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Characterization of neural stem cells on electrospun poly(ε-caprolactone) submicron scaffolds: evaluating their potential in neural tissue engineering

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Abstract—Development of biomaterials with specific characteristics to influence cell behaviour has played an important role in exploiting strategies to promote nerve regeneration. The effect of three-dimensional (3D) non-woven electrospun poly(ε-caprolactone) (PCL) scaffolds on the behaviour of rat brain-derived neural stem cells (NSCs) is reported. The interaction of NSCs on the randomly orientated submicron (PCL) fibrous scaffolds, with an average fibre diameter of 750 ± 100 nm, was investigated. The PCL scaffolds were modified with ethylenediamine (ED) to determine if amino functionalisation and changes in surface tension of the fibrous scaffolds affected the proliferation and differentiation characteristics of NSCs. Surface tension of the fibrous scaffold increased upon treatment with ED which was attributed to amine moieties present on the surface of the fibres. Although surface treatment did not change the differentiation of the NSCs, the modified scaffolds were more hydrophilic, resulting in a significant increase in the number of adhered cells, and increased spreading throughout the entirety of the scaffold. When the NSCs were seeded on the PCL scaffolds in the presence of 10% FBS, the stem cells differentiated primarily into oligodendrocytes, indicating that electrospun PCL has the capacity to direct the differentiation of NSCs towards a specific lineage. The data presented here is useful for the development of electrospun biomaterial scaffolds for neural tissue engineering, to regulate the proliferation and differentiation of NSCs.

Key words: Electrospinning; neural tissue engineering; nerve regeneration; neural stem cells; oligodendrocyte.

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INTRODUCTION

The spinal cord is the main communication channel between the brain and the rest of the body. Consequently, when the central nervous system (CNS) is damaged by neurodegenerative diseases or trauma there is minimal-to-no functional recovery with severe physiological consequences for the patient [1–4]. Despite extensive research there is still no definitive treatment for neural tract regeneration in the CNS. Hence, an active area of neural tissue engineering strategies strive to develop a more permissive environment to promote nerve regeneration [3, 5]. Moreover, recent advances in neurology, such as the discovery that neurogenesis occurs in adults [6–8], coupled with neural tissue engineering advances in combination strategies may provide a more optimistic outlook.

A wide variety of biomaterials have been evaluated for use in neural tissue engineering to provide a relevant matrix, for cell adhesion and guidance. Poly(α-hydroxy esters) have received considerable attention, as they are bioresorbable and have been shown to be biocompatible with many cell types [9–12]. Recent studies have focused on fabricating complex 3-dimensional (3D) scaffolds or hydrogels such as macroporous scaffolds that mimic the extracellular matrix (ECM) [13, 14] and injectable hydrogels for localized drug delivery [15]. Other complex 3D scaffolds were fabricated with ledges and fibres in an attempt to guide cell and neurite outgrowth [16, 17]. These strategies seek to provide cells with a permissive environment to optimize regeneration. Moreover, nanofibrous scaffolds are particularly attractive in neural tissue engineering as they are inherently porous and have large surface-area-to-volume ratios [18]. Thus, these fibrous scaffolds encourage cellular adhesion and proliferation by offering a variety of topographical features to guide an advancing growth cone to adhere. Such scaffolds can be fabricated in a variety of different ways, including self assembly [19] and thermally-induced phase separation [9]. Fibrous scaffolds of desired sizes and dimensions can be easily tailored for selected tissue engineering applications using electrospinning [16].

NSCs may play a pivotal role in cell replacement therapies for the repair of the spinal cord and only a handful of studies have been conducted involving the culture of NSCs on electrospun scaffolds. Recent work by Yang *et al.* cultured NSCs (from cerebellum, C17.2) in the presence of 10% foetal calf serum and 5% horse serum on electrospun poly(L-lactic acid) scaffolds, and evaluated differentiation on aligned and random nanofibres [16]. They investigated the differentiation of NSCs into neurons and found that when the electrospun fibres were aligned, neurites grew along the aligned fibres. It was also discovered that the differentiation of NSCs into neurons depended on the fibre diameter, with more cells differentiating into neurons on nanofibres than microfibres. This study showed considerable evidence that electrospun scaffolds can promote the preferential differentiation of NSCs based on fibre size.

