Surface chemical modification of PCL films
for peripheral nerve repair

A thesis submitted to the University of Manchester
for the degree of Doctorate of Philosophy (PhD)
in the Faculty of Medical and Human Science

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School of Medicine
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<th>Description</th>
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<tbody>
<tr>
<td>BDNF</td>
<td>Brain-derived Neurotrophic Factor</td>
</tr>
<tr>
<td>BS</td>
<td>Bottenstein and Sato’s medium</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CDI</td>
<td>Carbodiimide</td>
</tr>
<tr>
<td>CEA</td>
<td>2-chloroethylamine</td>
</tr>
<tr>
<td>CNBr</td>
<td>Cyanogen Bromide</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CTNF</td>
<td>Ciliary Neurotrophic Factor</td>
</tr>
<tr>
<td>dADSCs</td>
<td>Derived Adipose Stem Cells</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Minimum Eagle’s Medium</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal Root Ganglia</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared Spectroscopy</td>
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<tr>
<td>GA</td>
<td>Gluteraldehyde</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell line-derived Neurotrophic Factor</td>
</tr>
<tr>
<td>GGF</td>
<td>Glial Growth Factor-2</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
</tr>
<tr>
<td>HMD</td>
<td>Hexamethyldiamine</td>
</tr>
<tr>
<td>HMDS</td>
<td>Hexamethyldisilazane</td>
</tr>
<tr>
<td>IPA</td>
<td>Isopropanol</td>
</tr>
<tr>
<td>MES</td>
<td>2-N-morpholinoethansulfonic acid</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve Growth Factor</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal Goat Serum</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimidyl ester</td>
</tr>
<tr>
<td>NT-3</td>
<td>Neurotrophin-3</td>
</tr>
<tr>
<td>NT-4/5</td>
<td>Neurotrophin-4/5</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>PCL</td>
<td>Poly(ε-caprolactone)</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
</tr>
<tr>
<td>Pdl</td>
<td>Poly-D-lysine</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyhydroxybutyrate</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PGA</td>
<td>Poly(glycolic acid)</td>
</tr>
<tr>
<td>PHB</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PLA</td>
<td>Poly(lactic acid)</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral Nervous System</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>RSF</td>
<td>Relative Sensitivity Factor</td>
</tr>
<tr>
<td>SCs</td>
<td>Schwann Cells</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris(2-carboxyethyl)phosphine hydrochloride</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Tranforming Growth Factor-β</td>
</tr>
<tr>
<td>Th</td>
<td>Thiol</td>
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<tr>
<td>XPS</td>
<td>X-ray Photoelectron Spectroscopy</td>
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Abstract

Abstract of a thesis entitled “Surface chemical modification of PCL films for peripheral nerve repair” and submitted by Alba Carla de Luca for the degree of Doctor of Philosophy (PhD) to The University of Manchester, August 2012.

Nerve injury is a very common trauma affecting 300,000 people in Europe every year. Although autografts are currently the gold standard in surgery, they can cause loss of sensation and scar tissue formation. Artificial nerve conduits are a valid alternative for peripheral nerve repair. They can provide a confined environment during the regeneration process, enabling axons sprouting from the proximal to the distal nerve segments as well as reducing scar tissue formation. Poly-ε-caprolactone (PCL) is a biocompatible and biodegradable polymer suitable for the fabrication of nerve guidances. In particular, previous works demonstrated that neural cells are able to adhere and proliferate on micropitted PCL films obtained through solvent casting. Also, short term studies showed that axons were able to bridge 1cm injury gap. In this work a 18 weeks long term in vivo experiment using a rat model was performed to investigate the reinnervation of end organ skin and muscle. PCL conduits were compared to autografts, with no significant differences in terms of regeneration and reinnervation. However, Schwann cells (SCs), the most important glial cells in the peripheral nervous system, showed poor attachment in vitro on PCL scaffolds; hence, surface modification was carried out in order to improve the material biocompatibility. The effect of both hydrophilicity and functional groups on SCs was first investigated. PCL films were then hydrolysed and aminolysed to modify the surface with carboxylic and amino groups respectively. Hydrolysed films increased remarkably the surface hydrophilicity, although topography and mechanical properties were not affected. Conversely, the tensile modulus and strength were significantly reduced by aminolysis, but still suitable for the desired application. The two treatments influenced also the morphology of SCs. It was demonstrated that cell elongation was induced by hydrophilic surfaces, whilst cells preferred cell-cell interaction when cultured on aminolysed films. However, cell proliferation was remarkably increased on the latter surfaces, confirming previous results obtained on substrates characterised by amino groups. These results confirmed that a good balance between hydophilicity and surface chemistry is necessary to guarantee the best cell response. In order to enhance both proliferation and morphology of SCs, arg-gly-asp (RGD) sequences were immobilised on the PCL film surface using two different reaction mechanisms. Carbodiimide chemistry was compared to a new mechanism developed in the present study based on the Thiol chemistry. Biological tests performed on these modified films demonstrated the improvement of SC response after the peptide immobilisation using the novel approach. Cell attachment and proliferation were three times higher compared to untreated PCL films. It was also observed that the presence of peptides on the film surface induced the formation of focal adhesion plaques by SCs, important for the perception of cellular signals when in contact with a particular substrate. Hence, a good balance between focal adhesion and adhesion forces was achieved after peptide immobilisation. Overall the results of this study showed that material functionalisation is very important for SC response and it will be fundamental for the production of artificial nerve conduits.
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**Author’s Contribution**

This thesis presents three experimental chapters, which have been submitted/published for publication in peer-reviewed journals. The thesis has been therefore submitted in the “alternative” format as it offers the most appropriate structure to present the results of the performed research. The three manuscripts included in the present thesis are related and follow a logical progression.

**Chapter 3. Paper I.**


(The author carried out about the 30% of the experimental work).

**Chapter 4. Paper II.**


(All the experiments included in this chapter were undertaken by the author).

**Chapter 5. Paper III.**


(The author carried out about the 90% of the experimental work).
Acknowledgments

I would like to express my sincere gratitude to my supervisors, Prof. Giorgio Terenghi and Prof. Sandra Downes, for giving me this wonderful opportunity to work with them. I am really grateful for their continuous support and suggestions that helped me develop an incredible sense of criticism in my work and increase my self-esteem. Also, this research project would not have been possible without the help of all the people working in the Blond and McIndoe Laboratories and in the Downes’s group.

I thank my mother, my father and my brother. They have been always there because they believed in me, even when I did not. It was during those days when I was thinking “I give up, I am not going to make it” that my family was there, supporting me and giving me the strength to keep going, keep going … like a personal coach when the athlete is exhausted and disheartened. And I could never forget to thank my grandfather, my example of life. He helped me see the beauty in my dreams and made them all the more worth dreaming.

Special thanks go to Francesco and his very great love and patience. There have been very difficult times and I believe I might have been a bit nervous and stressed… but he has been always on my side.

Finally, I would like to thank all my old best friends and all the wonderful people that I have met during this amazing experience called “PhD”.
1. Introduction
1.1 The Peripheral Nervous System

The Nervous System can be divided anatomically into the Central (CNS) and the Peripheral Nervous System (PNS), which joins the former to the periphery. The CNS includes the brain and the spinal cord, while the PNS is composed of nerves, ganglia and sensory organs (Schmidt & Leach 2003). One of the most important differences between the two systems, in the field of Tissue Engineering, is that PNS nerve fibres can regenerate in comparison to the ones of the CNS because of differences in cytological organisation (Ide 1996; Stoll et al. 2002). However, the two systems are not physically divided and their neurons can lie in either of the two nervous systems.

![Figure 1.1. Neural cell types. Oligodendrocytes and Schwann Cells are involved in the myelination process of axons.](image)

In the nervous system two different types of cells are present: neurons and glia. A representative scheme of the two major classes of cells is shown in Figure 1.1. Neurons
1. Introduction

represent about 10% of the cell population in the brain and they can respond to an external stimulation, generating an “action potential” which propagates through the neuronal network (Verkhratsky & Butt 2007). Neurons are constituted of a central cell body, also called “soma”, and two different types of extensions, “axons” and “dendrites” (Figure 1.2). Axons send impulses generated by neurons to the synaptic terminal, while dendrites are involved in the transmission of afferent signals to the soma (Johnson et al. 2005). Glia represent most of the cells in the brain (~ 90%) and, unlike neurons, they can undergo cellular division (Schmidt & Leach 2003; Verkhratsky & Butt 2007).

Figure 1.2. The neuron structure. A neuron is constituted of cell body (soma) and two different types of extensions: axons and dendrites. Axons can also be wrapped by myelin sheathes produced by SCs.
1.1.1 The PNS structure

In the PNS it is possible to discern three different type of nerves depending on fibre-type compositions, such as sensory, motor and mixed nerves (Geuna et al. 2009). The structure of the PNS is characterised by fascicles of nerve fibres, delegated to the propagation of the nerve impulses (Topp & Boyd 2006). A peripheral nerve fibre is usually constituted of an axon, wrapped in a myelin sheath, due to the presence of a particular type of cell, known as Schwann cells (SCs). In particular, the myelin sheath can be seen as a flat glial process that is spirally surrounding the axon, while the SC body is moved towards the outer layer (Geuna et al. 2009). Two sheath tips can be distinguished within the enwrapping formation: the inner and the outer mesaxon. Furthermore between them the different myelin lamellae (compacted myelin) are formed through the fusion of the phospholipid bilayers (Verkhratsky & Butt 2007). In correspondence of the contact points between the inner cytoplasmatic surfaces, a dense major line is formed (Quarles 2002). Thus the myelin results in concentric layers of darker lines, which represent the dense major lines. Sometimes it is also possible to notice the presence of oblique incisures along the myelin sheath, known as Schmidt – Lanterman incisures.

Nevertheless, not all of the axons are wrapped by the myelin sheath; hence, it is possible to discern two types of fibres: myelinated and unmyelinated (Ide 1996; Mirsky et al. 2008), both of them surrounded by SCs. In myelinated fibres, SCs enwrap one single axon and form different myelin sheaths connected by junctions, known as Nodes of Ranvier, constituted of non compacted myelin (Topp & Boyd 2006). It is just in this paranodal area that Schmidt – Lanterman incisures are found, ensuring the exchange of the inner layers of the sheath (Geuna et al. 2009). The Nodes of Ranvier cover an important role in the fast “saltatory conduction”, whereas unmyelinated fibres only
allow a slower conduction of the nerve pulse. If an axon is myelinated or not, it depends mainly on its size, as myelination occurs for a critical diameter above 1µm (Verkhratsky & Butt 2007). Besides, both types of axon are enwrapped by a similar basal lamina, constituted of type IV collagen, fibronectin, isoform of laminin, heparan sulphate proteoglycan and entactin/nidogen (Topp & Boyd 2006; Hall 1997).

Nerves are made up of bundles of fibres, separated from each other by laminae (up to 15 layers) of connective tissue (*perineurium*), in which blood vessels and capillaries are also included (Geuna et al. 2009; Topp & Boyd 2006). All axons forming a fascicle are included in the *endoneurium*, while the fascicles are encased in fatty material, known as *interfascicular epineurium*. Finally, the nerve is surrounded by another connective tissue layer, called *epineurium* (Figure 1.3), constituted of type I and type II collagen fibrils, elastic fibres and different types of cells, such as fibroblasts, mast cells and fat cells (Topp & Boyd 2006). Between these three sheaths, the perineurium is responsible for any molecule and cellular infiltrations into the endoneurium. It provides a blood – nerve barrier (Kaplan et al. 2009), which can withstands up to 750 mmHg of intrafascicular pressure (Geuna et al. 2009). The intraneural vascular system in the PNS is characterised by a series of coiled arteries in the epineurium, forming the “*vasa nervorum*”, crossing the perineurium and turning into venules and large-diameter capillaries within the endoneurium fluid (Geuna et al. 2009; Topp & Boyd 2006).
1. Introduction

1.1.2 Schwann Cells (SCs)

SCs are the principal glial cells of the peripheral nervous system. They develop from the neural crest and, when mature, they can be myelinating and non-myelinating (Mirsky et al. 2008). SCs *in vitro* can be bi- or tripolar, characterised by a typical phase-bright spindle-shaped morphology with a small cytoplasm/nucleus ratio (Wei et al. 2009).

One of the most important factors involved in SC development process is their interaction with both extracellular matrix (ECM) and axons (Mirsky & Jessen 1996). In fact, the ECM that supports all the cellular components of the peripheral nervous system

---

**Figure 1.3.** Structure of a peripheral nerve. Axons are encased in the *endoneurium* and wrapped by connective tissue (*perineurium*) forming a fibre. Nerves are then made up of bundles of fibres included in a fatty material (*interfascicular epineurium*) and surrounded by another connective tissue layer (*epineurium*).
contains several macromolecules, such as glycoproteins, collagen, proteoglycans, laminin and fibronectin (Chernousov & Carey 2000). Depending on axonal contact, SCs are able to produce these structural and adhesive macromolecules, which are particularly important for their adhesion and proliferation as well as for axonal regrowth after injury (Chernousov & Carey 2000; Webber & Zochodne 2010). Also, SCs express different types of cell adhesion molecules, divided into the Immunoglobulin superfamily (e.g. N-CAM, L1, P0) and the Cadherin superfamily, which regulate axonal-SCs and axonal-basal lamina attachments (Evans 2000).

SCs are essential for axonal regrowth after injury as they secrete neurotrophic factors, which encourage and stimulate nerve regeneration after injury (Ide 1996). Acting through their receptors, neurotrophic factors are involved in neuronal activity, promoting nerve regeneration (Terenghi 1999; Chen et al. 2007). Their expression is strictly dependent on time after axotomy and it biases the regenerative capacity of axons as well as the supporting activity of SCs (Gordon 2009). Neurotrophins constitute one of the most important family of factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neutrotrophin-3 (NT-3) and neutrophin-4/5 (NT-4/5) (Schmidt & Leach 2003). NGF is one of the most important neurotrophins involved in nerve regeneration and is up-regulated rapidly in the distal stump after injury (Kingham & Terenghi 2006). BDNF is up-regulated in denervated SCs in order to allow myelination and nerve regeneration (Kingham & Terenghi 2006). Besides neurotrophins, other factors are involved during the regenerative process, like ciliary neurotrophic factor (CNTF), glial cell line-derived neurotrophic factor (GDNF), fibroblast growth factor (FGF) and transforming growth factor-β (TGF-β) (Terenghi 1999; Chen et al. 2007). All the neurotrophic factors cooperate in order to enable neuronal survival and axon outgrowth (Terenghi 1999).
1.2 Pathophysiology of nerve injury

Nerve transection or crush results in neurons and SCs changing phenotype and morphology and the nerve dividing into two different and retracted segments, known as proximal and distal stumps. The proximal stump, still part of the neuronal soma, is the point from which the regenerative process takes place (Geuna et al. 2009; Kingham & Terenghi 2006). Axon sprouting is supported by the production of neurotrophic factors released by SCs in the distal stump (Schmidt & Leach 2003; Stoll et al. 2002) (Figure 1.4). Proximally, the cell body undergoes chromatolysis and a number of new proteins are synthesised, which are essential for the nerve regrowth (Johnson et al. 2005).

**Figure 1.4.** Phases of nerve regeneration. After axotomy occurs at the injury site (A), SCs, macrophages and monocytes are involved in the phagocytosis of axon and myelin debris (B). New proteins and growth factors are synthesised by the cells, encouraging axon outgrowth (C). After reinnervation a new myelin sheath can wrap the regenerated axon (D). (Figure adapted from Schmidt & Leach 2003).
The distal stump undergoes what is called Wallerian degeneration within hours (Stoll et al. 2002; Dubový 2011). More specifically, the axonal membrane breaks and the process spreads from the injury site to the distal segment (Johnson et al. 2005; Stoll & Müller 1999). This first stage of nerve degeneration lasts about 3 – 6 weeks, during which the debris of axon and myelin breakdown are phagocyted by macrophages and monocytes (Hall 2001; Geuna et al. 2009). During Wallerian degeneration, SCs proliferate, de-differentiate and become aligned, forming the well known “bands of Büngner” from their basal lamina and acting as a scaffold within the distal stump ready to guide the axons regrowing from the proximal stump (Webber & Zochodne 2010).

After few hours post-injury, axons start to sprout from the node of Ranvier, extending towards the distal stump (Schmidt & Leach 2003). After reinnervation a new myelin sheath can wrap the regenerated axon (Johnson et al. 2005). However, axon diameter and myelin thickness are two crucial factors for a fully functional nerve regeneration (Johnson et al. 2005).

The types of injury can be classified on the gravity of the nerve damage (Burnett & Zager 2004) (Figure 1.5).
Figure 1.5. Types of nerve injuries. Compression and sheath loss are considered transient injuries and they stop temporarily the conduction of the nerve pulse. Disconnection involves the complete lesion of the axon and the myelin sheath, still preserving the basement membrane. Finally, degeneration results in the complete loss of continuity due to the whole nerve disruption.

A transient concussion, following for example compression, temporarily stops the conduction of the nerve pulse, without causing Wallerian degeneration (Burnett & Zager 2004). The function can be restored after 6 – 8 weeks from the time of injury (Kaye 1991). Conversely, when axotomy occurs, Wallerian degeneration causes complete denervation (Burnett & Zager 2004). Furthermore, the interruption of all the neural components is characterised by the whole disruption of the nerve, resulting in the complete loss of continuity (Kaye 1991; Burnett & Zager 2004). In this case, the nerve repair does not always imply the functional recovery due to a combination of factors (Burnett & Zager 2004; Wiberg & Terenghi 2003).
1.3 Peripheral Nerve Repair

Nerve injuries are very common and about 30% of them are caused by traumatic accidents (Campbell 2008). Nerve repair allows axons from the proximal nerve segment to regrow into the distal stump after injury (Fisher et al. 2007; Siemionow & Brzezicki 2009).

Short gaps without loss of axon material (<0.5cm) are usually repaired with end-to-end nerve stumps suture (Siemionow et al. 2010). When this is not possible, for example in longer gaps, nerve grafts, also known as autografts, are used. Specifically, a portion of a sensory nerve is harvested from a different part of the body of the patient (usually from the leg or the forearm) and it is implanted at the injury site as graft material (Lassner et al. 1995). Autografts retain the basal lamina and are characterised by high biocompatibility, low risk of toxicity and presence of autologous Schwann cells, which in turn release neurotrophic factors that enhance the regeneration of the nerve (Schmidt & Leach 2003; Siemionow et al. 2010). Nerve autografts are currently the “gold standard” in peripheral nerve repair, but their availability is very limited and functionality has been regained in only 50% of patients (Belkas et al. 2004; Lee & Wolfe 2000). Also, as autografts involve a second surgical procedure, this can cause permanent denervation at the donor site (Lassner et al. 1995; Siemionow et al. 2010).

In order to overcome the limitations of the autografts, research has focused on the use of nerve conduits to bridge the injury gap. It was demonstrated that biological tissues could act as neural conduits. Blood vessels, degenerated muscle grafts (Lassner et al. 1995) and other acellular nerve matrices have been studied for nerve repair. They preserve ECM components and mimic its physical and mechanical properties, although
damage of ECM structures and inflammation could be caused during the process of
decellularisation (Lassner et al. 1995; Schmidt & Leach 2003; Fisher et al. 2007).

1.3.1 Artificial Nerve Conduits

Artificial nerve conduits are a valid alternative to autografts, as they do not involve
tissue harvesting and their biological and physical properties can be modified and
improved in order to mimic the natural environment of the peripheral nervous system.
The main goal of artificial nerve conduits is to bridge the nerve gap and join the
proximal and the distal stump, as shown in Figure 1.6.

![Figure 1.6](image)

Figure 1.6. Artificial nerve conduits act like guides during the regeneration process. Nerve regeneration
starts at the proximal end, propagating towards the distal end, as indicated by the arrow.

Artificial nerve conduits isolate the regenerative process within a confined
environment, enabling axons sprouting from the proximal to the distal nerve segments
as well as reducing scar tissue formation (Kingham & Terenghi 2006). Both natural and
synthetic materials have been used to realise artificial conduits (Schmidt & Leach
2003). Neurotrophic factors and cells can be added to the simple tube structure to enhance
axonal outgrowth and make the material more “cell friendly” (Kingham &
1. Introduction

Terenghi 2006; Schmidt & Leach 2003). It has been demonstrated that regeneration can be controlled by manipulating four essential factors: growth permissive substrates, neurostimulatory ECM proteins or peptides, trophic factors and glial cells (Bellamkonda 2006). Moreover, an anisotropic distribution of these components allow a faster or better regeneration (Bellamkonda 2006).

1.3.1.1 Physical requirements

Artificial conduits are characterised by specific properties in order to achieve the best performance in peripheral nerve repair (Belkas et al. 2004).

A conduit should be easily formed and implanted, as well as easily sterilised before implantation (Belkas et al. 2004). It should show the most suitable balance between flexibility and structural integrity in vivo (Belkas et al. 2004; Siemionow et al. 2010), assuring an adequate physical support during regeneration as well as avoiding tube compression, kinking, breaking or tearing (Chiono et al. 2009; de Ruiter et al. 2009; Siemionow et al. 2010). Using different types of polymers, varying molecular weight and copolymer ratios, it is possible to achieve the best output (de Ruiter et al. 2009). Inner surface texture is important for facilitating regeneration, as smooth surfaces enable better and more robust axonal outgrowth than rough surfaces (Belkas et al. 2004; Evans 2000). Also, the thickness and the inner diameter of the conduit are important in order to allow polymer swelling during degradation (Heath & Rutkowski 1998; de Ruiter et al. 2009; Belkas et al. 2004). Swelling and degradation are thus essential nerve conduit properties as occlusion of the lumen and compression of regenerated axons could occur (de Ruiter et al. 2009).

