

Axonal Regeneration Supported by Oligo[poly(ethylene glycol)fumarate] Cell-Loaded Hydrogel Scaffolds in the Transected Rat Spinal Cord

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Abstract

Spinal cord injury results in complete tissue destruction and irreversible loss of neurologic function below the level of the lesion in 40% of patients in Ireland. Tissue engineering using polymer scaffolds offers potential to rebuild neural tissue through the injury site and to re-establish functional connections. An introductory review highlights current tissue engineering strategies and novel therapeutic approaches to axonal regeneration. Given the patient morbidity associated with respiratory compromise, the discrete tracts in the spinal cord conveying innervation for breathing represent an important and achievable therapeutic target. A variety of naturally derived and synthetic biomaterial polymers have been developed for placement in the injured spinal cord. Axonal growth is seen to be supported by inherent properties of the selected polymer, the architecture of the scaffold, permissive microstructures such as pores, grooves or polymer fibres, and surface modifications to provide improved adherence and growth directionality. Structural support of axonal regeneration is combined with integrated polymeric and cellular delivery systems for therapeutic drugs and for neurotrophic molecules.

This thesis proposes that cell-seeded hydrogel polymer scaffolds in a thoracic cord transection model allow for separate and controlled manipulation of the architecture, surface properties, and the molecular and cellular micro-environment of the regenerating spinal cord. The ability to control these variables with precision may enable the scaffold implant to be informative about individual facets of the repair process. A novel hydrogel, oligo[poly(ethylene glycol)fumarate] (OPF) has been developed, integrating chemical modification for positive surface charge as a substrate for axon growth. OPF scaffolds are loaded with either Schwann cells or mesenchymal stem cells, derived from the bone marrow of transgenic rats with expression of the enhanced green fluorescent protein (eGFP-MSCs). The capacity of each cell type to influence the regenerating environment is compared. Control scaffolds contain extracellular matrix only.

Chapter 2 describes the isolation of rat eGFP-MSCs and their characterization as stem cells capable of phenotypic differentiation to mesenchymal lineages. The isolation and characterization of Schwann cells from

neonatal rat pups is also described. OPF polymer synthesis, scaffold fabrication, scaffold cell loading with eGFP-MSCs and Schwann cells, thoracic spinal cord transection surgery and scaffold implantation in rats and postoperative outcomes are shown. Gross pathology of spinal cord specimens demonstrates scaffold integration and alignment.

In Chapter 3, the architecture of tissue formed after 4 weeks in response to the implantation of each scaffold type is examined initially by means of a general histopathology overview. Detailed immunohistochemistry and stereology approaches are then applied to the model. An analysis of the cell types that are contributing to separate structural and functional compartments within scaffold channels is done using antibodies to glial fibrillary acid protein (GFAP), S-100, vimentin, and neuroglycan-2. Image analysis quantifies the proportional area occupied by each cell type. Established astrocytosis is seen in a peripheral channel compartment, involved in producing boundaries which may organize axon growth. A structurally separate channel core contains immature astrocytes, Schwann cells, eGFP-MSCs, blood vessels and regenerating axons. Schwann cells double stain with GFAP and S-100 antibodies and are seen to populate each scaffold type equally, demonstrating migration into the scaffold from the animal. eGFP-MSCs are shown to be distributed in close association to blood vessels, in keeping with their function as pericytes. We propose that the tissue formed in MSC scaffold channels is granulation tissue.

The distribution of inflammatory leukocytes, T-cells and microglia is detailed. Microglial cells dominate the channel core area, whereas leukocyte infiltrate is diffuse. Image analysis provides evidence of T-cell immunomodulation in the MSC group. Quantification of axonal counts demonstrates regeneration is augmented by the presence of Schwann cells in implanted scaffolds. MSCs placed in scaffolds do not support axon growth to any extent. Axon regeneration is analysed in relationship to the developing channel vasculature. Methods of unbiased stereology provide insight into physiologic parameters of blood vessels in scaffold channels, derived from estimations of volume fraction, length density, and surface density. Mean vessel diameter and cross sectional area for each channel type are calculated. Whereas Schwann cell channels have high numbers of small, densely packed vessels, infrequent and large vessels dominate the structure of MSC scaffold channels. Significant

correlations between axon counts and vessel length and surface density are shown. Axon number is also shown to statistically correlate with decreasing vessel diameter, implicating the importance of blood flow rate in channels. Radial diffusion distances in vessels correlated significantly to axon number as a hyperbolic function.

In Chapter 4 the development of a retroviral library for gene delivery of neurotrophic factors to Schwann cells and MSCs is described. Retroviral expression plasmids, encoding the cDNA transcripts for human neurotrophin 3 (NT-3), brain derived neurotrophic factor (BDNF), and glial derived neurotrophic factor (GDNF), were constructed by molecular cloning. Neurotrophin genes were cloned into the pLXSN backbone, which has been modified to contain an internal ribosomal entry site (IRES) for bicistronic expression of eGFP in target cells. DNA sequence accuracy and eGFP-neurotrophin co-expression were verified prior to the development of GP+E86 packaging cell lines for NT-3, GDNF and BDNF retrovirus production. Neurotrophins are secreted at physiologic levels from target cells following viral infection. Stimulation of neurite outgrowth from dorsal root ganglia is shown in response to conditioned media from target cells infected with NT-3 retrovirus. Stably transduced Schwann cell and MSC lines have been made following retroviral gene transfer and cell selection for use in OPF+ scaffolds.

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Dedication

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With all my love to Theresa, as we look forward to our lifetime together.

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Abbreviations

AA2-P	ascorbic acid 2-phosphate
cAMP	cyclic adenosine monophosphate
BBZ	bis benzamide
AEC	3-amino-9-ethylcarbazole
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
ch-ABC	chondroitinase ABC
CFU	colony forming units
CMV	cytomegalovirus
CNTF	ciliary neurotrophic factor
CSPG	chondroitin sulphate proteoglycan
DAB	3,3' diaminobenzidine
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
ECM	extracellular matrix
ECMV	encephalomyocarditis virus
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
eGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FITC	fluorescein
GAG	glycosylaminoglycan
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
H&E	hematoxylin and eosin
HA	hyaluronic acid

HAMC	hyaluronic acid methylcellulose
HCl	hydrochloric acid
HGF	hepatocyte growth factor
HLA	human leukocyte antigen
HRP	horseradish peroxidase
HUVEC	human umbilical endothelial cell
IBA1	ionized calcium binding adaptor molecule 1
IDO	indoleamine 2,3-dioxygenase
Ig	immunoglobulin
IL	interleukin
INF-γ	interferon-gamma
iPSCs	pluripotent stem cells
IRES	internal ribosome entry site
Kb	kilobase
LB	Luria-Bertani
Lv	length fraction/density
LTR	long terminal repeat
MCS	multiple cloning site
MoMuLV	Moloney murine leukemia virus
mRNA	Messenger RNA
MSC	mesenchymal stem cell
N-CAM	Neural Cell Adhesion Molecule
NG-2	neuroglycan-2
NGF	nerve growth factor
NT-3	neurotrophin-3
OPF	oligo[poly(ethylene glycol)fumarate]
OPF+	positively charged oligo[poly(ethylene glycol)fumarate]
PAN/PVC	poly(acrylonitrile-co-vinylchloride)
PFA	paraformaldehyde
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEI	polyethylenimine
pHEMA	Poly(2-hydroxyethyl methacrylate)

pHEMA-MMA	pHEMA and pHEMA-co-methyl methacrylate
PLGA	poly lactic and poly glycolic acid
PNS	peripheral nervous system
Ppy	polypyrrole
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SC	Schwann cell
SCI	spinal cord injury
siRNA	short interfering RNA
SOC	super optimal broth
Sv	surface fraction/density
TAE	tris/ acetate/EDTA
TGFβ	transforming growth factor beta
Th	T-helper
TNFα	tumour necrosis factor alpha
TRITC	rhodamine isothiocyanate
TU	transducing units
V	volume
VEGF	vascular endothelium growth factor
Vv	volume fraction

Chapter 1: Introduction

Current Tissue Engineering and Novel Therapeutic Approaches to Axonal Regeneration following Spinal Cord Injury using Polymer Scaffolds

1.1 The Rationale for Tissue Engineering Approaches in the Context of Respiratory Compromise

Current interventions offer little hope of functional recovery for patients after a spinal cord injury (SCI). In the United States, the incidence of SCI is 32 injuries per million population, approximately 11,000 new injuries per year, affecting a young group of people of median age 26, that is predominately male (82%). Road traffic accidents, acts of violence, falls, and sports injuries account for the majority of injuries. The average inpatient stay is 9 months, during and following which the patient's life, in virtually all aspects, is profoundly changed (Lali, Sekhon et al. 2001; Association 2009). 45.7% of the 253,000 persons living in the United States with the residual of spinal cord injury have permanent and complete paraplegia or tetraplegia, irreversible loss of neurologic function below the level of the injury (National Spinal Cord Injury Association, 2009).

The majority of patients die from respiratory complications. Injury at any level of the spinal cord will impair respiratory function, through the destruction of motor nuclei and descending motor tracts innervating diaphragmatic, thoracic, intercostal and abdominal accessory muscles. Equally impaired are ascending sensory signals for muscle control via stretch reflexes, for cough, vomit and secretion clearance and from peripheral respiratory chemoreceptors. These axons project through the spinal cord to and from a neural network in the brainstem comprising three interconnected centers, the pontine group, and the medullary dorsal and ventral respiratory group. The pontine group (parabrachial/Kölliker-Fuse complex) controls respiratory timing, receives input from lung stretch receptors, and links respiration to behavioural cues; the dorsal group receives

afferents from respiratory chemo and mechanoreceptors, and coordinates respiratory-cardiac reflexes; the ventral group (Bötzinger complex) projects inspiratory neurons, expiratory motor neurons rostrally, and includes a pre-complex generating the respiratory rhythm. Axons descend in the spinal cord in the anterolateral white matter to phrenic, intercostal and abdominal motor neurons, laterally in the high cervical cord near the spinothalamic tract for autonomic function and with the corticospinal tracts for voluntary respiratory control (Nogués and Benarroch, 2008).

Accordingly, respiratory failure with spinal cord injury occurs as a consequence of alternations in tidal volume, ventilation and its pattern, diminished responses to hypercapnia, reduced lung and chest wall compliance, and progressive respiratory muscle fatigue due to compensatory breathing rates. Hypoxia from respiratory compromise can further the neurologic injury. Common secondary pathology includes (aspiration) pneumonia, atelectasis and the complications of mechanical ventilation (Lane et al, 2008). Injury to the cord can also induce paralytic ileus worsening aspiration. More severe respiratory compromise occurs with higher levels of injury with risk to phrenic motor nuclei located in cervical spinal cord segments C3-C5 (occasionally as low as C7) (Zimmer et al., 2007).

