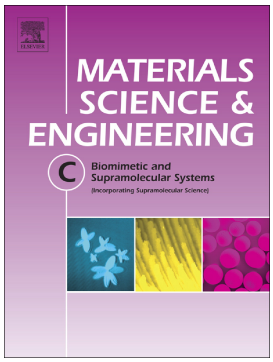

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Production and *in vitro* evaluation of macroporous, cell-encapsulating alginate fibres for nerve repair

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Abstract

The prospects for successful peripheral nerve repair using fibre guides are considered to be enhanced by the use of a scaffold material, which promotes attachment and proliferation of glial cells and axonal regeneration. Macroporous alginate fibers were produced by extraction of gelatin particle porogens from wet spun fibers produced using a suspension of gelatin particles in 1.5% w/v alginate solution. Gelatin loading of the starting suspension of 40.0, 57.0, and 62.5% w/w resulted in gelatin loading of the dried alginate fibers of 16, 21, and 24% w/w respectively. Between 45 and 60% of the gelatin content of hydrated fibers was released in 1 h in distilled water at 37°C, leading to rapid formation of a macroporous structure. Confocal laser scanning microscopy (CLSM) and image processing provided qualitative and quantitative analysis of mean equivalent macropore diameter (48–69 μm), pore size distribution, estimates of maximum porosity (14.6%) and pore connectivity. CLSM also revealed that gelatin residues lined the macropore cavities and infiltrated into the body of the alginate scaffolds, thus, providing cell adhesion molecules, which are potentially advantageous for promoting growth of glial cells and axonal extension. Macroporous alginate fibres encapsulating nerve cells [primary rat dorsal root ganglia (DRGs)] were produced by wet spinning alginate solution containing dispersed gelatin particles and DRGs. Marked outgrowth was evident over a distance of 150 μm at day 11 in cell culture, indicating that pores and channels created within the alginate hydrogel were providing a favourable environment for neurite development. These findings indicate that macroporous alginate fibres encapsulating nerve cells may provide the basis of a useful strategy for nerve repair.

Keywords: Alginate fibre; Dorsal root ganglion; Macropores; 3D image analysis; Nerve regeneration

1. Introduction

Nerve repair using tissue engineering principles seeks to regenerate the nerve fibre, which is composed primarily of axons that project from the neuron cell body and transmit sensory and motor nerve impulses, supported and protected by glial cells [Schwann cells (SCs) in the peripheral nervous system (PNS), astrocytes and oligodendrocytes in the central nervous system (CNS)]. Multiple nerve fibres are combined in a fascicular structure that is ensheathed by the epineurium of the nerve. The process of natural peripheral nerve repair involves the formation of longitudinal arrangements of proliferating SCs (bands of Büngner) that guide axonal growth from the damaged distal nerve segment coupled with the sprouting of new axons from myelinated and non-myelinated nerve fibres in the proximal segment. Neuronal axons elongate from the growth cone at the axon tip and eventually form synaptic terminals where release of neurotransmitters such as acetyl choline and monoamines occurs to allow communication between neurons and other cells. In addition to their guidance role in nerve repair, glial cells act to increase the speed of signal transmission and produce neurotrophic factors to modulate axon development [1, 2].

Nerve repair strategies in tissue engineering are generally based on a three-dimensional (3D) scaffold or ‘biological template’ that creates an environment which is designed to maintain cell viability and guide axonal development along the scaffold surface or within the scaffold [3, 4]. Refinements of this basic approach involve the incorporation of growth factors, stem cells, or transfected cells, which express axon growth-promoting molecules. The synthetic polyesters poly-ε-caprolactone

(PCL), poly (L-lactic acid) (PLLA) and poly (lactic co-glycolic acid) (PLGA) [5, 6] have been investigated extensively for production of nerve substitutes. Aligned, electrospun PCL fibres, for example, have been reported to encourage neurite outgrowth from rat pheochromocytoma PC12 cells in an oriented fashion compared with random fibres [6]. The natural polymers, including extra cellular matrix (ECM) components (e.g., laminin, collagen, and fibronectin) as well as polysaccharides (e.g., alginate and chitosan) have also been shown to be highly effective in promoting axonal development and nerve repair [7,8]

Alginate is an anionic, hydrophilic polysaccharide which has been investigated extensively for drug delivery and tissue engineering because of its excellent biocompatibility, biodegradability and favourable physicochemical properties [9]. Importantly, alginate forms hydrogels in aqueous solution under mild crosslinking conditions following exposure to divalent metal ions, principally Ca^{2+}. Thus, the polymer has been used frequently for cell encapsulation (e.g. pancreatic islets, recombinant cells) and has been fabricated in various forms for regeneration of neural tissue, including tubes [8], freeze-dried sponges [10, 11] and hydrogels [12, 13]. Hashimoto et al. [11] studied peripheral nerve regeneration through freeze-dried, covalently crosslinked alginate gel placed in 10mm defects in the rat sciatic nerve. Axons extended approximately 100μm into the material 4 days post-implantation, while SCs migrated into the gel at the distal lesion site over 8–14 days. Axons were observed to extend though the partially degraded gel rather than through the pore structure emphasising the importance of controlling pore connectivity and scaffold biodegradability. Matyash et al. [13] showed that non-functionalised alginate hydrogels formed by crosslinking with Ca^{2+}, Ba^{2+}, or Sr^{2+} support neural cell adhesion, and rapid and abundant neurite outgrowth on the surface and within the hydrogel *in vitro*.

Alginate hydrogels exhibit porosity on the 5–20 nm scale [14], which permits diffusion of nutrients and oxygen and the elimination of waste products of cell metabolism. However, the utility of the hydrogel as a tissue engineering scaffold also depends on the pore structure being suitable in terms of porosity, pore size and connectivity to permit cell proliferation and tissue development. Thus, a wide variety of techniques have been successfully applied to produce macroporous alginate scaffolds, including freeze-drying [15], particulate (porogen) leaching [16], emulsion templating [17] and micelle templating [18]. Partrap et al [18], for example utilized self-assembled micelles formed from cetyl trimethyl ammonium bromide (CTAB) surfactant to produce a template for alginate gelation resulting in hydrogels with controllable pore size ranging from 30 to 160μm. Alginate's lack of inherent cell adhesive properties, is a major limitation in scaffolding applications leading to arrested development of anchorage-dependent cells and tissue formation [19]. Attempts to resolve this shortcoming have involved modifying alginate or the finished scaffold with extra cellular matrix (ECM) proteins, such as fibronectin, vitronectin, laminin, and collagen for binding with cell adhesive receptors. Laminin-coated and Tyr-Ile-Gly-Ser-Arg (YIGSR) peptide-conjugated alginate hydrogel (peptide/alginate ratios of 1 mg/g) improved NB2a neuroblastoma cell attachment by 44 and 60%, respectively, compared with the control [20].

The objective of the present study was to develop a wet spinning technique for producing

macroporous alginate fibres containing nerve cells via extraction of incorporated gelatin particle porogens. The aim was to provide interconnected pores of sufficient size that would permit the proliferation of encapsulated glial cells and promote axonal outgrowth to re-establish nerve function. The 3D structure of cell-free fibres was characterised using confocal laser scanning microscopy (CLSM) and image analysis to provide quantitative estimates of macropore dimensions and porosity and to trace interconnected pores. Rat dorsal root ganglion (DRG) cells were encapsulated in macroporous alginate hydrogel fibres by wet spinning alginate solutions containing both gelatin particles and suspended cells. The gelatin particles were employed simultaneously as porogens and to provide cell adhesion molecules (CAMs) to promote nerve cell attachment to the hydrogel. Neurite outgrowth from encapsulated DRG cells was monitored over 15 days in cell culture using optical microscopy, and the neuron-specific mouse monoclonal anti-tubulin-III TU-20 marker was employed to confirm neurite formation.

2. Materials and Methods

2.1 Materials

Sodium alginate (Protanal RF 6650; G:M, 2:1) was obtained from IMCD Ltd, Brisbane, Australia; calcium chloride and gelatin from porcine skin (Type A, Bloom 300, average molecular mass 50–100 kDa), and Dulbecco's phosphate-buffered saline (D-PBS) and dimethyl sulphoxide were purchased from Sigma-Aldrich, Sydney, Australia. Cryopreserved rat DRG neurons and primary neuron growth

medium (PNGM^{TM}) BulletKit® (CC-4461) were purchased from Lonza, Australia. The mouse monoclonal anti-tubulin-III antibody (neuron-specific clone TU-20, MAB1637) was obtained from Chemicon, Australia. Cy3-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) and Calcein-AM were purchased from Invitrogen, Australia. Anti-fade Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (1.5 µg/mL) was purchased from Vector Laboratories Ltd, United Kingdom.

2.2 Production of macroporous cell-free alginate fibres

A schematic illustrating production of macroporous, cell-free alginate hydrogel fibres is shown in Figure 1. As received, gelatin powder was passed through a <90 micron mesh sieve and 100, 200, or 250 mg samples were homogenised (IKA Ultra-Turrax® T25 basic) in 1.5% w/v alginate solution (10 mL) for 30 s at 4°C, resulting in final gelatin particle-alginate suspensions of ratio 2:3, 4:3, and 5:3 w/w respectively. The gelatin-alginate suspensions (0.5 mL) were subsequently wet spun into 0.5 M $CaCl_2$ crosslinking solution (50 mL) using a syringe needle (0.6 × 32 mm) to produce alginate fibres containing increasing amounts of gelatin particles. A suspension flow rate of 2 mL/min was applied using a syringe pump (Harvard Apparatus PHD 2000). The resulting fibres were rinsed and triplicate samples were retained in distilled water (15 mL) at 37°C for 2 days to extract gelatin, thereby producing a macroporous structure.

