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Development and characterisation of compressed, macro-porous and collagen-coated poly-ε-caprolactone electrospun meshes

A thesis submitted to the National University of Ireland Galway for the degree of
Doctor of Philosophy

By
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Research Supervisor: Dr Dimitrios I. Zeugolis

September 2018

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Plagiarism statement

I certify that the thesis is all my own work and I have not obtained a degree in this University, or elsewhere, on the basis of this work.

Kieran P. Fuller
List of abbreviations

µg: Microgram
µl: Microliter
µm: Micrometre
µM: Micromolar
1L: 1 layer
2L: 2 layers
3L: 3 layers
302S25: A specific grade of stainless steel
3D: 3 dimensional
AO\textsubscript{HW}: Absorbance of the oxidised form at higher wavelength
AO\textsubscript{LW}: Absorbance of the oxidised form at lower wavelength
ASTM: American society for testing and materials
AA: Acetic acid
ADF: Adult dermal fibroblasts
AR: alamarBlue® reduced
ALP: Alkaline phosphatase
ANOVA: One way analysis of variance
BAT: Bovine Achilles tendon
BDNF: Brain-derived neurotrophic factor
bFGF: Basic fibroblast growth factor
BFP\textsubscript{1}: Bone-forming peptide-1
BMP\textsubscript{1}: Bone morphogenetic protein 1
BMP\textsubscript{2}: Bone morphogenetic protein 2
BSA: Bovine serum albumin
C: Circle in Chapter 2
C: Compressed in Chapter 3
C. officinalis: Calendula officinalis
CA: Cellulose acetate
Ca-P: Calcium phosphate
CD: Cluster of differentiation
CE: "Conformité Européene" aka CE mark
CFU: Colony forming unit
CFX-Na: Cefotamime sodium
ChroB: 2,6-dichloro-4′-hydroxy-3′,3″-dimethylfuchsonc-5′,5″-dicarboxylic acid disodium salt
cm: Centimetre
CO₂: Carbon dioxide
COL: Collagen
COL #: Collagen type #
C-P: Compressed and porous
CPM: Cross-polarisation microscopy
CTRL: Glass coverslip control for in vitro analysis
DAPI: 4′,6-diamidino-2-phenylindole
DF: Dermal fibroblasts
dsDNA: Double stranded deoxyribonucleic acid
DNA: Deoxyribonucleic acid
DNase: Deoxyribonuclease
DMAC: Dimethylacetamide
DMEM: Dulbecco’s modified Eagle’s medium

XVI
DMOG: Dimethyloxallyl glycine
DMSO: Dimethyl sulfoxide

*E. coli*: *Escherichia coli*

ECM: Extracellular matrix
EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA: Ethylenediaminetetraacetic acid
ELISA: Enzyme-linked immunosorbent assay
ETO: Ethylene oxide
ET: Ethanol
EU: European union
FBGCs: Foreign body giant cells
FBS: Fetal bovine serum
FBR: Foreign body response
FDA: Food and Drug Administration
F&R: Feulgen and Rossenbeck
G: Needle gauge
GAG: Glycosaminoglycan
GmbH: “Gesellschaft mit beschränkter Haftung” German equivalent of an LLC company
HA-DTPH: 3,3'-dithiobis (propanoic dihydrazide)-modified hyaluronic acid
HAP: Hydroxyapatite
HBSS: Hank's balanced salt solution
He: Helium
Hep: Heprasal™
hR: Human recombinant
hrs: Hours
HV: High voltage
IF: Immunofluorescence
IFN-γ: Interferon gamma
IHC: Immunohistochemistry
IL-#: Interleukin (#)
IMS: Industrial methylated spirits
Inc.: Incorporation, a business suffix
IRCSET: Irish Research Council for Science, Engineering and Technology
kN: Kilonewton
kV: Kilo volt
kDa: Kilo dalton
LDH: Lactate dehydrogenase
LHS: Left hand side
LPEI: Linear polyethyleneimine
LPS: Lipopolysaccharide endotoxin
M: Molar
MCP-#: Monocyte chemo-attractant protein-
MES: 2-(N-morpholino) ethanesulfonic acid
mg: Milligram
mins: Minutes
MIP-#: Macrophage inflammatory protein-
ml: Milliliter
mm: Millimetre
MMP: Matrix metalloproteinases
MMP-2: Matrix metalloproteinases-2
MPa: Megapascal
mRNA: Messenger ribonucleic acid
MT: Masson's trichrome staining
MW: Molecular weight
N: Newton
NAMSA: North American science associates
NaOH: Sodium hydroxide
NBF: Neutral buffered formalin
ng: Nanogram
NGF: Nerve growth factor
NHS: N-hydroxysuccinimide
NIH: National Institutes of Health
nm: Nanometre
O₂: Oxygen
°: Degrees in terms of angles
°C: Degrees Celsius
Op: operation
P: Porous
PBMCs: Peripheral blood mononuclear cells
PBS: Phosphate buffered saline
PCL: poly ε-caprolactone
PCR: Polymerase chain reaction
PDGF: Platelet-derived growth factor
PDLLA: Poly-DL-lactic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>pDNA</td>
<td>Plasmid Deoxyribonucleic acid</td>
</tr>
<tr>
<td>PDO</td>
<td>Polydioxanone</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
</tr>
<tr>
<td>PEGDA</td>
<td>Poly(ethylene glycol) diacrylate</td>
</tr>
<tr>
<td>PEI-HA</td>
<td>Poly(ethylenimine)-hyaluronic acid</td>
</tr>
<tr>
<td>PELA</td>
<td>Poly(ethylene glycol)-poly(DL-lactide)</td>
</tr>
<tr>
<td>PELCL</td>
<td>Poly(ethylene glycol)-b-poly(l-lactide-co-caprolactone)</td>
</tr>
<tr>
<td>PEO</td>
<td>Polyethylene Oxide</td>
</tr>
<tr>
<td>PEU</td>
<td>Polyester urethane</td>
</tr>
<tr>
<td>PEUU</td>
<td>Polyester urethane urea</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>pH</td>
<td>Acidity scale unit</td>
</tr>
<tr>
<td>PHB</td>
<td>Poly(3-hydroxybutyrate)</td>
</tr>
<tr>
<td>PHMB</td>
<td>Polyhexamethylene biguanide</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly(lactic acid)</td>
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<tr>
<td>PLCL</td>
<td>Poly (lactic acid-caprolactone)</td>
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<td>PLDL</td>
<td>Poly(L/DL)-lactide</td>
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<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
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<tr>
<td>PLLA</td>
<td>Poly(lactic acid)</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
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<tr>
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<td>PR</td>
<td>Picrosirius Red</td>
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<td>pRNA</td>
<td>Plasma ribonucleic acid</td>
</tr>
<tr>
<td>PS</td>
<td>Penicillin / Streptomycin</td>
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</table>
PTFE: polytetrafluoroethylene
PU: Poly urethane
PUR: Poly(ester urethane urea)
PVA: Polyvinyl alcohol
R: Rhomboid
®: Registered trademark symbol
RANTES: Regulated on activation, normal T cell expressed and secreted
RAW264.7: Mouse leukemic monocyte-macrophage cell line
RGD: Arg-Gly-Asp or arginyl-glycyl-aspartic acid
rhBMP-2: Recombinant human bone morphogenetic protein 2
RHS: Right hand side
ROI: Region of interest
RPM: Rotations per minute
RT: Room temperature
RPMI 1640: Roswell Park Memorial Institute medium
S: Square
*S. aureus: Staphylococcus aureus*
SD: Standard deviation
SEM: Scanning electron microscopy
SHE: Safranin-Haematoxylin-Eosin staining
T0: Time zero
TCC: Total COL content
TIR: Tissue ingrowth rate
TCH: Tetracycline hydrochloride
TCP: Tissue culture plastic
TE: Buffer of 10 mM Tris-HCl, 1 mM EDTA, pH 7.5
Tet: Tetracycline
TFE: 2,2,2-trifluoroethanol
TGF-β: Transforming growth factor beta
THP-1: Human derived leukemic monocyte cell line
TIMP: Tissue inhibitors of matrix metalloproteinases
™: Trade mark
TNF-α: Tumour necrosis factor alpha
TPPS: 5,10,15,20-tetraphenyl-21H,23H-porphinetetrasulfonic acid disulfuric acid
UV: Ultraviolet light
V: Volt
VEC: Vascular endothelial cells
VEGF: Vascular endothelial growth factor
VSMC: Vascular smooth muscle cells
v/v: Volume/volume
w/v: Weight/volume
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Abstract

Electrospun scaffolds are utilised in a diverse spectrum of clinical targets, with an ever-increasing quantity of work progressing to clinical studies and commercialisation. However, the very dense architecture and the low mechanical properties of the produced scaffolds limit their wide use in clinical practice.

Herein, firstly, a single-strep fabrication process for the development of electrospun scaffolds with variable porosity (0 %, 30 %, 50 %, 70 %) and pore shape (circle, rhomboid, square) was assessed and the structural, mechanical (tensile and ball burst) and biological (dermal fibroblast and THP-1) properties of the produced scaffolds were evaluated. The collector design did not affect the fibrous nature of the scaffold. Modulation of the porosity and pore shape offered control over the mechanical properties of the scaffolds. Neither the porosity nor the pore shape affected cellular (dermal fibroblast and THP-1) response. Overall, this work provides evidence that electrospun scaffolds of controlled architecture can be fabricated with fibrous fidelity, adequate mechanical properties and acceptable cytocompatibility for a diverse range of clinical targets.

The nano-fibrous architecture of electrospun meshes favours their use in biomedicine, but their low mechanical properties prohibit their wide use in clinical practice. Introduction of porosity, essential of tissue integration, decreases further mechanical integrity. Therefore, it was hypothesised that macro-porous electrospun meshes with adequate mechanical properties can be fabricated through layering and subsequent compression. Two and three layers electrospun poly-ε-caprolactone scaffolds were fabricated, compressed and subsequently 30 % circular porosity was introduced through laser cutting. Three-layered porous electrospun meshes exhibited mechanical properties similar to commercially available scaffolds without any
structural or cytotoxic effect. This study brings electrospun materials closer to clinical translation and commercialisation.

Electrospun meshes have small macro-porosity, which is associated with foreign body response, whilst macro-porous electrospun meshes have low mechanical integrity. Herein, compressed, macro-porous and collagen (bovine Achilles tendon and human recombinant) coated electrospun poly-ε-caprolactone scaffolds were developed and their biomechanical, in vitro and in vivo properties were assessed. Collagen coating, independently of the source, did not significantly affect the biomechanical properties of the scaffolds. Although no significant difference in cell viability was observed between the groups, collagen coated scaffolds induced significantly higher DNA concentration. In vivo, no signs of adverse tissue effect were observed in any of the groups and all groups appeared to equally integrate into the subcutaneous tissue. It is evidenced that macro-porous poly-ε-caprolactone electrospun meshes with adequate mechanical properties and acceptable host response can be developed for biomedical applications.

Collectively, these data suggest the feasibility to create highly defined electrospun meshes with improved mechanical properties, cytocompatibility and tissue integration, paving the way for further applications for various clinical targets.
Keywords
Electrospinning; Electrospun meshes; Controlled architecture scaffolds; Scaffold design; Mechanical properties; Compression; Porosity; Collagen coating; Bovine collagen; Human recombinant collagen; Subcutaneous implantation
Chapter 1 - Introduction

Sections of this chapter have been published in:


1.1. Introduction

The nanomaterial’s sector global market was worth $15.9 billion in 2012 and is expected to rise to $37.3 billion by 2017 with a compound annual growth rate of 19.1 % [1]. Top-down nano-processes, such as lithography, deposition and etching, reduce large pieces of materials to nano-scale components. However, such technologies require large amounts of starting materials and are often associated with excessive waste generation. On the other hand, bottom-up nano-fabrication approaches, such as electrospinning and self-assembly, are utilised to build three-dimensional hierarchical constructs by putting together atomic and molecular components in a hierarchical fashion. Such technologies have minimal processing waste and are particularly favoured for biomedical applications. Among the various bottom-up processes, electrospinning is favoured as means to fabricate sub-micron devices, largely attributed to its inherent versatility and controllability [2, 3]. Indeed, to-date, numerous natural and synthetic polymers [4, 5]; ceramics [6, 7]; and metallic materials [8, 9] have been electrospun that have found applications in textile [10], filtration [11] and biomedicine [12] sector / industries. The rationale of using electrospinning in biomedicine lays on the fact that this technology can create three dimensional fibrous scaffolds that closely imitate the nano- to micro-scale intertwined fibrillar meshwork of the extracellular matrix [12-15].

Electrospinning is considered one of the most facile scaffold fabrication processes available today [16, 17]. The compositional diversity, coupled with the ability to precisely control mechanical properties and structural features, along with the functionalisation potential have been instrumental for the wide utilisation of electrospinning in the biomedical field [18]. The successful alignment of electrospun fibres represented a landmark development, as we were able for first time to create
artificial hierarchical biomimicry (Figure 1.1). Over the years, numerous studies have demonstrated that fibre alignment promotes formation of long lamellipodia extensions parallel to the direction of the fibres, resulting in directional cell orientation and migration, via the mechanism of contact guidance, reminiscent of native tissue environmental signalling [19, 20]. Cellular orientation is particularly relevant to the biomechanics of musculoskeletal tissue, as it determines the pattern of ECM deposition [21], which is an essential factor in the functionality of bone, tendon, ligament and cartilage [22]. Specifically to bone tissue engineering, fibre alignment and consequent cellular alignment, with or without further functionalisation, have been shown to regulate cell adhesion and migration; to promote osteogenic phenotype; to differentiate stem cells towards osteogenic lineage; and to enhance mineralisation and osteogenesis in vitro and in vivo [23-25]; for a more detailed review in the topic, please refer to [26, 27]. Moreover, the fibre alignment associated with electrospun fibres closely resembles the orientation and architecture of collagen fibrils present in native tendon tissue. Such anisotropic constructs promote elongated physiological cell morphology; phenotype maintenance of tendon derived cells; trans-differentiation of other cell types towards tenogenic lineage; and parallel to the fibre axis neotissue formation in the presence or absence of functional molecules and/or cells [28-31]. The porous 3D nature of electrospun scaffolds, with or without functional molecules and / or cells, provides an ideal environment for chondrogenic phenotype maintenance; chondrogenic differentiation of stem cells; and neotissue formation in vivo in both cartilage and osteochondral defects [32-34]. Besides fibre alignment, fibre size has been demonstrated to be an important variable in maintaining cell phenotype and function. In cartilage tissue engineering, for example, nano-scale fibres, as opposed to micro-
scale fibres, have been show to maintain chondrogenic phenotype [35, 36]. Likewise, scaffold fibre diameter has been reported to be crucial in tenogenic phenotype maintenance and differentiation of bone marrow stem cells towards tenogenic lineage, even to greater extent than alignment, with fibre diameter greater than 2 µm to be more suitable for \textit{in vitro} development of tendon / ligament tissue [37].
Figure 1.1: Upon application of high voltage between the metallic needle and the metallic collector, the solvent evaporates and the electrospun nano- to micro-fibres are collected on the collector (a). Using a rotating collector, electrospun fibres can be produced in bidirectional fashion (b). Continuous extrusion can produce 3D implantable devices (c). Using a collector with predefined shape, 3D implantable devices can be produced with specific architecture (d). Such materials can be further functionalised either with particles (e) or with chemical / biological (f) linking systems (transglutaminase in this case). Bidirectionally aligned electrospun fibres facilitate parallel to the fibre axis cell attachment and elongation (g).
Electrospinning has also been successfully combined with other scaffold fabrication technologies with optimal outputs for various clinical targets. Electrospinning, for example, combined with freeze-dried materials and bone marrow stem cells enhanced osteochondral regeneration with improved compressive moduli in a rabbit model [38]. Bimodal and multiphasic scaffolds can be fabricated using electrospinning and additive manufacturing [39, 40]. The resultant multi-hierarchical scaffold contained large size pores, essential for cell and mass transportation, whilst the fibrous component provided suitable structures for cell attachment. The modular system allowed development of implantable devices suitable for interface (e.g. bone-ligament [41] and muscle-tendon [42]) tissue engineering. Electrospinning has also been extensively combined with hydrogels to enable localisation and sustained delivery of cells and therapeutics [43, 44]. Scalability issues are under intense investigation to enable clinical translation and commercialisation of electrospun scaffolds these include using multiple needle systems or needless systems [3].

1.1.1 Academic uses of electrospinning

Cells have been reported to sense and react to the topographical cues down to nanometre scale, resulting in changes to attachment, morphology, proliferation, migration, lineage commitment and differentiation. Fibre diameter (nano- to micro-scale) can be controlled via polymer solvent, polymer concentration, voltage intensity and distance between the needle and the collector, whilst fibre orientation can be controlled through collector rotation or collector architecture. Controlled size and bidirectional architecture is of particular importance for musculoskeletal and neural tissue engineering, as they have been shown, in in vitro conditions, to synergistically control osteoblastic cell migration and differentiation [45], regulate
tendon fibroblast growth and differentiation [46], enhance differentiation of mouse embryonic stem cells into neural lineages and promote and guide neurite outgrowth [47], promote the neuronal differentiation of human embryonic stem cells [48, 49] and regulate proliferation, differentiation and neurite outgrowth of neural stem cells in culture [50, 51]. It is worth pointing out that a recent study argues that fibre diameter seems to be even more important than fibre orientation in differentiated mesenchymal stem cells towards tenogenic lineage [37]. Further, collectors with controlled porosity enable production of porous scaffolds with better nutrient/waste exchange, better cell penetration [52] and better cell survival and angiogenesis [53] than traditionally derived electrospun scaffolds from a non-porous collector. The regenerative potential of electrospun scaffolds has been demonstrated in multiple preclinical models (e.g., peripheral nerve [54], tendon [55], cartilage [56]). Electrospun scaffolds have even been used to reduce the tumour volume in brain, by migrating away brain tumour cells [57]. The evolutionary progression of biomedical devices from basic implants that just about imitate the mechanical properties of the tissue to be replaced to biofunctional devices that interact with the host resulted in the development of several functionalisation strategies for electrospun scaffolds, (Table 1.1). Blending the desired molecule with the carrier polymer prior to electrospinning was the first functionalisation method described and is still widely used to deliver drugs [58, 59] and biologics [60-62] for multiple clinical indications. Dual electrospinning involves the use of two extrusion systems with different compositions, which are electrospun simultaneously to form a single device. This technique can be beneficial for tissue interfaces such as muscle–tendon [42]. Co-axial electrospinning creates a core-sheath fibre with highly controllable release pattern [63], as evidenced in bone [64], tendon [65] and vascular [63] indications.
Surface functionalisation based on plasma treatment [61, 66] or on immersion of the electrospun scaffold into a solution that contains the desired molecule [67] has shown promising results in cardiac [61] and bone [67] preclinical models. Multi-layer/sequential electrospinning gives rise to a device composed of distinct layers that can potentially release their cargo at different stages of the reparative process [63]. Electrospinning-co-electrospraying facilitates the integration of nanoparticles into the fibrous device, thus enhancing the regenerative potential of the final product [68, 69]. Unfortunately, the highly volatile solvents used are associated with denaturation of collagen [70, 71]. To this end, gelatin [72], elastin [73] and silk [74] have been extensively studied for various clinical indications, including neural [72], cardiovascular [74], musculoskeletal [75] and wound healing [73]. Further, although significant progression has been achieved in the orientation controllability for electrospun scaffolds through direct writing, rotating collectors, collectors with pre-defined architectural features and spatial distribution of the individual fibres remain a technological challenge.
Table 1.1: Chronological advancements of electrospinning in biomedicine.