Pathological (Quencer and Bunge 1996) and imaging studies (Bodley 2002) demonstrate tissue destruction with cysts and gliosis in the area of injury, along with atrophy in adjacent segments of cord. Strategies aimed at preventing immediate and delayed secondary damage need to be administered within minutes or hours of injury. Even if ideal protective agents were available, many patients would not be in circumstances where this would be available or successful. The area of cysts and glial scarring does not contain cells or tissue that contribute to regeneration and is consequently both a gap and a barrier to regeneration. There are, therefore, only two ways to re-establish neurologic function below the block: bypassing the area or rebuilding functional tissue within the cyst/scar. A functional bypass might be established by nerve autograft connections from areas above the lesion to distal effectors (cord or muscle) (Tadie, Liu et al. 2002). The second approach is to replace the cyst/scar with functional tissue, promoting the development of neural tissue bridges to carry regenerating axons from above to roots or muscles below the lesion (Friedman, Windebank et al. 2002). For future

use in patients, replacement of a segment of cord would be suitable for those with massive damage to the cord with no evidence of residual functional tissue in the area. Unfortunately, this accounts for a significant number of patients.

Animal models of spinal cord injury include complete transection (thoracic), hemisection (dorsal or unilateral), and contusion injuries (forceps and computer-controlled weight impact). These models approximate common human pathology, open cord laceration (1/4 of injuries) and closed compression/contusion injuries (3/4). Biomaterial polymers may be delivered as gels, suitable for contusion and small tears, as devices designed to fill larger defects (sponges) or to bridge large gaps and traverse the glial scar (tubes and multichannel scaffolds (Nomura, Tator et al. 2006). While deep tears or transections are rare in human injury, complete or partial transections in animal models are useful as proof of concept, and for the controlled study of axonal regeneration (Talac, Friedman et al. 2004). Animal models of respiratory dysfunction focus on high cervical injury producing diaphragm hemiplegia, but no studies to date have employed polymer-based tissue engineering strategies specifically in this context. Given the severity of patient morbidity and the rates of mortality associated with respiratory compromise, neurologic repair is an important therapeutic goal. A relatively short distance, from the medulla to phrenic C3-C5 or within the phrenic segments for example, needs be bridged by new neuronal tissue. Equally, respiratory innervation associates with discrete tracts, corticospinal and spinothalamic, and repair may often be unilateral given a lateral injury and diaphragmic hemiplegia. Such tracts represent ideal targets for polymer scaffold implantation given their limited scope and clinical importance.

1.2 Important Bioengineering Considerations

The classification of biomaterials for the spinal cord is based on whether the materials are naturally derived or synthetic, whether they are hydrogels, whether or not they are biodegradable, as well as other sub-classifications based on specific modification or functional adaptation (surface charged, drug-delivery etc.). Regardless of the source or application, the material must have properties which are compatible within the spinal cord environment (Kohane and Langer

2008). These properties in turn influence the regenerative capacity of engineered structural support for neurite outgrowth at a macro (i.e. fascicular) and micro (axonal) level.

1.2.1 Scaffold Placement within the Spinal Cord Environment

In relation to blood, cerebral spinal fluid is low in cellular nutrients. Scaffold permeability to various molecular sizes becomes crucial for access to oxygen and nutrients and removal of metabolic wastes. The degree to which a material swells within the aqueous environment of the spinal cord must be known if the scaffold is to maintain an appropriate alignment and not compress regenerating nerves.



Figure 1.1: Polymer scaffold in situ. Lateral radiograph showing a well aligned scaffold within the spinal canal after 4 weeks. The spine has been fixed and the scaffold contains barium contrast within the polymer. From (Rooney, Vaishya et al. 2008) with permission.

Degradation kinetics may be accelerated by the ingrowth of axons and by the deposition of extracellular matrix by support cells of the CNS or by the therapeutic cell line seeded within the scaffold. Stiffness, permeability, swelling, strength and degradation are of course specific to the particular polymer employed, are all readily modified through changes in polymer concentration or constituent ratios. (de Ruitter, Onyeneho et al. 2008) exemplify the type of *in vitro* characterization and methodology required to develop an implant of any polymer type. In this study,

the authors present a series of methods to characterize multichannel nerve tubes for properties of bending, deformation, swelling, and degradation and introduce a new method to test the permeability of multichannel nerve tubes from the rate of diffusion of different- sized fluorescent dextran molecules. Equally, the implantation methodology must be developed. While the material is placed within rigid spinal column, the spine may require further fixation (Figure 1.1) (Rooney, Vaishya et al. 2008). Scaffolds in unfixed spines have a greater tendency to produce scoliosis and become displaced (Figure 1.2). The material should be of sufficient softness not to physically damage the cord as the animal moves. The degradation products of the polymer, and any residual agents used in its fabrication, cannot be locally or systemically cytotoxic or elicit an immune response which will further gliosis, and be destructive both to the scaffold complex and any regenerating axons (Liu and Cao 2007).

Cells detect mechanical characteristics of the environment through adhesion complexes and the actin cytoskeleton, and the stiffness of the substrate may be of critical importance (Discher, Janmey et al. 2005). Finally the tensile strength of the material – its ability to hold a suture for example, will contribute to its clinical use.

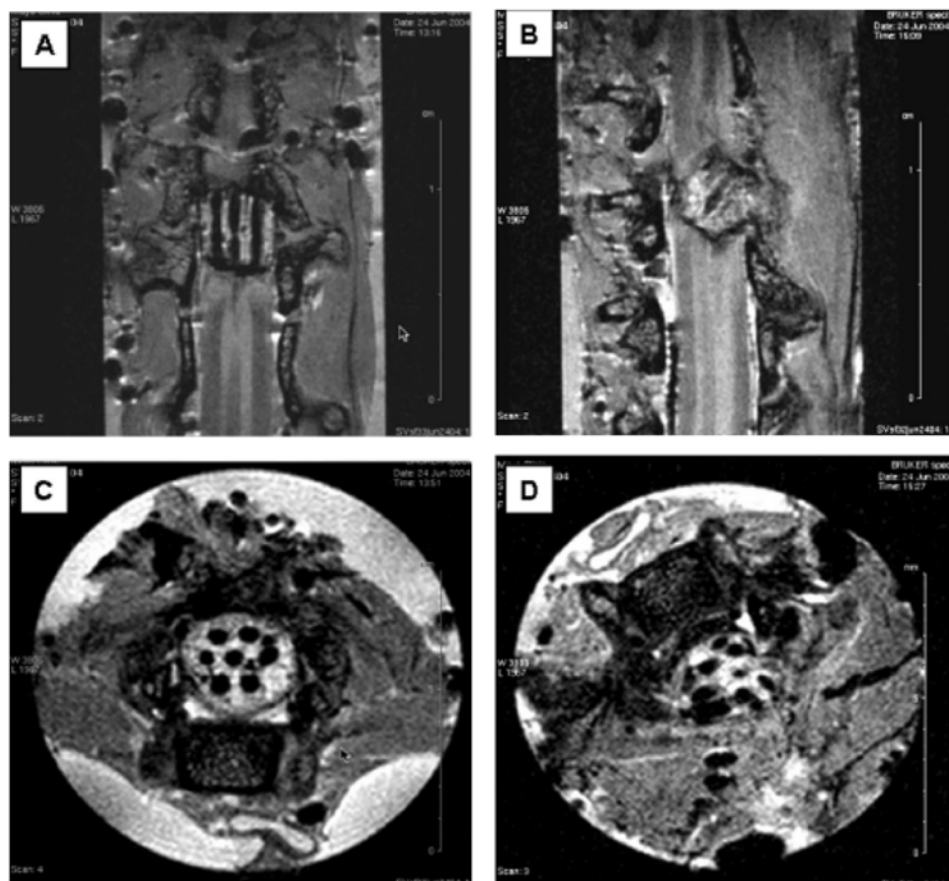


Figure 1.2: The effect of spine stabilization on scaffold alignment. 3-dimensional magnetic resonance microscopy (MRM) in coronal (A and B) and axial (C and D) images 4 weeks after scaffold placement into the transected cord with (A and C) and without (B and D) spine fixation. From (Rooney, Vaishya et al. 2008) with permission.

1.2.2 Material Fabrication and Porosity

Scaffold biomaterials for spinal cord placement are fabricated by dissolving the monomer in an aqueous or organic solvent to produce a liquid state that is polymerized into macromers by a chemical, thermal or photo-crosslinking reaction. Additional reagents may be added to enhance the crosslinking reaction. Synthetic polymers often employ the use of chemical initiators and accelerators to fine tune polymerization rates. The majority of spinal cord scaffolds are made by injection molding. To create pores in the structure, porogens are incorporated into the polymer mix. Commonly used porogens include sodium chloride crystals, ice crystals, gas bubbles introduced by peroxides or air-foaming, and gelatin composite materials. In each case, the polymer forms around the porogen, which

itself is removed from the final structure leaving only the space it occupied (Figure 1.3). The size of the pore therefore can be controlled by adjusting the variables that control crystal size and the direction of crystal growth (Madaghiele, Sannino et al. 2008), or by adjusting the water content of a hydrogel.

A continuous porous structure closely mimicking the intrinsic mechanical characteristics of the original tissue may provide a better environment for regeneration (Discher, Janmey et al. 2005; Deguchi, Tsuru et al. 2006). Material porosity is essential for cell attachments, allows for greater distances that may be

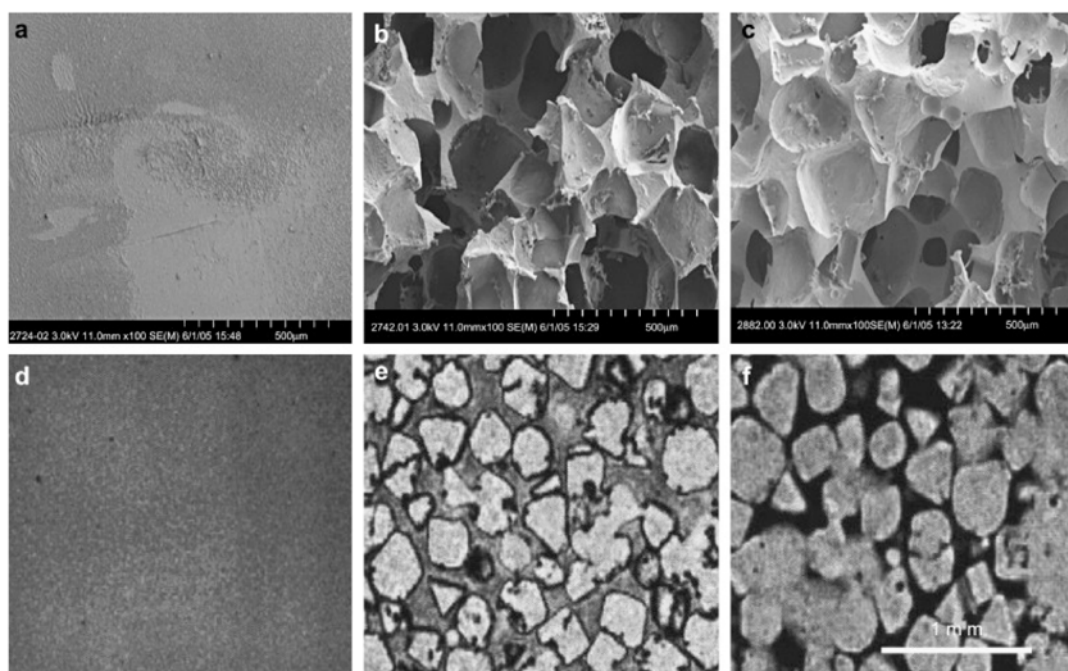


Figure 1.3: Scaffold polymer porosity. Porosity is controlled in a polyethylene glycol based hydrogel by varying the size of the porogen. Scanning electron microscopy (SEM) of lyophilized hydrogel (a-c) shows a highly porous structure with open, interconnected pores surrounded by polymer walls. Magnetic resonance microscopy (MRM) of scaffolds in their hydrated state (d-f) are compared. The distribution of sodium chloride particles was in the range of 100-500 μm . Scaffold with no porosity (a, d), porous scaffold with 75% porogen content and 300 μm particle size, (b,e), and porous scaffold with 75% porogen content and 500 μm particle size (c,f). Figure from (Dadsetan, Hefferan et al. 2008) with permission.

bridged, and improves functional recovery following transection (Jenq and Coggeshall 1987; Vleggeert-Lankamp, de Ruiter et al. 2007; Reynolds, Bren et al. 2008). Porosity also allows for tissue vascularization of the avascular scaffold

implant, influences cell migration and phenotype, and will improve implant stability at the cord-scaffold interface through interlocking between implant and cord tissue (Dadsetan, Hefferan et al. 2008).