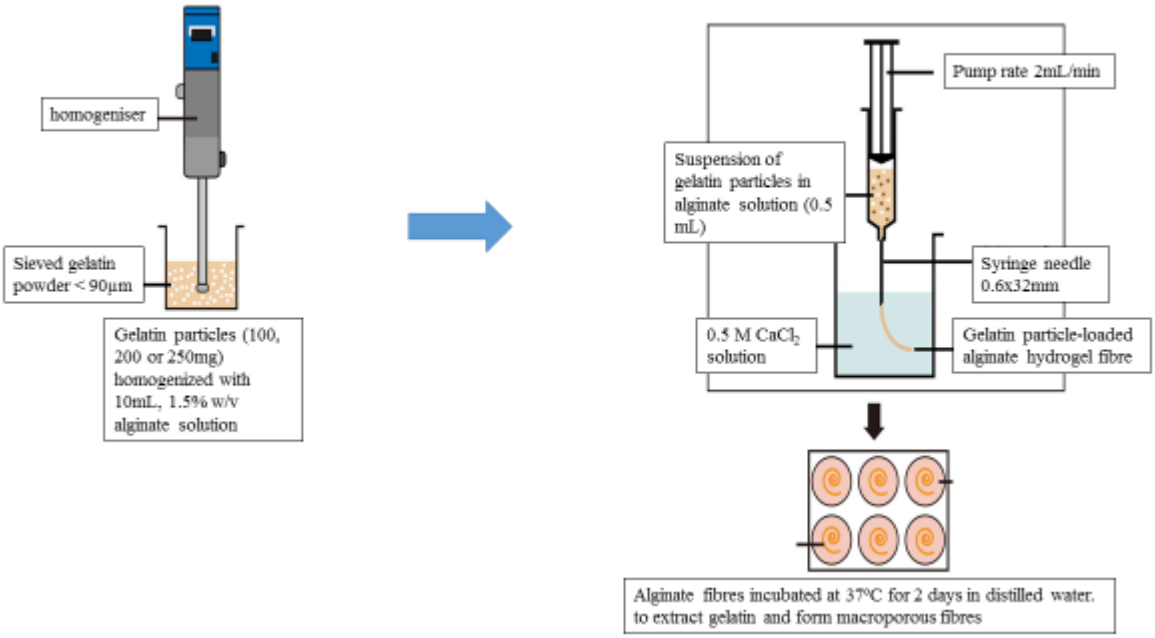


Figure 1: Production of macroporous, cell-free, alginate hydrogel fibres

2.3 Determination of gelatin content of alginate fibres and release behavior

Cumulative gelatin release from hydrated alginate fibres was investigated using triplicate samples (each sample was produced from 0.5 mL gelatin particle–alginate suspension) following immersion in 3 mL distilled water at 37°C. The gelatin concentration in the release medium was measured at 20, 40 and 60 min, and 24 and 48 h using the bicinchoninic acid protein assay (Sigma). After 48 h, the fibres were broken down using 0.2 M sodium citrate (1.5 mL) to assess the residual gelatin content. The total amount of gelatin released from the fibres at 48h and the residual gelatin content following breakdown of the fibres were determined using a calibration curve produced using a series dilution of gelatin in distilled water (220–1000 µg/mL).

The gelatin loading of the fibres was calculated as a percentage (w/w) of the dried fibres:

$$\textbf{Gelatin Loading of Fibre} = \frac{\textbf{Total Weight of Gelatin Released + Remaining Gelatin}}{\textbf{Weight of the Freeze-Dried-Gelatin-Loaded Fibre Fibre}} \times \textbf{100}$$

2.4 Surface morphology of dried and hydrated alginate fibres

The surface morphology of dried alginate fibres and gelatin-extracted alginate fibres was examined using a Philips XL 30 scanning electron microscope (SEM). Samples were mounted on aluminum stubs using adhesive carbon tabs and sputter-coated with platinum using an Eiko-Sputter coater automatic mounting press prior to examination in the SEM at a voltage of 10kV. The surface morphology of hydrated alginate fibres was examined using a Philips XL 30 SEM fitted with an Oxford CT1500 Cryo transfer system. Fibre samples were transferred to the cold stage of the preparation chamber, pre-cooled to approximately −180°C, and sputter-coated with platinum in an argon atmosphere inside the chamber before examination at a voltage of 5 kV.

2.5 Quantitative analysis of the macroporous structure of alginate fibres

A FluoView FV1000 confocal laser scanning microscope (Olympus Corporation, Japan) was used to visualize and record images of longitudinal sections of macroporous alginate fibres. Stacks of two-dimensional (2D) parallel images were acquired using laser excitation at 405 nm and emission detection at 415–515 nm in conjunction with a 10× objective lens (numerical aperture 0.4). The image size was 640 × 640 pixels, with each pixel corresponding to 1.9884 μm in the x and y directions. Images were recorded at a z-axis spacing of 4 μm.

Pore size data for two sections from an image stack (Fig 2A) were used to provide a measure of macropore size and the frequency distribution of equivalent pore diameters in the macroporous alginate fibre [21]. The two sections were located towards either end of the image stack and spaced apart by 26 image slices (104μm) to prevent inclusion of the same pores twice. Software was developed using MATLAB (The MathWorks Inc., Natick, MA, USA) to pre-process the 2D confocal images of the alginate fibres prior to 3D visualization of the pore structure. In brief, the 2D images were first converted to a greyscale and processed using thresholding to detect the pore boundary and form a binary image (Fig. 2B and 2C). The greyscale images were filtered and adjusted to detect the dark regions of the pores. The threshold level was adjusted manually for each image and pores on the image borders were not included because they may not be complete.

3D images of the macropore structure were constructed from sequential 2D greyscale images (typically 110 slices) using Amira v 5.3 software (Visage Imaging GmbH, Berlin, Germany). Additional visualisation methods were applied to display isolated macropores and interconnected macropores to provide an assessment of pore tortuosity. The macroporosity (%) of alginate scaffolds produced by extraction of gelatin particles from 16, 21, or 24% w/w (actual) gelatin-loaded fibres was analysed using software which compared the volume of the pore phase with the total volume of the fibre section.

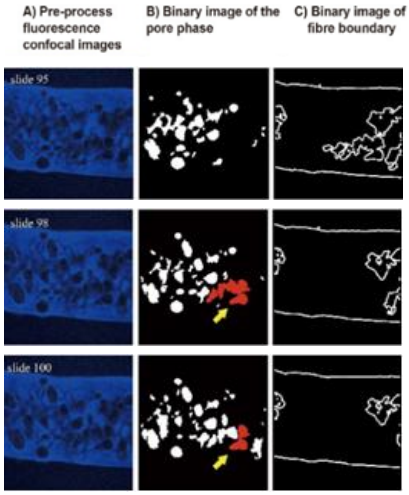


Figure 2. Confocal laser scanning microscopy (CLSM) 2D images of an alginate fibre selected from a z-axis stack. (A) fluorescence images (B) binary images of the pore phase (C) binary image of the fibre boundary. Data obtained using a hydrated (gelatin-extracted) macroporous alginate fibre produced by wet spinning a 62.5% (w/w) gelatin particle suspension in alginate solution. Actual gelatin particle loading of fibre = 24% w/w.

2.6 DRG cell encapsulation in non-macroporous alginate fibres

A vial of suspended rat DRG cells (2×10^5 cells/0.25 mL) was removed from liquid nitrogen and placed in a water bath preheated to 37°C. The vial was transferred to a laminar flow hood after 1.5 min following disinfection of the vial surface with 70% ethanol. The thawed cell suspension was transferred into a 15 mL centrifuge tube followed by drop-wise addition of 7.75 mL of preheated (37°C) PNGM^{TM} onto the cells while rotating the tube by hand. The cells were suspended by

pipetting up and down slowly with a 2 mL plastic disposable serological pipette and by carefully inverting the tube twice. One mL of the cell suspension contained 2.5×10^4 cells. Cell viability was monitored using Live/Dead Calcein-AM staining, and cell morphology was observed using an inverted optical microscope (CKX 41; Olympus) equipped with a digital camera (DP21; Olympus) and DP Controller software (Olympus).

Antiseptic techniques were strictly followed to prevent contamination. All equipment (homogeniser, and syringe pump) were sterilized by wiping the surfaces with 70% ethanol. Glassware and tools were autoclaved. Alginate solution (3 mL, 2% w/v) was sterilized using a syringe filter (0.2 µm, 33 mm, Millex ® Filter Unit, Millipore) and the prepared DRG cell suspension (1 mL, cell density 2.5×10^4 cells/mL) was added to achieve a final cell concentration of 6.25×10^3 cells/mL in a 1.5% w/v alginate solution. The cells were thoroughly dispersed in the alginate solution by pipetting slowly up and down with a 3 mL disposable plastic syringe prior to wet spinning (0.5 mL) into 50 mL of 0.5 M $CaCl_2$ solution. The resulting fibre was rinsed with distilled water and cut into 3 sections (approximately 5-cm each) that were each placed in individual wells of a 6-well plate. Samples were cultured in 5 mL of PNGM^{TM} culture medium for 4 h at 37°C under a 5% CO_2 atmosphere. The medium was replaced with fresh medium after 4 h and then every day up to 15 days.