<table>
<thead>
<tr>
<th>Study (year)</th>
<th>Advancement of electrospinning in biomedicine</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annis et al. (1978)</td>
<td>Electrospun scaffolds for vascular prosthesis</td>
<td>[76]</td>
</tr>
<tr>
<td>Fisher et al. (1985)</td>
<td>Electrospun scaffolds assessed in vivo for arterial prosthesis</td>
<td>[77]</td>
</tr>
<tr>
<td>Kenawy et al. (2002)</td>
<td>Electrospun scaffolds assessed in vivo for wound healing</td>
<td>[78]</td>
</tr>
<tr>
<td>Khil et al. (2003)</td>
<td>Electrospun scaffolds assessed in vivo for wound healing</td>
<td>[79]</td>
</tr>
<tr>
<td>Bini et al. (2004)</td>
<td>Electrospun scaffolds assessed in vivo for peripheral nerve repair</td>
<td>[80]</td>
</tr>
<tr>
<td>Shin et al. (2004)</td>
<td>Electrospun scaffolds assessed in vivo as vascularised bone grafts</td>
<td>[81]</td>
</tr>
<tr>
<td>Li et al. (2005)</td>
<td>First in vitro study to demonstrate stem cell lineage commitment on electrospun substrates</td>
<td>[82]</td>
</tr>
<tr>
<td>Liu et al. (2006)</td>
<td>Dermal fibroblast loaded onto electrospun scaffolds and assessed in vivo in a large animal model (porcine)</td>
<td>[28]</td>
</tr>
<tr>
<td>Stankus et al. (2006)</td>
<td>Concurrent electrospinning of biodegradable polymers and electrospraying of live cells</td>
<td>[83]</td>
</tr>
<tr>
<td>Dang and Leong (2007)</td>
<td>Electrospun thermo-responsive nano-fibres for cell sheet engineering</td>
<td>[84]</td>
</tr>
<tr>
<td>Szot et al. (2011)</td>
<td>Electrospun nano-fibrous scaffolds as an in vitro cancer model</td>
<td>[85]</td>
</tr>
</tbody>
</table>
1.1.2 Clinical data

Currently, there are four registered clinical trials (source: clinicaltrials.gov; term searched: electrospinning). The first trial [86] assessed the potential of a diabetic foot ulcer patch to release nitric oxide; infection of the ulcer and adverse effects relating to the patch application were reported. The second one [87] was a meglumine antimoniate patch, which re-epithelialised the wound, but affected the mucous membranes leading to termination of the trial. The third one [88] will assess the clinical potential of an electrospun vascular graft. When a polyurethane Tecophilic™ nano-fibrous mesh was used in 162 patients with chronic leg ulcers, it resulted in a 35% decrease in wound size and a 71% reduction in pain [89]. The fourth trial [90] assessed the use of an electrospun mesh as an full thickness scaffold for treatment of dermatological wounds following the removal of non-melanoma skin cancers.

1.1.3 Commercial activity

A patent search (source: Google patents; term searched: electrospinning in title only) returned 672 patents. Despite this high number of patents, the authors identified only a few electrospun products. Numerous companies have developed electrospun filtration systems (e.g., eSpin Technologies, HRV, United Air Specialists), whilst The Electrospinning Company Ltd, Nanofiber Solutions, SKE Advanced Therapies and 3D Biotek LLC produce tissue culture consumables. Specifically, to implantable devices, Nicast Ltd has developed the AVflo, an electrospun polycarbonate-urethane vascular access graft, which is CE certified and available in the EU market, in several Asian countries and Israel. Preliminary clinical data indicate enhanced primary graft patency compared to commercial predicates after 6 and 12 months, with complication rates equivalent to predicates in adult [91, 92] and in a single
paediatric patient [93]. NovaMesh, an electrospun polycarbonate-urethane ventral hernia mesh, and other products are under development. Medprin Biotech GmbH has developed ReDura, an anti-adhesive biodegradable mesh with similar patient outcomes to commercially predicates [94]. BIOTRONIK International has received CE approval for the PK Papyrus stent, which is a cobalt chromium alloy stent covered with a polyurethane membrane. Zeus Inc. has developed the BioWeb™ technology that allows electrospinning of polytetrafluoroethylene onto various implants. NanoNerve, Inc. produces neural conduits with anisotropic internal architecture and isotropic external architecture. PolyRemedy, Inc. has developed the only functionalised device, the HealSmart, which is an antimicrobial dressing based on hyaluronic acid and polyhexamethylene biguanide.

1.2. Electrospinning setup and parameters affecting electrospinability

Numerous modifications in the electrospinning setup allow (a) development of implantable devices with different architectures for specific clinical targets, (Figure 1.2), and (b) functionalisation through the ability to deliver therapeutic molecules (Figure 1.3).
Figure 1.2: Variations in the electrospinning setup allow development of three-dimensional implantable devices that closely imitate architectural features of the tissue to be replaced.
Figure 1.3: The superiority of electrospinning in regenerative medicine lays on the fact that offers several opportunities for sustained and localised delivery of therapeutics. These six functionalisation methods are Blending, Dual electrospinning, Co-axial electrospinning, Surface modifications, Multi-layering and Electrospinning–co-Electrospraying.
A typical electrospinning setup is made up of a syringe containing the material to be extruded suspended in a highly volatile solvent; a syringe pump that controls the flow rate of the extrudate; a power supply that controls the voltage; a metallic spinneret; and a metallic collector. However, regardless of the relatively simplistic setup numerous critical parameters dictate the fabricated fibre morphology (Table 1.2). During extrusion, the electrostatic force, applied by the high voltage power supply, overcomes the surface tension and forms a Taylor cone. A jet ejection is formed at the tip of the metallic spinneret and is subsequently attracted towards the grounded metallic collector. The initial ejection is in a uniaxial direction, which subsequently develops into a conical envelope through electrostatic and fluid dynamic instabilities [95]. As the solvent evaporates, there is a significant reduction in the ejected material size, which results in fibre formation that is collected on the metallic collector. An alternative to the traditional setups is needless electrospinning. This is a technique which is gaining increasing interest and there are various methods of electrospinning without a needle such as bubble electrospinning whereby a gas is passed through a solution and the bubbles disruption of the surface creates a Taylor’s cone [96], and another method uses a variety of spinnerets which rotate through a solution and when in close proximity to the collector where the electrostatic force is sufficient for Taylor cone formation, ejection of the solution occurs [97]. Operating parameters are crucial to produce reproducible electrospun products. Indeed, selection of an appropriate volatile solvent, compatible with the material to be electrospun is crucial for production of consistent fibres [98]. Temperature [99, 100] and humidity [99, 101] have been reported to significantly alter material properties and solvent evaporation rate. Material and solution properties, such as concentration and molecular weight [102]; viscosity [103];
conductivity [102]; and surface tension [102] are also crucial for homogeneous fibre production. Fibre diameter can be controlled by varying the extrusion rate [104]; the voltage / charge of the material [105]; the spinneret / needle gauge [103]; and the distance between the spinneret and the collector [106]. Alterations in the collector setup offer opportunities for the development of custom-made materials that closely imitate native extracellular matrix assemblies. Indeed, the first generation of electrospun setups utilised a flat and static collector that gave rise to random fibrous mats suitable for wound healing applications [107]. Subsequent use of rotating collectors gave rise to aligned mats, suitable for tendon [19, 108], cornea [69, 109] and neural [110, 111] applications. There are numerous modifications for the fabrication of aligned electrospun materials, such as parallel electrodes, rotating wire drum collectors and disc collectors [112]. Additionally, function specific collectors can be utilised for the development of more complex architectures. Indeed, a cylindrical tube can be used for the production of cylindrical, three dimensional electrospun constructs suitable for vascular prostheses [74, 113] or peripheral nerve repair [114, 115] and electrodes with a peripheral ring and central point have been used for dura mater formation [116]. The utilisation of porous collectors enabled production of macro-porous electrospun products that offer better cell infiltration capacity [52, 117, 118] and are bypassing multistep processes, such as physical removal of material [119]; salt leeching [120]; gas foaming [120]; and dissolution of one of the co-electrospun polymers [121]. Near field electrospinning allows for the fabrication of scaffolds, where finely controlled topographies are required [122]; however this technique is mostly employed for electronics applications.
### Table 1.2: Critical electrospinning parameters and the corresponding effect on fibre morphology

<table>
<thead>
<tr>
<th>Electrospinning parameters</th>
<th>Effect on the fibre morphology</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ambient parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td>Increasing temperature results in smaller fibre diameter</td>
<td>[4, 118, 123-126]</td>
</tr>
<tr>
<td>Humidity</td>
<td>Increasing relative humidity results in smaller fibre diameter</td>
<td>[4, 118, 124, 126-128]</td>
</tr>
<tr>
<td></td>
<td>Elevated humidity results in pores on the fibres</td>
<td></td>
</tr>
<tr>
<td><strong>Intrinsic solution</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polymer concentration</td>
<td>Increasing polymer concentration results in an increase in fibre diameter</td>
<td>[129-131]</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>Increasing molecular weight reduced the number of beads and droplets</td>
<td>[124, 131-135]</td>
</tr>
<tr>
<td>Viscosity</td>
<td>When viscosity is too low it can lead to beads and droplets formation</td>
<td>[10, 124, 130-133, 135-154]</td>
</tr>
<tr>
<td></td>
<td>Higher viscosity increases the fibre diameter</td>
<td></td>
</tr>
<tr>
<td>Conductivity</td>
<td>Increasing conductivity results in a reduction in fibre diameter</td>
<td>[129, 131, 132, 137, 138, 142, 143, 149, 153, 155, 156]</td>
</tr>
<tr>
<td></td>
<td>Increasing the conductivity can eliminate bead and droplet formation</td>
<td></td>
</tr>
<tr>
<td>Surface tension</td>
<td>Negligibly small to no link identified between the surface tension and fibre diameter/ morphology</td>
<td>[124, 134, 141, 143, 154, 157-159]</td>
</tr>
<tr>
<td></td>
<td>Elevated surface tension results in instability of jets.</td>
<td></td>
</tr>
</tbody>
</table>
## Operational conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Applied Voltage</td>
<td>Increasing voltage results in more fluid ejection in uncontrolled flow rate systems which increases the fibre diameter but decreases fibre diameter in controlled systems. Excessively high voltage may lead to beading.</td>
<td>[129, 131, 132, 134, 144, 146, 149, 160]</td>
</tr>
<tr>
<td>Distance to collector</td>
<td>When the distance is too short beading can form due to insufficient fibre drying. When the distance is too long beading can occur. Within the non-beading region, increasing the distance reduces the fibre diameter.</td>
<td>[136, 141, 143, 144, 149, 150, 154, 161, 162]</td>
</tr>
<tr>
<td>Flow rate</td>
<td>Increases in the flow rate result in an increase in the fibre diameter. Beading and droplets can occur when the flow rate is either too high or low.</td>
<td>[139, 141, 143, 146, 154, 162-164]</td>
</tr>
<tr>
<td>Spinneret orifice diameter</td>
<td>Increasing the spinneret orifice radius tends to increase the average fibre diameters (where applicable).</td>
<td>[103]</td>
</tr>
</tbody>
</table>
1.3 Electrospun scaffolds as delivery vehicles

Biomaterials design has evolved from basic constructs that imitate native tissue architecture and material properties to more sophisticated products that aim to interact with the host, promoting repair and regeneration. It has been postulated that sustained and localised delivery of therapeutics will alleviate issues associated with conventional drug delivery platforms, such as high dosage regime and drug toxicity. It soon became apparent that electrospun devices can act as ideal delivery vehicles, as appropriate selection of polymer will enable sustained delivery of the cargo through its controllable degradation rate, whilst the high surface area of the scaffold will allow delivery of much larger cargos or cargos with short half-life [164, 165]. To-date, electrospun scaffolds have been used to deliver numerous therapeutics / bioactive molecules, utilising a range of different delivery methods.

1.3.1 Blending

Blending the therapeutic into the extrudate solution was the first, and remains the simplest, technology having been assessed [165]. The elution rate of the therapeutic is dependent on the degradation rate of the carrier material. This delivery system has been extensively assessed in various clinical targets and bioactive molecules, including vascular tissue repair with heparin [166]; neural regeneration with growth factors/genes [62]; dermal [167], brain [168] and bone [169] tissue infection with various antibiotics (Table 1.3) [166-177]. Despite the efficacious pre-clinical data obtained to-date, the main limitation of this method stems from the uncontrolled release profile in heterogeneous solutions, which also results in pockets of decreased mechanical properties.
Table 1.3: Examples of blending electrospinning technologies as means to deliver therapeutics and biologics in a range of clinical targets.

<table>
<thead>
<tr>
<th>Clinical target</th>
<th>Polymer and molecule</th>
<th>Scaffold orientation</th>
<th>In vitro results</th>
<th>In vivo results</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wound healing</td>
<td>Hyper branched polyglycerol and C. officinalis extracts.</td>
<td>Random.</td>
<td>C. officinalis extracts enhanced the biohesive properties, tensile properties and swelling.</td>
<td>No difference between silicon in an irritation test. Full re-epithelisation after 6 days.</td>
<td>[170]</td>
</tr>
<tr>
<td></td>
<td>PVA and quaternised chitosan.</td>
<td>Random.</td>
<td>All growth of <em>S. aureus</em> and <em>E. coli</em> was prevented within 2 hours.</td>
<td>-</td>
<td>[171]</td>
</tr>
<tr>
<td></td>
<td>PEO: PDLLA 50:50 and nisin.</td>
<td>Random.</td>
<td>Sustained release of nisin for 4 days.</td>
<td>Enhanced wound closer and significantly reduced the CFU.</td>
<td>[167]</td>
</tr>
<tr>
<td>Cardiac</td>
<td>PCL with heparin.</td>
<td>Random.</td>
<td>Sustained heparin release for 14 days, without a pro inflammatory response or alteration in biological functionality.</td>
<td>-</td>
<td>[166]</td>
</tr>
<tr>
<td></td>
<td>POC and PLCL.</td>
<td>Random</td>
<td>The POC at 25 and 40% significantly increased cell proliferation.</td>
<td>-</td>
<td>[172]</td>
</tr>
<tr>
<td></td>
<td>PDO and Elastin.</td>
<td>Random</td>
<td>Varying the component ratios affect the tensile properties.</td>
<td>-</td>
<td>[173]</td>
</tr>
<tr>
<td>Section</td>
<td>Material System</td>
<td>Alignment</td>
<td>Release</td>
<td>Outcome</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>----------------</td>
<td>-----------</td>
<td>---------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>Neural</td>
<td>PLGA with Vancomycin.</td>
<td>Random.</td>
<td>Sustained release for 6 weeks.</td>
<td>No infection or inflammation for up to 8 week.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCL with BDNF.</td>
<td>Partially aligned.</td>
<td>-</td>
<td>BDNF improved microglial integration to a untreated injury levels but also enhanced neurite sprouting.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCL and Gelatin.</td>
<td>Random and aligned.</td>
<td>Gelatin significantly reduced tensile properties but increased cell viability.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Bone</td>
<td>PLDL with HAP.</td>
<td>Random.</td>
<td>The presence of HAP significantly unregulated mineralisation and osteoblastic differentiation.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Antibacterial</td>
<td>PLGA, Rifampicin and Fusidic acid.</td>
<td>Initial burst release followed by a sustained release for 35 days. Co loaded agents were fully bactericidal in 48hrs.</td>
<td>Co loading of the drugs significantly reduced CFU numbers.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not specified</td>
<td>PDO and PMeDO.</td>
<td>Random.</td>
<td>PMeDO addition did not increase the crystallisation rate for electrospun meshes unlike the properties in films, which correlated with mechanical properties and sustained degradation up to 5 weeks. The increased levels of PMeDO correlated with increased cell proliferation.</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
1.3.2 Dual electrospinning

Alternatively, pre-fabrication methods are based on modification of the electrospinning setup. For example, dual electrospinning utilises two separate spinnerets that produce fibres on the same collector simultaneously. The spinnerets can be loaded with different therapeutics to create an integrated fibrous hybrid structure. To avoid electrostatic interaction of the spinnerets, they are kept apart by 90° or more. This technology has tremendous potential in biomedicine (Table 1.4), [41, 42, 178-185] especially in clinical indications, where a delivery platform is required in conjunction with appropriate mechanical properties [178]. This method has been utilised for antibacterial delivery to promote dermal [179] and abdominal wall regeneration [180] and enhanced bone regeneration, through protein delivery [181]. Although dual electrospinning offers the possibility of eluting a molecule, whilst retaining some of the physical characteristics of the scaffold, reduction of mechanical properties, delamination and separation may occur, once the eluting material has degraded; these issues stemmed research for alternative strategies (Table 1.4).
Table 1.4: Examples of dual electrospinning technologies as means to deliver therapeutics and biologics in a range of clinical targets.

<table>
<thead>
<tr>
<th>Clinical target</th>
<th>Polymer and molecule</th>
<th>Scaffold orientation</th>
<th>In vitro results</th>
<th>In vivo results</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wound healing</td>
<td>CA and with PHMB.</td>
<td>Random.</td>
<td>CA enhanced hydrophobicity and 50% release of PHMB within 24h resulting in reduced microbial activity by 96% +.</td>
<td>In a burn model the co electrospun 4:1 PEU: CA with PHMB resulted in the most significant healing after 16 days.</td>
<td>[179]</td>
</tr>
<tr>
<td></td>
<td>PEO with HA-DTPH and PEGDA</td>
<td>Random.</td>
<td>Dual spinning of the crosslinked allowed in situ crosslinking.</td>
<td>-</td>
<td>[182]</td>
</tr>
<tr>
<td></td>
<td>PLLA with Lidocaine and mupirocin</td>
<td>Random.</td>
<td>The dual spinning allowed for an initial burst of lidocaine and a sustained release of mupirocin up to 72 hours.</td>
<td>-</td>
<td>[183]</td>
</tr>
<tr>
<td>Cardiac</td>
<td>PCL: Gelatin/ PLGA: Random.</td>
<td>The scaffold is cyto compatible for fibroblasts and endothelial cells.</td>
<td>-</td>
<td></td>
<td>[178]</td>
</tr>
<tr>
<td></td>
<td>Gelatin and PLGA: Chitosan.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone</td>
<td>PEO and PCL.</td>
<td>Random.</td>
<td>Tensile properties were a function of the polymer ratios. High PEO content increase cell integration once removed.</td>
<td>-</td>
<td>[184]</td>
</tr>
<tr>
<td></td>
<td>PLLA and PEO alone and blended.</td>
<td>Random.</td>
<td>The removal of PEO drastically enhanced cell integration due to higher porosities.</td>
<td>-</td>
<td>[185]</td>
</tr>
<tr>
<td></td>
<td>PLLA with rhBMP-2 and PLGA with Ca-P.</td>
<td>Random.</td>
<td>Fabricated evenly distributed bicomponent scaffolds.</td>
<td>-</td>
<td>[181]</td>
</tr>
<tr>
<td>Interfaces</td>
<td>Random.</td>
<td>Distinct mechanical properties of scaffold within the same scaffold for muscle and tendon. The scaffolds allowed cell adhesion and differentiation of myoblasts into myotubes.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>----------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLLA: Col and PCL: Col 1:1.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCL with HAP and PUR.</td>
<td>Random.</td>
<td>Bone and ligament scaffold. Gene expression of BMP-2 and osteopontin were upregulated on the mineral containing region and the opposite was seen for ALP mRNA.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-bacterial</td>
<td>Random.</td>
<td>Antibiotic activity retained up to 7 days. Addition of PEUU to PLGA reduced tensile properties but prevented shrinkage after 24hrs. The presence of tet significantly reduced skin dehiscence and abscess formation.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEUU/ PLGA with tet.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[42]
1.3.3 Co-axial electrospinning

Co-axial Electrospinning gives rise to a multi-compartmental fibre with a uniform [186] or interrupted [187] internal core or cores [188] and external sheath which can have a single [188] or multiple layers [189]. This set-up can be used to deliver multiple molecules in a single material, but it is more commonly used to deliver a material with an internal core, which provides structural support and a sheath containing a molecule to be released in a controlled manner. This method has been used for numerous clinical targets [186, 190] including in vivo delivery of antibacterial drugs [190]; nonsteroidal anti-inflammatory drugs to prevent adhesions in tendon defect model [191]; and growth factor delivery in wound healing models (Table 1.5) [192-199].
Table 1.5: Examples of co-axial electrospinning technologies as means to deliver therapeutics and biologics in a range of clinical targets.