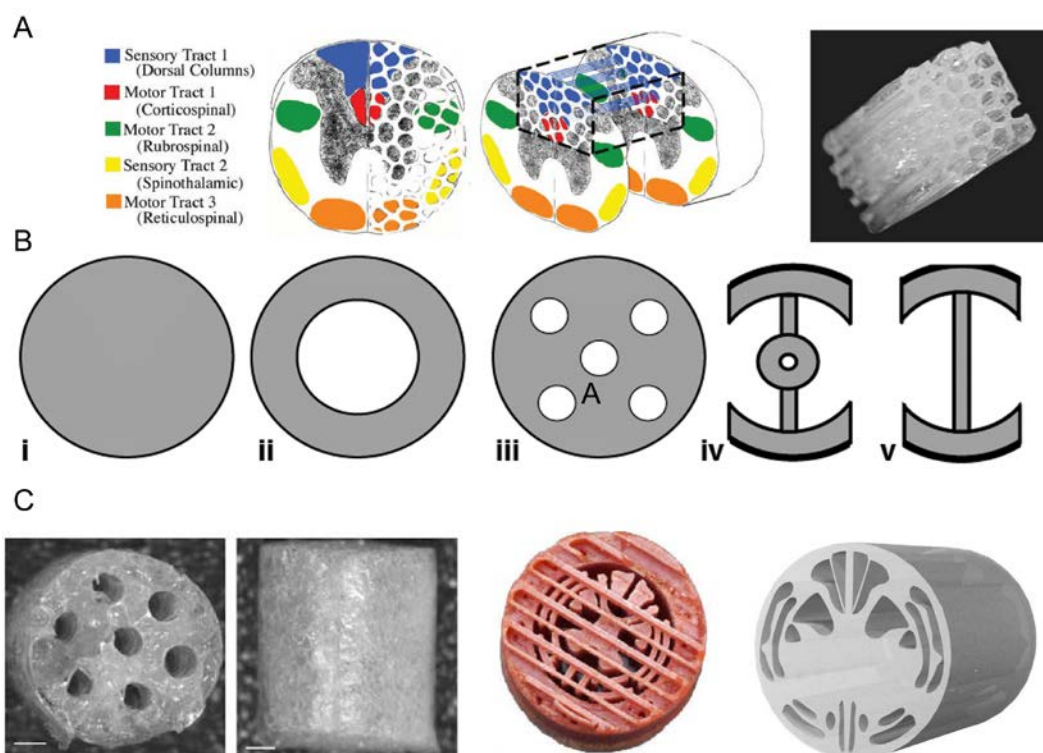


Figure 1.4: Macroarchitectural Design. Various injection-molding strategies for spinal cord scaffolds to align fascicular bundles. (A) Templated agarose cast over polystyrene fibres produces linear aligned channels of 200 μm diameter for a hemisection model (from (Stokols, Sakamoto et al. 2006) with permission). (B) Cylinder, tube, multichannel and open-path designs, from (Wong, Leveque et al. 2008) with permission, for casting in poly (ϵ -caprolactone). (C) PLGA multichannel scaffolds cast over parallel metal wiring provide dorsal and ventral channels, (left, bar 500 μm , from (Moore, Friedman et al. 2006) which may be elaborated into molds of complex anatomical design, (right, (Friedman, Windebank et al. 2002) both figures with permission).

1.2.3 Macroengineering

Nerve tracts in the human spinal cord have diameters of 100–1000 μm . It is the role of scaffold macro-engineering to design conduits whose architecture optimizes axonal growth potential through the alignment of fascicular groups (Figure 1.4). Channel sizes depicted in the templated agarose scaffold are on the order of 200 μm (Figure 1.4A), and 450 μm in the PLGA scaffold (Figure 1.4C).

In the first study of its kind, Wong *et al* (2008) directly compared porous poly (ϵ -caprolactone) synthetic polymer scaffolds cast in five different architectures, cylinder, tube, multichannel, and open-path design with and without a central core (Figure 1.4B). The findings demonstrated not only that open path designs were improvements over the other three designs in terms of regenerative capacity, but also that the other more closed designs adversely affected the surrounding cord, doubling the defect length. In a multichannel model, the channel size is of importance. Spinal cord scaffolds with multiple longitudinally-aligned channels of 450 and 660 μm were constructed from PLGA using injection molding. When seeded with Schwann cells, this scaffold design supported robust axonal growth (Moore, Friedman *et al.* 2006), although also demonstrated the formation of a rim of fibrous tissue surrounding the core of regenerated neurons. Further work evaluating the relationship between scaffold channel diameter and the number of axons regenerating showed a larger area of fibrous tissue and a reduced axon number in the larger channel size (Krych, Rooney *et al.* 2009).

1.2.4 Microengineering

A large myelinated axon in the CNS has a diameter of 15 – 20 μm . In order to create functional reconnections, the scaffold must also regionally align separated neuron groups at an axonal and growth cone level. Scaffold microengineering refers to designing features that are in the order of a few microns in at least one dimension (Khademhosseini and Langer 2007). In the context of the spinal cord, this includes micro-structures that will provide precise directionality to growth and improve axonal adherence at the level of the advancing growth cone. It is conceptually useful to consider the re-alignment of nerve fascicles as three dimensional bundles of axons which themselves prefer to grow or elongate along the planes of two dimensions (Bellamkonda 2006). Microgrooves can be placed in the polymer surface by laser etching, affecting contact guidance and alignment of neurites. *In vitro* work has demonstrated optimal sizes of 2 μm minimum groove depth (Clark, Connolly *et al.* 1991), with more narrow ridges (5 μm versus 10 μm) improving the number neurites aligned as well as the number of focal contact adhesions in a given cell (Goldner, Bruder *et al.* 2006). Further improvements in neurite outgrowth are seen with coating the

grooved surface with collagen or laminin peptides, Figure 1.5 (A and B) (Yao, Damodaran et al. 2010). Axonal growth cones preferentially advance up gradients of laminin (Adams, Kao et al. 2005). For the spinal cord, gradients have been made in natural and synthetic polymers with laminin (Dodla and Bellamkonda 2006).

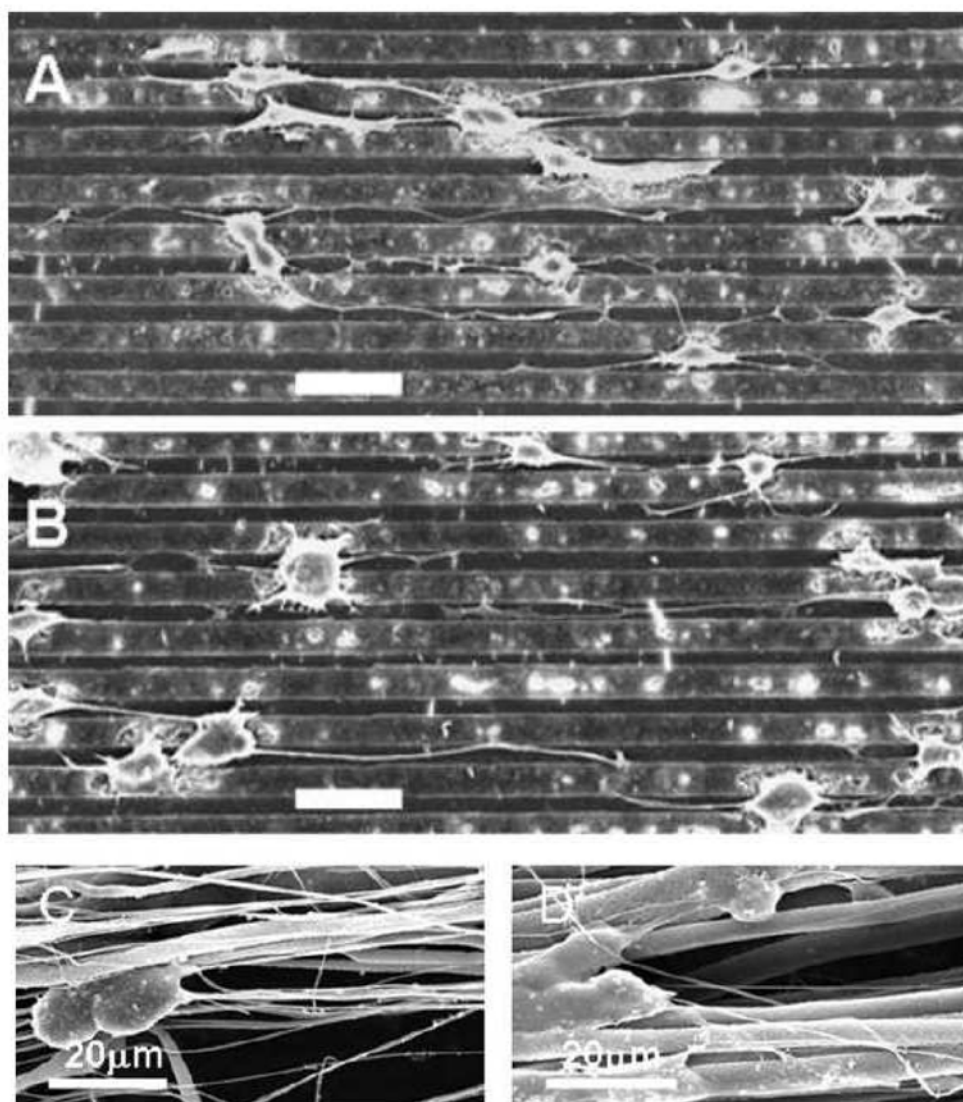


Figure 1.5. Oriented axonal growth. Microengineering strategies with oriented extension of neurite outgrowth in relation to micropatterned grooves and nanofibres. SEM imaging of PC12 cell growth on a collagen type I-coated PLGA film (A) and on a laminin peptide-coated PLGA film (B) each with a laser-etched groove size of 10 μm . Bar 40 μm , from (Yao, Wang et al. 2009). Neurite extension along the length of electrospun PCL fibres of a diameter ranging from 0.8 +/- 0.7 μm (C) and 3.7 +/- 0.5 μm (D), from (Yao, O'Brien et al. 2009) with permission.