2.7 DRG cell encapsulation in macroporous alginate fibres

A schematic illustrating production of macroporous, nerve cell-containing alginate hydrogel fibres is

shown in Figure 3. Gelatin powder was sieved through a 90-μm mesh sieve and the collected fraction was sterilized by UV irradiation in a cell culture hood for 20 min. The container was agitated during sterilization to ensure that all particles were exposed to UV. Alginate solution (2% w/v, 10 mL) was sterilized by filtration using a syringe filter (0.2 × 33 mm Millex filter units, Millipore). Sterilized gelatin particles (200 mg) were added to cold filtered 2% w/v alginate solution (10 mL) and homogenised using an IKA Ultra-Turrax® T25 Basic homogeniser in the cell culture hood. A gelatin:alginate w/w ratio of 4:3 was employed, corresponding to 57% w/w gelatin particle loading as a fraction of the total polymer content. DRG cell suspension (1 mL, cell density 2.5×10^4 cells/mL) was added to the gelatin particle/alginate solution (3 mL) to achieve a final cell concentration of 6.25×10^3 cells/mL in a 1.5% w/v alginate solution and dispersed by pipetting slowly up and down using a 3-mL disposable plastic syringe. The suspension of cells and gelatin particles in alginate solution (0.5 mL) was wet spun into 50 mL of 0.5 M $CaCl_2$ solution. The resulting fibre was rinsed with distilled water and cut into 3 sections (approximately 5-cm each) prior to incubation in PNGM™ culture medium (5 mL) for 4 h at 37°C under a 5% CO_2 atmosphere. The medium was replaced by a fresh medium after 4 h and then every day up to 15 days.

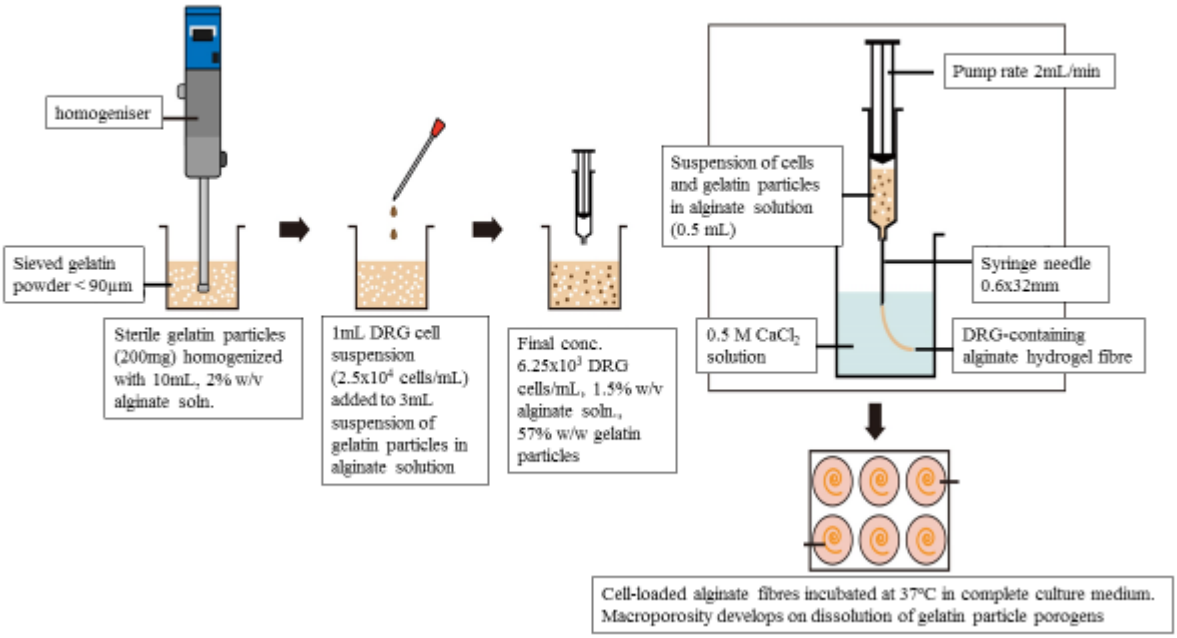


Figure 3: Production of macroporous, nerve cell-containing alginate hydrogel fibres

2.8 Immunolabelling of DRG cells encapsulated in alginate fibres

Class III β-tubulin is a microtubule element expressed predominantly in neurons of the central and peripheral nervous systems [22] and is a popular selective marker for neurons in nervous tissue. Hydrated alginate fibres encapsulating DRG cells that had been retained in culture medium for 15 days were fixed in 1 mL of 4% paraformaldehyde/D-PBS solution for 15 min. Immunolabelling of encapsulated DRGs was carried out with the primary antibody (mouse monoclonal anti-tubulin-III antibody, neuron-specific clone TU-20, MAB1637) at 1:250 dilution. The primary antibody solution was prepared in blocking solution by adding Triton-X (6 µL, 0.3% v/v, Sigma) to normal donkey serum (20 µL, 1% v/v, Jackson Immuno Research) and making up to 2 mL with PBS. Fixed fibre samples (approximately 0.5 cm in length) were rinsed with copious D-PBS, transferred into 1-mL

Eppendorf tubes, and incubated in 200 µL of the TU-20 primary antibody solution for 48 h at 4°C in a humid atmosphere. After incubation, the fibre samples were rinsed 5 times for 10 min each with copious cold D-PBS at 4°C. Immunolabelling of encapsulated DRGs with the secondary antibody [Cy3-conjugated Affinipure Donkey Anti-Mouse IgG (H+L)] was performed by adding 7.5 µL of the antibody solution to D-PBS (1.5 mL) to achieve a dilution of 1:200. Fibre samples were incubated in the prepared solution at room temperature for 50 min in the dark, rinsed 5 times for 10 min each with copious D-PBS in the dark to prevent the fluorescence from fading and preserved in one drop anti-fade Vectashield mounting medium with DAPI (1.5 µg/mL). Immunolabelled samples of DRG-loaded alginate fibres were stored in the dark at 4°C prior to imaging using laser excitation at 559 nm for Cy-3 with emission detection at 564–664 nm. The image size was 640 × 640 pixels, with each pixel corresponding to 1.9884 µm in the x and y directions. Images were acquired using a z-axis spacing of 8 µm.

3. Results and Discussion

3.1 Production and morphology of cell-free macroporous alginate fibres

Alginate fibres were successfully loaded with gelatin particles by wet spinning suspensions of particles in 1.5% (w/v) alginate solution into 0.5 M $CaCl_2$ cross-linking solution. The aim was to create a connected pore structure inside the alginate fibre by extraction of the porogen that would facilitate proliferation of encapsulated nerve cells and axonal extension throughout the fibre. The

gelatin powder was composed of particles of irregular shape (block, wedge and cuboid forms; Fig. 4A). Prior to fibre spinning, the gelatin powder was passed through a 90-µm mesh sieve to obtain a particle size range less than 90 µm since tissue engineering scaffolds featuring a mean pore size less than 150µm have been shown to permit infiltration by a variety of mammalian soft tissue cells [23-25].The particle size of the sieved powder was measured before and after homogenisation in alginate solution at 4°C to determine the effect of shear conditions and the aqueous environment on the size of the particles incorporated within the fibre and thus, on the expected macropore dimensions. Sieved gelatin particles exhibited dimensions as small as 57 ± 3 µm and as large as 102 ± 6 µm. However, following homogenisation in alginate solution for 30 s at 4°C (to prevent excessive swelling), the smallest and largest dimensions of the sieved gelatin particles increased by approximately three fold. This behaviour can be explained by the tendency of gelatin at temperatures less than 35–40°C to swell and absorb 5–10 times its weight of water [26].

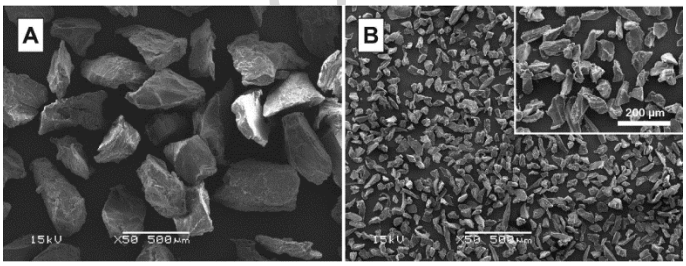


Figure 4. Scanning electron micrographs of (A) unsieved and (B) sieved gelatin particles in a size range <90 microns. Magnified view at top right.