<table>
<thead>
<tr>
<th>Clinical target</th>
<th>Polymer and molecule</th>
<th>Scaffold orientation</th>
<th>In vitro results</th>
<th>In vivo results</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wound healing</td>
<td>PELA with bFGF core.</td>
<td>Random.</td>
<td>After an initial burst release sustained release of bFGF occurred until 25 days.</td>
<td>Meshes significantly enhanced re-epithelisation and mature capillary vessel formation.</td>
<td>[192]</td>
</tr>
<tr>
<td></td>
<td>PLA with a PEG and salicylic acid core.</td>
<td>Random.</td>
<td>Porous sheaths were created by increasing the DMAC solvent concentration which facilitated faster drug release</td>
<td>-</td>
<td>[193]</td>
</tr>
<tr>
<td>Neural</td>
<td>PLCL with a BSA and NGF core.</td>
<td>Random.</td>
<td>-</td>
<td>The fibres with a BSA/ NGF core nerve reconstruction was like an autograft and significantly better to hollow PLLA fibres.</td>
<td>[194]</td>
</tr>
<tr>
<td>Sutures</td>
<td>PLLA with a CFX-Na core.</td>
<td>Aligned and subsequently braided.</td>
<td>Co-axially spun fibres had a more gradual release profile up to 250hrs and had better tensile properties than blended fibres.</td>
<td>The PLLA with CFX-Na showed less inflammation and infection than commercial silk or PLLA alone.</td>
<td>[190]</td>
</tr>
<tr>
<td></td>
<td>PLLA with a TCH core.</td>
<td>Aligned.</td>
<td>The encapsulated core prevented an initial burst release profile.</td>
<td>-</td>
<td>[195]</td>
</tr>
<tr>
<td>Not specified</td>
<td>Gelatin with a PCL core.</td>
<td>Random.</td>
<td>The PCL core significantly increased tensile properties and the sheath provided a porous structure for cell proliferation and adhesion.</td>
<td>[196]</td>
<td></td>
</tr>
<tr>
<td>PCL and PEG with a BSA core.</td>
<td>Random.</td>
<td>Heterogeneous release profile from blended meshes and homogeneous release profile from coaxial meshes.</td>
<td>[186]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCL/PEG with a BSA and PDGF core.</td>
<td>Aligned.</td>
<td>Rapid initial release occurred which plateaued after 15 days. The PEG levels allowed for controllable release.</td>
<td>[197]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCL with a PEG, pDNA and PEI-HA core.</td>
<td>Random.</td>
<td>The release profile was found to be most dependent on concentration and MW of the core polymer.</td>
<td>[198]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Various combinations of PHB and PDLLA were tested as either the core or sheath polymer with the location of DMOG also mixed.</td>
<td>Random.</td>
<td>Varying the thickness of the PHB sheath allowed for sustained release, while all other parameters resulted in a burst release.</td>
<td>[199]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.3.4 Surface treatment

Immersion of the electrospun mats into a solution containing the molecule of interest has also been extensively studied for numerous clinical targets (Table 1.6) [67, 118, 200-206]. Immersion has been used for a variety of pre-clinical applications, including delivery of BMP-1 [67], simvastatin [200] and collagen/nano hydroxyapatite [201] for guided bone repair and delivery of antibiotics to abdominal wall [202]. Although plasma treatment has been shown to increase cell proliferation, this treatment has been shown to have detrimental effect on the mechanical properties of meshes [207]. To this end, bi- and multi-functional conjugation strategies based on transglutaminase [208] and PEG-based systems [203, 204] respectively have been introduced.
Table 1.6: Examples of surface treatment of electrospun fibres as means to deliver therapeutics and biologics in a range of clinical targets.

<table>
<thead>
<tr>
<th>Clinical target</th>
<th>Polymer and molecule</th>
<th>Scaffold orientation</th>
<th>In vitro results</th>
<th>In vivo results</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wound healing</td>
<td>PCL and PEG with a LPEI /hEGF coating.</td>
<td>Random.</td>
<td>-</td>
<td>Burns in diabetic animals coated meshes had a rapid initial closure rate but were equivalent to controls at day 14</td>
<td>[204]</td>
</tr>
<tr>
<td></td>
<td>PCL and PEG with a LPEI /DNA coating.</td>
<td>Random.</td>
<td>DNA release was significantly higher in response to MMP-2.</td>
<td>Dorsal wounds in diabetic and normal animals showed highest DNA incorporation for coated samples.</td>
<td>[203]</td>
</tr>
<tr>
<td></td>
<td>PCL immersed in Biteral®.</td>
<td>Random.</td>
<td>100% release achieved after 24 hours.</td>
<td>The PCL with Biteral showed adhesion levels similar to a surgical sham.</td>
<td>[202]</td>
</tr>
<tr>
<td>Cardio</td>
<td>Plasma treated PU films were grafted onto electrospun PLGA which were again plasma treated with microwave induced argon.</td>
<td>Random.</td>
<td>Plasma treatment increased hydrophilicity and surface roughness correlating with and cell proliferation.</td>
<td>-</td>
<td>[205]</td>
</tr>
<tr>
<td>Neural</td>
<td>PCL and immersed in Aligned. fibronectin.</td>
<td>Aligned.</td>
<td>Radial alignment enhanced cell migration over randomly aligned. Coating enhanced cell elongation and cell migration speeds.</td>
<td>-</td>
<td>[116]</td>
</tr>
<tr>
<td>Tissue</td>
<td>Treatment</td>
<td>ALP activity and calcium deposition were significantly unregulated after 14 days with the mesh.</td>
<td>Cranial wounds after 2 months were $57.29 \pm 15.24%$ healed compared to $23.02 \pm 6.54%$ with fibres alone.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone</td>
<td>PLGA with a BFP-1 Random. coating.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCL with simvastatin Random. dropped onto it.</td>
<td></td>
<td>Simvastatin coating was not as effective as internalisation for longer term release. The cranial defect closure was enhanced by simvastatin compared to PCL control.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PLGA with collagen and HAP.</td>
<td></td>
<td>After 8 weeks in a cranial model, Col and HAP combined showed significant increase in regrowth.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ocular surface</td>
<td>PCL plasma treated with He/O$_2$.</td>
<td>The plasma treatment significantly enhance hydrophilicity.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

References:

[67] [200] [201] [206]
1.3.5 Multi-layering

Multi-layering of electrospun meshes can be beneficial for designing a material which will traverse multiple cell types for interfacial tissue engineering application and complex organs, such as cartilage-bone interface [209] and arterial wall reconstruction [210] respectively. This technique has the potential to utilise materials with different mechanical / degradation properties and biomimetic characteristics to form a functional composite that would closely imitate complex native extracellular matrix assemblies. Electrospun multilayers with different materials/cargos have been used in a variety of complex pre-clinical studies, including osteogenic regeneration in cranial defects [211]; cell delivery for sustained insulin release [212]; and subcutaneous inflammation [213]. Despite the wide potential of multi-layered electrospinning in biomedicine (Table 1.7) [63, 210-212, 214-216], it is worth pointing out that potential delamination issues should be addressed to avoid implant failure in vivo.
Table 1.7: Examples of multi-layered electrospinning technologies as means to deliver therapeutics and biologics in a range of clinical targets.

<table>
<thead>
<tr>
<th>Clinical target</th>
<th>Polymer and molecule</th>
<th>Scaffold orientation</th>
<th>In vitro results</th>
<th>In vivo results</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardio</td>
<td>PCL/ Elastin/Col in different ratios.</td>
<td>Random.</td>
<td>The triple layer scaffold combines to increase the overall mechanical properties where a single layer would fail.</td>
<td>-</td>
<td>[210]</td>
</tr>
<tr>
<td></td>
<td>Internal silicon tube or nylon mesh and nylon outer layers of 15 and 40% w/v with cells injected 5 weeks post op.</td>
<td>Random.</td>
<td>-</td>
<td>The implanted cells remained viable and within the scaffold for 6 weeks.</td>
<td>[212]</td>
</tr>
<tr>
<td>Cardio</td>
<td>Internal layer of chitosan hydrogel/ PELCL loaded with VEGF and an outer layer of emulsion/PELCL electrospun membrane-loaded with PDGF.</td>
<td>Random.</td>
<td>Proliferation rates of VECs increased and VSMC initially slowed followed by rapid increase after 6 days.</td>
<td>VECs and VSMCs developed on the lumen and exterior respectively with no thrombus or burst appeared.</td>
<td>[63]</td>
</tr>
<tr>
<td></td>
<td>PLA and PCL.</td>
<td>Aligned and Random.</td>
<td>Cells did not fully penetrate past the scaffold’s surface. Low collagen and GAG content were found compared to porcine pulmonary valve.</td>
<td>-</td>
<td>[214]</td>
</tr>
<tr>
<td>Category</td>
<td>Description</td>
<td>Random.</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------</td>
<td>-----------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone</td>
<td>PCL/PEG/ PCL with HAP</td>
<td>There was a slow initial degradation profile but it accelerated rapidly after 4 weeks.</td>
<td>[211]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>The treated group significantly increased bone formation up to 20 weeks in cranial bone with minimal inflammation.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-cancer</td>
<td>The four layers are PLCL with ChroB, PLCL, PLCL with TPPS and PLCL.</td>
<td>PLCL created a barrier causing distinct pause, thickness dependant, in the release of TPPS.</td>
<td>[215]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not specified</td>
<td>Ibuprofen intercalated layered double hydroxide with either PCL or PLA and with or without Pluronic.</td>
<td>Increased controllability of ibuprofen release profile achieved through polymer ratios alterations.</td>
<td>[216]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.3.6 Electrosprinning-co-electrospraying

Carrier systems can be added to the electrospun solution prior to electrospinning [217] or during the fabrication process, resulting in an integrated material [218]. Electrospraying has been studied extensively in biomedicine as a delivery platform in vitro [219, 220], but only few studies have been conducted in vivo (Table 1.8) [83, 218, 221-226]. We believe that multilayer electrospinning bypasses the need for creation of complex structures (particles and fibres), with potentially difficult or even impossible regulatory clearance.
Table 1.8: Examples of electrospinning-co-electrospraying technologies as means to deliver therapeutics and biologics in a range of targets.

<table>
<thead>
<tr>
<th>Clinical target</th>
<th>Polymer and molecule</th>
<th>Scaffold orientation</th>
<th>In vitro results</th>
<th>In vivo results</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wound healing</td>
<td>Electrospun PEUU with electrospayed porcine derived ECM.</td>
<td>Random.</td>
<td>Tuneable tensile properties based on component ratios.</td>
<td>In a full thickness abdominal wall defect no infection or herniation occurred after 4 weeks.</td>
<td>[221]</td>
</tr>
<tr>
<td>Cardio</td>
<td>Electrospun PCL with electrospayed PLGA microspheres with bFGF.</td>
<td>Random.</td>
<td>Release of bFGF created a gradient that increased in-depth cell migration.</td>
<td>In subcutaneous mice model it increased cell migration onto the scaffold and increased density of newly formed blood vessels.</td>
<td>[222]</td>
</tr>
<tr>
<td>Cell delivery</td>
<td>Electrospun PEUU with electrospayed vascular smooth muscle cells.</td>
<td>Random.</td>
<td>Significantly higher cell populations integrated into the scaffold at 7 days.</td>
<td>-</td>
<td>[83]</td>
</tr>
<tr>
<td>Increased cell infiltration</td>
<td>PCL/Col with PEO or Gelatin fibres or Hep nanoparticles</td>
<td>Random.</td>
<td>PCL/Col with Hep coating had significantly deeper cell infiltration after 10 days.</td>
<td>-</td>
<td>[218]</td>
</tr>
<tr>
<td></td>
<td>Electrospun PLGA/PEGDA or PUR/PEGDA and electrospayed C3H10T1/2.</td>
<td>Random.</td>
<td>Composite scaffold was cytocompatible, cell distribution uneven throughout the scaffold.</td>
<td>-</td>
<td>[223]</td>
</tr>
<tr>
<td>Bone</td>
<td>Sample Description</td>
<td>Effect</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>-------------------------------------------</td>
<td>----------------------------------------------------------------------</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>PHB/gelatin electrosprayed or blended with HAP.</td>
<td>Random.</td>
<td>Electrosprayed samples were significantly higher in ALP activity and mineralisation rates.</td>
<td>[224]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCL electro-sprayed with PLGA micro-particles.</td>
<td>Random.</td>
<td>Decrease in initial burst release in electrosprayed particles. Cells spread and remained viable.</td>
<td>[225]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLCL/Gelatin electrosprayed or blended with HAP.</td>
<td>Gelatin Random.</td>
<td>Electrospraying enhanced cell proliferation, mineralisation and ALP activity.</td>
<td>[226]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.4 Project rationale and hypotheses

Electrospinning has revolutionised the field of regenerative medicine due to its innate ability to create implantable devices that closely imitate native extracellular matrix architectures and its ability to develop scaffolds with controlled and sustained delivery of therapeutics. However, the fundamental limitations of electrospun meshes are the lack of macro-porosity and their low mechanical properties.

To develop macro-porous and high in mechanical properties, electrospun meshes, this project was divided into three phases: identification of the optimal pore shape and porosity in single step electrospinning (Phase I), development of porous electrospun meshes with clinically relevant tensile properties (Phase II) and functionalisation of the porous electrospun meshes with collagen (Phase III).

**Overall hypothesis:**

Electrospinning can produce macro-porous meshes with suitable mechanical properties for biomedical applications.

**Phase 1**

**Hypothesis**

The tensile properties and *in vitro* immune response of porous electrospun meshes can be modulated by porosity and pore shape.

**Specific objectives**

1. Fabricate electrospun PCL meshes with 0, 30, 50 and 70 % porosity and pore shapes of squares, circles and rhomboids;

2. Evaluate the biophysical properties of the produced electrospun meshes;
3. Evaluate the \textit{in vitro} inflammatory response using macrophage cells.

**Phase 2**

**Hypothesis**

Electrospun non-toxic meshes with physiologically relevant mechanical properties can be produced following post-fabrication processing.

**Specific objectives**

1. Fabricate compressed and porous electrospun meshes.
2. Evaluate the biophysical properties of the produced electrospun meshes.
3. Evaluate the \textit{in vitro} response of the produced electrospun meshes.

**Phase 3**

**Hypothesis**

Functionalisation of the optimised electrospun mesh with collagen will enhance tissue integration and reduce inflammatory response in a subcutaneous rat model.

**Specific objectives**

1. Functionalisation of the optimised electrospun meshes with bovine Achilles tendon and human recombinant collagens.
2. \textit{In vitro} assessment of the electrospun meshes.
3. \textit{In vivo} assessment of the electrospun meshes.

\textbf{Note:} The \textit{in vivo} assessment was conducted at NAMSA, France.
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Electrospun Scaffolds with Loaded Electrosprayed Microparticles.

Sections of this chapter have been published at:

2.1. Introduction

Provision of an appropriate structural support with adequate biomimicry that would modulate cell phenotype and function has been postulated to be a key facet in current regenerative therapies [1, 2]. This has led to the development of top-down (e.g. lithography [3], laser etching [4], imprinting [5] and bottom-up (e.g. self-assembly [6], direct writing [7], melt-spinning [8], 3D printing [9], electrospinning [10]) nano- and micro- fabrication techniques. While top-down fabrication technologies can provide clearly defined topographies down to the nano-scale, the majority of these techniques have considerable drawbacks in terms of scalability, functionalisation potential and clinical relevance [11-13]. In contrast, bottom-up techniques are favoured for clinically relevant tissue engineering, as they allow increased functional versatility with a more scalable and economical production [14-17].

Electrospinning [18, 19] is a facile method that allows fabrication of fibrous constructs with fibre diameter similar to native extracellular matrix assemblies and with significant potential in modern tissue engineering, for various clinical indications, including tendon [20], ligament [21], muscle [22], cardiac [23], corneal [24], dermal [25] and bone [10]. Although early studies could produce only randomly orientated fibres, advances in the fabrication process (e.g. introduction of a rotating collector) are now allowing production of aligned and orientated fibres [26]. However, there is limited progression in developing electrospun scaffolds with predesigned architectural features. The use of mandrels for creating conduits has been utilised for neural [27] and vascular [28] applications. However, these electrospinning methods typically create pores of about 3-5 μm, dependent on fibre diameter, resulting in a pseudo 3D material, which limits cell integration and subsequent vascularisation [10].
Current 3D fabrication methods that facilitate cell and tissue integration typically require post fabrication processing, such as laser etching and use of sacrificial components. However, laser etching induces thermal ablation, jeopardising scaffold’s architecture and cell attachment and function [29]. The use of sacrificial components introduces waste products and increases fabrication costs [30, 31]. To this end, a single-step process, based on patterned collectors has been proposed [32, 33]. However, a detailed study on the influence of pore shape and porosity on structural, physical and biological properties of the produced scaffolds has yet to be elucidated. Herein, we ventured to assess the influence of predefined collector architecture (variable pore shape and porosity) on the structural, mechanical (tensile and ball burst) and biological (dermal fibroblast and THP-1) properties of the produced electrospun scaffolds.
2.2. Materials and Methods

2.2.1. Scaffold fabrication

Typical protocols for electrospinning were utilised, as described previously [34-36]. Briefly, 200 mg/ml of poly ε-caprolactone (PCL, PURASORB® PC12, IV 1.2 dl/g, 70,000 MW, Corbion, The Netherlands) were dissolved in 2,2,2-trifluoroethanol (TFE; 99.8 %, Acros Organics, Ireland) and the solution was extruded at 10 µl/min through an 18 G stainless steel blunt needle (EFD Nordson, Ireland). Upon application of high voltage (15 kV) between the needle and the collector (20 cm distance), the fibres were collected on a rotating mandrel (60 revolutions per minute, RPM). The porous collectors, consisting of circle (C), rhomboid (R) and square (S) pores with porosities of 0 %, 30 %, 50 % and 70 %, were fabricated through chemical etching of a 0.1 mm thick hard rolled stainless steel 302S25 (Qualitetch Components Ltd., UK). These collectors were placed on top of a polytetrafluoroethylene (PTFE) sheet during the fabrication procedure. Optimisation of the electrospinning process to achieve a porous construct will be further discussed in the appendix. Solvent cast films were also fabricated by pouring the PCL solution onto a level PTFE mould that was allowed to evaporate overnight at ambient temperature and pressure.

2.2.2. Morphology assessment

Gross visual observations were performed with a stereomicroscope. For finer detail, the electrospun scaffolds were mounted onto a carbon disk, gold sputter coated and imaged with a Hitachi S-4700 scanning electron microscope (Hitachi High-Technologies Europe GmbH, Germany). Fibre diameter analysis was conducted using NIH ImageJ software.
2.2.3. Biophysical assessment

Mechanical properties were assessed using ball burst and uniaxial tension tests, using a Zwick/Roell (Leominster, UK) Z005 testing machine, loaded with a 10 N load cell, as has been described previously [37, 38]. The ball burst samples were prepared in accordance with ASTM D3787-15 and the uniaxial samples were cut into a dog-bone shape, as per ASTM D882-2010 guidelines. Prior to testing, all samples were incubated overnight at room temperature in phosphate buffered saline (PBS) and tissue paper was used to remove excess PBS. The sample thickness was measured using digital callipers (Scienceware®, Digi-Max™, Sigma-Aldrich, Ireland). The ball burst samples were placed between two layers of vulcanised rubber, and subsequently placed between the appropriate sample grips and hand tightened around the circumference. The uniaxial samples were hand-tightened between the vertical grips at a 5 cm gauge length. The extension rate was 20 mm/min for both methods. Scaffolds that broke at contact points with the grips were rejected from the analysis. The following definitions were used to calculate mechanical data: stress at break was defined as the load at failure divided by the original cross-sectional area (engineering stress), strain at break was defined as the increase in scaffold length required to cause failure divided by the original length and elastic modulus was defined as the slope of the stress-stain curve in the elastic deformation region.

2.2.4. Cell culture

Adult dermal fibroblasts (DF, ATCC®, UK), passage 6-9, were cultured in Dulbecco’s modified Eagle’s medium (4500 mg glucose, Sigma Aldrich, Ireland) with 10% foetal bovine serum (FBS, Sigma Aldrich, Ireland) and 1% penicillin
streptomycin (PS, Sigma Aldrich, Ireland) at a cell density of 25,000 cells/cm² for 1, 3 and 7 days on Nunc™ non-treated flasks (Dublin, Thermo Scientific, Ireland). A human monocyte cell line (THP-1 cells, ATCC, UK), passage 7 to 9, was cultured in RPMI-1640 (Sigma Aldrich, Ireland) with 10 % FBS (Sigma Aldrich, Ireland) and 1 % PS (Sigma Aldrich, Ireland), at a cell density of 25,000 cells/cm² for 1, 2 and 3 days on Nunc™ non-treated flasks (Dublin, Thermo Scientific, Ireland). Phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich, Ireland) dissolved in DMSO 100 ng/ml was used to stimulate the activation of monocytes to macrophages. Lipopolysaccharide endotoxin (LPS) from *E. coli* (Sigma Aldrich, Ireland) at 100 ng/ml concentration was used as positive control for inflammation. Both cells types were maintained at 37 °C in a humidified 5 % CO₂ incubator. The scaffolds were cut to dimension and then fixed into a 24 well tissue culture plate with a silicone O-rings (Ace O-rings, Sigma Aldrich, Ireland). Glass coverslips (CTRL), also contained O-rings, were used as control. Sterilisation was conducted in 70 % IMS for 24 hours, followed with three washes in Hank’s Balanced Salt Solution (HBSS, Sigma Aldrich, Ireland), followed by ultraviolet irradiation for 1 hour.

### 2.2.5. Metabolic activity assessment

Cell metabolic activity was determined using alamarBlue® assay (Invitrogen™, BioScience, Dublin, Ireland), as per the manufactures protocol. Briefly, alamarBlue® dye was diluted in HBSS (Sigma Aldrich, Ireland) to a 10 % (v/v) solution. The media was removed and the cells were gently washed with HBSS and the alamarBlue® and HBSS solution was added to each well. The cells were incubated for 3 and 6 hours for fibroblast and macrophages respectively, and then the absorbance was measured at wavelengths of 570 nm and 600 nm using a microplate
reader (Varioskan Flash, Thermo Scientific, Ireland). The level of metabolic activity was calculated using the simplified method of calculating % reduction, according to the supplier’s protocol.

