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Biomaterials for Spinal Cord Regeneration: Outgrowth of Presumptive Neuronal Precursors on Electrospun Poly(ε)-caprolactone Scaffolds Microlayered with Alternating Polyelectrolytes

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Abstract—**The aim of this study was to assess the feasibility of electrospun poly(ε)-caprolactone (PCL) scaffolds treated with alternating poly-electrolytes as a controllable three-dimensional adhesive substrate for neuronal progenitors. Unmodified PCL surfaces were generally not supportive of mouse embryonic stem cell (mESC) colony adhesion. However, scaffolds surfaced using layer-by-layer (LbL) deposition of heparin/poly-L-lysine encouraged better local adhesion of mESC colonies, and networking of monolayers containing nestin-positive presumptive neurons, similar to laminin coated controls, as observed by immuno-fluorescence microscopy. Confocal microscopy further revealed depth-wise penetration of mESC nestin-positive cell populations, and orientation along gross topographical features in the LbL scaffolds. LbL deposition therefore appears to provide a satisfactory adhesive substrate for contact and mechanical guidance of neuronal outgrowth in three-dimensions.**

I. INTRODUCTION

Sites of acute spinal cord injury often present clinically as mechanical damage (eg bending or compression) leading to contusions, and a stage-wise inflammation process [1]. In the long-term, injury sites can become infiltrated by fibrous scar tissue, which forms a physical barrier to cell body and axonal reconnection. Tissue engineering strategies in the repair of spinal cord injury have therefore been aimed at construction of synthetic porous scaffold biomaterials that allow in-growth and physical support of regenerating axons during the repair process [2]. These strategies are now overlapping heavily with stem cell biology, allowing the development of more integrated cell-based models and treatment approaches including the use of scaffolds as stem cell carriers, sources of growth factors or pharmacological agents to encourage local axon regeneration [3]. Biocompatability of polymer scaffolds has therefore become a parameter of major importance, for support of appropriate local biochemical and biomechanical cues, and suppression of acute inflammatory responses [4].

Electrospun polymer scaffolds have been become widely used for these applications, as a tool for soft tissue engineering. The method allows the formation of deformable three-dimensional meshes of controlled porosity, thickness, fibre size, biodegradability and biomolecular activity via functionalization with side groups such as growth factors, signalling peptides or matrix molecules [5]. As a biocompatible synthetic substrate, electrospun PCL has been demonstrated to successfully support the mechanical responses of cell types of the adult central nervous system such as cortical neurons, glial cells and Schwann cells [6]. Our group recently showed the polymer to be particularly useful in directing adult neuronal stem cell differentiation in a rat model [7].

The present investigation examined the feasibility of the same polymer to support neuronal progenitor outgrowth from murine embryonic stem cells, following surface modification with multilayered deposition of polyelectrolytes heparin (glycosaminoglycan) and poly-L-lysine (polypeptide). Both molecules are integral components of basement membrane substrates [8], with differential cell adhesion effects. LbL surfacing is not a new approach [9], but has been effectively applied in this study as a method for controlling micro-surface thickness and composition of a unique polymer-polyelectrolyte combination, in a three dimensional fibrous scaffold.

II. MATERIALS AND METHODS

A. Electrospinning of Scaffolds

PCL beads (80,000 kDa, Birmingham Polymers, USA) were dissolved in a 3:1 mixture of chloroform and methanol (Sigma-Aldrich, USA) and electrospun from an 18-gauge needle (flow rate = 0.6 ml/hour, working distance = 15 cm, gain = 15 kV). Randomly oriented fibres were deposited and collected on aluminium foil. After 3 hours, the fibres were carefully removed from collection surfaces and stored in plastic storage bags until required. Scaffolds were trimmed manually to a spherical disc shape of approximately 0.75 mm diameter in preparation for surface treatment and subsequent cell culture.

B. Layer-by Layer Deposition of polyelectrolytes

LbL surface treatment of scaffolds was achieved using alternating layers of polyelectrolytes poly-L-lysine (PLL)

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(net positive charge) and heparin (net negative charge). PLL was chosen because of its relatively low cytotoxicity, defined hydrophilicity and cell-surface adhesivenss, as determined previously [10]. Electrospun scaffolds were first surface-activated with 10 mg/ml polyethyleneimine (PEI) (Sigma-Aldrich, USA) for 1 hour and washed three times in a bioshaker (BioSam, USA). Scaffolds were then soaked in solutions of 4 mg/ml heparin in PBS solution (Sigma-Aldrich, USA) for 15 minutes, then rinsed for 5 minutes in PBS only. Scaffolds were then soaked in 3 mg/ml PLL (Sigma-Aldrich, USA) in PBS for 20 minutes, then rinsed. This process was repeated for 5 bilayers. Upon completion of LbL deposition, treated scaffolds were cross-linked in 2% ethyl-3-3- dimethyl- amino- propyl-carbodiimide hydrochloride solution (EDC, Aldrich, USA). All samples were washed in three times in PBS, followed by distilled water.