Macroporous alginate fibres were successfully produced by extraction of the gelatin particles in distilled water at 37°C. Particle loadings of 40.0, 57.0, and 62.5%, respectively, in the starting suspension used for fibre spinning resulted in particle loadings of 16, 21 and 24% (w/w) in the dried alginate fibres. The low efficiency of incorporation of gelatin particles in the alginate fibres suggests that particles are excluded from the extruded liquid filament or thread on passage through the syringe needle. Hydrated fibres exhibited "burst release" of around 45–60% of the gelatin content on immersion in distilled water at 37°C for 1 h and only minor additional amounts of gelatin were released in 48 h, suggesting that a macroporous structure would be rapidly established in cell culture to help facilitate proliferation of encapsulated cells or neurite outgrowth. Moreover, the plateau of gelatin release *vs.* time for the hydrated fibres indicated that a large fraction (around 50%) of the gelatin content remained bound within the alginate fibre (approximating to 10% w/w loading), where it may function to improve cell adhesion.

SEM examination of alginate fibres (without gelatin particles), which were dried by immersion in ethanol followed by solvent evaporation, exhibited a diameter of approximately 200 μm and a rough-textured oriented or fibrillar surface (Fig. 5A). Cryo-SEM revealed the microporous morphology of hydrated alginate fibres, with pore sizes of approximately 1μm (Fig. 5B), which is advantageous for the transport of metabolites, nutrient supply and oxygen exchange to maintain the viability of encapsulated cells. In comparison, fibres produced by wet spinning alginate solution

containing suspended gelatin particles, exhibited cross-sectional dimensions of approximately 350 µm (Fig 5C). The irregular fibre shape results from 'moulding' of the alginate polymer around the gelatin particles (Fig. 5C, arrowed). A macropore is partly visible in the fibre cross-section (Fig. 5D, arrowed).

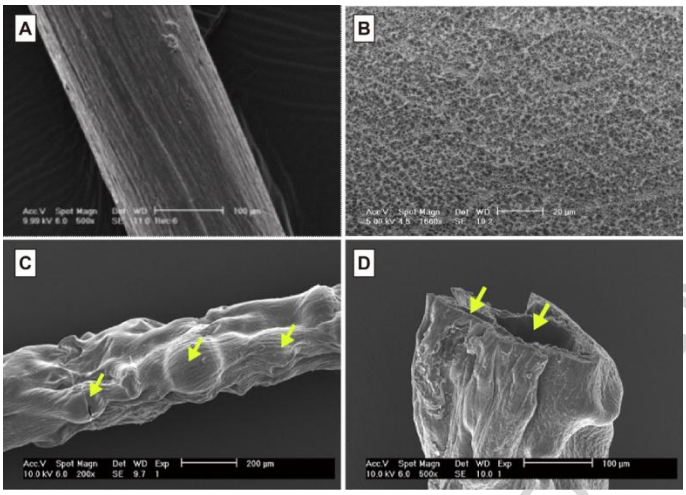


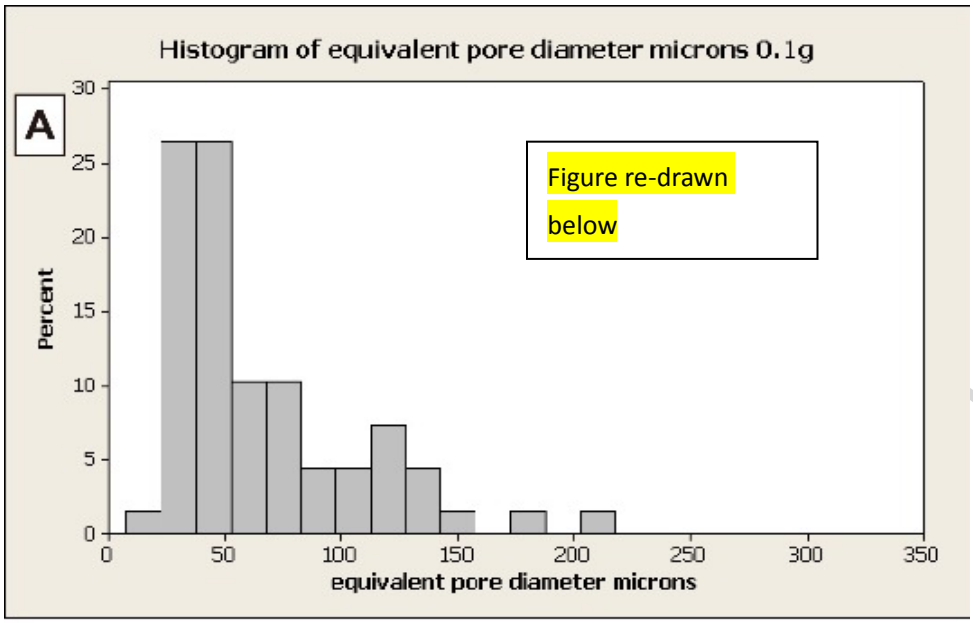
Figure 5. Scanning electron micrographs (A) dried alginate fibre (without gelatin particles) (B) Cryo-SEM of hydrated fibre (without gelatin particles) (C and D) dried gelatin-extracted alginate fibre produced by wet spinning a 57% w/w suspension of gelatin particles in alginate solution.

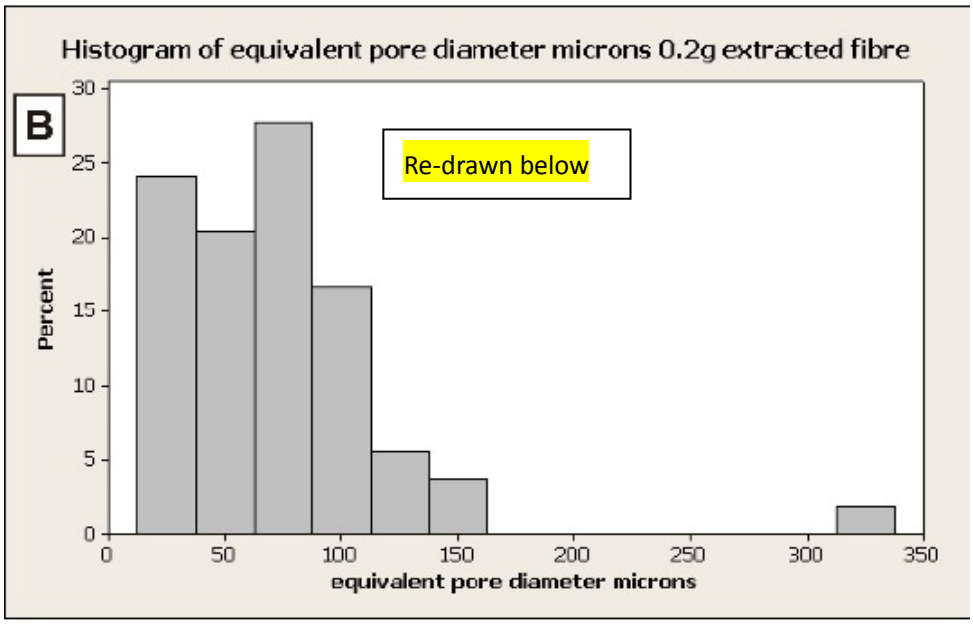
3.2 Analysis of macropore size and size distribution in alginate fibres using CLSM

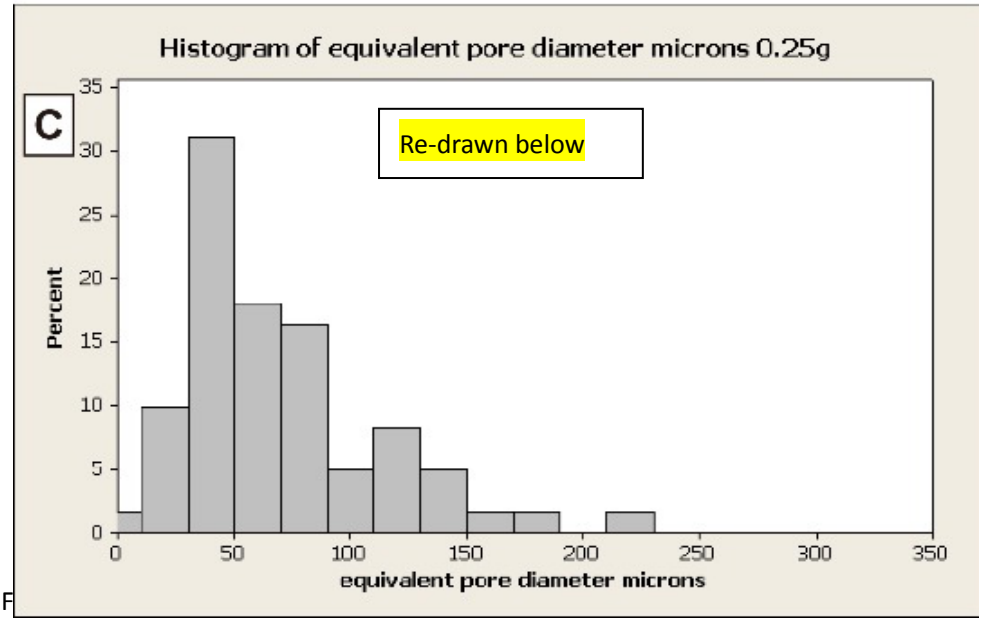
Analysis of sequential CLSM image slices provided quantitative information on the range of pore sizes (in terms of equivalent pore diameter) and frequency distribution of pore sizes in alginate fibres following gelatin particle extraction (Fig. 6). The 24% w/w gelatin particle-loaded fibres after gelatin

extraction exhibited a median pore diameter of 60 μm and a wide range of pore diameters (24 –228μm), with the majority between 39-86 μm (Table 1). The 16%w/w particle-loaded fibres after gelatin extraction exhibited a pore diameter of 48μm and similar pore size range, while the equivalent macropore diameter of 21% gelatin particle-loaded alginate fibres following gelatin extraction was found to measure around 70 μm. It is also apparent on inspection of the frequency distribution curves in Fig. 6 that the majority of the macropores (approximately 80-90%) are grouped in the 25–100 μm range for all gelatin loadings. and approximately 50% of the macropores are <50 μm in equivalent diameter.The analysis demonstrates the potential for controlling macropore size and size distribution by adjusting fibre spinning conditions.