Finally, permeability is one of the most important properties for conduit design (Vleggeert-Lankamp et al. 2007; Siemionow et al. 2010). Nerve regeneration is indeed
improved when a porous and permeable tube is used, as nutrients, growth factors and oxygen diffuse into the site of regeneration and waste products outside (de Ruiter et al. 2009; Vleggeert-Lankamp et al. 2007; Chiono et al. 2009).

1.3.1.2 Natural-based materials

Natural polymers have been used because of their excellent biocompatibility properties and ability to support cell attachment and functions (Wang & Cai 2010; Chiono et al. 2009).

Purified natural ECM proteins have been used to obtain nerve tube conduits, such as laminin, collagen and fibronectin (Siemionow & Brzezicki 2009; Evans 2000; Ciardelli & Chiono 2006). They are classified as “biodegradable polymers” and are able to support axons outgrowth until nerve is completely regenerated (de Ruiter et al. 2009; Wang & Cai 2010). Alternatively, ECM proteins can also be used as gels (transplantation matrices) to fill artificial conduits, promoting axons outgrowth and cell proliferation (Evans 2000), as well as binding neurotrophic factors (de Ruiter et al. 2009; Evans 2000). In this particular case the concentration of the gel and porosity of the conduit are the main parameters that can influence the regenerative process (de Ruiter et al. 2009).

Other natural polymers used for nerve regeneration include hyaluronic acid, fibrin gels, self-assembling peptides, alginate, agarose and chitosan (Chiono et al. 2009; Wang & Cai 2010; Ciardelli & Chiono 2006). By modifying their polymerisation procedure, it is possible to control cell attachment and neurite extension, as well as the their rate of degradation (Schmidt & Leach 2003).
However, natural polymers are characterised by poor mechanical properties and undergo too fast degradation *in vivo*, hence copolymers are required to improve the scaffold properties (Xie et al. 2008; Wang & Cai 2010).

### 1.3.1.3 Synthetic materials

Synthetic conduits can be classified as “inert” and “active”, according to their behaviour. The former just provide a suitable environment for regenerative process, whilst the latter are able to release also growth factors or other biological molecules, improving regeneration (Aldini et al. 2000).

Silicone has been one of the most used materials for the formation of artificial nerve conduits, also used in humans for clinical applications (Siemionow & Brzezicki 2009). However, it is not degradable and inflammation can occur *in vivo* (Siemionow et al. 2010). Polytetrafluoroethylene (PTFE) has also been used for clinical applications, but, like silicone, it is not absorbable and this can interfere with the regeneration of the nerve fibres that can be compressed (Siemionow & Brzezicki 2009). Research has therefore focused the attention on degradable and absorbable polymers for the production of the nerve conduits.

Polyesters, such as poly(lactic acid) PLA, poly(glycolic acid) (PGA), poly(lactic-co-glycolic acid) (PLGA) and poly(e-caprolactone) (PCL), are characterised by good degradation due to hydrolysis, which leads to the formation of low molecular by-products (Pitt et al. 1981). These by-products are easily metabolised or expelled from the body, reducing the risk of foreign body reactions. *In vivo* experiments demonstrated that polyester conduits enable nerve regeneration, showing comparable results to autografts (Nectow et al. 2012). Finally, although characterised by a very low density,
they showed good mechanical properties, which makes them suitable for tissue engineering applications.

In addition to polyesters, polyhydroxybutyrate (PHB) has shown very good capacity for nerve regeneration, thanks to its good mechanical and degradation properties (Hazari et al. 1999). It is also quite flexible and easy to handle, and its fibrous composition allows directional axonal regeneration (Boccaccini & Gough 2007).

Finally, other potential materials for peripheral nerve repair, like polyphosphazene, poly(ethylene glycol) (PEG), biodegradable poly(urethane) and methacrylate-based hydrogels have also demonstrated suitable nerve regenerative capacities (Aldini et al. 2000; Schmidt & Leach 2003).

Principal characteristics (Table 1.1) and repetitive units (Figure 1.7) of the most commonly used materials are shown below.

Although biodegradable polymers are preferred in nerve regeneration to avoid removal post-implantation in long-term applications, cytotoxic reactions can occur because of degradation products released (Belkas et al. 2004). However, degradation rate can be controlled by copolymerisation between different types of polymers, by the molecular weight as well as by the microgeometry of the conduit (Heath & Rutkowski 1998). This is one of the important aspect to be considered in conduit design, especially when growth or trophic factors are included in the structure and have to be delivered in a specific site (Heath & Rutkowski 1998).
Table 1.1. Main characteristics of the most commonly used materials for nerve regeneration. [* In the table, upper and lower values of mechanical and physical properties per each polymer are shown. (ρ = polymer density; σ = tensile strength; E = tensile modulus; ε = ultimate strain; Tg = glass transition temperature; Tm = melt point)] (Van de Velde & Kiekens 2002; Fields et al. 1989; Craig 1995; Dawes 1988).
1.3.1.4 Use of Poly-ε-caprolactone for peripheral nerve regeneration

In this study PCL has been chosen in order to create a novel biomaterial suitable for nerve regeneration. PCL is a biodegradable aliphatic polyester with very good degradation and adsorption properties \textit{in vivo} (Pitt et al. 1981). It is biocompatible and bioresorbable, with a glass transition temperature (\( T_g \sim -60^\circ C \)) and a low melting point (\( T_m \sim 60^\circ C \)) (Van de Velde & Kiekens 2002). PCL is characterised by a very low density and could lead to the lightest composites with other biopolymers (Van de Velde & Kiekens 2002). Also, it is less toxic and cheaper than other biodegradable polyesters, so that it is usually preferred to others (Ha et al. 1997).

Polymerisation process of PCL involves the ε-caprolactone ring opening, using a catalyst and heating (Figure 1.8.a). The repetitive unit is then characterised by one ester group and five methyl groups (Figure 1.8.b) (Fisher et al. 2007).
One of the most important chemical properties of PCL is its degrading ability in physiological environment (at about 37 °C) \textit{in vivo} (Pitt et al. 1981; Ali et al. 1993). In particular, it can undergo hydrolytic degradation by bulk or surface, cleaving its ester bonds and forming by-products of caproic acid (Fisher et al. 2007). By-products are eventually metabolised via the tricarboxylic acid (TCA) cycle or eliminated by direct renal secretion (Kweon et al. 2003).

The process of PCL degradation can be divided in two distinct phases, involving first a non-enzymatic bulk hydrolysis of ester linkages and reducing the molecular weight, hence a weight loss is registered (Ali et al. 1993; Pitt et al. 1981). It has been demonstrated that sample geometry does not influence degradation rate and the degradation mechanism is independent of the molecular weight of polymer (Pitt et al. 1981). However, the overall degradation time of PCL is sufficient to avoid polymer degradation before the axonal regeneration is completed, so that the conduit is able to give the required physical support throughout the regenerative process.

The application of PCL in peripheral nerve repair has been tested on different types of conduits produced mainly by film rolling, electrospinning, dip-coating and extrusion (Wang & Cai 2010). Although injured nerves showed regeneration after the implantation \textit{in vivo} of these artificial conduits, immune responses has been observed and nerve functionality has not been fully recovered. Modification of the artificial
conduits is therefore necessary in order to provide an optimum environment to assist nerve regeneration.

1.4 Optimisation of Nerve Conduits

Cell interaction with the biomaterial surface is an important biological reaction that occurs when the cells encounter the material and it can affect the nerve regeneration (Ikada 1994; Roach et al. 2007). Cell adhesion, migration and proliferation are coordinated by intracellular signals related to a particular complex of proteins, known as *focal adhesions*, also defined as “dynamic actin-integrin links” (Geiger et al. 2009).

Integrins are heterodimeric transmembrane receptors, constituted of two non-covalently linked subunits, called $\alpha$ and $\beta$ (Schmidt & Friedl 2010). Their different combination is the key of the integrin specificity of binding to a particular ligand (Takada et al. 2007), affecting the formation of focal complexes at cell junctions as well as the stimulation of specific signalling cascades for cell growth (van der Flier & Sonnenberg 2001; von der Mark et al. 2010). More specifically, integrins bind to the ECM proteins adsorbed on the substrate by changing the conformation of the $\alpha$ and $\beta$ subunits, form clusters and transmit signals transmission to the actin filaments through several protein assemblies (Owen et al. 2005; Geiger et al. 2009).

Based on their association with the cytoskeleton actin bundles, focal adhesions can be classified in two different types: dots and dashes, both containing the linker protein vinculin. Dots are usually observed at the edge of the cell and are defined as “immature” focal points; dashes are instead characterised by elongated shape and are associated with the cytoskeleton actin bundles (Owen et al. 2005). Beside their different morphology, both the types of focal adhesions are able to transmit forces and it is through them that cells are able to perceive the substrate, acting like mechanosensors through an integrin-
focal adhesion-cytoskeleton pathway (Owen et al. 2005; Yim et al. 2010). If cells perceive positive signals from the substrate, they start to produce integrins and ECM proteins. Conversely, negative signals trigger the production of degrading proteases, which will cause cell detachment (Boudreau & Jones 1999). Cells can therefore respond as function of different topographies and chemistry of the substrates (Geiger et al. 2009). One of the most important proteins involved in the mechanotransduction is the focal adhesion kinase (FAK), which is activated after the ECM-integrin interaction occurs. FAK activation is then followed by a cascade of phosphorylation reactions that lead to the generation of mechanical forces and the transcription and regulation of specific genes, responsible for cell growth and differentiation (Owen et al. 2005; Geiger et al. 2009). Signals transduction can however change over time as function of the external stimuli and the cells can regulate their path through the activation/deactivation of the focal adhesion protein network.

The main biomaterial properties influencing the cell-material interaction are topography, chemical composition, hydrophilicity and ionic charge (von der Mark et al. 2010; Lee et al. 2005; Gopal et al. 2007). Therefore, physical and chemical surface modifications are often required in order to obtain a more “cell friendly” scaffold.

1.4.1 Physical surface modification

The topography of a scaffold can influence cell adhesion and migration, as well as morphology and the formation of focal adhesions (Roach et al. 2007; Hoffman-Kim et al. 2010). In vitro experiments demonstrated that neurons can decrease neurite branching, reduce the number of neurites and increase neurite length as function of different topographical cues (Hoffman-Kim et al. 2010). For examples, grooved surfaces induce neurite alignment along the groove direction, reducing branching. This
could be explained as a confinement effect and neurites avoid crossing the features. Similar results were also obtained by using electrospun fibres, confirming the necessity of creating topographical cues for neurite outgrowth (Wang et al. 2009).

The inner surface of the conduit can be physically modified with grooves, channels, pores or fibres (Figure 1.9), in order to support migration and proliferation of SCs and direct the axonal outgrowth (de Ruiter et al. 2009). The performance in vivo of these modified conduits has remarkably improved and longer gaps have been able to be repaired after injury (de Ruiter et al. 2009; Daly et al. 2012).

![Figure 1.9. Physical modifications of the conduit structure.](image)

*In vitro* tests demonstrated that the presence of microgrooves on the inner surface induces cellular alignment along the grooves (Sun et al. 2010; Ni et al. 2010). However, cell and neurite alignment is strictly related to the size, the angle and the scale of the
grooves (Roach et al. 2007; Hoffman-kim et al. 2010), and it was demonstrated that the optimal range for the width and the spacing of the grooves is 10 – 20µm for NG108-15 cells (Sun et al. 2010). Grooved conduits with high degree of porosity showed not only cell alignment but also good permeability to proteins, which are important for the regenerative process (Ni et al. 2010).

The multi-channel structure of the conduit was developed to simulate the natural structure of nerve fibres (Daly et al. 2012). Agarose multi-channel conduits were shown to allow axonal growth after injury, and vascularisation occurred after 10 weeks in vivo (Tansey et al. 2011). Another example of multi-channel conduit was produced by using collagen. It showed good resistance to collapse, enzymatic degradation and good stability after swelling tests (Yao et al. 2010).

Nerve conduits fabricated with electrospun aligned fibres act in a similar way to grooves, influencing cell migration and nerve fibre alignment after regeneration (Hoffman-kim et al. 2010). Fibre diameter and distribution influence cell response, the latter affecting also the permeability and the porosity of the neural tube (Daly et al. 2012). Alignment of Schwann cells on collagen/PCL nanofibres was demonstrated as well as dorsal root ganglia (DRGs) neurons were elongated along the fibre direction with the neuritis extending in the same way (Schnell et al. 2007).

Recently, fillers, also known as intraluminal guidance structures, have been used in order to create internal frameworks that can mimic the natural structure of nerve tissues, or to encapsulate growth factors that will encourage nerve regeneration following release and uptake (Wang & Cai 2010; Hoffman-kim et al. 2010; Daly et al. 2012). Gels, sponges and fibres can be used in combination with topographical cues, sometimes at different nano- and micrometre-scales (Wang & Cai 2010; Daly et al.
However, the fillers may hinder the regenerative process impeding the diffusion of proteins and growth factors, hence a controlled study of the permeability of the construct is necessary in order to overcome this problem (de Ruiter et al. 2009; Wang & Cai 2010). Also, the “packing density” and the distribution of these intraluminal guidance structures can have a strong impact on the ability of the nerve to regenerate (Daly et al. 2012). High density of intraluminal structures may cause the inhibition of the nerve regeneration, whilst a low density may not be sufficient to support physically the regeneration of the nerve fibres.

1.4.2 Chemical surface modification

Chemical surface modification can be used when biomaterial surfaces are poorly biocompatible and are characterised by very high hydrophobicity. Indeed, surface chemistry is important for the formation of cell focal adhesion points, which are responsible for integrin binding between cell and substrate, as well as for cell signalling (Keselowsky et al. 2004).

Hydrophilicity is one of the most important properties for a scaffold to be used in tissue engineering. Most cells showed optimal attachment to surfaces with intermediate wettability between 55° and 85° (Lensen et al. 2008). Surface chemistry may be used to improve this property of the material. Substrates with similar wettabilities but different functional groups, such as –COOH, –CH₂OH, –CONH₂ and –CH₂NH₂, can influence cells adhesion and proliferation in a different way, while –NH₂ groups induce a better cell adhesion and spreading compared to –OH groups (Lee et al. 1994). The importance of amino groups on a biomaterial surface was confirmed by assessing electrostatic forces in cells adhesion on different polymer substrates (Lee et al. 2005). However, surface modification may affect the mechanical properties of the material.
Thick modifications may influence negatively mechanical and functional properties of materials, since they can modify completely the bulk properties of the scaffold. Hence, thinner modifications are preferred, in a range of 10 – 100 nm (Atala 2009). Nevertheless requirements such as uniformity, durability, functionality and stability are necessary in order to obtain suitable biological properties (Atala 2009).

Simple functional groups, different biomolecules, such as proteins, saccharides, lipids and drugs, as well as cells or microorganisms, can be added to the surface of biomaterials (Ratner et al. 2004). In the last years a wide variety of techniques have been developed in order to modify and functionalise tissue engineered scaffolds and the most important ones are listed in the table below (Table 1.2).

<table>
<thead>
<tr>
<th>Physical methods</th>
<th>Chemical methods</th>
<th>Radiation</th>
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<tbody>
<tr>
<td>Physical adsorption</td>
<td>Oxidation by strong acids</td>
<td>Plasma (glow and corona discharge)</td>
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<tr>
<td>Langmuir Blodgett film</td>
<td>Ozone treatment</td>
<td>Photo-activation (UV)</td>
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<td>Etching</td>
<td>Chemical modification</td>
<td>Laser</td>
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<td>Mechanical roughening and polishing</td>
<td>Flame treatment</td>
<td>Ion beam</td>
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<td>Patterning</td>
<td>Chemical vapour deposition</td>
<td>Electron beam</td>
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<td></td>
<td>Wet–Chemical oxidation treatment</td>
<td>γ-irradiation</td>
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1.4.3 Peptide immobilisation

Peptide immobilisation is a chemical method for surface modification which involves the covalent binding of specific amino acid sequences on the material surface.

Specific binding sequences found in the ECM have been immobilised on polymer surfaces in these last decades (Hersel et al. 2003; Perlin et al. 2008). Peptide-modified biomaterials, for example using RGD (Arg-Gly-Asp), IKVAV (Ile-Lys-Val-Ala-Val) and YIGSR (Tyr-Ile-Gly-Ser-Arg), have been found to facilitate the interaction between ECM proteins and integrins on cell membranes and to enhance cell adhesion and proliferation (von der Mark et al. 2010). Each amino acid sequence enables to target particular cell lines and elicit specific cell responses, as each motif is recognised by specific receptors on cell membranes (von der Mark et al. 2010; Schmidt & Friedl 2010). However, ligands concentration and binding forces should be correctly balanced. In fact, the lack of specific ligands does not allow cell adhesion nor migration on the biomaterial surface, but high concentrations may cause a very strong adhesion that can reduce or stop migration (Hubbell 1998). It has been demonstrated that a minimum peptide concentration of 1 fmol/cm$^2$ enables cell spreading, while a higher concentration of 10 fmol/cm$^2$ can induce also the formation of focal contacts and stress fibres (Massia & Hubbell 1991). However, these concentrations are strictly related to the specific cell line and the biomaterial surface used.

Compared to proteins, peptides are characterised by higher stability during sterilisation, heat treatments and changing in pH (Hersel et al. 2003). Their high selectivity in cell binding could be explained as they contain only a single specific motif recognisable by cells and they are packed at high density on the surface (Hersel et al. 2003). It can be asserted that, apart from the physical properties, the specific chemical
signals from peptides epitopes and the binding sites microdistribution are two main factors to be considered when designing a biomimetic surface (von der Mark et al. 2010). Different peptide sequences and lengths have also been analysed in order to find the best binding affinity (Hersel et al. 2003; Perlin et al. 2008). Nevertheless, there are no specific rules that define perfectly the affinity of different sequences, as cells attachment can be influenced by many factors, such as nature of the substrate, presence of a spacer that can increase the entropy of the system, peptide surface density, cell line or culture conditions (Hersel et al. 2003).

One of the most studied and used peptide for biopolymer functionalisation is RGD as it can trigger adhesion and proliferation of different cell lines (Hersel et al. 2003). However, RGD has shown affinity with many different cell lines, not being specific with particular cultures. On the contrary, two other peptide sequences derived from laminin, IKVAV and YIGSR, showed more specific affinity with neuronal cells (Tashiro et al. 1989; Hersel et al. 2003; Cargill et al. 1999). It was demonstrated that laminin plays an important role in neurite extension of NG108-15 cells, influencing also cell attachment and spreading (Smalheiser 1991; Cargill et al. 1999). In a laminin-derived peptide study, IKVAV-modified surfaces showed a significant improvement in stem cell adhesion (Santiago et al. 2006). However, the specific substrate has to always be taken into account, as it can affect cell-surface interaction in different ways. In facts, it has been found that YIGSR-modified surfaces were characterised by the highest amount of attached cells, while IKVAV-modified surfaces presented the longest neurite extension (Tong & Shoichet 1998).

Different approaches have been used to immobilise peptide sequences on polymer surfaces. Among them, glutaraldehyde (GA) chemistry and carbodiimide (CDI) chemistries are the most commonly used functionalisation techniques.
It has been demonstrated that functional groups, such as –COOH, –NH₂ or –OH, are necessary for the formation of specific binding sites for biomolecules (Ratner et al. 2004). However, some biomaterials are not characterised by any functional group, hence previous surface activation could be crucial in order to proceed with the final coupling step (Hersel et al. 2003).

An important point for covalent immobilisation of biomolecules on biomaterial surfaces is the presence of spacers, also known as “arm” or “leash” (Ratner et al. 2004). They could be, for example, carbodiimides, gluteraldehyde, cyanogen bromide (CNBr), N-hydroxysulfosuccinimide (Atala 2009; Zhu et al. 2002). The importance of the spacers is due to improvement of both steric hindrance and specific activity; also, they can present amino or carboxyl endings, avoiding the activation step (Ratner et al. 2004). However, the spacer length should be controlled, as too long arms could reduce cells attachment (Hersel et al. 2003).

1.4.3.1 Carbodiimide (CDI) chemistry

1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) is an N-substituted carbodiimide widely used in peptide immobilisation and it is well-known as a zero-length crosslinker (Grabarek & Gergely 1990). It is water-soluble and stable at pH between 5 and 7 (Gilles et al. 1990). During the amide binding formation with carboxylic groups, it forms an unstable reactive intermediate, known as O-acylisourea derivative, which is easily hydrolysed in the absence of amino groups (Grabarek & Gergely 1990). This adduct is stabilised by the addiction of N-hydroxysuccinimidyl esters (NHS). The product, a semi-stable amine-reactive NHS-ester, can then react with the amino groups present in the peptide sequence for the further immobilisation (Figure
1. Introduction

For that reason, this functionalisation method is known as a “two-step zero-length cross-linking procedure” (Grabarek & Gergely 1990).

