Evaluation of Xenogenic Extracellular Matrix Scaffolds for use in tissue engineered vascular grafts

Mr. Daniel Nicholas Martin Coakley MB. BMedSc, MRCSI, MRCOphth.

MSc Biomedical Engineering

Supervisors: Dr. Jeremy Robinson, Prof. Michael Larvin.

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Abstract

Existing synthetic vascular grafts have unacceptably high failure rates when replacing below knee arteries. In vitro endothelialisation is an emerging procedure that has been shown to enhance the patency rates of below knee synthetic vascular grafts. Here autologous endothelial cells are harvested and cultured on the lining of the graft prior to implantation. The technique is limited by the narrow time frame available for culture and the finite cells obtainable following harvest. As well as this, existing synthetic materials are poor cellular substrates and must be combined with coatings to promote cellular growth and attachment. The most common coated graft used clinically is fibrin-coated polytetrafluoroethylene (ePTFE).

The study aim was to compare the endothelialisation of fibrin-coated ePTFE with emerging novel extracellular matrix (ECM) scaffolds, which we hypothesise will provide a superior substrate for cell growth by mimicking the in vivo environment more closely. We also construct and verify a novel subatmospheric bioreactor in order to enhance the proliferation of cultured cells. Finally, we examine the culturing of endothelial cells inside the lumen of small diameter ECM vascular grafts.

Our Results showed that cells remained viable and produced von Willebrand factor on all substrates tested. There was no difference in adhesion rates between ECM scaffolds and fibrin-coated ePTFE (p = 1.00). Endothelial cells proliferated fastest on ECM scaffolds when compared to all other materials tested (p <0.001). When seeded scaffolds were exposed to subatmospheric pressures in a closed bioreactor a mechanical strain was exerted on the construct. This was shown to alter cellular morphology and enhance cellular proliferation. We found we could rapidly form a confluent endothelial lining on the luminal surface of small diameter ECM grafts.

In conclusion, ECM bioscaffolds offer a superior substrate for promoting rapid endothelialisation compared to existing fibrin-coated ePTFE by combining firm cellular anchorage and rapid cell expansion. Proliferation rates were positively influenced by a subatmospheric bioreactor. This work suggests that ECM materials are promising scaffolds for use in small vessel tissue engineering.
Declaration

I hereby declare that, except where otherwise indicated, this document is entirely my own work and has not been submitted in whole or in part to any other university.

Signed: ……………………………… Date: ……………..
I firstly wish to thank Prof. Pierce Grace and Prof. Tim McGloughlin for giving me the opportunity to carry out this research and for his limitless enthusiasm throughout. I would also like to thank my supervisors, Dr. Jeremy Robinson, Prof. Michael Larvin for all their hard work and encouragement. Thank you to Faisal Shaikh and Anthony Callanan for help with lab work. A special thank you to Kathleen O’ Sullivan in the Statistical Consultancy Unit, University College Cork for her time and expert advice on the statistical analysis of the data. To Suzanne Crotty in the BioSciences Institute, Univeristy College Cork for her endless SEM analysis of vascular grafts. Thank you to all the suppliers of materials, most of whom supplied materials gratis; Prof. S. Badylak, University of Pittsburgh, Extracellular Matrix Materials, Conor Fleetwood (Fleetwood Healthcare Ltd), supply of Dacron Materials, Bard/IMPRA Ltd., supply of ePTFE Materials, Margo Barry (KCI Ltd.), supply of VAC ® units and materials, and LeMaitre Vascular – supply of embolectomy catheters. I would also like to say thank you to Prof. Stuart Walsh for his input and also to Prof. Peter Zilla and Prof. Stephen Badylak for their expert opinions on the subject matter. Finally thank you to the Royal College of Surgeons Ireland for funding this research.
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<td>α-Gal</td>
<td>Galalpha1-3Galbeta1-(3)4GlcNAc-R</td>
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<td>α1,3GT</td>
<td>α1,3galactosyltransferase</td>
</tr>
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<td>Arg</td>
<td>Arginine</td>
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<tr>
<td>Asp</td>
<td>Aspartic</td>
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<td>bFGF</td>
<td>Basic Fibroblast Growth Factor</td>
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<td>Calcein AM</td>
<td>Calcein acetoxymethyl</td>
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<td>CaCl₂</td>
<td>Calcium Chloride</td>
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<td>CVD</td>
<td>Cardiovascular disease</td>
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<td>Endothelial Cell</td>
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<td>ECM</td>
<td>Extracellular Matrix</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<td>Ear Nose And Throat</td>
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<tr>
<td>EPC</td>
<td>Endothelial Progenitor Cell</td>
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<tr>
<td>ePTFE</td>
<td>Expanded Polytetrafluoroethylene</td>
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<td>EthD-1</td>
<td>Ethidium Homodimer – 1</td>
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<td>Ethylene Oxide</td>
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<tr>
<td>EU</td>
<td>European Union</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>Fn</td>
<td>Fibronectin</td>
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<td>Glu</td>
<td>Glutamic</td>
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<tr>
<td>HBSS</td>
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<td>HUVEC</td>
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<td>Il</td>
<td>Interleukin</td>
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<td>Ile</td>
<td>Isoleucine</td>
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<td>Inter nodal distance</td>
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<tr>
<td>KGF</td>
<td>Keratinocyte Growth Factor</td>
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<tr>
<td>MAP</td>
<td>Mitogen-activated Protein</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cells</td>
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<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate (reduced)</td>
</tr>
<tr>
<td>NPWT</td>
<td>Negative-pressure wound therapy</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline.</td>
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<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
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<td>PEI</td>
<td>Polyetherimide</td>
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<td>PES</td>
<td>Phenazine Ethosulfate</td>
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<td>PET</td>
<td>Polyethylene Terephthalate</td>
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<tr>
<td>PGA</td>
<td>Polyglycolic Acid</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly (lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>POE</td>
<td>Poly(orthoester)</td>
</tr>
<tr>
<td>PU</td>
<td>Polyurethanes</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomised Controlled Trial</td>
</tr>
<tr>
<td>REDV</td>
<td>Arginine -Glutamic- Aspartic -Valine</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-Glycine-Aspartic Acid</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation.</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
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<tr>
<td>Ser</td>
<td>Serine</td>
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<tr>
<td>SIS</td>
<td>Small Intestinal Submucosa</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>SMC</td>
<td>Smooth Muscle Cell</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package for the Social Sciences</td>
</tr>
<tr>
<td>TBS</td>
<td>Tissue Buffered Saline</td>
</tr>
<tr>
<td>TE</td>
<td>Tissue Engineering</td>
</tr>
<tr>
<td>TH1</td>
<td>T-helper 1</td>
</tr>
<tr>
<td>TH2</td>
<td>T-helper 2</td>
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<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor Beta</td>
</tr>
<tr>
<td>TRAC</td>
<td>Therapeutic Regulated Accurate Care</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
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<tr>
<td>UBM</td>
<td>Urinary Bladder Matrix</td>
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<tr>
<td>VAC</td>
<td>Vacuum Assisted Closure</td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Cell Growth Factor</td>
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<tr>
<td>vWF</td>
<td>Von Willebrand Factor</td>
</tr>
<tr>
<td>YIGSR</td>
<td>Tyrosine-Isoleucine-Glycine-Serine-Arginine Acid</td>
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1

Tissue engineering vascular grafts
1.1 Introduction

Cardiovascular disease (CVD) accounts for 48% of all mortality in the European Union (EU) and is the leading cause of death in women [1]. €192 billion per year is spent on treatment and prevention, equating to a cost of €223 per annum for each EU resident [1]. The principal cause of CVD is atherosclerosis; a complex, incompletely understood process of vascular cell malfunction, inflammatory reaction and accumulation of lipoproteins and cholesterol within the vessel wall [2-4]. Subsequent vascular remodelling leads to acute and chronic vessel narrowing, disruption of haemodynamics and reduced nutrient supply to the organ or tissue. The medical management of this disease is an intensely researched area of medicine and has advanced greatly in the last four decades. Medical treatment involves addressing risk factors (smoking, hypertension cholesterol, raised body mass index and diabetes) through lifestyle changes as well as pharmaceutical interventions in the form of antihypertensives, antiplatelet therapy and 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors.

Upon failure of medical therapy, two surgical options are available. The first, percutaneous angioplasty involves endovascular widening of a narrowed artery using a balloon catheter sometimes combined with intraluminal stenting. The second, bypass grafting, involves major open surgery, bypassing the obstructed or narrowed vessel using either autologous vessels (arteries or veins) or prosthetic vascular grafts. Despite the significant advancements in medical treatment and endovascular technology, bypass surgery continues to be an important therapeutic option. 2,242 vascular bypass operations were carried out in the Republic of Ireland in 2013 alone [5] and this figure is set to rise as peripheral vascular disease and kidney failure requiring haemodialysis is increasing [6].
It is universally accepted among vascular surgeons that autologous grafts, either in the form of veins for lower limb arterial bypass [7] or arteries for coronary bypasses [8] remain the “gold standard” since their introduction by Kunlin in 1949 [9]. However, almost a third of individuals do not have adequate veins or arteries available for reconstruction [10]. This can be due to concurrent vessel pathology (varicose veins), trauma or previous harvesting. The problem is compounded by the chronicity of vascular disease with patients often requiring secondary bypass grafting or revision procedures. Hence there is a pressing requirement for an alternative, readily available graft that can approximate the physical and biological characteristics of natural arteries and maintain adequate patency. The development of such a graft is considered the Holy Grail amongst vascular surgeons and is an intensely focused area of research.

Today the most widely used alternatives to autologous bypass conduits are synthetic grafts, most commonly consisting of either polyethylene terephthalate (Dacron ®) or expanded polytetrafluoroethylene (ePTFE). Prosthetic grafts deliver near equal patency rates over the short term in the above knee infrainguinal region when compared to autologous grafts (five year patency rate 91.0%) [11]. However meta-analysis has shown a marked deterioration in patency for below knee bypass grafting (five year patency rate 38.4%) [12]. Furthermore, Jackson and associates demonstrated that prosthetic graft occlusions had worse clinical outcomes than autologous graft occlusions [13]. Current synthetic grafts are hindered by poor elasticity, inferior compliance [14] and thromogenicity [15]. These properties lead to three recognised sources of prosthetic graft failure; acute thrombosis due to lack of an endothelial layer; intimal hyperplasia from compliance mismatch and infection [16-20]. Thus, prosthetic grafts are widely regarded as inferior to autologous vessels
and tend mainly to replace high-flow/low resistance vessels i.e. large calibre vessels (6 – 10mm) where they have met with considerable success [21,22].

The ideal bypass conduit should possess a number of mechanical and biological properties. These include strength, elasticity, biocompatibility and biostability [23]. It should also be able to adjust to differing haemodynamic environments encountered in the short and long term. Despite extensive research efforts, existing prosthetic bypass grafts are not able to fulfil these requirements [24] and no superior graft has gained widespread clinical acceptance since the introduction of ePTFE by Matsumoto in 1973 [25].

The extensive and diverse properties required in this field have lead researchers to investigate nanotechnology and tissue engineering (T.E.) for possible solutions. Nanotechnology involves combining manmade molecules with existing materials to enhance specific properties such as anti-thromobgenicity or biocompatibility [26]. Tissue engineering is “an interdisciplinary field that applies the principles of engineering and life sciences to the development of biological substitutes in order to restore, maintain, or improve tissue functions” [27]. Early vascular tissue engineering attempts, involved seeding human endothelial cells (EC) onto the luminal surface of non-absorbable synthetic grafts. It was hoped that by creating a confluent, well-attached endothelial layer, the graft would be non-thrombogenic and thus increase patency. However current synthetic grafts are poor substrates for the attachment and proliferation of human cells [28-31]. Two studies in the 1980’s and 1990’s demonstrated that endothelial attachment rates on untreated ePTFE approached 4% [32, 33], with later studies reporting a retention rate of approximately 14% [34, 35]. Studies with uncoated Dacron showed similarly poor results, likely due in part to its high porosity and inert synthetic fibres [36].
The chief aim of graft endothelialisation is to promote cell proliferation and attachment in order to create a confluent monolayer and limit cellular loss upon implantation. To improve the biocompatibility of existing grafts, several mechanisms have been investigated, including coating with extracellular matrix (ECM) proteins (collagen, fibronectin, laminin, gelatine, poly-l-lysine etc.) [37-41], pre-clotting of the graft with blood [42], plasma [43, 44], serum [45] or fibrin glue [46] and more recently, surface modification with ECM adhesion peptides (heparin, arginine-glycine-aspartic acid, lectins, cell adhesion peptides) [47-52]. Of these methods, ePTFE grafts coated with fibrin glue has provided the most robust clinical evidence with the longest follow up times. Kadletz et al. reported improved cellular attachment and migration of endothelial cells on ePTFE materials pre-coated with fibrin glue [40]. Magometschnigg [53] and Zilla [54] have both demonstrated enhanced patency rates using endothelialised ePTFE/fibrin bypass grafts in long-term human trails. Even though these hybrid grafts have demonstrated improved patency rates, they are not widely used. They still rely on existing 1970’s synthetic grafts with their known limitations, including suboptimal compliance, propensity to infection and chronic inflammatory reactions. Advancements in tissue engineering and our understanding of cell physiology have raised the possibility of developing a graft that could obviate these issues. A tissue-engineered graft could incorporate a confluent endothelial layer and possess the same mechanical properties of the vessel it replaces. It would lack synthetic materials that could prompt a foreign body reaction or promote bacterial colonisation. These vessels could be patient tailored with no bifurcations or disease and be free of harvest-related damage [55]. They would eliminate the co-morbidity; operative time and cost associated with vessel harvesting [56, 57].
Hence the aim of this thesis is to evaluate a novel biomaterial – decellularised xenogenic extracellular matrix for use in a tissue engineered vascular graft. To this end, I will compare the endothelialisation of these scaffolds with existing coated synthetic graft materials, assessing biocompatibility and the stability of the endothelium. The current chapter gives a description of the anatomy and physiology of human arteries we aim to replace. I will outline existing bypass graft materials and the tissue engineering advances made to date in the area of vascular surgery. This chapter will also detail the emergence of extracellular matrix technology in medicine.

1.1.1 The Cardiovascular System - Overview of Anatomy & Physiology

The purpose of the cardiovascular system is to provide nutrients and collect waste products from tissues in order to maintain a suitable environment for tissue survival and function. It consists of a four-chambered pump; the heart and a collection of tubes (blood vessels), which channel the blood. Vessels that leave the heart are called arteries or conductance vessels, which transport blood under high pressures to the tissues. Those that return blood to the heart are called veins. Arteries proximal to the heart are large in calibre and contain a high proportion of elastic laminae. These arteries are adapted to transport large quantities of blood and accommodate the pressure fluctuations of the cardiac cycle. They are sometimes are referred to as elastic arteries. As the arteries leave the heart and approach their target organs (brain, liver, pancreas etc.) or other end points (mesenteries, pia mater, muscle, connective tissue etc.) their calibre progressively decreases and their walls become thinner, with higher amounts of smooth muscle cells, the finest of which is called arterioles. Arterioles still have relatively thick walls but a very narrow lumen. The function of
arterioles is to dampen the hydrostatic pressure of the blood prior to entering the capillaries. The capillaries are the smallest diameter blood vessels and are composed of endothelium only with a thin basement membrane. They are one cell in diameter and have one-cell thick walls. It is here that homeostasis is maintained by the exchange of nutrients, oxygen and waste products such as carbon dioxide. The blood from the capillaries then empties into venules, which have the same structure as capillaries but are slightly larger in diameter. These vessels then drain into progressively larger veins until they return to the heart. The cardiovascular system can therefore be thought of as a closed circuit comprising the heart, arteries, veins, capillaries, venules and arterioles. The vessels of the circulatory system all share the same basic histological structure, namely three concentric coats or tunicae with variations in composition according to location and function (Fig. 1).

Fig. 1. The layers of a blood vessel.
1.1.2 Tunica Intima

The innermost lining of a vessel wall comprises an elongated confluent layer of simple squamous epithelial cells known as the endothelium, which is attached to an underlying basement membrane (Fig. 2). A variable amount of connective tissue and internal elastic lamina is present beneath the endothelium providing structure support and flexibility. The endothelium is orientated according to the flow of blood and has essential roles in haemocompatibility and anti-thrombogenicity of the vessel. The endothelial cells produce glycoproteins, which inhibit cells and proteins from the plasma entering the vessel wall, hence acting as a selective barrier. They also produce nitric oxide, which relaxes the smooth muscle cells and hence have a role in regulating blood flow. The endothelium can therefore be considered the most important vessel layer. It lines the entire vascular system including the heart where it is known as the endocardium.

Fig 2. Illustration; cross section of blood vessel wall.
1.1.3 Tunica Media

Most of the vessel wall is made up of the tunica media layer, which gives the vessel the majority of its mechanical characteristics. Its principle cells are smooth muscle cells (SMC), which produce collagen, elastin, and proteoglycans. These are arranged circumferentially around the vessel and are especially well developed in arteries where large volumes of blood are transported, i.e. large calibre vessels, close to the heart. This layer can constrict and relax when stimulated by neural and hormonal factors, thus assisting in the transport of blood and maintaining blood pressure. The medial layer is divided from the inner most layers by the internal elastic lamina.

1.1.4 Tunica Adventitia

This outer most coat is predominantly composed of loose connective tissue with some smooth muscle cells in larger vessels. The bulk of this layer is primarily made up of collagen types I and III. Other components include elastin and to a lesser extent proteoglycans (versican etc.), hyaluronan and glycoproteins. This layer conveys physical and structural integrity to the vessel wall. Connective tissue fibres allow the vessel wall to stretch in response to pressure exerted by blood flow whilst preventing overexpansion. It has its own blood supply; the vasa vasorum, which also supplies some of the medial layer beneath the external elastic lamina.
1.2 Existing Synthetic Grafts

1.2.1 Expanded polytetrafluoroethylene (ePTFE) Graft

PTFE (-CF₂-CF₂-) is an inert porous fluorocarbon polymer of tetrafluoroethylene. It was first used medically to replace cardiac valves in the 1960’s and later as a vascular graft for haemodialysis in the 1970’s (Fig. 3a). Vascular expanded PTFE is made by a process of expanding, heating and extruding to produce a graft of irregular-shaped solid membranes or “nodes” (Fig. 3b) [58]. It is durable because of the strength of the carbon–fluorine bonds and evokes minimal tissue reaction when implanted in vivo [59,60]. Due to the electronegativity of fluorine it is highly hydrophobic, thus minimising it’s reactivity with blood components. Its structure is made up of circumferential irregular-shaped solid membranes interconnected by a meshwork of fine fibrils (Fig. 3b). The porosity is determined by the “inter nodal distance” (IND), which is typically 30-90µm, however in reality it is the distance between each fibril that determines the porosity of the material and hence the ingrowth space available. Experimentally low-porosity
is defined as \( \leq 30\mu m \) IND and high-porosity is \( \geq 45\mu m \) IND. Standard ePTFE provokes a similar response as Dacron when implanted in vivo: a thin fibrin layer develops over time and no endothelialisation is seen. The grafts are encapsulated within 6 months with minimal cellular infiltration [59]. Over the years, a plethora of alternations have been made to ePTFE grafts to improve functionality (ultrathin walled grafts, tapered ends, high porosity, carbon-coated, heparin bounded, reinforced external rings to prevent kinking and increase crush resistance) however most haven’t improved outcomes [61-63]. Increasing the porosity of ePTFE was investigated as a means to increase patency rates. It was thought that this would enhance capillary ingrowth and encourage endothelialisation of the graft lumen. In primate studies, capillary ingrowth did occur and a confluent complete endothelium was formed [64,65]. These findings were replicated in subsequent canine studies with high-porosity grafts achieving enhanced patency rates [66, 67]. Unfortunately human trails did not replicate these findings and no increase in patency rates was seen [68, 69]. More recently expanded cuffed ePTFE grafts (Venaflow ®), which reduce turbulent outflow, has increased patency rates to some degree [70]. EPTFE grafts perform well in replacing large diameter, high flow vessels. In aortic replacement it achieves five year primary patency rates of 91 – 95% [71,72]. In below knee lower limb arterial replacement, patency drops to 38.4% at five years [12]. A recent systematic meta-analysis involving nine randomised controlled trials comparing the patency rates of ePTFE and Dacron in above and below knee bypass did not show any significant advantage of either synthetic material [73].
1.2.2 PET/Dacron (Polyethylene terephthalate)

Polyethylene terephthalate (-O-C=O-C₆H₄-O-C=O-CH₂-CH₂-) is a thermoplastic polymer resin first introduced in 1939. This was further refined and the common Dacron fibre was patented in 1950 [74] with its first use in a vascular graft reported in 1957 by Julian [75]. In vascular grafts, round Dacron cross sectional fibres are arranged as multifilament fibres available in woven or knitted forms (both widely available) (Fig. 4a). Woven Dacron is produced in an over-and-under fibre arrangement to reduce creep and porosity (Fig. 4b). This improves strength but reduces compliance. The threads of knitted Dacron grafts are looped in a textile fashion, which enhances radial distensibility and increases porosity. In the past the high porosity of knitted grafts necessitated pre-clotting to prevent blood extravasation upon implantation. Several coatings have since been incorporated into knitted grafts to avoid the need for pre-clotting including gelatine [76], collagen [77] and albumin [78]. These coatings have not been shown to enhance patency rates [72, 79]. Dacron can be crimped to enhance distensibility, flexibility and kink-resistance. External rings are occasionally incorporated into the grafts to provide mechanical...
stability and resist compression. Despite the differences in physical properties, the patency rates between knitted or woven grafts in aorto-iliac bypasses are similar [80]. Dacron is known to dilate over time, particularly knitted Dacron, but complications owning to this are extremely rare [81]. Upon implantation and restoration of blood flow, a host tissue and graft reaction begins immediately. Within hours fibrin containing platelets and blood cells is deposited onto the luminal graft and interstices and builds up over several days [82, 83]. After 18 months this layer matures to form a compacted fibrin lining [84]. The porosity of knitted and to a lesser degree woven Dacron does allow for limited tissue ingrowth. However, the compacted fibrin layer appears to be a barrier to the ingrowing capillaries to reach the luminal surface [85 - 87]. Autopsy studies involving knitted Dacron grafts fail to show any substantial areas of endothelialisation up to 11 years post implantation [88]. A confluent endothelium is only found in animal studies and this appears to be due to transanatomtic ingrowth rather than mid-graft healing [89 - 91]. Aortic Dacron grafts achieve five year patency rates of 93% [71]. This rate reduces to 43% for femoropopliteal bypass grafts and further still for distal below knee procedures [92].