While the longer-term goal is to utilize cell–scaffold constructs that will assist in functional recovery of the damaged spinal cord *in vivo*, this study investigates the differentiation profile of NSCs within 3D electrospun poly(ε-caprolactone) (PCL)

scaffolds *in vitro*. Stem cell morphology, differentiation and distribution through the electrospun PCL scaffolds were investigated as a function of aminolysis of the PCL fibres. The introduction of amino groups changes the hydrophilicty of the fibres and is also known to affect the level of adhesion and differentiation of hematopoietic stem/progenitor cells [20]. In this study, we are particularly interested in neural stem/progenitor cells derived from the subependyma of the lateral ventricle forebrain in rats because they are capable of self-renewal and are multipotent differentiating into neurons, astrocytes and oligodendrocytes [21, 22]. For a regenerative neural tissue engineering strategy of the spinal cord, a combination of cell types will likely be required. For example, the close interaction of axons and oligodendrocytes required for functional regeneration highlights this point [23].

MATERIALS AND METHODS

All chemicals were used as received unless otherwise stated. Distilled and deionized water was obtained using a Millipore Milli-RO 10 Plus and Milli-Q UF Plus system, and used at 18 MΩ resistance.

Materials and scaffold fabrication

Poly(ε-caprolactone) (PCL) was obtained from Absorbable Polymers. Polymer solutions of 10% (w/v) were prepared by dissolving PCL in 2 ml chloroform (Merck) and methanol (Merck) at a ratio of 3:1 (v/v). The solutions were placed into a glass syringe (10 ml) with a 21-guage needle for electrospinning and a flow rate of 0.397 ml/h was set. A 20 kV accelerating voltage was applied by a high voltage power supply and a 15 cm working distance was utilized. The scaffolds were collected in an aluminum container (10 cm × 10 cm × 2 cm) filled with 20 ml phosphate-buffered saline (PBS). The non-woven nanofibrous scaffolds were then dried in a vacuum oven for 2 days at 30°C. The scaffolds were then aminolysed in 0.05 M ethylenediamine (ED, Sigma–Aldrich) in 2-isopropanol (IPA, Caledon Laboratories) and allowed to react at 20°C for 10 to 40 min. The treated samples were then washed 3 times in ice water and soaked for 1 h in distilled water on ice. The samples were dried overnight in a vacuum oven, and cut into 5 mm square scaffolds and disinfected in 70% ethanol for cell-culture preparation.

Scaffold characterisation

The non-woven scaffolds were characterised by scanning electron microscopy (SEM). Scaffolds were gold-coated using a sputter coater (Balzers SCD-005, BAL-TEC). A 25 mA current was used for a total coating time of 180 s. The SEM (Hitachi S-570) was operated at 15 kV. The average diameter of the fibres was determined by measuring a total of 20 fibres on three different samples.

The surface tensions of the nano-fibre scaffolds were determined by a modified contact angle measurement using a VCA — optima™ (AST Products) at room temperature. A modified sessile drop method was employed using solutions of different IPA concentrations with MilliQ water to detect the changes in drop penetration through the scaffold and the surface tension determined through comparison with standard curves [24, 25]. Statistical analysis was conducted using SigmaStat for Windows version 3.0.3©, on a sample size ($n = 6$). A one-way analysis of variance was employed using Tukey's post-hoc testing.

Surface functionalization was characterized using X-ray photoelectron spectroscopy (XPS) equipped with a monochromatic Al Kα source operated at 12 kV and 25 mA. The samples were placed on round coverslips in preparation for analysis. The XPS was conducted using a Leybold MAX 200 X-ray Photoelectron Spectrometer. The samples were measured at an emission angle of 90° from the surface. Three different areas on each sample were measured.

The surface functionalization of the scaffold was also characterized by fluorescence using 3-(4-carboxybenzoyl)-quinoline-2-carboxaldehyde (CBQCA, Invitrogen™). The PCL scaffolds that were previously aminolysed were dissolved in a DMSO/THF (19:1) solution at a concentration of 2.6 mg/ml to which 5 mg/ml of sodium cyanide (NaCN) in 0.1 M triethanolamine (TEA) buffer was added. The CBQCA was dissolved in DMSO at a concentration of 10 mg/ml. 100 µl of polymer solution and 25 µl of CBQCA and NaCN in buffer were placed in a 96-well plate in triplicate. The samples were then incubated for 48 h while shaking at 37°C. They were then visualized at 560 nm using a fluorescence plate reader (Spectra MAX Gemini EM, Molecular Devices).