Axons also show a preference to grow along the length of micro and nano-fibres of polymers such as collagen and synthetic polymers, Figure 1.5 (C and D). Polymer fibres are made by monomer self-assembly, producing a randomized fibre orientation of larger caliber fibres, or by electrospinning for parallel alignments of nanometer scale fibres (50 nm – 30 μ m). This technique uses electric charge to draw and elongate threads of liquid polymer (collagen, PLGA, PCL) from a syringe pump source. The solvent evaporates from the airborne filament which is laid down upon an electrically grounded plate or rod, or onto a spindle apparatus (Yang, De Laporte et al. 2005). Collagen filament bridges with fibres of 20 μ m show promise as 2 dimensional surfaces to guide axons, improving axonal density and motor function, but appear also to have significant cytotoxicity with high animal mortality in a rabbit model (Yoshii, Oka et al. 2004; Yoshii, Ito et al. 2009). A goal of scaffold microengineering is to pack larger channels of scaffolds with these fibrous substrates to approximate normal axonal densities (Schnell, Klinkhammer et al. 2007).

Table 1.1

The major natural polymers in use in animal models of spinal cord injury and examples of their applications

Natural Polymers	Structure	Examples of Applications for Spinal Cord Repair
Natural Hydrogels		
Collagen, type I	Glycine-X-Y polypeptide fibrils	Collagen gels as extracellular support growth matrix. (Marchand, Woerly et al. 1993; Ma, Fitzgerald et al. 2004) Collagen gels with embedded neurotrophic factors [NT-3, BDNF] (Houweling, Lankhorst et al. 1998) Collagen filament bridges as growth substrates (Yoshii, Oka et al. 2004; Pfister, Iwata et al. 2006) Linear ordered collagen scaffolds with collagen binding BDNF (Han, Sun et al. 2009) Schwann Cell Delivery (Dewitt, Kaszuba et al. 2009) Electrospun nanofibres and stem cell differentiation (Xie, Willerth et al. 2009) Gels for drug delivery [EGF and FGF-2] (Jimenez Hamann, Tator et al. 2005) Growth factor infused matrix for synthetic polymer channels (Tsai, Dalton et al. 2006) Collagen coating of PLGA for gene delivery (De Laporte, Yan et al. 2009) Guidance gradients with incorporated laminin (Yao, Damodaran et al. 2010)
Agarose/alginate	Linear polysaccharide β -D-mannuronic acid and α -L-guluronic acid	Agarose gels as growth matrix (Bellamkonda, Ranieri et al. 1995; Balgude, Yu et al. 2001) Microencapsulation of neurotrophic factors (Maysinger, Krieglstein et al. 1996) Agarose gels with neurotrophic gradients (Cao and Shoichet 2001). Freeze-dried alginate sponges as neurite growth substrate (Kataoka, Suzuki et al. 2004) Cell encapsulation into alginate hydrogel (Novikova et al. 2006) Linear guidance pore scaffolds (freeze dried agarose) (Stokols and Tuszynski 2006) Uniaxial multichannel scaffolds with BDNF-secreting MSCs (Stokols, Sakamoto et al. 2006) Scaffolds with neurotrophin integration (Jain, Kim et al. 2006; Stokols and Tuszynski 2006) Guidance gradients with incorporated laminin (Dodla and Bellamkonda 2006) Steroid delivery with agarose embedded PLGA nanoparticles (Chvatal, Kim et al. 2008)
Hyaluronic acid	Dissacharide units of glucuronic acid and N-acetylglucosamine	Intrathecal drug and growth factor delivery with as a methylcellulose composite gel. (Gupta, Tator et al. 2006; Shoichet, Tator et al. 2007; Kang, Poon et al. 2009)
Chitosan	Co-polymer of N-acetylglucosamine and N-glucosamine	Chitosan scaffolds supporting cell adhesion and growth (Freier, Koh et al. 2005; Freier, Montenegro et al. 2005) Schwann cell encapsulation (Yuan, Zhang et al. 2004) Protein elution (Kim, Tator et al. 2008) Extra and intramedullary tissue bridge conduits with neural stem cell differentiation (Nomura et al. 2008; Nomura et al. 2008)
Fibronectin	Plasma glycoprotein dimer	Fibronectin mats for oriented axonal growth (King et al. 2003) Neurotrophin and drug delivery (Phillips, King et al. 2004)
Fibrin	Fibrillar glycoprotein polymer	Neurotrophin elution from heparin complexes (Taylor, Rosenzweig et al. 2006) Embryonic stem cell differentiation (Willerth, Fixel et al. 2007) Gliosis inhibition (Johnson, Parker et al. 2009)

1.3 Biomaterials for the Spinal Cord

1.3.1 Natural Polymers

The spinal cord lacks a support matrix equivalent to the endoneurium and perineurium in peripheral nerve, one that can act as conduits to approximate disconnected axonal groups. Furthermore the axonal density of the spinal cord far surpasses that of peripheral nerve with far less extracellular matrix support. The rationale for polymer implants is to replace a damaged area of the cord with just such a structural matrix. Natural polymers are biological fibrillar protein, polysaccharide, or glycosylaminoglycan (GAG) carbohydrates which form hydrogels. These polymers already have an intrinsic function such as extracellular matrix or structural support, and a degradation profile by *in vivo* enzymes that is in keeping with their natural role. Hydrogels are mesh networks of insoluble polymer fibres, through which water can freely flow to osmotically swell or shrink the overall structure. Hydrogels are attractive materials for use in the spinal cord. They are macroporous, soft materials readily allowing cell adhesion and migration, while nutrients and wastes are easily exchanged. They can be easily shaped to fit the defect, their elasticity and degradation may be adjusted by component density. Table 1 details the main natural polymers and highlights examples of their application within the spinal cord. Two spinal cord extracellular matrix (ECM) components are used, collagen and hyaluronic acid (HA); two polymers are derived from marine plants, agarose and alginate, and chitosan is derived from insect or crustacean shells.

Extracellular matrix-based polymeric systems

Being the predominant extracellular matrix protein, type I collagen has intrinsic properties including molecular sites for cell adhesion and migration, inherent signalling transduction for proliferation and differentiation, and mechanical properties similar to soft tissue. Antigenicity is low, provided the origin species is the same as the host. Solutions of collagen are polymerized by adjusting pH, or with the addition of ionic salts. Whereas early use focused on its application as a three dimensional matrix growth, it was soon realized that collagen itself has a limited capacity to support axon growth (Marchand, Woerly et al. 1993), and that its use required further functionalization. An important trend

for the use of collagen is in combined strategies, particularly as a growth or elution matrix within or on the surface of other polymer types (Tsai, Dalton et al. 2006) or as a composite material (Cheng, Deng et al. 2003). Axonal extension onto collagen can be improved through covalent modification, or the incorporation of cell-adhesion molecules such as laminin, to provide directional guidance as a gradient along collagen fibres (Yao, O'Brien et al. 2009). Collagen also has the advantage of being thermoresponsive, gelling at physiologic temperatures, making it attractive for use as an injectible polymer delivery system. This property enables the incorporation of neurotrophic factors, drugs or cells at the time of gelation without thermal damage to the factor or cell line. Incorporation of neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF) (Houweling, Lankhorst et al. 1998) improved axonal counts and animal function, including a specific regional improvement of corticospinal tract density with the use of NT-3. Degradation of the collagen *in situ* allows for sustained release of the growth factor, as well as an improved surface for cell attachments.

The glycosaminoglycan HA was thought to be a good material candidate given its role as extracellular matrix in the brain, but its success in supporting axonal growth is modest. As a scaffold material, HA has been used with benefit as a matrix for cultured embryonic spinal cord tissue placed into transected cord (Rochkind, Shahar et al. 2002). HA has however been developed into an extremely useful co-polymer gel with methylcellulose for intrathecal drug delivery (Gupta, Tator et al. 2006). Whereas methylcellulose gels at increasing temperature, unmodified HA quickly disperses *in vivo*. The combination of acetate-modified HA with methylcellulose (HAMC) has the distinctive property of already gelling at room and physiologic temperature prior to its delivery, but become liquid when subjected to the mechanical shear forces involved with syringe and needle injection (Katz and Burdick 2009). Collagen embedded epidermal growth factor (EGF) (Shoichet, Tator et al. 2007), and erythropoietin (Kang, Poon et al. 2009), have been safely delivered with sustained release *in situ* from HAMC. The latter agent enhanced neuroprotection with reduced cavitation size and increased neuron numbers following clip compression of the spinal cord.

Polymers extracted from marine life

Agarose is used in many of the same ways as collagen for spinal cord repair. It is a linear polysaccharide derived from seaweed and cross-linked by temperature gradients through hydrogen bonding. Agarose is thermoresponsive, but at temperatures lower than 37°C. It has been used as an injectable system when it can be rapidly cooled *in situ* using liquid nitrogen vapour (Jain, Kim et al. 2006). Such a system is now being developed for direct topical delivery of dexamethasone onto the injury site, from drug eluting nanoparticles suspended within an agarose implant (Chvatal, Kim et al. 2008). Early inflammatory infiltrates and lesion size were reduced by day 7. Like collagen, agarose itself is relatively impenetrable by axons, but serves as an excellent axonal growth substrate, particularly when functionalized with laminin gradients (Dodla and Bellamkonda 2006). Tuszynski and colleagues have used a freeze drying method to form agarose scaffolds containing linear guidance pores with a mean diameter of 120 µm (Stokols and Tuszynski 2004; Stokols and Tuszynski 2006). This process involves the formation of ice crystals whose size and direction of growth can be controlled by the temperature gradient (Tabesh, Amoabediny et al. 2009). Pore size in the scaffold can also be controlled by the freezing rate and pH, with the faster rate creating smaller sizes (Sachlos and Czernuszka 2003). Integrating BDNF into the scaffold material (Figure 1.6) and in separate experiments, BDNF-secreting mesenchymal stem cells scaffold channels, significantly improved the scaffold's capacity to promote regeneration (Stokols and Tuszynski, 2006). BDNF within lipid microtubules has also been incorporated into agarose scaffolds, enhancing axonal growth for the length of the scaffold but not into the distal cord (Jain, Kim et al. 2006).

Alginate is obtained from algae and the polymer solution is crosslinked by calcium into a sponge-like structure. Such a structure supported axonal extension in the spinal cord and limited gliosis (Kataoka, Suzuki et al. 2001). Hippocampal neurospheres and BDNF-secreting fibroblasts have been seeded onto alginate and placed into the transected cord (Nomura, Katayama et al. 2006). Agarose and alginate requires ultrapurification prior to use, given that commercial preparations often contain mitogens and cytotoxic byproducts.

Chitosan is a glycosaminoglycan polymer derived by chemical deacetylation of chitin, the major structural polysaccharide found in crustacean,

shellfish and insect shells. Cell adhesion to the structure is determined by the extent of its positive charge, itself a function of the degree of alkaline deacetylation (Nisbet, Crompton et al. 2008). Further improvements in cell attachment are seen with the addition of poly-L-lysine to the polymer mix, and a thermoresponsive polymer can be made with the addition of glycerol phosphate salts (Crompton, Goud et al. 2007). Chitosan scaffolds support axonal growth in the spinal cord (Freier, Montenegro et al. 2005), and the polymer may be used to encapsulate therapeutic cell lines (Yuan, Zhang et al. 2004), Figure 1.7 (lower). Recent work by Shoicet and colleagues demonstrates chitosan's use as extramedullary and intramedullary conduits capable of supporting neural stem cell differentiation in the transected cord (Nomura, Zahir et al. 2008; Zahir, Nomura et al. 2008).