### 1.2.3 Polyurethane Grafts

Polyurethanes (PU) are a family of elastic copolymers made up of three monomers. They comprise a soft chain, usually polyol that confers malleability and flexibility. A hard crystalline component imparts strength and rigidity and a third monomer, acts as a chain extender [93]. Differing ratios of these monomers alter the
mechanical characteristics of the material, which can be tailored for a specific requirement. This makes PU a very appealing biomaterial in tissue engineering. PUs were first used medically in ventricular assisted devices and as coatings for heart valves, demonstrating excellent biocompatibility [94]. Their physical properties, including enhanced elasticity and compliance made them a natural choice for use in a vascular graft. It was proposed that PU may offer superior mechanical properties to the relatively non-compliant ePTFE and Dacron grafts and hence increased patency rates. The first generation PU vascular grafts underwent excessive hydrolytic biodegradation and in one study eight out of 15 below knee grafts failed at one year [95, 96]. Second generation PU grafts were polyester-based and resistance to hydrolysis, however clinical studies demonstrated oxidation biodegradation leading to high failure rates and the abandonment of human trials [97]. The latest generation use polycarbonate based PUs and are hydrolytically and oxidatively more stable [98]. In preclinical rat trials, they were superior to ePTFE in terms of faster endothelialisation and decreased rates of neointimal formation in small calibre vessels [99]. In subsequent canine studies in the aorto-iliac position, PU grafts displayed neointimal formation in the mid graft and anastomotic sites with no significant degeneration seen [100]. Although PU grafts have demonstrated favourable results in animal studies, there is currently a lack of trials to determine if PU grafts offer superior outcomes to existing Dacron and ePTFE grafts in humans [101].
1.3  Tissue engineering of blood vessels

1.3.1  The ideal tissue engineered arterial replacement

Before investigating vascular graft materials, it is important to understand the properties tissue engineers strive for. Arteries serve a critical function; they are complex, multilayered structures that must adapt to prevailing pressure changes. Despite several decades of intensive research, vascular surgeons are still relying on technology from the 1960’s and 70’s in the form of ePTFE and Dacron grafts, with no widely accepted alternative. This fact illustrates the immense challenge facing tissue engineers and the many obstacles to overcome. Thomas et al. set out the ideal principles of a vascular substitute [102]. He states, that a potential replacement should:

1. Contain a confluent, well adherent and quiescent endothelium.
2. Be resistant to infection.
3. Be biocompatible.
4. Possess specific mechanical properties (strong, durable, compliant, good suturability, kink resistant).
5. Low manufacturing costs.
7. Available in differing lengths and calibres.

As previously discussed, researchers have failed to deliver a graft that meets all of these requirements. Current grafts operate well when replacing large calibre, high flow vessels; however they have unacceptable failure rates in smaller calibre vessels.
A primary requirement of any tissue-engineered replacement is biocompatibility. Biocompatibility refers to a material's ability to perform a set task with an appropriate response from the host in which it is implanted [103]. More specifically, it describes the ability of a product to accommodate cellular activity including molecular signalling and allow for tissue regeneration without provoking an undesirable local or systemic host reaction. There is no single experiment to determine biocompatibility, as it is a term that encompasses many properties. It is generally accepted that in vivo animal and human trials are ultimately required to determine this property. Biocompatibility has always been a major hurdle when developing tissue engineered medical devices and has frequently hampered progress. A plethora of methods to reduce the host’s reaction to implants have been investigated, but none to date have completely eliminated the host response. This is a particular concern in vascular graft tissue engineering, as provocation of an undesirable host reaction such as an immune response or coagulation cascade could lead to graft failure and possible death.

1.3.2 Biomechanical considerations: compliance match

Mechanical properties required by a graft include burst strength, compliance and viscoelastic characteristics. A burst strength of 2000 mmHg, which is greater than that of venous grafts (1680 +/- 307mmHg) is generally considered as acceptable to resist the continuous haemodynamic stresses upon implanted [104]. Along with adequate burst strength a potential graft must function in and adapt to the prevailing haemodynamic forces. The structure of arteries; particularly the multi-laminar organisation of collagen and elastin is well suited for this purpose. The arterial wall
is highly elastic at low pressures thus providing recoil energy for distal profusion. At higher pressures more collagen fibres contract and the wall becomes stiffer and less distensible [105,106]. Gupta et al. showed that up to 120mmHg, elastic fibres were stretched, above 120mmHg; further pressures were exerted exclusively on collagen fibres, which prevented excessive stretch [107]. These mechanical properties are known as compliance, which is defined as the expansion and contraction of the blood vessel wall secondary to alternating intraluminal pressures. Compliance mismatch can occur at the anastomosis of native artery and a poorly compliant vascular graft [108]. This mismatch results in increased velocity gradients, turbulent flow, low shear stress rates and decreased distal perfusion [109]. Overtime these adverse haemodynamic changes cause weakening of the vessel wall and endothelial injury resulting in intimal hyperplasia or aneurysmal formation [110-112]. Compliance mismatch associated with intimal hyperplasia is one of the leading causes of bypass failure. Current synthetic grafts (ePTFE) have a compliance of 1% per mmHg × 10^{-2} compared to 4-8% per mmHg× 10^{-2} of the human femoral artery [104]. Several studies have demonstrated a clear link between compliance mismatch decreased patency rates [113,114].

1.3.3 Thromboresistance and endothelialisation

It is known that compliance mismatch induces intimal hyperplasia, however there is an increasing body of evidence to suggest that the presence of an intact endothelium is also critical in preventing this [115]. In humans, prosthetic grafts do not develop an endothelial lining when implanted [116,117]. This contrasts to animal studies, which demonstrate neovascularisation of the graft lumen from the
perianastomic area, ingrowth of capillaries into graft interstices or from circulating progenitor endothelial cells [65]. This lead to promising results of early prosthetic grafts in animal trails, which was not replicated in human studies. There is growing consensus that a confluent stable endothelium is essential for a successful small calibre vascular graft and this theory was well demonstrated in a large human trial by Zilla et al., where endothelialised ePTFE grafts achieved equal patency rates to autologous venous graft in the infrainguinal area [115].

1.3.4 Off-the-shelf availability

Off-the-shelf availability is another major hurdle in graft design and is a significant obstacle in achieving widespread clinical acceptance of tissue-engineered grafts. The issue arises due to the significant proportion of surgical revascularisations that are acute. In terms of endothelialised grafts; prolonged cell culturing times as well as the need for cellular harvesting are the main limiting factors [118]. A recent avenue of research to solve this problem was the potential harvesting or in vivo recruitment of endothelial progenitor cells (EPCs) [119]. EPCs are CD34++ cells that are released into the systemic circulation at times of ischemic injury. They are not stem cells but they are committed to a vascular linage and can differentiate into endothelial cells [120]. They appear to be a potentially rich source of endothelial cells, which are released at the time of vessel injury and can be harvested by simple venesection. This non-depleting, self-renewing source is ideal and several animal trials using EPCs have shown promise. Tepper et al. demonstrated EPCs forming new vessels via vasculogenesis at an ischemic soft tissue site in a rat model [121]. Kaushal et al., seeded ECM scaffolds with EPCs and
implanted them in the common carotid artery of seven sheep [120]. All grafts remained patent at the end of the study (15 - 130 days). Upon explantated, grafts were completely endothelialised. Other avenues currently being investigated include more efficacious harvesting methods and faster in-vitro seeding times using erythropoietin and hydronymethylglutaryl-coenzyme A reductase inhibitors [122,123]. Despite these promising areas of research, an off the shelf tissue engineered grafts is currently not possible.

1.4 The Development of Tissue Engineered Vascular Grafts – The Scaffold

The scaffold is a critical component in all areas of tissue engineering. Its 3-dimensional structure provides support and protection for indwelling cells and a framework for cellular growth, migration, differentiation as well as orientation. Ideally a scaffold should only act as a temporary template till populating cells manufacture a replacement ECM. It should degrade at a predictable rate thus allowing for tissue regeneration and avoiding host tissue reactions whilst also minimising the risk of infection. Numerous different types of graft materials have been examined for this purpose including natural proteins and synthetic biopolymers.

1.4.1 Natural Scaffolds

Proteins such as elastin and collagen are structural components of natural cellular scaffolds – the extracellular matrix; hence they are ideal components for cellular adhesion and differentiation. Indeed collagen was the scaffold used in the first tissue engineered bypass graft in 1986 [124]. Collagen is a favoured scaffold for
its low antigenicity and excellent cellular biocompatibility properties. However it is mechanically weak and fails to provide support against the hemodynamic forces found in vivo. Researchers have attempted to strengthen these scaffolds using several methods including glycation [125], incorporating meshes or “sieves” [126-129] and mechanical precondition [130,131]. Collagen scaffolds have also been used in conjunction with Dacron or polyurethane grafts to provide mechanical support [129]. This technique was further refined by using biodegradable “wrappings” including cross-linked collagen and elastin [127,128]. Electrosprinning technology is a method to alter the physical characteristics of natural proteins; creating biomimetic vascular grafts with increased burst strength [132]. Along with collagen, elastin is thought to be crucial in tissue engineering an artery. Elastin provides elasticity to the arterial wall along with regulatory and structural functions [133]. Smooth muscle cells produce elastin and these have been incorporated into collagen and fibrin scaffolds for this purpose [134]. Elastic blood vessels have been created using a fibrin scaffold and these have shown promise in early pre-clinical studies [135-137]. These scaffolds demonstrated improved mechanical strength following implantation and exposure to arterial haemodynamics similar to that seen with autologous venous grafts.

1.4.2 Biodegradable polymer scaffold

Biodegradable polymers have previously been evaluated for use in potential tissue engineered grafts. Most of these materials are already employed in other areas of surgery for example in meshes, and have been adapted for use in tissue engineering. They are relatively inexpensive, widely available and can be easily manufactured to any graft dimension. Polymer scaffolds are particularly attractive in
tissue engineering because their mechanical properties can be specifically adjusted to optimise tissue growth and remodelling i.e. elasticity, degradation rate, microstructure and biocompatibility. For example, the physical characteristics of polyurethane can be altered via the ratio of monomers as previously described. The main concept in utilising these materials is that they act as a temporary cellular framework, which slowly degrades in vivo allowing implanted cells to form a permanent replacement ECM. However the harsh chemical and mechanical processes used to create these materials are detrimental to cell integration during scaffold formation. Subsequent cell seeding and invasion can result in inadequate cell population [138]. This can be a significant problem; an inability of cells to construct a robust ECM before the polymer degrades could result in a mechanically unstable graft, which would prove disastrous in a clinical setting. Several polymer scaffolds have been researched, however polyglycolic acid (PGA) is by far the most extensively studied in vascular graft design [139,140]. PGA scaffolds rapidly degrade, losing their strength and frequently require mechanical preconditioning or chemical strengthening prior to implantation for example; copolymerising with poly-L-lactic acid [141,142), polyhydroxyalkanoate [143] or polyethylene glycol [144].

PGA grafts cultured with endothelial cells and preconditioned using a biomimetic perfusion system were implanted in pigs and remained patent at 24 days [144]. Watanabe et al. implanted canine vascular cells onto grafts of PGA combined with polycaprolactone-co-polylactic acid [145]. After one week of in vitro growth the hybrid grafts were grafted onto the inferior vena cava of a canine. The scaffold degraded within three months and the graft remained patent at 13 months. A similar scaffold was used by Shin’oka et al. to repair a re-occluded right intermediate pulmonary artery in a four year old girl following repair for ventricle and pulmonary
Autologous cells were harvested from the patient’s peripheral vein and cultured on the graft for 10 days. At seven month follow up the vessel was patent and displayed no evidence of aneurysm formation. This case illustrates the important role tissue engineered grafts play in paediatric vascular reconstruction where a graft is expected to grow and adapt as the patient grows. Shin’oka subsequently used bone marrow cells seeded onto copolymers of lactide and ε-caprolactone to repair congenital heart defects in 22 patients [25]. All grafts showed no evidence of thrombosis, stenosis or aneurysm formation and remained patent throughout the study. Despite the success of these early clinical trails, long term follow up data is lacking and very little is know about possible late complications such as calcification or abnormal remodelling. Also the grafts in these studies were placed in a low-pressure circulation (20-30 mmHg systole), which is not as demanding as the high-pressure environment of coronary artery (100 – 140mmHg) or peripheral distal arteries. As well as this, PGA degradation products are acidic which could potential illicit a host inflammatory reaction. Higgins et al. found the breakdown products of PGA inhibited the proliferation of SMCs and lead to their de-differentiation [147]. Other biodegradable polymer scaffolds tested include poly (lactic acid) [142], Poly(orthoester)s (POE) [148] and Poly(caprolactone) [149]. However these scaffolds lack the biocompatibility properties of PGA and frequently need to be modified by chemical coatings [150-152].
1.4.3 Decellurised Tissues

Cells can be removed from xenogenic vessels or other tissues by biological, physical and chemical processes [153-155]. What remains is a non-immunogenic natural ECM material that maintains most of the mechanical properties of the original tissue [156]. An advantage of decellularised ECM materials is that they retain a rich mixture of growth factors and cytokines, which is extremely important in encouraging cellular proliferation, attachment and differentiation. Typically these materials are implanted without cells in the hope that native cells will repopulate the material and transform into host tissue. Several animal studies have shown successful tissue remodelling including vascular reconstruction. In 2003 Komeda et al. used decellularised porcine carotid artery to replace the abdominal artery in a canine [157]. At 18 weeks, the graft had endothelialised and was patent. Fibroblasts and macrophages had infiltrated the graft and the neointima contained normal layers of smooth muscle cells. Teebken et al. created a biocompatible and mechanically stable graft by seeding human venous ECs and myofibroblasts onto decellularised porcine aorta [158]. A confluent monolayer was formed which was stable under flow conditions using at physiological bioreactor in vitro. An advantage of these materials is that they are available off shelf, are in abundant supply and cheap to manufacture. However, although decellularised vessels maintain satisfactory mechanical characteristics and have shown promise in animal trials much less is known about their behaviour when replacing human tissue. Their thrombotic proteins are still poorly understood and it is likely that they would need to be repopulated with recipient cells either in vivo or vitro for long term success, particularly when replacing small diameter vessels.
1.5 Promoting Endothelialisation on Biomaterials

1.5.1 Introduction

The endothelium is a dynamic lining of the blood vessel, not only assisting laminar flow but also regulating the coagulation cascade. It senses and responds to prevailing haemodynamic forces and is central in the metabolism of the vessel [159]. As cardiovascular research progresses, the roles of the endothelium are better understood, placing it at the centre of pathological processes such as intimal hyperplasia and vascular graft failure [160,161]. The importance of a stable, healthy endothelium is demonstrated by investigating the failure of autologous vascular grafts in coronary artery bypass surgery. Coronary bypass grafts are usually arterial, typically using the internal mammary artery [162,163] or occasionally the saphenous vein [164]. 10-year patency rates for arterial verses venous bypass grafts are 80% and 40% respectively [165,166]. Pathology studies involving failed venous grafts demonstrated endothelial loss, neutrophil infiltration and altered smooth vascular cell phenotype [167]. Scanning electron microscopy studies of failed venous grafts showed damaged/absent endothelium with exposed sub-endothelial collagen, whereas the arterial grafts demonstrated healthy endothelium, free of atherosclerotic damage or harvest injury [168]. This study was corroborated by Segesser et al., who examined endothelial function in surgically acquired vascular grafts [169,170]. The arterial grafts demonstrated superior endothelial function compared to the venous conduits, showing greater endothelial dependent relaxation when exposed to acetylcholine, thrombin and adenosine diphosphate. The results of these studies taken together highlight the crucial role of endothelium in graft patency and have lead to the pursuit of graft endothelialisation.
Spontaneous endothelialisation can occur within existing synthetic grafts by three methods. Firstly perianastomotic spread can occur by direct migration of the endothelial cells from the host artery. This however is limited, occurring at a rate of approximately 0.1mm per week in rat models [171]. Intensive research has been conducted to encourage perianastomotic spread and understand the effects of haemodynamics have on endothelial migration and proliferation [172-175]. However no intervention to date has been successful in promoting perianastomotic spread. The second method of endothelialisation is transmural migration of endothelial cells and this is the principle method seen in animal models. Edwards first demonstrated this in 1957 when he reported the growth of a cellular lining while using perforated polyethylene grafts [176]. Subsequent animal studies using high porosity ePTFE (60-90um intermodal distance) also reported complete endothelialisation as compared to limited perianastomotic endothelialisation in lesser permeable grafts [68]. Unfortunately these findings were not replicated in human studies, which although reported increased capillary transmural ingrowth, no significant increase in endothelialisation was seen and patency rates were not affected [71]. Although spontaneous endothelialisation is an exciting area of research, it is still in its infancy. Studies are limited to animals such as rats and rabbits which endothelialise much more rapidly than humans. Circulating endothelial cells are not likely to attach and propagate under intravascular flow conditions. The most likely source of endothelial cells would be from capillary infiltrate, which in human studies has not yielded satisfactory endothelial coverage. The significant obstacles encountered with spontaneous endothelialisation, has lead researchers to investigate a third method; the endothelial seeding of vascular grafts in vitro.
Initial research began by examining the seeding of endothelial cells on uncoated ePTFE and Dacron. However these synthetic materials do not adequately support the adhesion and growth of human cells [28-31]. Early studies demonstrated endothelial retention on untreated ePTFE to approach 4% [32, 34], while later studies reported a retention rate of approximately 14% [35]. Rosenman et al. demonstrated a 70% cell loss from pre-seeded ePTFE grafts immediately upon implantation, which continued over 24 hours [177]. Early studies with uncoated Dacron showed similarly poor results [36] with later experiments reporting an adhesion rate of only 17% [34]. Several different graft coatings have been investigated to try and improve cellular retention, adhesion and proliferation. These include fibronectin [178], collagen [179], plasma [180], basic fibroblast growth factor [181], fibrin [182], vibronectin [183] and combination coatings [40,184]. Although the majority of these coatings enhance endothelialisation to some degree, there is no consensus as to the optimal coating [51]. Coatings with collagen demonstrate up to three times more endothelial attachment when compared to uncoated grafts [185,186]. Subsequent studies by Anderson et al. using fibronectin reported superior cell retention and adhesion when compared to collagen coatings [184]. Combinations of fibronectin/collagen and laminin/collagen appear to increase retention rates compared to using single coatings [40,45]. Wissink et al. found that collagen mixed with basic fibroblast growth factor decreased the cell density needed to achieve confluency [187]. Fibronectin (Fn) is an extensively utilised coating in tissue engineering [188]. Fibronectins are large glycoproteins that are centrally involved in cellular attachment, spreading and growth [189,190]. As coatings, they enhanced cellular attachment and retention [191,192] and have shown superior cell attachment in comparative studies with other proteins such as collagen [185], gelatine [185], laminin [193] and albumin [194].
Subsequent studies combining Fn with collagen, laminin, gelatine and fibrin increased cell-seeding efficacy [193].