Fibre macroporosity was estimated using 3D image visualization by comparing the pore volume with the corresponding fibre volume (Fig. 7). A maximum macroporosity of 14.6% was obtained following gelatin extraction from 21% w/w gelatin particle-loaded fibres (Table 1). Contrary to expectations, increasing the gelatin loading of the alginate spinning solution did not increase the fibre porosity. It is interesting to note that the maximum porosity coincided with fibres showing the narrowest pore size distribution (i.e., 90% pore < 100μm, Fig. 6B). The relatively low porosity reflects the low efficiency of incorporation of gelatin particles in the alginate fibres (16–24% w/w).







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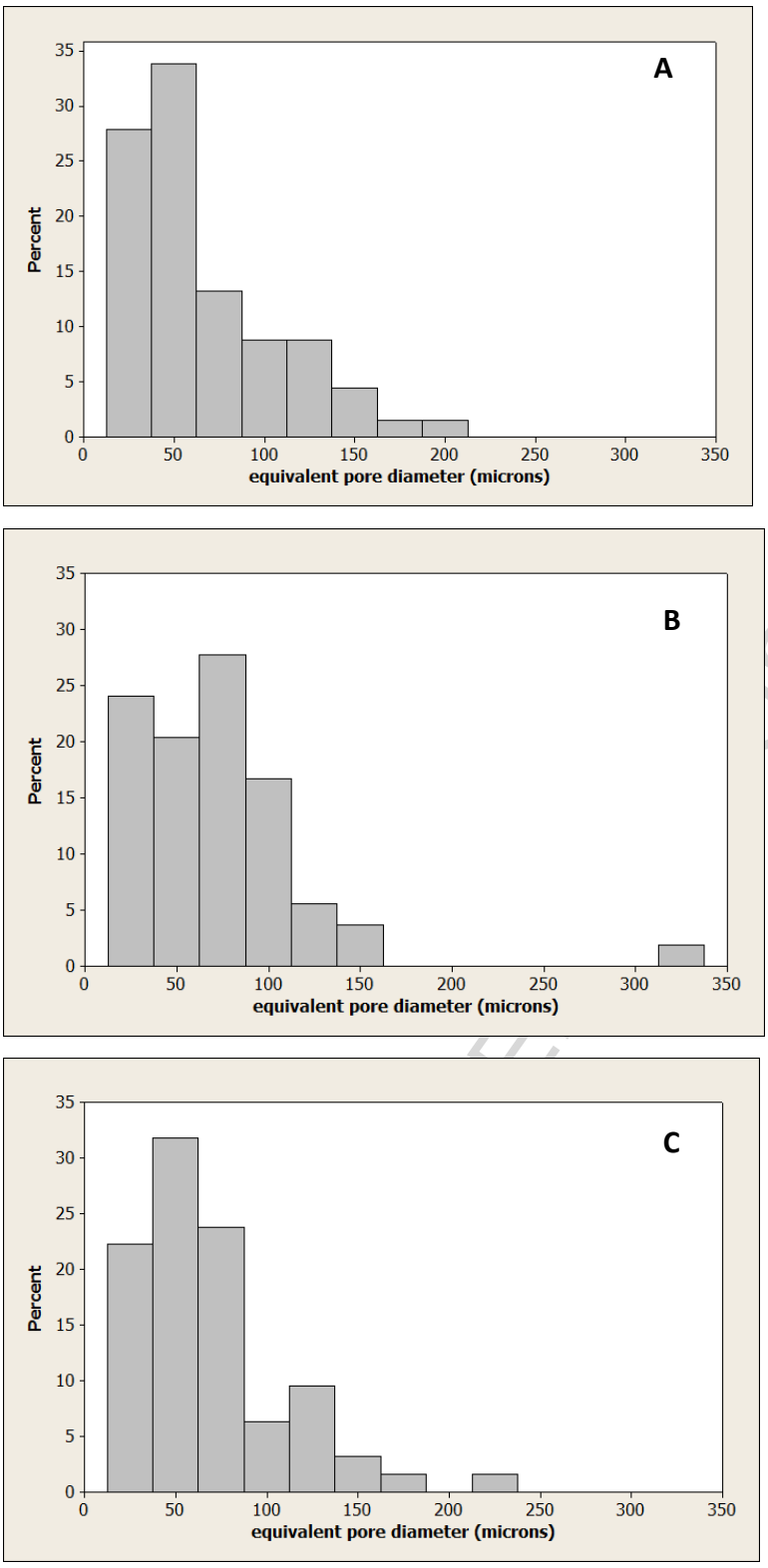

Figure 6. Frequency distribution of equivalent macropore diameter in hydrated macroporous alginate fibres following porogen extraction from (A) 16%, (B) 21% and (C) 24% w/w gelatin particle-loaded

alginate fibres. Data generated using image analysis of sequential CLSM micrographs.

Table 1. Equivalent pore diameters and porosity of macroporous alginate fibres measured using CLSM image analysis.

Gelatin loading of fibre (%w/w)	Sieved particle size (μm)	No. of pores analysed	Median equivalent pore diameter (μm) (first and third quartiles)	Total range equivalent pore diameter (μm)	Porosity (%)
16	<90μm	68	48 (35–87)	22–209	11.3
21		54	69 (40–90)	23–332	14.6
24		63	60 (39–86)	24 61–228	12.1

Macropore connectivity in alginate fibres was evaluated by tracing regions of macropore coalescence in selected 3D image presentations (Fig. 8). As expected, alginate fibres with high gelatin particle loadings displayed a greater probability of pore coalescence. Interconnected pore lengths of 252, 607 and 509 μm were measured following extraction of 16, 21 and 24% gelatin-loaded fibres respectively. Again, it is interesting to note that the 21% w/w gelatin particle-loaded fibres displaying the narrowest pore size distribution (90% <100 μm, Fig. 6B) resulted in the highest connectivity. This finding indicates opportunities for improving cell infiltration or axonal extension by controlling the particle size distribution and content of porogens. However, it should be borne in mind that the dimensions of interconnecting channels (connects) and fenestrations or windows in the pore surface

must also be of sufficient size to allow cell ingrowth and these structural features can be difficult to analyze.

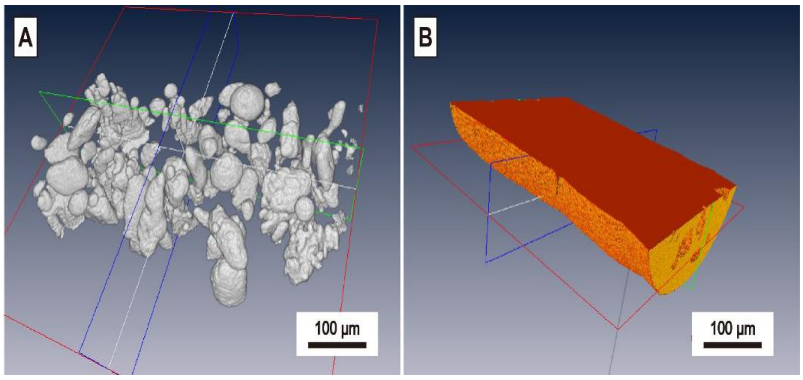

Figure 7. 3D visualization of hydrated 24% w/w gelatin particle-loaded alginate fibre following gelatin extraction. (A) pore volume and (B) corresponding fibre volume

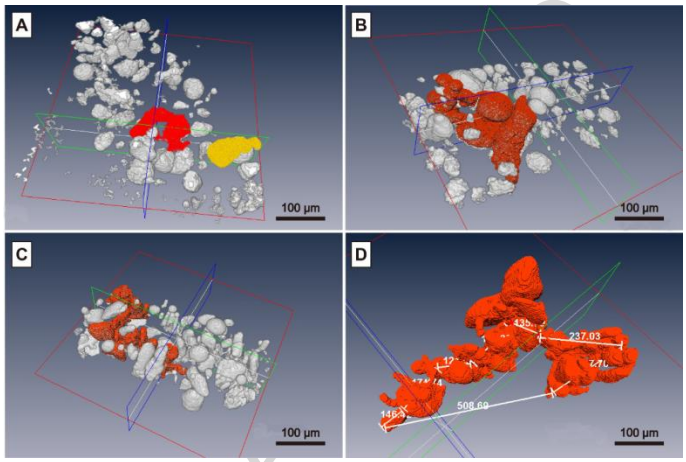

Figure 8. Regions of pore coalescence (highlighted in red) identified by CLSM analysis of (A) 16%, (B) 21%, and (C) 24% (w/w) gelatin-loaded alginate fibres after protein extraction. (D) Magnified view of a region of pore coalescence.

