Directionality and bipolarity of olfactory ensheathing cells on electrospun nanofibres.

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OLFACTORY ENSHEATHING CELLS ON ELECTROSPUN NANOFIBRES

Abstract

Aims: As a preliminary to construction of an olfactory ensheathing cells (OECs) bearing scaffold for bridging larger lesions in spinal cord, we have investigated the response of purified cultured OECs to varying diameter nanoscale fibres using FDA-approved, biodegradable poly(lactic-*co*-glycolic)-acid. **Materials & Methods**: Conventional electrospinning produced fibres of ≈700nm diameter (Nano-700) while nanocomposite electrospinning with quantum dots produced significantly more uniform fibres of a reduced diameter to ≈237nm (Nano-250). OECs from adult rat were FACS purified, cultured at low density on either a flat surface or a meshwork of randomly orientated Nano-700 and Nano-250 fibres, and assessed using cytomorphometric analysis of immunofluorescent confocal images and by scanning electron microscopy. **Results & Conclusion:** Compared with flat surface, culture on Nano-700 mesh increases cell attachment. Cells change from rounded to stellate forms in random orientation. Further size reduction to the Nano-250 favours bipolarity in cells with unidirectional orientation, observed in, previously reported, transplanted OECs bridge of small rat lesions.

INTRODUCTION

Experimental studies have shown the ability of transplanted adult olfactory ensheathing cells (OECs) to induce regeneration of severed nerve fibres across lesions of the spinal cord and spinal roots [1−7]. Current transplantation technology is to inject a suspension of cultured cells. Introduced in this way into small lesions of the rat spinal cord, the transplanted cells elongate for more than 100μm to form tubular structures which ensheathe the nerve fibres and carry them across the site of the lesion [8]. To produce such a bridge the transplanted cells depend on cues in the host tissue microenvironment which enable them to integrate with the host astroglial pathways so as to permit the entry of regenerating CNS axons into the bridge and their exit from it.

While these observations provide a proof of principle, the scale of the damage which is caused by human spinal cord injuries is much larger. If we are to be able to translate the small scale repairs achieved in experimental lesions to such a clinical setting, the numbers of cells which can be produced by current protocols is a serious limitation, and even if adequate numbers could be obtained, the large mass of transplanted cells would lack alignment cues. To cross this translational gap, the possibility of deploying the cells on synthetic bridging material may help to maximize the effect of limited number of cells available and at the same time provide physical cues for their alignment [9−12].

To achieve biocompatibility and effectiveness such materials should be able to simulate features of the *in vivo* microenvironments. In the olfactory system *in situ*, OECs lie in a structured microenvironment. Their outer surface, facing the olfactory nerve fibroblasts, is clothed by extracellular matrix (ECM; [13]. After transplantation OECs re-establish the same relationships with fibroblasts. Microarray studies show that OECs secrete a rich array of ECM molecules [14−17].

An accumulation of information in a number of different biological systems has shown the rich three-dimensional surface topography of the extracellular matrix (ECM; [18]) on the submicron scale. The interaction of cells with the ECM is a two way process. The ECM provides a scaffold for cell guidance, and conversely, cells remodel the ECM [19]. The adhesive and mechanical interactions of ECM with the cell surface link to intracellular mechanisms leading to migration, proliferation and differentiation [20,21]. The type of interaction between cells and surfaces has shown to be influenced by the spatial domains, structural composition and mechanical forces at the nanoscale [22].

Over the last few years nanotechnology has made it possible to fabricate biodegradable structures at a controlled submicron scale. Their application in biological systems has shown the effect of nanotopography on the behaviour of a variety of cells, including fibroblasts, astrocytes, oligodendrocytes and epithelial cells [23–26]. In the present study we used electrospinning of PLGA (poly(lactic-co-glycolic acid; [27–29], an FDA approved, biodegradable material, to produce fibres on the nanoscale. The addition of quantum dots, which has been reported to produce nanosized fibres [30], to the PLGA has enabled us to fabricate fibres in dimensions approaching those with which cells are associated in their natural environment in tissues.

Here we compare the attachment, morphology and directionality of purified OECs seeded at low density on flat surfaces with those on meshes of sterilized and functionalized nanoscale PLGA fibres.