2.2.6. Cell viability assessment

Cell viability was assessed using lactate dehydrogenase (LDH; CytoTox 96®, Promega, MyBio Ltd., Ireland) assay. In brief, a standard curve was prepared form 0 to 50,000 cells and media samples containing released LDH were transferred to a 96 well plate, to which the reaction mixture was added. After 30 minutes incubation in dark, at room temperature, the stop solution was added and the absorbance was read at 490 nm using a microplate reader (Varioskan Flash, Thermo Scientific, UK).

2.2.7. Cell proliferation assessment

Cell proliferation was assessed using DNA quantification PicoGreen® (Invitrogen™, Bio-Science, Ireland) assay, as per the manufactures protocol. Briefly, the media was extracted from the samples and replaced with 200 µl water. Equal quantities of the samples and PicoGreen® dye were added to a 96 well plate and incubated in dark, at room temperature for 5 minutes. A standard curve of 0 to 500 ng/ml DNA was utilised. Samples and standards curves were read on a microplate reader (Varioskan Flash, Dublin, Thermo Scientific, UK) at 485/535 nm.

2.2.8. Statistical analysis

Numerical data are expressed as mean ± standard deviation (SD). Analysis was performed using statistical software (Minitab version 17, Minitab Inc., USA). One-way analysis of variance (ANOVA) for multiple comparisons and 2-sample t-test for
pair wise comparisons were employed after confirming the following assumptions: (a) the distribution from which each of the samples was derived was normal (normality test) and (b) the variances of the population of the samples were equal to one another (test for equal variances). Non-parametric statistics were used when either or both of the above assumptions were violated and consequently Kruskal-Wallis test for multiple comparisons or Mann-Whitney test for 2-samples were carried out. Statistical significance was accepted at $p < 0.05$. 

2.3. Results

2.3.1. Morphology assessment

The electrospun scaffolds accurately reproduced the macro porosity of the stainless-steel collectors across all porosities and pore shapes (Figure 2.1). Optimisation of the electrospinning process to achieve a porous construct will be further discussed in the appendix. The fibre diameter was not affected as a function of pore shape and porosity and was in the range of 1.0 µm and 1.6 µm (Figure 2.2).

Figure 2.1: Optical analysis indicates that the produced electrospun scaffolds accurately reproduced the morphology of the collectors. Scale bar: 5 mm. Porosity: 0\%, 30\%, 50\%, 70\%. Pore shape: C: circle, R: rhomboid, S: square.
Figure 2.2: Neither the pore shape (C: circle, R: rhomboid, S: square) nor the porosity (0 %, 30 %, 50 %, 70 %) affected fibre diameter, as evidenced by fibre diameter histograms. Sample size: 50.
2.3.2. Biophysical assessment

Ball burst (Figure 2.3) and tensile (Figure 2.4) tests yielded typical stress-strain curves of materials that yield and undergo plastic deformation. The 70 % R exhibited the lowest strain, stress and force at break values. Ball burst test (Table 2.1) revealed that the 0 % porosity scaffolds exhibited the highest strain, stress and force at break values and the 0 % porosity, and the 30 % circle exhibited the highest modulus values ($p < 0.001$). The lowest at strain, stress, force and modules values were obtained from the 70 % R, 70 % R and 70 % S, 70 % R and 70 % S, respectively ($p < 0.001$). Tensile testing (Table 2.1) revealed that the 0 % porosity exhibited the highest modulus values ($p < 0.001$). The 30 % C exhibited the highest stress, strain and force at break values ($p < 0.001$). The lowest at strain, stress, force and modules values were obtained from the 70 % R, 70 % C, 30 % S and 70 % C and 70 % C, respectively ($p < 0.001$).
Figure 2.3: Typical ball burst test stress-strain curves were obtained, independently of the porosity (0 %, 30 %, 50 %, 70 %) and pore shape (C: circle, R: rhomboid, S: square).
Figure 2.4: Typical tensile test stress-strain curves were obtained, independently of the porosity (0 %, 30 %, 50 %, 70 %) and pore shape (C: circle, R: rhomboid, S: square).
Table 2.1: Ball burst (sample size: 9) and tensile (sample size: 5) test mechanical data of electrospun scaffolds, as a function of porosity (0 %, 30 %, 50 %, 70 %) and pore shape (C: circle, R: rhomboid, S: square). Green background denotes highest values ($p < 0.001$), whist red background indicates lowest values ($p < 0.001$).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Thickness (mm)</th>
<th>Maximum Strain (%)</th>
<th>Maximum Stress (MPa)</th>
<th>Maximum Force (N)</th>
<th>E modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ball burst test</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 %</td>
<td>0.04 ± 0.02</td>
<td>9.06 ± 4.13</td>
<td>0.76 ± 0.17</td>
<td>0.58 ± 0.13</td>
<td>15.17 ± 3.82</td>
</tr>
<tr>
<td>30 % C</td>
<td>0.07 ± 0.02</td>
<td>6.71 ± 2.25</td>
<td>0.29 ± 0.13</td>
<td>0.47 ± 0.16</td>
<td>16.28 ± 6.97</td>
</tr>
<tr>
<td>50 % C</td>
<td>0.08 ± 0.03</td>
<td>5.61 ± 3.13</td>
<td>0.25 ± 0.05</td>
<td>0.52 ± 0.15</td>
<td>12.04 ± 2.91</td>
</tr>
<tr>
<td>70 % C</td>
<td>0.11 ± 0.03</td>
<td>4.10 ± 0.83</td>
<td>0.16 ± 0.04</td>
<td>0.42 ± 0.11</td>
<td>8.05 ± 2.65</td>
</tr>
<tr>
<td>30 % R</td>
<td>0.07 ± 0.02</td>
<td>5.13 ± 1.55</td>
<td>0.22 ± 0.09</td>
<td>0.38 ± 0.15</td>
<td>11.34 ± 5.77</td>
</tr>
<tr>
<td>50 % R</td>
<td>0.07 ± 0.03</td>
<td>6.02 ± 1.73</td>
<td>0.20 ± 0.07</td>
<td>0.33 ± 0.14</td>
<td>10.12 ± 3.47</td>
</tr>
<tr>
<td>70 % R</td>
<td>0.08 ± 0.01</td>
<td>3.72 ± 0.78</td>
<td>0.14 ± 0.07</td>
<td>0.25 ± 0.14</td>
<td>10.43 ± 7.19</td>
</tr>
<tr>
<td>30 % S</td>
<td>0.06 ± 0.02</td>
<td>7.34 ± 4.07</td>
<td>0.20 ± 0.07</td>
<td>0.32 ± 0.13</td>
<td>11.20 ± 5.55</td>
</tr>
<tr>
<td>50 % S</td>
<td>0.10 ± 0.06</td>
<td>5.99 ± 2.52</td>
<td>0.19 ± 0.21</td>
<td>0.41 ± 0.30</td>
<td>11.49 ± 12.28</td>
</tr>
<tr>
<td>70 % S</td>
<td>0.08 ± 0.02</td>
<td>6.33 ± 2.58</td>
<td>0.14 ± 0.06</td>
<td>0.29 ± 0.10</td>
<td>7.01 ± 2.96</td>
</tr>
<tr>
<td>0 %</td>
<td>0.09 ± 0.01</td>
<td>52.17 ± 16.55</td>
<td>0.45 ± 0.34</td>
<td>0.44 ± 0.03</td>
<td>7.95 ± 1.03</td>
</tr>
<tr>
<td>Tensile test</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 % C</td>
<td>0.10 ± 0.03</td>
<td>258.02 ± 15.42</td>
<td>0.76 ± 0.02</td>
<td>0.72 ± 0.22</td>
<td>2.25 ± 0.14</td>
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<tr>
<td>50 % C</td>
<td>0.12 ± 0.01</td>
<td>209.71 ± 28.02</td>
<td>0.52 ± 0.17</td>
<td>0.63 ± 0.21</td>
<td>1.19 ± 0.56</td>
</tr>
<tr>
<td>70 % C</td>
<td>0.27 ± 0.01</td>
<td>104.63 ± 71.11</td>
<td>0.18 ± 0.17</td>
<td>0.25 ± 0.20</td>
<td>0.36 ± 0.20</td>
</tr>
<tr>
<td>30 % R</td>
<td>0.08 ± 0.01</td>
<td>199.19 ± 35.06</td>
<td>0.60 ± 0.02</td>
<td>0.46 ± 0.04</td>
<td>1.35 ± 0.14</td>
</tr>
<tr>
<td>50 % R</td>
<td>0.10 ± 0.01</td>
<td>143.38 ± 26.37</td>
<td>0.33 ± 0.02</td>
<td>0.33 ± 0.02</td>
<td>1.84 ± 0.44</td>
</tr>
<tr>
<td>70 % R</td>
<td>0.10 ± 0.03</td>
<td>18.95 ± 3.42</td>
<td>0.57 ± 0.10</td>
<td>0.58 ± 0.08</td>
<td>4.42 ± 1.49</td>
</tr>
<tr>
<td>Sample</td>
<td>Thickness (mm)</td>
<td>Maximum Strain (%)</td>
<td>Maximum Stress (MPa)</td>
<td>Maximum Force (N)</td>
<td>E modulus (MPa)</td>
</tr>
<tr>
<td>--------</td>
<td>----------------</td>
<td>-------------------</td>
<td>---------------------</td>
<td>------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>30 % S</td>
<td>0.09 ± 0.01</td>
<td>87.58 ± 20.21</td>
<td>0.29 ± 0.01</td>
<td>0.25 ± 0.01</td>
<td>1.62 ± 0.43</td>
</tr>
<tr>
<td>50 % S</td>
<td>0.22 ± 0.01</td>
<td>239.86 ± 15.03</td>
<td>0.59 ± 0.04</td>
<td>1.30 ± 0.10</td>
<td>3.40 ± 0.66</td>
</tr>
<tr>
<td>70 % S</td>
<td>0.10 ± 0.07</td>
<td>131.48 ± 46.92</td>
<td>0.30 ± 0.10</td>
<td>0.29 ± 0.09</td>
<td>1.70 ± 1.08</td>
</tr>
</tbody>
</table>
2.3.3. Biological assessment

DF metabolic activity, viability and DNA concentration were not affected \( (p > 0.05) \) as a function of pore shape and porosity. However, the metabolic activity of all electro-spun samples and the CTRL are significantly elevated compared to the film at day 7 (Figure 2.5). With respect to THP-1 (Figure 2.6), the metabolic activity was significantly reduced \( (p < 0.001) \) at days 2 and 3 (in comparison to day 1) for all electrospun scaffolds, whilst no significant difference \( (p > 0.05) \) in viability and DNA concentration were observed, independently of the of pore shape and porosity.
Figure 2.5: Metabolic activity, viability and DNA concentration of DFs. Porosity: 0%, 30%, 50%, 70%. Pore shape: C: circle, R: rhomboid, S: square. (p > 0.05).
Figure 2.6: Metabolic activity, viability and DNA concentration of THP-1 cells. LPS addition is indicated with *. Porosity: 0 %, 30 %, 50 %, 70 %. Pore shape: C: circle, R: rhomboid, S: square. (p > 0.05).
2.4. Discussion

Traditional electrospinning technologies produce random or bidirectionally aligned fibrous mats, suitable for wound healing [39] or peripheral nerve [40], tendon [41] and cornea [42] repair and regeneration, respectively. Although studies have reported the development of controlled porosity electrospun scaffolds using melt electrospinning [43] or controlled porosity collectors [44, 45], no work has assessed in detail the influence of controlled porosity and pore shape, induced by predesigned collectors, on the properties of the derived electrospun scaffolds.

Structural analysis made apparent that the circular collectors resulted in electrospun scaffolds with very precise circular features. The accuracy was reduced when angular shapes (rhomboids and squares) were used. The lack of precision may be due to an imbalance in electrostatic attraction of the fibres to the charged collectors where an angle is present, as the distance to the fibre may not be equivalent, thereby attracting the fibre towards the nearest charged surface, potentially covering corners or edges [46]. In addition, all collectors produced fibrous mats with diameter range in the region of 1.0 µm to 1.6 µm, which is within the low inflammatory response fibre diameter region (500 nm to 2 µm) [47, 48].

Typical stress-strain curves of viscoelastic materials, such as extruded collagen fibres [49], tissue grafts [50], synthetic meshes [51] and electrospun scaffolds [52] that yield and undergo plastic deformation were obtained under ball burst and tensile tests. Under ball burst test, removal of material by introduction of porosity resulted in decreased mechanical properties, whilst under tensile deformation, the introduction of porosity, especially the 30 % circle, resulted in increased mechanical properties (stress, strain and force at break). Previous studies have demonstrated that by increasing the concentration of porogen, the mechanical properties of PCL-
hydroxyapatite composites were compromised, whilst larger porogen particle size led to increased tensile strength and reduction in Young’s modulus [53]. The compressive strength of PLGA scaffolds, fabricated by solvent casting/salt-leaching method, has also been shown to increase, as the pores became smaller [54]. The hardness and elastic modulus of macro-porous nickel materials increased as the pore size decreased, suggesting that ‘smaller is stronger’ due to the combined effects of sharing the load with more and smaller pore compartments and larger surface area to volume ratio [55].

The *in vitro* controls for the DF cells consisted of a general in vitro baseline control in the form of glass coverslips, a surface chemistry control in the form of the solvent cast PCL films, and a topographical and surface chemistry combined control in the form of the non-porous electrospun meshes. Utilising these control groups we were able to compare the implications of the electrospun meshes to planar. The THP-1 cells had additionally positively primed pro-inflammatory controls using LPS to allow for comparative inflammatory analysis of the samples. With respect to biological analysis, not only porosity and pore shape did not affect dermal fibroblast function, but also all electrospun scaffolds and the CTRL samples consistently exhibited higher metabolic activity than films. This is in agreement with previous publications [56-61], where electrospun fibrous scaffolds, by imitating the native extracellular matrix structural milieu, maintained cellular function *ex vivo*. As far as the THP-1 response, we observed that the viability and DNA concentration were not altered as a function of time in culture for all electrospun scaffolds, whilst the metabolic activity was reduced. In contrast, cells seeded on glass coverslips, glass coverslips and LPS and PCL films, consistently increase metabolic activity and DNA concentration, suggesting inflammatory response. This is in accordance to
previous publications [62, 63], where electrospun scaffolds have been shown to modulate immune response. Further, other studies have shown that electrospun scaffolds suppress immune response, in comparison to films made from the same material, whilst fibre diameter, primarily, and fibre alignment, secondary, have been also shown to influence in vitro macrophage activation and secretion of pro-inflammatory molecules (nano-fibrous scaffolds minimised inflammatory response when compared to micro-fibrous scaffolds and films) [64]. Other studies have also shown a correlation between increased fibre / pore size and increased expression of M2 markers and decreased expression of M1 markers and increased secretion of angiogenic cytokines at scaffolds with large fibre / pore size [65]. This is in agreement with other studies, were thick fibres tended to polarise into the immunomodulatory and tissue remodelling (M2) phenotype, whilst thin fibres expressed pro-inflammatory (M1) phenotype [66]. Overall, the work presented herein, offers opportunities for tailoring mechanical properties to specific tissue engineering applications by controlling the architectural features of the scaffold and by reducing the amount of material used, it complies with lean manufacturing principles [67] and strategies to control immune response [68, 69].
2.5. Conclusion

Development of reproducible ways to fabricate electrospun scaffolds with controlled architectural features will substantially increase applications of electrospinning in regenerative medicine. Pre-designed collectors offer the opportunity to tailor the mechanical properties of the produced scaffolds, as a function of porosity and pore shape, and to design tissue-specific implantable devices. Indeed, the adjustable nature of porosity and pore shape can customise the construct between multi-axial tensile requirements, such as hernia repair and wound healing, or uniaxial applications, such as tendon and neural repair. Concurrently, the fibrous nature of the produced constructs promotes fibroblast growth, whilst hindering inflammatory response, as compared to planar substrates. Overall, this study opens up new avenues in the rational design of electrospun scaffolds for tissue engineering applications.
2.6 References


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63. Toncheva, A., Paneva, D., Manolova, N., Rashkov, I., Electrospun Poly (L-Lactide) Membranes Containing a Single Drug or Multiple Drug System for


Chapter 3 - Multi-step porous electrospinning

Sections of this chapter are under submission at:

3.1. Introduction

Electrospun scaffolds are under intense investigation for multitude clinical indications [1-3]. However, the high mechanical loads often required by most tissues restrict their clinical translation and commercialisation. Multiple methods have been employed during or post fabrication to increase the mechanical resilience of electrospun scaffolds with variable level of success. For example, the use of carbon nano-tubes or cellulose nano-crystals during the fabrication process has been shown to significantly increase the mechanical properties of the produced scaffolds [4-7]. Post-fabrication methods, such as solvent assisted compression moulding, cross-linking, stretching and drawing have also been shown to increase the interfibrous bonding and to improve the molecular orientation and crystallinity, resulting in enhanced mechanical properties [8-13]. However, multi-component approaches hinder regulatory approval, chemical cross-linking approaches are often cytotoxic and stretching induces bi-directional fibre orientation that jeopardises elasticity and multi-axial mechanical properties and reduces fibre diameter that impairs cell attachment and function [14-16]. Evidently, it is imperative to engineer electrospun scaffolds that would meet the high mechanical requirements of target tissues.

Another important parameter in the development of implantable electrospun meshes is the introduction of controlled porosity. Pore size and pore shape have been shown to control cell function in vitro and to modulate tissue integration in vivo [17-19]. Although low or high in porosity electrospun meshes can be developed via low and high flow rate, respectively [20], controlled pore size and pore shape can be introduced either by electrospinning onto collectors with controlled porosity or by laser cutting the electrospun meshes post-fabrication [21-23]. However, inevitably, the introduction of porosity affects the overall structural integrity of the mesh [23,
24], imposing the need to engineer porous electrospun scaffolds that would meet the high mechanical requirements of target tissues.

Herein, we hypothesised that electrospun meshes with adequate mechanical properties can be developed through layer stacking and compression. We subsequently assessed the influence of porosity on the mechanical properties of the produced scaffolds.
3.2. Material and methods

3.2.1 Scaffold fabrication

Electrospinning was performed as previously described [23, 25-27]. Briefly, 200 mg/ml of poly-ε-caprolactone (PCL, PURASORB® PC12, IV 1.2 dl/g, 70,000 MW, Corbion, The Netherlands) were dissolved in 2,2,2-trifluoroethanol (TFE; 99.8 %, Acros Organics, Ireland) and was extruded at 100 µl/min from three syringes simultaneously through an 18 G stainless steel blunt needle (EFD Nordson, Dublin, Ireland). Upon application of high voltage (20 kV) between the needle and the collector (20 cm distance), the solvent evaporated, and the fibres were collected on a rotating mandrel [60 revolutions per minute (RPM)]. Single, double and triple layer electrospun scaffolds, stacked perpendicularly and parallel to each other to avoid bias introduced by the relative position to the collector mandrel and the rotating speed of the mandrel (Figure 3.1), were then compressed at 282 N/cm for 20 minutes at 25 °C (Proxy Biomedical, Ireland). 30 % circular porosity was then introduced through laser cutting. Solvent cast films, acted as control, were also fabricated by pouring a PCL solution onto a level polytetrafluoroethylene (PTFE) mould and allowing to evaporate overnight at ambient temperature and pressure.
Figure 3.1: Fabrication process of the layered and compressed electrospun meshes.
3.2.2 Morphology assessment

Gross visual observations were performed with a stereomicroscope (Nikon, Japan). For finer details, the electrospun scaffolds were mounted onto a carbon disk, gold sputter coated and imaged with a Hitachi S-4700 scanning electron microscope (Hitachi High-Technologies Europe GmbH, Germany). Fibre diameter analysis was conducted using NIH ImageJ software (N=100).