Figure 1.10. Carbodiimide (CDI) chemistry for peptide immobilisation on surfaces that expose free amino groups.

Different groups adopted EDC chemistry for polymer functionalisation, immobilising various biomolecules on the surface and resulting in a higher biocompatibility of the material (Santiago et al. 2006; El-Ghannam et al. 2004; Ho et al. 2005; Chunli et al. 2009).

Although CDI chemistry on PCL has been reported in literature, there are some disadvantages inherent of this technique. First of all, it does not guarantee repeatability. This is due to the preliminary activation step of the PCL surface necessary for functionalisation, which involves the addition of amino groups using hexamethylenediamine. Indeed, both of the –NH₂ functionalities could react with the polymer backbone, causing the formation of “molecular bridges” with no free amino groups on the surface. Furthermore, side reactions could take place instead of the desired peptide immobilisation as peptides could be characterised by carboxylic and guanidine groups within the amino acid sequence, hence these would be able to react
with the free –NH₂ on the aminolyzed-PCL surface (Vladkova 2010). Finally, in order to avoid the formation of the unstable reactive O-acylisourea derivative, which undergoes fast hydrolysis, the use of one more chemical, such as N-hydroxysuccinimide, is necessary (Grabarek & Gergely 1990).

1.4.3.2 Thiol (Th) chemistry

Thiol chemistry can be defined as a thiol-halogen “click reaction”, which involves the very strong nucleophilic substitution of halogens by thiols, also known as C-Sulfanylation (Figure 1.11) (Koval’ 2007).

![Figure 1.11. Thiol (Th) chemistry, also defined as thiol-halogen “click reaction”, for peptide immobilisation on surfaces that expose halogens (in this particular case, Chlorine).](image)

“Click chemistry” has been defined as a powerful, selective and modular set of blocks (Kolb et al. 2001). Specific requirements were needed for the process to occur, such as simple reaction conditions, readily available starting materials and reagents, use of non-toxic solvents and simple product isolation (Kolb et al. 2001).
Thiol-halogen reaction fulfils all these requirements (Becer et al. 2009; Hoyle et al. 2010). It is characterised by high specificity due the high reactivity of those chemical groups. In facts, thiols are characterised by high nucleophilicity, hence high reactivity with halogens (Fox & Whitesell 2004; Roth et al. 2011). Also, due to the lower electronegativity of sulphur compared to oxygen, halogens are selectively displaced by thiols even in presence of alcohols (Hoyle et al. 2010). In the C-X bond, where X is a halogen atom, carbon appear to be partially positively charged and X partially negatively charged. Carbon is therefore electron deficient and it is easily attacked by nucleophilites, resulting in the halide ion displacement.

In the literature there is no mention of using this reaction for peptide immobilisation, although the halogen-thiol substitution has been widely studied (Koval’ 2007). The mechanism is quite simple and it could overcome some drawbacks of CDI chemistry. Particularly, cysteine has been recently investigated for protein modifications thanks to its high nucleophilic thiol group (Smith et al. 2010; Tedaldi et al. 2009).

1.5 Hypothesis and Aims

The micropitted scaffold prepared by the solvent casting mechanism using poly-ε-caprolactone (PCL) was found to be suitable for adhesion and proliferation of Schwann cells (SCs) and derived adipose stem cells (dASCs), and consequently as artificial nerve conduit (Sun et al. 2010; Tse et al. 2010). However it was necessary to further improve the PCL scaffold by modifying surface morphology, chemistry and physical properties in order to enhance cell compatibility of the tissue-engineered construct for peripheral nerve repair.
The hypothesis of the present study was that surface modification of PCL films would improve SC response. This could be achieved by using different functional groups and by developing a new mechanism to immobilise RGD moieties.

The overall aims of this project were as follows:

- To compare PCL conduits to autografts, also known as the “gold standard” in nerve repair, using an *in vivo* rat model of peripheral nerve injury in order to assess the level of axonal regeneration and functional recover.

- To improve PCL films by functionalisation of the material surface in order to enhance SC response. As PCL is a hydrophobic polymer, it was investigated whether the surface hydrophilicity of the material could be increased through either hydrolysis or aminolysis reactions. Also hydrophilic properties were compared and related to the chemical properties of the films surface.

- To functionalise the surface of the conduits with RGD sequences with a new developed mechanism (thiol chemistry), which was compared to the conventional carbodiimide chemistry. A new immobilisation mechanism was also used to increase the specificity of the coupling reaction.

- To assess the cyto-compatibilities of SCs on these modified scaffolds and to analyse cells morphology and proliferation in comparison to a non-functionalised scaffold.
2. Materials and Methods
2. Materials and Methods

All chemicals and reagents were purchased from Sigma-Aldrich Ltd. (Dorset, UK) unless otherwise specified.

2.1 Fabrication and characterisation of the substrates

2.1.1 Preparation of PCL films

PCL films were fabricated by solvent casting. In this particular study, PCL pellets (Mn ~ 70000 – 90000) were dissolved in dichloromethane (DCM)) to a final concentration 3% w/v. PCL/DCM solution (300µl) was then spread on a glass coverslip (18x18 mm²) and the solvent was left to evaporate overnight at room temperature. The complete evaporation of DCM led to the formation of thin PCL films that were easy to peel off the substrate for further investigation and modification (Figure 2.1).

Figure 2.1. Scheme of the fabrication process of PCL films. The polymer solution was spread on glass coverslip and the solvent evaporation led to the formation of thin PCL films.

2.1.2 Surface modification of PCL films

2.1.2.1 Hydrolysis and Aminolysis

The effect of different functional groups and different wettabilities was investigated by treating PCL films with hydrolysis and aminolysis. Also, the effect of two hydroxide solutions was compared to investigate any differences that may occur during the
hydrolysis reaction. During these treatments the characteristic ester bond of PCL is cleaved, resulting in the formation of carboxylic groups (hydrolysis), and amino groups (aminolysis) respectively (Figure 2.2).

![Figure 2.2. Scheme of Hydrolysis and Aminolysis of PCL. After cleaving PCL ester bond, carboxylic and amino groups are introduced on the surface of PCL films.](image)

Two hydroxide solutions were prepared by dissolving sodium (NaOH) and potassium hydroxide (KOH) in distilled water (dH₂O) to the concentration 10M (pH=12). PCL films were then immersed in either of these solutions for 1 hour at room temperature, shaking continuously. To stop the reaction and return the pH to neutral (pH~7), films were carefully rinsed in dH₂O. They were finally dried before further investigations.

A similar procedure was used to introduce amino groups on the film surface through aminolysis. A solution of hexanemethylendiamine (HMD) in isopropanol (IPA) was prepared at the concentration 10% wt/v (pH=7). PCL films were then immersed and continuously shaken for 1 hour at room temperature. Finally, they were accurately
rinsed in dH₂O to stop the reaction and to wash off all the unreacted HMD residues, and dried.

The parameters used for all the treatments are summarised in the following table:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Conc.</th>
<th>Time</th>
<th>Temp. (ºC)</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH in dH₂O</td>
<td>10M</td>
<td>1 h</td>
<td>RT</td>
<td>Hydrolysis</td>
</tr>
<tr>
<td>KOH in dH₂O</td>
<td>10M</td>
<td>1 h</td>
<td>RT</td>
<td>Hydrolysis</td>
</tr>
<tr>
<td>HMD in IPA</td>
<td>10% wt/v</td>
<td>1 h</td>
<td>RT</td>
<td>Aminolysis</td>
</tr>
</tbody>
</table>

2.1.2.3 Immobilisation of RGD sequences

Peptide sequences were immobilised on the surface of PCL films using two different chemistries. Specifically, the films were functionalised with RGD sequences using CDI and Th chemistries.

CDI chemistry:

![Scheme of CDI chemistry for the immobilisation of RGD sequences on PCL film surface.](image)
PCL films were first aminolysed for 3 hours at ~40°C to create amino groups on the surface, necessary for the functionalisation through CDI chemistry (time and temperature for the aminolysis of PCL films were optimised as reported in Appendix II). Aminolysed films were then rinsed carefully in dH₂O and left to dry before further modification.

1-ethyl-3-(dimethylaminopropyl)carbodiimide (EDC) was dissolved in 2-N-morpholinoethanesulfonic acid (MES) buffer 0.1M (pH~5.5). An EDC solution 0.1M was then applied on the aminolysed PCL film and left to react for 3 hours at room temperature. The previous solution was then removed and a new solution was prepared with tri-peptide RGD and N-hydroxysuccinimide (NHS) in MES buffer 0.1M. According to Santiago et al., the final peptide concentration to spread on film surface was 0.2µg/mm² (Santiago et al. 2006). The peptide solution was left to react for 24 hours at room temperature. Samples were finally washed in dH₂O and dried.

Th chemistry:

Th chemistry is based on the nucleophilic substitution of chlorine atoms by thiol groups, which are present in cystein residues.

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**Figure 2.4.** Scheme of Th chemistry for the immobilisation of RGD sequences on PCL film surface.
In order to introduce chlorine atoms on the surface of PCL films, they were immersed in a 10% wt/v 2-chloroethylamine hydrochloride (CEA)/dH$_2$O solution (pH 9) for 3 hours at ~40°C, stirring continuously. Chlorinated films were then rinsed carefully in dH$_2$O and left to dry before further modification.

The pentapeptide RGDSC (Biomatik Corporation, Cambridge, Canada) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were dissolved in MES buffer 0.1M (pH~5.5) in a molar ratio of 1:5, to obtain again a final peptide concentration of 0.2µg/mm$^2$ on the film surface. The solution was then applied on chlorinated films and left to react for 24 hours at room temperature. Samples were finally washed in dH$_2$O and dried.

2.1.3 Characterisation of film properties

2.1.3.1 X-ray Photoelectron Spectroscopy (XPS)

XPS analysis was carried out at the School of Chemical Engineering and Analytical Science by Dr. Joanna S. Stevens and Dr. Sven L. M. Schroeder.

Films were flattened and stuck on a metal bar using a double-sided tape, hence loaded in the instrument chamber under vacuum conditions. XP spectra were recorded with a Kratos Axis Ultra instrument employing a monochromatic Al Kα source (1486.69eV). Data analysis was carried out with Casa XPS software using a linear background and GL(30) line shape (Fairley & Carrick 2005). Samples were referenced to adventitious hydrocarbon contamination at 285eV (Stevens & Schroeder 2009; Stevens et al. 2010). Repeatability of the peak positions was ±0.1eV.
2. Materials and Methods

2.1.3.2 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR analysis of hydrolysed and aminolysed PCL films was conducted using a Nicolet 5700 FTIR spectrophotometer (Thermo Electron Corporation), equipped with Smart Orbit accessory. Films were blocked on the diamond stage and data were collected in a range of 400 – 4000 cm\(^{-1}\) at 4 cm\(^{-1}\) resolution with 32 scans.

2.1.3.3 Detection of amino groups using Ninhydrin Assay

Ninhydrin assay is a colorimetric assay which allows detection of amino groups. This test was therefore carried out on aminolysed PCL films to evaluate the amount of amino groups linked to the surface after treatment. Ninhydrin was dissolved in ethanol to a final concentration 1.0M. Firstly, a calibration curve was made at different HMD concentrations in IPA, in a range of 0.1 – 2.0 mM (see Appendix II). Aminolysed-PCL films were then cut into small pieces (6 x 18 mm\(^2\)) and placed into small vials with 100µl of ninhydrin solution. Vials were warmed gently at ~70°C in a water bath and 500µl of DCM were added to dissolve the film. Finally, 500µl of IPA were added into each vial and absorbance was read at 562nm by using a microplate reader (Multiskan Ascent V1.22).

2.1.3.4 Scanning electron microscopy (SEM)

The topography of untreated and modified PCL films was investigated through SEM (VPSEM, Zeiss EVO60). Films were stuck on aluminium stubs using double-sided carbon tape and gold sputter coated (Edwards, Sussex, UK). Samples were then mounted on a metal support and loaded in the vacuum chamber of the instrument. Images were captured at different magnifications with an accelerating voltage in a range of 5 – 20 kV.
2. Materials and Methods

2.1.3.5 Contact angle measurements

Contact angle measurements were carried out to investigate the different hydrophilic properties of the films. All the tests were performed using the contact angle analyser DSA100 (Kruss, Hamburg, Germany).

The sessile drop contact angle was measured using the Kruss Drop Shape Analysis software (Kruss, Hamburg, Germany). Films were stuck on glass slides with a double-sided tape to keep them flat during the analysis. Four drops of 10µl of dH₂O were placed on each film surface (n=5) and the software evaluated the contact angle through the camera mounted on the instrument (Kruss, Hamburg, Germany).

The advancing and receding contact angles were evaluated by respectively increasing and decreasing the water droplet size of 5µl each time and measuring the contact angle as previously described (n=5). Gravity effects were not counted for drop sizes less or equal than 10µl (Bachmann et al. 2000).

2.1.3.6 Mechanical testing

In order to measure the mechanical properties of the films, longer films (76x26mm²; 1830µl) were prepared and cut in a dumbbell shape (width = 3.8mm; gauge length = 20mm). Thicknesses were measured by using an electronic digital micrometer (Farnell, UK). Each sample was mounted on a table top tensile testing machine (Instron Corporation) using a grip distance of 35mm and a full scale load range of 10N. The crosshead speed was set up at 50mm/min. During all tests (n=5), humidity and temperature were kept constant respectively at (50±5)% and (23±1)°C.

Data were finally analysed using Series IX Automated Materials Testing System and Kaleidagraph software (version 4.0, Synergy software), giving the maximum load, tensile strength (T.S.), yield stress (Y.S.) and the Young’s modulus (E).
2. Materials and Methods

2.2 Assessment of the films biocompatibility

2.2.1 Schwann cell harvest and culture

All the experiments involving animals were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986. Rat sciatic nerves were harvested from adult Sprague-Dawley rats. Under a dissecting microscope (Wild Heerbrugg M3Z, Switzerland), the nerves were aseptically desheathed with tweezers and sectioned in short segments (~3mm). The sectioned nerves were then moved into a Petri dish and incubated at 37°C, 5% CO$_2$ for 2 weeks in a medium containing Dulbecco’s Minimum Eagle’s Medium (DMEM), 10% (v/v) foetal bovine serum (FBS; Biosera, UK), 1% (v/v) penicillin-streptomycin (PAA, UK), 14µM forskolin and 63ng/ml glial growth factor-2 (GGF-2; Acorda Therapeutics, NY, USA) (SC medium). The migration of cells was daily observed under a light microscope and medium was changed every 3 – 4 days. After incubation, the nerve segments were incubated in 1ml of SC medium containing 0.0625% collagenase IV (Worthington, Biochemicals, USA) and 0.5mg/ml dispase (Invitrogen, UK) for 24 hours at 37°C, 5% CO$_2$ for digestion. Nerves were transferred into falcon tubes and accurately triturated using a glass pipette. After adding 5 ml of SC medium without growth factors, the cell suspension was filtered through a 100µm cell strainer to remove debris and centrifuged at 900rpm for 5 minutes. The pellet was then resuspended in 10 ml of SC medium and the cells were plated on poly-D-lysine (pdl) coated flasks and incubated at 37°C, 5% CO$_2$. SC medium was changed every 3 days and cells were moved into new pdl-coated flasks.

2.2.2 SC immunodepletion

In order to purify the SC population from fibroblast contamination, immunodepletion was performed. Confluent cells were washed with 5ml of Hank’s
Balanced Salt Solution (HBSS) to remove traces of serum. Trypsin/EDTA (Invitrogen, UK) was then added to the flask (3ml for a 75cm$^2$ flask) and incubated for 5 minutes at 37°C. To stop the enzymatic reaction, 7ml of SC medium were finally added and cell suspension was centrifuged at 1000rpm for 5 minutes. The supernatant was aspirated and the resulting pellet was resuspended in 500µl of medium containing mouse anti-Thy 1 (1:500; Serotec, Oxford, UK). The cell suspension was incubated for 10 minutes at 37°C. 250µl of rabbit anti-mouse complement (Serotec, Oxford, UK) were then added and the cell suspension was incubated for other 30 minutes, gently shaking every 10 minutes. 5ml of SC medium were finally added and the cell suspension was centrifuged at 800rpm for 5 minutes. The supernatant was aspirated and, after resuspending the pellet in 10ml of SC medium, the cells were plated onto new poly-D-lysine coated flasks and incubated at 37°C, 5% CO$_2$. This procedure for immune-depletion should guarantee a SC purity of 99% (Brockes et al. 1979) and can be repeated if necessary.

2.2.3 Investigation of cell attachment

Treated and untreated PCL films were mounted on plastic supports (Scaffdex, Finland) to make them flat and avoid them floating during cell culture. They were then transferred into 24 well plates and sterilised under UV light overnight prior to cell seeding.

For the investigation of cell attachment, SCs were trypsinised and diluted to the desired density after counting. Specifically, few drops of cell suspension were pipetted on a haemocytometer (Weber Scientific International Ltd, UK) and, using the grid, cells were counted with an inverted microscope (Olympus IX-50, Olympus, UK).

The cells were seeded on the PCL films at a density of 10000 cells/film in 200µl. The samples were then incubated at 37°C, 5% CO$_2$ for 2, 4 and 6 hours (n=3 each film
type and time point). At each time point, the medium was aspirated and the cells were fixed in 4% paraformaldehyde (PFA) for 20 minutes at room temperature. The cells were then carefully washed in PBS twice for 5 minutes and the seeded area of the films was cut out from the scaffdex using a scalpel. The films with adherent cells were finally mounted on glass slides with Vectashield containing DAPI (Vector Labs, Peterborough, UK).

In order to count the cell nuclei, 6 random images per each film were captured at 10x using a fluorescence microscope (BX60; Olympus, Japan). ImageJ software (National Institute of Health, NIH, USA) was used for the cells counting. The average number of the attached cells was finally calculated as the percentage of seeded cells per film.

2.2.4 Analysis of SC proliferation

2.2.4.1 MTS Assay

MTS Assay was performed to assess Schwann cell proliferation. PCL films were mounted on scaffdex and sterilised overnight under UV light. The cells were seeded at a density of 5000 cells/film in 200 µl on the surface of the scaffolds (n=3) and incubated for 2 hours to allow attachment. The growth medium was then topped up to the final volume of 1ml per each well and the cells were incubated at 37°C, 5% CO₂. Also, the growth medium was added to three empty wells without cells. After 1, 3 and 5 days, medium was aspirated, the films were removed from the scaffdex and moved into new 24 well plates. 300µl of MTS solution (20% MTS in DMEM without phenol red) were added to each well and incubated at 37°C for 4 hours. Aliquots of 100µl (in triplicate) of the incubated MTS solution were then aspirated and moved into a transparent 96 well
plate. Absorbance was recorded at 490nm ($A_{490}$) and 650nm ($A_{650}$) with a plate reader (Biochrom Asys UVM 340 Microplate Reader, Biochrom Ltd., UK).

The absorbance registered at 650nm is usually associated to fingerprints, scratches or other background that could alter the reading, hence $A_{650}$ was subtracted from that at $A_{490}$ to reduce the background. The absorbance contribution of the culture medium (control without cells) registered at 490nm was also adjusted for background reading. Data were finally normalised and expressed as a percentage of the values detected in controls (untreated PCL films).

2.2.4.1 DNA Assay

DNA assay was also performed to quantify the number of cells on the surface of the films. After seeding the cells as previously described (5000 cells/film) ($n=3$), they were incubated for 1, 3 and 5 days in their growth medium containing growth factors. At every time point the cells were washed in PBS to eliminate non-attached cells and any residue of FBS, and scaffdex were removed. 500µl of dH$_2$O were then added to each well and frozen rapidly to -80°C. The samples were freeze-thawed three times and aliquots of 50µl (in triplicate) were moved in a clear flat bottom 96 well plate. 50µl of TNE buffer were added to each well before the final addition of 100µl of Hoechst stain solution (0.02mg/ml). Fluorescence was recorded at 355nm (excitation) and 460nm (emission) with a fluorescence plate reader (Fluostar Optima). Data were expressed as number of cells evaluated through a standard curve obtained with known cell densities (see Appendix III).

2.2.5 Investigation of cell morphology through SEM

SEM analysis was performed to investigate the morphology of SCs on the surface of untreated and treated films. Cells were seeded as previously described at a density of
5000 cells/film and they were cultured for 5 days without changing the culture medium. The medium was then aspirated and the cells were carefully fixed in 1.5% gluteraldehyde for 30 minutes at 4°C. The cells were washed in PBS twice and dehydrated through a series of ethanol gradients taking care to prevent cells from drying out in between. The cells were therefore washed twice for 3 minutes in ethanol solutions at the following concentration: 50%, 70%, 90% and 100%. To complete dehydration, the cells were then washed twice in hexamethyldisilazane (HMDS) for 5 minutes under the fume hood and left overnight to dry before mounting for SEM analysis.

Scaffdex were removed and the films were stuck on aluminium stubs as previously described. The samples were finally gold sputtered and loaded in the SEM chamber under vacuum. Images were captured at different magnifications with an accelerating voltage in a range of 5 – 20 kV.