MATERIAL AND METHODS

Fabrication of fibrous meshes using conventional and nanocomposite electrospinning

The various concentrations of 50/50 DL-lactide/glycolide copolymer (PLGA) (Purasorb® PDLG 5010; PURAC Biomaterials, Netherlands) were prepared using hexafluoroisopropanol, (HFIP; Merck, UK) as the solvent. The electrospinning protocol was previously described [31]. Briefly, the dissolved PLGA solution at the given concentration (below) was fed at a constant controlled feed rate through a stainless steel needle. The polymer solution was electrospun in an electrically charged environment at a fixed voltage. The tip-to-collector format was orientated in the horizontal direction at a constant distance of 30cm. Voltage was adjusted within the range of 8kV to 25kV at feed rates from 100µl/hr to 600µl/hr. After adjustment, a stable Taylor cone was formed at 9.7kV with feed rate 300µl/hr. Each electrospinning process was completed in two hours. Among the range of concentrations (3.0-7.2%; wt/wt) tested, 6% was found to be optimal for nano-scale fibres.

The strategy employed for nanocomposite electrospinning process is similar to that implemented in CdTe-PVA electrospun nanofibres with minor modifications [30,32]. Briefly, CdSe/ZnS (5.2nm in diameter and 610nm (i.e. red in colour)) quantum dots (core-shell type, Sigma-Aldrich UK) were added into fully dissolved 6% PLGA/HFIP. The nanocomposite solution was then stirred vigorously with a stirrer bar magnet overnight to ensure an even dispersion of the quantum dots in the solution. Concentrations of 1% and 2% CdSe/ZnS were tested. To prevent quantum dots aggregation within the viscous PLGA/HFIP solution, the solution was used immediately for electrospinning. The nanofibres were deposited using the same format as described above. A stable Taylor cone was formed at 11.2kV with feed rate of 400µl/hr. The distance of the tip to the electrode collector was set at 30cm. Each

electrospinning process was completed in two hours, and the mesh was placed on a membrane insert which was placed in a plastic culture dish.

Fibre Characterization using Scanning Electron Microscopy (SEM) and Digital Image Analysis

The electrospun fibre meshes were carefully mounted on the SEM stubs and sputter coated with platinum (Emitech K650TB, UK) prior to scanning microscopy imaging. SEM images were obtained using electron beam metrology and scanning electron microscopy system (Leica EBL40, Cambridge). Digital image analyses were performed using ImageJ (NIH) processing software. SEM micrographs were calibrated to a standardized size of 1280 x 1040 pixels. Briefly, fiber diameter and length were measured at minimum 35 different points along a randomly drawn straight line across the image. The method adopted to characterize the fibre diameter is length weighted mean fiber diameter, where the mean is based on a frequency distribution previously described [33,34], with some minor modifications. Briefly, taking into account the different lengths which exist among the fibres, each fiber diameter (d_i) was weighted to its length (L_i).   The log mean diameter, $\ln(\bar{d}_g)$ and the log standard deviation ($\ln \sigma_g$) of the frequency distribution of the length weighted fiber diameters were expressed as

$$\ln(\bar{d}_g) = \frac{1}{L_{total}} \sum L_i \ln(d_i)$$

and

$$\ln \sigma_g = \sqrt{\frac{\sum L_i (\ln d_i - \ln \bar{d}_g)^2}{L_{total}}}$$

Both values were used to compare the fibre diameters in various experimental conditions in electrospinning parameters. Graphs were plotted based on the lognormal probability density, using the mathematic software, Wolfram Mathematica (UK).

Sterilization and Surface Functionalization of Fibre Meshes

Three sterilisation processes were tested: ultraviolet (UV) radiation, ethanol and ethylene oxide (EtO). For UV sterilization, the PLGA fibre mesh was treated with ultraviolent ray type B, using a UV oven (Luzchem, UK) for 15min to 1h. For ethanol sterilization, the PLGA fibre mesh was soaked in a series of incremental percentages of ethanol: 30%, 50%, and 70% for 15min respectively. For EtO sterilization, the PLGA fibre mesh was contained in Seal and Peel® packaging (Andersen Products, UK) and sealed with an impulse heat sealer. The sealed membrane inserts were then packed in designated liner bag together with a 5ml EtO ampoule (Andersen Products, UK). In an allocated fume hood, the ampoule was broken, and the package was placed in tight-lid container for overnight gas diffusion sterilization. Samples remained in the fume hood for another 24h to allow any EtO residues to be removed completely.