3.2.3 Mechanical properties assessment

Mechanical properties were assessed via ball burst test, using a Zwick/Roell (Leominster, UK) Z005 testing machine, loaded with 1 kN load cell, as described previously [23, 28, 29]. The ball burst samples were prepared in accordance with ASTM D3787-15. Prior to testing, all samples were incubated overnight at room temperature in phosphate buffered saline (PBS), immediately prior to testing tissue paper was used to remove excess PBS. The sample thickness was measured using digital callipers (Scienceware®, Digi-Max™, Sigma-Aldrich, Ireland). The ball burst samples were placed between two layers of vulcanised rubber, and subsequently placed between the appropriate sample grips and hand tightened around the circumference and the extension rate was 20 mm/min. Five replicates were used throughout. The following definitions were used to calculate mechanical data: stress at break was defined as the load at failure divided by the original cross-sectional area (engineering stress) and strain at break was defined as the increase in scaffold length required to cause failure divided by the original length.
3.2.4 Cell culture

Dermal fibroblasts (DF), passage 6 - 9, were cultured in Dulbecco’s Modified Eagle’s Medium (high glucose, Sigma Aldrich, Ireland) with 10 % foetal bovine serum (FBS, Sigma Aldrich, Ireland) and 1 % penicillin streptomycin (PS, Sigma Aldrich, Ireland) at a cell density of 30,000 cells/well for 1, 3 and 7 days on Nunc™ non-treated flasks (Thermo Scientific, Ireland). The cells were maintained at 37 °C in a humidified 5 % CO₂ incubator. The meshes were cut to dimension and then fixed into a 24 well tissue culture plate with a silicone O ring (Ace O-rings, Silicon from Sigma). Glass coverslips (CTRL) also contained O rings. Sterilisation was conducted through immersion in 70 % industrial methylated spirit (IMS) for 24 hours, then rinsed three times in Hanks balanced salt solution (HBSS, Sigma Aldrich, Ireland), followed by ultraviolet light for 1 hour.

3.2.5 Cell metabolic activity assessment

The metabolic activity was determined using the alamarBlue® assay (Invitrogen™, Bio-Science, Ireland), as per manufacturer’s protocol. Briefly, alamarBlue® dye was diluted in HBSS to a 10 % (v/v) solution. The media were removed, and the cells were gently washed with HBSS and the alamarBlue® solution was added to each well. The cells were incubated for 3 hours and the absorbance was measured at wavelengths of 570 nm and 600 nm using a microplate reader (Varioskan Flash, Thermo Scientific, Ireland). The level of metabolic activity was calculated using the simplified method of calculating % reduction, according to the supplier’s protocol.
3.2.6 Cell viability assessment

Cell viability was assessed using quantification of lactate dehydrogenase (LDH) assay (CytoTox 96®, Promega, UK), as per manufacturer’s protocol. In brief, a standard curve was prepared from 0 to 50,000 cells and samples’ media containing released LDH was transferred to a 96 well plate, to which the reaction mixture was added. After 30 minutes incubation in dark, at room temperature, the stop solution was added and the absorbance was read at 490 nm using a microplate reader (Varioskan Flash, Thermo Scientific, UK).

3.2.7 Cell proliferation assessment

Cell proliferation was assessed with DNA quantification through PicoGreen® assay (Invitrogen™, Bio-Science, Ireland), as per manufacturer’s protocol. Briefly, the media was extracted from the samples and replaced with 200 µl of water, which was frozen at the appropriate time points and freeze / thawed three times. Equal quantities of the samples and PicoGreen® dye were added to a 96 well plate and incubated in dark at room temperature for 5 minutes. A standard curve of 0 to 500 ng/ml of DNA was utilised. Samples and standards were read using a microplate reader (Varioskan Flash, Thermo Scientific, UK) at 485 / 535 nm.

3.2.8 Statistical analysis assessment

Statistical evaluation was conducted using Minitab® (Minitab® version 17, Minitab® Inc., USA). One way analysis of variance (ANOVA) with a Tukey post-hoc test for multiple comparisons were employed after confirming the following assumptions: (a) the distribution from which each of the samples was derived was normal (normality test); and (b) the variances of the population of the samples were
equal to one another (test for equal variances). Non-parametric statistics were used when either or both of the above assumptions were violated and consequently Kruskal-Wallis test for multiple comparisons or Mann-Whitney test for 2-samples were carried out. Numerical data is expressed as mean ± SD. Statistical significance was accepted at $p \leq 0.05$. 
3.3. Results

Compression of single layer electrospun meshes significantly \((p < 0.001)\) increased force, strain and stress at break, whilst it significantly \((p < 0.001)\) reduced thickness (Table 3.1). The force at break, the stress at break and the thickness of compressed electrospun meshes were significantly \((p < 0.001)\) increased, whilst the strain at break did not appear to change as the layers were increased from 1 to 2 and from 2 to 3 (Table 3.1). The orientation of the layers did not appear to affect \((p > 0.05)\) the mechanical properties of the compressed electrospun meshes (Table 3.1).

30 % circular porosity significantly \((p < 0.001)\) decreased force and stress at break, significantly \((p < 0.001)\) increased strain at break and did not affect \((p > 0.05)\) the thickness of compressed electrospun meshes of same number of layers (Table 3.2). Compression and porosity did not appear to affect the overall macroscopic appearance of the electrospun scaffolds and their fibrillar structure was maintained (Figure 3.2).

However, the laser ablation procedure there are truncated the electrospun fibres terminating in the pore with localised melt back (data not shown). In vitro cell response was analysed using dermal fibroblasts on all compressed and porous groups and a smooth film (Figure 3.3). Initially, all groups presented similar metabolic activity to the tissue culture plastic control group (CTRL) but, at days 3 and 7, the porous meshes exhibited significantly higher metabolic activity than non-porous ones \((p>0.05, \text{Figure 3.3A})\). Cell viability was not significantly affected by the increase of PCL sheet layers or introduction of porosity \((p>0.05, \text{Figure 3.3B})\).

Regarding cell proliferation, electro-spun meshes presented lower DNA concentration than CTRL at days 3 and 7 \((p>0.05, \text{Figure 3.3C})\). However, also at
days 3 and 7, all compressed (C) samples were significantly lower than compressed and porous (C-P) meshes.
Table 3.1: Mechanical properties of different layered and compressed electrospun meshes determined by ball burst testing. All compressed electrospun meshes exhibited significantly higher ($p < 0.001$; indicated with *) stress at break values than the non-compressed meshes. The force at break increased proportionally to the number of layers. N = 5.

<table>
<thead>
<tr>
<th>Compressed</th>
<th>Number of Layers</th>
<th>Layer orientation</th>
<th>Force (N)</th>
<th>Strain (%)</th>
<th>Stress (MPa)</th>
<th>Thickness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>1</td>
<td>-</td>
<td>122.8 ± 40.1</td>
<td>28.30 ± 6.85</td>
<td>1.52 ± 0.50</td>
<td>1.27 ± 0.00</td>
</tr>
<tr>
<td>Yes</td>
<td>1</td>
<td>-</td>
<td>159.6 ± 35.0</td>
<td>32.44 ± 8.05</td>
<td>5.51 ± 0.97*</td>
<td>0.45 ± 0.03</td>
</tr>
<tr>
<td>Yes</td>
<td>2</td>
<td>┴</td>
<td>253.9 ± 36.0</td>
<td>25.16 ± 0.95</td>
<td>5.80 ± 0.79*</td>
<td>0.69 ± 0.02</td>
</tr>
<tr>
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<td>2</td>
<td>┴</td>
<td>259.7 ± 38.0</td>
<td>27.43 ± 4.48</td>
<td>5.78 ± 0.98*</td>
<td>0.71 ± 0.02</td>
</tr>
<tr>
<td>Yes</td>
<td>3</td>
<td>┴</td>
<td>415.9 ± 61.7</td>
<td>25.43 ± 1.00</td>
<td>6.11 ± 0.65*</td>
<td>1.17 ± 0.07</td>
</tr>
<tr>
<td>Yes</td>
<td>3</td>
<td>┴</td>
<td>440.0 ± 46.0</td>
<td>32.64 ± 9.65</td>
<td>6.70 ± 0.50*</td>
<td>1.03 ± 0.04</td>
</tr>
</tbody>
</table>
Table 3.2: Mechanical properties of layered and compressed electrospun meshes before and after introduction of 30 % circular porosity determined by ball burst test. Three-layers, compressed and with 30 % circular porosity electrospun meshes exhibited force and strain at break values similar to one layer, compressed and 0 % porosity electrospun meshes, which are similar to commercially available scaffolds. N = 5.

<table>
<thead>
<tr>
<th>Number of Layers</th>
<th>Force (N)</th>
<th>Strain (%)</th>
<th>Stress (MPa)</th>
<th>Thickness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – 0 % Porosity</td>
<td>159.6 ± 35.0</td>
<td>32.44 ± 8.05</td>
<td>5.51 ± 0.97</td>
<td>0.45 ± 0.03</td>
</tr>
<tr>
<td>1 – 30 % Porosity</td>
<td>62.56 ± 9.10</td>
<td>45.70 ± 2.12</td>
<td>2.08 ± 0.12</td>
<td>0.47 ± 0.05</td>
</tr>
<tr>
<td>2 – 0 % Porosity</td>
<td>259.7 ± 38.0</td>
<td>27.43 ± 4.48</td>
<td>5.78 ± 0.98</td>
<td>0.71 ± 0.02</td>
</tr>
<tr>
<td>2 – 30 % Porosity</td>
<td>116.03 ± 12.03</td>
<td>41.54 ± 1.69</td>
<td>2.19 ± 0.26</td>
<td>0.84 ± 0.06</td>
</tr>
<tr>
<td>3 – 0 % Porosity</td>
<td>440.0 ± 46.0</td>
<td>32.64 ± 9.65</td>
<td>6.70 ± 0.50</td>
<td>1.03 ± 0.04</td>
</tr>
<tr>
<td>3 – 30 % Porosity</td>
<td>141.32 ± 33.14</td>
<td>38.03 ± 2.16</td>
<td>2.14 ± 0.43</td>
<td>1.03 ± 0.03</td>
</tr>
</tbody>
</table>
Figure 3.2: Compression and introduction of porosity did not affect the macro-structure (A; 20X, scale bars = 2 mm) and the micro-structure (B; 1000X, scale bars = 50 µm) of the electrospun meshes.
Figure 3.3: Human dermal fibroblast metabolic activity (a), viability (b) and proliferation (c) were not affected ($p > 0.05$) as a function of layering, compression and introduction of porosity. Compressed: C, compressed and porous: C-P, 1 layer: 1L, 2 layers: 2L, 3 layers: 3L. All data were normalised to CTRL (glass coverslip) at day 1.
3.4. Discussion

Although electrospinning is widely used in tissue engineering and regenerative medicine (6103 papers are available; Source: PubMed, Term Searched: electrospinning, Date: 08/07/2018), it has not been translated to clinical practice as the produced scaffolds are of low mechanical resilience and not adequate porosity. Herein, we ventured to assess the influence of compression and porosity on the structural, mechanical and biological properties of electrospun scaffolds.

Two- and three- layers electrospun scaffolds were fabricated via compression and macro- and micro- scopic analysis revealed that the fibrous sub-structure was not affected. This is of particular importance as heat compression treatments, compression moulding and solvent-assisted compression moulding are often associated with fibre fusion and damage or loss of the three-dimensional fibrous structure [8, 30, 31].

Compression increased the mechanical properties of the scaffolds, as compared to the non-compressed ones, and layering led to a proportional increase in the mechanical properties. This is in agreement with previous observations, where layered electrospun materials had significantly higher maximum stress than single layer materials [32]. Layering is commonly used with electrospun materials for vascular grafts [33], tendon repair [34] and abdominal mesh applications [35]. However, this is usually achieved through sequential electrospinning of different synthetic and natural material or through the combination of layers with different characteristics (e.g. woven and non-woven or nano- and micro- fibres) [34, 36-38].

The subsequently introduced porosity to the layered and compressed meshes resulted in decreased force and stress at break and increased strain at break. It is worth noting though that the porous three-layer electrospun mesh reached properties similar to
commercially available meshes (e.g. monofilament knitted polypropylene meshes have force at break in the region of 150 N). this is of significant importance as porosity modulates tissue integration and foreign body response after implantation [39]. Further, micro-porous structures have been associated with an elevated risk of encapsulation, which causes pain and impedes normal abdominal movements [40, 41]. On the other hand, macro-porosity has been linked to reduced fibrosis and foreign body response in mouse and rat subcutaneous models [42-44].

Having successfully fabricated meshes with clinically relevant mechanical properties, we proceeded to assess their cytocompatibility. There were minimal differences in cell viability, metabolic activity and proliferation between the different experimental groups, which is expected from PCL considering its wide use in tissue engineering and regenerative medicine and acceptable environmental fate [45-49].

Compressed meshes samples had the lower metabolic activity levels, similarly to the films, and the lowest DNA concentration, suggesting the compression compromises the advantages of the biomimetic surface topography. However, the introduction of porosity significantly increased the metabolic activity to comparable levels to the CTRL samples and both metabolic activity and DNA concentration were equivalent or higher than the non-compressed meshes.
3.5. Conclusion

The purpose of this study was to develop porous electrospun meshes with mechanical properties suitable for biomedical applications. Three layers compressed electrospun meshes with 30% circular porosity exhibited mechanical properties similar to commercially available meshes without any negative effect in fibre morphology and cytocompatibility. This study paves the path for wider acceptance, clinical translation and commercialisation of electrospun meshes.
3.6 References


7. Chuenjitkuntaworn, B., Inrung, W., Damrongsri, D., Mekaapiruk, K., Supaphol, P., Pavasant, P., Polycaprolactone/Hydroxyapatite Composite
Chapter 3 – Multi-step porous electrospinning


46. Lau, C.L., Kovacevic, M., Tingleff, T.S., Forsythe, J.S., Cate, H.S., Merlo, D., Cederfur, C., Maclean, F.L., Parish, C.L., Horne, M.K., Nisbet, D.R., Beart, P.M., 3D Electrospun Scaffolds Promote a Cytotrophic Phenotype of


Chapter 4 - *In vivo* characterisation

Sections of this chapter are under submission at:

4.1. Introduction

Electrospun meshes are extensively used in biomedicine due to their controllable mechanical properties and immune response and their ability to promote cell adhesion and directional migration / growth; to either maintain cell phenotype or to control cell differentiation; and to direct neotissue formation [1-4]. Additionally, numerous methods have been developed to functionalise electrospun meshes (e.g. blending, multi-layering, dual electrospinning, co-axial electrospinning, electrospinning-co-electrospraying) enabling them that way to deliver bioactive / therapeutic cargos in a controlled and localised manner [5-9]. Poly-$\varepsilon$-caprolactone (PCL) is a biodegradable hydrophobic semi-crystalline polyester commonly used in the biomedical applications due to its plasticity, ductility, slow degradation rate, relative cheap manufacturing cost and FDA approval [10, 11]. Environmental fate analysis has shown that electrospun meshes are transferred to amended soils [12], whilst in vitro PCL degradation has been shown to be a two-stage process: the first involves a non-enzymatic hydrolytic cleavage of the ester groups and the second initiates when the polymer becomes more crystalline and of low ($\leq$3,000 kDa) molecular weight (primarily intracellular degradation by macrophages and giant cells and to a lesser extent by fibroblasts) [13]. Yet again, the relatively low mechanical properties of electrospun meshes in combination with their dense structure have jeopardised their wide acceptance and use in clinical practice. Considering that the dimensionality and the shape of pores of hernia meshes have been shown to modulate tissue integration in vivo [14, 15], recent efforts are directed towards the development of mechanically resilient and micro-porous electrospun meshes. Although electrospun meshes with controlled porosity can now be developed [16-19], the introduction of porosity affects the overall structural integrity of the mesh
[19, 20]. Using compression and thermal ablation, we have developed electrospun meshes with circular pores and 30% porosity with clinically relevant mechanical properties [19, 21]. However, due to the hydrophobicity [22] and surface chemistry [23] of PCL, cell adhesion and the subsequent *in vitro* and *in vivo* response can be impeded [23-26]. Therefore, functionalisation with collagen (COL) is commonly used, considering that COL is the most abundant extracellular matrix (ECM) protein and it is favoured for biomedical applications [27]. One should note though that electrospun COL alone scaffolds are not suitable for biomedical applications due to their poor mechanical properties [28]. Electrospun co-extruded COL / PCL scaffolds are also not suitable, as the addition of COL has been shown to significantly weaken the overall tensile properties in comparison to PCL alone scaffolds [28, 29]. Further, the fluoroalcohols that are customarily used in the electrospinning process have been shown to irreversibly denature COL [30, 31]. Post-fabrication COL coating not only does not compromise mechanical integrity and does not denature collagen, but it has also been shown to promote cell response [32-34].

Considering the above, herein we developed electrospun macro-porous PCL meshes, which were subsequently coated with human recombinant collagen type I (hR COL) or bovine Achilles tendon collagen type I (BAT COL). The produced scaffolds were assessed *in vitro* (morphological, mechanical and cytocompatibility analyses) and *in vivo* (rat subcutaneous implantation).
4.2. Material and Methods

4.2.1 Scaffolds fabrication

Electrospinning was performed as has been described previously [19, 21, 35-37] with slight modifications. Briefly, 200 mg/ml of PCL (PURASORB® PC12, IV 1.2 dl/g, 70,000 MW, Corbion, The Netherlands) were dissolved in 2,2,2-trifluoroethanol (TFE; 99.8 %, Acros Organics, Ireland) and were extruded at 100 µl/min from three syringes simultaneously through an 18 G stainless steel blunt needle (EFD Nordson, Dublin, Ireland). Upon application of high voltage (20 kV) between the needle and the collector (20 cm distance), the solvent evaporated and the fibres were collected on a rotating mandrel [60 revolutions per min (RPM)]. The electrospun scaffolds were compressed at Proxy Biomedical (Spiddal, Ireland) at 282 N/cm for 20 min at a temperature of 25 °C. Some compressed electrospun scaffolds were coated with 5 mg/ml hR COL (CollPlant, Israel) or BAT COL (Vornia Biomaterials, Ireland). After coating, the scaffolds were allowed to air dry in a laminar flow hood at room temperature (RT) overnight and then were crosslinked with 1-ethyl-3-[3-dimethy-aminopropyl]carbodiimide (EDC): N-hydroxysulfosuccinimide (NHS) in 0.05M 2-(N-morpholino)ethanesulfonic acid (MES) buffer at pH 5.5 in a 3:1:5 ratio respectively, as has been described before [38-40]. The following day, three phosphate buffered saline (PBS) washes were carried out and then the meshes were allowed to dry at RT. Electrospun scaffolds (coated and uncoated) were then laser cut to introduce porosity (circular pores, 30 % porosity).

4.2.2 Morphology assessment

Gross visual observations were performed with a stereomicroscope (Olympus, Japan). For finer details, the electrospun scaffolds were mounted onto a carbon disk,
gold sputter coated and imaged with a Hitachi S-4700 scanning electron microscope (Hitachi High-Technologies Europe GmbH, Germany).

### 4.2.3 Mechanical properties assessment

Mechanical properties were assessed via a ball burst test, using a Z005 Zwick/Roell (Leominster, UK) testing machine, loaded with 1 kN load cell. The samples were prepared as per ASTM D3787-15 guidelines. Prior to testing, all samples were incubated overnight at RT in PBS and immediately prior to testing, tissue paper was used to remove excess PBS. The samples were placed between two layers of vulcanised rubber and subsequently placed between the appropriate sample grips and hand tightened around the circumference. The extension rate was 20 mm/min. The following definitions were used to calculate mechanical data: stress at break was defined as the load at failure divided by the original cross-sectional area (engineering stress), strain at break was defined as the increase in scaffold length required to cause failure divided by the original length and modulus was defined as the ratio of stress to strain.

### 4.2.4 Cell culture

Dermal fibroblasts (DF), passage 6 - 9, were cultured in Dulbecco’s Modified Eagle’s Medium (high glucose, Sigma Aldrich, Ireland) with 10 % foetal bovine serum (FBS, Sigma Aldrich, Ireland) and 1 % penicillin streptomycin (PS, Sigma Aldrich, Ireland) at a cell density of 30,000 cells/well for 1, 3 and 7 days on Nunc™ non-treated flasks (Thermo Scientific, Ireland). The cells were maintained at 37 °C in a humidified 5 % CO₂ incubator. The meshes were cut to dimension and then fixed into a 24 well tissue culture plate wells with a silicone O ring (Ace O-rings,
Sigma Aldrich, Ireland), the glass coverslips (CTRL) also contained O rings. Sterilisation was conducted through immersion in 70 % industrial methylated spirits (IMS) for 24 hrs, then rinsed three times in Hanks balanced salt solution (HBSS, Sigma Aldrich, Ireland), followed by ultraviolet light for 1 hrs.

4.2.5 Cell viability assessment

Cell viability was assessed using quantification of lactate dehydrogenase using supplier’s protocol (LDH; CytoTox 96®, Promega, MyBio Ltd.). In brief, a standard curve was prepared from 0 to 50,000 cells and samples media containing released LDH were transferred to a 96 well plate, to which the reaction mixture was added. After incubating for 30 min in the dark, at room temperature, the stop solution was added and the absorbance was read at 490 nm using a microplate reader (Varioskan Flash, Thermo Scientific, UK).