CLSM micrographs of both gelatin-free and gelatin particle-loaded alginate fibres displayed intense autofluorescence (Fig. 9), which enabled FluoView CLSM analysis to resolve the gelatin particles and subsequently, their size and distribution. Autofluorescence emission is commonly reported in cells and originates from entities, such as mitochondria and lysosomes, when certain molecules are excited by UV/visible radiation of suitable wavelengths [27]. However, the extracellular matrix can also contribute to autofluorescence because of the intrinsic properties of proteins, such as collagen and elastin [28] which are related to the aromatic amino acids tryptophan, tyrosine, and phenylalanine. Gelatin emits an intense blue fluorescence at 415 nm resulting from tyrosine. In the case of alginate, autofluorescence emission at 450nm is caused by phenolic compound impurities or residues present in most commercially available alginates [29].

CLSM revealed that the gelatin particle-loaded alginate fibres exhibited higher emission intensity than alginate. This behaviour advantageously caused the gelatin particles to stand out against the background fluorescence of the alginate phase (Fig. 9D), thus, facilitating analysis of particle distribution in the fibres. Moreover, CLSM micrographs of gelatin-extracted fibres (Fig. 10B) clearly showed a bright blue zone around the macropores (black colouring), suggesting that protein residues lined the pore cavity after particle extraction and infiltrated into the body of the alginate fibre to a depth of approximately 20–30 μm. This finding is consistent with the protein release behaviour recorded for gelatin particle-loaded hydrated fibres where around 50% of the gelatin content was

retained at 48 h. Gelatin exhibits amphoteric behaviour due to the presence of terminal amino and carboxyl groups and is positively charged at a pH below its isoelectric point (IEP) (Gelatin Type A exhibits an IEP at pH 7–9) [30]. On the other hand, alginate is uncharged at pH 2 due to complete protonation and becomes more negatively charged with increasing pH until it is fully deprotonated at pH > 6.5. The negative charge of the hydrogel arises due to the carboxyl groups that are not involved in binding with Ca^{2+} counter ions (crosslinking). Thus, it is expected that gelatin binds electrostatically with the alginate fibre to modify the surface of the macropores and provide cell adhesion molecules that would be highly advantageous for attachment and growth of encapsulated nerve cells and axonal extension. The presence of acidic and positively charged lysine and arginine residues in the gelatin macromolecule promotes electrostatic binding with negatively charged cell surfaces. Moreover, specific cell adhesion (RGD) sequences along the protein molecule bind with cell surface integrin receptors.

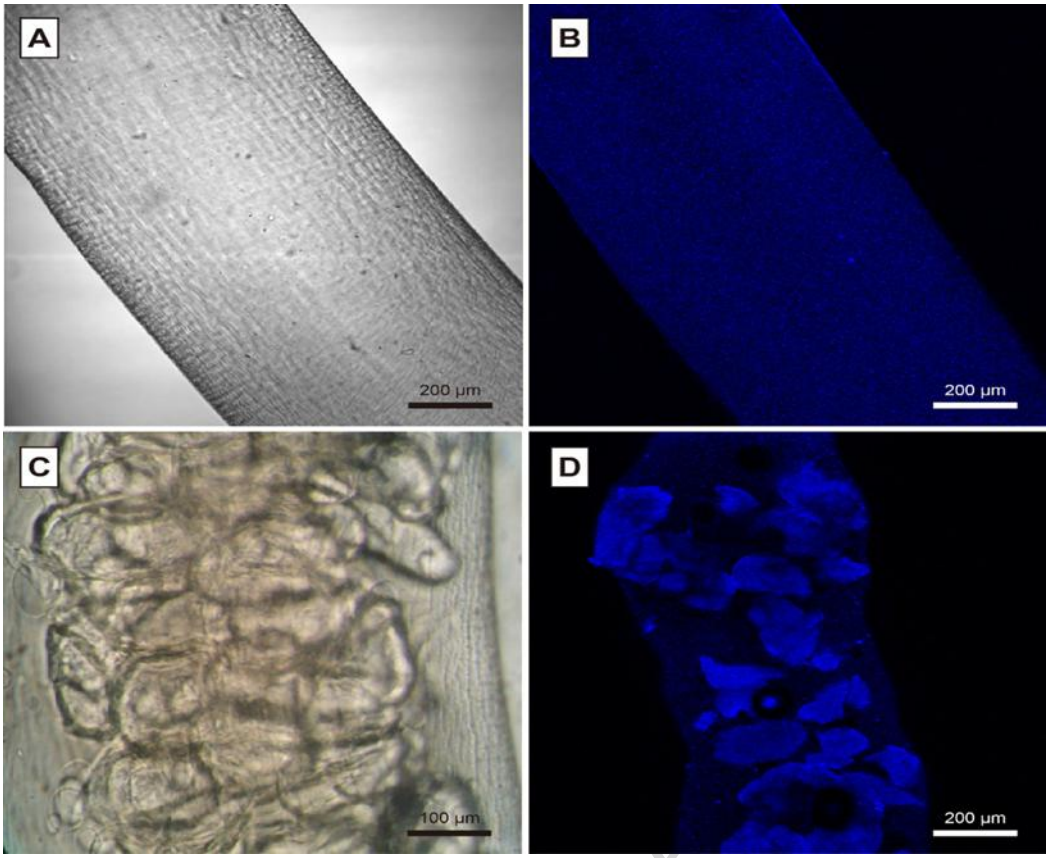

Figure 9. CLSM images of hydrated alginate fibre spun from 1.5% alginate solution into 0.5 M $CaCl_2$ solution. (A) differential interference contrast (DIC) and (B) autofluorescence. CLSM images of 21% w/w gelatin particle-loaded alginate fibre. (C) DIC and (D) autofluorescence

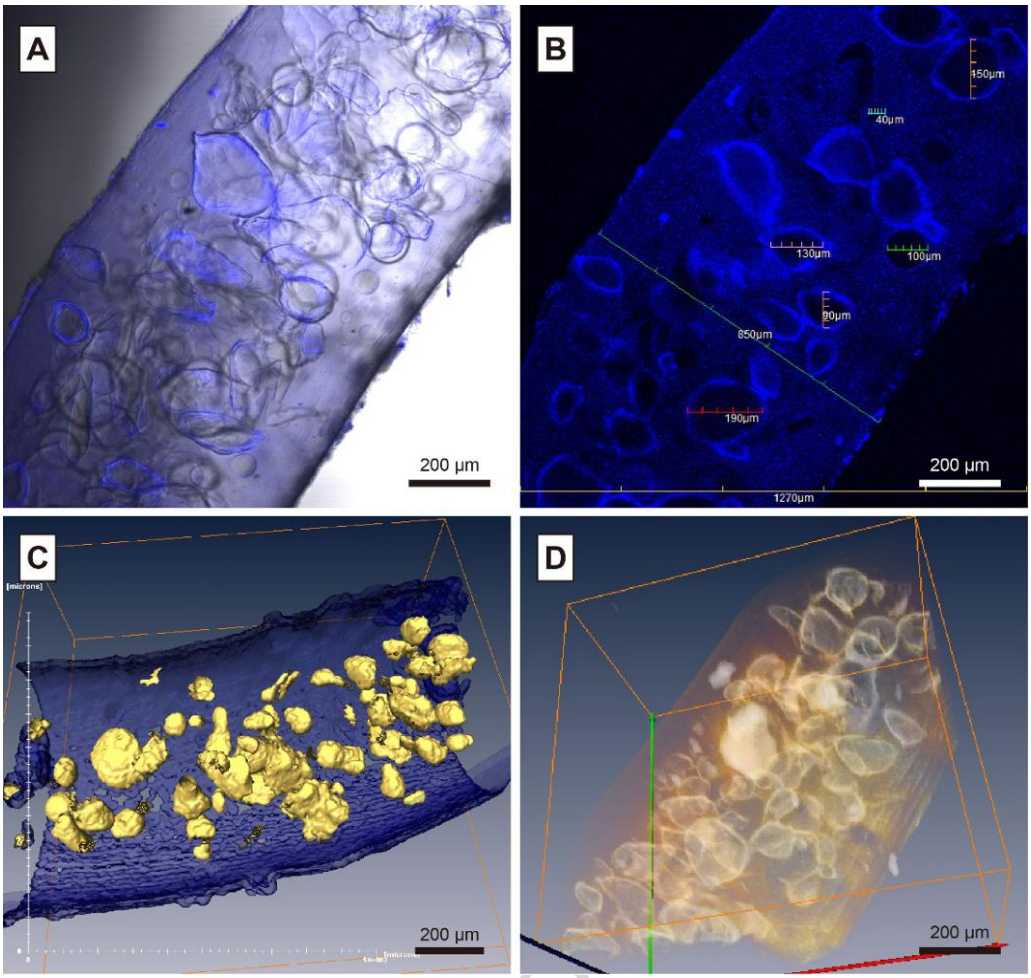

Figure 10. Confocal laser scanning microscopy images of hydrated macroporous alginate fibres. (A) Differential interference contrast, (B) autofluorescence, (C) 3D visualization showing isolated regions of macropore coalescence, and (D) 3D projection view.

Fibre produced by extracting gelatin particles from a 16% w/w particle-loaded alginate fibre.