2.2.6 Immunocytochemistry

After mounting the films with the Scaffdex and sterilising them, 5000 cells were seeded on each film as previously described and they were cultured for 5 days without changing the culture medium. Afterwards, the medium was aspirated and the cells were carefully fixed in 4% wt/v paraformaldehyde (PFA) for 20 minutes at room temperature. The cells were carefully washed three times in PBS for 5 minutes and permeabilised in 0.2% v/v Triton-X/PBS for 20 minutes. The cells were washed again three times in PBS for 5 minutes and non specific antigens were blocked with 1% wt/v bovine serum albumin (BSA) in antibody diluent for 1 hour at room temperature. The primary antibody (Mouse Anti-S100 Antibody; 1:500 or Anti-Vinculin antibody (hVIN-1, Abcam, UK); 1:400) was applied overnight at 4°C. The following day, cells were washed three times in PBS for 5 minutes before the secondary antibody (Alexa Fluor
568 donkey anti-mouse IgG, Life Technologies, UK; 1:1000) was applied for 1 hour at room temperature in the dark to avoid bleaching. The cells were washed again three times in PBS for 5 minutes and incubated with phalloidin (Alexa Fluor 488, Life Technologies, UK; 1:40) for 20 minutes at room temperature in the dark. The cells were finally washed again three times in PBS for 5 minutes and the films were cut off the scaffdex before mounting on glass slides with Vectashield containing DAPI. Images were acquired at different magnification using a fluorescence microscope and processed using Image-Pro Plus (V 6.0.0.260, Media Cybernetics).

2.2.7 Quantification of the spreading and the elongation of SCs

In order to evaluate the spreading of the SCs on untreated and treated PCL films, the cells were stained against vinculin with the protocol previously described (cf. 2.2.6). 15 – 20 fluorescence images were captured at 40x and analysed using ImageJ software. After setting the metric scale using a calibrated graticule, images were transformed into 8 bit and then converted into greyscale. The cell area was then highlighted by tracing a line around the cell, including the cellular processes. An intensity threshold was applied to differentiate bright immunostaining from any low-level background autofluorescence, and the immunostaining area (expressed in $\mu m^2$) was calculated by the software using the “analyse particle” function (Figure 2.5).
2. Materials and Methods

Figure 2.5. Quantification of cell spreading using ImageJ. a) Image conversion into 8 bit, grayscale; b) setting of the threshold; c) analysis of the cell area using the “analyse particle” function.

A similar procedure was applied to evaluate the elongation of the cellular processes. SCs were stained against S100 as previously described. After setting the scale and converted the image into greyscale, cellular processes were manually measured and expressed in µm.

2.3 In vivo experiment using a rat model

All the procedures were carried out in compliance with the regulations specified in the Animals (Scientific Procedures) Act 1986 (UK Home Office).

2.3.1 Conduit preparation

PCL films previously treated with 10M NaOH were rolled up by using a 16G intravenous cannula (16G Abiocath®-T, Abbott Ireland, Sligo, Republic of Ireland), the inner side of the conduit being characterised by micropits. The films were then sealed by heating up the polymer around 40 – 50 °C, which is below the melting point of PCL. The resulting conduit was 1.4cm in length with an internal diameter of 1.6mm. The conduits were sterilised under UV light before implantation in vivo.
2.3.2 Conduit implantation and harvesting

The surgical procedures were carried out on adult male Sprague-Dawley rats by Dr Adam Reid in the School of Medicine, University of Manchester.

The animals (n=10) were anaesthetised with isofluorane gas and the absence of reflexes was tested before starting the operation. Skin and muscle of the left gluteal region were incised to expose the rat sciatic nerve. Under an operating microscope (Zeiss, 10x magnification), a standard nerve injury (1 cm gap) was created in the sciatic nerve and the conduit was placed at the injury site (n=5). In control rats (n=5) the excised nerve was reversed and sutured at the injury site (nerve autograft). At each end the injured nerve was sutured to the conduit or to the autograft with a 9-0 epineural suture. Muscle and skin were then closed with subcuticular suture points. The animals were regularly monitored for general health during the duration of the experiment.

After 18 weeks, the animals were killed in accordance with Schedule I of the Animals Act 1986. The rats were killed by CO₂ narcosis and termination was finally confirmed by cervical dislocation. Grafted conduits and nerve autografts were harvested from the rats together with the proximal and distal sections of the nerve. The nerve was then carefully removed from the conduit and pinned on a plastic sheet to prevent shrinkage and distortion. Footpad skin from the lateral half of the plantar surface on the operated and controlateral (control side) of each animal was also harvested and pinned on plastic sheets in a similar way.

2.3.3 Tissue fixation and sectioning

The harvested tissues were fixed in Zamboni’s solution at 4°C overnight. For the following 3 days, the tissues were washed in a PBS/sucrose solution until the solution became clear.
Small boats were prepared with aluminium foil. The nerves were orientated longitudinally in the boat and they were embedded with a cryomatrix agent (OCT; Raymond A Lamb-Laboratory Supplies, UK) with a piece of pre-fixed rat liver at the proximal end to orient the tissue during the microscopy analysis. The nerves were rapidly frozen by dipping the boats into liquid nitrogen. The samples were stored overnight at –40°C, before sectioning at the cryostat (Hacker Bright Model OTF Cryostat, Bright, UK). The frozen tissue was then moved into the cryostat chamber and it was fixed with few drops of OCT on a metal support. Longitudinal nerve sections were cut with a thickness of 14µm and collected by using Vectabond™ coated glass slides (Vector Laboratories, UK). The nerve sections were left to dry overnight on a warm plate before staining.

A similar procedure was followed for the footpad skin. After creating the aluminium boats, the footpad skin was placed inside with the epidermal surface facing the base of the boat. The tissues were then covered with OCT and frozen in liquid nitrogen for cryosectioning. Sections were cut at a right angle from the epidermal surface of the skin in order to obtain sections comprising all skin layers.

2.3.4 Tissue Immunohistochemistry

In order to minimise any solution spreading from the sections during the staining procedure, a water-repellent circle was drawn around the nerve sections on the glass slides using Dako Pen (Dako, UK). The sections were then immersed in 0.2% Triton-X/PBS solution for 1 hour at room temperature to permeabilise the tissues and they were rinsed twice in PBS for 5 minutes. The sections were blocked with Normal goat serum (NGS; 1:100) for 1 hour at room temperature in a humid chamber to avoid drying. The primary antibody was then applied to the sections (relative to the protein
under investigation, Table 2.2) and incubated overnight at 4°C. The following day, the sections were washed three times with PBS for 5 minutes and the secondary antibody (Fluorescein (FITC) goat anti-rabbit (conjugated secondary antibody, Vector Laboratories, UK; 1:100) was applied for 1 hour at room temperature in a dark room to avoid florescence bleaching. The sections were washed again in PBS three times for 3 minutes before being mounted under coverslips with Vectashield medium to minimise fluorescence fading. Tissue sections were finally analysed using a fluorescence microscope.

Table 2.2. Primary antibodies used for immunohistochemistry.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Primary antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nerve</td>
<td>Protein gene product 9.5 (PGP 9.5 anti-rabbit polyclonal, Dako, UK)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Skin</td>
<td>PGP 9.5</td>
<td>1:1000</td>
</tr>
<tr>
<td>Skin</td>
<td>Calcitonin gene-related peptide (CGRP, anti-rabbit polyclonal, Sigma, UK)</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

2.3.5 Quantification of nerve regeneration

Immunostaining area was used as an indication of the quantity of axonal regeneration. Measurements were carried out at a fixed point (5mm from the proximal stump) to allow direct comparison between autografts and PCL conduits. The quantification of the regeneration was carried out through the analysis of images at 20x magnification using Image-Pro Plus software (Figure 2.6). A calibrated graticule was then used to measure the distance of regeneration.
2. Materials and Methods

Figure 2.6. Quantification of nerve regeneration. Images were captured at 20x magnification 5mm after from the beginning of the proximal stump.

Images were first converted to 8 bit greyscale and converted to black and white images. A threshold was then applied to each image to select the stained regenerating nerve, maintaining the thresholding range constant for all the specimens and avoiding operator bias. In the preview window, the software was set on “class color on transparent”. The staining area of the regenerating axons was evaluated automatically by the software (expressed in $\mu m^2$). After the evaluation of the total frame area, the staining area, defined as the fraction of the area of interest within which the axons lay, was calculated according to the following formula:

$$\% \text{Staining area} = \frac{\text{Staining area} (\mu m^2)}{\text{Total frame area} (\mu m^2)}$$

2.3.6 Quantification of skin reinnervation

As for the quantification of nerve regeneration, immunostaining area was used to calculate the amount of cutaneous innervation. For each antibody, six random pictures
were captured at 20x magnification, including epidermis and dermis. Using Image-Pro Plus software, images were converted into 8 bit greyscale and converted to black and white images. The immunostained area within epidermis and dermis was then highlighted by tracing a line around its margins. The images were then thresholded as previously described and the immunostaining area was automatically quantified by the software. The immunostaining area was finally expressed as fraction of the total frame area of epidermis + dermis, according to the same formula as above (cf. 2.3.5).

2.4 Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical significance for material characterisation studies was estimated by one-way ANOVA test in Prism 5 (GraphPad Software, Version 5.03). Also, Dunnett’s post test was chosen to compare groups. Cell work data were analysed by two-way ANOVA test and Bonferroni’s post test was performed. Levels of significance were expressed as p values vs. controls (nerve autografts and controlateral foot pad skin for the in vivo work; untreated PCL films for the material characterisation and in vitro tests) (*p<0.05, **p<0.01, ***p<0.001). Statistical analysis on the data obtained from the in vivo experiment was performed by t-test comparing the two groups, autografts and PCL conduits.
3. Paper I

Long term peripheral nerve regeneration using a novel bioengineered PCL nerve conduit

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Long term peripheral peripheral nerve regeneration using a novel bioengineered PCL nerve conduit

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Abstract

Peripheral nerve injury is a real common trauma affecting more than 300,000 people every year in Europe. Autologous nerve graft (autograft) is the gold standard in surgery, but it can cause loss of sensation and scarring at the donor site. Artificial conduits aim to overcome the drawbacks of nerve autografts. Polycaprolactone (PCL) has shown good physical and biocompatible properties to support nerve regeneration after injury. In this study, autografts were compared to PCL conduits in a 18 weeks in vivo experiment. Axonal myelination and regeneration were analysed by immunohistochemistry, and no significant differences were observed between the two groups. After a morphometric analysis of muscles, the results obtained for muscle fibre area or diameter were also comparable between the autografts and the PCL conduits. However, the wet muscle weight of the medial gastrocnemius muscle supplied by the nerve repaired with autograft was significantly heavier than the wet weight of medial gastrocnemius muscle supplied by the nerve repaired with PCL nerve conduit. Finally, the skin reinnervation was analysed and no statistical difference was observed. After 18 weeks, the artificial PCL conduits did not show degradation, demonstrating the ability of supporting axonal regeneration over long periods of time. PCL conduits could be therefore a valid alternative to autologous nerve grafts for peripheral nerve repair.

Keywords

Peripheral nerve repair; autograft; conduit; polycaprolactone; biodegradable polymer.

1. Introduction

The management of peripheral nerve gap following injury is a surgical challenge. The gold standard in clinical practice for surgical repair of a peripheral nerve gap remains an autologous nerve graft [1;2]. However, the use of autologous nerve graft confers morbidity such as loss of sensation and scarring at the donor site, can be time-
consuming and a technical challenge for the surgeon, is a finite resource, and nerve size mismatch has to be overcome by using multiple lengths of nerve graft within the defect. The functional recovery of this reconstruction remains deficient and these injuries can have a profound and permanent impact on the patient and their ability to perform activities of daily living as well as preventing return to work [3;4]. Poor outcomes may be a direct consequence of slow, insufficient and misdirected axonal outgrowth at the site of injury [5;6]. Whilst innovative experimental strategies have been employed to engineer a synthetic nerve conduit to bridge the defect, nothing yet has proven good enough to be used routinely in clinical practice [7-9].

Recently, a novel synthetic conduit made of poly-ε-caprolactone (PCL) has demonstrated experimental promise [10;11]. This material possesses many features that would suggest an ideal nerve conduit; in particular it is biodegradable, flexible with sufficient strength for clinical handling, sterilizable, non-toxic, non-immunogenic, and may incorporate supportive cells into its structure. In short-term studies, excellent nerve regeneration has been demonstrated with the use of PCL conduit to bridge a 1cm nerve gap such that a large volume of axons consistently bridged the 1cm gap at only 2 weeks post-injury [12]. In order to understand the potential for clinical translation of this bioengineered conduit, it is crucial to demonstrate long-term outcomes such as re-innervation of end organ skin and muscle and compare this to the current clinical gold-standard. This study sought to test the long-term regeneration of an in vivo peripheral nerve injury when repaired with a PCL nerve conduit as compared to an autologous graft across a 1cm nerve gap in a rat sciatic nerve injury model.
2. Materials & Methods

2.1 Experimental design
We compared experimental peripheral nerve repair of a 1cm gap using empty PCL conduit versus autologous nerve graft. Outcome measures compared were axonal counts and myelination in the conduit or autograft and in the distal stump; end organ muscle fibre size and weights; end organ skin re-innervation.

2.2 Preparation of PCL conduits
Poly ε-caprolactone (PCL), conduits were prepared as previously described [10]. Briefly, 3% (w/v) of PCL (Sigma Aldrich, US) was dissolved in dichloromethane (Fisher Scientific, UK) and spread evenly on degreased borosilicate glass cover slip. Following complete solvent evaporation, the films were treated in 10N NaOH for 1 hour with gentle shaking and washed in distilled H2O. Films were cut into rectangular sheets and rolled around a 16G intravenous cannula (Abbocath®, Abbott Ireland, Republic of Ireland), which standardized the internal diameter of the conduits at 1.6mm, more than 1.5 times the diameter of rat sciatic nerve, thus allowing space for post-injury swelling. Conduits were sealed by controlled heating while still mounted on the cannula and then sterilized using UV radiation.

2.3 Surgical procedures and groups
The animal care and experimental procedures were performed in accordance with the terms of the United Kingdom Animals (Scientific Procedures) Act 1986 and the number of animals used was kept to a minimum. Surgical procedures were performed under isofluorane general anesthesia on young adult male Sprague-Dawley rats (180g-220g). All animals underwent sciatic nerve transection and a 10mm nerve gap was created at the level of the mid-femur. In the autologous nerve graft group (n=5) under microscope vision the resected 10 mm of sciatic nerve was reversed such that the distal end was
sutured to the proximal nerve stump and the proximal end to the distal stump; in the PCL conduit repair group (n=5), the proximal and distal nerve stumps of the transected nerve were secured 2mm within the PCL conduit using 4 interrupted 9-0 Ethilon epineurial sutures (Ethicon, US). The conduit had been cut to 14mm length in order to maintain a 10mm gap between the nerve stumps. The wound was closed in layers and post-operative analgesia was given as 4µg buprenorphine intramuscularly. The animals were caged in a temperature and humidity controlled room with a 12-hour light/dark cycle, and food and water provided immediately. After 18 weeks survival, animals were deeply anaesthetized with isoflurane and perfused transcardially with 0.9% heparinized saline (0.9% NaCl, 50 units/ml heparin) followed by 4% paraformaldehyde in 0.1M phosphate buffer solution (PBS). The sciatic nerves including PCL conduit were harvested and post-fixed in 4% paraformaldehyde before being frozen in OCT embedding medium (VWR, UK) and stored at -40°C. For axon counts, 2-3mm long sciatic nerve specimens were excised at 3mm distance from the proximal and distal nerve graft/conduit interfaces. These nerve segments were fixed in 3% glutaraldehyde, post-fixed in 1% osmium tetroxide (OsO4) in 0.1M cacodylate buffer (pH7.4), dehydrated in acetone and embedded in Vestopal. Both ipsilateral and contralateral medial gastrocnemius muscles were weighed and then frozen in OCT using isopentane cooled with liquid nitrogen and foot pad skin was post-fixed in Zamboni’s solution before being frozen in OCT embedding medium (VWR, UK) and stored at -40°C.

2.4 Axon counts

Semi-thin transverse sections of proximal and distal nerve segments were cut on a 2128 Ultratome (LKB, Sweden) and counterstained with Toluidine Blue. Myelinated axons in the these segments were counted at 1000x final magnification using the fractionator probe in Stereo Investigator™ 6 software (MicroBrightField, Inc.,USA).
2.5 Axonal regeneration

Random longitudinal nerve sections were cut with a thickness of 14µm and collected onto glass slides coated with Vectabond (Vector, Peterborough, UK). The sections were permeabilized in 0.2% (v/v) Triton-X in PBS and washed twice in PBS for 5 minutes. They were then blocked with normal goat serum (Sigma, UK; 1:100 dilution) and the primary antibody (protein gene product 9.5, PGP 9.5, Dako, UK; 1:1000) was applied overnight. The following day, the sections were washed three times in PBS and a fluorescein goat anti-rabbit conjugated secondary antibody (Vector Laboratories, UK; 1:100) was applied. The sections were finally washed carefully in PBS and mounted on glass slides with Vectashield. After staining, each section was examined by fluorescence microscopy using a 20x objective. Measurements were carried out at 5mm from the proximal stump and images were captured using an Olympus BX60 inverted fluorescence microscope and a monochrome camera (Evolution QEi, MediaCybernetics, Bethesda, USA). The immunostaining area was then quantified using Image Pro Plus Imaging Software (Media Cybernetics). Images were converted into grayscale and thresholded. The immunostaining area was finally evaluated automatically by the software and expressed as µm$^2$. The final staining area was expressed as the ratio between the staining area and the total frame area.

2.6 Muscle analysis

Sixteen micron transverse sections of gastrocnemius muscles from the contra-lateral and operated sides were cut on a cryostat and samples were then fixed in 4% (w/v) paraformaldehyde for 15 min at room temperature, washed briefly in phosphate buffered saline (PBS) and permeabilized in 0.1% (v/v) Triton X-100 in PBS for 30 min. Samples were blocked with normal serum and then incubated with monoclonal primary antibodies raised against fast and slow myosin heavy chain protein (NCL-MHCf and
NCL-MHCs, Novocastra, Peterborough, UK; both 1:20) for 2 h at room temperature. Each slide was also co-incubated with rabbit anti-laminin antibody (Sigma, Poole, UK; 1:200). After rinsing in phosphate-buffered solution, secondary goat anti-rabbit and goat anti-mouse antibodies Alexa Fluor 488 and Alexa Fluor 568 (Invitrogen, The Netherlands; 1:100) were applied for 1 h at room temperature in the dark. The slides were cover-slipped with Prolong anti-fade mounting medium containing 4’-6-diamidino-2-phenylindole (DAPI; Invitrogen). Morphometric analysis of muscle sections was performed in a blind manner using randomly assigned codes as previously described [13]. Five random fields were chosen (using the x16 microscope objective) and images for the immunolocalisation of each myosin heavy chain type plus that for laminin were captured using the appropriate emission filters, and combined to provide dual-labelled images. Each image contained at least 25 individual muscle fibers for analysis. Image-Pro Plus software was calibrated to calculate the mean area and diameter (in µm) for each muscle. The injured side was expressed relative to the contralateral control side and the relative mean % ± SEM calculated for each group.

2.7 Skin re-innervation

Systematic random samples of 15µm cryosections from ipsilateral foot pad skin were collected onto glass slides coated with Vectabond. Sections were permeabilized in 0.2% Triton-X (BDH Laboratory Supplies, Bristol, UK), and stained by indirect immunohistochemistry using primary antisera against polyclonal rabbit antisera for protein gene product 9.5 (PGP 9.5; 1:1000) and polyclonal rabbit calcitonin gene-related peptide (CGRP; Sigma, UK; 1:1000). PGP antibodies identify all nerve fibre types whilst CGRP antibodies are specific for C and Aδ sensory fibres. Staining was visualised using a fluorescein isothiocyanate-conjugated secondary antibody (conjugated goat anti-rabbit serum; 1:100). After staining, each section was examined.
by fluorescence microscopy using a 20x objective. For each antibody, a random sample of six visual fields, including epidermis and dermis was captured for analysis using an Olympus BX60 inverted fluorescence microscope and images acquired using a monochrome camera. The immunostaining area in each captured image was quantified by image analysis using Image-Pro Plus Imaging software as previously described [14]. First the colour immunofluorescent image was converted into greyscale; then the epidermis and dermis was outlined by tracing its margin. An intensity threshold was applied to differentiate bright immunostaining from any low-level background autofluorescence, and the immunostaining area was calculated automatically.

2.8 Statistics

GraphPad Prism 4© software (GraphPad Software, San Diego, CA, U.S.A.) was used to calculate statistics. An unpaired student t-test was used to compare the two experimental groups. All data was expressed as mean±S.E.M. A value of \( P<0.05 \) was considered to be statistically significant.

3. Results

No adverse events were encountered in the use of the PCL conduit in the 18 week survival period and there was no evidence of conduit degradation at this time point.

3.1 Axon counts

Specimens of the distal nerve were harvested 3mm from the graft/conduit nerve interface and contained numerous myelinated nerve fibers (Fig 1). There were not any marked differences in appearance and distribution of myelinated fibers between the autograft and PCL conduit experimental groups (Fig 1A) and quantitative analysis demonstrated that similar numbers of axons were found in the distal stumps of both groups (Fig 1B). Proximal stump axon counts were significantly higher for both groups.
In order to measure and compare the extent of axonal regeneration within the nerve repaired with conduit or autograft, the area highlighted by PGP immunohistochemical staining was expressed as a proportion of the area within the conduit or autograft (Fig 1C). This demonstrated that there was no significant difference between the volumes of regenerating axons within the nerve repaired with conduit and the nerve repaired with autograft.