The electrospun fibres were coated with 10µg/ml poly-L-lysine (pLL for mucosal cells) or poly-D-lysine (pDL for bulbar cells; both from Sigma). pLL and pDL were reconstituted in sterile tissue culture grade water. To ensure uniform coating, the sterilized scaffolds were soaked in PBS overnight and then transferred to the respective coating material for 1h and returned to PBS to remove excess coating molecules, prior to cell seeding.

Purified OEC from primary olfactory cell culture using flow cytometry

Primary OEC Cultures

Purified OECs were first prepared from primary rat olfactory bulbar and mucosal culture as described in [35]. Briefly, the olfactory mucosa was collected from the upper posterior surface of the adult rat nasal septum using a scalpel blade. After washing to remove mucus, tissue was chopped using a McIlwain tissue chopper, digested using 0.05% collagenase (Type I, 2.5g/ml; Sigma-Aldrich UK) for 5min at 37°C, and triturated to obtain single cell suspension. The mucosal cell suspension was seeded at a density of 27,000 cells/cm^2 on pLL coated tissue culture dishes. Olfactory bulbs were obtained through a dorsal craniotomy. After careful detachment of meninges around the bulb, the olfactory nerve fibre and glomerular layers were dissected and cut into fragments. These tissue fragments were incubated in 0.25% trypsin/EDTA solution (TE; Invitrogen) and DNase I (Sigma) at 37°C for 10min with intermittent trituration to obtain a cell suspension which was seeded at 2 bulbs per 60mm^2 on pDL coated tissue culture dishes.

All primary cultures were maintained in the complete culture medium (10% foetal bovine serum (FBS) in DMEM/F12, supplemented with 1% insulin-transferrin-selenium and 1% penicillin-streptomycin (all from Invitrogen, UK)) in a humidified incubator enriched with 5% CO_2 at 37°C for 10 days in culture (DIV). Culture medium was replaced every 3d. At the first two medium changes the cells containing supernatant was spun down, and the cells re-suspended in fresh medium and replated.

Flow Cytometry

At 10DIV, cells from both cultures were sorted using flow cytometry, based on two surface markers: p75 and Thy1, to obtain purified OEC, as described in [35]. Briefly, cultures were detached from dishes using TE to obtain a single-cell suspension which was blocked with fresh cold bovine serum albumin (BSA)/PBS base solution prior to fluorescent antibody labelling to tag for OECs.

The cell suspension was incubated with mouse anti-p75 (1:150, Chemicon), diluted in the BSA/FBS base solution for 45min on ice. After two washes, the incubation continued with Alexa Fluor 488 goat anti-mouse (1:200, Molecular Probes, Invitrogen) in the dark on ice for 30min and further blocked in mouse serum overnight at 4°C. For Thy1 labelling, the blocked cell suspension was labelled with the phycoerythrin-conjugated, mouse anti-Thy1 (Thy1-PE, 1: 150; clone MRC OX-7, AbCam, UK) for 45min on ice in the dark. After a quick wash with ice cold PBS, the cells were re-suspended in cold BSA/FCS base solution, and filtered using 40μm cell strainers (BD Biosciences), ready for sorting. The concentration was adjusted to 10^6 cells/ml.

Cell sorting of the living cells was performed on a MoFlo XDP (Beckman Coulter) with Summit software. 70μm nozzles were used at sheath pressure of 30psi and flow rate of 250-400 events per second with the sort precision mode set for purity, and plate voltage at 5.0. Cells sorted on the basis of p75 or Thy1 were collected within defined gates based on appropriate positive and negative controls (Figure 5 in [35]). Cells were collected in complete culture medium and a small aliquot put through verification sorting.   Cells meeting 95-98% purity were centrifuged and re-suspended in medium. Cells were plated at density of 10,000 cells/cm^2 on to either the flat surface of the chamber slides (NUNC Labtek II-CC^2) or the surface of the PLGA electrospun Nano-700 and Nano-250 fibres coated with poly-lysine, and cultured for a

further four days, after which the OEC bearing meshes were detached from the membrane insert and divided into two portions: one for scanning electron microscopy (SEM) and the other for immunofluorochemistry (IFC).