Isolation and culture of adult brain-derived stem cells

Brain-derived neural stem cells (NSCs) were isolated from the subependymal region of the lateral ventricles of adult rat forebrains as previously described [22]. Brain-derived neurospheres appeared within 2–3 weeks of harvesting, after which cells were passaged every week at a 1:3 dilution. The stem/progenitor cells were used after 3–5 passages.

The PCL scaffolds (about 200 µm thick) were placed into sterile 24-well polystyrene culture dishes in triplicate, and were cut to completely cover the bottom of the wells to ensure that the cells remained on the scaffold during seeding. They were then soaked in Neurobasal Media (NBM, Invitrogen™) in the absence of mitogens overnight. The NBM contains 2% B27 Supplement (Gibco-Invitrogen™), 2 mM L-glutamine (Gibco-Invitrogen™), and 100 ug/ml penicillin–streptomycin (P/S, Gibco-Invitrogen™). The neurobasal media was removed immediately before the NSCs were seeded.

Laminin was used as a positive control because it has been shown to promote cell attachment. Laminin (0.15 µg/ml) was coated on glass cover slips, by submerging the glass cover slips overnight in the solution and removing it 4 h prior to culture to allow for drying. These cover slips were placed in the culture dishes in triplicate and

served as positive controls. NSCs were then dissociated and seeded at a density of 5×10^5 cells/ml on the electrospun scaffolds in proliferating media that consisted of the above NBM with 20 ng/ml each of recombinant human epidermal growth factor (EGF) and recombinant human basic fibroblast growth factor (bFGF), and 2 ng/ml heparin (Sigma–Aldrich). For the laminin controls a density of 5×10^4 cells/ml was employed to avoid over-population, as this is a 2D environment. A separate plate with controls was used to study the effect of differentiation media on NSCs. The same cell densities were used; however, 10% foetal bovine serum (FBS) was added to the NBM instead of the mitogens. 1 ml of NBM with dissociated cells of the above densities was added to each of the wells.

The NSCs were then cultured for 7 days at 37°C, 5% CO_2 and 100% humidity, before being fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature. The samples were then washed 3 times in PBS (pH 7.4) for 5 min while being gently rocked. The scaffolds were then mounted on their end using frozen section medium (Stephens Scientific), before being sectioned into 20 μm slices using a cryostat (Leica CM 3050S).

Immunocytochemistry

The immunocytochemistry procedures were modified specifically for this application. Sections were firmly attached to glass slides by baking at 37°C for 30 min. The NSCs were again fixed using 4% PFA for 2 min and washed in PBS 3 times for 5 min in a static environment. The scaffolds were then permeabilised in 0.1% Triton X-100 for 30 min and washed 3 times in PBS. Non-specific antibody binding was then blocked with a solution of 4% normal goat serum (NGS) for 1 h and were then exposed to the following antibodies: mouse anti-nestin (1:1000; BD Pharmingen™) for undifferentiated cells, mouse anti-GFAP Cy3 conjugate (1:400; Sigma–Aldrich) for astrocytes, mouse anti-CNPase (1:100; Chemicon) for oliodendrocytes, and rabbit anti-neurofilament (NF 200) (1:500; Chemicon) for neurons. The primary antibodies were added to PBS containing 1% NGS and 0.1% Triton X-100 and were allowed to react at 4°C overnight. The sections were then washed 3 times with PBS with 1% Tween (Sigma–Aldrich) added. The secondary antibodies, AlexaFluor 546 Cy3 (1:500; Molecular Probes) for CNPase and nestin, AlexaFluor 647 (1:500; Molecular Probes) for the NF200, were applied for 1 h before again being washed with the PBS-Tween solution. The nuclei were then stained with 10 μM Hoechst 33342 for 5 min, followed by the final 3 washes with the PBS-Tween solution. ProLong Gold anti-fade reagent (Molecular Probes) was then added to the samples and coverslipped. The cells were imaged using a laser scanning confocal microscope (LSCM, Leica TCS SP2) with a 50× air lens, NA 0.8. The LSCM images presented in this paper (Fig. 4) were pseudo-coloured, meaning that the Cy3 stain used to mark some of the cells (e.g., astrocytes in Fig. 4C and 4D) are depicted as green rather than the orange-red that it emits. The cells were counted manually and the differentiation statistics were conducted using SigmaStat for Windows version 3.0.3© and reported as mean ± standard deviation ($n = 9$). A pair wise Tukey's

post-hoc test was also employed. The cell numbers are presented as a percent differentiation, calculated as a ratio of the number of positively marked cells to the total number of nuclei.

