts growth on 3ABAPANI-PLA 45:55 blend fibers is shown in Figure 5.

Figure 6 presents cell attachment and proliferation on 30:70 3ABAPANI-PLA nanofibrous mat, visualized by phase-contrast microscope and after appropriate staining using phalloidin-texas red and DAPI by epifluorescence microscopy. COS-1 fibroblast cells grown on the nanofibrous mat demonstrate the ability of the cells to adhere and proliferate on the nanofibers, confirming their biocompatibility.

Antimicrobial Properties of Electrospun Nanofibers

We performed an experiment to visually examine the antimicrobial effect of the nanofibers on the viability of bacteria using BacLight (Figure 7). The green stain (SYTO-9) is able to penetrate intact bacteria, while the red stain (PI) can penetrate the bacteria only through damaged membranes of dead cells. The viable *S. aureus* 6838 bacteria on the PLA and glass controls completely cover the field of view, and there are very few dead bacteria in the control samples. There were clearly fewer viable bacteria on the 3ABAPANI-PLA nanofibers 45:55 (31%) compared to the glass (98%) or PLA (95%) controls which confirms the antimicrobial activity of the 3ABAPANI-PLA nanofibers.

The ability of the 3ABAPANI-PLA nanofibrous blends to both provide a scaffold for normal cell growth and kill pathogenic bacteria, makes it feasible for these materials to be used as the basis for wound dressings that perform both of those functions, and act as a physical barrier to prevent further tissue damage. The disadvantage of conventional antimicrobial wound dressings is that they usually contain silver or iodine which are non-adherent, and a secondary dressing is needed. Additionally, they cannot be used for patients with sensitivity to iodine or silver.[20] Silver-based dressings are also not recommended for use together with topical medications, as silver turns black when oxidized and may stain or discolor periwound tissue.

Iodine and silver-based antiseptics have been used widely to reduce bacterial colonization of wounds, but they bind quickly to organic matter and so become inactive, and in addition resistance of microorganisms to silver and iodine has been reported.[21,22] Moreover, iodine and silver-based antiseptics may also delay the healing process. Studies have been performed to examine the

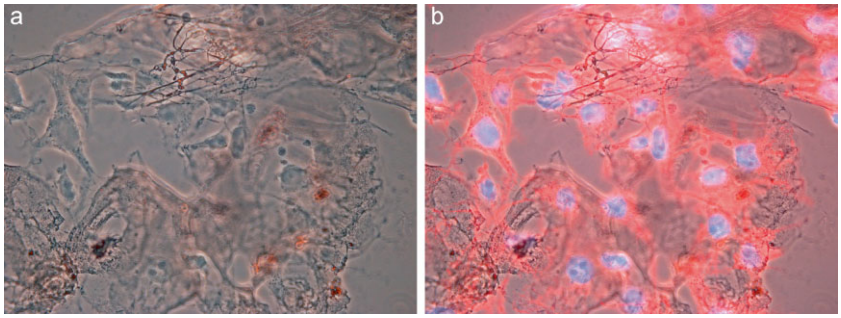

Figure 6. COS-1 cell attachment and proliferation on 3ABAPANI-PLA nanofibers visualized by (a) phase-contrast microscope and (b) after staining using phalloidin-texas red and DAPI by epifluorescence microscopy.