Characterisation of OEC Fibre Meshes Using Scanning Electron Microscopy

For SEM, OEC bearing meshes were fixed overnight in 3% glutaraldehyde, 1% paraformaldehyde in 0.08M sodium cacodylate buffer at pH 7.4. After three 10 minute rinses of 0.1M sodium cacodylate buffer, followed by secondary fixation in 1% aqueous osmium tetroxide for 45min the samples again received three 10 minute rinses of 0.1M sodium cacodylate buffer followed by dehydration through a series of ascending alcohols for ten minutes each: 50%, 70%, 90% and three times at 100% . This step was followed by chemical drying with hexamethyldisilazane (HMDS – Merck, VWR, UK; which gave superior preservation to critical point freezing). After about 30 minutes the fibre mesh turned opaque, indicating the completion of air drying. Then the samples were mounted on the Agar stub to be sputter coated with platinum using Emitech platinum sputter-coater. Scanning electron micrographs were taken using a Jeol JSM-6700F scanning electron microscope.

Immunofluorochemistry

For IFC, the OEC bearing meshes were fixed with 4% paraformaldehyde (TAAB, UK) for 20 minutes, washed three times in PBS, permeabilised and blocked with 2% skim milk in PBS containing 0.1% Triton X-100 (TAAB). Primary antibodies were applied for two hours at room temperature (RT) or overnight at 4°C. Cells were washed three times and incubated with appropriate species specific fluorescent secondary antibodies for one hour at RT in the dark. After washing twice, cells were counterstained and mounted using ProLong® Gold Antifade with DAPI (Invitrogen).

Primary antibodies were 1:250 mouse anti low affinity nerve growth factor receptor (anti-p75; clone 192-IgG Chemicon, Millipore, UK), 1:200 mouse anti-Thy1 (IgG clone MRC OX-7; AbCam, UK) and 1:500 rabbit anti-S100β Ig (Dako, UK). Secondary antibodies were Alexa Fluor 488 and Alexa Fluor 546 goat anti-rabbit IgG (all 1:400; Molecular Probes, Invitrogen).

Cell Morphology Imaging and Characterization

Images of fluorescent labeled cells, described above, were captured using LSM 510 Meta confocal microscope (Zeiss, Jena, Germany) at 200x magnification. Laser power and wavelength intensity settings were kept consistent across all the randomized micrographs taken in each session. Images were then adjusted for brightness and contrast, if need be, and subsequently exported using LSM Image Browser software.

Cytomorphometric analyses of the purified OEC from the confocal images were performed in two steps: morphological identification of single cells in each image, followed by cytomorphometric analysis of the identified cells. The image processing software used was Fiji (http://fiji.sc/wiki/index.php/Fiji), developed from NIH ImageJ and each image was magnified at an additional digital zoom of 9.6 times. Morphological identification of bipolar and stellar OECs was performed using the "simple neurite tracer" plugin. The cell tracings were aided by the colocalisation of DAPI positive nuclei with the p75+ cell body and cytoplasmic branches. For images with more flattened cells, tracing using freehand selection with the aid of "ROI manager" was used in conjunction with "simple neurite" plugin. Once identified, all singly traced cells were measured for various morphological parameters using the "Measure" plugin to obtain perimeter (P), surface area (A), minimum Feret diameter, and the major axis (r_1). Major axis is obtained from the best fitted ellipse (determined by the shape

descriptors, one of default function within "Measure" plugin) of the cell body. These measurements were used to calculate the circularity (C) and roundness (R) of each cell using the following formula:

i.   circularity,

$$C = \frac{4\pi A}{P^2}$$

ii. roundness ,

$$R = \frac{4A}{\pi r_1{}^2}$$

As the value of circularity and roundness approach 0.0, the morphology of the cells would be increasingly elongated. For elongated cells, at a given surface area, a stellar cell (i.e. a cell with more than two elongated processes) would have a shorter major axis, but a longer perimeter when compared to those of spindle-like, bipolar cell. Based on the equations above, a stellar cell has a higher roundness index but a lower circularity index than a bipolar cell.