RESULTS AND DISCUSSION

Electrospinning and surface modification.

The electrospun PCL scaffolds consisted of randomly orientated non-woven fibres shown in Fig. 1, with the average and standard deviation of fibre diameter throughout the scaffold being 750 ± 100 nm ($n = 3$). To investigate the effect of surface hydrophilicity and chemistry on NSCs adhesion, differentiation and colonization, the surface of the PCL scaffold surfaces were modified by partial aminolyzation for 10–40 min. While it has been previously shown on 2D surfaces of poly(lactic-co-glycolic acid) (PLGA) that the overall amount of aminolyzation did not change after 10 min of reaction with ED [26], we were uncertain whether this would translate to the 3D electrospun PCL scaffold. To assess changes in surface hydrophilicity, we used a modified contact angle measurement technique to calculate the surface tension on the 3D electrospun scaffolds [25]. Using a series of water-IPA solutions of different surface tensions, it has been shown that there is a critical surface tension when a liquid shows no adsorption delay, and that this is analogous to critical wetting surface tension of a solid surface, including fibrous membranes [27]. Table 1 shows the change in surface tension of the PCL electrospun scaffolds after treatment with ED. When the PCL was subjected to treatment for 10 min the surface tension increased from 36 ± 1 dyne/cm to 42 ± 2 dyne/cm. This change was likely to have resulted from nucleophilic attack of carbonyl carbon, which forms a positively charged tetrahedral intermediate [26], and formation of amine (NH_2) moieties on the surface of the fibres. With increasing treatment times the surface

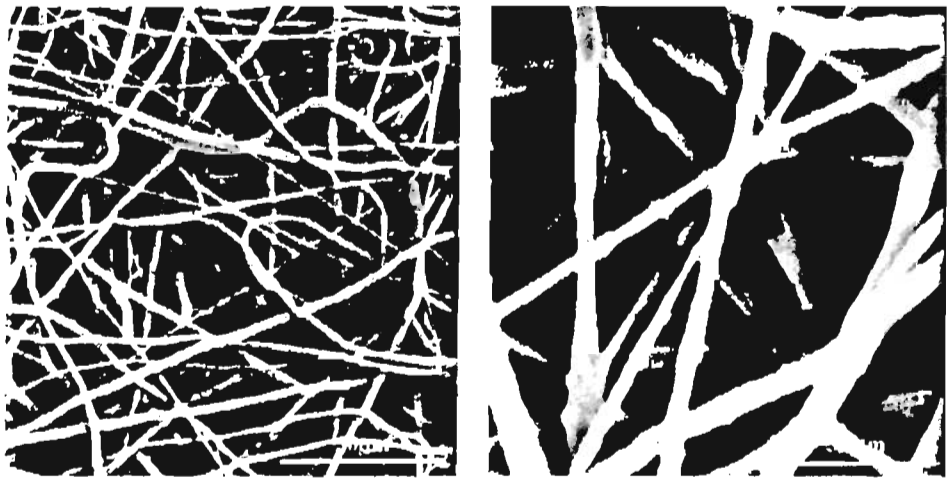

Figure 1. SEM images of the electrospun PCL scaffolds at different magnifications. The average fibre size in the PCL scaffolds is 750 ± 100 nm (mean ± standard deviation, $n = 3$).

Table 1.
Surface tension of the PCL scaffolds after ED modification

Material	Treatment time in ED (min)	Surface tension (dyne/cm)
PCL	No treatment	36 ± 1
	10	$42 \pm 2^*$
	20	$42 \pm 2^*$
	40	$42 \pm 2^*$

Surface tension is represented as mean ± standard deviation ($n = 6$). ANOVA with Tukey's post-hoc test revealed significant differences between each of the treatments and the no treatment.
$^*P < 0.05$.

tension remained at 42 ± 2 dyne/cm, indicating that 10 min of aminiolysation is sufficient to achieve the maximum surface energy using this method.