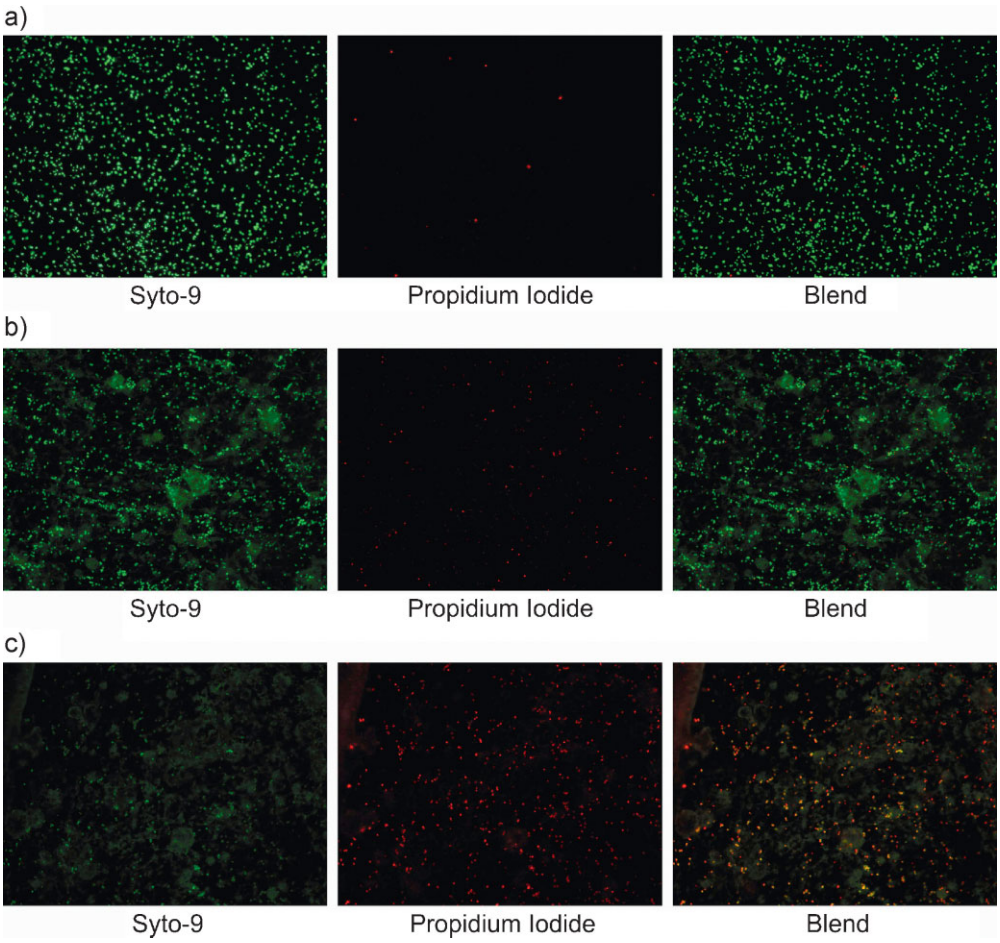

Figure 7. Antimicrobial activity against *S. aureus* 6838 at 10^6 CFU · mL^{-1}. Substrate [% live cells]: (a) glass [98], (b) PLA [95], (c) 45:55 3ABAPANI-PLA [31].

effect of these antiseptics on wound healing using in vitro and in vivo models. Povidone-iodine has been shown to be lethal to fibroblasts and keratinocytes at levels that are bactericidal in vitro,[23,24] and application of povidone-iodine to wounds has been shown to delay healing in vivo.[25] Silver ions have been shown to inhibit fibroblast and keratinocyte proliferation at bactericidal levels[26] and oxidize disulfide bonds present in matrix metalloproteinases, inhibiting the activity of a group of enzymes that play an important role in tissue remodeling.[27] Very recently, it has been reported[28] that silver nanoparticles are directly involved in mitochondrial toxicity and DNA damage. There is no evidence, to date, that the 3ABAPANI-PLA nanofibrous blends possess any of these disadvantages.

Conclusion

In conclusion, 3ABAPANI nanofibrous blends with PLA, prepared from a solution of the polymers in DMSO/THF mixed solvent, showed honeycomb structures that appear, from preliminary tests on wounds, to expedite healing of damaged tissue. A variety of CPs have been utilized to provide electrical stimulation of cells, including PANI in the form of electrospun PANI/gelatin blend nanofibers. The conductive honeycomb structure provides a continuous conducting path through the whole three-dimensional network, which can be expected to facilitate electrical stimulation of damaged tissue. The nanofibrous blends allow mammalian cells to attach and proliferate, while killing pathogenic bacteria cells, and are novel conductive materials that are potentially well suited for use as biocompatible scaffolds for tissue engineering and as antimicrobial wound dressings that have the advantage of being able to kill microorganisms without use of an antiseptic.

Acknowledgements: The authors acknowledge financial support from the *Foundation for Research, Science and Technology, New Zealand* (Contract nos. UOAX0606 and UOAX0812).

DOI: 10.1002/mabi.201000237

Received: June 11, 2010; Revised: June 27, 2010; Published online: September 14, 2010; DOI: 10.1002/mabi.201000237

Keywords: biomaterials; conducting polymers; nanofibers; tissue engineering

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