For the directionality, the "Directionality" plugin, based on the Fast Fourier Transform algorithm, was used to determine the pixel directionality of all traced cells in each image. The computation of the algorithm was set on 180 bins, producing a directionality histogram of 180°. Each image was described by the dispersion index (i.e. standard deviation of the Gaussian distribution) and the goodness of the Gaussian fit to the histogram (1 good fit, 0 bad fit with multiple peaks.

RESULTS

Fabrication and characterisation of the PLGA nanofibres using traditional and nanocomposite electrospinning

Meshes of randomly woven nanofibres were produced by electrospinning from 50:50 PLGA solutions in hexafluoroisopropanol (HFIP). A range of polymer concentrations from 3.0 to 7.2% (wt/wt) was studied. Lower concentrations resulted in beading, and higher concentrations gave an increased fibre diameter (Fig. 1). 6% was found to be the optimal concentration to produce beadless, uniform and continuous fibres. A voltage of 9.7kV with a feed rate of 300μl/hr was found to produce homogenous uniform fibres with a length weighted average diameter of 707.82±1.646nm (Fig. 2). These were defined as Nano-700.

To achieve lower diameter fibres 6% PLGA/HFIP with added CdSe/ZnS 5.2nm diameter quantum dots was electrospun at a voltage of 11.2kV with a feed rate of 400μl/hr. These nanocomposite fibres had a similar surface texture, but with an increased uniformity of diameter as reflected in the narrower dispersion of the length weighted lognormal average fibre diameter frequency distribution (Fig. 2). A 1% concentration of quantum dots yielded fibre diameters of 549.77±1.311. A 2% concentration produced a further decrease to 237.22±1.390 nm; these fibres were defined as Nano-250.

In preparation for seeding with OECs the electrospun PLGA fibres were sterilised. Of three different sterilisation methods tested (Fig. 3), chemical sterilisation with ethylene oxide vapour followed by functionalisation by coating with poly-lysine was found to maintain the consistency and high fidelity of the submicron scale features and dimension of the fibres

Cell adhesion and growth of OECs

(a) cultured on flat surfaces: Dissociated cells from olfactory bulbar or mucosal tissue samples cultured for 10 days were FACS purified to 98% p75+Thy1- (verified on a second sort) and cultured for a further 4 days on flat surface poly-lysine coated chamber slides. The purified cells attached and continued to grow. As described in previous study [31], cells plated at high density ($\approx$ 81,000 cells/cm^2) adopt a radial morphology of elongated branches with narrow cell bodies. However under low density culture conditions ($\approx$10,000 cells/cm^2) the cells adopted a flattened, wide bodied morphology with no distinction between branches and cell body (Fig. 4A, D). Immuno-staining confirmed the consistent expression of p75 and S100. Bulbar and mucosal OECs were indistinguishable in their individual morphology and antigenic expression.

(b) cultured on Nano-700 and Nano-250 fibres: FACS purified bulbar and mucosal OECs were seeded at low density ($\approx$10,000 cells/cm^2) on sterilised meshes of randomly orientated Nano-700 and Nano-250 fibres. After culture for 4 days the cells were examined by immunofluorescence microscopy. On uncoated fibres the OECs adhered poorly and remained as small rounded cells. Functionalisation by coating with poly-lysine enhanced cell attachment, spreading and growth. There were no detectable differences in the numbers of adherent OECs between the Nano-700 and Nano-250 fibres (Fig. 4B-F).

Cytomorphometric comparison of OECs cultured on flat surfaces with Nano-700 and Nano-250 fibres

Compared with low density OEC cultures on the flat surfaces, immunofluorescence and SEM images suggest that OECs cultured at the same density on the Nano-700 and Nano-250 fibre meshes responded by increased elongation (Fig. 4G-L). Cytomorphometric analysis based on the p75 immunofluorescence images demonstrated a major reduction in surface area and minimum Feret diameter (an indication of narrowing of the cell body; Fig. 5A, B).