3.2 Muscle fibre size and weights

The area and diameter of both fast and slow muscle fibre types was measured after immunohistochemical staining of the medial gastrocnemius muscle (Fig 2). There were no statistically significant differences between the autograft and the PCL groups for muscle fibre area or diameter. The wet muscle weight of the medial gastrocnemius muscle supplied by the nerve repaired with autograft was significantly heavier than the wet weight of medial gastrocnemius muscle supplied by the nerve repaired with PCL nerve conduit (60.24±2.6% and 48.58±3.1% of contralateral sides respectively, \( P<0.05 \)).

3.3 Skin re-innervation

Skin re-innervation was measured by CGRP and PGP immunohistochemical staining of the ipsilateral footpad skin (Fig 3). There was nerve regeneration into the dermis of all sections examined. There were no statistically significant differences between the autograft and the PCL groups for the immunostaining area of CGRP or PGP.

4. Discussion

Autologous nerve grafting remains the clinical gold standard for repair of a nerve gap; however, much promising work has been reported in the development of synthetic nerve conduits to fulfill this role. This study provides evidence of comparable long-term histological outcomes in \textit{in vivo} nerve gap repair with autograft or a novel PCL nerve conduit; thereby stimulating further work on the potential use of this synthetic
biodegradable PCL nerve conduit in the clinical setting. In our previous in vivo study of this nerve conduit, we demonstrated that a large volume of axons consistently bridged a 1 cm rat sciatic nerve gap at only 2 weeks post-injury [12]. At 18 weeks there was a similar volume of regenerating axons within the nerve autograft and PCL conduit repair groups, and similar numbers of myelinated axons in the distal stump of both groups. Furthermore, there was evidence of comparable re-innervation of end organ muscle and skin with the only significant difference the lower wet weight of the muscle from the PCL conduit nerve repair group. Given that there is a similar muscle fibre size and number of myelinated axons between the two groups, the mechanism of a different muscle weight is unclear; however, the long survival period in this study may conceal differences in aspects of regeneration that could have been detected at earlier time points, and may be more critical in a human model [15]. However, these results are favourable when compared with recent studies examining collagen and fibrin nerve conduits versus autologous nerve grafting [16;17]. After 18 weeks survival, there were no adverse incidents and the PCL conduit remained intact with no evidence of degradation. This indicates that the conduit could provide support for axonal regeneration over longer distances and longer periods of time. This is useful because regeneration is slower in humans than in rats and a nerve conduit may need to provide support for many months of nerve guidance.

It is clear that further experimental studies and innovation will be required before a bioengineered conduit demonstrates superiority to an autologous nerve graft. This may be realised with the advent of stem cell integration within conduits and the manipulation of the conduit lumen to bear physical or chemical cues in order to aid axonal regeneration [11;18;19]. Features such as this will attempt to recreate the cellular and
molecular ‘regenerative milieu’ of an autologous nerve graft within a biomaterial nerve conduit.

References


Fig 1. Myelination morphology is very similar between the autograft and PCL conduit nerve repair groups (A, Scale=20µm). Myelinated axon counts demonstrated similar numbers in the distal stumps of both groups 7729±1308 and 6414±1544 axons in autograft and conduit respectively (B). The proportion of regenerating axons within the autograft (0.48±0.02) and PCL nerve conduit (0.52±0.03) were not significantly different (C).
Fig 2. Immunohistochemistry of fast and slow muscle fibres from the medial gastrocnemius muscle demonstrating similar sizes of muscle fibre (A, Scale=50µm). The area (B) and diameter (C) of fast and slow muscle fibre types was expressed as a percentage of the contralateral side and did not demonstrate a significant difference between the autograft and the PCL nerve conduit repair groups. Fast muscle fibre area was 84.0±3.3% in the autograft group and 72.6±7.0% in the PCL conduit group; slow muscle fibre area was 71.2±7.0% in the autograft group and 76.5±7.8% in the PCL conduit group. Fast muscle fibre diameter was 84.8±1.6% in the autograft group and 77.8±5.7% in the PCL conduit group; slow muscle fibre diameter was 74.3±3.6% in the autograft group and 78.0±4.7% in the PCL conduit group.
Fig 3. PGP and CGRP immunohistochemistry demonstrating nerve regeneration into dermis of ipsilateral foot pad skin 18 weeks after sciatic nerve repair with autograft or PCL conduit (A). PGP immunostaining (B) was present in 0.29±0.07% of the skin area in the autograft group compared to 0.35±0.05% of the skin area in the PCL conduit group. CGRP immunostaining (C) was present in 0.16±0.03% of the skin area in the autograft group compared to 0.11±0.02% in the PCL conduit group.
4. Paper II

*Chemical surface modification of poly-ε-caprolactone improves Schwann cell proliferation for peripheral nerve repair*

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Chemical surface modification of poly-ε-caprolactone improves Schwann cell proliferation for peripheral nerve repair

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Abstract

Poly-ε-caprolactone (PCL) is a biodegradable and biocompatible polymer used in tissue engineering for various clinical applications. Schwann cells (SCs) play an important role in nerve regeneration and repair. SCs attach and proliferate on PCL films but cellular responses are weak due to the hydrophobicity and neutrality of PCL. In this study, PCL films were hydrolysed and aminolysed to modify the surface with different functional groups and improve hydrophilicity. Hydrolysed films showed a significant increase in hydrophilicity while maintaining surface topography. A significant decrease in mechanical properties was also observed in the case of aminolysis. In vitro tests with Schwann cells (SCs) were performed to assess film biocompatibility. A short-time experiment showed improved cell attachment on modified films, in particular when amino groups were present on the material surface. Cell proliferation significantly increased when both treatments were performed, indicating that surface treatments are necessary for SC response. It was also demonstrated that cell morphology was influenced by physico-chemical surface properties. PCL can be used to make artificial conduits and chemical modification of the inner lumen improves biocompatibility Copyright © 2012 John Wiley & Sons, Ltd.

Keywords polycaprolactone; surface modification; wettability; biocompatibility; Schwann cells; nerve regeneration

1. Introduction

Schwann cells (SCs) play an important role in nerve regeneration and repair. Their basal lamina are responsible for the myelin sheath that enwraps axons. In addition, they can release neurotrophic factors and cellular adhesion molecules that have been known seen to support the regenerative process (Hall, 1997; Wiberg and Terenghi, 2003). After nerve transaction or crush, the phenotype and morphology of SCs change throughout the nerve and two different segments are generated (Hall, 1997). Macrophages and monocytes are involved to phagocyte all axon and myelin debris and together, SCs release neurotrophic factors, encouraging axon outgrowth (Schmidt and Leach, 2003). It was demonstrated that regeneration was possible for interstump gaps shorter than 1 cm even though functionality was not assured. Gaps longer than 5 cm are hard to repair after injury; therefore, artificial nerve conduits were studied to provide a clinical solution (Hall, 1997).

The biocompatibility of a material is influenced by several factors. The method by which materials are made can be controlled and modulated through processing parameters to optimise the biomaterial for a particular tissue application (Schmidt and Leach, 2003). Cell-material interactions are related to surface microstructure (von der Mark et al., 2009). Scaffold surface area, porosity and interconnectivity of the pores affect cell migration and division, as well as the transport of nutrients and metabolic products (Fisher et al., 2007). Permeability, wettability and ionic charge are all properties to consider in the design of
b biomaterials (Ikada, 1994; Lee et al., 1994; Tamada and Ikada, 1994; Gopal et al., 2007; Vleggeert-Lankamp et al., 2007). It was also found that samples with same wettabilities but different charged functional groups such as –COOH, CH₃OH, -CONH₂ and -CH₂NH₂ had a significant effect on cell adhesion, differentiation and proliferation (Lee et al., 1994; Lee et al., 2005). Scaffold permeability and porosity are important in the exchange of nutrients, growth factors, oxygen diffusion and waste products through the device (Vleggeert-Lankamp et al., 2007; de Ruiter et al., 2009).

Aliphatic polyesters are frequently used as synthetic materials in tissue engineering (Pit et al., 1981). Specifically, polycaprolactone (PCL) is a biodegradable aliphatic linear polyester with semi-crystalline properties (Fisher et al., 2007). It is biocompatible and bioresorbable and characterised by a glass transition temperature (Tg) at ~60 °C and a low melting point (Tm) at ~60 °C (Van de Velde and Kiekens, 2002). One of the most important chemical properties of PCL is its degradability in physiological environments (at 37 °C) in vivo (Pit et al., 1981; Ali et al., 1993). PCL can undergo hydrolytic degradation by bulk or surface mechanisms with cleavage of the ester linkages and formation of monomeric caproic acid (Fisher et al., 2007). Monomeric and polymeric products are then metabolised via tricarboxylic acid (TCA) cycle or eliminated by direct renal excretion (Kweon et al., 2003). Previous studies showed that PCL films provide surface pores/pits with homogeneous pore distribution (Tang et al., 2004; Sun and Downes, 2007). In particular, Khor et al. demonstrated that PCL cast films allowed human keratinocytes to attach and proliferate on the surface (Khor et al., 2002). Biocompatibility studies demonstrated that PCL films supported neuroglioma NG108-15 cell growth and allowed their differentiation (Sun and Downes, 2007). However, SC adhesion and proliferation was still poor and surface treatments of PCL films were necessary to improve their biocompatibility. On one hand, hydrolysis using a sodium hydroxide (NaOH) is one of the most useful methods to provide a significant improvement in cell attachment and proliferation (Lee and Gardella, 2000; Ang et al., 2006; Chong et al., 2006; Ng et al., 2007; Tan and Teoh, 2007; Sun and Downes, 2009; Yeo et al., 2010). Hydrolysis also leads to the formation of carboxylic groups by cleaving the ester bond of PCL. On the other hand, aminolysis is a very fast reaction used for the formation of amino groups on polyester surfaces with minimal degradation (Zhu et al., 2002; Croll et al., 2004; Santiago et al., 2006). Hydrolysis and aminolysis where used in the present study to modify the surface of PCL films. The effects of hydrophilicity and surface chemistry on SC responses were investigated to improve their attachment and proliferation on the biomaterial, which will be used for the future formation of artificial conduits for nerve repair.

2. Materials and methods

2.1. Materials

All chemicals and reagents were purchased from Sigma-Aldrich Ltd. (Dorset, UK) unless otherwise specified.

2.2. PCL film preparation

PCL pellets (Mn ~ 70 000–90 000) were dissolved in dichloromethane (DCM) (Fisher Scientific, Leicestershire, UK) to a final concentration of 3% wt/v (Sun and Downes, 2007). Once PCL was totally dissolved, 300 μl of solution was casted on a glass coverslip (18 x 18 mm²) on an open bench at room temperature, allowing solvent evaporation overnight.

2.3. Surface treatments

Three different solutions were prepared to evaluate the effect of the treatment on the polymer surface. NaOH and KOH solutions (10 M; pH = 12) were prepared and samples were then soaked in either of these solutions for 1 h and shaken gently at room temperature. Finally, they were rinsed twice with distilled water to return the pH to neutral (pH = 7). The aminolysis reaction was conducted by soaking the films in hexamethylenediamine (HMD)/2-propanol (CHROMASOLV™ for HPLC) 10% wt/v (pH = 7) (Sigma-Aldrich). This reaction was conducted for only 1 h at room temperature to keep time and temperature constant for all three different treatments. Finally, films were carefully rinsed in distilled water to wash off HMD residue on the surface. After hydrolysis, PCL films were characterised by the formation of carboxylic and hydroxyl groups on the surface (PCL-OH) due to the polyester chains scission. Aminolysed-PCL films were instead characterised by the formation of an amide binding and free amino and hydroxyl groups on the surface (PCL-NH₂) (Figure 1).

2.4. Material characterisation

2.4.1. Ninhydrin Assay for amino group detection

Ninhydrin assay is a colorimetric assay that allows amino group detection and was used to confirm the effectiveness of aminolysis reaction. First, a solution of ninhydrin in ethanol 1.0 M was prepared. Untreated and aminolysed-PCL films were then cut into small pieces (6 x 18 mm²) and placed into small vials (n = 3) where 100 μl of ninhydrin solution was then added. Vials were warmed gently at ~70°C in a water bath and 500 μl of DCM was added to dissolve the film. Finally, 500 μl of IPA was added into...
each vial and absorbance was read at 562 nm by using a microplate reader (Multiskan Ascent V1.22).

2.4.2. Fourier Transform Infrared spectroscopy (FTIR)

FT-IR analysis was conducted using a Nicolet 5700 FT-IR spectrophotometer (Thermo Electron Corporation, OH, USA) equipped with Smart Orbit™ accessory. Data were collected in a range of 400 – 4000 cm⁻¹ at 4 cm⁻¹ resolution with 32 scans.

2.4.3. Topographical analysis: scanning electron microscopy (SEM)

To investigate the topography of treated and untreated PCL films, they were mounted on aluminium stub, gold sputter coated (Edwards, Sussex, UK) and analysed using SEM (VPSEM, Zeiss EVO60; Carl Zeiss Ltd, Hertfordshire, UK). Images were captured at different magnifications. The dimension of micropits on PCL film surfaces was quantified through ImageJ software (National Institutes of Health, MN, USA) (n = 5).

2.4.4. Contact angle measurements

Wettability tests on PCL, PCL-OH and PCL-NH₂ were carried out by the contact angle analyser DSA100 (Krüss, Hamburg, Germany). First, four drops of 10 μl of distilled water were placed on each film surface (n = 5) and the sessile drop contact angle was evaluated using Krüss Drop Shape Analysis software (Krüss, Hamburg, Germany). The advancing and receding contact angles were evaluated by increasing/decreasing the water droplet size by 5 μl each time and measuring the contact angle as previously described (n = 5). Gravity effects were not counted for drop sizes ≥ 10 μl as reported by Bachmann et al. (Bachmann et al., 2000).

2.4.5. Mechanical testing

To measure the mechanical properties of the films, longer films (76 x 26 mm²; 1830 μl) were prepared and cut in a dumbbell shape (width = 3.8mm; gauge length = 20 mm). Thicknesses were measured by using an electronic digital micrometer (Farnell, UK). Each sample was mounted on a table tensile testing machine (Instron Corporation, Bucks, UK) using a grip distance of 35 mm and a full-scale load range of 10 N. The crosshead speed was set up at 50 mm/min. During all tests (n = 5), humidity and temperature were kept constant respectively at (50 ± 5)% and (23 ± 1)°C. Data were analyzed using the Instron Series IX Automated Materials Testing System (Instron) and Kaleidograph software (version 4.0, Synergy software; Reading, PA, USA) giving the maximum load, tensile strength (TS), yield stress (YS) and Young's modulus (E).

2.5. In vitro cell tests

2.5.1. Schwann cell harvest and culture

All experiments involving animals were carried out in accordance with the U.K. Animals Scientific Procedures Act, 1986. The sciatic nerves of the rats were harvested from adult Sprague–Dawley rats. Briefly, they were desheathed, sectioned and incubated for 2 weeks in SCs medium consisting of Dulbecco’s Minimum Eagle’s Medium (DMEM), 10% (v/v) fetal bovine serum (FBS, Invitrogen, UK), 1% (v/v) penicillin-streptomycin solution (Invitrogen, UK) and 14 μM forskolin and 63 ng/ml glial growth factor-2 (GGF-2; Acorda Therapeutics, NY, USA). Tissues were digested with collagenase and dispase for 24 h, filtered and centrifuged. The pellet was resuspended in SC medium and cells were plated on poly-D-lysine coated flasks. Once confluent, for purification of SCs, cells were trypsinized and resuspended in medium containing 1:500 anti-Thy 1 (Serotec, Oxford, UK) and incubated for 10 min at 37°C and a further 30 min after 250 μl rabbit anti-mouse complement (Serotec, Oxford, UK) was added. Cells were centrifuged, replated onto new poly-D-lysine coated flasks and incubated at 37°C and 5% CO₂.

2.5.2. Cell attachment

Prior to cell seeding, scaffolds were mounted on plastic supports (Scaffdex, Finland) and transferred into 12-well plates (Corning Inc., Corning, NY) prior to sterilisation overnight under UV light. Schwann cells were trypsinized and seeded at a density of 10 000 cells/film in a volume of 200 μl. Samples were incubated at 37°C in a 5% CO₂ humidified atmosphere for 2, 4 and 6 h. After each time point, the medium was removed and cells fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature. Scaffolds were then washed twice in PBS and mounted on glass slides with Vectashield containing DAPI (Vector Labs, Peterborough, UK) to visualise the nuclei. Six random images at 10x were captured across the seeded area of each film with a BX60 fluorescence microscope (Olympus, Japan). Cell nuclei were finally counted with ImageJ and the percentage of seeded cells calculated.

2.5.3. Proliferation assessment

MTS assay was performed to assess Schwann cell proliferation. Cells were seeded at a density of 5000 cells/film on the surface of the scaffolds (n = 3) and incubated in their growth medium containing growth factors. After 1, 3 and 5 days, medium was aspirated and cells were incubated at 37°C for 4 h with MTS solution (20% MTS in DMEM without phenol red). Absorbance was recorded at 490 nm and 650 nm with a Biochrom Asys UVM 340 microplate reader (Biochrom Ltd., UK). Data were expressed as a percentage of the values detected in controls (untreated PCL films).

DNA assay was also performed to quantify the number of cells on the film surface. After seeding the cells (5000 cells/film) (n = 3), they were incubated for 1, 3 and 5
days in their growth medium containing growth factors. At each time point, cells were washed in PBS, covered with distilled water and frozen at −80°C. Cells were froze-thawed three times and aliquots of 50 µl (in triplicate) were moved into a clear flat bottom 96-well plate. Fifty µl of TNE buffer (10 mM Tris, 2M NaCl, 1 mM EDTA, pH 7.4) and 100 µl of Hoechst stain solution (stock solution: 1mg/ml Hoechst 33258 in water; dilution 1:50 in TNE buffer) were added to each well. Fluorescence was recorded at 355 nm excitation and 460 nm emission with a fluorescence plate reader (FLUOstar Optima). Data were expressed as number of cells evaluated through a standard curve obtained with known cell densities.

### 2.5.4. Immunocytochemistry

Five-thousand Schwann cells were seeded on the surface of each film and maintained in their culture medium with growth factors for 5 days. Medium was then aspirated and the cells were carefully fixed in 4% PFA for 20 min at room temperature. Cells were washed three times in PBS for 5 min, permeabilized in 0.2% Triton-X in PBS for 5 min at room temperature and washed again three times in PBS for 5 min. Non-specific antigens were blocked with 1% (w/v) bovine serum albumin (BSA) for 1 h at room temperature. A primary antibody (Mouse Anti-S100 Antibody; 1:500) was applied overnight at 4°C. Next, samples were washed three times in PBS for 5 min, incubated with a secondary antibody (Cy3 Donkey Anti-Mouse IgG Antibody, Jackson Immunoresearch Labs; 1:100) for 1 h at room temperature in the dark and washed again three times in PBS for 5 min. Films were finally mounted on glass slides with Vectashield containing DAPI. Images were acquired using a fluorescence microscope (Olympus BX60).

### 2.5.5. SEM fixation

Five-thousand Schwann cells were seeded on the surface of each film and maintained in their culture medium with growth factors for 5 days. Medium was then aspirated and the cells were carefully fixed in 1.5% glutaraldehyde for 30 min at 4°C. Cells were washed twice in PBS and dehydrated through a series of ethanol gradients (50%, 70%, 90% and 100%). Finally, cells were immersed twice in hexamethyldisilazane (HMDS, Molekula, UK) for 5 min at room temperature. Samples were left overnight under the hood to allow complete evaporation of HMDS and mounted for SEM analysis.

### 2.6. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical significance for material characterisation studies was estimated by one-way ANOVA test in Prism 5 (GraphPad Software, Version 5.03). In addition, Dunnett’s post test was chosen to compare groups. Cell attachment and proliferation data were analysed by two-way ANOVA test and Bonferroni’s post test was performed. Levels of significance were expressed as p values vs. controls (untreated PCL films) (*p < 0.05, **p < 0.01, ***p < 0.001).

### 3. Results

#### 3.1. Confirmation of surface modification and functional groups detection

Figure 2 shows the infrared spectra of untreated and treated PCL films in the region of 530 – 2000 cm⁻¹. The characteristic bands of PCL are well delineated in the first spectrum. Strong adsorption peaks were, indeed, expected at 1727 cm⁻¹ (C = O carbonyl stretching), 1293 cm⁻¹ (C–O and C–C stretching mode in the crystalline phase) and 1170 cm⁻¹ (C–O and C–C stretching mode in the amorphous phase) (Elzein et al., 2004; Khatiwala et al., 2008). NaOH treatment is well known to cause an effective hydrolysis reaction of PCL (confirmed through contact angle analyses). Finally, the aminolysed-PCL spectrum showed the formation of three new peaks at −3340 cm⁻¹ (not shown in the figure), 1640 cm⁻¹ and 1560 cm⁻¹, corresponding respectively to N–H stretching of NH₂ group, C = O stretching and N–H bending of amide group. These results are similar to the amide bands found by Causa et al. (Causa et al., 2010). The amide bind formation between PCL and HMD was also confirmed by Ninhydrin assay. As expected, the dye solution turned from yellow into purple and the absorbance was read at the microplate reader equal to 0.522 ± 0.061.