4.2.6 Cell proliferation assessment

Cell proliferation was assessed with DNA quantification through PicoGreen® (Invitrogen™, Bio-Science, Ireland) as per manufacturer’s protocol. Briefly, the media was extracted from the samples and replaced with 200 µl of water which was frozen at the appropriate time points and freeze / thawed three times. Equal quantities of the samples and PicoGreen® dye were added to a 96 well plate and incubated in the dark at room temperature for 5 min. A standard curve of 0 to 500 ng/ml of DNA was utilised. Samples and standards curves were read on a microplate reader (Varioskan Flash, Thermo Scientific, UK) at 485 / 535 nm.
4.2.7 Animal study

Adult male Sprague Dawley rats (Charles River Laboratories, France), weighting 291 - 332 g at T0, were used for subcutaneous implantation procedure. The animals were feed *ad libitum*; animal husbandry complied with the European directive 2010/63/EU. The facility where the procedure was conduct was a NAMSA facility, which is registered with the French Department of Agriculture. Prior to implantation, the fur of the rats was clipped, and the area of implantation was cleaned with 70% isopropyl alcohol (Laboratoire Pharmaceutique Galénique, France) and povidone iodine (Vetedine® savon, Vetoquinol, France). A neutral veterinary ophthalmic ointment (Ocrygel®, TVM, France) was applied to the eyes to prevent drying and the rats were placed in ventral recumbency. The protocol was approved by the NAMSA Ethical Committee before the beginning of the study. The rats were anesthetised by inhalation of an O₂–isoflurane mixture (IsoFlo®, Axience, France). Four incisions (two per side) large enough to accommodate each scaffold were made through the skin and parallel to the vertebral column (Figure 4.1). Pockets placed at appropriately spaced intervals were formed by blunt dissection of the subcutaneous tissues. COL coated or uncoated scaffolds that had been sterilised with ethylene oxide were introduced in each pocket and the skin was closed with stainless steel wound clips (Figure 4.2). The rats were observed until recovery from the anaesthetic procedure and then returned to their respective cages.
Figure 4.1: The four skin incisions large enough to accommodate each scaffold parallel to the vertebral column.
Figure 4.2: The subcutaneous impanation procedure performed. Representative skin incisions (A). Creation of the pocket by blunt dissection in the subcutaneous tissue (B). Implantation of the electrospun meshes (C). Visual inspection before skin closure (D). Closure of the site with stainless steel wound clips (E).
At 10-, 28- and 56-days post-implantation, the designated rats were anesthetised with an intra-muscular injection of tiletamine-zolazepam (Zoletil® 100, Virbac, France), weighed and sacrificed by a lethal intravenous injection of pentobarbital (Dolethal, Vetoquinol, France). The subcutaneous tissues were then macro-scopically examined and excised, allowing a sufficient area around the site for proper histologic preparation. Any gross changes in tissues surrounding the implant were recorded using the following parameters: size, shape, colour, consistency, distribution, presence/absence of implant encapsulation, tissue integration, degradation and any other observations, as appropriate. Macro-scopic pictures of each implanted site were taken. The sites were collected including epidermis, dermis, panniculus carnosus and the subcutaneous tissue surrounding the implant with at least 5 mm excess all around.

4.2.8 Histopathologic, histologic and histomorphometric analyses

Samples for histopathologic analysis from each site were fixed in 10 % neutral buffered formalin (NBF, Sigma Aldrich, France), as was a spleen biopsy from one rat (control tissue marking to serve as a positive control for Picrosirius Red (PR) Staining. The draining axillary and inguinal lymph nodes were excised, macro-scopically examined and any gross changes of the lymph nodes were recorded using the following parameters: size, shape, colour, consistency, distribution and any other observations, as appropriate. The lymph nodes were fixed in 10 % NBF. After 24 h to 72 h fixation in 10 % NBF, the implanted together with a piece of each unimplanted sample (T0) were dehydrated in alcohol solutions of increasing concentration, cleared in xylene and embedded in paraffin. Two central longitudinal cranio-caudal cross sections (4-7 µm thickness) were prepared for each site using a
microtome (MICROM®️, France). Sections were stained with modified Masson’s Trichrome (MT, Sigma Aldrich, France) (Figure 4.3). Two additional serial central longitudinal cross-sections per sample were prepared for histomorphometric analysis: one section was stained with PR and the other one was stained with Feulgen & Rossenbeck (F&R) stain.

Histologic sections of the pieces of the unimplanted articles were prepared as described above for structural characterization and comparison with the implanted materials.
Figure 4.3: Scheme of histologic sections.
Histopathologic analysis focused on the following parameters, which were graded according to pathology report: inflammatory cells, necrosis, fibrosis, neovascularisation, fatty infiltrate, fibrin, haemorrhage, cell or tissue degeneration, fibroplasia, tissue integration, tissue ingrowth, encapsulation and scaffold degradation, using the scoring system outlined (Table 4.1). Example of the scoring system are indicated (Figure 4.4).
<table>
<thead>
<tr>
<th>Cell type/Response</th>
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</tr>
</thead>
<tbody>
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<td></td>
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<tr>
<td>Macrophages</td>
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<tr>
<td>Lymphocytes</td>
<td>0</td>
</tr>
<tr>
<td>Plasma cells</td>
<td>0</td>
</tr>
<tr>
<td>Polymorphonuclear cells</td>
<td>0</td>
</tr>
<tr>
<td>Giant cells</td>
<td>0</td>
</tr>
<tr>
<td>Necrosis</td>
<td>None</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>None</td>
</tr>
<tr>
<td>Neovascularization</td>
<td>None</td>
</tr>
<tr>
<td>Fatty infiltrate</td>
<td>None</td>
</tr>
<tr>
<td>Fibrin</td>
<td>None</td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>None</td>
</tr>
<tr>
<td>Cell or tissue degeneration</td>
<td>None</td>
</tr>
<tr>
<td>Fibroplasia</td>
<td>None</td>
</tr>
<tr>
<td>Tissue integration</td>
<td>None</td>
</tr>
<tr>
<td>Tissue ingrowth</td>
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</tr>
<tr>
<td>Material degradation</td>
<td>None</td>
</tr>
<tr>
<td>Encapsulation</td>
<td>None</td>
</tr>
<tr>
<td>Mast cells</td>
<td>0</td>
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</tbody>
</table>
Figure 4.4: Grading system examples.
Histomorphometric analysis used a region of interest (ROI) (4 x 8 mm length x width) encompassing the scaffolds and located in the centre or near the centre of the site was determined to measure the tissue in growth rate and quality of the collagen. A light microscope (Nikon Eclipse 80i, Japan) equipped with an image analyser system (Tribun, France, IPS version 4.06) was used for the analysis.

4.2.9 Collagen quality and tissue ingrowth assessment

To measure the tissue in growth rate and quality of the collagen, the following parameters were measured: the total collagen content percentage within the implanted scaffolds was determined with the PR stained sections via quantitatively analysis. Results were expressed in percent of total collagen area measured within the ROI. Collagen polymorphism (COL I and COL III), using PR stained sections, were assessed by cross-polarisation microscopy (CPM). Under CPM, a section of the rat spleen was used as the positive control to accurately set the angle of polarisation before measurement of the COL III area and COL I was confirmed with the spleen using the same angle of polarisation to analyses the quantitative histomorphometric. Results were expressed as the ratio of the COL I and COL III surface area. The F&R stained slides were used to determine the cell area density (%) corresponding to the cell area (µm²) / ROI area (µm²) x 100. The tissue in growth rate was defined by the total collagen content plus the cell area density (%).

4.2.10 Statistical analysis

Statistical evaluation was conducted using Minitab® (*in vitro* data analysis) (Minitab® version 17, Minitab® Inc., USA) or SPSS (*in vivo* data analysis) (Software SPSS version 19.0, SPSS Inc.). One way analysis of variance (ANOVA)
with a Tukey’s post-hoc test for multiple comparisons were employed after confirming the following assumptions: (a) the distribution from which each of the samples was derived was normal (normality test); and (b) the variances of the population of the samples were equal to one another (test for equal variances). Non-parametric statistics were used when either or both of the above assumptions were violated and consequently Kruskal-Wallis test for multiple comparisons or Mann-Whitney test for 2-samples were carried out. The local tissue effects, integration and the degradation evaluations were based on qualitative macroscopic observation of the implanted sites at termination. Qualitative and semi-quantitative histopathologic evaluation of the mean score comparison of the semi-quantitative parameters and comparison of the qualitative histopathologic findings. Quantitative histomorphometric analysis of the collagen content, cellularity and tissue ingrowth rate. The histomorphometric individual data were presented in percentages. The histomorphometric individual data in percentages calculated based on the histomorphometric individual data in μm before being rounded-off. Numerical data is expressed as mean ± SD. Statistical significance was accepted at $p \leq 0.05$. 


4.3. Results

4.3.1 Structural and biomechanical assessment

Macroscopic analysis revealed that all scaffolds had the same macro-porosity, whilst microscopic analysis made apparent that the collagen coating, independently of the origin (BAT COL I or hR COL I), completely covered the fibrous topography and created a smooth layer (Figure 4.5). COL coating, independently of the origin (BAT COL I or hR COL I), did not significantly ($p>0.05$) affect the mechanical properties of the scaffolds (Table 4.2).
**Figure 4.5:** Macro- and microscopic images of non-coated and collagen [bovine Achilles tendon (BAT) and human recombinant (hR)] coated electrospun scaffolds. No particular differences were observed between the groups.
Table 4.2: Ball burst test results of non-coated and collagen [bovine Achilles tendon (BAT) and human recombinant (hR)] coated electrospun scaffolds. No significant difference was observed in any of the measured values.

<table>
<thead>
<tr>
<th>Group</th>
<th>Maximum Strain (%)</th>
<th>Maximum Stress (MPa)</th>
<th>Maximum Force (N)</th>
<th>E Modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-spun (n=5)</td>
<td>4.53 ± 1.50</td>
<td>1.11 ± 0.17</td>
<td>47.61 ± 3.16</td>
<td>63.15 ± 7.61</td>
</tr>
<tr>
<td>BAT COL I (n=5)</td>
<td>6.08 ± 2.03</td>
<td>1.27 ± 0.15</td>
<td>40.02 ± 5.67</td>
<td>78.07 ± 4.10</td>
</tr>
<tr>
<td>hR COL I (n=5)</td>
<td>4.95 ± 1.65</td>
<td>1.14 ± 0.11</td>
<td>36.74 ± 3.69</td>
<td>67.06 ± 7.31</td>
</tr>
</tbody>
</table>
4.3.2 In vitro assessment

No significant ($p>0.05$) differences were found in cell viability between the groups (Figure 4.6 A). At day 7, collagen coated groups exhibited significantly ($p<0.01$) higher DNA concentration than their non-coated counterparts, although no significant ($p>0.05$) difference was observed between the different (BAT COL I and hR COL I) collagens (Figure 4.6 B).
Figure 4.6: Cell viability was not affected as a function of the groups (A). Collagen coating significantly increased DNA concentration at day 7 (B). ($p > 0.05$).
4.3.3 *In vivo* assessment

No clinical abnormalities or adverse events occurred during the post-operative period. All rats gained body weight between the surgery and the termination (up to $14 \pm 3\%$ at day 10, up to $34 \pm 4\%$ at day 28 and up to $46 \pm 8\%$ at day 56).

No major abnormalities were observed on the skin above the implanted sites or at the soft tissues surrounding the scaffolds for all groups at all time points. The macroscopic integration at 10 days was equivalent for all samples. A premature tissue envelope was observed for all scaffolds with similar adhesion to the fascia muscle. After 28 days of implantation, the major part of the scaffolds was well integrated with the surrounding tissue with a thin and transparent to pinkish envelop. After 56 days of implantation, a thicker and transparent to whitish / pinkish envelop was developed (**Figure 4.7**).

The COL coating appeared as a 300 to 800 µm-thick membrane delineated by a collagen-like membrane and filled with finely granular eosinophilic material consistent with a proteinaceous matrix (**Figure 4.8**). All groups showed slight to moderate vascularisation, which decreased over time post implantation. Further, there was no sign of scaffold degradation throughout the implantation period (**Table 4.3, Table 4.4, Table 4.5, Table 4.6** and **Figure 4.9**). Partial envelopes (no envelope on the central part) were observed with an increased occurrence for the electrospun and BAT COL I coated groups at day 28, but this observation was not significant according to semi-quantitative histopathologic analysis of the MT staining (**Figure 4.9, Figure 4.10, Figure 4.11, Figure 4.12** and **Table 4.3**) or quantitative morphometric and histomorphometric analysis of the F&R staining (**Table 4.8, Figure 4.13** and **Figure 4.14**) and PR staining (**Figure 4.15** and **Figure 4.16**). However, BAT COL I and hR COL I samples had slightly higher integration mean
scores in comparison to the non-coated scaffolds after 56 days of implantation (Table 4.3 Table 4.4, Table 4.5, Table 4.6 and Figure 4.9). All scaffolds were well integrated, as evidenced by macroscopic and microscopic analyses, as well as all other macroscopic observations (e.g. adhesion and neovascularization) were considered normal and to be related with the surgical procedure or to the healing process.

Statistically significant differences were observed in the mean ratio COL I / COL III at day 28 between non-coated and BAT COL I coated scaffolds. However, it was not considered to be biologically relevant, as it was an isolated occurrence and the collagen polymorphism mean ratios for all three groups at day 56 were similar. The cell area density parameter did not show significant differences between the groups at each time point or over time (Table 4.7 and Figure 4.17).
Figure 4.7: The macroscopic integration at 10 days post-implantation was equivalent for all samples. At 28 days post-implantation, the major part of the scaffolds was well integrated with the surrounding tissue. At 56 days post-implantation, a thick and transparent to whitish/pinkish envelop had been developed.
Figure 4.8: Microscopic images of the scaffolds, SH&E stained unimplanted scaffolds and MT stained unimplanted scaffolds.
Figure 4.9: Histopathologic analysis on non-coated and collagen [bovine Achilles tendon (BAT) and human recombinant (hR)] coated electrospun scaffolds.
Table 4.3: Summary of histopathologic Evaluation Scoring Systems used for the subcutaneous implantation study

<table>
<thead>
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<th>Time period</th>
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<th>Lymphocytes</th>
<th>Plasma cells</th>
<th>Polymorphonuclear cells</th>
<th>Giant cells</th>
<th>Necrosis</th>
<th>Fibrosis</th>
<th>Neovascularization</th>
<th>Fatty infiltrate</th>
<th>Fibrin</th>
<th>Hemorrhage</th>
<th>Cell or tissue degeneration</th>
<th>Fibroplasia</th>
<th>Tissue integration</th>
<th>Tissue ingrowth</th>
<th>Encapsulation</th>
<th>Material degradation</th>
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Table 4.4: Histopathologic Evaluation Scoring Systems used for the subcutaneous implantation study at Day 10

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Table 4.5: Histopathologic Evaluation Scoring Systems used for the subcutaneous implantation study at Day 28

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<th>Plasma cells</th>
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*: reserve site included in the semi-quantitative analysis (because included in the histomorphometric analysis in replacement).
Table 4.6: Histopathologic Evaluation Scoring Systems used for the subcutaneous implantation study at Day 56

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*: reserve site included in the semi-quantitative analysis (because included in the histomorphometric analysis in replacement).
Figure 4.10: Representative Masson’s Trichrome histological images at termination on Day 10 at 20X magnification. No signs of local adverse tissue effect and no degradation were observed in any of the groups at any time point. All groups appeared to similarly integrate into the subcutaneous tissue at the time points tested.
Figure 4.11: Representative Masson’s Trichrome histological images at termination on Day 28 at 20X magnification. No signs of local adverse tissue effect and no degradation were observed in any of the groups at any time point. All groups appeared to similarly integrate into the subcutaneous tissue at the time points tested.
Figure 4.12: Representative Masson’s Trichrome histological images at termination on Day 56 at 20X magnification. No signs of local adverse tissue effect and no degradation were observed in any of the groups at any time point. All groups appeared to similarly integrate into the subcutaneous tissue at the time points tested.
Figure 4.13: Representative Feulgen & Rossenbeck histological images at termination at 50X magnification. No signs of local adverse tissue effect and no degradation were observed in any of the groups at any time point. All groups appeared to similarly integrate into the subcutaneous tissue at the time points tested.
Figure 4.14: Representative Feulgen & Rossenbeck histological images at termination at 2.01X magnification. No signs of local adverse tissue effect and no degradation were observed in any of the groups at any time point. All groups appeared to similarly integrate into the subcutaneous tissue at the time points tested.
Figure 4.15: Representative Picrosirius red histological images at termination at 50X magnification. No signs of local adverse tissue effect and no degradation were observed in any of the groups at any time point. All groups appeared to similarly integrate into the subcutaneous tissue at the time points tested.
Figure 4.16: Representative Picrosirius red histological images at termination at 2.01X magnification. No signs of local adverse tissue effect and no degradation were observed in any of the groups at any time point. All groups appeared to similarly integrate into the subcutaneous tissue at the time points tested.
Figure 4.17: Quantitative morphometric and histomorphometric analysis. Total COL content (TCC) % and the ratio of COL I / COL III were obtained from PR stains, while the cell area density % and the tissue ingrowth rate (TIR) % were obtained from the F&R stains.
Table 4.7: Semi-quantitative histopathologic analysis of non-coated and collagen [bovine Achilles tendon (BAT) and human recombinant (hR)] coated electrospun scaffolds.

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<th>Time period</th>
<th>Group</th>
<th>Macrophages</th>
<th>Lymphocytes</th>
<th>Plasma cells</th>
<th>Polymorphonuclear cells</th>
<th>Giant cells</th>
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<th>Fibrosis</th>
<th>Neovascularization</th>
<th>Fatty infiltrate</th>
<th>Fibrin</th>
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<td>0.0</td>
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<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>±</td>
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Table 4.8: Quantitative morphometric and histomorphometric analysis. Total COL content (TCC) % and the ratio of COL I / COL III were obtained from PR stains, while the cell area density % and the tissue ingrowth rate (TIR) % were obtained from the F&R stains. * indicates statistically significant difference.

<table>
<thead>
<tr>
<th>Time period</th>
<th>Group</th>
<th>TCC %</th>
<th>Ratio COL I / COL III</th>
<th>Cell area density (%)</th>
<th>TIR %</th>
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<tr>
<td>Day 10</td>
<td>E-spun (n=5)</td>
<td>14.1 ± 8.3</td>
<td>6.9 ± 2.8</td>
<td>4.7 ± 1.4</td>
<td>18.8 ± 9.3</td>
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<td>BAT COL I (n=4)</td>
<td>14.1 ± 3.6</td>
<td>3.6 ± 1.0</td>
<td>4.2 ± 0.4</td>
<td>18.3 ± 3.6</td>
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<td>hR COL I (n=5)</td>
<td>19.7 ± 6.5</td>
<td>4.3 ± 2.5</td>
<td>4.3 ± 0.8</td>
<td>24.0 ± 7.0</td>
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<tr>
<td>Day 28</td>
<td>E-spun (n=5)</td>
<td>15.3 ± 3.1</td>
<td>4.2 ± 1.3*</td>
<td>3.2 ± 1.1</td>
<td>18.5 ± 3.5</td>
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<td>BAT COL I (n=5)</td>
<td>10.5 ± 3.8</td>
<td>2.1 ± 0.3*</td>
<td>4.0 ± 1.5</td>
<td>14.5 ± 4.4</td>
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<tr>
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<td>hR COL I (n=5)</td>
<td>17.1 ± 5.3</td>
<td>2.0 ± 1.1</td>
<td>3.3 ± 0.9</td>
<td>20.4 ± 6.0</td>
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### In vivo characterisation

<table>
<thead>
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<th>Time period</th>
<th>Group</th>
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<th>Ratio COL I / COL III</th>
<th>Cell area density (%)</th>
<th>TIR %</th>
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<tr>
<td>Day 56</td>
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<td>17.6 ± 4.5</td>
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<td>3.4 ± 0.3</td>
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<td>BAT COL I (n=5)</td>
<td>17.3 ± 5.5</td>
<td>2.9 ± 1.3</td>
<td>3.6 ± 2.4</td>
<td>21.0 ± 7.1</td>
</tr>
<tr>
<td></td>
<td>hR COL I (n=5)</td>
<td>19.4 ± 6.5</td>
<td>3.1 ± 1.0</td>
<td>3.9 ± 0.9</td>
<td>23.4 ± 7.3</td>
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</table>
4.4. Discussion

Porosity has been shown to have modulatory effect on tissue integration and foreign body response (FBR). Small pores have been associated with an elevated risk of encapsulation due to bridging scars and FBR [41] resulting in impeded abdominal movements [42]. Meshes with larger pores have been shown to result in elevated tissue integration and correspondingly lower FBR [43]. In fact, macro-porous and light weight meshes have shown remarkable biocompatibility in mice models [44]. Traditional electrospun meshes, although very light, they have small porosity, which results in FBR in preclinical models [45]. Although macro-porous electrospun meshes are under investigation, no in vivo data are available. Thus, herein, we ventured for first time to assess in vivo the influence of macro-porous electrospun meshes without and with collagen (bovine Achilles tendon and human recombinant) coating.