On the Nano-700 meshes the majority of the elongated OECs exhibit multiple, radiating cell protrusions. On the Nano-250 meshes the majority of the cells were shorter, and virtually all had adopted a bipolar geometry (e.g. Fig. 4I). Cytomorphometric evaluation of the cell shapes based on the particle analysis concepts of 'circularity' (this estimates the extent to which the cell extends its perimeter into processes) and 'roundness' (this estimates the overall compactness of the cell) confirmed the statistical significance of these differences between the OECs grown on these two substrates (Fig. 5C,D).

Strikingly, the population of elongated bipolar OEC grown on Nano-250 fibres showed more unidirectional growth when compared with the anisotropic growth on Nano-700 fibres. This is despite the randomness of the orientation of the fibres in the mesh. The histogram of fast fourier transfer analysis of cell pixel directionality indicates a clear peak of a particular angle for Nano-250 fibre meshes, compared to the multiple peaks of random angles for OECs grown on both flat surfaces and Nano-700 fibre meshes (Fig. 6).

Scanning electron microscopy (SEM)

The Nano-700 and Nano-250 fibres are recognised as unbranched, smooth-surfaced, and of uniform diameter (Fig. 7). The OECs remain on top of the fibre mesh so that their processes are seen running in continuity over the underlying fibre mesh. On the Nano-700 fibres the OEC cell bodies are elongated oval shapes, occasionally tripolar or stellate (Fig. 7A) with long smoothly tapering processes, and which (unlike fibroblasts, *unpublished observations*) do not weave into the underlying fibre mesh. The overall population of OECs shows a tendency to lie in the same orientation. On the Nano-250 mesh only tapering, bipolar cells were seen, and, as in the confocal micrographs, the uniform directionality becomes dominant.

Two examples of the mode of termination of the OEC process on Nano-250 mesh are shown in Fig. 7B and C. In B the process terminates in a small lamellipodial expansion generating a

mass of irregular fine filopodia. In C a long, fine filopodial process terminates as an expanded

claw-like structure wrapped over a point of intersection of the underlying nanofibres.

DISCUSSION

Injections of suspensions of cultured adult olfactory ensheathing cells are able to provide pathways for regenerating axons to cross small lesions in rat spinal cord and allow restoration of function. The fabrication of a scaffold for the transplanted cells provides a possibility to bridge larger lesions.

While macroengineering of an artificial bridge provides a overall biomimetric structure [36] at the microengineering level, the precision in nanotopography serves as a tool to modulate contact guidance, such as cell elongation, directional growth and changes in cellular function [37], often specific to cell types [38]. Anisotropic stress generated from nanotopography is generally considered as one of the mechanism of morphological response [39,40].

Our results confirm that the attachment and growth of the OECs on the nanofibres required coating of the scaffold [41], where without this surface functionalisation, there is evidence of no cells attached (data not shown). Surface functionalisation with a range of ECM molecules and short peptides (biological macromolecules, together with changes in the nanotopography, have been reported [42,43]. In the present study we used the positively charged macromolecule, poly-lysine [44] to demonstrate the basic response of OECs to nanotopography without the use of adhesion enhancing molecules such as laminin or collagen [45,46].

Compared with growth of the artificial flat surfaces of standard tissue culture, growth on the nanoscale fibers engendered a morphological change from rounded to elongated stellate cells and an increase in the numbers of cells attached. This increase maybe due either to increased adhesion (i.e retention of a larger proportion of the seeded suspension) or to increased cell survival or proliferation [47,48].

Further improvements in growth were observed in OECs on fibre meshes, produced by nanocomposite electrospinning PLGA with quantum dots. This technique enabled us to increase the uniformity of the size distribution of the nanocomposite nanofibres and achieve diameters down to the 237.22±1.390nm. At this dimension the fibres are approaching the scale of surface structures which the OECs encounter in their microenvironment. The reduction in the fibre diameter from 707.82±1.646nm (Nano-700) to 237.22±1.390nm (Nano-250) elicited a step change in the response of the OECs from the elongated, randomly arranged stellate population to the unidirectional arrangement of small bipolar cells.