Although the number of coatings investigated to date is vast, the coating with the most clinical success in vascular graft tissue engineering is fibrin. Fibrin is a natural adhesive, which participates in the processes of clotting and wound healing. It has several characteristics, which make it an ideal cellular material. It is biocompatible and biodegradable and already utilised in a range of clinical applications [195]. As well as this, fibrin contains several cell binding sites, which promote strong adhesion [196]. These inherent properties of fibrin where first exploited by Schrenk et al. in 1987, who demonstrated enhanced endothelialisation with fibrin coated ePTFE grafts [197]. Subsequent studies by Kumar demonstrated less platelet adhesion to fibrin when compared to gelatine and also superior cell retention under shear stress [198]. In 1989, Zilla et al. cultured adult human endothelial cells on fibrin coated ePTFE grafts and exposed them to pulsatile shear stresses in a bioreactor for 48 hours [46]. After 48 hours the majority of cells remained attached to the graft surface. Gosselin et al. compared the cellular retention of fibrin-coated grafts with fibronectin-coated grafts exposed to shear stress in vitro. He also concluded that cells seeded on fibrin grafts had superior resistance to shear stress [182]. The success of these early in-vitro studies and the proven biocompatibility of fibrin lead to human clinical trials [199]. In 2001, Zilla et al. described a seven year follow up study involving 153 endothelialised ePTFE grafts in the lower limb (102 above knee and 51 below knee). 6-mm and 7-mm fibrin coated ePTFE grafts were used. These were preseeded with venous endothelial cells harvested from the cephalic vein. The team found a patency rate of 62.8% after seven years (60% - above knee and 70.8% - below knee), demonstrating superior patency rates for endothelialised vascular grafts.
The same group more recently published a 15-year (mean 9.6 years) follow-up study involving 341 endothelialised fibrin coated ePTFE grafts (308 femoropopliteal and 33 femorodistal bypasses), which were carried out in a non-tertiary hospital [200]. ECs were harvested from subcutaneous veins including the cephalic, basilic, external jugular, and saphenous veins. They were propagated in a lab for approximately nine days then seeded onto 6mm and 7mm fibrin coated ePTFE grafts. The 7 mm and 6 mm grafts achieved 78% and 62% at 5 years and 71% and 55% at 10 years respectively. The results of the study reaffirmed the increased patency rates of endothelialised grafts, which approached that of autologous venous grafts. The study also demonstrated the procedure could be carried out safely in a tertiary hospital and that fibrin was a suitable vascular coating.

More recent techniques to enhance adhesion rates involved the isolation of protein peptide sequences such as the Arginine-Glycine-Aspartic acid (Arg-Gly-Asp) or RGD sequences used in conjunction with polymers [201]. The RGD peptide sequence is an important site for cellular attachment and is found in a wide range of ECM proteins. It is recognised by half of all cellular integrins [202]. Other sequences being investigated include YIGSR (Tyr-Ile-Gly-Ser-Arg), a sequence found in laminin [203] and REDV (ARG-GLU-ASP-VAL), a domain of fibronectin [204]. The appeal of these peptides lies in the fact that they selectively bind endothelial cells without attracting platelets or smooth muscle cells.
1.5.2 Single-Stage Seeding

Single-stage seeding involves firstly isolating autologous ECs and then seeding the cells onto a vascular graft lumen in a single procedure. It was primarily designed to make endothelialised grafts available within the time frame of a bypass procedure, allowing their use in patients requiring immediate grafting. These types of acute procedures make up a substantial proportion of the total bypass operations carried out. Single stage seeding simplifies the technique of endothelialisation by reducing the number of steps involved. It was thought that this would reduce costs and the potential for complications such as infection. The simplified technique could also be carried out in non-specialist centres, without the need for costly culturing labs and equipment. Herring et al. initially reported the concept in 1978 [205]. He carried out several bypass operations in animals using saphenous vein ECs on Dacron conduits. At six weeks, histological and electron microscopy studies, revealed the grafts were endothelialised. The procedure was later refined using collagenase to increase harvest yield and using endothelialised ePTFE grafts instead of Dacron [206]. Stanley et al. reported enhanced patency rates at four weeks with endothelialised 4mm Dacron grafts in canine studies (73% endothelialised grafts V’s 27% control) [207]. Further canine studies in the aortic and carotid arteries with longer follow up times reaffirmed the enhanced patency rates of endothelialised grafts [208-210]. However, not all of these early animal studies proved successful when carried out in humans. Zilla et al. demonstrated higher incidence of activated platelets in patients with seeded or non-seeded PTFE grafts compared with a venous grafts [211]. The same team examined the outcome of 18 human distal femoropopliteal bypasses with autologous endothelialised grafts. Their study found that seeded grafts did not develop a confluent endothelialised surface after 14 weeks, a period three times as
long as the one required for confluent cell coverage in dogs [212]. Further human studies by Walker in 1995 investigated endothelial coverage by measuring fibrinolytic activation markers. The study, involving 32 patients with endothelialised grafts (n = 15) or control (n = 17), concluded that that endothelial coverage remains limited at 1-year follow up [213]. A more recent larger randomised controlled trial (RCT) compared low-density endothelialised synthetic above knee lower limb grafts with autologous venous grafts [214]. Thirty-month patency rates reached approximately 40% and 90% respectively.

It is clear that the early animal trials did not translate into similar success in humans. Possible reasons cited for the poor performance were low cell yield from harvesting, inadequate cellular adhesion, and lower potential for endothelialisation in humans than animal models. All the above studies used cells harvested from autologous saphenous or external jugular veins. These sources are technically difficult to harvest from and have a low yield of cells. This low initial density of cells in the single stage procedure has been cited as a possible cause for the disappointing clinical outcomes [215]. This has lead researchers to search for a source of endothelial cells that would rapidly yield higher cell densities. Microvascular cells, harvested from easily accessible fat sources are an alternative source of endothelial cells that would yield a high density of cells, suitable for single stage seeding [216,217]. Park et al. placed a single stage endothelialised prosthetic vascular graft in the splanchnic venous system in a patient with Budd Chiari syndrome [218]. Microvascular endothelial cells in the subcutaneous adipose tissue were harvested. At nine months the graft was explantation due to a mechanical stricture, however the luminal layer was thrombus free and displayed a monolayer of endothelial cells. In 1998, Sharp et al. conducted a small-randomised control trial
involving nine patients using microvessel cell seeded ePTFE grafts for haemodialysis access. Using serial graft biopsies he found the intima of the "seeded" graft to be twice as thick as that of the "non-seeded" grafts and because of this the study was terminated [219]. Meerbaum reported on 34 lower extremity revascularisations with seeded ePTFE grafts. Microvascular endothelial cells were used. The patency rates were disappointing, although the author did suggest the many patients had severe peripheral vascular disease and several patients subsequently had limb salvage operations [220].

1.5.3 Double Stage Seeding

The disappointing human trials involving single-stage seeding were attributed in part to low EC harvest yield, poor cellular adhesion and low shear-stress resistance. Leseche [221] and Miyata [222] proposed a two-stage seeding protocol, whereby harvested endothelial cells were expanded in culture prior to seeding. The two-stage procedure would increase the initial seeding density and give cells time to attach and acclimatise to the graft. This method also allowed for the possibility of mechanical stimulating cells to alter their phenotype so that they’d be better adapted for conditions encountered in-vivo. Zilla et al. studied 49 patients requiring lower limb bypass but lacking a suitable autologous graft. Patients were randomised to seeded and un-seeded ePTFE graft groups [54]. Cultured autologous endothelial cells from the external jugular vein were confluenctly lined onto fibrin coated polytetrafluoroethylene grafts. The double-stage procedure took approximately four weeks. At 32 months, primary patency was 84.7% with endothelialised conduits and 55.4% with un-seeded grafts. This study contrasted to Herring et al. who compared
single staged seeded ePTFE and un-seeded ePTFE in femopliteal bypass surgery, and reported no significant difference between the two grafts [214]. The findings of Zilla et al. were replicated by Leseche et al. in 1995 [223] and Deutsch and Meinhart in 1999 [199]. The latter was an important study involving 113 in vitro endothelialised ePTFE grafts with a nine-year follow up. Cell source was generally the cephalic vein and all grafts were pre-coated with fibrin glue. The patency rates over the nine years approached that of autologous venous grafts proving that graft endothelialisation enhances the patency of vascular grafts. Although these studies all show increased patency rates with seeded grafts, the practice still has not gained widespread clinical acceptance. The reasons for this are likely to be multifactorial. The two-stage procedure is unsuitable in the acute setting. The harvesting of endothelial cells adds a second surgical procedure with associated risks and cost. The culturing of endothelial cells requires laboratory facilities and expertise, not readily available in most hospitals. Despite this, these studies do show that endothelialisation of grafts is an exciting area of vascular surgery with research groups around the world are continuing to explore new materials for endothelialisation, new sources of cells and more efficient seeding techniques such as electrostatic seeding [224,225] and mechanical preconditioning [175].

1.6 Extracellular Matrix Materials

1.6.1 Introduction

The extracellular matrix is nature’s own cellular scaffold. It is a complex structure produced by resident cells and is specifically designed for the functional needs of the tissue from which it is harvested. It acts not only as a mechanical
framework, but also has a critical role in cellular physiology. It provides attachment sites for cells and contains a milieu of cell signalling factors, which guide cellular migration, proliferation, differentiation and inflammatory pathways. It is often stated that ECM is in a state of “dynamic equilibrium” with resident cells, providing informational cues, which influence their behaviour [226]. ECM is composed of a range of proteins, glycoproteins and proteoglycans, which are organised into a complex three-dimensional ultrastructure. Attached to the protein components of ECM are a vast range of cytokines and growth factors, which can affect such diverse processes as angiogenesis, vasculogenesis, inflammation and wound healing [227]. ECM is arranged and constructed according to the physiological needs of its resident tissue [228]. For example, ECM of tendons serves a mechanical function; hence this ECM is rich in collagen and provides tensile strength. ECM in highly metabolically active tissue such as the submucosa of the small intestine are rich in cell growth factors such as basic fibroblast growth factor (bFGF), transforming growth factor beta (TGF-β), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), platelet derived growth factor (PDGF) epidermal growth factor (EGF) and vascular endothelial cell growth factor (VEGF) [229-231]. Hence ECM from this tissue promotes cellular growth and attachment.

In 1966 Hauschka et al. discovered that ECM was involved in the transformation of myoblasts into myotubes [232]. Wessells et al. subsequently reported that ECM components such as glycosaminoglycans played a pivotal role in salivary gland morphogenesis [233]. These studies lead researchers to hypothesis that ECM scaffolds were not simply inert scaffolds but had the ability to alter important tissue processes such as inflammation, wound healing and vascularisation. This property is now widely accepted and has coined the term “constructive
remodelling” [234-236]. ECM is found across all species with the amino acid sequences and quaternary structure of its components remaining remarkably consistent [237]. This allows decellularised forms of the material to be used in humans with low antigenic potential. Several sources of ECM, each with specific properties have been processed and investigated for application in surgery including: the aorta [238], veins [239], bladder [240], tendons [241] and amniotic membrane [242]. Multi-layering by vacuum pressing can increase tensile strength and durability [243]. More malleable forms of ECM including powder suspensions and gels have also been developed [244, 245]. These expand the application of ECM materials and allow for minimally invasive placement. Freeze drying processing methods have been introduced to increase shelf life and availability [246].

ECM scaffolds have been employed in reconstructing a range of specialised tissues in pre-clinical and clinical trials including body wall [247-249], urinary bladder [250-252], rotator cuff [253-256] intestine [257,258] urethra [259-263], ureter [264,265] and diaphragm [266,267]. These studies continually demonstrated ECMs the remarkable ability to change a tissue’s response to injury from one of scar formation into one of host tissue regeneration - “constructive remodelling”. The mechanisms behind this are presently unclear, however the process is characterised by rapid angiogenesis, repopulation of the scaffold with host cells and the formation a neo-matrix. ECM scaffolds are now widely used in range of clinically specialities. A list of available products is shown in Table 1.
Table 1. A selection of ECM based products is listed below as identified by Badylak et al. [268].

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
<th>Material</th>
<th>Chemical Modification</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acellular</td>
<td>Health point</td>
<td>Porcine small intestinal submucosa (SIS)</td>
<td>Natural</td>
<td>Partial &amp; Full thickness wounds; Superficial and second degree burns</td>
</tr>
<tr>
<td>Oasis ®</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xelma TM</td>
<td>Molnlycke</td>
<td>ECM protein, PGA, water</td>
<td>Cross-linked</td>
<td>Venous leg ulcers</td>
</tr>
<tr>
<td>Alloderm</td>
<td>Lifecell</td>
<td>Human skin</td>
<td>Cross-linked</td>
<td>Abdominal wall, breast, ENT/head &amp; Neck reconstruction, grafting</td>
</tr>
<tr>
<td>Cuffpatch TM</td>
<td>Arthrotek</td>
<td>Porcine small intestinal submucosa (SIS)</td>
<td>Cross-linked</td>
<td>Reinforcement of soft tissues</td>
</tr>
<tr>
<td>Tissuemend ®</td>
<td>TEI</td>
<td>Fetal bovine skin</td>
<td>Natural</td>
<td>Surgical repair of damaged or ruptured soft tissue in rotator cuff</td>
</tr>
<tr>
<td>Durepair ®</td>
<td>TEI</td>
<td>Fetal bovine skin</td>
<td>Natural</td>
<td>Repair of cranial or spinal dura</td>
</tr>
<tr>
<td>Xenform TM</td>
<td>TEI</td>
<td>Fetal bovine skin</td>
<td>Natural</td>
<td>Repair of colon, rectal, urethral, and vaginal prolapse, pelvic reconstruction, Urethral sling</td>
</tr>
<tr>
<td>SurgiMend TM</td>
<td>TEI</td>
<td>Fetal bovine skin</td>
<td>Natural</td>
<td>Surgical repair of damaged or ruptured soft tissue membranes</td>
</tr>
<tr>
<td>PriMatrix TM</td>
<td>TEI</td>
<td>Fetal bovine skin</td>
<td>Natural</td>
<td>Wound Management</td>
</tr>
<tr>
<td>Product</td>
<td>Manufacturer</td>
<td>Type</td>
<td>Source</td>
<td>Cross-linked</td>
</tr>
<tr>
<td>--------------</td>
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<td>-----------------------------</td>
<td>-------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Permacol™</td>
<td>Tissue Science Laboratories</td>
<td>Porcine skin</td>
<td>Cross-linked</td>
<td>Soft connective tissue repair</td>
</tr>
<tr>
<td>Graft Jacket ®</td>
<td>Wright Medical Tech</td>
<td>Human skin</td>
<td>Cross-linked</td>
<td>Foot ulcers</td>
</tr>
<tr>
<td>Surgisis ®</td>
<td>Cook SIS</td>
<td>Porcine small intestinal submucosa SIS</td>
<td>Natural</td>
<td>Soft tissue repair and reinforcement</td>
</tr>
<tr>
<td>Durasis ®</td>
<td>Cook SIS</td>
<td>Porcine small intestinal submucosa SIS</td>
<td>Natural</td>
<td>Repair dura matter</td>
</tr>
<tr>
<td>Stratasis ®</td>
<td>Cook SIS</td>
<td>Porcine small intestinal submucosa SIS</td>
<td>Natural</td>
<td>Treatment of urinary incontinence</td>
</tr>
<tr>
<td>OrthADAPT™</td>
<td>Pegasus Biologicals</td>
<td>Horse pericardium</td>
<td>Cross-linked</td>
<td>Reinforcement, repair and reconstruction of soft tissue in orthopedics</td>
</tr>
<tr>
<td>DurADAPT™</td>
<td>Pegasus B</td>
<td>Horse pericardium</td>
<td>Cross-linked</td>
<td>Repair dura matter after craniotomy</td>
</tr>
<tr>
<td>Axis™ dermis</td>
<td>Mentor</td>
<td>Human dermis</td>
<td>Natural</td>
<td>Pelvic organ prolapse</td>
</tr>
<tr>
<td>Suspend ™</td>
<td>Mentor</td>
<td>Human fascia lata</td>
<td>Natural</td>
<td>Urethral sling</td>
</tr>
<tr>
<td>Restore ™</td>
<td>De Puy</td>
<td>Porcine small intestinal submucosa SIS</td>
<td>Natural</td>
<td>Reinforcement of soft tissue</td>
</tr>
<tr>
<td>Veritas ®</td>
<td>Synovis Surgical</td>
<td>Bovine pericardium</td>
<td>Soft tissue repair</td>
<td></td>
</tr>
</tbody>
</table>
1.6.2 Composition

The architecture and composition of extracellular materials is unique and cannot be replicated in a laboratory [269,270]. It is entirely different depending on the origin of ECM and will influence how it can be adapted for clinical use. For example, ECM containing a basement membrane, i.e. UBM, contains structural and functional proteins appropriate for culturing epithelial or endothelial cells, hence UBM may have possible applications in tissue engineering of vascular grafts or skin.

ECMs consist of a wide variety of proteins, carbohydrates and growth factors each providing a specific function. Collagen makes up the bulk of components found in ECM with almost 30 different subtypes identified. It accounts for up to 90% of dry weight and interacts with surrounding glycosaminoglycans, growth factors and other proteins [237]. By far the most common subtype is type I; which confers axial strength and is abundant in load baring tissues, i.e. tendons and ligaments [241]. Other subtypes are found in much smaller quantities and have their own specific properties. Collagen Type III is probably the second most abundant subtype, it is less rigid and is found predominantly in tissues which require a degree of flexibility or compliance i.e. small intestine and blood vessels [271]. Type IV and type VII is
found in basement membranes and forms important attachment sites of epithelial and endothelial cells [272,273]. Collagen Type VI serves as a linker between collagen type I and glycosaminoglycans [273]. In nature collagen interacts with surrounding glycosaminoglycans and other proteins. The diverse characteristics of so many different collagen subtypes explain in part, the complexity of ECM and the extreme challenge in replicating it.

The second most abundant ECM component is fibronectin, which is a high-molecular weight glycoprotein. It has critical roles in attachment such as binding to cell membrane-spanning receptor proteins called integrins, as well as linking other ECM constituents such as collagen and proteoglycans [274-276]. It actively partakes in several biological processes such as tissue healing, coagulation, cellular spreading and attachment. Due to its ability to bind to a diverse range of cells, fibronectin has been extensively utilized in the field of tissue engineering [277]. More recent work has focused on replicating an important ECM sequences within fibronectin (e.g. arginine-glycine-aspartate (RGD) which mediates cell adhesion via integrin receptors to a number of different cell types [278,279].

Laminin is also an important adhesion glycoprotein found primarily in the basement membrane [275]. It is a trimeric protein, containing α, β, and γ-chains; the ratio of which determines the specific subtype. It is critical in the repair and survival of tissues and has a central role in the embryogenesis of the basement membrane [280]. Basement membranes are specialised extracellular sheets providing a substratum for all epithelia and endothelia. They provide mechanical stability and assist in the spatial orientation of cells in tissue. They also influence cellular differentiation and migration and have critical roles in the development and repair of vascular tissue [276,277]. It is because of this, that laminin is considered an
extremely important component ECM. Vascularisation of the scaffold upon implantation is a major hurdle in scaffold design and is critical to the success of the scaffold.

   ECMs also contain a variable amount of glycosaminoglycans. These are carbohydrates made up of long un-branched polysaccharides including hyaluronic acid, chondroitin sulphate, heparin and heparan sulphate. Hyaluronic acid is present in high concentrations in foetal tissue and has been investigated extensively for its wound healing properties [281]. Glycosaminoglycans absorb large quantities of water, enabling them to maintain the structural integrity of the ECM scaffold. They interact with several ECM components including functional proteins and can influence their behaviour. They act as co-receptors for growth factors and take part in cellular signalling [282].