To gain a greater understanding of the surface chemistry, modified surfaces were characterized by both X-ray photoelectron spectroscopy (XPS) and fluorescence using CBQCA. While the N_{1s} peak was detected by XPS, the difference relative to the background was minimal. This is due to the random error of XPS being 1–2% of the measured value of the elements, and the low concentration of amino groups using ED. For this reason the surface chemistry functionalization was characterized by fluorescence using CBQCA. From the fluorescence intensity of CBQCA, 10 min of aminolyzation using 0.05 M ED resulted in 0.1 nmol NH_2/g PCL polymer. A significant effect on surface tension can be achieved with a low concentration of amines on the scaffold surface.

NSCs response to electrospun scaffolds

To better understand how cells would respond to electrospun PCL scaffolds, dissociated NSCs were plated on PCL scaffolds in either the presence of serum (a differentiation factor) or EGF/FGF2/heparin (mitogens). The survival and colonization of the cells were investigated relative to laminin controls, in an attempt to determine how the dissociated NSCs interacted with the fibrous scaffold. Figure 2 highlights the distribution of cells (marked with Hoechst 33342) within the unmodified (Fig. 2A) and modified (0.05 M ED; Fig. 2B) scaffolds. On the unmodified scaffold, there is minimal penetration of the NSCs within the scaffold. Furthermore, the cells do not remain in their dissociated state, instead forming large spheres on the surface of the scaffold. In contrast, on the modified scaffold, the cells penetrate throughout the entire scaffold and remain dissociated. The greater cell interaction within the modified *vs.* unmodified scaffold is likely due to the increased hydrophilicity although the concurrent effect of changes in surface chemistry cannot be discounted. As a result there may either be a greater amount of adsorbed proteins on the modified surfaces or the conformation of proteins may be optimal for cell adhesion, spreading and proliferation. As shown in Table 2, more cells adhered to modified than unmodified scaffolds in both proliferation and differentiation culture conditions. As expected, cells cultured in the mitogens

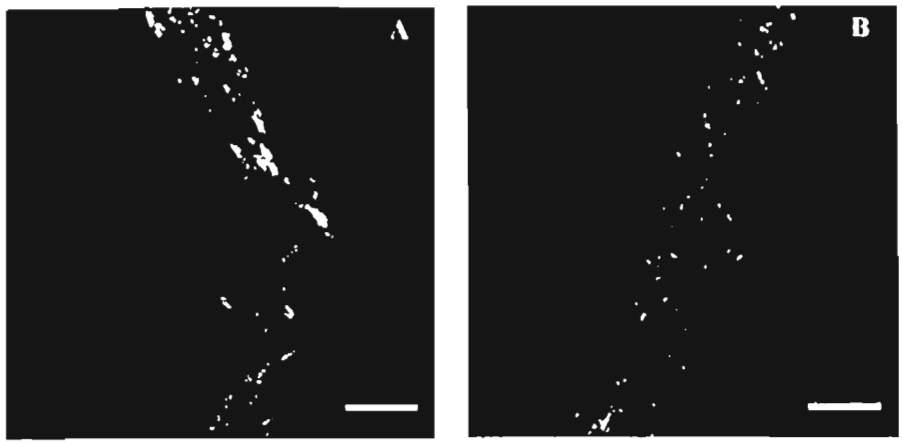

Figure 2. The circular shapes represent the nuclei (stained blue with Hoechst 33342) of NSCs seeded on the PCL scaffolds (imaged using transmission, shown as strands in red). (A) A neurosphere is re-formed on the surface of the unmodified PCL scaffold. (B) Cell spreading occurs throughout the PCL scaffold after modification with ED. Scale = 50 μm. This figure is published in colour at http://www.ingenta.com

Table 2.
Cell number on the unmodified and modified scaffolds that were subjected to different culture conditions

Material (PCL scaffold)	Culture condition	Mean cell number per 84 100 μm^2
Unmodified	Mitogens	190 ± 50
Modified	Mitogens	480 ± 130
Unmodified	10% FBS	30 ± 15
Modified	10% FBS	140 ± 30

The results are reported as mean ± standard deviation ($n = 9$). ANOVA with pair-wise Tukey's post-hoc testing revealed all groups are significantly different ($P < 0.05$), apart from the cells cultured on the unmodified PCL scaffold in the presence of mitogens compared to the modified PCL in the presence of 10% FBS.

(EGF/bFGF/heparin) had a significantly greater number of adhered cells than those cultured in the differentiating FBS media.