We suggest that the morphological response of the OECs to the decreasing diameter of the nanofibres reflects increased density of the surface features presented by the nanofibres to the cells - either the fine grain discontinuity of the molecular interaction and/or the charge presentation.

Shen et al, culturing OECs on randomly orientated silk fibre scaffolds, similarly reported unidirectionality was produced when the fibre diameter was 300nm as compared with 1,800nm fibres [48]. OECs cultured on naturally occurring ECM fibres [47] or self-assembled IKVAV peptides [49], both of which were <100nm diameter, also achieved unidirectionality, although an additional factor in these studies could be the high density culture.

Taken together these results suggest that unidirectionality is influenced both by the density of attachment and the diameter of the substrate fibres. In the present study, cell unidirectionality occurred on flat surface culture where the attached cell density results in close cell to cell contact. Culture on our Nano-700 (707.82±1.646nm) fibres increased cell attachment as

compared with flat surface culture, but unidirectionality at the same attachment density was only achieved on Nano-250 (237.22±1.390nm) fibres.

In our study, as in those of [47], [48] and [49], unidirectionality of OECs was achieved on randomly oriented (isotropic) fibre substrates. The most likely source of this unidirectionality is cell-cell interactions. The use of aligned (anisotropic) fibre or patterned substrates would enable the orientation of alignment to be controlled [10,36].

Conclusion

The ability of nanofibres to determine the attachment, growth, morphology and directionality of OECs *in vitro*, shown in this study, provides an indication of the requirements for the biomaterials to be developed for bridging the larger lesions currently under investigation in clinical trials of OEC transplantation [50]. Our development of a viable of ethylene oxide vapour sterilisation process and subsequent surface functionalisation which preserve the nanotopography of the biodegradable PLGA scaffold and enhance cell adhesion and viability provide indications of a possible route for future GMP. The increased attachment of cells from the seeded suspension improves the efficiency of retention of the limited numbers of cells available. The nanofibres mediate the transformation of OECs to a bipolar morphology such as has been observed when transplants bridge small rat lesions (Figure 7,8 and 11 in [51] and Figure 1 and 2 in [52]), and provide a situation in which the cells can assemble in a uniform direction.

FUTURE PERSPECTIVE

Animal models have established the principle that transplantation of OECs can provide pathways for the regeneration of severed nerve fibres in CNS injuries. We have explored growing OECs on nanofibres as a method to enable limited numbers of cells to bridge large injuries. The nanofibers scaffold will harbor the unique natural assembly of the limited OEC cells. Decreasing the fibre diameter to the nano-scale induces OEC alignment. Elongation of OECs is needed to provide pathways for regeneration of nerve fibres. The next step would require a compatible macroarchitecture, designed to also sNanofibres bearing OECs will be used to construct prosthetic devices for repairing CNS injuries. Reparative prostheses will require mixtures of different cell types. These prostheses will be tested in in vivo animal

models. Following successful results in animal studies these devices will be implanted in

clinical injuries of the spinal cord and CNS.

Executive Summary

- We have explored growing OECs on nanofibres as a method to enable limited numbers of cells to bridge large injuries.

Nanocomposite Electrospinning using Poly PLGA

- Conventional electrospinning produced up to 700nm PLGA fibres (Nano-700), while the addition and even dispersion of the CdSe/ZnS Quantum dots in the PLGA/HFIP solution resulted in a significantly more uniform population of fibres diameter and enabled a reduction in diameter to 237nm (Nano-250).

Cytomorphometric Assessment of Purified OECs

- Compared with culture on a flat surface, culture on Nano-700 mesh increases cell attachment and the cells change from rounded to stellate forms with elongated branches in random orientation. Further size reduction to the Nano-250 favours the formation of bipolar cells.

- Despite the randomly orientated nanofibres mesh, the reduction of fibre diameter from 700nm to 250nm encourages unidirectional orientation in the growth of these bipolar OECs.

GMP Procedures

- Our development of a viable of ethylene oxide vapour sterilisation process and subsequent surface functionalisation which preserve the nanotopography of the biodegradable PLGA scaffold and enhance cell adhesion and viability provide indications of a possible route for future GMP.

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