![Figure 2. FT-IR spectra of (a) untreated and treated PCL films (b – HMD treated; c – KOH treated; d – NaOH treated) in the region of 530 – 2000 cm⁻¹. At 1640 cm⁻¹ and 1560 cm⁻¹, the C = O stretching and N–H bending of the amide group were detected for the HMD treated (b). No differences were found between untreated and hydroxide treated films](image-url)
3.2. SEM analysis

PCL films were characterised by micropits spread homogeneously across the surfaces. Micropits were formed during solvent evaporation; they are important for cell attachment as already demonstrated in previous studies (Sun and Downes, 2007). After treatment, there was no significant degradation of the film surfaces as shown by SEM images (Figure 3) and the pit structure was maintained after all treatments. Chemical treatments affected only the chemistry without modifying the characteristic topography of the film surfaces.

3.3. Investigation of film hydrophilicity

Wettability of the PCL films increased significantly after hydrolysis treatments (Figure 4). The more hydrophilic films were those treated with NaOH (54.13 ± 2.73°) followed by those treated with KOH (56.83 ± 2.88°) (**p < 0.001). Hence, data were statistically significant compared to untreated PCL films (76.58 ± 1.25°). HMD treatment slightly decreased the sessile drop contact angle (*p < 0.05) (Table 1). A further analysis was performed to characterise the wettability of the films. Advancing and receding contact angles were evaluated for each surface. The difference between the advanced and receded contact angle was defined as hysteresis and it was possible to draw a loop as function of the drop volume. The hysteresis of the samples was evaluated, as related to both roughness and chemical heterogeneities (Sherratt et al., 2004). However, in this specific study, roughness did not affect the hysteresis since data were comparable and not statistically significant. Figure 5 did not show a closed hysteresis loop of the PCL films. Therefore, even though the material was chemically homogenous, the presence of the pits affected hysteresis. Since the treated materials were obviously chemically heterogeneous, they all showed a large contact angle hysteresis effect as expected. Furthermore, it is noteworthy that the very hydrophilic NaOH and KOH treated surfaces showed a partial water adsorption and it was not possible to evaluate the final receding contact angle (5 μl).

3.4. Evaluation of mechanical properties

Using a tensile machine the Young’s modulus (E), tensile Stress (TS) and yield stress (YS) were evaluated. All treatments affected PCL films as shown in Table 2. Specifically,
only aminolysis significantly reduced the Young’s modulus of PCL films (\(p < 0.05\)) from 105.50 ± 5.91 MPa to 69.60 ± 13.83 MPa, while samples were not affected after hydrolysis (102.30 ± 3.77 MPa and 108.90 ± 7.13 MPa). Even though hydroxide treated samples conserved their elasticity, the tensile stress, which is the maximum strength of a material, dropped in both cases (\(p < 0.05\)) from 19.80 ± 1.70 MPa to 12.71 ± 1.77 MPa and 12.81 ± 2.19 MPa, respectively. Aminolysed films were even more affected, reducing the tensile stress to 7.27 ± 0.78 MPa (***\(p < 0.001\)). A similar decreasing trend was observed for the yield stress, although not as dramatic. Only HMD treatment data were statistically significant (5.55 ± 0.78 MPa, **\(p < 0.01\)). In fact, the mechanical properties of aminolysed PCL films were reduced by ~30% compared to untreated and hydrolysed films.

### 3.5. Schwann cell attachment

A preliminary attachment test was carried out for 6 h to investigate the adhesion time of Schwann cells on untreated and treated films. SCs showed very low attachment on untreated materials (only 8% after 6 h incubation), which significantly improved after chemically treating the surface of the films. After 2 h, 21% of cells were completely attached on the aminolysed films compared to 7% on untreated PCL films (***\(p < 0.001\)). In addition, the hydroxide treatments increased cell adhesion up to 13% on KOH treated surfaces (*\(p < 0.05\)) and 11% on NaOH treated surfaces (Figure 6). After 4 h, 12% of cells were attached on hydrolysed surfaces (*\(p < 0.05\)) and a decrease of 17% of cell attachment was observed on

![Figure 5](image-url)
aminolysed surfaces (***p < 0.001). Unexpectedly after 6 h, the number of attached cells decreased on treated surfaces. Data were statistically significant only in the case of aminolysed PCL films (16%, **p < 0.01) but the percentage of attached cells dropped to 10% on hydrolysed surfaces.

3.6. Cell proliferation

SC proliferation was first assessed by MTS assay (Figure 7a), which demonstrated a significant increase after 3 days of culture when PCL films were treated (***p < 0.001). After 5 days of culture, cell proliferation was still higher on treated films compared to untreated PCL films. Interestingly, no differences were found between the two different treatments even when cell adhesion was higher on aminolysed films in the early attachment study. A similar trend was observed for the quantification of cells through DNA assay (Figure 7b). After 24 h culture, the number of cells evaluated on treated films was higher compared to untreated surfaces. The most significant increase in cell proliferation was observed after 3 days of culture, since all treatments showed a significant difference in cell number compared to untreated PCL (***p < 0.001 for NaOH and HMD treatment; **p < 0.01 for KOH treatment). After 5 days, HMD treated surfaces presented a higher number of cells (*p < 0.05).

3.7. Investigation of cell morphology

Cell morphology was investigated by immunocytochemistry and SEM analysis after 5 days of culture. As already demonstrated by proliferation studies, fewer cells were observed on microscopy and SCs were characterised by a different morphology, especially when a different functional group was present on the PCL film surface (Figures 8, 9). SCs were stained with phalloidin (green) and S100 (red) and analysed by fluorescence microscopy. Cells appeared better spread on treated surfaces (Figure 8h, lp) compared to untreated PCL films (Figure 8d). SEM analysis confirmed the difference in cell morphology. Particularly, SCs showed longer extensions, characteristic of their spindle shape, on hydrolysed surfaces (Figure 9b; red arrows highlight cell bodies). No difference in cell morphology was observed between NaOH and KOH treated films. Conversely, Figure 9c showed how SCs on a aminolysed surface presented a rounder body and cell-cell interaction seemed to be stronger compared to other materials. However, the characteristic spindle shape of SCs with long neural extension was conserved as shown in Figure 10.

Discussion

Surface treatments can be used to optimise the biocompatibility of materials in medical devices. It has been demonstrated that hydrophilicity and surface charge can affect cell behaviour (Lee et al., 2005). The hydrophilicity of the PCL polymer surfaces was affected by treatments with alkali (negative charge) and HMD (positive charge). To find the best outcome between different treatments, PCL solvent cast films were soaked in three different solutions (10M NaOH, 10M KOH, 10% HMD/IPA). Sodium hydroxide was used to introduce –COOH groups on the polymer surface (Ang et al., 2006; Choong et al., 2006; Ng et al., 2007; Tan and Teoh, 2007; Sun and Downes, 2009; Yeo et al., 2010). We also investigated...
the potassium hydroxide effect on film chemistry and structure. When a different cation (K⁺) was involved in the reaction instead of a Na⁺ ion, there was no difference observed. Lee et al. demonstrated that –NH₂ terminal groups on a polymer surface can improve cell adhesion (Lee et al., 2005) due to the interaction between NH₃⁺

Figure 8. SCs on PCL films were stained with S100 (red) and phalloidin (green). In addition, nuclei (blue) and the merged images are shown: a-d) PCL; e-h) NaOH treatment; i-l) KOH treatment; m-p) HMD treatment.

Figure 9. SEM analysis of SCs on: a) untreated PCL; b) hydrolysed and c) aminolysed films. SCs appeared better spread on hydrolysed surfaces, while they seemed to prefer cell-cell interaction on aminolysed films. Cell bodies are highlighted by red arrows.
of the molecular weight of the polymer chains. Despite the decrease in mechanical properties, the aminolysis treatment could help the degradation of PCL in vivo since a faster degradation rate is usually associated to shorter polymer chains.

*In vitro* tests using SCs confirmed the biocompatibility of PCL films. SCs are the most important cells in the peripheral nervous system since they are significantly involved in the process of nerve repair. After injury, they start to phagocyte myelin debris and secret chemokines to recruit macrophages (Schmidt and Leach, 2003; Vargas and Barres, 2007; Wei et al., 2009). They also support axon regrowth by producing neurotrophic factors during the regeneration process (Ide, 1996), which in turn are involved in neuronal activity, promoting nerve regeneration (Terenghi, 1999; Chen et al., 2007). Since gaps greater than 5cm are hard to repair, research is now focused on artificial conduits. However, since results are still poor, conduits could be preseeded with SCs to enhance axonal outgrowth and complete nerve regeneration. The ability of SCs to adhere and proliferate on different film surfaces could be related to both hydrophilicity and surface chemistry, which most influence cell response (Lee et al., 2003; Liu et al., 2007; Soria et al., 2007; Lensen et al., 2008). Accordingly, it was necessary to find a good compromise between these two factors to enhance the response of this particular kind of glial cells. SC adhesion was remarkably higher on aminolysed surface (c.a. 70.33 ± 0.87°), probably due to its functional groups. It well known that SC attachment on tissue culture plastic improves when surfaces are coated with poly-D/L-lysine, which forms positive charges on the substrate and the attachment of anionic receptors of SC membranes is promoted (Yavin and Yavin, 1974). In addition, Kim et al. demonstrated that wettability was not a significant factor for early cell adhesion (Kim et al., 2007). SC behaviour could be therefore attributed to the positive charge introduced by amino groups across the PCL film surface.

Regarding the proliferation of SCs on the films, treated surfaces significantly improved the cell response. This could be attributed to both surface chemistry and wettability. Most cells showed optimal attachment to surfaces that also allowed higher protein adsorption; important for cell adhesion, attachment and proliferation. Immunohistochemical studies confirmed the viability of cultured SCs on different surfaces since they expressed the typical glial marker S100. However, SC morphology varied on the different substrates. Specifically, carboxylic groups allowed more cell spreading. The cells adopted a more well-spread morphology, possibly due to the hydrophilicity of the surface. Conversely, the aminolysed film, characterised by higher contact angle and NH₂ decorated chemistry, allowed more cell-cell interactions with additional

**Figure 10.** SCs on aminolysed PCL film surface. Neural extensions were very well elongated, even though the cell body was bigger compared to hydrolysed surfaces. Cell bodies are highlighted by red arrows.
long extensions of the cells. Lee et al. demonstrated that PC-12 cells showed longer neurite formation when seeded on hydrophilic surfaces. Particularly, the longest neurites were observed on substrates characterised by a contact angle of 55° (Lee et al., 2003), close to the values obtained for hydroxylated PCL films (54.13 ± 2.73° NaOH treatment; 56.83 ± 2.88° KOH treatment). The different cell morphology could be therefore attributed to surface wettability. The present study demonstrated the importance of both hydrophilicity and surface chemistry as the main factors affecting cell responses. It could be therefore be asserted that surface treatments are necessary for adhesion and proliferation of SCs, which are highly involved in the process of nerve regeneration. Further studies will involve the use of treated PCL films to form artificial nerve conduits for peripheral nerve repair.

Conclusions

Cell interaction with polymer surfaces is one of the most important factors affecting cells in tissue engineering. In this study, we analysed the chemical, physical and biological properties of the material in detail. Hydrolysis and aminolysis were carried out on PCL films that altered the surface charge of the films without affecting topography. However, both treatments influenced material properties. Hydrolysis significantly affected hydrophilicity of the PCL films, improving their biocompatibility. On the other hand, aminolysis caused a decrease in the mechanical properties, which could be beneficial in terms of material degradation. In vitro tests demonstrated a dramatic increase of SCs attachment and proliferation on all modified PCL films compared to untreated films. Surface treatments also had a significant effect on cell morphology, allowing more cells spreading. Optimisation of these properties could lead to improved biomaterial design for peripheral nerve conduits.

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5. Paper III

**Immobilisation of cell-binding peptides on poly-$\varepsilon$-caprolactone film surface to biomimic the peripheral nervous system**

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Immobilization of cell-binding peptides on poly-\(\varepsilon\)-caprolactone film surface to biomimic the peripheral nervous system

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Abstract: Cell-material interactions are crucial for cell adhesion and proliferation on biomaterial surfaces. Immobilization of biomolecules leads to the formation of biomimetic substrates, improving cell response. We introduced RGD (Arg-Gly-Asp) sequences on poly-\(\varepsilon\)-caprolactone (PCL) film surfaces using thiol chemistry to enhance Schwann cell (SC) response. XPS elemental analysis indicated an estimate of 2–3% peptide functionalization on the PCL surface, comparable with carbodiimide chemistry. Contact angle was not remarkably reduced; hence, cell response was only affected by chemical cues on the film surface. Adhesion and proliferation of Schwann cells were enhanced after PCL modification. Particularly, RGD immobilization increased cell attachment up to 40% after 6 h of culture. It was demonstrated that SC morphology changed from round to very elongated shape when surface modification was carried out, with an increase in the length of cellular processes up to 50% after 5 days of culture. Finally RGD immobilization triggered the formation of focal adhesion related to higher cell spreading. In summary, this study provides a method for immobilization of biomolecules on PCL films to be used in peripheral nerve repair, as demonstrated by the enhanced response of Schwann cells. © 2012 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A:000–000, 2012.

Key Words: nerve regeneration, Schwann cell, surface modification, RGD peptide, polycaprolactone

INTRODUCTION

Peripheral nerve injury is one of the most common traumas, affecting over 300,000 people per year in Europe. 1 Despite autografts are the “gold standard” in peripheral nerve repair, there is a limited availability of graft material and nerve grafting is often related to neurological defect at the donor site. 2–4 The most common repair techniques reported in literature nowadays include grafting using natural materials and entubulization using artificial nerve conduits. 4

The ultimate approach in the fabrication of biomimetic scaffolds is the immobilization of particular extracellular matrix (ECM) moieties, such as fibronectin, laminin, or elastin, which are able to bind to specific integrins present on cell membrane. 5,6 However, proteins are difficult to purify, may degrade and they do not have the ability of being appropriately orientated on the biosurface. 5 Smaller amino acids sequences, like RGD, YIGSR, or IKVAV, are more stable than proteins and are able to elicit specific cell responses. 7 Lack of specific ligands on the surface does not allow strong adhesion nor migration. Conversely, an overconcentration of ligands could bring a very strong adhesion, resulting in slow migration of the cells. 8 Massia et al. evaluated a minimum peptide concentration of 1 fmol/cm² for cell spreading, while a higher concentration of 10 fmol/cm² can also guarantee formation of focal contacts and stress fibres. 5 However, these concentrations may change for different cell lines and biomaterial surface.

RGD (Arg-Gly-Asp) is one of the most studied and used peptide for biopolymer functionalization and can trigger different cell lines to adhere and proliferate on the surface. Half of the 24 known types of integrins are able to bind ECM molecules, such as \(\alpha\beta\)1, \(\alpha\varepsilon\beta_1\), \(\alpha\beta\)3, \(\alpha\varepsilon\beta_3\), \(\alpha\beta\)5, \(\alpha\varepsilon\beta_5\), \(\alpha\varepsilon\beta_6\), \(\alpha\beta\)1, and \(\alpha\varepsilon\beta_1\). Different techniques have been used to perform the peptide immobilization, such as surface-initiated atom transfer radical polymerization, 10 protonation with HCl, 11 pegylation, 12 glutaraldehyde, and carbodiimide chemistry. 13,14

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In previous work we have demonstrated that aminolysis of poly-e-caprolactone (PCL) is advantageous for cell attach-
ment. However, to improve on this film treatment in the present study we functionalized films with RGD sequences in order to improve Schwann cell (SC) response on the biomate-
rial surface. Peptides were immobilized through the carbodi-
mide (CDI) chemistry, known as “zero-length cross-linking reaction,” which is one of the most used reactions for mate-
rial functionalization. However, CDI chemistry requires the use of N-hydroxysuccinimide to avoid the formation of the unstable reactive O-aclylisourea derivative. We also used an innovative method based on the thiol (Th) chemistry to enhance the stability of the reaction for the peptide immobili-
zation on PCL films. Demonstration of the effectiveness of this reaction was given by in depth surface chemical char-
acterization using XPS, which provides a powerful way to monitor the chemical modification of a polymer surface with peptide functionalization. Schwann cell cultures were used to test the improvement of their attachment and proliferation following different treatments of PCL.

MATERIALS AND METHODS

All chemicals and reagents were purchased from Sigma-
Aldrich Ltd. (Dorset, UK) unless otherwise stated.

Film preparation

A PCL solution (3% wt/v) was prepared dissolving the polymer pellets ($M_n \sim 70,000–90,000$) in dichloromethane (DCM) (Fisher Scientific, Leicestershire, UK). The solution was cast on glass coverslips and the solvent was left to evaporate overnight at room temperature, leading to film formation as explained previously.

Films were then modified with two different methods in order to immobilize RGD sequences on the surface: carbodi-
imide (CDI) chemistry and thiol (Th) chemistry.

CDI chemistry [Fig. 1(a)]: PCL films were immersed in a 10% wt/v hexamethylenediamine (HMD)/2-propanol solution (pH 7) for 3 h at 40°C, stirring continuously. Amino-
lyzed films were then washed carefully in distilled water and left to dry overnight.

1-ethyl-3-(dimethylaminopropyl)carbodiimide (EDC) was dissolved in 2-N-morpholinoethanesulfonic acid (MES) buffer 0.1M (pH ~ 5.5) to increase its reactivity. An EDC so-
lution 0.1M was then applied on the aminolyzed PCL film and left to react for 3 h at room temperature. The previous solution was then removed and a new solution was prepared with tripeptide RGD and N-hydroxysuccinimide (NHS) in MES buffer 0.1M (pH ~ 5.5). According to Santiago et al., the final peptide concentration to spread on film sur-
face was 0.2 $\mu$g/mm². The peptide solution was left to react for 24 h at room temperature. Samples were finally washed in distilled water and dried.

Th chemistry [Fig. 1(b)]: PCL films were immersed in a 10% wt/v 2-chloroethylamine hydrochloride (CEA)/water solution (pH 9) for 3 h at 40°C, stirring continuously. Chlori-
nated films were then washed carefully in distilled water and left to dry overnight.

The pentapeptide RGDSC (Biomatik Corporation, Cam-
bridge, Canada) and tris(2-carboxyethyl)phosphine hydro-
chloride (TCEP) were dissolved in MES buffer 0.1M (pH ~
5.5) in a molar ratio of 1:5, to obtain again a final peptide concentration of 0.2 $\mu$g/mm² on the film surface. The solu-
tion was then applied on chlorinated films and left to react for 24 h at room temperature. Samples were finally washed in distilled water and dried.

Material characterization

X-ray photoelectron spectroscopy (XPS) investigation. XP spectra were recorded with a Kratos Axis Ultra instrument employing a monochromatic Al $K_\alpha$ source (1486.69 eV). Surveys were measured with 80 eV pass energy in steps of 0.25 eV, with 300 ms dwell time per data point. High resolution spectra were measured within the spectral range of interest (ca. 20 eV around core level emission peaks) with a 20 eV pass energy, 0.1 eV steps, and 300 ms dwell time per data point. For the functionalized films, the dwell time was increased up to 500 ms for C 1s and O 1s and 2000 ms for N 1s and S 2p spectra to increase the sensitivity of detection.

Analysis of the data was carried out with Casa XPS soft-
ware using a linear background and GL(30) line shape. The elemental compositions (relative atomic %) were calcul-
ated using an O 1s relative sensitivity factor (RSF) of 2.52 relative to C 1s. Surfaces were referenced to adventitious hydrocarbon contamination at 285 eV. Repeatability of the peak positions was ±0.1 eV. In the remaining text, when reporting binding energies for atoms in functional groups the atom referred to is underlined.

Scanning electron microscopy. The topography of untreated and modified PCL films was investigated through SEM (VPSEM, Zeiss EV060). Films were mounted on aluminum stubs and gold sputter coated (Edwards, Sussex, UK). Images were captured at different magnifications with an accelerating voltage of 5 kV.
Contact angle. The hydrophilicity of the different films was investigated using a contact angle analyser DSA100 (Krüss, Hamburg, Germany). The sessile drop test was carried out on four drops of 10 μL of distilled water per film (n = 5) and the contact angle evaluated using Krüss Drop Shape Analysis software (Krüss, Hamburg, Germany).

Investigation of the film biocompatibility

Schwann cell harvest and culture. All the experiments involving animals were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986. Rat sciatic nerves were harvested from adult Sprague-Dawley rats. They were desheathed, sectioned, and incubated for 2 weeks in a medium containing Dulbecco’s Minimum Eagle’s Medium (DMEM), 10% (v/v) foetal bovine serum (FBS, Invitrogen, UK), 1% (v/v) penicillin-streptomycin (Invitrogen, UK), 14 μM forskolin and 63 ng/ml glial growth factor-2 (GGF-2; Acorda Therapeutics, NY) (SC medium). The sectioned nerves were incubated with collagenase and dispase for 24 h for digestion. After filtering through a 100 μm cell strainer and centrifuging, the pellet was resuspended in SC medium and cultured for 5 days. Afterwards, the medium was aspirated and the cells were carefully fixed in 1.5% glutaraldehyde for 30 min at 4°C. Cells were washed in PBS twice prior to dehydration through a series of ethanol gradients (50, 70, 90, and 100%). Samples were then washed twice in hexamethyldisilazane (HMDS) for 5 min and left overnight under the hood to completely dry before mounting and gold sputtering for SEM analysis. Images were captured at different magnifications with an accelerating voltage of 5 kV.