C. *Characterization of LbL scaffolds*

Scaffolds were mounted on aluminium sample holders and gold sputtered (Dynavac, Australia). Imaging was obtained using a SEM S570 (Hitachi, Japan) with a working distance of 7mm and gain of 15kV. Fibre diameters and angles (relative to the x-axis of micrographs) were characterised by measuring 20 fibres from 3 separate fields of view taken using ImageJ software (NIH Image). LbL treated scaffolds were also analysed using X-ray photoelectron spectroscopy (XPS) for elemental composition profiles.

D. *Preparation of scaffolds for cell culture*

After distilled water rinses with hourly changes, scaffolds were further washed and sterilized in 100% ethanol (Merck, Australia) and left to dry overnight under HEPA filtered, laminar air-flow conditions. Scaffolds (pre-cut from larger electospun sheets) were then soaked overnight in Neurobasal A medium (NBM, Invitrogen, Australia) without supplements, in individual wells of 48-well culture plates (Falcon, Becton Dickinson, USA). Scaffolds were weighed down with custom made, pre-sterilized tubular glass inserts (Monash Scientific Glassware, Australia). Control wells were prepared by aliquoting 100 µl of 7.1 µg/ml laminin (Sigma, USA) and also allowed to air-dry prior to addition of NBM on the day of culture.

E. *Embryonic stem cell culture*

Immediately before cell seeding, the NBM soaking medium was replaced by 150 µl of fresh NBM culture medium (supplemented with 1% ITS-G (Invitrogen), 200mM L-glutamine, 1% N2, 1% B27, penicillin (50IU/ml) and streptomycin (50 µg/ml), 10 ng/ml mFGF). Plates were then pre-equilibrated under standard culture conditions (37°C, 5% CO2 in humidified air within a thermostatted incubator). Briefly, cultures were prepared from frozen stocks of mouse (129 strain) embryonic stem cells (mESCs). mESCs were passaged to 50-60% confluency the day before generating embryonic bodies (EBs). EBs were cultured for 5 days in IMDM plus F12 nutrient mix (Invitrogen, Australia), supplemented with BSA fraction V (0,5%; Sigma, Australia), synthetic lipids (Invitrogen) 1% ITS-X (Invitrogen), MTG (SIGMA), L-Glutamine (2mM/L), penicillin (50IU/ml) and streptomycin (50 µg/ml). EBs were disaggregated mechanically, then re-seeded to control (uncoated and laminin coated wells) and experimental (LbL scaffold substrate) culture groups for a further 5-7 days (period of embryoid body formation) at an average density of $3\text{-}5x10^4$ viable cells per well, as determined previously by manually counting of disaggregated cells stained with Trypan Blue dye.

F. *Immunostaining of cultured scaffolds*

Plates were removed from culture, the NBM was carefully aspirated and wells were rinsed several times with phosphate buffered saline (PBS, Invitrogen). Disturbances to well contents were minimised when adding or aspirating solution (no mechanical agitation was used). Scaffolds were fixed with 4% paraformaldehyde in PBS, incubated with blocking buffer (PBS supplemented with 3% normal goat serum and 2% Triton X100 (Sigma, Australia) for 1 hour at room temperature to prevent non-specific antibody binding, followed by rinsing with PBS. Wells were then incubated (2 hours, 37°C) with a solution of primary mouse anti-Nestin antibody, to detect the expression of nestin, a marker of cytoskeletal intermediate filaments in presumptive neurones of the developing brain and spinal cord [11]. After careful repeated rinsing in PBS, a solution of secondary antibody (1/1000 dilution in PBS, see appendix A for details) was then added (2 hours, 37°C). After further rinsing, all wells were incubated with a solution of blue fluorescent nuclear stain (DAPI, Invitrogen, Australia) for 10 minutes, followed by further rinsing in PBS before mounting. Immediately after fixation and staining, scaffolds were carefully removed from wells using microforceps and mounted on glass microscope slides. Individual scaffolds were moistened with a few drops of Dako aqueous mountant (Dako, USA), overlaid with a glass coverslip and allowed to set for 30 minutes. Mounted scaffold samples were then stored in the dark at 4°C before imaging.