To gain a greater perspective of the impact of aminolyzation of PCL fibres on NSC differentiation, the cells were labeled with antibodies against GFAP (for astrocytes), CNPase (for oligodendrocytes), NF200 (for neurons) and nestin (for neural progenitor cells). As shown in Fig. 3, PCL modification did not impact stem cell differentiation. Interestingly, in differentiating serum, there was evidence of astrocytes, oligodendrocytes, and neural progenitor cells, but not neurons on PCL scaffolds; however, in control samples cultured on laminin, there was evidence of neurons and the proportion of cells was different. Importantly, there were 75–80% oligodendrocytes on PCL fibres, which is significantly greater than the 40–41% oligodendrocytes on laminin controls. The differentiation to oligodendrocytes in the presence of serum was not significantly different on modified *vs.* unmodified PCL fibres, suggesting that PCL fibres promote the preferential differentiation to

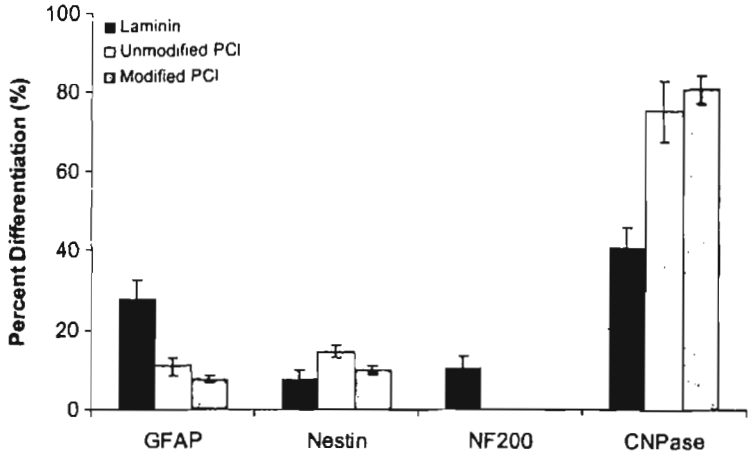

Figure 3. Stem cell differentiation on PCL scaffolds and controls cultured in the presence of serum after 7 days *in vitro*. GFAP stains for astrocytes, Nestin for neural progenitor cells, NF200 for neurons and CNPase for oligodendrocytes.

oligodendrocytes. Oligodendrocytes are important for remyelination, making this phenotypic response on PCL of interest. This process of remyelination has recently been observed after adult neural precursor cells were engrafted within the white matter of an injured spinal cord [28]. Delivery of NSCs to a damaged region within the CNS with a highly porous 3D scaffold may be a desirable approach for regeneration.

While surface modification did not alter the differentiation profile of the NSCs (*cf.*, Fig. 3), the morphologies of the differentiated NSCs cultured on the PCL scaffold were investigated and compared to laminin control sample using LSCM as shown in Fig. 4. Morphologies of the differentiated NSCs, as shown in Fig. 4, agree with the differentiation profile shown in Fig. 3. NSCs differentiated into neurons on laminin control as shown in Fig. 4A by NF200 staining, but no neurons were found on the PCL scaffold (see Fig. 4B). The morphology of astrocytes on laminin and PCL scaffolds have similarities, as shown in Fig. 4C and 4D. NSCs that have differentiated to astrocytes show long thin processes extending from cell bodies regardless of the culturing surface, i.e., laminin or PCL scaffold. However, there was increased cellular spreading on the 2D positive control (Fig. 4C) compared to the 3D PCL scaffold. This is possibly due to there being superior interfacial effects between the cells and the laminin 2D control. Furthermore, the density of the cells seeded on the 2D control is much lower, which also results in the appearance of greater spreading. The morphology of the differentiated oligodendrocytes on laminin or PCL scaffold (as shown in Fig. 4E and 4F, respectively) are similar. Thus, the differentiation study suggests that neither surface chemistry nor hydrophilicity of PCL has a significant influence on directing stem cell differentiation and morphology, but rather PCL fibre geometry has the dominant effect.