Primary DRG cell cultures or explants derived from the peripheral nervous system of animals are commonly utilised as a neuronal model to study neuron function and disease-related axonal degeneration and repair. Primary rat DRG cells were successfully encapsulated in macroporous alginate fibres using wet spinning wherein a suspension of cells (6.25×10^3 cells/mL) and gelatin

particles (<90 μm, 57% w/w) in alginate solution (1.5% w/w) was extruded through a syringe needle into 0.5 M $CaCl_2$ solution used as a crosslinking/gel forming agent for the alginate phase. The gelatin particles were employed simultaneously as porogens and to provide cell adhesion molecules (CAM). The aim was to provide space and a cell-adhesive pore surface to promote nerve cell attachment and axonal growth within the polysaccharide fibre structure.

Neurons differentiate in cell culture and form neurites (membrane projections, extensions or outgrowths), the precursors of dendrites or axons. However, DRG cells contained in non-macroporous alginate fibres showed no significant outgrowth over 15 days. At day 5 in cell culture, the translucent appearance of the fibre with characteristic "chevron-like" flow markings was apparent (Fig. 11) together with the presence of rounded, highly separated cells (arrowed). Only isolated examples of neurite extension and branching by DRG cells (arrowed) were observed. Krantis et al. [31] reported extensive neurite outgrowth from cryopreserved DRG neurons on day 2 and 3 of cell culture on micro-ruled Cellattice™ coverslips. Ribeiro et al. [32] recorded neurite outgrowth of 180μm in 2 days from mouse embryo DRGs on collagen-coated glass coverslips. Matyash et al. [13] showed that alginate hydrogel supports neural cell adhesion *in vitro*, resulting in abundant neurite outgrowth (on the surface) and penetration into the hydrogel (up to 1000 μm), to create a 3D network by day 10. The virtual absence of neurite outgrowth over 15 days in the present study indicates that the nerve cells are efficiently encapsulated within the alginate hydrogel matrix,

and there is an absence of pore and channel structures of dimensions sufficient to accommodate neurite extension. Additionally, biodegradation of the matrix is not adequately advanced at day 15 to allow neurite growth through a weakened hydrogel.

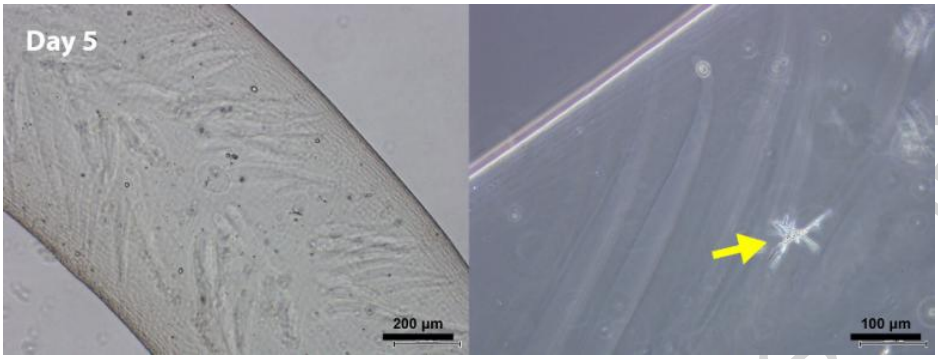

Figure 11. Bright field microscopy images of primary rat DRG cells encapsulated in a non-macroporous alginate fibre at Day 5 in cell culture. DRG-containing fibre produced by wet spinning 1.5% w/v alginate solution containing DRGs (cell density 6.25×10^3 cells/mL) into 0.5 M $CaCl_2$ solution.

In contrast, DRG cells encapsulated in macroporous alginate fibres developed a branched neurite structure (Fig. 12). On day 1 (Fig 12A), undissolved gelatin particles of size range 50–200 µm were evident in the fibres and the majority were extracted by day 2 (Fig 12B), resulting in a macroporous fibre structure. This finding correlates with rapid release of approximately 50% of the gelatin content of alginate fibres over 24 h in distilled water. Similar to non-macroporous fibres, the first evidence of neurite outgrowth and branching was observed on day 5 (Fig 12C, arrowed) over a distance of approximately 40µm and extensive neurite outgrowth and spreading within the alginate fibre was

confirmed at day 11 (Fig. 12D). This behaviour indicates formation of a pore and channel structure within the alginate hydrogel which supports neurite extension. Furthermore, it appears that the macropore size distribution within alginate fibres produced by extraction of 21% w/w gelatin particles (the majority are grouped in the 25–100 μm size range, Fig 6B) is conducive to neurite outgrowth.

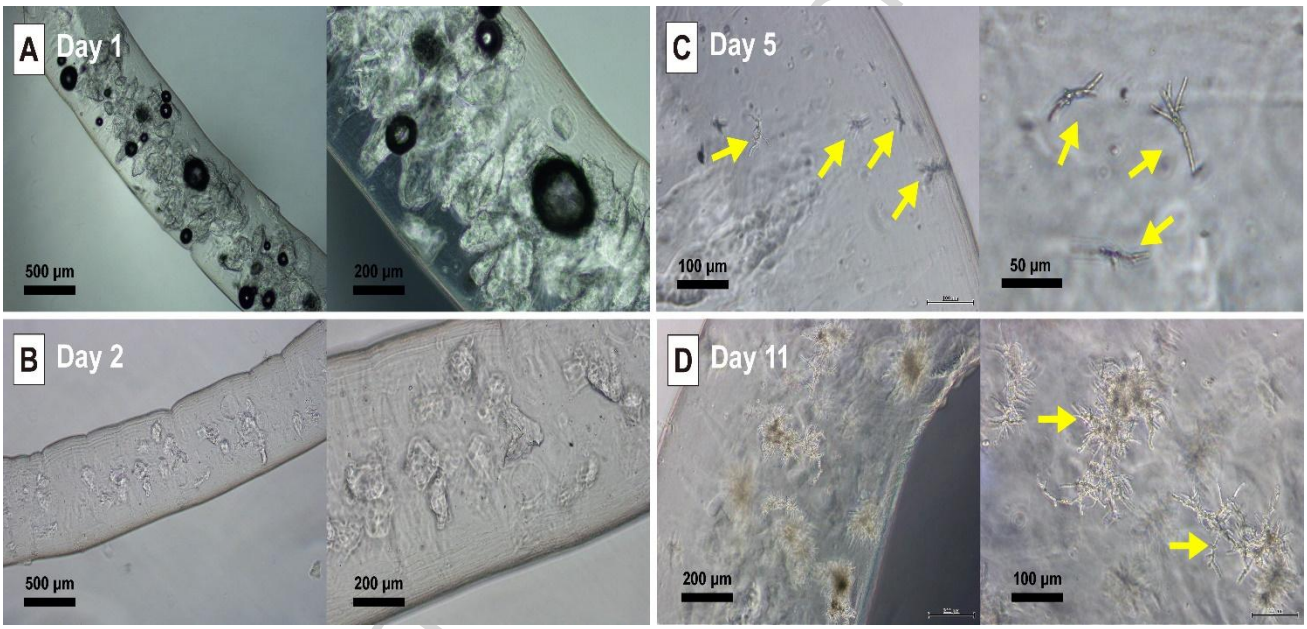

Figure 12. Bright-field microscopy of cultures of rat dorsal root ganglia (DRGs) cells encapsulated in an alginate fibre. DRG-containing fibre produced by wet spinning 1.5% w/v alginate solution containing suspended DRGs (cell density 6.25×10^3 cells/mL) and gelatin particles (57% w/w) into 0.5 M $CaCl_2$ solution.

Alginate hydrogels are known to be non-adhesive for cells inculture and have thus been modified with molecules, such as peptides (eg. Tyr-Ile-Gly-Ser-Arg) [20] or fibronectin [33] to enhance

neuron growth. The extensive neurite outgrowth observed in macroporous alginate fibres produced by wet spinning alginate solution containing DRGs and gelatin particles indicates that the gelatin particles function not only as porogens, but also provide cell adhesion molecules (CAMs) for modification of the pore surfaces to promote cell attachment and growth. CLSM analysis of cell-free macroporous alginate fibres (Fig 10B) clearly revealed protein residues lining the pore cavities formed by gelatin extraction. However, the extensive neurite outgrowth observed on day 11 (Fig. 12D) suggests that the pores and channels in the alginate hydrogel, external to the macropores, have also been surface modified. This condition may have been produced by adsorption of CAMs from the culture medium (e.g., fibronectin) and gelatin molecules during solubilisation of the gelatin particles. Release experiments demonstrated that 50% of the original gelatin content of the fibres was retained following 2 days in the release medium.

Further observations of the morphology of DRGs encapsulated in alginate fibres are presented in Fig. 13. The early stage of neurite outgrowth (days 2 and 5, Fig. 13A and B) appears to show a pseudo-unipolar-DRGsensory neuron. These specialized nerve cells feature a cell bodysited above two axon branches, one of which extends centrally toward the spinal cord, the other towards the periphery. . After 8 days, DRG cells aggregate in balls (probably when located within a macropore) and extend multiple neurites, becoming a densely branched network (Fig. 13 C and D). Evidence of neurite convergence at day 9 is shown in Fig. 13E and F.