Polymers derived from the blood

Plasma derived polymers, fibronectin and fibrin, are being used as spinal cord scaffolds. Fibronectin mats are formed with linearly aligned fibres which can orient axonal growth (King, Henseler et al. 2003) and sequester growth factor within its pores for gradual release (Phillips, King et al. 2004). Fibrin scaffolds are formed from monomers following fibrinogen cleavage by thrombin and crosslinked with Factor XIIIa (Willerth and Sakiyama-Elbert 2007). It is a natural matrix for wound repair having inherent cell-binding sites. Heparin has been crosslinked to fibrin scaffolds using a bidomain Factor XIIIa-heparin linker peptide for use as an affinity-based delivery system for growth factors, including NT-3 (Taylor and Sakiyama-Elbert 2006) and for factors to differentiate embryonic stem cells seeded within the matrix. A recent study implanting fibrin polymer scaffolds into a dorsal hemisection model demonstrated delayed astrocytosis and improved neuron fibre extension (Johnson, Parker et al. 2009), Figure 1.7 (upper panels).

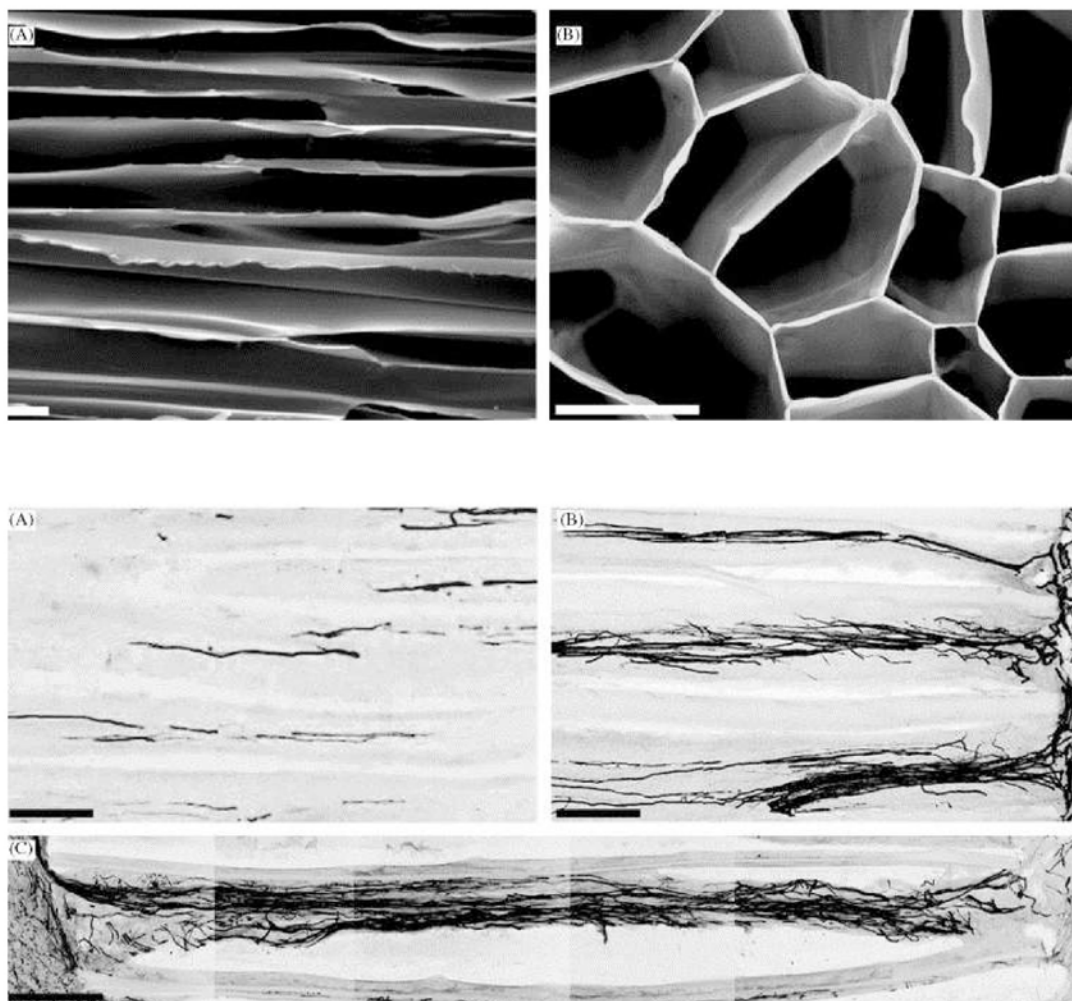


Figure 1.6: Freeze Dried Agarose Scaffolds in a complete transection model, figure from (Stokols and Tuszynski 2006) with permission. Upper Panel: Scanning electron microscopic images of scaffolds in (A) longitudinal or (B) cross-sectional orientation shows the arrangement of channels in a honeycomb structure. Scale bar is 100 µm. Lower Panel: Neurofilament labeling demonstrates penetration and linear growth of axons within channels of scaffolds. (A) Scaffold lacking growth factor. (B) Scaffold loaded with 2 µg recombinant human BDNF into walls and matrix-filled lumen of individual channels. Magnitude of linear axonal growth is significantly increased. (C) Best example of linear axonal growth through complete length of channel. Scale bars=100 µm.

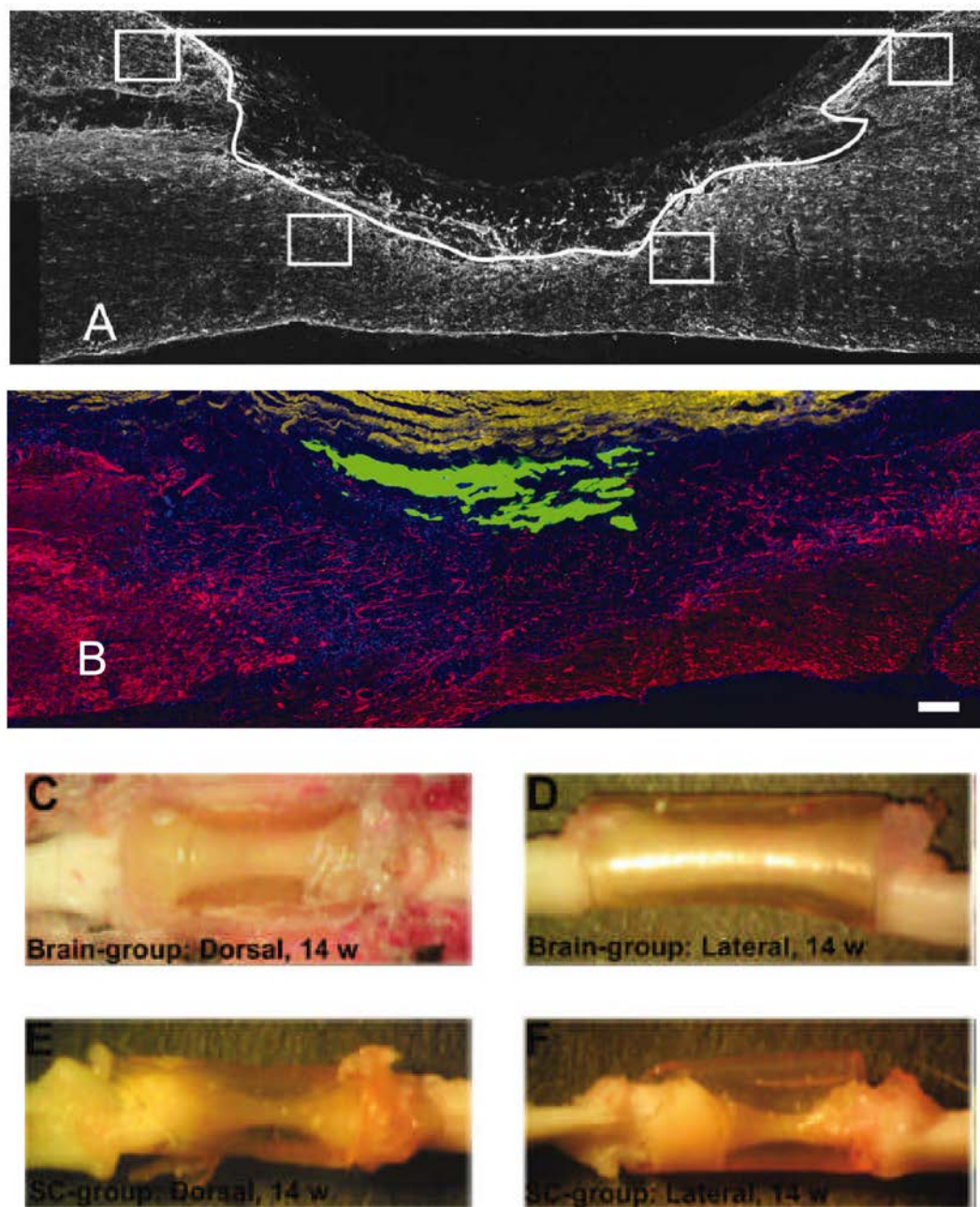


Figure 1.7: Scaffolds in situ. A dorsal hemisection injury (A) is filled with a fibrin matrix, as visualized with fibrinogen immuno-fluorescence (B). Diverse applications have been developed with this polymer, including affinity-based drug elution from heparin complexes and embryonic stem cell differentiation. Bar is 200 μ m. Adapted from (Johnson, Parker et al. 2009) with permission. (C-F) A complete spinal cord transection is bridged with chitosan scaffolds loaded with brain-derived neural stem cells (C and D) or Schwann cells (E and F) as seen from a dorsal and lateral aspect. Neuronal tissue bridges have developed from the transected cord stumps. (Zahir, Nomura et al. 2008) with permission.

1.3.2 Synthetic Polymers

Whereas the use of natural polymers in the spinal cord takes advantage of their inherent properties, their natural role to some extent being the basis of their function or their modification, using synthetic polymers offers wider scope to design and control the characteristics of the material. The synthetic polymers used thus far in the spinal cord are either biodegradable materials based around polyesters of lactic and glycolic acid (PLA and PGA), are biodegradable hydrogels based on polyethylene glycol (PEG), or are non-biodegradable hydrogels based on methacrylate. Early spinal cord scaffolds were made from the same materials as were in common clinical use for surgical repair of peripheral nerve and skin grafting. Rapid advances in hydrogel chemistry have produced materials more suited to the spinal cord in their mechanical properties. The trend now is towards using these highly aqueous, soft polymers given the similarity of their properties to spinal cord tissue, and the versatility with which their chemistry and architecture can be adjusted. Functionalized synthetic polymers have included gradients and surface charge modification for cell adhesion, neurotrophic gradients, and have opened the field to using scaffolds themselves as drug delivery and gene delivery vehicles to novel extent. The technical ability to rapidly photocross-link synthetic hydrogels has also enabled a remarkable degree of sophistication in macro and micro-architecture through the use of photolithography. Please see Table 2 for a listing of synthetic polymers and examples of their use within the spinal cord.