G. *Fluorescence and confocal microscopy*

Higher resolution fluorescence images were obtained using a motorised epi-fluoresence microscope (Provis AX70, Olympus, Japan) with a motorised stage using filter cubes for red, green and blue emission spectra. Scanning of entire scaffold areas was performed using a second epifluoresence microscope (Dot-slide XL20, Olympus, Japan) with automatic scanning and rastering capability. Cross-sectional fluorescence images were taken using an argon laser scanning confocal microscrope (Leica TCS NT, Germany) using an argon laser and red, green emission filter cubes. Emission wavelengths for red and green secondary antibodies were set at 488 and 568 nm. Acquisition of DAPI was obtained at 330nm excitation and 450 nm emission wavelengths. Image analysis and 3D imagine reconstruction of z-stacks were by compiled using ImageJ (NIH), AnalySIS (Leica) and Imaris software. Alternative imaging to detect both PCL fibres and attached cells was performed using a multi-photon confocal microscope (Leica SP, Germany) with input wavelength of

633nm. This wavelength provided an alternative method of reflectance visualization of individual nanofibres in three dimensions.

III. RESULTS

A. Structural characterization of electrospun PCL

SEM of electrospun scaffolds revealed intrinsic details of fibre morphology (Fig. 1). Mean fibre diameters of scaffolds used in the present study were determined to be in 100-500nm, with mean inter-fibre angles of 98° ± 40. Multiphoton microscopy revealed a similar range of fibre orientations, although angle calculations were less reliable due to shadow effects. Surface characterization of the same scaffolds after LbL deposition revealed spectroscopic evidence of elemental sulphur (indicating Heparin) and nitrogen (evidence of poly-L-lysine), with relatively large oxygen peaks, also corresponding the presence covalently bonded oxygen residues of both polyelectrolytes, relative to baseline (Fig. 2).

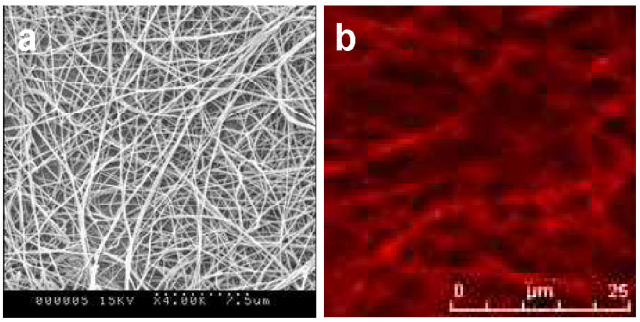

Fig. 1. (a) SEM (Mag = x1300) and (b) multi-photon images of a randomly aligned electrospun PCL scaffold.

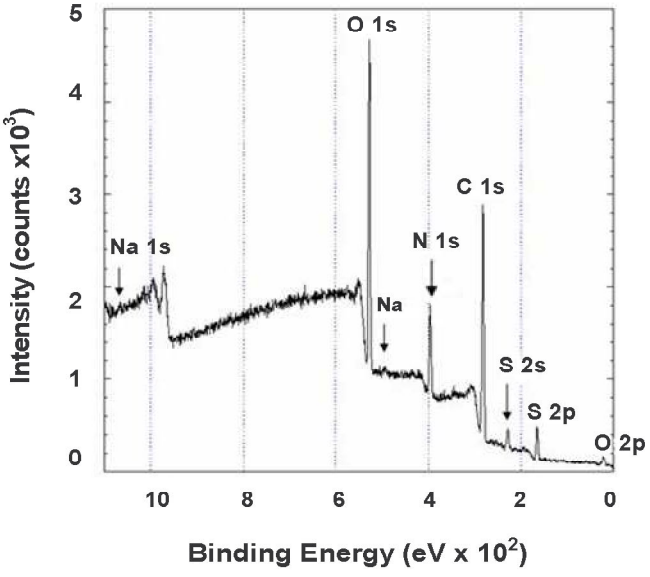

Fig. 2. XPS scan of a PCL scaffold after surface deposition of polyelectrolytes. S = sulphur (corresponding to residues on heparin; N = nitrogen (poly-L-lysine amino residues).

B. Neurite outgrowth on scaffolds in vitro in two- and three- dimensions

Compared to the polystyrene and laminin coated multi-well plate controls (Fig. 3a, b) uncoated PCL scaffolds revealed very low densities of colonies remaining adherent to the PCL scaffolds. Fluorescence microscopy of LbL scaffolds revealed adherent colonies with interconnecting processes containing nestin-postive presumptive neurons (Fig. 3c, d). Closer investigation of specific areas using confocal microscopy revealed that nestin-positive monolayers also orientated themselves with respect to topographical features in the LbL scaffolds, with depth-wise penetration and anchorage (Fig. 4).