Biomechanical analysis made apparent that the collagen coating, independently of the source, did not affect the structural and biomechanical properties of the scaffolds. Although previous studies have shown composite scaffolds to have superior mechanical properties than their single component counterparts [46, 47], this was not observed here, possibly due to the fact that the collagen was not co-extruded or that the collagen layer was too thin to induce any notable difference in mechanical properties. One should also note that ECM materials, such as collagen, elastin, gelatin, hyaluronic acid, are not used for mechanical integrity, but to improve biological response. Indeed, herein, collagen coating, independently of the source, improved in vitro cell response by day 7. Electrospun hybrid synthetic polymer and collagen [32, 48, 49], gelatin [50, 51], collagen / gelatin [52], elastin [53] and hyaluronic acid [37] scaffolds have been previously shown to have enhanced cell
attachment, proliferation and growth, when compared to synthetic polymer alone scaffolds.

Post-implantation macro- and microscopic analysis of the scaffold integration suggests minimal differences between the groups. By day 28, the majority of the scaffolds were well integrated, with full integration by day 56. All other macroscopic observations were indicative of a normal post-operative healing process. Collagen coated and uncoated samples showed no relevant differences in terms of local tissue effects. The inflammatory cell response was consistent with that of a normal healing response, whereby basal inflammatory response was observed at day 10 and dissipated almost completely by day 28 and 56. The exception to this was the presence of giant cells, which were heavily infiltrated by day 56. The presence of giant cells around the electrospun PCL meshes after 28 and 56 days is typical of FBR and is consistent with the literature [54, 55]. The amount of giant cells has been shown to be significantly lower in electrospun PCL meshes compared to solid PCL constructs, which indicates initially elevated M1 macrophages with subsequently M1/M2 macrophages modulation and upregulation of pro-healing cytokines compared to the pro-inflammatory cytokines of the solid construct [56]. Similar results for inflammation have been reported in a porcine hernia model, with heavy macrophage inflammation after 90 days, including giant cell infiltration with fibrosis around individual mesh macro-fibres and periodic bridging across mesh interstices [15]. Electrospun PCL fibres have also been shown to elicit minimal inflammatory response in a rodent ligament reconstruction model; although high number of monocytes and macrophages were observed at 2 weeks, their number peaked at 6 weeks and significantly decreased from week 6 to week 12 post-implantation [57]. The limited inflammatory response is consistent with finding comparing solid
materials to electrospun material, where a significantly thicker encapsulation layer formed around films compared to electrospun meshes [54]. While the presence of new vascularisation around the implant was not observed inside the scaffold, mature vascularisation was evidenced around the implant from the termination site images for all time points. Neovascularisation in knitted meshes has been seen within the mesh, without crossing through the interstices of the mesh after 90 days [15]. No degradation was observed \textit{in vivo}, which is consistent with reports of 1 to 7% mass loss after 6 months [58] and with complete degradation after 30 months [10] for PCL electrospun meshes. A tubular PCL/PTMC electrospun scaffold has also been reported to remain intact for up to 4 weeks post-implantation and its micro-porous structure allowed for infiltration of autologous cells, whilst, although at the periphery giant cells were present, there was no obvious fibrous encapsulation [59].

With respect to animal extracted versus human recombinant collagen, no particular differences were observed in \textit{in vitro} and \textit{in vivo} setting, further corroborating a previous suggestion that a niche area should be identified to justify its cost, although at the low amounts used in this study, still makes it a viable option [60]. Several studies have shown scaffold functionalisation to improve host response. For example, PCL and PCL/gelatin electrospun fibres loaded with metronidazole have been shown to evoke a less severe inflammatory response after 8 months of subcutaneous rabbit implantation than pure PCL nanofibers [61, 62]. Further, PCL / bFGF electrospun scaffolds exhibited minimal inflammatory response 16 weeks post-implantation in an athymic rat model for anterior cruciate ligament reconstruction [63]. Electrospun PCL/collagen scaffolds facilitated penetration of tissue cells into the fibrous scaffolds at day 7, whilst no such cell penetration was noticed in the PCL alone scaffolds [64]. This different host response between our
work and previous studies may be attributed to the cross-linking method employed that has been shown to be crucial in foreign body response [65].
4.5. Conclusion

Macro-porous, compressed and collagen (bovine Achilles tendon and human recombinant) coated poly-ε-caprolactone electrospun scaffolds were fabricated and their biomechanical, biological and host response properties were assessed. Collagen coating, independently of the origin, did not affect the biomechanical properties of the scaffolds. Although cell viability was not affected as a function of the collagen coating, DNA concentration was increased after 7 days in culture. Implantation studies showed no differences between the groups. Electrospun macro-porous and compressed scaffolds have been produced that even without collagen coating demonstrate suitable properties for biomedical applications.
4.6. References


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59. Jiang, T., Zhang, G., He, W., Li, H., Jin, X., The Tissue Response and Degradation of Electrospun Poly(ε-Caprolactone)/Poly(Trimethylene-


5.1. Summary

Among the various bottom-up processes, electrospinning is favoured for the development of sub-micron devices [1-4]. Indeed, to-date, numerous natural and synthetic polymers [5, 6]; ceramics [7, 8]; and metallic materials [9, 10] have been electrospun that have found applications in textile [11], filtration [12] and biomedicine [13] sector / industries. The rationale of using electrospinning in biomedicine lays on the fact that this technology can create three dimensional fibrous scaffolds that closely imitate the nano- to micro- scale intertwined fibrillar meshwork of the extracellular matrix [13-16]. While electrospun scaffolds are utilised in a multitude of clinical targets, fabrication of scaffolds suitable for higher mechanical applications such as sutures [18] and hernia [19] are not always feasible. Our aim was to assess if electrospinning can produce macro-porous meshes with suitable mechanical properties for biomedical applications.

The development of reproducible ways to fabricate electrospun scaffolds (Chapter 2) with controlled architectural features substantially increases applications of electrospinning in regenerative medicine. Pre-designed collectors offered the opportunity to tailor the mechanical properties of the produced scaffolds, as a function of porosity and pore shape, and to design tissue-specific implantable devices. Indeed, the adjustable nature of porosity and pore shape can customise the multi-axial tensile requirements, for applications such as hernia repair and wound healing, or uniaxial applications, such as tendon and neural repair. Concurrently, the fibrous nature of the produced constructs promoted fibroblast growth, whilst hindering inflammatory response, as compared to planar substrates. Overall, this opens up new avenues in the rational design of electrospun scaffolds for tissue engineering applications.
Optimal layering and porosity conditions (Chapter 3) exhibited mechanical properties similar to commercially available meshes without any negative effect in fibre morphology and cytocompatibility. This study paves the path for wider acceptance, clinical translation and commercialisation of electrospun meshes. Macro-porous, compressed and collagen (bovine Achilles tendon and human recombinant) coated poly-ε-caprolactone electrospun scaffolds were fabricated and their biomechanical, biological and host response properties were assessed (in Chapter 4). Collagen coating, independently of the origin, did not affect the biomechanical properties of the scaffolds. Although cell viability was not affected as a function of the collagen coating, DNA concentration was increased after 7 days in culture. Subcutaneous implantation studies showed no differences between the groups independently of collagen coating.

Overall, we provide evidence of single step fabrication methods for highly conformable electrospun meshes and methodology for the fabrication of mechanically robust scaffolds suitable for biomedical applications. Furthermore, the inherent properties of the enhanced scaffolds are provide comparative data to that of functionalised scaffolds when implanted.
5.2. Future studies

Electrospinning has revolutionised the field of regenerative medicine due to its innate ability to create implantable devices that closely imitate the nano- to micro-scale intertwined fibrillar network of the native extracellular matrix architectures [13-16], coupled with an array of functionalisation methodologies suitable for a range of clinical targets [4]. Among the various bottom-up processes, electrospinning is favoured as a facile scaffold fabrication method [3, 4] with inherent versatility and controllability [1, 2] of sub-micron devices. This has resulted in an exponential growth in number of electrospinning paper published pre-annum over the last few decades. However, translation from academic publications to clinical and commercial applications is still in its infancy, with only a handful of products successfully receiving CE marks, such as AVflo by Nicast Ltd, LimFlow stents by LimFlow and PK Papyrus stent by BIOTRONIK.

5.2.1. Functionalised electrospun meshes as a hernia repair platform

Hernia repair is the most frequent abdominal surgery, with particularly high disease prevalence in males. In the mid-20th century, with the advent of biomaterials and appropriate surgical techniques, development of a cure for this disease reached rates close to 100%. However, while successful in curing the underlying morbidity numerous issues are have been linked to the prostheses itself. These include foreign body reactions, infections, migration, erosion, discomfort, shrinkage of the prosthesis [9, 10], stiffing of the abdomen and chronic pain [11]. It can be hypothesised that electrospinning can produce bioreabsorbable meshes of adequate mechanical properties, that will facilitate hernia repair. A fundamental requirement in hernia repair meshes is to provide sufficient tensile strength so as to contain the
herniating tissue, 27 N/cm in healthy adults [12]. Having achieved tensile properties in excess of the physiological requirements with a functionalised biodegradable electrospun mesh platform, assessment of the platform in a full thickness parietal defect rat model would increase scope and potential ramifications of this thesis. The comparative analysis of the electrospun meshes functionalised with COL type’s v’s a commercial predicate in relation to the inflammatory response and functional repair would develop a benchmark upon which further optimisation can be investigated. Furthermore, the long term characterisation of the balance between the biodegradable mesh and the tissue ingrowth in terms of tensile properties would define the feasible reabsorption rate required for future prostheses.

5.2.2. Functionalised electrospun meshes with antibacterial agents

The implantation of prostheses are associated with bacterial infiltration resulting in acute and chronic infections. While systemic antibacterial doses can mitigate some of the underlying symptoms, this puts an unnecessary burden on the system as a whole, while localised concentrated treatments can have a more profound outcome directly at the required site. Concurrently, antibacterial coatings can also have anti-adhesion properties, the use of an antibiotic dip loaded onto an electrospun PCL mesh has been shown to reduce adhesions in an abdominal rat model [17]. It can be hypothesised that complimentary antibacterial functionalisation of the platform developed, will further enhance the capabilities for a multitude of clinical applications. A comparative evaluation of an antibacterial agents encapsulated within an electrospun PCL meshes suitable for sustained long term release verses a shorter term burst release from an antibacterial agents mixed into the COL coating to assess the effects on acute and chronic infections. Initially evaluation will consist of drug
release profiles and bactericidal efficiency when comparing the higher surface area of the electrospun topography v’s the lower surface area of planar COL coating in the short to longer term, and in vitro analysis of the ramifications to non-bacterial cells. Subsequently, a subcutaneous rat infection model will facilitate the efficiency of localised verse systematic approach in terms of system wide response to the treatment, local recovery rate and morbidity.

5.2.3. Functionalised electrospun meshes to achieve enhanced the tensile properties

While electrospun scaffolds are utilised in a multitude of clinical targets, application requiring higher mechanical requirements such as sutures [18] and hernia [19] are not always feasible with an electrospun material. Multiple methods have been employed both during fabrication or post fabrication treatments to increase the overall mechanical properties. Methods applied during the fabrication process include functionalisation with co-polymers [20], such as carbon nanotubes [20], cellulose nanocrystals [21], hydroxyapatite [22] or combinations of such [20]. Reduction of the fibre diameter to ≤ 700-800 μm [23, 24], can substantially increase the loading capacity of a mesh [25], while inducing anisotropic fibre orientation can substantially increase the loading capacity [26], facilitating a more uniform load distribution [27]. Post fabrication methods for increasing the tensile properties can include interfibrous bonding via solvent assisted compression moulding [28], cross-linking [20], or thermal treatments [29], while utilising annealing treatments can improve the molecular orientation and crystallinity [30, 31]. It can be hypothesised that a systematic evaluation of the various methodologies for enhancing the tensile properties will result in a definitive method for further development. Numerous
methods exist to enhance the tensile properties of an electrospun mesh, comprehensive comparative evaluation of these methods in isolation and in combination with each other have yet to be undertaken.
5.3. References


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Chapter 5 - Appendices: Protocols and supplementary information
## A. List of components and reagents

**Table A.1:** List of components and reagents.

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<td>2-(N-Morpholino) ethanesulfonic acid</td>
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<td>18 G stainless steel blunt needle</td>
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<tr>
<td>N-hydroxysuccinimide (NHS)</td>
<td>Sigma Aldrich, Ireland</td>
</tr>
<tr>
<td>Penicillin / streptomycin</td>
<td>Sigma Aldrich, Ireland</td>
</tr>
<tr>
<td>Phorbol 12-myristate 13-acetate</td>
<td>Sigma Aldrich, Ireland</td>
</tr>
<tr>
<td>Phosphate buffered saline</td>
<td>Fischer Bioreagents, Ireland</td>
</tr>
<tr>
<td>Poly ε-caprolactone</td>
<td>Corbion, The Netherlands</td>
</tr>
<tr>
<td>Polytetrafluoroethylene sheet</td>
<td>Proxy Biomedical</td>
</tr>
<tr>
<td>Quant-iT™ PicoGreen® dsDNA kit</td>
<td>Invitrogen, Thermo Fisher Scientific, Ireland</td>
</tr>
<tr>
<td>RPMI-1640 medium</td>
<td>Sigma Aldrich, Ireland</td>
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<tr>
<td>Trypan blue</td>
<td>Gibco, Thermo Fisher Scientific, Ireland</td>
</tr>
<tr>
<td>Trypsin/ EDTA</td>
<td>Sigma Aldrich, Ireland</td>
</tr>
</tbody>
</table>
B. Scaffold fabrication

1. Electrospun scaffolds fabrication general

- Samples should be prepared as following:
  - Weigh the polymer.
  - Place it in an appropriately sized falcon tube.
  - Add required amount of solvent to the polymer in an appropriate fume hood.
  - Secure cap on tube.
  - Cover with tube cap with Parafilm™.
  - Place in rotating mixer until completely dissolved.
- Load a syringe with the excess of the required volume inside a fume hood.
- Add a blunt tip needle of the required diameter to the syringe.
- Switch on the extraction and filter system.
- Install the syringe in the syringe pump, (Figure B.1).
- Set the distance between the tip of the needle and the mandrel by moving the syringe pump platform.
- Set the pump’s parameters (syringe diameter, flow rate).
- Attach the HV (high voltage) electrode to syringe the needle (for multiple syringe systems attach a HV electrodes to each relevant syringe needle) (Figure B.1).
- Check that there is no spillage of the solution and that there are no conducting parts in connect with the HV electrode other than the needle.
- Orientate the mandrel to the desired location and where required securely fasten to the rotating motor by completely closing the mandrel chamber.
- Turn the syringe pump on.
- Close the Safety hood (Verify that the interlock is completely closed).
• On the control panel (bottom left), restart the apparatus and engage the rotating motor to the speed desired level, (Figure B.1).

• Turn on the HV supply and rotate the HV dial to the required level. Check for any signs (visible or audible) of malfunction.

• Continue to be vigilant during the electrospinning process.

• When processing is complete, stop all components in the following order Voltage, mandrel rotation and syringe pump.

• Open the safety hood and remove the mandrel and separate the sample from the mandrel.

• Remove the syringe from the pump.

• To make multiple scaffolds repeat the steps as required.

• When finished completely dispose of the blunt tipped needle in the sharps bin and place the spent syringe in the normal waste disposal bin.

• Make sure that the electrical supply is turned off at the mains, that the facility is cleaned and switch off lights.
Figure B.1: Electrospinning utilises a high voltage to induce an electrostatic charge in the PCL/Solvent solution. This charge acts in opposition to the surface tension of the polymeric solution. As this force overcomes the surface tension a polymer jet is eject from the tip of the needle toward the rotating collector. As the jet travels from the tip of the needle to the collector plate, the solvent evaporates resulting in the deposition of polymer fibres.
2. Porous collector optimisation

The initial phase of porous optimisation involved the identification of an appropriate collector design. Therefore under and over woven collector and chemically etched collector were selected, (Figure B.2).

The under and over woven collector was dropped after the preliminary evaluation due to the obvious potential weakness visible after SEM imaging, (Figure B.3).

The topography of overall structure of the mesh highlights the conformation of the electrospun fibres at the point of deposition. However, the heterogeneous deposition on a localised level also indicates that the structure would have reduced tensile properties compared to a more uniform fibre deposition.

The chemically etched meshes were either 0.0127 mm or 0.1 mm thick. Theses thicknesses dimensions were chosen due to the material thickness limitations of chemically etched stainless steel being on the low level and a fold difference, which would allow practical manipulation for use with the electrospinning facilities, to investigate if an increase in thickness increased the attraction of the fibre during the flight. While, the thickness did not appear to have any impact on the porosity % of the meshes it did appear to increase the thickness of the meshes, indicating that an increased attractive force may be present in the thicker meshes (Table B.1, Figure B.4).

Initially the collectors were applied directly on top of the rotating metal mandrel. However, this caused the fibre deposition on the pore area as well as the metallic struts of the collectors. This was improved by insulating the collection area underneath the collector so that the only conductive area was the collector itself. The reduction of the collector rotation speed allowed the electrospun fibres to be attracted to the collector due to the conductivity in a more organised manner, rather than being
forced into position due to the rotation speed. The third factor that facilitated the
development of porous electrospun meshes was the reduction of the extrusion speed
of the polymer/solvent solution so that less material would be dispensed. These
three factors combined to produce single step fabrication of porous electrospun
meshes (Table B.1, Figure B.4).
Figure B.2: The under and over woven collector density can be clearly seen to be more than the chemically etched collector.
Figure B.3: The definition of overall structure of the electrospun mesh, at higher magnification the flanking areas of the collector show a reduced fibre deposition.
Table B.1: Electrospinning parameters for the initial porous scaffold fabrication.

<table>
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<tr>
<th>#</th>
<th>Extrusion rate (μl/min)</th>
<th>Rotation speed (RPM)</th>
<th>Thickness (mm)</th>
<th>Insulated drum</th>
<th>Mesh thickness N=5</th>
<th>Porosity % N=5</th>
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</thead>
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<tr>
<td>1</td>
<td>100</td>
<td>750</td>
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<td>No</td>
<td>0.1</td>
<td>NA</td>
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<td>0.354</td>
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</tr>
</tbody>
</table>
Figure B.4: The resulting electrospun meshes which correspond with the parameters from Table B.1.
With the initial proof of concept work concluded 9 chemically etched collectors of differing pore shape (circle, square and rhomboid) and three differing porosity (30%, 50% and 70%) were used to fabricate the various porous meshes for the second chapter (Figure B.5).
**Figure B.5:** Collectors on the LHS and corresponding porous electrospun meshes fabricated from the collectors on the RHS.
3. Parameters for electrospinning onto porous collectors

- Polymer concentration: 200 mg/ml of poly ε-caprolactone.
- Solvent: 2,2,2-trifluoroethanol.
- Flow rate: 10 µl.
- Extrusion time: 45 mins.
- Needle gauge 18 G.
- Voltage: 15 kV.
- Distance between spinneret and collector: 20 cm.
- Collector rotation speed: 60 RPM.

4. Parameters for electrospinning onto thick meshes

- Polymer concentration: 200 mg/ml of poly ε-caprolactone.
- Solvent: 2, 2, 2-trifluoroethanol.
- Flow rate: 100 µl.
- Extrusion time: 4 hrs.
- Needle gauge 18 G.
- Voltage 20 kV.
- Distance between spinneret and collector: 20 cm.
- Collector rotation speed: 60 RPM.
- Number of syringes running simultaneously: 3.

5. Solvent casting

- Prepare a solution of 200 mg/ml of PCL in TFE. Allow the solution to fully dissolve until it is fully homogeneous.
• Securely affix the polytetrafluoroethylene (PTFE) sheet to a movable flat surface (board).
• Place the PTFE and attached board into a chemical grade fume hood.
• Using a level / inclinometer position the board so that the surface is perfectly level. Failure to make the sheet level will result in films of varying thickness.
• Clean the PTFE sheet with 70% ethanol and allow 5 minutes for evaporation.
• Pour the PCL solution onto the sheet ensuring as even a distribution as feasibly possible is achieved.
• Allow the solvent to evaporate off overnight at ambient temperature and pressure.

6. **Collagen coating and cross-linking**

NOTE: All solutions have to be kept at 4-8 °C. Therefore, keep them in ice during the film preparation.