   An extensive range of growth factors has been identified within ECM scaffolds following processing. These include; VEGF, FGF, EGF, TGF- β, KGF, hepatocyte growth factor (HGF) and platelet derived growth factor (PDGF) [229-231]. These growth factors are released as the ECM scaffolds degrade and encourage rapid angiogenesis and cellular proliferation [283, 284]. Importantly, their release has shown to be sustained until the scaffold is fully degraded and their attachment to ECM proteins appears to protect them from degradation in vivo [285]. Researchers have struggled to integrate these growth factors into existing synthetic scaffolds [286]. Difficulties in the release and formulation of the correct combinations have lead to disappointing clinical results. In natural ECM biomaterials, these growth factors appear to be present in the correct ratios.
1.6.3 Biomechanical Properties

The mechanical properties of a given scaffold are determined primarily by the collagen content, the orientation of the protein fibres and the tissue source. Our understanding of the physical characteristics of ECM scaffolds comes principally from experiments involving UBM and SIS. SIS has a predominantly longitudinal orientation of its collagen fibres. Orberg et al. reported two distinct fibre populations with ±30° fibre directions from the longitudinal axis [287-289]. This structural layout is attributed to the peristaltic actions of the small intestine. This alignment confers biaxial strength to SIS scaffolds, which have been exploited in orthopaedic surgery to reconstruct load barring tissues [290]. In contrast, UBM has more uniform longitudinal fibre alignment due to the mechanical characteristics of the urinary bladder. This material has been investigated as a potential material in bladder reconstruction [291].

Several studies have shown the method of harvesting and sterilization can also affect the mechanical properties of a scaffold. Gilbert and his team stated that the technique of delamination influences fibre orientation of UBM [292]. The group found that by mechanically delaminating across the longitudinal axis of the bladder (apex to neck) as opposed to a circumferential direction makes the collagen fibres more longitudinally anisotropic. This orientation of fibres gives UBM stiffer mechanical properties when compared to the alternative preparation. Other methods of processing can also alter mechanical properties; two studies looked at the effect of lyophilized SIS and demonstrated altered fibre kinematics [293,294]. The process of sterilisation may also alter the physical characteristics of ECM scaffolds. In 2008, Freytes et al. investigated the outcomes of three different sterilisation procedures on the physical properties of UBM [295]. Sheets of UBM underwent ethylene oxide
(ETO), gamma irradiation or electron beam irradiation. Each scaffold was then tested for a range of mechanical properties including; ball-burst strength, stiffness and energy dissipation. The team demonstrated that each sterilization procedure altered the mechanical property in some way. ETO had the least effect while gamma and electron beam irradiation reduced the uniaxial and biaxial strength and tangential stiffness of the ECM scaffolds. To date there have been several attempts to alter or enhance the physical properties of ECM scaffolds. Layering of ECM sheets by vacuum has been shown to dramatically increase strength; a four-layered scaffold increases mechanical strength 150%. [243].

Finally it should be noted that ECM scaffolds are degradable and that their mechanical properties change depending upon the stage and rate of degradation. As well as this, populating cells remodel the scaffold following implantation, which also affects the physical characteristics of the material. This process was reported by Badylak et al. when examining the degradation of multilaminate SIS in the canine body wall [296]. The study demonstrated a progressive decrease in strength of the scaffold up to day 10, after which time the scaffold was remodelled and increased its strength over two years to twice the biaxial load-bearing capability compared to pre-implantation measurements.

1.6.4 Immune reaction to extracellular matrix scaffolds

The employment of xenogenic materials as implants in humans raises the possibility of a host immune response. This is primarily due to the α-gal epitope, which is synthesised by non-primate mammals via the enzyme \( \alpha 1,3 \text{galactosyltransferase} \) (\( \alpha 1,3 \text{GT} \)) [297]. Humans however, do not produce this epitope because the \( \alpha 1,3 \text{GT} \)
gene was inactivated in Old World primates. Humans do however; produce the anti-Gal antibody, which acts as an immunological defence mechanism, preventing whole xenogenic transplantation in humans. ECM scaffolds are decellularised prior to implantation and hence are absent of any xenogenic cellular epitopes that might elicit an immune response. The standard decellularisation technique is repeated emersion in hypotonic saline followed by treatment with peracetic acid and finally ethylene oxide or gamma irradiation [298,299]. This process removes all cells and nuclear matter above 300 base pairs [300]. Despite this, small amounts of gal epitope have been detected on ECM scaffolds following processing, however they do not seem to be in sufficient quantity to provoke a damaging immune rejection [301]. Human patients implanted with ECM materials do not produce a rise in anti-gal antibodies [226]. However, even in the event that this was to happen, it is now possible to produce ECM from transgenic gal knockout animals or to treat ECM with x-Galactosidase to prevent a gal evoked immune response.

Despite the extensive processing to decellularise ECM scaffolds, it extremely difficult to completely remove every cell component or nuclear material. To date there has been exhaustive studies involving ECM to understand host tissue reaction to these materials. In transplant immunology, two pathways are of particular interest; the T-helper 1 (TH1) and T-helper 2 (TH2) pathways [302,303]. The TH1 pathway is characterised by the production of Interferon γ; Interleukin-2 and tumour necrosis factor-β. These switch macrophages to a bactericidal state and activate B-cells to produce opsonising antibodies [304,305]. This pathway produces proinflammatory signals responsible for perpetuating autoimmune responses and is associated with transplant rejection [306,307]. The TH2 pathway is characterised by the release of IL-4, IL-5, IL-6 and IL-10, which induces B-cells to form neutralizing antibodies.
This pathway is active in transplant acceptance [308,309]. Early animal studies involving mice implanted with SIS demonstrated the TH2 response [310]. The purpose of the TH2 immune reaction in response to ECM is presently unclear. One theory is that it may be due to the host remodelling process [226]. An important point to note is that ECM scaffolds rapidly degrade in vivo. Studies involving SIS in Achilles tendon reconstruction and urinary cystoplasty showed that over 50% of the scaffold degrades by within 28 days and the remainder by day 60 [311-313]. Previous studies have shown that chronic low levels of Gal-epitope are implicated in cases of delayed graft rejection [314]. Therefore, there may not be a persistent stimulus to evoke an immune rejection of the scaffold.

1.6.5 Degradation

Biodegradability is the key property of ECM scaffolds making them particularly attractive in tissue engineering applications. Biodegradable materials act as a temporary implant allowing host cells to integrate whilst minimising the risk of infection and chronic host immune reaction. Several animal studies involving a variety of specialised tissues, have demonstrated by histological and immunohistochemical methods that ECM is rapidly degraded once implanted [315-318]. Two more recent animal studies have investigated the degradation of SIS scaffolds in Achilles reconstruction and bladder augmentation using $^{14}$C labelled SIS and accelerator mass spectrometry. This is an extremely accurate method of determining degradation, capable of tracking minute quantities of the scaffold. Gilbert et al. employed this method to examine the degradation of SIS when used in canine Achilles reconstruction [319]. The study demonstrated rapid degradation of
the scaffold with 60% mass lost at one month. The rate of degradation increased as
host cells began to invade the scaffold, which was completely degraded by three
months. An important result was that the tendon maintained its structural integrity
throughout the study and at explantation the reconstructed tendon was histologically
and mechanically similar to that of normal tissue. The explanted graft demonstrated
new unlabelled ECM deposited by host cells indicating rapid host cellular invasion
of the scaffold and subsequent organization of the tissue as the scaffold degrades.
Similar findings were documented by Record et al. using the same method, this time
involving SIS scaffolds in porcine bladder augmentation [313]. The study showed
the scaffold being fully degraded at 90 days. Both studies demonstrated host tissue
regeneration, site specific for the tissue that was being replaced. An interesting
aspect of both studies was that the quality of host tissue regeneration was dependent
on the presence of biomechanical factors of the tissues being reconstructed. For
example, in the bladder reconstruction study, if a urinary catheter was placed thus
elimination bladder expansion and contraction, host tissue remodelling was almost
completely lost, leading to weak, disorganised tissue regeneration and scar
formation. A similar finding was shown the case in the Achilles reconstruction when
load bearing axial forces were not applied. This highlights the importance of
mechanical conditioning during constructive remodelling. In studies examining the
degradation rate of ECM, an important follow up question is where the by-products
are excreted or deposited. Record et al. demonstrated that SIS was completely
eliminated from the body with no significant deposition in any parenchymal tissues.
The route of excretion is haemogenous initially with subsequent urinary excretion
[313].
As more and more studies are being carried out on ECM reconstruction it is becoming more apparent that biodegradability is a significant factor in their capacity to induce constructive remodelling [320-322]. Peptides and growth factors like VEGF, TGF and FGF-2 are released as the ECM scaffolds degrade which encourage angiogenesis and be mitogenic and chemoattractant for a variety of cell types [321]. Importantly, their release has shown to be sustained until the scaffold is fully degraded. ECM scaffolds not only induce the local migration of host cells, but increasing reports suggest that cells from the bone marrow are also recruited to the area of remodelling [141,320]. Badylak et al. demonstrated this in experiments with labelled bone marrow-derived cells in mice [320]. This study proved that endothelial and fibroblasts found in remodelled tissue were bone marrow derived and that they participated in host tissue remodelling and repair.

1.6.6 Antimicrobial Resistance

Infection of an implant is a serious and potentially fatal complication of tissue reconstruction [323,324]. In vascular graft surgery it is a particularly serious complication with infection rates ranging from 1-6% for prosthetic vascular grafts [323]. The constituents of a biological implant are important in determining its susceptibility to bacterial colonisation. Different implant materials can affect bacterial adherence and the host’s ability to mount an immune defence [325]. Several studies have examined the antimicrobial resistance of ECM scaffolds and compared them to existing synthetic implants. Badylak et al. used Dacron and SIS grafts to reconstruct canine stifle joint and deliberately contaminated the scaffolds with staphylococcus aureus bacteria [326]. The study found that the ECM scaffolds
were more resistant to infection and importantly continued to support constructive host tissue remodelling despite the presence of pathogenic organisms. Dacron scaffolds demonstrated persistent infection with its synthetic fibres showing increased bacterial load compared to the ECM scaffolds. A fibrous tissue formed around the synthetic graft in response to the infection, thus preventing vascularisation and blocking any host immune response. There are probably several reasons why ECM scaffolds display greater microbial resistance. Firstly, the scaffolds rapidly degrade, thus limiting attachment sites for pathogens. Secondly, ECM as previously noted accommodates rapid neovascularisation, thus supporting an immediate host immune response. This rapid angiogenesis has been attributed to the release of angiogenic growth factors as the scaffold degrades and the favourable endothelial attachment sites on ECM components [321]. A third mechanism is the discovery of antimicrobial peptides in the by-products of ECM degradation [327,328].
1.7 Thesis Aims

The aim of this thesis is to evaluate a novel biomaterial – decellularised xenogenic extracellular matrix for potential use in a tissue engineered vascular graft.

1. To compare the endothelialisation of flat ECM scaffold sheets with existing coated and uncoated synthetic graft materials in static culture.

2. To construct and validate a novel subatmospheric bioreactor which exerts a mechanical strain on a tissue engineered ECM vascular construct in order to enhance endothelialisation of this construct.

3. To examine the effects of varying subatmospheric pressures on a tissue engineered ECM vascular construct by examining cellular proliferation, morphology and phenotype.

4. To culture endothelial cells inside the luminal surface of 6mm acellular extracellular vascular grafts and existing fibrin coated ePTFE grafts via a rotating seeing device and compare the rate of endothelialisation.
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Comparing the Endothelialisation of Extracellular Matrix Bioscaffolds with Coated Synthetic Vascular Graft Materials.
2.1 Introduction

The endothelial lining of an artery plays a central role in vascular physiology and its importance in maintaining vascular functionality cannot be over emphasised. The endothelium regulates vascular permeability, responds to mechanical signals from the blood and maintains a non-thrombotic homeostatic surface [1]. Grafts without an intact stable endothelium are essentially passive pro-thrombotic conduits, unable to respond to the haemodynamic environment. This lack of a confluent endothelial lining has been repeatedly cited as one of the most common causes of conduit failure, particularly in small diameter grafts [2, 3]. There is now a growing consensus that the presence of an intact functional endothelium is crucial in the design of a successful small diameter graft. Methods investigated to achieve this include: promoting in vivo endothelialisation via circulating endothelial or endothelial progenitor cells [4-6], tissue engineering complete de-novo vessels [7] or in vitro culturing of endothelial cells onto the graft lumen prior to implantation [8]. Of these methods, in vitro graft endothelialisation has shown the most promise and is backed by long-term clinical trials, which have shown increased patency rates in humans [9-14]. Using this method, a patient’s own ECs are gathered from superficial veins or adipose tissue and seeded onto the graft lumen prior to implantation. Thus, the antithrombotic properties of an intact endothelium are combined with existing approved vascular graft materials. This method was first described in the 1970’s to enhance the patency rates of synthetic ePTFE and Dacron grafts [15] and has since been expanded to improve the functionality of artificial cardiac valves [16,17], arteriovenous grafts [18] and intravascular stents [19].

However, this process is not without difficulty. Existing synthetic graft materials are poor substrates for cellular culture, resulting in slow
proliferation and prolonged culture times [20, 21]. As well as this, weak cellular attachment results in significant cell loss upon implantation. An example of this was well illustrated by Rosenman et al. who demonstrated only 20% of cells initially adhered to ePTFE and upon implantation; 30% were immediately lost with continued detachment up to 24 hours [22]. Researchers have explored several methods to try to improve the endothelialisation of these materials with mixed results. Altering vascular wall chemical structure [23], cell sodding techniques [24], mechanical preconditioning [25, 26], electrostatic seeding [27, 28] and coating the luminal surface with adhesion proteins or peptides [29-35] have all been evaluated as methods to enhance graft endothelialisation. Despite the plethora of graft coatings evaluated, there is no common consensus as to the optimum. The principle requirements of a successful coating are biocompatibility, rapid cell proliferation and adequate cellular attachment whilst also minimising platelet adhesion. Sufficient cellular adhesion is required to resist shear stresses upon restoration of blood flow and cells must be able to rapidly proliferate to attain a confluent layer with limited culture times and seeding density. Several ECM constituent like proteins and peptides have been investigated as potential luminal coatings and all have been shown to enhance the seeding of vascular grafts to some degree. But perhaps the most extensively studied luminal coating clinically is fibrin glue [36-39]. Fibrin has several inherent properties, which make it an ideal cellular substrate. It is a natural physiological scaffold, which supports the adhesion and proliferation of ECs and is already widely used in a variety of clinical settings. As fibrin forms it releases peptides, which are mitogenic for several cell types [40, 41]. It naturally accommodates angiogenesis and tissue repair in vivo and has been shown in several studies to be a highly versatile biomaterial [40, 42]. However, due to its mechanical
weakness and low suture retention strength it must be combined with existing synthetic graft materials to withstand physical forces found in vivo. This brings with it, well known synthetic graft limitations such as inflammatory reactions, infection risk and compliance miss-match, which can promote intimal hyperplasia. Investigators have also reported poor proliferation on fibrin and raised concern regarding embolisation or detachment of the coating in vivo [43]. Added to this, Swartz et al. demonstrated almost immediate platelet deposition to a fibrin coated vascular graft in an ovine model, raising concerns regarding the thromobogenity of this scaffold [44].

These limitations have in part, impeded the introduction of endothelialised fibrin coated grafts into widespread clinical use and has lead researcher to search for alternative graft materials. Previous studies reported that ECM components enhance cellular attachment on synthetic materials and reduce cell loss when exposed to shear stresses [29- 35]. ECM scaffolds are already used safely in wide range of clinical specialities and have demonstrated excellent mechanical characteristics suitable for use as a vascular graft [45-47]. This raises the question whether a graft consisting of a complete ECM may offer a superior substrate for endothelialisation. The aim of this study is to compare the endothelialisation of emerging ECM scaffolds with fibrin-coated ePTFE. We are specially examining cellular viability, phenotype, attachment, growth and morphology.

The graft materials we are examining in this study are decellularised xenogenic extracellular matrix materials derived from the porcine urinary bladder wall [Urinary Bladder Matrix (UBM)] and porcine jejunum [small intestine submucosa (SIS)]. These materials have already been outlined in detail in chapter one, section 1.6. Briefly, they are xenogenic extracellular matrix scaffolds
which have been decellularised rendering them non-immunogenic. They consist of structural and functional proteins including collagens [48], laminins [49], fibronectin [50] and elastin [51]. Attached to these are a vast range of growth factors; fibroblast growth factor-2 (FGF-2), epithelial growth factor (EGF), transforming growth factor – β (TGF-β), platelet derived growth factor (PDGF) and vascular endothelial cell growth factor (VEGF) which upon implantation are released, encouraging rapid angiogenesis and cellular proliferation [52, 53]. ECM scaffolds are naturally occurring cellular substrates and hence should be ideally suited to cellular culturing. They have already proven successful in reconstructing a range of specialised tissues in pre-clinical and clinical trials [54-56]. SIS in particular, was extensively evaluated for use in vascular applications. It has been shown to possess suitable mechanical properties for vessel reconstruction with superior compliance compared to contemporary synthetic grafts [57]. Several animal studies have shown promising long term patency rates using SIS vascular grafts [58, 59].

UBM is an emerging ECM scaffold produced from the tunica mucosa and tunica propria layer of the porcine urinary bladder wall. It has been successfully used to reconstruct a range of specialised tissues including the urinary bladder, oesophagus, larynx and myocardium [60-68]. It is most notable in possessing an intact basement membrane following processing. This specialised ECM ultrastructure has pivotal roles in cellular growth and influences cell differentiation and migration during wound healing [69]. It is the natural substrate for the growth of endothelial cells and has potential for combining rapid endothelialisation and firm cellular anchorage. We hypothesis that ECM materials could offer a superior scaffold for in vitro endothelialisation when compared to fibrin coated ePTFE.
2.2 Methods & Materials

2.2.1 Overview of experimental design

Adult human umbilical venous endothelial cells (HUVEC) were grown on the surfaces of five different vascular material sheets: UBM, SIS, fibrin coated ePTFE. Uncoated woven Dacron and ePTFE served as controls. The samples were prepared as 2.5 cm diameter discs (Fig. 11a) and seeded constructs were placed in static culture for up to nine days (Fig. 11b). Each test was performed in triplicate. The constructs were serially tested for viability, cellular attachment, morphology, proliferation and bacterial contamination.

2.2.2 Preparation of ECM scaffolds

Urinary Bladder Matrix (UBM) Bioscaffolds

UBM and SIS in the form of 6×10 cm-sterilised sheets (Fig. 5 & 6) were kindly supplied by Prof. Stephen Badylak, University of Pittsburgh, Pittsburgh, U.S.A. A urinary bladder was obtained from market-weight pigs following euthanasia. Urothelial cells were removed by soaking the bladder in normal saline solution. The bladder was expanded with water under high pressure to aid in the stripping of the muscle layer and tunica submucosa. The bladder was then incised via its apex and halved. The external layers of the bladder wall (tunica serosa, tunica muscularis externa, tunica submucosa, and the muscularis mucosa) were removed by mechanical delaminated. The remaining bilayered material including the basement membrane of the tunica mucosa layer and the subjacent tunica propria layer constitute UBM. The basement membrane of the tunica mucosa will henceforth be
referred to as the luminal surface (Fig. 7a) and the tunica propria will be referred to as the abluminal surface (Fig. 7b). The material was decellularised by soakage in 0.1% (v/v) peracetic acid, 4% (v/v) ethanol and 95.9% (v/v) sterile water. The sheet was then soaked in distilled water with phosphate buffered saline (pH 7.4) to remove any acidic residues and return the PH to neutrality. Finally UBM is terminally disinfected by 10-kGy-gamma irradiation.

Fig. 5. Macroscopic view of UBM following possessing. Note the semitranslucency and structural reminisce of blood vessels.

Fig. 6. A 6×10cm terminally disinfected UBM sheet.
Fig. 7a. A scanning electron micrograph of the luminal surface of UBM sheet. The luminal layer is characterised by a smooth contour, characteristic of a basement membrane (× 500 magnification).