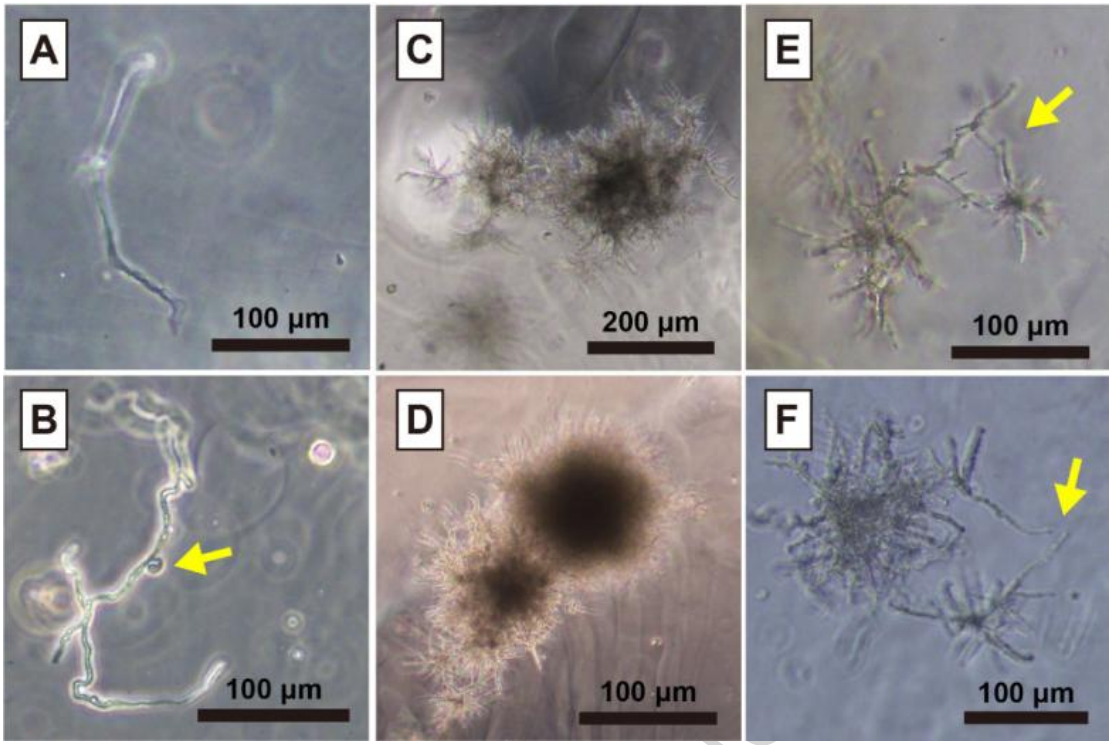

Figure 13. Morphology of rat dorsal root ganglia (DRG) cells encapsulated in alginate fibre. (A) Early stage of neurite outgrowth at day 2 in cell culture (B) pseudo-unipolar-DRG-like neuron at day 5 (C, D) DRG cells aggregate in balls and extend multiple neurites, becoming a densely branched network at day 8 (E, F) evidence of neurite convergence at day 9. DRG-containing fibre produced by wet spinning 1.5% w/v alginate solution containing DRGs (cell density 6.25×10^3 cells/mL) and gelatin particles (57% w/w) into 0.5 M $CaCl_2$ solution.

Nicotinamide adenine dinucleotide and flavin adenine dinucleotide are found to contribute in major part to rat DRG neuron autofluorescence [34]. In the present study, DRG cells encapsulated within the alginate fibre autofluoresced when excited with UV at a wavelength 350 nm and emission maximum at 460 nm. Regions of neurite branching and extension over a distance of approximately

150 µm in the alginate fibre at day 15 in cell culture are clearly visible in Fig.14.

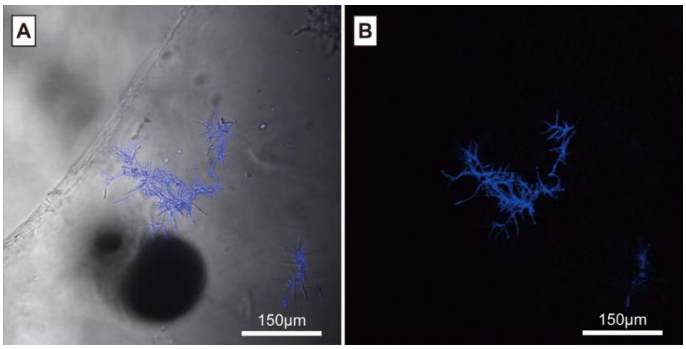

Figure 14. CLSM images of neurite outgrowth from rat dorsal root ganglia (DRG) cells encapsulated in a macroporous alginate fibre at day 15 in cell culture. (A) Superimposed differential interference contrast/autofluorescence and (B) autofluorescence produced at an excitation wavelength of 350 nm and emission maximum of 460 nm. DRG-containing fibre produced by wet spinning 1.5% w/v alginate solution containing DRGs (cell density 6.25×10^3 cells/mL) and gelatin particles (57% w/w) into 0.5 M $CaCl_2$ solution.

The results of immunolabelling DRG cells within a macroporous fibre at day 15 of cell culture using the neuronal selective marker, TU-20, are shown in Fig. 15. Neurite outgrowth from DRG cells forms isolated networks that extend approximately 200 µm in the hydrogel structure (white arrows). The DIC and fluorescence image (Fig. 15A) corresponds with the TU-20 labelled neurites and reveals the separation of the networks to be approximately 500 µm. These observations suggest that nerve cell density within the alginate fibres should be increased by a factor of at least two to increase the probability of neurite convergence.

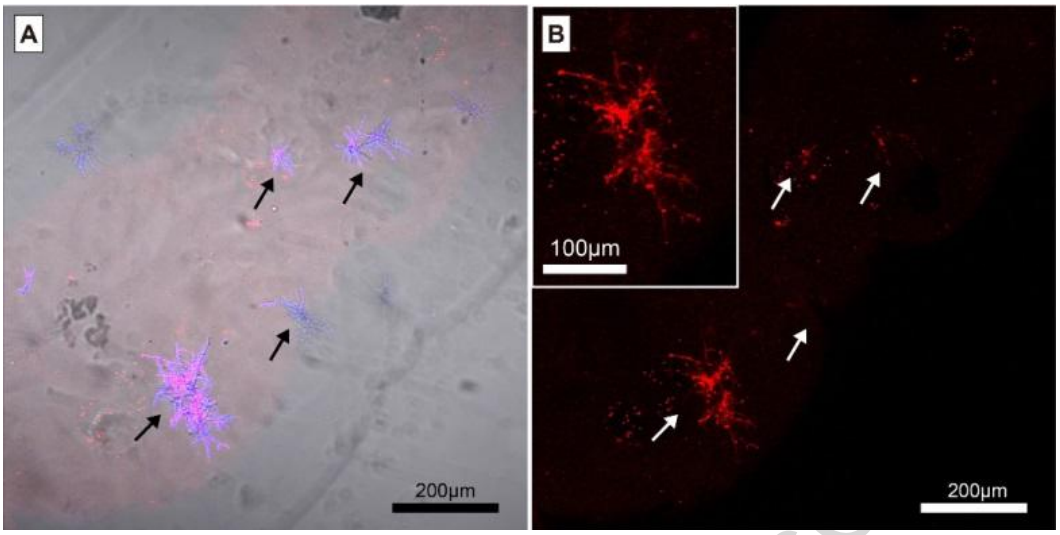

Figure 15. Mouse monoclonal anti-tubulin-III antibody (TU-20)-labeled dorsal root ganglia cells in a macroporous alginate fibre at day 15 of cell culture. (A) Superimposed differential interference contrast and fluorescence images (arrowed black) (B) TU-20 labelled images reveal neurites (arrowed white) encapsulated in the alginate fibre. Magnified view of highly branched neurites displayed in the selected field. DRG-containing alginate fibre produced by wet spinning 1.5% w/v alginate solution containing DRGs (cell density 6.25×10^3 cells/mL) and gelatin particles (57% w/w) into 0.5 M $CaCl_2$ solution.

4. Conclusion

Macroporous alginate fibres were produced for application in nerve tissue engineering by wet spinning suspensions of gelatin particles in alginate solution followed by extraction of gelatin. CLSM image analysis enabled 3D visualisation of the pore structure and provided estimates of

equivalent macropore diameter (48–69 μm), pore size distribution, maximum porosity (14.6%) and pore connectivity. CLSM also revealed that gelatin residues lined the macropore cavities and had migrated into the body of the alginate fibres thus, providing CAMs, which are potentially advantageous for promoting nerve cell proliferation and axonal extension. Encapsulation of primary DRGs in macroporous alginate fibres resulted in marked neurite outgrowth over a distance of 150 μm on day 11 in cell culture, indicating that pores and channels created within the alginate hydrogel provided a favourable environment for neurite extension. Evidence of neurite convergence was obtained at day 9. These findings indicate that macroporous alginate fibres encapsulating nerve cells may provide the basis of a useful strategy for nerve repair.

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31st October 2016

Re: Highlights for paper titled *'Production and in vitro evaluation of macroporous, cell-encapsulating alginate fibres for nerve repair'*.

- Nerve cells were encapsulated in macroporous alginate fibres for use in nerve repair.
- Fibres were produced from alginate solution containing gelatin porogens and cells.
- Pores and channels created within the fibre encouraged nerve cell development.
- Extensive neurite outgrowth of 150 µm occurred at day 11 in cell culture.