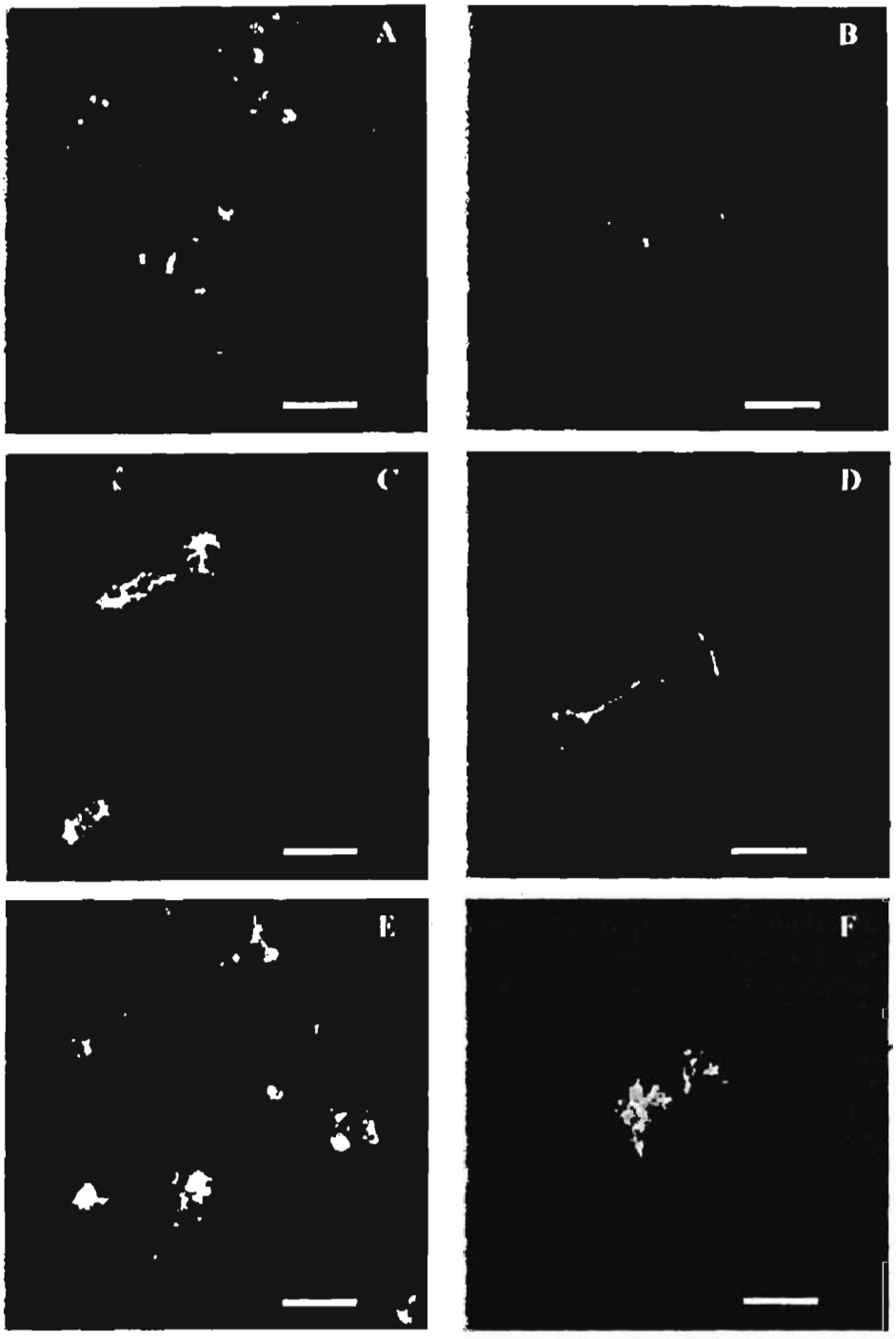

Figure 4. Morphology of the differentiated NSCs on the unmodified PCL scaffold culture in the presence of 10% FBS. The images have been pseudo-coloured. (A) Control sample that was dual stained with nestin (green) and NF200 (red). (B) NSCs cultured on the PCL scaffolds, which were also dual stained but only expressed nestin. (C) Control sample that expresses GFAP (green). (D) NSCs cultured on the PCL scaffolds, which also expressed the GFAP marker. (E) Control sample that expressed CNPase (green). (F) NSCs cultured on the PCL scaffold, which also expressed the CNPase marker. Scale = 50 μm. This figure is published in colour at http://www.ingenta.com

CONCLUSIONS

In this study, 3D non-woven PCL scaffolds were prepared by electrospinning and chemically modified with ED. While this surface modification did not influence NSC differentiation, it had a profound effect on the number of adherent cells. Interestingly PCL electrospun fibre scaffolds influenced the differentiation of NSCs primarily into oligodendrocytes, demonstrating lineage specificity as a function of the scaffold's physical and not chemical properties.

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