Fig. 7b. The abluminal surface of UBM, a highly porous matrix of connective tissue (× 500 magnification).
Preparation of Small Intestine Submucosa (SIS) Bioscaffolds

SIS was harvested from the porcine jejunum immediately after euthanasia. The preparation process of SIS has been presented in detail previously [58]. In brief, sections of rinsed porcine jejunum were longitudinally split to form an elongated sheet. The superficial mucosal and external muscular layers with surrounding serosa were extracted by physical delamination. The remaining layers; submucosa, muscularis mucosa and basilar layers of the mucosa, the most superficial of which is the stratum compactum [the laminar layer (Fig. 8a)], constitute SIS (approximately 80 – 100 µm thick). The side from which the muscular layers were removed will be the abluminal surface of SIS. This tissue was rinsed with phosphate buffered saline (pH = 7.0) and distilled water to lyse any remaining cells and remove residual cellular debris. This was then sterilised with

Fig. 7c. A scanning electron micrograph of the cross-section of UBM sheet. Clearly there are pores and interstices present that can serve as entry points and migration channels for invading cells (× 200 magnification).
0.1% peracetic acid and 20% ethanol and finally 1.5 MRad gamma irradiation. The SIS sheet was stored in lyophilized form.

Fig. 8a. Scanning electron microscope picture of the dense stratum compactum or luminal surface of SIS (× 35 magnification).

Fig. 8b. Scanning electron microscope picture of the submucosal or abluminal surface of SIS (× 500 magnification).
2.2.3 Preparation of synthetic vascular materials

Sterile uncoated knitted Dacron grafts (16mm × 20 cm) were kindly provided by Intervascular, La Ciotat, France. They were terminally sterilised by Gamma irradiation. Uncoated ePTFE grafts (16mm × 35 cm), 30µm intermodal distance, 600 µm thick were provided by IMPRA, Bard Ltd (West Sussex, England). They were terminally sterilised with Ethylene Oxide gas. The grafts were incised longitudinally and 2.5cm diameter circular segments were prepared using an autoclavable cylindrical piece of Perspex ®, which was manufactured at University Limerick (Fig. 9).

Fig. 9. Machined Perspex ® utensil.
2.2.4 Fibrin Gel preparation

The formation of fibrin gel has already been documented [70]. I will summarize the production method here. Fibrin is formed by the polymerisation of fibrinogen by the enzyme thrombin and cofactor calcium chloride (CaCl$_2$) (Sigma-Aldrich). Human fibrinogen (Sigma-Aldrich) is added to tissue buffered saline (TBS) to make a concentration of 10mg/ml. Thrombin (Sigma-Aldrich) solution is similarly made to get a final concentration of 40IU/ml. Prior to use, 75µl of 50mM CaCl$_2$ in TBS was added to 75µl of 40IU/ml thrombin and 350µl of TBS. Then 500µl of fibrinogen solution was added and mixed with gentle shaking. The gel is left for one hour to polymerise in the incubator at 37°C with 5% CO$_2$ and > 95% humidity environment. Fibrin glue was applied evenly to the graft surface using a sterile syringe.

2.2.5 Sample preparation

Every sample was placed under a sterile stainless steel ring to prevent cell leakage (Fig. 10). The rings have inner diameters of 2.2 cm such that 3.8 cm$^2$ of the luminal graft surface was exposed. The luminal surfaces of UBM and SIS were used for seeding of cells.
2.2.6 Cell culturing technique

HUVEC cell lines were purchased from Cascade Biologics/Invitrogen ® (Cat. No. C-003-5C). They were obtained from human umbilical veins as documented by Jaffe et al. [71]. The HUVEC culture medium 200 was obtained from Cascade Biologics/Invitrogen ® (Cat. No. M-200-500) and supplemented with low serum growth supplement (Cascade Biologics/Invitrogen ®, Cat. No. S-003-10). It is a mixture of 10% Iron supplemented calf serum, 1% L-glutamine, sodium bicarbonate, and HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, with 10 000 UI/ml penicillin–streptomycin, 20 mg/ml endothelial cell growth supplement and 90 mg/ml heparin. The media was replaced every 48 hours till cells reached confluency. The
cells were then split in a ratio 1:3 with 3mls buffered saline solution containing 0.25% trypsin and 0.09% ethylenediaminetetraacetic acid (EDTA). The seeded 75cm² flasks were placed in a 37°C, humidified (> 95%), 5% CO₂ incubator.

2.2.7 Seeding Protocols

2.5cm diameter discs of each material were placed in six well culture dishes (Fig. 11a & b). The Sterile stainless-steel culture rings were placed over the scaffolds to prevent cell leakage during seeding. The cells were seeded onto the each sample at a density of $7 \times 10^4$ cells per cm² in 2ml of culture media. The constructs were then left in an incubator for two hours to accommodate for cell adhesion. Thereafter the chambers were flooded with media and the constructs were returned to the incubator. The culture medium was replaced every two days.

Fig. 11a. 2.5cm diameter discs of each material were placed in six well culture dishes.
2.2.8 Cellular Viability

The cytotoxicity of the constructs to HUVECs was assessed by determining cellular viability using a live/dead viability assay (Invitrogen ® Corp.). The live/dead assay differentiates viable and dead cells via two colour fluorescent markers; calcein acetoxymethyl (calcein AM) and ethidium homodimer-1 (EthD-1). Calcein AM is capable of diffusing across viable cell membranes and is metabolised by cellular esterases to yield a green highly fluorescent by-product; calcein which is retained in the cells. EthD-1 is a nucleic acid stain unable to penetrate live cell membranes, but can diffuse across damaged membrane walls of non-viable cells where it binds to nucleic acid to give a red fluorescence. Images were taken with a Nikon Eclipse TE200 inverted microscope.
2.2.9 **Immunofluorescence analysis of von Willebrand Factor (vWF)**

Endothelial cells seeded on each substrate were fixed with 3.7% paraformaldehyde in Phosphate buffered saline (PBS). This was then rinsed with PBS and mixed for 20 min with 0.27% NH4Cl/0.38% glycine in PBS and permeabilised with Triton X-100 (0.5%) in PBS. Von Willebrand Factor was confirmed via fluorescein-labelled antibodies -conjugated mouse anti-human vWF antibodies (2 μg/mL⁻¹).

2.2.10 **Cellular Attachment**

Cellular adhesion was determined by examining the percentage of attached cells over time. Construct discs were rehydrated for 20 minutes with Hanks balanced salt solution (HBSS). Uniform passages HUVECs (P7) of 7×10⁴ cells per cm² were seeded separately onto each material surface in a drop wise manner and incubated for a maximum of 120 minutes. At 30 minute intervals, the surface of the constructs were rinsed with 5ml phosphate buffer solution (PBS) containing 0.4% v/v trypan blue to wash off any unattached cells. The detached cells were counted with a standard hemocytometer (Fig. 12a & b) and expressed as a fraction of the original seeding density.

![Fig. 12a. Haemocytometer gridlines showing the 16 corner squares used for counting cells. × 10 microscope objective.](image)

![Fig. 12b. The chambers of the haemocytometer are carefully filled with a Gilson pipette.](image)
2.2.11 Cellular Proliferation

Cellular proliferation was assessed on days 1, 3, 5, 7 and 9 using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega Corporation, Cat. # G3580) (Fig. 13). Essentially the assay incorporates a tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and an electron acceptor phenazine ethosulfate (PES). Live metabolic cells produce reducing agents such as NADH or nicotinamide adenine dinucleotide phosphate (reduced) (NADPH). These pass their electrons to an intermediate electron transfer reagent (PES) in the cytoplasm, which reduces the MTS tetrazolium compound “Owen’s reagent” into a soluble formazan product in the culture. The volume of formazan produced equates to the quantity of live cells present and is measured using a spectrophotometer at absorbance at 490 nm. The MTS assay is considered the backbone of biocompatibility studies in vitro [72].

Fig. 13. A 96-well microtiter plate used in MTS assay. As can be seen, higher numbers of cells results in more formazan production, illustrated here by a stronger purple colour.
2.2.12 Scanning Electron Microscopy (SEM)

Each construct with or without HUVECs was primarily fixed with 2.5% glutaraldehyde/paraformaldehyde phosphate buffer solution. After primary fixation, the constructs were post-fixed in 2% osmium tetroxide then dehydrated through a graded series of acetones up to 100%. After critical point drying, the specimens were mounted onto aluminium stubs and then coated with gold. Samples were then analysed using the scanning electron microscope (JEOL 5510) at University College Cork.

Fig. 14. Scanning electron microscope JSM-5510 (Jeol Ltd., Tokyo, Japan) at the Electron Microscopy Facility, Biosciences Institute University College Cork.
2.2.13 Statistical Analysis

Statistical analysis was carried out with Statistical Package for the Social Sciences (SPSS) 20.0 software for windows. Each quantitative experiment was performed in triplicate. Data is summarised using means and standard deviations (SD). A value of \( P < 0.05 \) was considered to be statistically significant. For cell attachment, a 5x3 factorial analysis of variance was conducted and for cell proliferation a 5 x 5 factorial analysis was conducted to examine the effects of materials, test days and the interaction between these two factors. Where a significant interaction between materials and test days was found, this was explored using simple main effects analysis. Initially, simple main effects of materials at each test day were conducted. The significant simple main effects of materials were further analysed by pairwise comparisons employing a Bonferroni adjustment for multiple comparisons. Secondly, for each material the simple main effects of test days were explored. Significant simple main effects of test days were further analysed by pairwise comparisons using a Bonferroni adjustment for multiple comparisons.

2.3 Results

2.3.1 Cellular Viability

Live/Dead assays showed that synthetic grafts, fibrin/ePTFE hybrid and the ECM scaffolds (UBM and SIS) could all support the viability of HUVEC cells in static culture (Fig. 15a-e). Cells proliferated on all scaffolds without evidence of contact inhibition and remained viable till the end of the study, demonstrating that neither the scaffolds nor their coatings were cytotoxic to the HUVECs.
Fig. 15 (a-e) showing live dead assays of HUVEC cells on each scaffold. Green colour indicates live cells, red colour indicates dead cells. Original magnification $\times 10$ microscope objective.
2.3.2 Validation of endothelial cells

In the immunofluorescence staining, intracellular vWF of HUVECs was confirmed on all materials. Figures 16a-e shows that ECs expressing vWF when cultured on all scaffolds examined, confirming that ECs were functioning appropriately.

Fig. 16a. Dacron.

Fig. 16b. ePTFE.

Fig. 16c. UBM.

Fig. 16d. SIS.

Fig. 16e. Fibrin/ePTFE.

Fig. 16a –e. Endothelial Cells producing vWF on each graft material.
Original magnification × 20 microscope objective.
2.3.3 Cellular Attachment

Data showing the proportion of cells attaching to each substrate over 120 minutes in culture is tabulated in figure 17. ECs seeded on both ECM scaffolds and fibrin-modified ePTFE achieved statistically higher attachment efficiency at each time point when compared to both synthetic graft materials ($p \leq 0.001$). The primary adherence of HUVECs to ECM scaffolds and fibrin/ePTFE hybrid after 60 minutes was almost three times the amount of cells adherent to the synthetic materials. The adhesion rates between the ECM scaffolds and the fibrin-coated ePTFE was statistically similar at each time point ($p = 1.00$). For all materials examined, cell attachment was significantly higher at 90 minutes and 120 minutes compared to 60mins. There was no difference in attachment rates between 90 and 120 minutes.

![Adhesion Rates of Vascular Scaffolds](image)

Fig. 17. Comparison of the percentage of HUVEC attached to different substrates. The values are mean of three replicates in the case of each matrix ($\pm 1$ Standard Deviation).
2.3.4 Cellular Proliferation

Cell Proliferation for each material is illustrated in figures 18 and 19 (a-e). For days 3, 5, 7, and 9, cell proliferation was significantly higher on UBM, SIS, and fibrin/ePTFE when compared to Dacron and ePTFE (p < 0.001). From day three on, cells grew faster on the ECM scaffolds when compared to fibrin-coated ePTFE (p < 0.001). ECs grown on ECM scaffolds achieved confluency the fastest (day seven) with no significant growth after this time (p = 1.000). There was no difference in cell proliferation at any time point between UBM and SIS (p = 1.000). There was also no difference between ePTFE and Dacron at any time point (p = 1.000). The numbers of cells seeded onto fibrin/ePTFE hybrid or uncoated synthetic grafts (7 x 10^4 cells per cm²) were not enough for cells to reach confluency by the end of the study (day nine).

![Cellular Proliferation](image)

Fig. 18. Cellular proliferation graph.
Fig. 19 a-e. Immunofluorescence studies showing the HUVEC growth on all types of scaffolds after seven days of culture. Magnification × 10 microscope objective.

2.3.5 Cellular Morphology

On ePTFE and Dacron (Fig. 20 & 21), cells appeared as small spheroid shapes with single point contact to the material, suggesting that these cells have not yet acclimatised to the substrate and are poorly attached, essentially in a quintessential
phase. They were inhomogeneously distributed on the surface and aggregate in clusters. As illustrated, cells seeded on ePTFE did not cover the prosthetic fibres uniformly and produced isolated clumps of cells with large sections of uncoated fibres exposed. In this specific photo the cell number is clearly lower than on the ECM scaffolds or fibrin/ePTFE hybrid. Fibrin/ePTFE hybrid appeared to be almost completely covered by a homogeneous layer of cells (Fig. 24). The cells were well spread on the surface with a large mean cell–material contact area. Cells grown on ECM scaffolds produced a monolayer, showing a typical cobblestone structure (Fig. 22 & 23). The cells were homogeneously distributed and well spread.

Fig. 20. Endothelial cell on Dacron SEM × 1700 magnification.
Fig. 21. Endothelial cells on ePTFE. SEM × 1000 magnification.

Fig. 22. Endothelial cells on UBM. SEM × 1300 magnification.
Fig. 23. Endothelial cells on SIS. SEM × 1500 magnification.

Fig. 24. Endothelial cells on Fibrin/ePTFE. SEM × 1500 magnification.
2.4 Discussion

In vitro endothelialisation is an emerging technique to enhance the patency rates of small diameter vascular grafts. A major stumbling block is delayed cellular proliferation as well as inadequate cellular adhesion. Dacron and ePTFE are poor substrates for the adhesion and proliferation of human cells [73-76]. Vohra et al. [77] and Williams et al. [78] reported that the EC adhesion rate on untreated ePTFE approaches 4%, while later studies demonstrated a retention rate of approximately 14% at seeding densities of $1 \times 10^4$ cells/cm$^2$ [79,80]. To improve the biocompatibility of synthetic materials several coatings have been evaluated, including ECM proteins (collagen, fibronectin, laminin, gelatine etc.) [81-85], preclotting of the graft with blood [86], plasma [87,88], fibrin [89,90] and more recently, surface modification with ECM adhesion peptides (heparin, arginine-glycine-aspartic acid, lectins, cell adhesion peptides) [80, 91]. Of these methods, ePTFE grafts coated with fibrin have provided the greatest clinical success in human trials. In a randomised control trial, Zilla et al. demonstrated that 6 and 7mm ePTFE endothelialised grafts coated with fibrinolitically inhibited fibrin had superior primary patency rates compared to untreated ePTFE (84.7% and 55.4% respectively) at three year follow up [14]. A subsequent larger study involving a cohort of 341 infrainguinal endothelialised ePTFE grafts reported primary patency rates of 6 mm verses 7 mm grafts as 78% / 62% at 5 years and 71% / 55% at 10 years respectively [9].

Cells adhere to surfaces via transmembrane integrins interacting with adhesive ECM proteins, which are absent on untreated synthetic materials [92]. The findings of previous studies show that individual ECM components increase to some degree endothelial attachment and resistance to shear
stress on synthetic materials. We hypothesis that a vascular coating consisting of a complete and intact ECM would replicate the natural vascular wall architecture and provide a superior surface for in vitro endothelial seeding. In vivo, endothelial cells are arranged as a monolayer, which rest on a basement membrane layer. This layer acts to support the architecture and functionality of the overlying endothelium. It is consists of structural proteins such as collagen and adhesive proteins such as laminin and fibronectin. These and other ECM components regulate cellular adhesion and proliferation via RGD (arginine-glycine-aspartic acid) based integrin-mediated receptors [93].

The xenogenic scaffolds we examine (UBM & SIS) are composed of a variety of structural and functional proteins including collagens, laminins, fibronectin and elastin as outlined in detail in chapter one, section 1.6. Attached to these are a vast range of growth factors including fibroblast growth factor-2 (FGF-2), epithelial growth factor (EGF), transforming growth factor – β (TGF-β), platelet-derived growth factor (PDGF) and vascular endothelial cell growth factor (VEGF). Upon seeding, these growth factors are released, encouraging rapid angiogenesis and cellular proliferation [53]. ECM materials have been employed in reconstructing a range of specialised tissues in pre-clinical and clinical trials [54 - 56]. They have repeatedly demonstrated the remarkable property of “constructive remodelling” – converting a tissues response to injury from scar formation to host tissue regeneration [45]. Porcine small intestine submucosa (SIS) is the most utilised ECM biomaterial and has so far been used in over one million patients in reconstructing a variety of specialised tissues [46]. SIS has also been extensively studied for use in a potential vascular graft. Badylak et al. demonstrated excellent long-term patency in replacing the infrarenal aorta in 13 canines using SIS. All grafts
remained patent during the study (four days to eight years) [58]. There was no
evidence of immune reaction, aneurysms or thrombosis. Examination of the grafts
following euthanasia demonstrated a fully confluent endothelial layer on the graft
surface within 28 – 42 days. SIS grafts have also been used to replace arteries in
primates with similar outcomes seen in non-primates. ECM scaffolds for
reconstructing veins [46] and venous valves have also demonstrated encouraging
results [47] UBM is an emerging ECM scaffold and is most notable for possessing
an intact epithelial basement membrane (Fig. 7a) following processing [94].

In this study cell/scaffold interactions were examined,
specifically, cellular adhesion, proliferation, migration and differentiation, which are
fundamental biocompatibility properties required for successful scaffold design. The
results of the live/dead assay indicate that all scaffolds could support the viability of
human endothelial cells. These results are in agreement with previous studies
involving other ECM analogues, particularly porcine small intestinal submucosa
(SIS), which demonstrated the ability to grow several different cell types including
endothelial cells, fibroblasts and keratinocytes [95]. These results indicate that the
materials, coatings or sterilisation methods were not cytotoxic to endothelial cells.
Cells cultured on all materials stained positive for VWF. This is widely accepted to
be an indicator of normally functioning endothelial cells [96]. The VWF
immunofluorescent staining appears different in each group due to the differing
topographies of the substrates.

The behaviour of cells is guided by the physical and
biochemical make up of their surroundings [97,98]. The adherence of ECs to the
luminal surface must be able to resist in vivo forces and this is important for long-
term patency. We found cell attachment to Dacron was negligible, possibly due to
the high porosity of the material resulting in leakage of the seeding solution. EPTFE proved to be no better despite the different nature of the graft lumen at a fibre level, with a flatter surface and smaller pore size. All biological scaffolds including fibrin/ePTFE hybrid supported superior adhesion when compared to uncoated ePTFE and Dacron at each time point ($p \leq 0.001$). EC adhesion on ECM scaffolds and fibrin coated ePTFE showed that fibrin supported similar cellular attachment rates to ECM scaffolds at each time point (Fig. 17). ECM scaffolds and fibrin coated ePTFE retains at least three times the number of cells as uncoated ePTFE. The finding that fibrin vastly enhances endothelial attachment to ePTFE grafts agree with those of Zilla et al. [90]. Fibrin possesses several important inherent features that make it an attractive tissue engineering material. It has excellent biocompatibility properties and is biodegradable with a high affinity for biological surfaces [99]. It performs crucial roles during wound healing supporting angiogenesis and tissue repair, making it an ideal material for cell scaffolding [100]. As well as this, fibrin possesses several cellular attachment sites, which have been shown to enhance cell seeding [101]. The improved endothelialisation associated with fibrin may lie in its thrombin component. Bar-Shavit showed that pre-incubation of alpha-thrombin prior to coating a plastic surface enhances cellular adhesion, spreading [102]. The attachment of endothelial cells to ECM scaffolds is likely to be complex and multifactorial. Both ECM materials displayed similar high rates of cellular adhesion, without any significant difference between the two scaffolds even though the culturing surfaces of SIS and UBM are different. SIS possesses an interstitial like surface whilst UBM contains a basement membrane. Baker et al. while investigating graft endothelialisation reported that cells attached equally well to the basement membrane surface (collagen types IV and V) and interstitial surface (collagen types I
and III) of human amnion [103]. Adhesion proteins contained in ECM scaffolds, such as fibronectin, collagen type I and laminin are widely recognised as important attachment substrates for endothelial cells, encouraging growth and “sprout” formation [104,105]. Proteins are not the only ECM components known to facilitate cellular attachment. Growth factors identified in SIS and UBM enhance and possibly play a synergic role in promoting cellular attachment. Fibroblast growth factor 2, transforming growth factor beta and vascular endothelial growth factor are proven to encourage the adherence of endothelial cells [106].