Poly α -hydroxy acid polymers

At the time of our previous review (Friedman, Windebank et al. 2002), much of the work in scaffold design focused on the use of biodegradable synthetic polymers, particularly the poly (α -hydroxy acids) PLA, PGA and their copolymer PLGA. These polymers are polyester links of lactic and glycolic acid which are hydrolyzed *in vivo* to release lactide and glycolide, dissolving the material. The pH around the grafted site accordingly will become more acidic. These compounds were a good initial choice for spinal cord placement, having a long track record of FDA approved clinical use as an absorbable suture material, and as grafting material for skin and for peripheral nerve repair (Mackinnon and Dellon 1990; den Dunnen, Van Der Lei et al. 1993). The transition to CNS

applications was based on the idea that such scaffolds could provide a corresponding PNS-like endoneurial and perineurial guidance structure to regenerating spinal cord axons, improved with the addition of myelinating Schwann cells within the scaffold (Hadlock, Sundback et al. 2000).

Table 1.2

The main synthetic polymers in use in animal models of spinal cord injury and examples of their applications.

Synthetic Polymers	Structure	Examples of Applications for Spinal Cord Repair
<i>Biodegradable</i>		
PLA	Poly(D,L lactic acid)	Single and multichannel scaffolds seeded with Schwann Cells.
PGA	Poly(glycolic acid)	PLA: Single channel (Oudega, Gautier et al. 2001)
PLGA	Poly(lactic-co-glycolic acid)	Freeze dried macroporous foam scaffold with incorporated BDNF (Patist, Mulder et al. 2004) Freeze dried macroporous scaffold with BDNF-secreting Schwann cells (Hurtado, Moon et al. 2006) PLGA: Multichannel scaffolds seeded with Schwann cells (Moore, Friedman et al. 2006) Multichannel scaffolds seeded with neural stem cells (Teng, Lavik et al. 2002) Neurotrophin-eluting micro and nanospheres within scaffolds (Yang, Murugan et al. 2005; Wang, Wu et al. 2008) Protein release from chitosan scaffolds (Kim, Tator et al. 2008)
PCL	Poly- ϵ -caprolactone	Nanofibre spinning for axonal growth orientation (Schnell, Klinkhammer et al. 2007)
PCLF	Poly- ϵ -caprolactone fumarate	Multichannel scaffolds (Wang, Mullins et al. 2009)
<i>Hydrogels</i>		
PEG	Polyethylene Glycol	Intravenous solution (Lavery, Leskovar et al. 2004) Immunoprotective sealant gel (Borgens, Shi et al. 2002; Duerstock and Borgens 2002) Injectable gel with PLA for NT-3 delivery (Piantino, Burdick et al. 2006) and BDNF (Soderquist, Milligan et al. 2008) 3-Dimensional neurite growth matrix (Namba, Cole et al. 2009) Intravenous PEG with Magnesium Sulfate (Ditor, John et al. 2007; Kwon, Roy et al. 2009)
<i>Non-biodegradable hydrogels</i>		
pHEMA	Poly(2-hydroxyethyl methacrylate)	pHEMA sponges (Giannetti, Lauretti et al. 2001) Guidance channels (Tsai, Dalton et al. 2004) Fibre templated scaffolds (Flynn, Dalton et al. 2003) Cell adhesion gradients (Yu and Shoichet 2005) Neurotrophic gradients (Moore, MacSween et al. 2006) Co-polymer with chitosan including cell adhesion peptides (Yu, Kazazian et al. 2007) Coil (Nomura, Katayama et al. 2006) or multilayered pHEMA (Carone and Hasenwinkel 2006) scaffold reinforcement Surface charge modification for cell adhesion (Lesny, Pradny et al. 2006) and axonal growth (Hejcl, Urdzikova et al. 2008)
pHEMA-MMA	pHEMA-co-methyl methacrylate	NGF-release from PGLA microspheres and pHEMA-NGF polymer bilayer (Piotrowicz and Shoichet 2006) Matrix filled channels for acidic fibroblast growth factor (FGF-1) and NT-3 delivery (Tsai, Dalton et al. 2006)
pHPMA	Poly[N-(2-hydroxypropyl) Methacrylamide]	pHPMA colloid gel (Woerly, Pinet et al. 1998) Colloid gel with cell adhesion peptides (Woerly, Pinet et al. 2001) (Neurogel™) Colloid gel with CNTF and BDNF (Loh, Woerly et al. 2001)
PAN/PVC	Poly(acrylonitrile-co-vinylchloride)	Schwann cell seeded scaffolds with BDNF & NT-3 cord infusion (Bamber, Li et al. 2001) Schwann cell seeded scaffolds with GDNF Matrigel™ (Iannotti, Li et al. 2003)

PLA has been used to make scaffolds. Having shown the resorbability and biocompatibility of PLA with Schwann cells and the spinal cord (Gautier, Oudega et al. 1998), the University of Miami group placed single channel Schwann cell loaded scaffolds into the transected rat spinal cord. The PLA materials used were structurally unstable, fragmenting and collapsing, but proved to support the extension of axons and vascular growth into the graft (Oudega, Gautier et al. 2001). More recently, macroporous PLA foam scaffolds made with longitudinally aligned pores were fabricated using a freeze drying technique. BDNF was dissolved into the scaffold matrix but did not improve an overall low yield in axon numbers through the graft (Patist, Mulder et al. 2004). This study was extended to incorporate Schwann cells into the foam that had been genetically engineered to secrete a bi-functional neurotrophin (D15A) with BDNF and NT-3 activity (Hurtado, Moon et al. 2006). Axonal regeneration was modest at 6 weeks, and few Schwann cells survived the first week of scaffold placement. There has been recent interest in the use of PLA nanofibres as a cell substrate (Wang, Mullins et al. 2009).

The degradation rate of PLA can be somewhat controlled in the copolymer PLGA by altering the ratios of the PLA and PGA in composite. The *in vitro* characteristics (bending, swelling, deformation, degradation and permeability) of varying PLA:PGA ratios have been extensively determined for PLGA spinal cord scaffolds implants (de Ruitter, Onyeneho et al. 2008). Multichannel PLGA scaffolds have been demonstrated to support robust axonal regeneration when seeded with Schwann cells without functional improvements (Moore, Friedman et al. 2006). As detailed in the section below, PLGA degradation kinetics are the basis for drug delivery via microspheres embedded in polymer scaffolds.

Synthetic hydrogels

Polyethylene glycol is a biodegradable synthetic polymer of ethylene oxide units. Its role is somewhat unique in that it is an 'exclusionary' compound, immunoprotecting the areas to which it is applied by keeping out cell infiltrates, and equally is used as a delivery system for cells, neurotrophins and genetic constructs. It has been formulated into gels for topical application onto the injured

spinal cord (Borgens, Shi et al. 2002), combined with PLA for neurotrophin delivery of a photo-inducible gel, or as a formulation of 'pegylated' BDNF as an intrathecal infusion (Soderquist, Milligan et al. 2008). Intravenous solutions with magnesium sulfate are being investigated as a first line immunomodulatory therapy in acute spinal cord injury (Kwon, Roy et al. 2009).

Poly(2-hydroxyethyl methacrylate) (pHEMA) polymeric systems were initially used as non-biodegradable materials for soft contact lenses. Professor Shoicet's group in Toronto has extensively developed pHEMA and pHEMA-co-methyl methacrylate (pHEMA-MMA) for use as spinal cord scaffolds. Early applications included pHEMA sponges (Giannetti, Lauretti et al. 2001), evolving to guidance channels (Tsai, Dalton et al. 2004), with a variety of surface modifications to improve cell adherence and axonal extension (Moore, MacSween et al. 2006; Tsai, Dalton et al. 2006) including neurotrophic gradients and adhesion gradients within a chitosan composite (Yu, Kazazian et al. 2007). Microfluidic techniques are employed to create functional concentration gradients in scaffolds, again providing cues for directionality of axonal growth. The technique involves casting a scaffold using 2 or more inlet ports coupled with rapid polymerization. An important advantage of this polymer class is the modification of surface charge with the addition of quaternary amine groups or of a second methacrylate subtype. Many cell types adhere better to a positively charged surface. Lesny *et al.* (2006) evaluated the in growth of neural tissue in a dorsal hemisection injury bridged with four pHEMA composites, demonstrating an improvement in axonal regeneration into the core of the scaffold and a reduction in astrocyte infiltration in the positively charged scaffold.

Very sophisticated architecture is possible with these compounds. The scaffolds have been reinforced with coils, as well as being made as with neurotrophin PLGA microspheres. Multi-layered macroporous pHEMA composites, including innermost channel layers that elute neurotrophic factors, have been made by liquid-liquid centrifugal casting. Thin layers of the polymer liquid are forced against the side of the mold by the centrifugal force and polymerized. The polymers can also be crosslinked with light with the addition of a photoinitiator. Using photolithography, in which discrete sections of the polymer mix are exposed to light while others are masked, open channel size and

pore size was recently controlled with remarkable precision (Bryant, Cuy et al. 2007).

1.4 Biomaterials as Therapeutic Cell Systems in the Spinal Cord

As one of the field's principal contributors, Bellamkonda advocates that scaffold strategies for nerve regeneration should combine four main components, a permissive growth substrate (hydrogel or micro/nano fibre), a neurostimulatory extracellular matrix (protein or peptide), the provision of neurotrophic factors, and glial or support cells (Schwann cells, neural stem cells). Cell therapies can be delivered to the spinal cord by direct injection into the cord substance, intrathecal infusion, or by polymeric microspheres or scaffolds. It has been shown that a variety of cells support axonal regeneration within polymer scaffold models in the cord. Cells can be loaded onto polymer scaffolds when suspended in a support matrix such as fibrin or Matrigel™. If the scaffold is a macroporous hydrogel, the cells migrate and become resident within that porous structure, and if the scaffold is designed to be multichannel, select channels can be seeded with different cell types allowing for regional topography (Friedman, Windebank et al. 2002).

1.4.1 Schwann Cells

Early work in the spinal cord was again derived from peripheral nerve repair strategies. Schwann cells myelinate peripheral nerve, and will naturally migrate from peripheral-central nerve junctions such as the dorsal root ganglia during times of cord injury to support repair (Oudega, Moon et al. 2005). Here they play a structural role, lay down extracellular matrix proteins like laminin, and provide paracrine trophic support through secretion of nerve growth factor (NGF), NT-3, BDNF, ciliary neurotrophic factor (CNTF) and basic fibroblast growth factor (bFGF) (Willerth and Sakiyama-Elbert 2008). Xu *et al* (1995) placed Schwann cells expanded in culture from harvested rat sciatic nerve into a poly(acrylonitrile-co-vinylchloride) (PAN/PVC) nerve conduit and into a complete cord transection model. The study demonstrated axonal extension and myelination of about a quarter of the regenerated sensory nerves as seen with

electron microscopy, and that the extent of the repair could be enhanced by administering methylprednisolone in the acute phase of the injury (Chen, Xu et al. 1996). The Miami group also utilized peripheral nerve grafts placed directly into the transected spinal cord serving as natural conduits for axonal extension and as a source of Schwann cells (Oudega and Hagg 1996; Oudega and Hagg 1999). This work was extended to cell-seeding polymer scaffolds, demonstrating evoked nerve conduction potentials across the graft only in the Schwann cell group (Pinzon, Calancie et al. 2001). In all applications, it is important to understand whether cells survive in the grafted material (natural or synthetic). We have demonstrated cell survival for up to 6 weeks after transplantation through a multichannel scaffold, and that in this model, Schwann cells have a higher capacity than stem cell neurospheres to enhance axonal regeneration in the transected cord, Figure 1.8 (Olson, Rooney et al. 2009).