Immunocytochemistry. The morphology and the ability of Schwann cells to form focal adhesion on treated and untreated PCL films were investigated through immunocytochemistry. Five thousand cells were seeded on each film and cultured for 5 days. Afterwards, the medium was aspirated and the cells were carefully fixed in 4% PFA for 20 min at room temperature. Cells were washed in PBS, permeabilized in 0.2% Triton-X/PBS and washed again in PBS. Non-specific antibodies were blocked with 1% (w/v) bovine serum albumin (BSA) for 1 h at room temperature. The primary antibody (Mouse Anti-S100 Antibody; 1:500 or Anti-Vinculin antibody (hVIN-1, Abcam, UK); 1:400) was applied overnight at 4°C. Samples were washed in PBS before the secondary antibody (Alexa Fluor 568 donkey anti-mouse IgG, Life Technologies, UK; 1:1000) was applied for 1 h at room temperature in the dark. Samples were washed again in PBS and incubated with phallolidin (Alexa Fluor 488, Life Technologies, UK; 1:40) for 20 min at room temperature in the dark. Films were finally washed again in PBS and mounted on glass slides with Vectashield containing DAPI. Images were acquired at different magnification using a fluorescence microscope (Olympus BX60).

Quantification of the length of cellular processes using S100 staining and the cell spreading expressed as cell area using Vinculin staining was performed with ImageJ software.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical significance for material characterisation studies was estimated by one-way ANOVA test in Prism 5 (GraphPad Software, Version 5.03). Also, Dunnett’s post test was chosen to compare groups. Cell work data were analysed by two-way ANOVA test and Bonferroni’s post test was performed. Levels of significance were expressed as p values versus controls (untreated PCL films) (*p < 0.05, **p < 0.01, ***p < 0.001).

RESULTS

Peptide immobilization on PCL films

XPS is a surface-sensitive technique, with probing depths of typically <5 nm for organic materials,29 that provides element-specific chemical state information.
Analysis of untreated PCL (Fig. 2) showed an elemental composition close to that expected from its known bulk composition (Table I). A carbon content slightly higher than expected arose from adventitious carbon contamination that is ubiquitous in any laboratory environment and a known feature of surface analysis of organic materials. The carbon 1s (C 1s) emission spectrum was deconvoluted into four peaks corresponding to increasingly electronegative chemical environments. These are (i) aliphatic C–C at 285 eV, (ii) carbon neighboring ester groups C–COO at 285.3 eV, (iii) C–O species at 286.5 eV, and (iv) ester groups O=CC=O at 289.0 eV (Fig. 2, Table II; note that the underlined element is the moiety responsible for the observed emission feature). Two photoemission peaks were observed in the O 1s spectrum, reflecting the decreasing electron density at the two ester oxygen environments, C–O at 532.1 eV and O–CC=O at 533.5 eV (Fig. 2).

Aminolysis of PCL through nucleophilic substitution at the ester bonds leads to formation of an amide bond (Fig. 1). The resulting chemical modification of the surface was observed by XPS through alteration in the elemental composition (Fig. 3, Table I), which became evident through additional carbon, nitrogen, and chlorine photoemission features in the spectra (Table II). The nitrogen 1s photoemission signal around 400 eV is characteristic of an amide bond (Fig. 3), comprising approximately two-thirds of the total nitrogen and chlorine intensity. Removing these contributions from the relative elemental composition led us to estimate that ~1.0 nitrogen atomic % was present on the polymer film (Table I).

Functionalization with a peptide leads to further chemical modification of the surface. Nucleophilic substitution of the chlorine of PCL-CEA with the thiol sulfur of the cysteine residue of RGDSC provided additional chemical indicators of reaction progress in the form of S presence and loss of Cl (Fig. 4, Table I). A significant increase in nitrogen resulted from attachment of the peptide (Table I), with photoemission at 400.1 eV representative of the additional amide peptide linkages and the arginine side-chain, while the slight asymmetry to high E0 values reflected the presence of NH3+ at the free arginine N-terminus. The sulfur photoemission around 160.5 eV (Fig. 4, Table II) arose from the C=S–C

![FIGURE 2. XPS elemental survey, C 1s, and O 1s spectra of PCL and peak assignments.](image)

### TABLE I. Relative Elemental Concentrations of the PCL Films from XPS

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>O</th>
<th>N</th>
<th>S</th>
<th>Cl</th>
<th>C/O</th>
<th>O/N</th>
<th>O/S</th>
</tr>
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<tr>
<td>PCL</td>
<td>77.7</td>
<td>22.3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>3.49</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PCL-CEA</td>
<td>79.9</td>
<td>18.1</td>
<td>1.0</td>
<td>1.0</td>
<td>4.41</td>
<td>18.10</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PCL-CEA-RGDSC</td>
<td>74.3</td>
<td>23.4</td>
<td>2.0</td>
<td>0.3</td>
<td>3.18</td>
<td>11.70</td>
<td>78.00</td>
<td></td>
</tr>
</tbody>
</table>

* Unreacted CEA values removed.

Since every CEA attachment leads to the hydrolysis of an ester group according to:

\[ O = C - O - O = C - N + C - OH \]

and chemical shifts from X–C=O or X–C–O oxygens with different substituents X are generally too close to be resolved. There was some signal arising from unreacted CEA hydrochloride (NH3+ and Cl–, Fig. 3), comprising approximately two-thirds of the total nitrogen and chlorine intensity. Removal of these contributions from the relative elemental composition led us to estimate that ~1.0 nitrogen atomic % was present on the polymer film (Table I).

### TABLE II. XPS Chemical Shifts and Assignments of the Untreated and Treated PCL Films

<table>
<thead>
<tr>
<th></th>
<th>PCL</th>
<th>PCL-CEA</th>
<th>PCL-CEA-RGDSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>C–C</td>
<td>285.0</td>
<td>285.0</td>
<td>285.0</td>
</tr>
<tr>
<td>C–COO</td>
<td>285.3</td>
<td>285.4</td>
<td>285.4</td>
</tr>
<tr>
<td>C–N</td>
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<td>C–RHD(3/2,1/2)</td>
<td>–</td>
<td>168.3</td>
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</table>

De Luca et al. 4  IMMOBILIZATION OF CELL-BINDING PEPTIDES
FIGURE 3. XPS elemental survey, C 1s, N 1s, and Cl 2p spectra of PCL after aminolysis with CEA, showing the presence of amide nitrogen and covalently bound chlorine with attachment of CEA. The presence of some unreacted CEA is reflected in the N 1s and Cl 2p spectra from NH$_3^+$ and Cl$^-$.  

FIGURE 4. XPS elemental survey, O 1s, N 1s, and S 2p spectra of PCL-CEA after functionalization with RGDSC, showing the presence of nitrogen and sulfur specific to peptide immobilization on the PCL surface. There is some slight degradation/X-ray damage visible towards low binding energy in the O 1s spectrum.
linkage between PCL-CEA and the cysteine residue of RGDSC, while the absence of a chlorine signal indicated no unreacted CEA residues on PCL within the sensitivity of detection (0.1 atomic %, 1ppth). Increased intensity was also observed for amide-specific binding energies in the C1s and O1s spectra compared with PCL, along with the presence of C–N and COO− from the peptide.23 This was particularly noticeable for oxygen (Fig. 4).

Because nitrogen is not present in PCL, the relative atomic % of N found with XPS for the functionalized film (2.0 %, Table I) was used to estimate the number of peptide moieties attached per PCL monomer unit, and thereby the surface coverage. Assuming one RGDSC attaches to each chlorine terminus of PCL-CEA (suggested by the complete loss of chlorine signal with functionalization) we obtained an experimental O:N ratio of 11.7:1 (Table I) compared to the O:N ratio of 1.22:1 that would occur if every PCL ester group (monomer unit) was functionalized. Accounting for the number of oxygen and nitrogen atoms in PCL (–C6H10O2−) and -CEA-RGDSC (C20H31N9O9S) units, this corresponds to an estimate of 2.1 ± 0.4 covalently bound RGDSC moieties per 100 PCL monomer units (~2% coverage). As sulfur is specific to the peptide, the O:S ratio (Table I) could be used to independently estimate the coverage of the functionalized film: this results in 2.9 ±1.0 RGDSC per 100 PCL monomer units, in good agreement with the estimate from the O:N ratio.

XPS analysis was also performed on functionalized PCL films modified through CDI chemistry. Data (not shown) demonstrated the effectiveness of the reaction, as already found in previous work.13,14

Physical characterization of different treated films
The topography of PCL films was characterized by micropits spread across the surface [Fig. 5(a)], with diameter size ranging between 0.2 and 2 μm. Larger more dispersed pits were also detected on the surface, with a diameter up to 16.40 μm, as also found by Tse et al.24 PCL modification and peptide immobilization did not affect the micropitted structure of the films, as shown in Figure 5.

Material hydrophilicity was also investigated and water contact angle was measured for each type of surface. Aminolysed films caused an increase in hydrophilicity, as the contact angle dropped from 75.85 ± 0.54° to 65.62 ± 4.04° (*p < 0.05) (Table III). RGD immobilization did not affect material wettability, as the contact angle was stable after

<table>
<thead>
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<th>Sample</th>
<th>Contact Angle (°)</th>
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<tr>
<td>PCL</td>
<td>75.85 ± 0.54</td>
</tr>
<tr>
<td>HMD</td>
<td>65.62 ± 4.04*</td>
</tr>
<tr>
<td>CEA</td>
<td>69.94 ± 0.83</td>
</tr>
<tr>
<td>RGD</td>
<td>65.98 ± 0.95*</td>
</tr>
<tr>
<td>RGDSC</td>
<td>70.63 ± 2.30</td>
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</table>

Hydrophilicity was improved when samples were modified following CDI chemistry (*p < 0.05). Only slight reduction of the contact angle was observed in case of Th chemistry.
aminolysis (65.98 ± 0.95) was recorded after 6 h of culture. Samples which underwent CDI chemistry attached cells, up to 40% in the first 2 h of culture (**p < 0.01) and RGDSC functionalized films. However, no statistical significance was found.

Schwann cell attachment and proliferation

An early attachment test was carried out on untreated and treated PCL films in order to investigate the ability of the SCs to adhere on the different substrates. Cells showed a very low attachment on untreated surfaces, as demonstrated in Figure 6(a). Less than 10% of the seeded cells was attached after 2 h and no remarkable increase in adhesion was observed after 6 h of culture. Treatments were found to be necessary to enhance cell attachment. The preliminary modification of PCL with amino or chlorine groups increased the percentage of attached cells up to ~30% after 2 h (***p < 0.001). Peptide immobilization showed the higher number of attached cells, up to ~40% in the first 2 h of culture (***p < 0.001). An interesting and unexpected result was observed after 6 h of culture. In unmodified PCL, cells showed very short processes and poor adhesion to the substrate. After PCL modification with amino or chlorine groups on the film surface, a slight enhancement in cell elongation was observed. This was due to the presence of the new functional groups on the activated substrates, which are positively sensed by SCs. However, especially in presence of NH2 groups, cells showed a very large nuclear area compared with the cellular process size. A dramatic change in cell shape was observed after peptide immobilization. At 5 days of culture the cells perfectly adhered on the film surface, showing a very similar morphology to the SCs cultured on PdL-coated glass.25 SCs flattened on the functionalized films due to the presence of the immobilized peptides and the cell body was more stretched in the direction of the cellular processes. This was particularly observed on RGDSC functionalized films.

Cell morphology was investigated with SEM and fluorescence microscopy, and quantification of the length of the cellular processes was performed on S100 stained cells using ImageJ software [Fig. 8(a)]. As already mentioned, SCs cultured on untreated PCL films showed short elongation (processes length < 50 μm after 5 days of culture). Longer processes were observed after the introduction of NH2 and Cl groups on film surfaces. Cellular process length increased up to 50% after 5 days of culture on chlorinated films (89.21 ± 6.63 μm; *p < 0.05). Unexpectedly, no remarkable difference in SC process length was observed when the cells were cultured for 5 days on RGD-modified films compared to chlorinated films (81.37 ± 8.07 μm; *p < 0.05). The longest cellular processes of SCs were observed after the immobilization of RGDSC on PCL films, increasing their length up to 97.38 ± 20.19 μm (***p < 0.01).

Influence of peptides in the formation of focal adhesion of SCs

To assess the results of Vinculin staining in SCs, these were firstly compared with those of fibroblasts (results not shown), which are known to have several vinculin adhesion points. In contrast, SCs are generally characterized by lesser

FIGURE 6. Attachment and proliferation of SCs. Peptide immobilization remarkably improved cell response compared to untreated films. (a) Cell attachment was expressed as percentage of attached cells after seeding. All values vs. PCL control were statistically significant (**p < 0.05). (b) MTS assay performed on SCs over 6 days of culture. Absorbance values were normalized and expressed as percentage of the value recorded for untreated PCL after 24 h (*p < 0.05, ***p < 0.01, ****p < 0.001).
focal adhesion, with shorter focal adhesion plaques, especially distributed at the end of the cellular processes, as shown by Vinculin staining. As SCs have a characteristic "spindle" shape, they present a relatively large nuclear area with thin processes, flat at their end where the focal adhesion plaques are present.

As SCs showed a shorter and often rounder shape on untreated PCL films, a lack of focal adhesion was also noted [Fig. 9(b)]. In addition, the cell spreading was quantified using Vinculin staining and expressed as cell area [Fig. 8(b)]. SC area on PCL films was relatively small (923.166 ± 110.06 μm²) compared with all the other treatments. In presence of NH₂ and Cl functional groups the cells appeared more elongated with longer processes, but in each case they were characterized by very few small focal adhesion plaques as defined by the Vinculin staining [Fig. 9(c–f)]. This poor improvement in focal adhesion did not enhance cell spreading and cell area values did not show statistical significance (p > 0.05). The peptide immobilization on the film surface triggered the formation of focal adhesion plaques as shown in Figure 9(g–j). Vinculin was particularly expressed at the end of the cellular processes, where cells flatten and attach clearly to the surface. SCs cultured on films modified through the Thiol chemistry showed the highest number of focal adhesion plaques, which allowed better cellular adhesion. The cells were also characterized by the largest area when the Th method was applied compared to the CDI chemistry (1952.58 ± 315.71 μm² and 1609.98 ±
DISCUSSION

Previous work demonstrated that PCL is a suitable material for peripheral nerve repair.\textsuperscript{15,26–29} Particularly, PCL films fabricated by solvent casting are characterized by micro-pits which improve cell response.\textsuperscript{30,31} However, SCs have shown very poor attachment on PCL films and the possibility of improving their attachment was investigated using surface modification. Different reactions can lead to biomolecule immobilization on biomaterial surfaces, altering both chemical and physical properties. Spacers are often required in the covalent linkage of biomolecules, such as carbodiimides,\textsuperscript{14} gluteraldehyde,\textsuperscript{13} CNBr, or N-hydroxysulfosuccinimide,\textsuperscript{32} improving both steric freedom, and specific activity.\textsuperscript{33} However, the length of the spacer should be controlled as too long arms could reduce the cell attachment.

Peptides are characterized by high stability during sterilization, heat treatments and changes in pH.\textsuperscript{7} Peptide sequences are very selective in ECM binding, containing only single specific motif mediating cell-specific adhesion and function.\textsuperscript{7,14} In fact, different types of receptors are involved in signal recognition from the ECM, such as integrins.\textsuperscript{5,35,36}

In this study we immobilized RGD sequences on PCL film surface using an innovative method based on thiol chemistry and we compared it to the CDI chemistry, which is the most used reaction for materials functionalization. However, CDI chemistry may be at times variable and non-specific, as it firstly involves the activation of PCL with

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure8.png}
\caption{(a) Length of cellular processes evaluated on SCs stained with S100 after 24 h and 5 days of culture. (b) SC area evaluated on SCs stained with vinculin after 24 h and 5 days of culture. Levels of significance were expressed as $p$ values vs. untreated PCL ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).}
\end{figure}

123.04 $\mu m^2$, respectively, which could probably explain the highest and more stable attachment already shown in Fig. 6(a).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure9.png}
\caption{SCs on different PCL films, stained with vinculin (red), phalloidin (green), and DAPI (blue) (left column: merged images; right column: vinculin staining in gray scale; 100$\times$ mag.). (a,b) PCL; (c,d) HMD; (e,f) CEA; (g,h) RGD; (i,j) RGDSC. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]}\end{figure}
hexamethylenediamine, characterized by two free amino groups. This could cause the formation of "molecular bridge" when both the free –NH₂ react with the polymer surface, hence no functional group will be available for further peptide immobilization. Also, any carboxylic or guanidine group that may be present in the amino acid sequence will be able to react with the free –NH₂ on the aminolysed-PCL surface.³⁷ This will cause the activation of side reactions instead of the peptide-binding in the correct position. Finally, CDI chemistry requires the use of N-hydroxysuccinimide to avoid the formation of the unstable reactive O-acetyl-sourea derivative, which undergoes fast hydrolysis.¹⁶ The new immobilization reaction used in this study involves firstly PCL activation with chlorine groups via aminolysis. The reaction is performed in water, which is a less permeating solvent than isopropanol and it reduces polymer degradation. Afterwards no additional chemical is required for the peptide immobilization as a thiol-halogen "click" reaction takes place.³⁹–⁴¹ In fact, the functionalization mechanism is simply based on the very strong nucleophility of the sulfur atom present in the cysteine residue. Sulfur is less electronegative than oxygen and therefore it generates higher nucleophilicity, hence higher reactivity to halogens.⁶² The thiol chemistry is therefore very specific and straightforward, with no side reactions occurring.

XPS provided a powerful way to monitor the chemical modification of the polymer surface with peptide functionalization. For the immobilization of the peptide sequence via thiol chemistry, the nitrogen and sulfur signals acted as elemental indicators for successful attachment as well as evidence of changes in chemical environment, along with more subtle effects in the carbon and oxygen spectra. Comparison of the XPS elemental concentrations indicated an estimate of 2.8 ± 1.1% peptide functionalization on the PCL surface. As no remarkable changes in surface topography and hydrophilicity were observed, it could be asserted that the improvement in cell response was due to the modified surface chemistry after immobilization of cell-binding peptides.

Schwann cells are the most important cells in the peripheral nervous system. They are characterized by a spindle shape with a small cytoplasm to nucleus ratio⁴³ and poor or absent focal adhesion⁴⁴ when cultured on standard culture substrates in standard conditions. Their adhesion and proliferation was remarkably improved in presence of peptides in comparison to untreated PCL films. Particularly, SCs detach from PCL surface after a preliminary phase of attachment, as also demonstrated in previous work.¹⁵ This is possibly due to the hydrophobicity of the films (ca., 75.85 ± 0.54°) which also do not contain any functionality in the polymer backbone. SC low adhesion on PCL surface was also confirmed by lack of vinculin staining, which is the protein responsible for focal adhesion.⁴⁵ It is through focal adhesion points that cells are able to perceive the substrate to which they are attached and transmit force, acting like "mechanosensors".⁴⁶,⁴⁷ If cells perceive positive signals from the substrate, they are triggered in producing integrins and ECM proteins. Conversely, negative signals trigger the production of degrading proteases, which will cause cell detachment.⁴⁸ Even though cell adhesion was improved in the first 2 hours of culture on aminolysed PCL films, lack of focal adhesions and vinculin staining resulted in cell detachment after 6 h. Unexpectedly, the introduction of chlorine in the polymer background increased cell attachment and stimulated the formation of focal contacts, although RGD functionalized films showed the best performance. After peptide immobilization, SCs were characterized by a good spreading with the formation of visible focal adhesion plaques, and cell adhesion was particularly stabilized when modification was carried out via thiol chemistry. We could thereby state that focal adhesion formation and adhesion forces were well balanced as proliferation increased continuously over 5 days.

CONCLUSIONS

We have presented an innovative chemical mechanism for peptide immobilization on PCL films based on quick thiol-halogen click chemistry. It was demonstrated that the novel method overcomes the drawbacks found in the CDI chemistry, enhancing the reactivity and the specificity of the peptide binding to the polymer surface. RGD-modified PCL films supported and improved SC adhesion and proliferation. Also, surface chemical modification influenced cell morphology, with remarkable changes in cell spreading and elongation of the cellular processes. Focal adhesion formation was finally observed on RGD-surfaces, preventing cell detachment and increasing attachment and spread area. Results demonstrated that PCL films modified via thiol-chemistry could be used as a potential material for peripheral nerve repair.

ACKNOWLEDGMENTS

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REFERENCES

ORIGINAL ARTICLE
6. Discussion and Conclusion
6.1 Discussion

Peripheral nerve injury can easily occur and, although many techniques have been shown to achieve nerve regeneration in recent years, it is still impossible to predict any results about functional repair. Since the 17th century, many attempts have been performed in order to repair nerve injuries (Belkas et al. 2004). The most common repair techniques reported in the literature nowadays include grafting using natural materials and entubulisation using artificial nerve conduits (Fisher et al. 2007). All these strategies have the same aim in common, which is to allow the sprouting of sensory, motor and autonomic axons from the proximal nerve segment into the distal stump after injury (Siemionow & Brzezicki 2009).