As well as the components of ECM scaffolds, the topography may also influence cellular adhesion. Several studies have reported changes in cellular behaviour depending on the roughness of their substrates. This has lead to nanoscale surface engineering to alter cellular orientation, growth, attachment and migration [107-110]. Miller et al. reported the enhancement of attachment and growth of ECs on poly (lactic-co-glycolic acid) (PLGA) materials by mimicking the surface roughness of natural extra-cellular matrix [111]. The luminal surface of SIS and UBM comprise a dense compact basement membrane like surface following the removal of the epithelial layer. In SIS, this represents the lamina propria of the tunica mucosa and is described as interstitial like, while in UBM it is a true epithelial basement membrane [94]. The topography found in ECM, may provide cells with a more natural nanosurface, resembling that found in vivo and positively influencing cell growth and attachment. More recent studies suggest the stiffness of ECM may also play an important role in cell-matrix adhesion [137]. The specific elasticity of ECM triggers a mechanosensing mechanism within the cell via cytoplasmic integrin adapter proteins. These ensure firm and consistant cellular anchorage via integrin receptors to the ECM. This so called “a mechan- osensitive
“switch” may in part help to explain the superior attachment rates to ECM scaffolds [137].

The findings of our research show that the majority of cells attached to ECM scaffolds and fibrin/ePTFE scaffolds between 60 and 90 minutes. The optimum time for attachment appears to be 90 minutes with no advantage gained after 120 minutes of incubation time. Currently there is no consensus regarding the optimum attachment period with various studies reporting a range of times including 15 minutes [112], 45 minutes [113], 60 minutes [114] and 90 minutes [115]. Sugawara et al. studied the attachment times of endothelial cells on fibronectin-coated ePTFE and collagen-coated knitted Dacron grafts using similar cell densities as used in this study (5.59 +/- 1.1 to 6.69 +/- 1.5 x 10⁴ cells/cm²) [116]. The knitted Dacron grafts demonstrated maximum cellular adhesion of 88 +/- 5% following eight hours, while ePTFE grafts achieved this at 24 hours (83 +/- 6%). This compares to a retention rate after 90 minutes of 80.23 +/- 3.6% for UBM and 81.6 +/- 3.0% for SIS found in our study. This comparison highlights the advantage of a complete ECM substrate versus single ECM components. The excellent attachment rates seen with ECM scaffolds are supported by several other ECM studies mainly involving SIS and support its potential use in future vascular graft development [117-119]. This rapid cellular attachment within a short time frame could be advantageous in a clinical setting and may permit a single stage endothelialisation procedure. Exhaustive research has been undertaken to replicate the properties of ECM scaffolds and improve the biocompatibility of synthetic grafts. Methods include extracting and purifying adhesion proteins, synthesising de novo oligopeptides or genetically engineering ECM components. A more recent development is to alter the chemical composition of graft materials to incorporate the
arginine-glycine-aspartic acid (RGD) or tyrosine-isoeucine glycine-serine-arginine (YIGSR) peptide sequences to mediate cellular attachment with the graft material [120-124]. These processes are known as synthetic mimetics of ECM. However these methods don’t replicate the complexity of ECM and their mechanical characteristics differ substantially. Impurity, mechanical weakness and immune reactions have all been attributed to replicated ECM components [125]. While this approach has shown promise, isolated peptide sequences or proteins do not fully replicate the function of intact natural ECM [126].

To observe the morphological characteristics of ECs grown on each scaffold, different sections of seeded construct were analysed by scanning electron microscopy. On ePTFE and Dacron (Fig. 20 & 21), cells appeared as small spheroid shapes with single point contact to the material. Cells initially attach in a spherical morphology and later gain a larger, more flatter shape. The morphology observed on the synthetic scaffolds suggests that these cells have not yet acclimatised to the substrate and are poorly attached, essentially in a quintessential phase. They are inhomogeneously distributed on the surface and aggregate in clusters leaving large sections of uncoated fibres exposed. The morphological difference between cells grown on synthetic grafts and biological grafts (SIS, UBM, fibrin/ePTFE) is striking. Cells grown on the biological substrates exhibited a larger, flatter cell shape with increased cytoplasmic extensions to the materials (Fig. 22, 23 & 24). These cells have a greater number of attachment bonds to the substrates, thus increasing attachment strength. All cells examined on the biological substrates were beginning to form the typical cobblestone monolayer morphology as seen in vivo. This type of morphology is known to correlate with high levels of attachment and facilitate enhanced cellular proliferation and migration [127]. Flattened phase
endothelial cells exhibit increased amounts of contact regions with their substrate and are also in a shape most capable of allowing the streamlining of blood flow thus minimizing cell loss.

In vitro endothelialisation not only requires adequate cellular attachment, but cells also need to proliferate at an acceptable rate. Slow proliferation increases incubation times, leading to higher rates of contamination and increased costs. This limits the procedure to specialist centres, delays implantation and increases the initial harvest density required. In this study we used a limited amount of HUVECs to obtain a confluent endothelial layer in a short time frame. These results can be extrapolated for other cell types including human saphenous vein endothelial cells and microvascular endothelial cells to evaluate the technology for clinical use. Our results demonstrated (Fig. 18) that all materials studied, supported cellular proliferation to some extent. Although cells grown on synthetic grafts remained viable, displaying slow but consistent growth, they did not achieve confluency by the end of the study (day nine). The high porosity and poor cellular attachment may have initially delayed cell-cell contact formation and therefore had a negative impact on cell growth. Furthermore, a smooth surface is required to allow for uninterrupted cellular growth [128]. The fibrillar interstices of ePTFE and the Dacron fibres may hinder cellular growth and migration. Despite the excellent attachment rates achieved with fibrin-coated ePTFE, this was not replicated when examining cellular proliferation. The reason for this is not fully understood. Theoretically, fibrin is an excellent scaffold; it supports angiogenesis and tissue repair in vivo [129,130]. It also releases fibrinopeptides, which are known to be mitogenic for ECs [129,131]. Henrich examined two types of fibrin glues and also discovered high levels of attachment but weak proliferation [132]. Although fibrin
did increase the proliferation rate when compared to uncoated ePTFE, this was insufficient to form a confluent endothelium within the time frame of this study.

Of all the vascular materials studied, cells proliferated fastest on ECM scaffolds with both reaching 100% confluency at day seven (Fig. 18 &19). Thereafter there was no significant growth (p=1.000) due to contact inhibition. Each had statistically higher cell numbers from day three on, when compared to fibrin/ePTFE hybrid. It is generally accepted that cellular growth on biomaterials is highly reliant on the topography and composition of the substratum [133]. ECM is crucial during angiogenesis, which involves endothelial growth and spreading [134 - 136]. It is rich in substances known to enhance angiogenesis such as the growth factor VEGF [52] and functional proteins such as laminin and fibronectin, which accelerate endothelial proliferation. Glycosaminoglycans present in ECM bind to growth factors and cytokines and encourage cellular growth. In vivo, the luminal surface of SIS and UBM support a continually dividing cellular layer; the small intestine enteriocytes in SIS and transitional epithelium in UBM. These layers are repeatedly subjected to mechanical and infectious trauma, hence it’s reasonable to assert, that ECM harvested from such areas has evolved to optimise rapid cellular growth and differentiation to aid repair. Thus the composition of intact ECM appears to be an ideal substrate for promoting rapid endothelialisation. In a clinical setting where a finite amount of cells are available to form a complete monolayer within a short time frame; firm cellular attachment combined with rapid endothelialisation will be important criteria in determining the success of this technology.

We chose the seeding density of $7 \times 10^4$ cells per cm$^2$ as previous experience in our lab demonstrated that this is an ideal density in order to examine cellular proliferation. If you have too high a density the cells would rapidly reach confluency
and this makes it difficult to compare different growth rates. Too low a density would require a longer experiential timeframe to accurately judge cell growth on different materials. HUVECs were chosen for several reasons. Firstly, we have a long established history of using this cell line at our lab in UL. Hence the experience and expertise were in place and expense was saved on consumables such as media and growth factors. HUVECs are a very robust cell line and are easy to culture in a lab setting. Therefore they are ideal for initial in-vitro experiments. As well as this, HUVECs are the most commonly used cell types in similar studies. Therefore by using this cell line, it allowed us to compare our results with other studies. Future experiments will involve alternate cell lines including endothelial progenitor cells and microvascular endothelial cells to verify and compare these initial results.

In summary, we demonstrated the rapid expansion and firm anchorage of endothelial cells to ECM scaffolds. Cells grown on ECM scaffolds displayed significantly stronger attachment when compared to existing synthetic grafts. Cells proliferated at a significantly faster rate when compared to the leading endothelialisation substrate fibrin coated ePTFE. This work suggests that ECM materials are promising scaffolds for small vessel tissue engineering.
2.5 References


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Construction of a subatmospheric pressure bioreactor for tissue engineered vascular graft constructs
3.1 Introduction

Tissue engineering a vascular graft can either involve the growth of a de novo vessel from cells alone [1] or more commonly combining cells with synthetic [2-6] or biological [7,8] scaffolds. Limitations of the latter include fragile structure in biological grafts, poor cellular attachment, proliferation in synthetic grafts. One method to overcome this is to pre-condition tissue engineered grafts with mechanical stimuli, which is increasingly being recognised as an essential step in producing a successful tissue engineered replacement [9]. Vunjak-Novakovic et al. stated in a review article in 2006 that; “It becomes quite apparent from the progress in functional tissue engineering in the past decade that mechanical stimulation should be an integral part of any attempt to engineer a functional tissue where mechanical function is physiologically important.” [10]. This is particularly true in the tissue engineering of blood vessels, which must possess a complex array of mechanical and biological properties that are absent in statically seeded constructs. In vivo, blood vessels are exposed to four different haemodynamic forces: shear stress as a result of the flow of blood, luminal pressure due to blood pressure, as well as longitudinal and circumferential stretch secondary to the cardiac cycle. To date, several studies have been conducted to examine these forces in the preconditioning of tissue engineered vascular grafts [11-16]. Each of these forces, whether alone or synergistically can affect the behaviour of vascular cells [17-20]. Several different types of bioreactors have been constructed to generate a range of mechanical environments. These can be simple, also known as deconstructive models; mimicking a single mechanical stimulus i.e. shear stress or axial stretch [21,22]. More complex systems, also known as reconstructive models attempt to completely replicate in vivo conditions, for example explanted vessel systems and mock arteries [23-27].
Simple bioreactors investigated to date, include mechanically stirred flasks [28], rotating wall reactors [29], perfusion systems [30] and mechanical strain bioreactors [22]. These have met with variable results in improving cell proliferation, adhesion and construct strength [31,32] and presently, there is no consensus regarding the optimal preconditioning protocol. It is also unknown whether complete replication of all physiological stresses is required or only selected forces to optimise cell growth. In vascular graft tissue engineering exposure to shear stress is by far the most common form of mechanical pre-conditioning [33, 34]. This technique requires expensive and complex closed circuit bioreactors that are particularly vulnerable to technical failure or contamination [35]. They also require a high level of expertise to operate. Reports have cited increased cell loss and weakened grafts following shear stress preconditioning [36]. There is also limited data on the acute response of shear stress preconditioned endothelial cells to circulating blood once implanted in vivo.

Most tissues in the body undergo some form of mechanical stretching and in response to this, researchers have developed a wide range of mechanical strain bioreactors. These types of strain bioreactors can be categorised as uniaxial or multiaxial systems. Uniaxial bioreactors exert strain in one axis only and may be constrained (whereby an axis is fixed to prevent strain in this direction – the Poisson effect) or unconstrained. Multiaxial systems are more complex and exert strain in more than one direction. They include biaxial and equiaxial (stretch is applied equally in all directions). In addition to this, both uniaxial and multiaxial bioreactors can be further subdivided into in-plane (the stretch is applied in the same plane as the construct) or out-of-plane systems. Multiaxial bioreactors are generally much more complex to build and may not confer any advantage with respect to vascular tissue
engineering [37]. It is felt the uniaxial stretch systems may replicate the vascular in vivo environment better and be more relevant to vascular constructs [38,39]. Uniaxial systems are also easier to construct, monitor and modify for a particular experiment. One difficulty with uniaxial systems is that researchers have found it difficult to produce a homologous strain on the construct [40].

This study, explores a simple novel bioreactor, which induces mechanical strain on tissue engineered vascular constructs via the application of subatmospheric pressure. The subatmospheric pressure is generated via commercially available Vacuum assisted closure ® unit (V.A.C. ®) which is EU and Food and Drug Administration (F.D.A.) approved since 1995. We hypothesize, that this mechanical preconditioning could enhance the cellular seeding of vascular graft materials prior to implantation. Vacuum assisted closure treatment is a widely used therapy that enhances granulation tissue production in acute and refractory wounds [41,42]. It applies negative pressures, typically 125mmHg (range 50 – 200mmHg) to the wound via a GranuFoam ® Dressing (Fig 25a & b). It has been proven to promote healing in part by exerting a physical strain on the wound bed [43]. In a recent study, Saxena et al. using finite element modelling proved that V.A.C. therapy produced between 5 – 20% tissue strain [44]. This degree of cellular stretch is known to stimulate cell growth in vitro [45, 46]. Hence, we sought to develop a simple novel bioreactor, capable of producing subatmospheric pressures in a closed system thereby inducing a mechanical strain on vascular tissue constructs with an aim to enhance in vitro endothelialisation. The current study is a technical description of the construction and validation of the bioreactor. When designing a bioreactor, certain design criteria need to be followed. For example; the bioreactor should be simple to use, allowing for repeated consistent experiments. It should be small in size to reduce the amount
of culture media used and save on incubator space. It needs to be constructed with biocompatible components amenable to repeated sterilising. Lastly it should permit continuous experimental observation and maintain a constant controllable environment.

Fig. 25a. GranuFoam: an open cell dressing, which can be cut to a specified size.

Fig. 25b. SEM image of the reticulated foam dressing having 400-600 um pore diameters. × 20 magnification.
3.2 Methods

3.2.1 Culture Platform

Urinary Bladder Matrix Cellular Scaffolds were used in this study. Their preparation has already been outlined in chapter two, section 2.2.3.

3.2.2 Cells

Human Umbilical Endothelial Cells were used in this study, purchased from (Cascade Biologics/Invitrogen, Cat. # C-003-5C). Their isolation and culturing has previously been described; chapter two, section 2.2.6.

3.2.3 Biochamber design

The biochamber was manufactured from Ultem ® 1000 polyetherimide (PEI) obtained from Quadrant Plastics, Lenzburg, Switzerland with other components (screws, nuts, and washers) made from stainless steel as described by Shaikh et al. [47]. Polyetherimide is an amorphous, amber-to-transparent thermoplastic. It is an autoclavable polymer characterized by high heat resistance and strength that remains stable over a wide range of temperatures. The biochamber was manufactured as a three-part detachable box; height: 7.5cm, length: 7.5cm and width: 6.5cm, total volume: 111.375 cm³. The transparency of the polyetherimide allows for observation during experiments (Fig. 26). The middle part and lower sections were designed to accommodate 6 x 4 cm scaffolds and media, thus providing cell-media contact above and below the scaffold (Fig. 27). An outlet was incorporated into the lower chamber to allow for media drainage via a valve (Fig. 29). A sterile GranuFoam dressing (Kinetic Concepts Inc. [KCI]) was trimmed to size, and positioned over the seeded
scaffold in the second chamber (Fig. 29). GranuFoam is hydrophobic, reticulated, open-cell polyurethane foam approved for direct contact with cells (Fig 28a & b). The pore size of 400–600 microns and the open-cell structure have been found to optimise granulation tissue formation. V.A.C. GranuFoam allows the even distribution of applied pressure to all parts of the matrix in contact with the foam. A sterile polyurethane cover was then stretched over the GranuFoam dressing creating an airtight seal (Fig. 30). The drape is made of a sterile polyurethane sheet backed with adhesive, which is supplied by K.C.I. It provides flexible pneumatic covering over the foam and is gas permeable; allowing the constant exchange of air through the drape and to the cultured cells. A small hole (1cm diameter) was made in the drape and the Therapeutic Regulated Accurate Care (T.R.A.C ®) Pad fastened over this (Fig. 30). Tubing from T.R.A.C Pad was attached to the VAC Freedom ® System (Fig 32). The lid of the bioreactor was specifically designed for the support and mechanical fixation of two ports; a media supply port and port for the tubing running to the vacuum pump (Fig. 33). Culture media was infused into a sterile IV drip bag and fed into the biochamber via a hypodermic needle through the foam dressing. To maintain a seal the needle was inserted via a self-sealing polyurethane adhesive backed pad (3M) (Fig. 31). This provided an airtight seal and dissipated mechanical forces during the application of negative pressure. Culture media was supplied via an infusion pump at a rate of 100mls of medium per 24 hours (Fig. 34). The culture plate lid is positioned over the Granufoam dressing which fixes the media support needle and CO₂ permeable silicone tubing to the VAC Freedom ® System. The three parts of the chamber are fixed via sterile stainless steel bolts and nuts. The biochamber is positioned in a standard incubator (37°C and 5% CO₂, 95%
humidity) to maintain a constant environment conducive to cell growth. The tubing from the biochamber is fed via an external incubator port to the VAC unit. A schematic of the fully constructed bioreactor can be found in Appendix 5 (page 316).

Fig. 26. The biochamber; a 3-part detachable cube manufactured from transparent polyetherimide.

Fig. 27. Insertion of a 6 x 4 cm scaffold into the biochamber. The chambers were then filled with media.
Fig. 28a. V.A.C. ® Granufoam ® Sterile Large Dressing 25.6 × 15 × 3.3 cm.

Fig. 28b. Left to Right: V.A.C. ® Adhesive Drape, T.R.A.C Pad ® with tubing, clamp and connector, GranuFoam ® Sterile Large Dressing.
Fig. 29. A Granufoam dressing placed over and in contact with the seeded scaffold. Note the drainage outlet for media.

Fig. 30. Polyurethane adhesive film was stretched over the Granufoam dressing and the T.R.A.C Pad ® placed.
Fig. 31. Culture media is fed into the biochamber via a hypodermic needle through a self-sealing polyurethane adhesive backed pad.

Fig. 32. VAC Freedom ® System.
Fig. 33. Upper chamber was specially designed for the support and mechanical fixation of 2 ports; a media supply port and port for tubing running to the vacuum pump.

Fig. 34. Culture media was supplied via an infusion pump.
3.2.4 Cell Culture and Seeding

HUVEC cell lines were obtained from Bio Cascade Biologics/Invitrogen, Cat. # C-003-5C and cultured as previously described in chapter two, section 2.2.6. The media was replaced every 48 hours until cells reached confluency. The cells were then split in a ratio 1:3 with 3mls buffered saline solution containing 0.25% trypsin and 0.09%. EDTA. The seeded 75cm$^2$ flasks were placed in a 37°C, 95% humidified, 5% CO$_2$ incubator. At confluence, HUVECs were trypsinised and resuspended in fresh media before being seeded onto the luminal surface of 6×4 cm UBM scaffolds at a density of 2×10$^4$/cm$^2$. Seeded UBM sheets were submerge in media via sterile steel plates.

3.2.5 Application of negative pressure

Seeded UBM scaffolds were exposed to a selected continuous pressure (range 75mmHg - 200 mmHg) for up to two days. Media was supplied into the biochamber via an automated pump at a rate of 100 mL per day. Seeded UBM scaffolds at atmospheric pressure in the bioreactor acted as a control. All experiments were carried out in a standard incubator maintaining a constant environment (37°C, 95% humidity, 5% CO$_2$). Each experiment was carried out in triplicate.

3.2.6 Assessment of Cellular Viability

The cytotoxicity of the constructs to HUVECs was assessed by determining cellular viability using a live/dead viability assay
(Invitrogen™). The live/dead assay was already described in detail in chapter two, section 2.2.8. Photos were taken with a Nikon Eclipse TE200 inverted microscope.