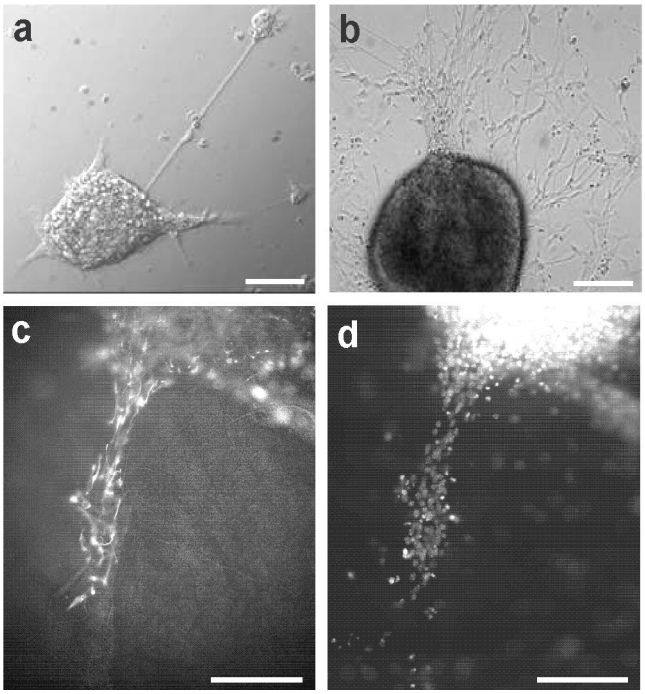

Fig. 3. Phase contrast micrographs of neuron-like branching from mESCs on (a) uncoated and (b) laminin-coated polystyrene. Immunofluoresceonce images of (c) nestin-positive cells with neurite-like morphology (d) DAPI counterstained cell extension differentiating from the edge of a mESC colony. (Scale bar – 150 µm)

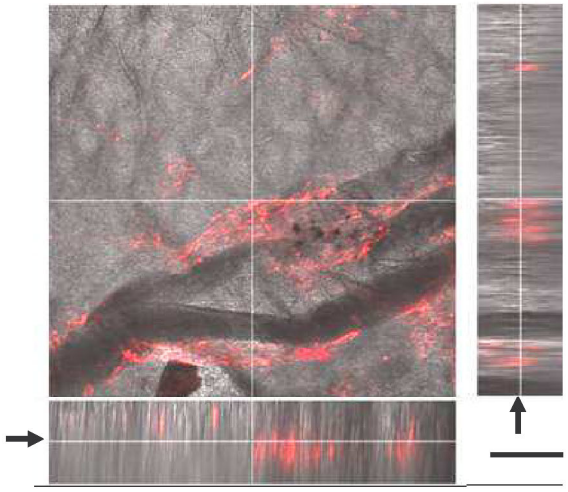

Fig. 4. Confocal micrograph of a LbL-coated PCL scaffold at sites containing nestin-positive cells orientated along topographical folds, with sub-surface penetration evident in side-views. (Scale bar =30 µm, arrowheads = surface level).

IV. DISCUSSION

Electrospun PCL scaffolds treated with LbL deposition of heparin/poly-L-lysine are supportive of two- and three-dimensional outgrowth of presumptive neuronal precursors from embryonic stem cells in vitro. Surface functionalization of a hydrophobic polymer with charged, hydrophilic coatings has been previously observed by our group to enhance surface adhesion and penetration [10]. The present result is novel because this observation has no previously been reported for this polymer/polyelectrolyte combination, in the context of differentiating presumptive neurons in vitro.

Of particular interest is the way in which cell monolayers containing presumptive neuronal precursors responded to the surface topography of the microlayered scaffolds. This response is relevant to recent reports of directional modulation of neurite outgrowth by scaffolds with micro-surface modifications such as conduits [12-14] for which the LbL is highly amenable as an alternative approach. Indeed, those studies used PCL and poly-L-lysine in alternative spatial configurations. The random nature of electrospun fibres remains one of the key points requiring optimization, toward the provision of geometrically well-aligned channels or conduits to mechanically entrain axons connections as in the native spinal cord.

The question of regulation of differentiation by alterative scaffold composition is more difficult to address, particularly owing to the heterogeneous cell fates present in differentiated cultures of embryonic stem cells. While neuronal differentiation is the default pathway for embryonic stem cells [15], it provides a useful testing ground to study the neurogenesis and contact guidance in vitro. Further investigations to support the present data would include the use of other immunotypic markers, particularly those of mature neurons (eg β_{III}-tubulin) combined with quantitative imaging of neuronal branching (planned). This marker was used, however non-specific immuno-labeling was observed in co-stained samples with strong nestin-positive staining (data not shown).

Directed differentiation of stem cell populations into identifiable neuronal progenitors requires depends in vitro relies on complex morphogenetic cues provided by growth factors and extracellular matrix molecules [15]. Both cues provide biochemical and biomechanical signalling that can have synergistic benefits on nerve regeneration in vivo [16]. Future studies will examine the utility of electrospun PCL with polyelectrolyte microlayering as a binding substrate for such factors, to provide local cues to adherent stem cell colonies. These would be useful in parallel with implantation studies in animal models of spinal cord damage.

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