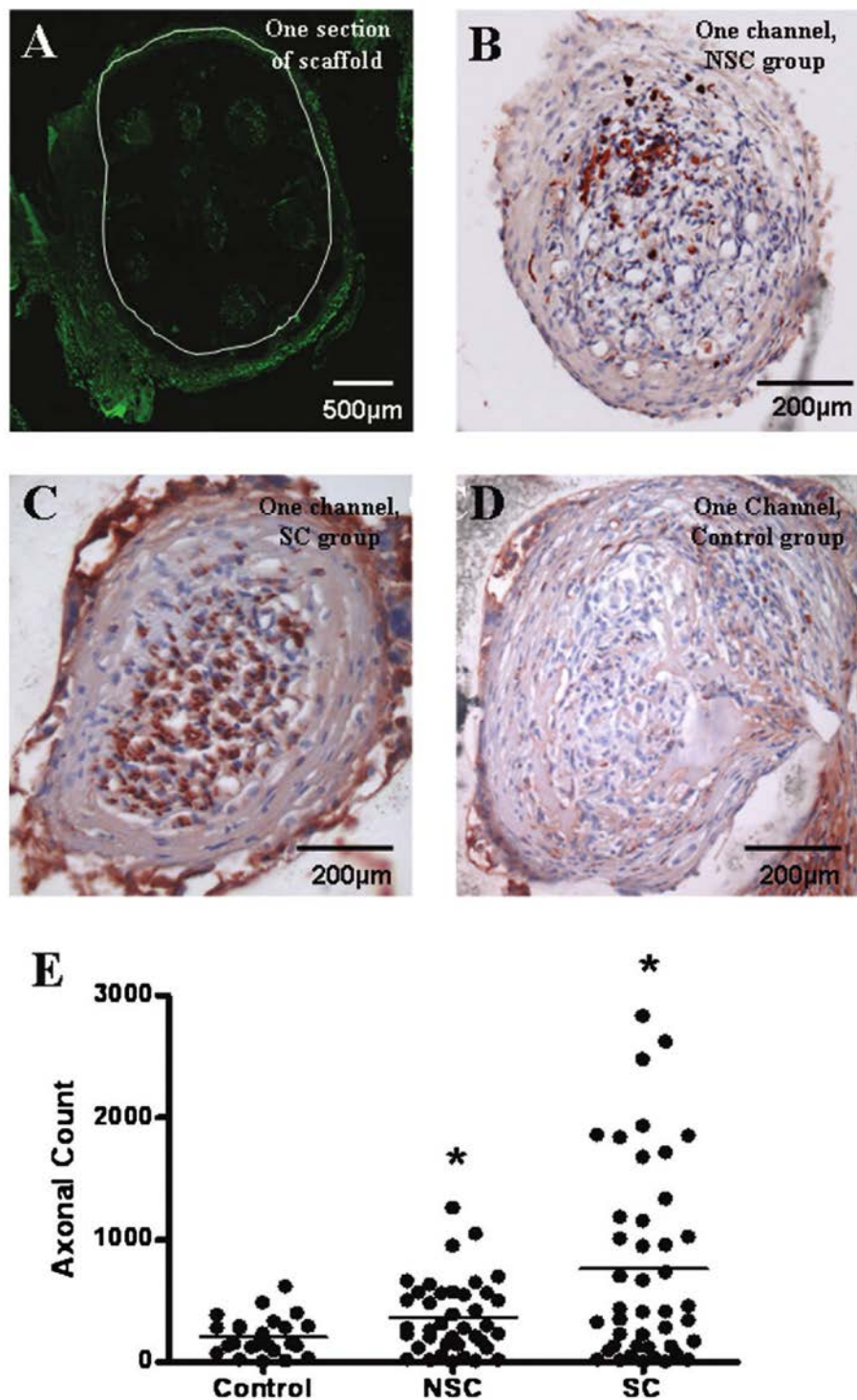


Figure 1.8: Neurofilament staining of axons in transverse sections through a PLGA scaffold after 1 month in vivo. Fluorescent microscopy image of one transverse section of a scaffold (loaded with NSCs) stained with an antibody against neurofilament with the region in which axons were counted encircled by a white line (A). Transverse sections of one channel from the NSC group (B), one from the SC

group (C), and one from the control group (D) stained for neurofilament using an AEC chromogen. Magnification: 25 x(A); 100 x (B–D). Dot plots of axonal counts per cord in the NSC-, SC-, and control-treated groups (E). Data points represent individual animals. 95% Confidence interval was used for each group. *Medians vary significantly ($p < 0.05$). (Olson, Rooney et al. 2009) with permission.

1.4.2 Olfactory Ensheathing Cells (OECs)

Olfactory ensheathing glia are cells within the peripheral and central component of the olfactory system that contribute to the regenerative capacity of olfactory neurons. The average lifespan of olfactory receptor neurons is four weeks. These neurons are bipolar cells projecting to the nasal epithelium and to the olfactory bulb. New receptor neurons are derived from a stem cell layer at the base of the nasal epithelium, from where axons are projected through the cribriform plates into olfactory bulbs. The role of OEC is to enfold and guide growing axons to the bulb in bundles of unmyelinated axons, and once at the bulb to interact with resident astrocytes and fibroblasts to finalize the connections (Franssen, de Bree et al. 2007). While extensive work (over 40 *in vivo studies*) has been done with cell injections into the cord, few studies have utilized polymer scaffolds. In one of the first and most successful studies Bunge's group used Schwann-cell seeded PAN/PVC conduits in a thoracic transection model, now in combination with stereotactic injection of OEC's at four midline depths. The OEC's induced axonal growth in through the SC-containing channel and for distances of 2.5 cm, the longest distance observed thus far for OECs (Ramon-Cueto, Plant et al. 1998). Followup studies showed functional recovery without the use of scaffolds (Ramon-Cueto, Cordero et al. 2000). Lu and Ashwell (2002) used collagen matrix soaked with OECs to bridge a dorsal transection and with similar efficacy to pieces of olfactory lamina propria and OEC injections. Chuah *et al* (2004) encapsulated OEC's into polyvinylidene fluoride particles for injection following dorsal transection which increased the numbers of collateral branches from the intact ventral cord. A strategy which used Schwann cells embedded in a Matrigel™ bridge, injections of OEC's into the distal and proximal cord stumps following transection, and alternate day delivery of Chondroitinase

ABC via an intrathecal catheter, resulted in select fibre regeneration and some recovery of function (Fouad, Schnell et al. 2005).

1.4.3 Neural Stem Cells

Neural Stem Cells (NSCs) are a pluripotent, self-renewing population of precursor cells that give rise to astrocytes, oligodendrocytes, and neurons in the CNS. Their role in regenerative medicine is therefore to remyelinate axons growing from the injured cord, to themselves become neuronal links within the injury (Lowry and Temple 2007), and to elaborate neurotrophic factors to stimulate regrowth (Lu, Jones et al. 2003). In animal models, they are derived from fetal brain homogenates, from which they are cultured as spherical aggregates (neurospheres), which can be in turn be subcultured for *in vitro* differentiation or *in vivo* transplantation. Stem cells for use in humans pose several problems. Their nature as multipotent cells risks unrestrained proliferation to desired or to unwanted cell lineages. They must be derived from fetal tissue in order to preserve the full range of pluripotency, or be obtained in the adult from brain or spinal cord biopsy; the latter as a source may provide cells whose lineage is more restricted to a neuronal phenotype. Cross-species implantation will not avoid immune surveillance. When injected into the cord following contusion injury (Cao, Zhang et al. 2001) or a hemi-section model (Chow, Moul et al. 2000), neurospheres tend to adopt an astrocyte morphology and the neurite connections which do form tend to create pain circuits (Hofstetter, Holmstrom et al. 2005). That indeed no study has shown large scale neuronal differentiation of engrafted stem cells suggests the injured cord, regardless of the injury model, is not a permissive environment (Enzmann, Benton et al. 2006). There have been numerous efforts to control cell lineage with growth factors, location and timing of cell harvest, and genetic transduction prior to implantation.

Many researchers have turned to polymer scaffold substrates as a mechanism to differentiate the cells into neurons either *in vitro* prior to implantation, *in vivo* in conjunction with a scaffold implant, or to encapsulate the cells with polymer for immune protection (Zhong and Bellamkonda 2008). Spinal cord scaffolds then may well play a central role should neural stem cells become useful for human clinical therapy. Recent *in vitro* work has set out to define which polymer compounds and structures are permissive or inhibitory to stem cell

viability and differentiation. The most promising substrates include the use of 3-dimensional fibrin scaffolds, seen *in vitro* to support and differentiate mouse embryonic stem cells when cell culture and scaffold fabrication conditions were optimized (Willerth, Arendas et al. 2006). Further work evaluated the effects of growth factors, their doses and combinations on stem cell differentiation to neurons and oligodendrocytes (Willerth, Faxel et al. 2007). PLGA has also been shown to support NSC viability and neurite outgrowth to a greater extent than poly- ϵ -caprolactone (PCL) and PLA (Bhang, Lim et al. 2007). *In vivo* work done by the Langer lab has used PLGA scaffolds seeded with NSCs in a hemisection model: this scaffold had complex architecture, with an inner layer seeded with NSCs approximating the gray matter, and an outer layer whose pores were oriented longitudinally for axon guidance and radially for permeability. Scaffolds were left in the animal for up to a year with persistent functional improvement, including hindlimb weight bearing and improved coordination seen between 2-3 months post implantation (Teng, Lavik et al. 2002). Our group has also seeded neurospheres into multichannel PLGA scaffolds in a direct comparative study with Schwann cells, Figure 1.9, demonstrating the capacity of PLGA to support neurosphere differentiation (Olson, Rooney et al. 2009).

Micro and nano structures influence stem cell growth and differentiation *in vitro*. A micropatterned polystyrene surface provided growth differentiation and direction to hippocampal stem cells with laminin (Recknor, Sakaguchi et al. 2006) and with growth factor cues without adhesion molecules (Oh, Recknor et al. 2008). Fibrous nanostructures differentiate NSCs and may even control cell lineage through the structure itself. Silva *et al* showed self-assembled nanofibres could rapidly differentiate NSCs to neurons and inhibit the development of astrocytes (Silva, Czeisler et al. 2004) Similarly, electrospun nanofibres of PCL polymer differentiated cells and provided a stimulus and directionality to neuron growth (Xie, Willerth et al. 2009).

1.5 Biomaterials as Drug and Cell Based Delivery Systems in the Spinal Cord.

The therapeutic potential of spinal cord scaffolds is enhanced by the development of integrated polymer drug delivery systems and cell lines that are genetically modified to secrete neurotrophic factors. Polymeric delivery from scaffolds is achieved by means of the materials inherent properties: its porosity and permeability for the sequestration and diffusion of a drug its degradation kinetics for release of the entrapped drug, its chemical affinity to a drug by means of a linker moiety, or by being a non-biodegradable material for the preservation of a gradient (Willerth and Sakiyama-Elbert 2007). We have seen in the discussion of individual polymer types a number of ways each polymer type can be used to deliver neurotrophins or drugs. The drug or factor can be released from the material itself, from integrated micro- or nano- spheres or tubules of a different material, or by means of a scaffold's capacity to support a genetically-modified cell line *in vivo*.