3.2.7 Immunofluorescence analysis of von Willebrand Factor

Endothelial cells seeded on each substrate were fixed with 3.7% paraformaldehyde in Phosphate buffered saline (PBS). This was then rinsed with PBS and mixed for 20 min with 0.27% NH4Cl/0.38% glycine in PBS and permeabilised with Triton X-100 (0.5%) in PBS. Von Willebrand Factor was confirmed via fluorescein-labeled Antibodies-conjugated mouse anti-human vWF antibodies (2 µg.mL⁻¹).

3.2.8 Contamination assays

Sterility of the bioreactor was critical, as any bacterial growth would harm to growth of cells and invalidate the bioreactor. Contamination in a clinical setting at final implantation could also have fatal consequences. Antibiotics (streptomycin) were added to all perfusion media as standard (see section 2.2.6). Following each experiment the turbidity of the medium was inspected visually. Medium samples were retrieved and sent in sterile specimen containers to University Hospital Limerick Microbiology laboratory for evaluation for bacterial or fungal contamination.
3.2.9 Optical and scanning electron Microscopy

The methods for Scanning Electron Microscopy have already been outlined in section 2.2.12.

3.2.10 Statistical Analysis

Statistical analyses were conducted using SPSS 20. Data was summarised using means and standard deviations. Statistical significance was determined as p<0.05. Differences in cell viability (Bioreactor) between treatment and control groups were tested by independent two-sample t-test.

3.3 Results

3.3.1 Biochamber

Prior to culturing experiments, the biochamber was fully constructed, infused with water mixed with typan blue dye and examined for air leaks at six hourly intervals up to 48 hours. These experiments were carried out under a range of subatmospheric pressures (75-200mmHg). No leaks were found.

3.3.2 Scaffold Viability

The UBM scaffold structure was examined following 48 hours of continuous subatmospheric pressure at 125mmHg. Optical and SEM examinations of the scaffolds demonstrated that the shear and tensile forces generated by VAC dressing
did not result in attrition or rupture of the scaffold. Dehydration or contracture of the UBM samples was also not seen. These findings were confirmed with SEM imaging (Fig. 35). Following exposure of UBM to subatmospheric pressures for 24 hours surfaces indentations were seen over the scaffold surface. This corresponded to the topography of the GranuFoam dressing indicating a stress was being applied.

3.3.3 Cellular Viability

Live/Dead assays showed the cells remained viable in both the control culture and when cells were grown under subatmospheric pressure (Fig 36 a & b). Cells remained viable on the scaffolds at the conclusion of the experiment day two and there was no difference in cellular viability between static or subatmospheric pressure (p=0.602) (Fig 37). The mean viability of cells +/- standard deviation under static atmospheric pressure was 92.59% +/- 2.96127% and under continuous negative pressure (125mmHg) 91.22% +/- 2.99303%.
3.3.4 Validation of endothelial cells

In the immunofluorescence staining, intracellular vWF of HUVECs was confirmed both on static and cells exposed to subatmospheric pressures after 24 hours. VWF is known to be an indicator of normal functioning endothelial cells.
Figures 38a and 38b shows ECs expressing vWF demonstrating endothelial cells to be functioning.

3.4 Discussion

In vivo, vessels adapt to haemodynamic changes by cellular proliferation and the formation of new extracellular matrix, a process termed adaptive vascular remodelling. Several different mechanical forces are present in vivo, which act independently and synergistically on the behaviour of vascular cells. The constant passage of blood creates shear stresses on the endothelial cells. The rhythmical contraction and relaxation of the heart generates axial stresses and cyclical torsion on the blood vessel wall. The use of bioreactors to promote adaptive vascular remodelling in vitro and improve the functionality of constructs is increasingly being employed in tissue engineering. Over the last decade, researchers have focused on developing bioreactors that mimic the chemo-mechanical environment of the tissues they aim to replace. These systems are commonly refered to as biomimetic systems.
In vascular tissue engineering this most commonly involves complex shear stress bioreactors, which mimic the flow of blood. However such bioreactors are complex and some studies have reported inhibition of endothelial and smooth muscle cell growth [47]. Along with this, studies investigating human embryologial development have shown that vascular embryogenesis is not dependent on large amounts of shear stresses [48]. In this study, we have designed a simple bioreactor that generates mechanical strain via negative pressure over the newly developing vascular construct. The subatmospheric pressure compresses the granufoam dressing and draws it down onto the vascular construct, which is lifted upward, thus creating a mechanical strain. The granufoam dressing ensures an equal distribution of strain to the construct [50].

Our results showed that following 24 hours of subatmospheric pressure, surface indentations were visible on the UBM surface, which corresponded to the geometry of the Granufoam dressing. This illustrated that a strain was being applying to the matrix via the VAC system as described by Saxena [44]. These GranuFoam indentations are also seen clinically in wounds treated with subatmospheric pressure [49]. Cell viability testing demonstrated that neither the bioreactor, GranuFoam dressing nor the strain applied by the subatmospheric pressure was harmful to the cells. Von Willebrand factor analyses demonstrated that after the application of subatmospheric pressure, endothelial cells continued to produce von Willebrand factor, indicating that the bioreactor was not harmful to the endothelial cells, which were continuing to function. These results were crucial in validating the bioreactor.

To function properly a bioreactor must maintain tight controls of the culturing environment (O₂, CO₂, temperature, humidity) suitable for cell. A bioreactor must also allow for sterile experimentation. These properties are vital for controlled,
reproducible experiments and large-scale manufacturing. The bioreactor described here is a small unit with limited components, capable of fitting inside standard incubators and laminar flow benches. The VAC Freedom® Unit is battery operated and compatible with all standard incubators found in most laboratories. The vacuum pump and GranuFoam dressings are readily available commercially with FDA and EU approval, thus lowering potential costs. Although the biochamber described here is small, it is easily adaptable to larger scale units as is the GranuFoam dressings. Because of the simplicity of design, contamination risk is minimised as demonstrated by repeated negative cultures. All biochamber parts (polyetherimide, silicone and stainless steel) are durable with excellent mechanical strength. They can all be sterilized in an autoclave and are reusable. The design of the biochamber components is simple and easily constructed. The sterilisation process is also straightforward. The GranuFoam dressing, adhesive drape and tubing are disposable and come already sterilised by Kinetic Concepts Inc. They are commercially available and hence are easily replaceable. The complete unit can be constructed in a standard laminar flow hood with limited expertise. An appealing aspect of this bioreactor is that the VAC unit can be programmed to different pressure settings (range 50mmHg – 200mmHg) either continuously or intermittently. This allows us to study cellular behaviour at a variety of subatmospheric pressure settings. The VAC unit also automatically regulates the pressure ensuring a constant pressure is being applied with warning alarms if pressure is lost via leaks in the seal or if the tubing is blocked. The pressure is regulated via Sensa TRAC ® technology which monitors target pressure at the wound via outer sensing lumens in the tubing.

One disadvantage of the bioreactor is the large quantity of medium that must be used during the experiments to replace that lost in the vacuum. This could be a
particular problem for lengthier studies and could also make it difficult to examine subtle metabolite changes in the cells. One solution would be to scale down the bioreactor chamber, as much of its volume was not required in this experiment.

Further experiments are needed to confirm initial experimental findings and to quantify the rate of cellular proliferation, cellular morphology and attachment; these will be explored in the next chapter. It would also be interesting in future studies to quantify the effects of subatmospheric pressure on the formation of cell-to-cell junctions, orientation and polarisation. From this, our understanding of endothelial cell physiology under subatmospheric pressure may lead to improvements in vascular graft endothelialisation. The simplicity of the bioreactor would allow for experimentation in other areas of cell physiology for example, it is particularly suited to examining the mechanisms behind negative-pressure wound therapy (NPWT).

In summary, we have constructed and validated a novel bioreactor to examine the effects subatmospheric pressure has on cells. Following the application of subatmospheric pressure UBM matrixes displayed characteristic undulations similar to what is observed clinically indicating a strain had been applied to the cells and underlying scaffold. The bioreactor maintained a continuous subatmospheric pressure to the vascular construct in a stable and constant atmosphere for up to two days. The cells remained viable throughout the study and the structure of the scaffold was not damaged during the application of subatmospheric pressure. There was no evidence of bacterial contamination during the study. Thus we have constructed a stable bioreactor capable of reproducible in vitro experiments on the effects of subatmospheric pressure has on cells and cellular scaffolds.
3.5 References


The preconditioning of endothelial seeded vascular graft materials with subatmospheric pressure.
4.1 Introduction

It is generally accepted that physical stimulation metabolically activates cells and this phenomenon has been utilized in medicine for over a century [1-4]. Plastic surgeons use tissue expanders to mechanically induce tissue growth for soft tissue reconstruction and skin augmentation [5]. Orthopaedic surgeons utilise mechanical straining of fractured bone to induce osteogenesis which leads to faster healing times [6, 7]. Therapies such as traction and tissue expansion generate microstrain [8, 9], which is thought to evoke a cellular physiological response through a process called mechanotransduction [10]. Cells appear to be capable of sensing and reacting to physical stimulation through the activation of specific genes and cellular pathways. Several papers have demonstrated that cells, which are physically strained, are more capable of responding to mitogenic growth factors than cells grown in static culture [11, 12]. More specific to our discussion, vascular endothelial cells have been shown to up regulate differing combinations of genes depending on the type of shear stress they are exposed to i.e. laminar, oscillatory or turbulent [13-15]. The mechanisms behind this remain unclear, but it is thought to involve physical stimulation of the cytoskeleton (the structural framework of a cell), which somehow stimulates signalling pathways [16].

Mechanical preconditioning is widely used in tissue engineering. It has been employed to accelerate tissue growth and promote the formation of improved functional constructs. Currently, the most common method employed is to attempt to replicate the in vivo physiological environment of the tissue being replaced. In vascular surgery, this has lead to the recreation of complex bioreactors aimed to replicate the exact physiological conditions of blood vessels [17-19]. Despite the development of complex bioreactors, the optimum conditioning protocol currently
remains unclear and it is still unknown whether developing tissues should be preconditioned to all physical stimuli present in vivo or if it is better to only partially recreate the physiological environment. For example, several studies have reported encouraging results when preconditioning vascular grafts with dynamic wall straining alone without employing shear stress [20-22].

The most common examples of mechanical tissue stimulation in modern medicine are negative pressure dressings. They are efficacious and extensively employed in the treatment for acute and chronic wounds with several randomised controlled trials demonstrating their efficacy at promoting increased granulation tissue formation [23-25]. In porcine wound models, negative-pressure wound therapy promotes angiogenesis and granulation tissue whilst reducing oedema and bacterial load at the wound [26]. As well as this, recent finite element analysis of negative-pressure wound dressings has shown that they induce a mechanical strain on the wound bed [27]. It is hypothesised that this may influence cellular physiology, thus creating a strong stimulus for increased cellular growth and angiogenesis. Several studies have investigated the preconditioning of tissue engineered vascular grafts via shear stress; however far less is known about the role mechanical stretch has on endothelial function.

Several groups have speculated that the application of mechanical stretch may influence endothelial behaviour independently from shear stress forces [28]. Negative-pressure wound therapy has been shown to increase microvascular density and promote angiogenesis [29]. The mechanical strain created from NPWT promotes cellular proliferation and is known to activate endogenous VEGF, which is mitogenic for endothelial cells [39]. Hence, the current study examines the effects of subatmospheric pressure on seeded vascular graft materials using a novel
subatmospheric bioreactor. We hypothesise that the application of mechanical stimulation via negative pressures could lead to enhanced vascular graft endothelialisation.

4.2 Methods

4.2.1 Experimental Overview

Changes in cellular morphology, viability and proliferation in response to negative pressures were investigated. Human umbilical endothelial venous cells seeded on ECM scaffolds were placed into the bioreactor under different protocols: 1. Static pressure. 2. Continuous subatmospheric pressure 75mmHg, 3. Continuous subatmospheric pressure 125mmHg 4. Continuous subatmospheric pressure 200mmHg. Cells were then examined in terms of morphology, viability and proliferation.

4.2.2 Extracellular matrix preparation

UBM in the form of 6×10cm-sterilised sheets were kindly supplied by Dr. Stephen Badylak, University of Pittsburgh, Pittsburgh, U.S.A. Their production has already been described in chapter two, section 2.2.3.

4.2.3 Bioreactor Set Up

Briefly, cell constructs were placed into the well of the bioreactor as outlined in chapter three, section 3.2.3. GranuFoam dressings which facilitate the equal distribution of pressure were cut to the size of the chamber and positioned over the
construct. The V.A.C. Drape was then stretched over the GranuFoam and attached to the outside of the bioreactor to create an airtight seal so that the vacuum can be applied. The tubing from the drape was threaded through the bioreactor lid and connected to a V.A.C. Freedom unit located outside the incubator. Culture medium was delivered to the construct as described in chapter three section 3.2.3 at a rate of 100mls per 24 hours. The V.A.C.s Freedom system was programmed to run continuously at 75mmHg, 125mmHg or 200mmHg. As a control, a bioreactor was also set up as described above, but under static environmental conditions. For every experiment the biochamber was placed inside a standard incubator to maintain an environment of 37 °C, 95% humidity and 5% CO₂. Each experiment was carried out in triplicate.

4.2.4 Cells

HUVEC cell lines were obtained from Bio Cascade Biologics/INVITROGEN, Cat. # C-003-5C. Culturing technique has already been described in chapter two, section 2.2.6.

4.2.5 Cell Culture and Seeding

HUVECs were seeded onto 75cm² flasks, which were placed in a 37°C humidified 5% CO2 incubator. The media was replaced every 48 hours till cells reached confluency. The cells were then split in a ratio 1:3 with 3mls buffered saline solution containing 0.25% trypsin and 0.09%. Ethylenediaminetetraacetic acid (EDTA) At final confluence, HUVECs were resuspended in fresh media before being seeded onto the luminal surface of 6×4cm UBM scaffolds at a density of 2×10⁴/cm². Seeded UBM sheets were fixed by steel
plates in order to submerge them in media in the petri dishes. We chose a lower cell density \((2 \times 10^4 \text{ cells per cm}^2)\) than the static experiments in chapter 2 to help us conclude if we could reach confluency with a lower cell harvest by using mechanical stimulation.

4.2.6 Cell Viability Testing

The viability of cell constructs placed in the bioreactor under static and subatmospheric pressures at 12 hours. After 12 hours of exposure to subatmospheric pressure of 75mmHg, 125mmHg and 200mmHg cell viability was assessed using a live/dead viability assay (Invitrogen™). The method has already been described in chapter two, section 2.2.8. Cells grown in static culture acted as a control. Images were captured with a Nikon Eclipse TE200 inverted microscope. The amount of viable and dead cells was calculated from a minimum of three fields of view.

4.2.7 Cellular Proliferation Assays

The rate of cellular proliferation was measure using an MTS cell count kit. Cell numbers were measured at 24 and 48 hours under static and subatmospheric conditions. The MTS assay has been described in chapter two, section 2.2.11.

4.2.8 Cellular Morphology

Each construct with HUVECs was primarily fixed in cold 2.5% glutaraldehyde/paraformaldehyde phosphate buffer solution. After primary fixation,
the constructs were post-fixed in 2% Osmium Tetroxide then dehydrated through a graded series of acetones up to 100%. After critical point drying, the samples were attached to aluminium SEM specimen mounting stubs and then sputter coated with gold. Following processing, samples were analyzed using the Scanning Electron Microscope (JEOL 5510) at University College Cork.

4.2.9 Contamination Assays

The cell cultures were tested following each experiment for bacterial or fungal contamination. Media and scaffold samples were sent in sterile containers to the University Hospital Limerick Microbiology lab for analysis.

4.2.10 Statistical Analysis

Statistical analysis was performed using SPSS 20.0 software for windows. Each quantitative experiment was performed in triplicate. Data is expressed as the mean ± standard deviation (SD). A value of P < 0.05 was considered to be statistically significant. For cell proliferation, a 4x2 factorial analysis of variance was conducted to examine the effects of sub-atmospheric pressures, test days and the interaction between these two factors. Where a significant interaction between sub-atmospheric pressures and test days was found, this was explored using simple main effects analysis. Initially simple main effects of sub-atmospheric pressures at each test day were conducted. The significant simple main effects of sub-atmospheric pressures were further analysed by pairwise comparison using a Bonferroni adjustment for multiple comparisons. Secondly, the simple main effects of test days at each sub-
atmospheric pressure were explored. Significant simple main effects of test days were further analysed by pairwise comparisons employing a Bonferroni adjustment for multiple comparisons. One-way analysis of variance was carried out to examine differences in cell viability between sub-atmospheric pressure groups. The underlying assumption of Normality was examined using Normal probability plots and the Shapiro-Wilk test. The homogeneity of variance test was examined by plotting the residuals of the model against the fitted values and the Levene’s test. There were no concerns for departures from the statistical assumptions of Normality and constant variability.

4.3 Results

4.3.1 Cellular Viability

After 12 hours, cells exposed to negative pressures and static controls displayed similar degrees of cell viability (Fig. 39). This indicated that the bioreactor kept the cells viable with adequate amounts of media and the pressure it created was not harmful to the cells. It also demonstrated that the higher negative pressures of 200mmHg did not have a detrimental effect on cellular viability.
4.3.2 Cellular Proliferation

Our experiments demonstrated that cells subjected to subatmospheric pressures of 125mmHg and 200mmHg exhibited higher levels of growth than cells in atmospheric pressure at 24 (p ≤ 0.016) and 48 hour (p ≤ 0.001) (Fig. 40). Cells grown at 75mmHg did not grow faster at 24 hours (p=0.077) than the control but did proliferate faster at 48 hours (p=0.001). No statistical difference was found between the subatmospheric groups at 24 hours (p=1.000). Cells grown at 125mmHg (p ≤ 0.05) and 200mmHg (p ≤ 0.02) grew faster than those at 75mmHg from 24 hours until the conclusion of the experiment. There was no difference in cell growth between cells exposed to 125mmHg and 200mmHg at 24 (p=1.00) or 48 hours (p=1.00). At 48 hours, cellular activity at 125mmHg was over 50% greater than that of the control.
Fig. 40. Cellular Proliferation under differing subatmospheric pressures

![Cellular Proliferation graph]

Absorbance (490nm) vs. Time (Hours)

- Static
- 75 mmHg
- 125mmHg
- 200mmHg

Fig. 41. Immunofluorescence staining of cells at 48 hours. × 10 magnification.

Static

75mmHg

125mmHg

200mmHg
4.3.3 Cellular Morphology

Inspection of the cells reveals subtle morphological differences between the static group and cells grown under subatmospheric conditions. Cells under static conditions tended to be slightly more spherical and sparse with more areas of exposed substrate (Fig. 42a). Cells exposed to negative pressure were more organised, elongated and expanded (Fig. 42b). This is consistent with actively growing cells and would be indicative of that seen in vivo. These differences reflect the increase in cell numbers but may also be reflective of physiological changes in the cells in response to mechanical stimulation.

Fig. 42a. Scanning Electron Microscope endothelial cells in static culture 48 hours.
4.4 Discussion

The application of mechanical preconditioning is an increasingly employed technique in tissue engineering to enhance the functionality of tissues. Here, we examined the feasibility of a novel subatmospheric bioreactor to generate mechanical strain for applications in vascular tissue engineering. This mechanical strain approximates the stretch vascular cells experience from pulse pressure in vivo. We found equal rates of cellular viability rates in cells grown in static cultures and those exposed to negative pressures. This demonstrated that neither the bioreactor, nor the mechanical forces had a damaging effect on the cells; these results were in agreement with results outlined in chapter three and were crucial in validating the
bioreactor. Cells remained viable through all ranges of negative pressure tested which is important in helping to determine optimum pressure settings.

The scaffold remained structurally intact throughout the study even at the higher subatmospheric pressure levels (200mmHg). The seeded scaffold remained fixed and in the correct position in the biochamber at the end of each experiment. Previous studies have found that cells, which are not fixed or grown on a floating matrix, undergo apoptosis [28]. Results taken together suggest that the bioreactor and the mechanical forces it creates were not harmful to the cells and that the matrix was stable, even at the higher pressures level of 200mmHg.