• Invert and agitate the collagen solution to homogenise the solution.
• Using a pipette add 1ml of collagen (5 mg/ml) to the sample and spread homogeneously.
• Place the coated samples in a laminar flow hood and allow them to dry at RT for 3 hrs.
• Flip the sample over and apply collagen to the other side of the sample. Allow 3 hrs to dry in a laminar flow hood at RT.
• When the samples are fully dried, immerse them in a crosslink solution of 1-ethyl-3-[3-dimethy- laminopropyl]carbodiimide (EDC): N-hydroxy-sulfosuccinimide (NHS) in 0.05M 2-(N-morpholino) ethanesulfonic acid (MES)
buffer at pH 5.5 in a 3:1:5 ratio respectively. The pH is neutralised with 1M NaOH.

NOTE: NHS and EDC were mixed immediately before using it (5-15 mins).

- Place the sample with the crosslinker solution on rocker overnight.
- Wash films three times with 1x PBS for 20 mins (each time).
- Dry films in a laminar flow hood at RT.

C. **In vitro analysis**

All cell culture activity should be performed in aseptic conditions. All equipment or material which will come in contact with the cell should be sprayed with 70 % ethanol prior to putting them into the biological safety hoods.

1. **Culture medium preparation for human dermal fibroblasts**
   - Dulbecco’s Modified Eagle’s Medium (DMEM) high glucose (4500 mg/L) (Sigma Aldrich, Ireland).
   - 10 % Foetal bovine serum (FBS) (Sigma Aldrich, Ireland).
   - 1 % Penicillin streptomycin (PS) (Sigma Aldrich, Ireland).
   - Nunc™ non-treated flasks (Thermo Scientific, Ireland).
   - Silicone O ring (Ace O-rings, Silicon from Sigma).
   - Glass coverslips.
   - Hanks balanced salt solution (HBSS, Sigma Aldrich, Ireland).
   - IMS 70 %.

2. **Cell thawing and passaging human dermal fibroblasts**
   - Remove vial from liquid nitrogen container and thaw in water bath at 37 ºC.
• Transfer contents to culture flask of appropriate size and add pre-warmed culture medium.
• Transfer the flasks to the incubator at 37°C and 5 % CO₂.
• Change medium every 2-3 days and monitor cell proliferation with a phase contrast microscope.
• When cells cover more than 80 % of the culture flask, remove culture medium, wash cell layer with Hank’s Balanced Salt Solution (HBSS) and add 5 ml of trypsin / EDTA ensuring complete coverage of the flask. Incubate at 37 °C for 5 mins.
• Examine the flask under the microscope to see if cells detach from the surface and are floating in the media. If not, lightly tapping.
• Once cells are detached, add 5ml of culture medium to neutralise the action of trypsin / EDTA and transfer flask contents into a tube and centrifuge at 1200 RPM for 5 mins.
• Discard the supernatant ensuring that the pellet is not disturbed.
• Counted the number of cells using a Neubauer chamber. Re-suspend cells in desired amount of medium and seed into new flasks.

3. Cell freezing
• Aspirate culture medium and wash cell layer with HBSS.
• Add trypsin / EDTA and incubate at 37 °C for 5 mins.
• Add culture medium to neutralise the action of trypsin, collect flask contents into a tube and centrifuge at 1200 RPM for 5 mins.
• Re-suspend supernatant in 1 ml of medium and count cells using a haemocytometer and trypan blue.
• Re-suspend cells in necessary amount of freezing medium (90 % growth medium to 10 % DMSO) to have 1 million cells per millilitre of medium.

• Add 1 ml of cell suspension per cryogenic vial and place in Mr. Frosty overnight at -80 ºC.

• Move to liquid nitrogen for long term storage.

4. **Culture medium preparation for THP-1 macrophages**

• Roswell Park Memorial Institute (RPMI).

• 10 % Foetal bovine serum (FBS).

• 1 % Penicillin streptomycin (PS).

• 1 % glutamine.

5. **Cell thawing and passaging THP-1 macrophage**

• Remove vial from liquid nitrogen container and thaw in water bath at 37 ºC.

• Transfer contents to culture flask of appropriate size and add pre-warmed culture medium.

• Daily observations morphology checks on the optical microscope are required.

• Maintain the cell concentration between 500,000 and 800,000 cells/ml (Do not exceed 1,000,000 cell/ml during expansion).

• Spin down every 7 days and replace all the media.

6. **THP-1 macrophages Differentiation**

• Use THP-1 cells when their density reaches 800,000-1,000,000 cells/ml.

• Dilute PMA in DMSO at 5 µg/ml.
• Add 100 µl PMA at 5 µg/ml for each 50 ml supplemented RPMI 1640 medium (10 ng/ml).
• Spin down the cells and re-suspend them with the medium of differentiation.
• Count the THP-1 cells with hematocytometer and trypan blue.
• Adjust the cell density at 100,000 cells/ml.
• Seed cells in a 24 well plate by adding 0.5 ml of cell suspension per well.
• Incubate cells at 37 °C for 6 hrs.
• Check the differentiation by observation at the microscope. If you see floating cells (undifferentiated), incubate for 6 hrs more.
• Then, remove the media and replace it by normal medium or activation medium (1 ml/well). Activation medium is supplemented RPMI 1640 medium with 100 ng/ml LPS (2.5 µl of LPS stock solution at 2 mg/ml for 50 ml medium).
• Incubate the cells for 24 hrs at 37 °C.
• Replace by normal medium (this is time point 0).

7. Cell freezing THP-1 macrophages

• Centrifuge the media at 1200 RPM for 5 mins.
• Re-suspending the pellet in the necessary amount of freezing medium (90% growth medium to 10% DMSO) to have 1 million cells per millilitre of medium.
• Add 1 ml of cell suspension per cryogenic vial and place in Mr. Frosty overnight at -80 °C.
• Move to liquid nitrogen for long term storage.
8. **alamarBlue® assay**

- Prepare a 10% alamarBlue® solution in HBSS.
- Remove culture medium from the cells and wash with HBSS.
- Add 0.4 ml of the diluted alamarBlue® solution to the cells and a negative control of alamarBlue® at 10% alone.
- To obtain the background absorbance, add HBSS to empty wells.
- Incubate for 3 hrs at 37 ºC, 5% CO₂.
- Transfer 100 µl of the alamarBlue® solution and of the negative control and background to a clear 96 well plate.
- Measure the absorbance at 570 nm and at 600 nm.
- Subtract the values of HBSS to the values of alamarBlue® alone from both absorbance values to obtain the absorbance of alamarBlue®. For 550 nm this value is called absorbance of the oxidised form at lower wavelength (AO₁₅₀) and for 595 nm it is called absorbance of the oxidised form at higher wavelength (AO₉₅₅).
- Calculate the correlation factor:

\[ R_0 = \frac{AO_{150}}{AO_{955}} \]

- To calculate the percentage of alamarBlue® reduced (AR) by the cells use the following:

\[ AR = \frac{ALW - (AHW \times R_0)}{100} \]

9. **Cell metabolic activity assay using Quant-it™ PicoGreen®**

- Remove the media and gently rinse with HBSS.
- Add 200 µl of DNase free water.
- Repeatedly freeze-thaw cells three times.
• Prepare a 1X TE buffer from the 20X stock solution.
• Prepare a standard of DNA using DNase free water and the Lambda DNA standard at 100 μg/ml in TE.
• Make up a 2 μg/ml DNA solution (dilution 1:50 from 100μg/ml DNA standard).
• Make up a 50 ng/ml DNA solution (dilution 1:80 from 2μg/ml DNA solution).
• Transfer 100 μl of each sample and DNA standard curve into a 96 well plate for fluorescence assays, (Table C.1).
• Make up diluted PicoGreen solution: 9 ml 1x TE + 45 μl concentrated PicoGreen (enough for a 96 well plate).
• Add 100 μl of diluted PicoGreen to each well.
• Incubate at room temperature 2-5 mins in the dark.
• Read the plate using fluorescent channel (excitation 485 nm, emission 535 nm).
**Table C.1:** Volume for preparing the DNA standard curve.

<table>
<thead>
<tr>
<th>DNA Concentration (ng/ml)</th>
<th>Water volume (µl)</th>
<th>Volume of 2µg/mL DNA stock (µl)</th>
<th>Volume of 50ng/mL DNA stock (µl)</th>
</tr>
</thead>
<tbody>
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</tr>
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</tr>
<tr>
<td>0</td>
<td>400</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
10. Cell viability assay using cytotoxicity kit

- At each time point transfer the supernatant of each well to eppendorf tubes.
- Centrifuge at 250 x g for 4 mins.
- Transfer supernatant to a new eppendorf tube and keep it at – 80 °C until testing.
- Transfer 50 μl of the supernatant to the corresponding well of a flat-bottom 96-well plate.
- Add 50 l of a 1:5,000 dilution of LDH Positive Control to 3 separate wells.
- Reconstitute Substrate Mix using Assay Buffer from the kit.
- Add 50 μl of the reconstituted Substrate Mix to each well of the plate.
- Cover the plate and incubate at room temperature, protected from light, for 30 mins.
- Add 50 μl of the Stop Solution to each well of the plate (NOTE: only perform this step if media is phenol red free).
- Record absorbance at 490 nm using a microplate reader.

D. Biophysical analysis

1. Gross visual observations

- Turn on the Olympus SZX16 Microscope and open the ‘Stream Basic’ Program.
- Click on <View>, <Tool Windows> and <Camera Control>.
- Click on <LIVE> in Camera Control window to initial the image acquisition.
- Prior to taking any images verify the accuracy of the measurements using a reticule. If the measurement is more than 2 % off recalibrate the microscope.
- Set magnification to the required level and select the required lens.
2. Scanning electron microscope (SEM) and fibre diameter analysis

Note: Gloves must be worn at all times when handling the samples.

- Samples of sufficient size were cut from the electrospun meshes using a scalpel.
- These electrospun scaffolds were mounted onto a carbon disk which is attached to an SEM stub.
- The samples were placed into an Emitech K-550X sputtering system to coat them with gold sputter coating using for 2 mins under an argon atmosphere.
- The samples were then placed into a Hitachi S-4700 SEM (Hitachi High-Technologies Europe GmbH, Germany) and imaged in accordance with the established protocol.
- Once the images were collected the fibre diameter was analysed using NIH ImageJ software.
  - Open ImageJ software.
  - Select the appropriate image, “File > Open > Select Image”.
  - Set the scale bar by drawing a straight line along the SEM scale bar which is on all the images.
  - Define the length of the line “Analyse > Set Scale”. Input the known distance of the line that you drew and unit of length.
  - “Analyse > Measure” (or Ctrl M) to take measurement of selected area.
  - The measured parameters will appear in a separate screen.
  - Repeat until N=100 for each sample.
  - Copy the measurement data into excel and calculate the average fibre diameter.

3. % porosity calculation

- Open the file to be measured in imageJ.
• Select the “straight” line option and draw a line over the scale bar on the SEM image go to “Analyze>Set Scale”. The pop up window will show the length of distance in pixels of the line drawn, put in the known distance acquired from the SEM image scale. Change the “unit of length” as appropriate. If a batch of images is to be processed at the same magnification click “Global>OK”, this negates having to repeat this step per image.

• Cropping the image: Remove the SEM information bar from the image as failure to do so will result in inaccurate data acquisition. Draw a rectangle using the rectangle option to include just the SEM image. Then use menu “Image> Crop”.

• Convert the image to bmp or tiff format.

• Select the whole picture by clicking “control + A” on the keyboard, then get the area of the picture by clicking “Analyze> Measure”. The results pop up appears.

• Binarisation: The image must be binarised using thresholding procedure. Click “Image>Adjust> Threshold”. A pop up will appear with 2 sliding bars, optimise the red area is only in the pores using these bars. Selecting “auto” is possible but manual optimisation is required subsequently. Click “Apply” changing the image to black and white.

• Click “Analyze> Set measurements…” select the “Limit to threshold” option and click “ok”. This allows for the calculation of the area of the threshold only rather than the area of the entire image again.

• After thersholding, select the whole picture by clicking “control + A” then get the area of the picture click “Analyze> Measure”.

• With the overall area and the area of the pores it is possible to work out the % area from that calculation.

4. **Weighing samples**

• Power on weighing scales and printer.

• Press the “On/Zero” button, to turn weighing scales on.

• Prior to using the weighing scales for the first sample calibrate the weighing scales by pressing the ‘Cal’ button. Ensure that all of the glass doors are closed prior to performing the calibration. Wait for the scales to Zero. (Incal ‘busy’ flashes until calibration is complete. If Incal ‘Abort’ is displayed press the ‘Cal’ button again).

• Open the glass slide door on the balance.

• Place the sample to be measured on the balance and close the sliding glass door.

  **Important:** Be careful to ensure there is no interaction with the scales/table that could interfere with the weight recording during the weighing process.

• Wait for the result to stabilise on the display. This stabilisation is indicated by the ‘*’ symbol appearing on the display. (If the weighing scale has not stabilised the * symbol will not appear and a “?” symbol will be printed out next to the weight on the receipt. This is not accurate and therefore is not acceptable for the weight measurement so the weight must be retaken. Only when the asterisk ‘*’ is displayed should the print button be selected).

• Open a glass slide door and return the unit to its container.

• Repeat steps until all units are weighed.
D.2. Thickness measurement

- To accurately assess the thickness of the compressible electrospun meshes the meshes were placed between two panes of glass.
- The thickness of the glass and sample were measured $N=5$ times with a micrometer / callipers.
- The sample was then removed, and the glass alone was measured with micrometer / callipers.
- The average thickness of the sample between the glass panes was taken away from the average thickness of the glass alone to give the thickness of the sample alone.
- Repeats as required for the number of samples to measure.

E. Biophysical assessment uniaxial tensile testing

1. Materials

- Scalpel and Blades, preferably an 11 blade.
- Cutting board.
- Cardboard.
- Digital callipers Scienceware®, Digi-Max™, Sigma-Aldrich, Ireland.
- Two glass panes.
- PBS.
- Zwick/Roell (Leominster, Herefordshire, UK) Z005 testing machine.
- 10 N or 100 N load cell available.
- Standard grips.
- A Laptop with excel.
- Optional:
Appendices

- Sand paper to enhance the fiction generated by the sample grips. Utilisation of sand is material type dependent but is generally beneficial in preventing sample “slipping”.
- A marker to identify the location where sample exits the grips. This allows for monitoring of potential sample “slipping”.

2. Sample preparation

- Creating a template out of cardboard with the dimensions the dog bone (Figure E.1), which are based on the ASTM D882-2010 guidelines. Place the sample on top of a cutting board, place the template on top and cut out the desired sample using a scalpel with a sharp blade, any defects in the test region should result in an automatic rejection of the sample.
Figure E.1: This is the dog bone shape which allows for the correct analysis of the materials without perverting the results due to fixation method required for the test, units in mm.
• The samples should be hydrated overnight through emersion in PBS.

• Prior to commencement of testing the thickness (or diameter where relevant) must be measured so the area can be calculated for stress vs. strain curves. Using the callipers take five measurements from the thickness of the glass panes alone, insert the sample between the panes and repeat as per sample. The glass panes are used to give a consistent thickness measurement for the sample by reducing potential bias introduced when measuring. Additionally, the glass protects the sample from potential defects created by the callipers.

3. Testing procedure

Physical setup

• Turn on the power at the main switch on the side of the machine.

• On the front panel of the device you have the manual control for up and down, the on button and emergency stop. Press the on button on the front of the machine to activate the device.

• Identify the grips with the knurled surface inserts and place them on the tensile tester so that the sample will align in a perfectly vertical direction, if not done correctly a rotational force will be incorporated into the results.

• Insert the sample into the upper grip and tighten. It is essential to ensure that the placement of the sample is at a 90° angle to the grip so as to negate potential bias upon test.

• Using the manual override bring the crosshead into an appropriate range for the sample and affix the sample to the lower grip ensuring slack is present so that the test will accurately measure the initial point of the test.
Software setup

- Turn on the computer.
- Select the testXpertII program.
- When it is open select “File> Open> program name” (the program you wish to run).
- Then click “OK”, this will load the test parameters required for that tensile test.
- Select the “Wizard” to define the sample specifics.
  - The Pre-test; auto calculate the grip to grip separation; define the specimen cross sectional shape and pre load value.
  - Test parameters; define the test speed of 20 mm/min.
  - Results; the parameters to be measured.

Test commencement

- Zero the force of the system.
- Input the sample thickness and width.
- Click the “Start” icon. When the test is completed click the “Stop” icon and select the “Actions after the test” in the pop up that appears.
- Export the data to Excel and this will allow the data to be analysed.

Sample analysis and processing

The following definitions are used to calculate mechanical data: stress at break is defined as the load at failure divided by the original cross-sectional area (engineering stress); strain at break is defined as the increase in scaffold length required to cause failure divided by the original length, and elastic modulus is defined as the slope of its stress-stain curve in the elastic deformation region.
F. Biophysical assessment ball burst tensile testing

1. Materials

- Scalpel and Blades.
- Cutting board.
- Digital callipers Scienceware®, Digi-Max™, Sigma-Aldrich, Ireland.
- Two glass panes.
- PBS.
- Zwick/Roell (Leominster, Herefordshire, UK) Z005 testing machine.
  - 1000 N load cell.
- Ball burst testing rig; Custom built to ASTM D3787-15 specifications.
- A Laptop with excel.
- Vulcanised rubber.
- Allen keys.

2. Sample preparation

- Cut a rough 65 mm diameter circle in the sample material.
- The samples should be hydrated overnight through emersion in PBS.
- Prior to commencement of testing the thickness must be measures so the area can be calculated for stress v stain curves. Using the callipers take five measurements from the thickness of the glass panes alone, insert the sample between the panes and repeat as per sample. The glass panes are used to give a consistent thickness measurement for the sample by reducing potential bias introduced when measuring. Additionally, the glass protects the sample from potential defects created by the callipers.
3. Testing procedure

Physical setup

- Turn on the power at the main switch on the side of the machine.
- On the front panel of the device you have the manual control for up and down, the on button and emergency stop. Press the on button on the front of the machine to activate the device.
- Load the “ball burst testing rig” onto the machine.
- Insert the sample between two pieces of rubber onto the rig and securely close the clamps. Note: the vulcanised rubber provides grip to the sample to ensure the sample doesn’t slip during testing.
- Using the manual override bring the crosshead into an appropriate range for the sample so that it is just above the sample.

Software setup

- Turn on the computer.
- Select the testXpertII program.
- When it is open select “File> Open> program name” (the program you wish to run).
- Then click “OK”, this will load the test parameters required for that tensile test.
- Select the “Wizard” to define the sample specifics.
  - The Pre-test; auto calculate the grip to grip separation; define the specimen cross sectional shape and pre-load value.
  - Test parameters; define the test speed of 20 mm/min.
  - Results; the parameters to be measured.
Test commencement

- Zero the force of the system.
- Input the sample thickness and width.
- Click the “Start” icon. When the test is completed click the “Stop” icon and select the “Actions after the test” in the pop up that appears.
- Export the data to Excel and this will allow the data to be analysed.

Sample analysis and processing

The following definitions are used to calculate mechanical data: stress at break is defined as the load at failure divided by the original cross-sectional area (engineering stress); strain at break is defined as the increase in scaffold length required to cause failure divided by the original length, and elastic modulus is defined as the slope of its stress-stain curve in the elastic deformation region.
G. Research outputs

1. Awards
   - Awarded IRCSET Enterprise Partnership Scheme scholarship for PhD.

2. Manuscripts
   - K. P. Fuller, A. Pandit and D. Zeugolis, The Multifaceted Potential of Electrospinning in Regenerative Medicine, Pharmaceutical Nanotechnology, 2 (1) 23-34.
• L. M. Delgado, N. Shologu, **K. P. Fuller**, D. I. Zeugolis, Acetic acid and pepsin result in high yield, high purity and low macrophage response collagen for biomedical applications, Biomedical Materials, 12 (6) 065009.


• D. Gaspar, **K. P. Fuller**, D. I. Zeugolis, Polydispersity is key modulator of extracellular matrix deposition under macromolecular conditions, Submitted to Acta Biomaterialia on the 27th of Sep 2017.


3. Conferences participations


• Delgado LM, Gaspar D, **Fuller K**, Pandit A, Zeugolis DI. Collagen cross-linking enhances stability whilst induces pro-inflammatory macrophage response. UKSB 14th Annual Conference and Postgraduate Day. 2015, United Kingdom.

• Delgado LM, **Fuller K**, Gaspar D, Pandit A, Zeugolis DI. Collagen Cross-linking modulates scaffold stability and pro-inflammatory macrophage response. TERMIS World. 2015, USA.


• A Irizar, M. J. B. Amorim, **K. P. Fuller**, D. I. Zeugolis, J. J. Scott-Fordsmand, Environmental fate and effect of biodegradable electrospun scaffolds (Biomaterial) - A case study, ESB 2017, Athens.
4. Conference papers


5. Professional courses

- LAST course, 2012, Trinity College Dublin.