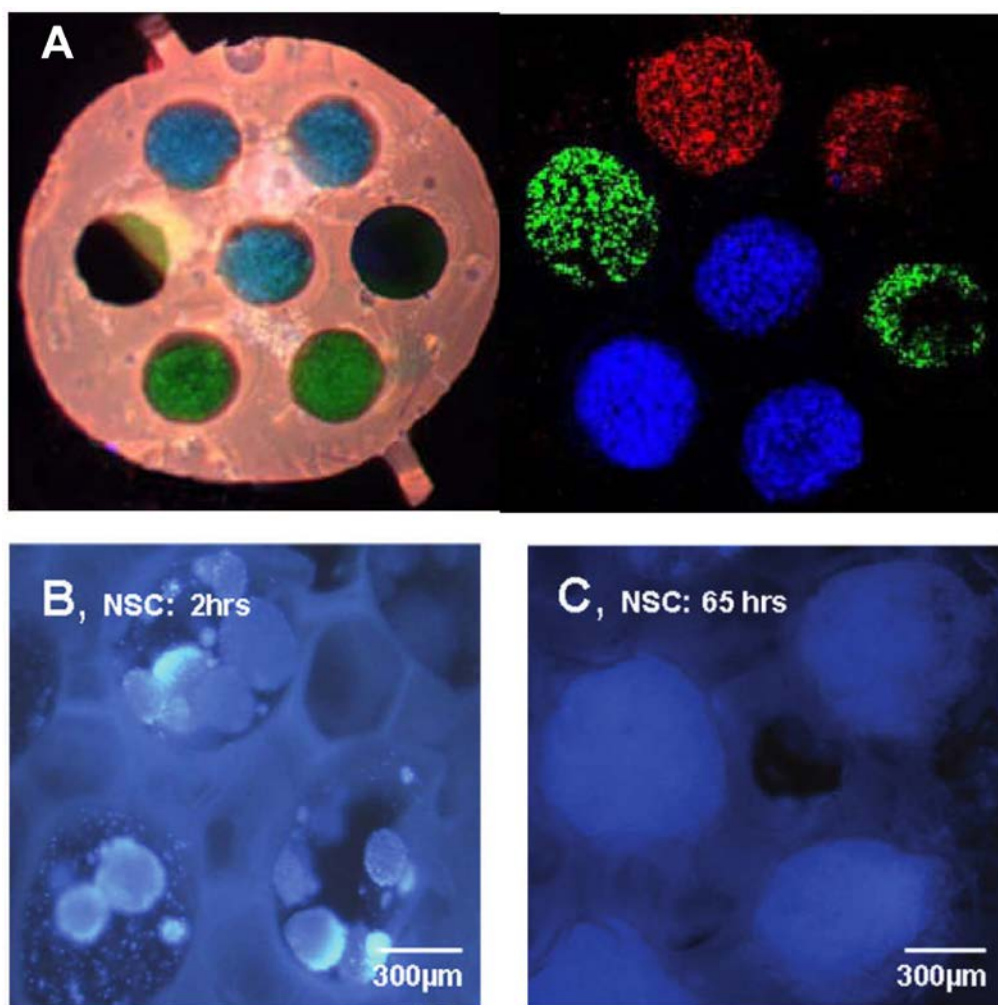


Figure 1.9: (A) Cells of different types can be placed in a scaffold. PCL-Fumarate scaffold of 2 mm diameter loaded with SpL201 cells (blue), Schwann Cells from a GFP rat (green) and rat Mesenchymal Stem Cells labeled with red quantum dots. Subsequently the scaffold can be placed in a way that ventral and dorsal orientation is retained. (B) Neurospheres of Neural Stem Cells suspended in scaffold channels at 2 hours grow to fill the channel diameter (C) within 3 days, figure from (Olson, Rooney et al. 2009) with permission.

1.5.1 Micro- and Nano-Spheres and Nanoshells

Micro- and nano-spheres refer to particulate synthetic polymer of the order of microns or nanometers in diameter. By far the most frequently used polymer is PLGA, as its degradation rate and thus drug release is readily controlled by the proportion of PLA to PGA. For example 85:15 PLA to PGA will degrade

significantly more slowly than a 50:50 ratio. PLGA microspheres are typically 2-45 μm in size. Alginate-chitosan microspheres are also used. Micro and nanospheres are produced by microemulsion techniques, whereby an aqueous solution is emulsified in an organic phase polymer solution to create spherical droplets which are then extracted into another external aqueous phase (Benoit, Faisant et al. 2000). The size of the droplet can be controlled by the emulsion agitation rate, the aqueous and organic phases used, and the addition of surfactants to modify the surface tension between the phases (Khademhosseini and Langer 2007). The drug is usually stabilized with protein (bovine serum albumen) or with zinc, added to the aqueous phase and becomes encapsulated upon polymerization of the droplets. Other techniques include aerosol freeze drying. The rate of drug delivery will depend on the initial concentration in the sphere and the size of the sphere for a polymer of given degradation kinetics. The particles are injected as a suspension, or can be suspended in a scaffold of another material, in which case the pore size of the material must enable diffusion or cell access within. Alternatively, as shown in chitosan scaffolds (Figure 1.10), the spheres can be localized directly to the channel wall by centrifugation (spin-coating) techniques (Kim, Tator et al. 2008). Nanoshells may be useful in the delivery of

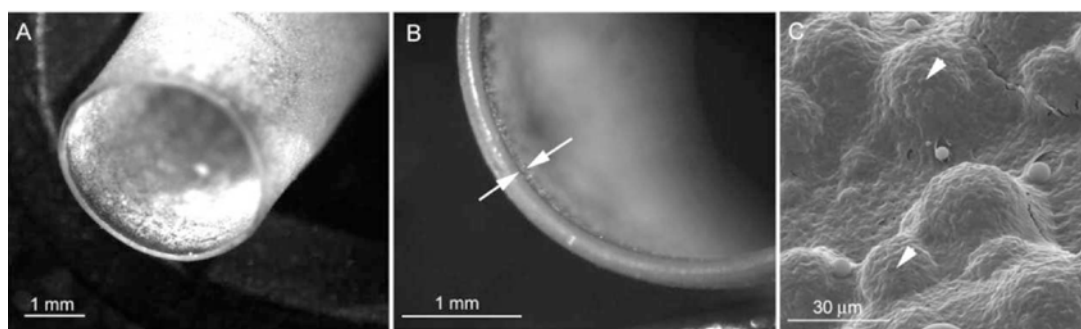


Figure 1.10: (A, B) Light micrographs of the microsphere-loaded chitosan channels. The thickness of the channels when hydrated is approximately 200 μm , of which the secondary chitosan layer (indicated by arrows) contributes about 20 μm . (C) Scanning electron microscopy shows microspheres (arrowheads) embedded by the secondary chitosan coating. Figure from (Kim, Tator et al. 2008) with permission.

hydrophobic drugs from scaffolds. These spherical particles combine the benefits of PEG liposomes with polymeric shell, and are fabricated from PLGA polymer, lecithin phospholipid and a PEG core using modified emulsion techniques (Chan, Zhang et al. 2009).

Microspheres in scaffolds allow the opportunity for sustained local delivery of therapeutic molecules within the blood-brain barrier. Neurotrophins embedded in PLGA microspheres have been used in a variety of polymer scaffolds with NGF (Mahoney, Krewson et al. 2006) being the most extensively characterized. Cyclic AMP has been shown in many models to induce or enhance axonal growth (Murray and Shewan 2008). Similarly the work of Bunge and colleagues has demonstrated that neurotrophins enhance axonal growth in regenerating spinal cord (Blits, Oudega et al. 2003) and that this may be further enhanced by c-AMP (Pearse, Pereira et al. 2004). Chondroitinase ABC (ch-ABC) an enzyme which degrades a variety of chondroitin and heparin sulfate proteoglycans (Plant, Chirila et al. 1998) as well as hyaluronan, disrupts the glial scar matrix and facilitates axonal repair. Treatment with ch-ABC significantly enhanced axon regeneration in vitro (Plant, Harvey et al. 1995) and following brain (Nash, Borke et al. 2002) or peripheral nerve lesions (Richardson, McGuinness et al. 1980) and, most importantly, SCI in rats (Stichel, Hermanns et al. 1999). Furthermore, functional recovery in rats after SCI has been observed (Bradbury, Moon et al. 2002). We have demonstrated sustained release of dibutyl cAMP for over 3 weeks when incorporated into microspheres. Finally, while the efficacy of systemic delivery of methylprednisolone in the acute setting for SCI remains an area of contention, polymeric delivery of the steroid is more beneficial in animal models than systemic steroid in reducing the lesion volume when delivered from a scaffold (Kim and Martin 2006) or from an injectible nanoparticle colloid (Kim, Caldwell et al. 2009).

1.6 Conclusions and Future Directions

This chapter has highlighted some of the novel tissue engineering approaches to spinal cord repair using implantable polymers in thoracic transection models. Restoration of respiratory function will be a critical application for biomaterial approaches given the degree of mortality and morbidity associated with respiratory compromise after spinal cord injury. Important bioengineering considerations, fabrication techniques for macro- and micro-engineering, along with some prominent applications and modifications of

the main materials in use for scaffolds, have been presented as an overview of the field. Regarding polymer materials, these continue to evolve rapidly in the sophistication of their chemistry and design. New applications with the polymer Polypyrrole (Ppy) represent an exciting avenue for nanofibre and drug delivery systems. Used in the past as coating for neuroelectrodes, Ppy is electrically conductive, and voltage stimulus can induce axonal growth and its direction, along with the release of integrated neurotrophic factors and dexamethasone from the polymer surface.

The integration of Schwann cells, olfactory ensheathing cells and neural stem cells into the polymer structures have improved their regenerative capacity, as has the use of scaffolds as delivery devices for therapeutic agents, particularly neurotrophic factors. Designing scaffolds and seeded cell lines to release neurotrophins for specific axonal locations and sensitive phenotypes may regionalize repair. The use of human embryonic stem cell implantation in polymer scaffolds will be directed by recent FDA approval for Phase 1 clinical trials, and the outcomes of renewed studies in animals. The absence of their use has influenced development of alternate stem cell types for scaffolds including mesenchymal stem cells and induced pluripotent stem cells (iPSCs) as potential sources both of new neurons as well as drug delivery cells. Further advances in polymers as genetic delivery tools, particularly for siRNA, non-viral and viral gene delivery with or without targeted genomic integration, is an exciting therapeutic prospect. It is hoped that combination strategies will maximize our ability to regenerate spinal cord tissue through the glial scar and recreate connections. The caveat of course is that the investigation of many possible combinations of material, geometry, functionalization strategies, and integrated drug or cell based therapies will need to be properly controlled and systematic. The ability to control these variables with increasing precision, through rapid technological advances such as were highlighted here, enables scaffold models of spinal cord repair to be highly informative about individualized facets of the repair process.