PCL has been widely used in tissue engineering due to its good biocompatibility and ability to degrade in physiological environment (Chasalow et al. 1981). Although previous work has demonstrated its suitability for peripheral nerve repair (Sun & Downes 2009; Sun et al. 2010), research has been recently focused on both physical and chemical modification that can improve the material biocompatibility (Hoffman-kim et al. 2010; Wang & Cai 2010; Daly et al. 2012; Bell & Haycock 2011). The inner surface of the conduit can be in fact modified with physical cues, like grooves or multichannels to align cells and regenerated nerve fibres, or pores to improve material permeability (Roach et al. 2007; Hoffman-kim et al. 2010; Daly et al. 2012). Also, fillers can be inserted to create internal frameworks or to encapsulate growth factors (Wang & Cai 2010; Hoffman-kim et al. 2010; Daly et al. 2012). Chemical surface modification can affect surface hydrophilicity and mimic the biological environment, hence cell response (Atala 2009; von der Mark et al. 2010; Perlin et al. 2008). Particularly, the immobilisation of specific peptide motifs can target specific receptors of different cell types, enhancing their adhesion and proliferation (von der Mark et al. 2010). Also, it
was demonstrated that, during the development and the regeneration of the PNS, as well as the myelination process of PNS axons, ECM molecules influence SC differentiation (Milner et al. 1997). SC behaviour could be therefore controlled and regulated through the interaction between ECM molecules and cell receptors.

Following preliminary studies on the properties of PCL films (Sun & Downes 2007; Sun & Downes 2009) and their use *in vivo* as artificial conduit for two weeks (Sun et al. 2010), a long term experiment was performed using a rat sciatic nerve injury model. PCL conduits were used to repair 1cm nerve gap and nerve regeneration was compared to autografts, which are currently the gold standard in surgery. Long term studies are very important to assess skin and muscle reinnervation. After 18 weeks, nerve regeneration was quantified through immunohistochemistry and no remarkable differences were observed between the two groups (cf. Chapter 3). Similar results were also found after analysing the myelination of fibres and the skin reinnervation. A similar trend was also confirmed by the area and the diameter of muscle fibres (cf. Chapter 3). These results showed improvement when compared to previous *in vivo* studies using collagen and fibrin conduits (Waitayawinyu et al. 2007; Pettersson et al. 2010). Importantly, after 18 weeks no significant degradation of the PCL conduits was observed, meaning that the material could provide support for longer regeneration times and distances, particularly needed for human applications.

The aim of this study was to fabricate a biomaterial for nerve regeneration. Although PCL conduits showed good results for peripheral nerve repair, surface modification of the inner surface is necessary to improve nerve regeneration. Indeed, it was noted that SCs attached poorly on the surface of this material, which could affect axonal regeneration over longer time. The short time *in vitro* experiment to test cell attachment demonstrated that less than 10% of cells attach after 6 hours of culture (cf.
Chapter 4, Chapter 5). Previous work showed that SC response is strictly related to surface hydrophilicity (Soria et al. 2007). The effect of different functional groups on SC response was therefore investigated. Particularly, a novel and more specific mechanism (Thiol chemistry) was developed to immobilise RGD sequences on the surface materials. The cytocompatibility of SCs on these modified scaffolds was finally assessed. This study demonstrated that both wettability and surface chemistry are important when optimising the surface biocompatibility for a specific cell type. High hydrophilic surfaces (54.13±2.73° and 56.83±2.88° for NaOH and KOH treated films respectively) allow good cell spreading and long process extension, but higher contact angle with free amino groups (70.33±0.87° for HMD treated films) results in better attachment and higher cell proliferation (cf. Chapter 4). This could be explained as the negatively charged glycosaminoglycans on cell membranes interacts with NH$_3^+$ species (Vandamme et al. 1994; Lee et al. 2005). Accordingly, it was in fact demonstrated that SC adhesion on tissue culture plastic is remarkably improved when coating the surface with poly-D-lysine (Pdl), which is positively charged (Yavin & Yavin 1974).

Although SC response was remarkably improved on aminolysed PCL films, immunohistochemical studies showed that cells prefer cell-cell interaction to cell spread as induced by hydrophilic surfaces (cf. Chapter 4). It was previously demonstrated that PC-12 cell line cultured on different substrates characterised by different wettabilities developed longer neurites on surfaces of low contact angle (Lee et al. 2003). It could then be concluded that SC behaviour is strongly influenced by surface hydrophilicity and chemistry, and a good balance between the two properties is necessary to obtain the best output.

In order to enhance both proliferation and morphology of SCs, peptide sequences were immobilised on the PCL film surface. In this study, RGD sequences were initially
6. Discussion and Conclusion

bound on PCL films using the carbodiimide (CDI) chemistry, which is one of the most common reactions for peptide functionalisation (Vladkova 2010). However, undesired side reactions can occur which may lead to different degrees of functionalisation. Peptides were therefore immobilised using a novel chemical reaction based on the Thiol chemistry, also known as a thiol-halogen “click reaction”. This mechanism involves a preliminary modification of PCL film surface, forming chlorine groups required for the peptide immobilisation. Due to the very high nucleophilicity of thiols, halogens are easily and selectivity displaced by them (Fox & Whitesell 2004; Hoyle et al. 2010), as shown in Figure 1.11 (cf. 1.4.3.2). As the peptide chain can be easily customised for a specific application, the RGD sequence was therefore modified by adding a cysteine residue, containing a thiol group. The effectiveness of the peptide immobilisation was investigated using XPS, which is a surface-sensitive technique with probing depths typically of less than 5nm for organic materials (Tanuma et al. 1994). Results showed that the total amount of bound peptides is comparable using both CDI and Thiol chemistry (~3 RGD molecules per 100 PCL monomer units).

The biological response was remarkably improved when SCs and DRG neurons were seeded on the modified films. SC attachment in presence of peptides was about four fold higher than untreated PCL films (cf. Chapter 5). Also, cells showed stable adhesion on films functionalised with the Thiol chemistry after 6 hours of culture, and no cell detachment was observed (cf. Chapter 5). Cell detachment on untreated films could be due to the hydrophobicity of the material and to the absence of chemical cues. The formation of focal adhesions was not observed on untreated films after staining the cells for vinculin, indicating poor cell-material interaction. The improved cell response on the modified films could be therefore explained as a consequence of the formation of focal adhesion plaques, as shown in Figure 9, Chapter 5. Indeed focal adhesion points
act like “mechanosensors” and help the cells to perceive the substrate (Owen et al. 2005; Yim et al. 2010). Films functionalised with the Thiol chemistry also allowed better cell spreading with SCs showing a spindle shape morphology comparable to the one observed on Pdl-coated coverslips (cf. Chapter 5). However, adhesion forces were not sufficiently strong to impede cell proliferation, which increased continuously over 5 days (cf. Chapter 5).

The importance of peptide sequences and the effectiveness of the Thiol chemistry were finally confirmed by culturing dissociated dorsal root ganglia (DRG) neurons on untreated and treated films. Neurites were remarkably longer on RGD-modified films compared to untreated PCL surfaces and glass coverslips (cf. Appendix IV). The presence of immobilised peptide moieties induced the formation of longer and more branched neurites when compared to untreated PCL films (cf. Appendix IV). Similarly to SCs, neurons responded to external chemical stimuli, a beneficial feature for future in vivo application of the bioengineered conduit.

6.2 Future work

In this study the immobilisation of the peptide was accurately confirmed through the XPS analysis and it was demonstrated that SC response was improved on the modified PCL films. The analysis of the co-localisation between integrins and peptides would be useful to investigate the effect of the peptide on the expression by SCs of specific cell receptors. The integrin family is the most important as cells show high affinity adhesion, helping cell anchoring, spreading and differentiation (Hersel et al. 2003; Tsang et al. 2010; Schmidt & Friedl 2010). It was demonstrated that αv integrins are receptors for specific proteins containing RGD sequences, like fibrin, laminin and fibronectin (Chernousov & Carey 2003; Milner et al. 1997). By adding a specific dye to
the peptide sequence, it would be therefore possible to identify the αv integrin on SCs and the peptide sequence previously immobilised on the PCL films.

RGD has affinity with many different cell types without specificity for particular cells. Instead, IKVAV shows specific affinity with neuronal and glial cells (Tashiro et al. 1989; Hersel et al. 2003; Cargill et al. 1999), and prospective work could address the effect of the immobilised penta-peptide on SC response. The length of the peptide chain can induce a different cell response on the material surface. It has been shown that every amino acid is characterised by different structures and functional groups (Fox & Whitesell 2004), which can both affect the overall hydrophilicity of the amino acid chain. Also the longer the chain, the higher the mobility and the ability in changing conformation, hence cells respond to chemical cues in a different way. It would therefore be interesting to compare different chain lengths and investigate their influence on cell response.

Following the optimisation of the surface functionalisation of the PCL films with the best peptide sequence, in vivo experiments will be necessary to assess their use in peripheral nerve repair. Nerve conduits can be easily formed by rolling the films around a cannula (cf. 2.3.1). A preliminary short term in vivo experiment of about 2 – 4 weeks using a rat model could be initially carried out to investigate the ability of these conduits to regenerate the injured nerve. Immunohistochemical analysis will be a useful tool for the evaluation of the length and the thickness of the regenerated fibres. However, a longer in vivo experiment will be then necessary to analyse the functional reinnervation of skin and muscles.
6. Discussion and Conclusion

6.3 Conclusion

In summary, this study demonstrated the ability of PCL conduits to support the regeneration of injured nerves with results comparable to autografts, the current gold standard in surgery. As SCs are involved during the regenerative process, it was important to test their response on PCL films in order to improve their performance. It was demonstrated that SCs adhesion and proliferation is influenced by surface chemistry. A novel method for the chemical immobilisation of peptides on the material surface was developed and the biocompatibility of the films was tested. RGD-modified surfaces resulted in better cell response, triggering the formation of focal adhesion points. This improvement of the scaffold material will allow a better regeneration of the nerve in future experiments.


References


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References


Appendix I. Buffers and solutions

**Antibody diluent**

30µl of Triton X-100 (0.03%)

0.1g of Bovine Serum Albumin (BSA) (0.10%)

0.1g of Sodium Azide (0.10%)

100ml PBS

To store at 4°C.

**Hoechst stain solution**

100mg of Hoechst 33258

100ml of dH₂O

This solution should be diluted 1:50 in TNE buffer. To store at 4°C in the dark.

**MES buffer (1.0M)**

19.5g of MES

100ml of dH₂O

pH = 5.5

**Paraformaldehyde (PFA) (4%)**

4g of Paraformaldehyde

80ml of PBS

Heat at maximum 60°C and add NaOH (10M) to clear the solution. Adjust the volume to 100ml.
Appendix I

Phosphate Buffer Saline (PBS) (X10)

439.5g of NaCl
13.75g of KH₂PO₄
53.67g of Na₂HPO₄
5L of dH₂O
pH 7.3 (adjust with NaOH/HCl)

Sucrose PBS

150g of Sucrose
1g of Sodium Azide
1L PBS

TNE buffer

0.121g of Tris (10mM);
11.688g of NaCl (2M);
0.0372g EDTA (1mM);
100ml of dH₂O;
pH = 7.4

Zamboni’s fixing solution

82ml of 2% PFA in PBS
15ml of Saturated Picric Acid
To store at 4°C.
Appendix II. Optimisation of aminolysis

By using Ninhydrin assay, amino group concentration on film surface was assessed after different treatments as described in section 2.1.3.4. Firstly, a calibration curve with known HMD concentration in solution was drawn by plotting concentration (mM) against absorbance (Figure A.1).

![Calibration curve of Ninhydrin assay. Equation: y = 1.9075x – 0.1815. R² = 0.85.](image)

Concentration of amino groups linked to the polymer surface after 1 hour at room temperature was then extrapolated from the calibration curve by using the equation of the trend line. Untreated PCL films were used as negative control. After aminolysis, the ninhydrin solution turned from yellow into purple and, using a microplate reader, it was possible to record an absorbance of 0.522±0.061, meaning a concentration of NH₂ groups of about 3.5 x 10⁻³ mM/mm². Conversely untreated PCL films did not affect the colour of the dye solution and no absorbance was recorded.
Different reaction times and two temperatures, room temperature (RT) and 37°C, were then tested in order to find the best parameters for the aminolysis reaction prior to peptide functionalisation. The treatment at 37°C was more efficient than the one at room temperature (Figure A.2). During the first three hours of reaction, NH₂ concentration at 37°C was always higher than at room temperature. Hence, by warming the soaking solution, the treatment was faster and more efficient. After 3 hours the two treatment curves showed a different trend. At room temperature, NH₂ concentration continued to increase, whilst at 37°C the concentration of amino groups started to decrease. After Ninhydrin assay, aminolysis before peptide immobilisation was performed at ~40°C for 3 hours.

Figure A.2. Concentration of amino groups on PCL films treated with HMD at different times and temperatures (RT = room temperature).
Appendix III. Standard curve of DNA Assay

Before performing the DNA Assay on PCL films as described in section 2.2.4.1, a standard curve with known cell densities was necessary for the evaluation of the cells number. A range of cell dilutions was prepared by mixing cell suspensions with media at different ratios, starting with $1 \times 10^3$ cells/well and increasing to $5 \times 10^5$ cells/well. The calibration curve was therefore obtained by reading the fluorescence for each cell density (Figure A.3).

Figure A.3. Calibration curve of DNA Assay. Equation: $y = 0.0127x - 1188.5$. $R^2 = 0.9996$. 
Appendix IV. Culture of Dorsal Root Ganglia on untreated and treated PCL films

An additional short experiment was performed to assess the biocompatibility of the untreated and treated PCL films with dissociated dorsal root ganglia (DRG) neurons.

Experimental section

DRG neurons harvest and culture

DRG were harvested from adult male Sprague-Dawley rats and placed into a petri dish containing F12 medium. An enzymatic dissociation was performed with two cycles of 0.125% wt/v collagenase type IV (Worthington Biochemical, UK) for 1 hour at 37°C. DRG were carefully washed and incubated in a solution containing 0.25% wt/v trypsin (Worthington Biochemical) for 30 minutes at 37°C. The DRG were then washed with a solution containing 33% FBS to stop the action of trypsin. The dissociated neurons were gently washed three times with F12 medium to remove all traces of serum and they were transferred into a falcon tube to be mechanically dissociated using a glass pipette. The cell suspension was filtered through a 100µm pore size strainer and centrifuged at 500rpm for 5 minutes. The pellet was resuspended and centrifuged one more time at 500rpm for 5 minutes. A 50% v/v BSA/F12 solution was prepared to create a gradient trail along the wall of a fresh falcon tube and the cell suspension was then slowly pipetted down the track and centrifuged at 2000rpm for 5 minutes. This creates two separate layers, with the neurons on the bottom layer and the unwanted material on the top layer. The supernatant was then aspirated and the pellet was resuspended in Bottenstein and Sato’s medium (BS; 1% N₂ supplement (v/v; PAA, UK) and 50ng/ml NGF (Millipore, USA) in F12 medium). The neurons were finally seeded on glass
coverslips and on untreated and RGD-modified PCL films, previously sterilised under UV light, and cultured for three days in BS medium containing NGF.

Adipose derived stem cells (ASCs): harvest and differentiation into a SC phenotype

Stem cells were harvested from the visceral and inguinal fat of adult male Sprague-Dawley rats. Fat was finely chopped using a sterile razor blade and transferred into a Falcon tube containing a 0.15% wt/v Collagenase type I solution (Gibco, UK). The solution was continuously shaken for 1-2 hours in a water bath at 37°C and then filtered through a 100µm strainer to remove undissociated tissue. Stem cell growth medium prepared with Eagle’s medium (α-MEM, 10% FBS, 1% PS) was added to neutralise the enzyme and the solution was centrifuged at 1000rpm for 5 minutes. The supernatant was then aspirated, the cell pellet constituting the stromal fraction was resuspended in 10ml of stem cell growth medium and transferred into 75cm² flasks. Cells were finally incubated at 37°C, 5% CO₂, and maintained at sub-confluent levels.

At passage 2, growth medium was removed and replaced with 10ml of fresh medium containing 1mM β-mercaptoethanol. After 24 hours, cells were washed with Hank’s Balanced Salt Solution (HBSS) and 10ml of fresh medium containing 35ng/ml all-trans-retinoic acid. After 72 hours, cells were washed with HBSS and incubated with the stem cell differentiation medium (stem cell growth medium supplemented with 14μM forskolin, 63ng/ml GGF-2, 5ng/ml platelet-derived growth factor (PDGF; Sera Lab. International, UK) and 10ng/ml basic fibroblast growth factor (bFGF; Sera Lab. International, UK)). The cells were cultured for 2 weeks under these conditions, changing the medium every 72 hours.
Co-culture of DRGs and dASCs

Twenty-four hours prior to DRG harvest, 20000 dASCs were seeded on each coverslip and on untreated and RGD-modified PCL film, previously sterilised under UV and incubated at 37°C, 5% CO₂. The differentiation medium was then aspirated and neurons plated over the dASCs seeded on each substrate. The final co-culture medium was composed of 50% BS and 50% differentiation media. However, the final concentration of FBS was reduced to 2.5% to avoid the proliferation of satellite cells derived from the DRG dissociation. Before incubating the cells at 37°C, 5% CO₂, 50ng/ml NGF were added to each sample.

Investigation of neurite formation and length by immunocytochemistry

Neurons were cultured for 3 days in the presence or absence of dASCs (n=3) after which the preparations were fixed in 4% wt/v PFA for 20 minutes at room temperature. The preparations were carefully washed three times in PBS for 5 minutes and permeabilised in 0.2% v/v Triton-X/PBS for 2 minutes. The cells were washed again three times in PBS for 5 minutes and non specific antigens were blocked with 1% wt/v BSA in antibody diluent for 1 hour at room temperature. The primary antibodies were then applied overnight at 4°C as specified in Table A.1. The following day, the cells were washed three times in PBS for 5 minutes and the secondary antibodies (Table A.1) were applied for 1 hour at room temperature in the dark. The cells were finally washed with PBS and the samples were mounted on glass slides with Vectashield containing DAPI.

In order to quantify the elongation of the neuritis formed by the DRGs, 20 – 25 fluorescence images were randomly captured at 10x and 20x and analysed using ImageJ software. After setting the metric scale using a calibrated graticule, the longest and the
average length of the neuritis as well as the number of neuritis per cell body were calculated.

Table A.1. List of the antibodies used for DRG and dASC staining.

<table>
<thead>
<tr>
<th></th>
<th>DRGs</th>
<th>DRGs/dASCs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary antibody</strong></td>
<td>β-tubulin III (anti-mouse monoclonal)</td>
<td>β-tubulin III (anti-mouse monoclonal)</td>
</tr>
<tr>
<td><strong>Secondary antibody</strong></td>
<td>FITC goat anti-rabbit (Vector Laboratories, UK; 1:100)</td>
<td>Alexa Fluor 568 donkey anti-rabbit (Life Technologies, UK; 1:1000)</td>
</tr>
</tbody>
</table>

**Results**

The effect of RGD peptides on the surface of PCL films was significant compared to the other two groups. RGD moieties induced the formation of longer neuritis (294.3±19.9 µm; longest = 562.5 µm) compared to untreated films (85.9±1.2 µm; ***p<0.001; longest = 259.7 µm) and glass coverlips (174.7±10.5 µm; **p<0.01; longest = 378.3 µm) (Figure A.4.a,b and Figure A.5). When DRGs were co-cultured with dASCs, results followed a similar trend, and the stem cells induced the formation of longer neuritis (356.0±25.2 µm RGD, 222.1±13.0 µm PCL, 331.0±7.3 µm Glass). Data were statistically significant in both cases.

The effect of the RGD peptides immobilised on PCL films was still significant in terms of number of neuritis per cell body when the stem cells were not previously plated on the substrates (Figure A.4.c). DRGs showed more neuritis on RGD-modified films.
Appendix IV

(4/cell body) than on glass coverslips (3/cell body) and untreated films (1/cell body; \( \ast \ast p < 0.01 \)). When DRGs and dASCs were co-cultured, the number of neuritis per cell body increased, particularly on glass coverslips (6/cell body), which showed a higher number compared to RGD-modified films (5/cell body) and untreated films (3/cell body). However, results showed a significant difference between the two different film surfaces (\( \ast p < 0.05 \)).

Figure A.4. Quantification of the neuritis formed by the DRG with and without dASCs on different substrates: glass coverslips, untreated and RGD-modified PCL films. a) Average of the length of neuritis; b) Longest neuritis per each group; c) Number of neuritis per cell body (\( \ast p < 0.05 \), \( \ast \ast p < 0.01 \), \( \ast \ast \ast p < 0.001 \)).
**Figure A.5.** Immunofluorescence images of DRG cultured with or without dASCs on different substrates: a,d) glass coverslips; b,e) untreated PCL films; c,f) RGD-modified PCL films. The cells were stained with vinculin (red), β-tubulin III (green) and DAPI (blue).