Scanning electron microscopy demonstrated subtle changes in cellular morphology between the control and cells exposed to subatmospheric pressures. Cells in static culture were less plentiful and slightly less elongated, being more oval in shape. Under subatmospheric pressures, cells gained a more sheet like appearance, covering more of the material and beginning to achieve the cobblestone morphology, which is seen in vivo. Of note, SEM analysis of the seeded scaffolds demonstrated that the UBM remained structurally intact with no visible evidence of rupture or erosion. These findings reaffirmed the results of visual and optical inspection of the scaffolds following each experiment. The more streamlined appearance of cells preconditioned with subatmospheric pressure may reflect physiological changes such as ECM production, gene regulation or cellular signalling [29, 30]. This flattened morphology has more cellular contact points with the material and allows streamlining of blood thus minimising cell loss upon implantation and reducing the risk of thrombosis. Collins et al. showed that endothelial cells under tensional forces upregulate platelet endothelial cell adhesion molecule-1 (PECAM-1), which causes a
change in the cytoskeletal structure by increasing focal adhesions and cytoskeletal stiffening [31].

Endothelial cells proliferated significantly quicker under subatmospheric pressures of 125 and 200mmHg than the controls at each time point (Fig. 40). Cells grown at 125mmHg and 200mmHg grew at a significantly faster rate after 24 hours compared to 75mmHg ($p \leq 0.05$). We found no difference between cells grown at 125 and 200mmHg at any stage, suggesting that 125mmHg may be the optimal pressure setting for this density of cells. In a clinical setting there will be a finite amount of cells available following harvesting. Hence, good cellular adhesions, as well as high proliferation rates are important. This could enhance the efficacy of a seeding protocol, allowing for faster monolayer formation and the possibility of single-stage seeding.

The exact mechanism as to how mechanical stimulation influences cell behaviour remains to be fully elucidated, but is likely to be multifactorial and complex. Our bioreactor induces a mechanical strain on the scaffold and the cells attached. In vivo, mechanical strain in blood vessels induces adaptive remodelling which is characterised by cellular proliferation and the synthesis of a wide range of ECM components [32]. Endothelial cells contain several transmembrane and intracellular receptors, which sense and respond to mechanical stimuli via a complex intracellular transduction cascades [33]. Examples of these include plasma membrane phospholipids, tyrosine kinases, platelet endothelial cell adhesion molecule-1, integrins and the endothelial surface glycocalyx. Several second messenger pathways, including mitogen-activated protein (MAP) kinases, protein kinase C as well as phospholipase C and D have been shown to be activated by mechanical stretch [34]. These in turn activate transcription factors and influence gene
expression. Mechanical stimulation of endothelial cells has been shown not only to influence cellular growth and alignment but also to regulate the production of nitric oxide, prostacyclin and calcium, which are crucial in maintaining homeostasis [35]. Kendal et al. documented several cytokines and their transcription factors that were up regulated by mechanical stretching [36]. These findings were replicated by Grinnell et al. who found significant reductions in DNA and ECM synthesis by fibroblasts grown on relaxed collagen matrices [37]. Ingber et al. compared the growth of capillary endothelial cells in static culture and under cytoskeletal tension [12]. The team found that cells under tension responded to soluble growth factors and proliferated faster compared to cells grown in static culture. Cells not under tension displayed a more rounded shape and were in an arrested growth phase. Rubbens et al. showed that dynamic straining of endothelial cells enhanced collagen cross-linking and the quality of the neomatrix produced [38].

The findings of our experiment suggest that subatmospheric pressure in a closed bioreactor exerts a mechanical strain on a seeded vascular graft construct. These strains alter cellular morphology and enhance cellular proliferation. This technology has potential use in vascular graft tissue engineering and may lead to more efficient in vitro endothelialisation. Future studies could look at the effect of subatmospheric preconditioning on different vascular constructs and its effect on ECM production.
4.5 References


Comparing the Endothelialisation of Small diameter Extracellular Matrix Vascular Grafts with Fibrin coated ePTFE grafts.
5.1 Introduction

Small diameter vascular grafts are the most sought after, yet technically challenging arteries to engineer. They operate at low blood flow velocities and have unacceptable failure rates primarily due to thrombosis formation and intimal hyperplasia [1]. To improve patency rates, researchers have attempted to line the graft lumen with an endothelial layer, a technique first described by Herring et al. in 1978 [2]. The endothelium has a central role in minimising platelet adhesion and thus preventing clot formation [3]. There is also evidence that it inhibits intimal hyperplasia via the release of TBF-β and PDGF [4]. The harvested autologous endothelial cells are generally venous in origin however more recently; microvascular endothelial cells [5] and endothelial progenitor cells [6] have been investigated as an alternative source. The use of autologous cells eliminates the chances of an autoimmune response from the host and the potential for cross infection.

Vascular grafts are most commonly endothelialised in a double seeding procedure as outlined in chapter one. Here the cells are allowed to expand in culture for several days or weeks and then seeded on to the graft lumen with or without additional mechanical preconditioning. Although labour intensive, this method allows time for cell expansion, adhesion and acclimatisation to the graft. Subsequent mechanical preconditioning can functionise the cells and prepare them for in vivo implantation. Single stage seeding involves soddng the grafts with a high density of cells just prior to implantation. There has been far less research carried out with this technique and so far clinical results have been disappointing. Herring and Ortenwall carried out the earliest single stage seeding studies using venous endothelial cells and reported encouraging results [7-10]. However subsequent larger studies by Walker [11,12] and Zilla [13] did not replicate these findings and cited poor initial cell
density and weak attachment as possible causes. Despite the increased costs, procedural steps and longer incubation times, double-stage seeding remains the most effective method in achieving adequate cell coverage and a mature well adherent endothelium [14-16]. Zilla et al. [17] and Meinhart [18] have achieved considerable long-term clinical success using this method, reporting increased patency rates approaching that of venous grafts. Meinhart described the technology as a means to “close the gap between vein grafts and synthetic prosthesis” [19]. In these clinical trials, 6 or 7mm ePTFE grafts coated with either fibrinolitically inhibited fibrin glue [20] or plasma [21] were endothelialised.

The seeding of cells onto a construct is a critical step in any tissue engineering application and forms the basis for subsequent remodelling and integration in vivo [22]. Tissue engineered constructs can be bulk seeded, i.e. cells are grown throughout the scaffold or surface seeded; cells are cultured on a particular surface as in our case. A major challenge of this technique is to culture a uniform homogenous confluent layer of endothelial cells inside the graft lumen and to do so in the shortest timeframe possible with minimal injury to the cells. The issues mainly lie with the mechanical forces involved which can be difficult to control and potentially have a detrimental effect on the physical characteristics of the scaffold or induce apoptotic cell death of the residing cells [23,24]. Several different types of bioreactors have been developed to seed tubular scaffolds employing a range of different mechanical forces including sedimentary, rotational, electric field, or vacuous strains [25-28]. Many of these systems are complex, requiring a number of protocol steps thus limiting their application in a clinical setting [29].

The properties sought in a seeding protocol include, the rapid efficient formation of a uniform layer of cells with minimal cell death. The technique should
be cost effective and reproducible with minimal expertise and equipment to operate. Cell-seeding techniques are divided into passive and dynamic seeding. Passive, also known as static seeding is the simplest and most widely employed yet is also the most inefficient with reports of 10-25% seeding efficiency \[30,31\]. It involves seeding cells directly onto a construct and culturing the cells for days or weeks in static culture. The drawbacks of this method include difficulty forming a uniform monolayer of cells on an irregularly shaped construct unless it is continuously moved or seeded for a lengthly period. As well as this, cells take a long time to acclimatise to the material resulting in poor attachment and potential cell loss when implanted in vivo \[30\]. Dynamic seeding employs physical forces to increase seeding efficiency and enhance uniformity of a cell layer. The two main forces employed include hydrostatic forces, most commonly rotatory \[25\] or systems that create pressure gradients such as vacuum seeding devices \[28\]. In rotational seeding systems the cellular scaffold is rotated along with the cells in a culture medium to promote the efficient even distribution of cells. Seeding efficiency using these techniques have been reported as 38 – 90% in various studies \[31,32\].

The aim of this study, was to examine the feasibility of in vitro endothelialisation of 6mm uncoated extracellular matrix vascular grafts which we hypothesis will provide an enhanced substrate for endothelial cells. We are specifically examining cellular morphology, phenotype and proliferation.
5.2 Methods

5.2.1 Decellularised Vascular Grafts

UBM in the form of four layered 6mm diameter 10cm sterilised tubes (Fig. 43a & b) were kindly supplied by Prof. Stephen Badylak, University of Pittsburgh, Pittsburgh, U.S.A. The preparation of UBM has previously been described in chapter two section 2.2.3. Multilayer tubular scaffold materials were prepared by wrapping hydrated sheets of UBM four times around a 6mm perforated tube that was covered with umbilical tape. The UBM graft was then inserted into a plastic pouch and connected to a vacuum pump (Leybold, Export, PA, Model D4B) with a condensate trap inline. The construct was exposed to pressures of 710 to 740 mm Hg for 10 to 12 h to dehydrate the construct forming a tightly wrapped four-layer laminate. The four-layered UBM graft was then terminally sterilized with ethylene oxide.

Fig. 43a. A 6mm ×10 cm UBM four-layer tube.
5.2.2. Synthetic Graft material

Thin-walled ePTFE (W.L. Gore & Associates, Inc., Livingston, Scotland) 6mm diameter grafts were used for each experiment (Fig. 44). Each graft was cut into lengths of 12cm using a sterile technique. These grafts were not coated with any substance; the lumen is purely ePTFE. The polytetrafluoroethylene molecule is neutral, but the fluorine atoms surrounding the carbon chain are electronegative and therefore are effective at attracting available electrons. At the time of seeding, the graft lumen was not charged.
5.2.2 Cell culturing technique

HUVEC cell lines were purchased from Bio Cascade Biologics/Invitrogen, Cat. # C-003-5C. Culturing techniques have already been described in chapter two, section 2.2.6.

5.2.3 Fibrin coating of hybrid-PTFE grafts

One end of a 12cm ePTFE grafts was clamped. The preparation of fibrin glue has already been described in chapter two, section 2.2.4. Human fibrinogen (32.1U/ml) and bovine thrombin (0.32U/ml) were made up in volumes twice that of the grafts. Prior to grossly observing polymerization (6mins 10secs), the fibrin glue preparations were injected into each graft under pressure using a syringe. Two minutes later the excess fibrin was removed using an inflated six Fr. embolectomy catheter (LeMaitre Vascular, Inc.) (Fig. 45). 1cm was cut from each end of the graft,
leaving 10cm grafts for seeding. Prior to the seeding experiments, three random lumen sections from three different grafts were analysed—using SEM imaging to verify the uniformity of the fibrin layer.

5.2.3 Seeding Protocols

We used a simple horizontally rotative seeding device (Fig. 46a & b) to seed a monolayer of endothelial cells on the inner lumen of ECM grafts. The seeding device has already been validated by our lab [33]. It is composed of two nylon rollers mounted on bearings, which are interconnected via a pulley-belt system. The pulleys are powered by a 24V low-speed motor (Radionics Ltd., Ireland) and gearbox. ECM
grafts were firstly submerged and soaked in media to allow the material to soften and optimise cellular attachment. One end of the graft was sealed with a sterile silicone stopper and o-ring. The grafts were then filled with endothelial cell suspension containing $4 \times 10^5$ EC/ml culture medium. The other end of the graft was then sealed. The seeded graft was placed in a sterile 250ml spinning glass tube with culture medium. The spinning flask was placed on top of the seeding device with the top partially open to allow for gaseous exchange. The entire apparatus was then placed inside a 37°C, 95% humidified 5% CO$_2$ incubator and set to rotate at six revolutions per hour (rph) for five hours. Following this the seeded grafts were left submerged in static culture for up to nine days to give cells time to adhere to and adapt to the underlying graft surface. The media in the spinning glass was changed every 48 hours. The seeding protocol was determined according to that described in several previous clinical studies by Zilla et al. [17,19,21].

![Seeding bottle with graft.](image)

**Fig. 46a.** Horizontal Rotative Seeding Device.
5.2.4 Cellular Viability

The cytotoxicity of the constructs to HUVECs was assessed on day two by determining cellular viability using a live/dead viability assay (Invitrogen™). The live/dead assay was outlined in chapter two, section 2.2.8.

5.2.5 Immunofluorescence analysis of von Willebrand Factor

On day two Endothelial cells seeded on each substrate were fixed with 3.7% para formaldehyde in Phosphate buffered saline (PBS). This was then rinsed with PBS and mixed for 20 min with 0.27% NH4Cl/0.38% glycine in PBS and permeabilised with Triton X-100 (0.5%) in PBS. Von Willebrand Factor was confirmed F via
fluorescein-labelled Antibodies-conjugated mouse anti-human vWF antibodies (2 µg/mL$^{-1}$).

5.2.6 Cellular Proliferation

Each graft was rinsed to remove unattached cells prior to cell counts. Cellular proliferation was assessed on days two, four, six and nine using the MTS colorimetric assay as described in chapter two, section 2.2.11.

5.2.6 Scanning Electron Microscopy (SEM)

After five days of static culture mid-graft specimens were analysed using SEM imaging to enable a visual examination of seeding density and morphological maturation. Each construct with HUVECs was primarily fixed with 2.5% glutaraldehyde/paraformaldehyde phosphate buffer solution. After primary fixation, the constructs were post-fixed in 2% Osmium Tetroxide then dehydrated through a graded series of acetones up to 100%. After critical point drying, the specimens were mounted onto aluminium stubs and then coated with gold. Samples were then analysed using the Scanning Electron Microscope (JEOL 5510) at University College Cork.
5.3 Results

5.3.1 Live Dead Assay

The study demonstrated that the fibrin/ePTFE hybrid (Fig. 47a) and the ECM grafts (Fig. 47b) supported the viability of endothelial cells in static culture and after rotational seeding. The seeding methods or seeding device did not appear to be cytotoxic to the cells. Cells proliferated on all scaffolds and remained viable till the end of the study.

Fig. 47a. Live/Dead assay Fibrin/ePTFE day two.
Fig. 47b. Live/Dead assay UBM day two.

5.3.2 Immunofluorescence analysis of von Willebrand Factor

Seeded HUVECs were shown to produce von Willebrand Factor on all materials. Figure 48a & b shows ECs expressing vWF in static culture on both graft materials. This result confirmed that the endothelialisation protocol did not result in changes to the endothelial phenotype and cells continued to function whilst growing on the graft lumen.
5.3.3 Cellular proliferation

Cell numbers were similar on both UBM and Fibrin/ePTFE grafts at day two (p=1.00). Thereafter cells proliferated at a significantly faster rate on UBM grafts at each time point measured (Fig. 47) (p≤0.05). ECs grown on ECM scaffolds achieved confluency the fastest (day six) with a shorter doubling time compared to Fibrin/ePTFE hybrid graft. Final confluent cell numbers on Fibrin/ePTFE and UBM scaffolds were similar (day nine). The number of cells seeded onto Fibrin/ePTFE hybrid and UBM at the outset (4 × 10^5 EC/ml) was enough for cells to reach confluency by the end of the study (day nine).

![Fig. 48a. Cells seeded on Fibrin/ePTFE grafts producing vWF.](image1)

![Fig. 48b. Cells seeded on UBM grafts producing vWF.](image2)

![Cellular Proliferation](chart)

Fig. 49. Proliferation of cells on each material day two, four, six and nine. The mean value of triplicate samples for each film was calculated with the standard deviation, and statistical error was calculated by the Student’s t-test (p≤0.05).
Fig. 50. Live/Dead Immunofluorescence cells grown on 6mm UBM & Fibrin/ePTFE grafts days two, six and nine.
5.5.4 Cellular Morphology

The fibrin layer was analysed on ePTFE following three separate preparations. SEM demonstrated a complete uniform fibrin layer on each specimen with no underlying ePTFE fibres visible (Fig 51 a- c). Following seeding of the grafts cellular morphology was determined using scanning electron microscopy (SEM). Sections from the mid-graft region were analysed on day four. Both Scaffolds demonstrated cobblestone morphology with cells in their flattened stage indicating that cells had acclimatised to the underlying matrix and were well attached (Fig.52 a & b).

Fig. 51a. Fibrin/ePTFE specimen one. Fig.51b. Fibrin/ePTFE specimen two. Fig. 51c. Fibrin/ePTFE specimen three.
5.4 Discussion

Our present study has two main objectives. Firstly to examine the feasibility of creating an endothelial layer on the luminal surface of 6mm acellular extracellular tubularised grafts and secondly to compare them to existing fibrin coated ePTFE grafts. The experiments were set up in order to replicate as much as possible the seeding protocols and materials used in previous clinical trials. Human umbilical venous endothelial cells were selected as they have been employed in the majority of
the preclinical trials and almost all cells used in clinical studies are venous in origin, usually cephalic [34]. More recent research has focused on microvascular cells as an easily accessible and more abundant source of ECs [35,36]. Park et al. described a case involving microvascular endothelialised mesoatrial Dacron graft implanted into a patient with Budd-Chiari syndrome. He found that the endothelial monolayer persisted in the mid graft region at nine months [37]. A larger study by Meerbaum, involving 34 patients with lower limb microvascular endothelialised ePTFE grafts reported poor patency rates [38]. There is currently no consensus as to the optimal endothelial cell type to use in a tissue engineered vascular graft. Venous endothelial cells have proven clinical efficacy in large-scale long-term clinical trials and there is simply not enough data on the newer cell types such as microvascular or EPC to definitively decide which is the optimum. Indeed there are studies that show that the original seeded cells reduce over time to be replaced by host endothelial cells [39]. We cannot be certain whether this is due to cell death or simply detachment due to shear stress forces. Hence the seeded endothelium may act as a temporary endothelium to be gradually replaced by host cells.

The main issue of using venous cells is the limited supply due to the short segments available, generally $0.56 \times 10^5 - 100 \times 10^5$ [40]. We chose seeding densities of $4 \times 10^5$ cells/ml, which was within the range used by Zilla et al. during their clinical study on infrainguinal ePTFE grafts [34]. It is difficult to know what the optimal seeding densities are as these will differ depending on the material being examined and the type of endothelial cells employed. Too low a density will result in delayed proliferation and poor attachment, too high a density will waste cells and limit the cell types suitable for harvesting [41,42]. Optimal seeding density for uncoated ePTFE has been reported as $2 \times 10^5$ /cm$^2$ and $3.5 \times 10^5$ /cm$^2$ for Dacron due
to its higher porosity [43]. The amount of cells generated from venous harvesting result in a low cell density on the graft surface and are not sufficient to enhance patency rates in a single stage seeding process [44]. Here up to 70% of cells are immediately lost upon implantation and continues thereafter up to 24 hours [45]. While venous cells do not appear to be suitable for single stage seeding they are suitable for double stage seeding whereby cells numbers can be expanded to sufficient quantities and given time to properly attach.

The coating of ePTFE with fibrin was carried out according to the protocol outlined by Greisler et al. [46]. Following the protocol the ePTFE graft surface was visualised using SEM and demonstrated a complete uniform fibrin coating on three separate experiments (Fig. 51 a-c). 6mm walled ePTFE grafts were chosen, as these were the same types of grafts used by Zilla et al. during clinical trials. A similar seeded protocol was also employed according to the same study – seeded grafts rotated at 6rph for five hours, followed by static seeding for nine days to allow for cell attachment and maturation.

Cytotoxicity studies on the biomaterials used and the seeding protocol was a fundamental test to determine biocompatibility. Two complimentary assays were used to investigate potential cytotoxicity; live/dead viability assay and the MTS colorimetric assay. These studies confirmed that neither the materials used nor the seeding protocols were injurious to the cells. This was an important finding as damage to the cells during the seeding process would increase the initial cell count required and render the entire protocol less efficacious. The cells continued to produce vWF following endothelialisation, which indicated that they were functioning normally.
SEM imaging allowed direct visual evaluation of the endothelialised graft lumen. The morphology of endothelial cells on Day four is presented in (Fig. 52 a & b). Cells were shown to be well attached on both grafts materials. These cells demonstrated a cobblestone morphology, which would limit cell displacement by shear forces upon implantation. Homogenous cellular covering is critical. Exposed areas of graft material slows de-novo ECM production as cells must migrate into these areas first. There is also a risk of thrombosis and graft failure with incomplete cell coverage. There were some areas of scaffold exposed on the fibrin/ePTFE graft, which could allow for platelet adhesion and thrombosis in vivo. This has always been a concern when using biological glues as a cellular scaffold. Results from chapter two showed that both materials promoted high levels of cellular attachment. ECM scaffolds are known to possess several recognition sites for integrin complexes of cells. For example, collagen type I contained in UBM has two recognized binding sites Arg-Gly-Asp site and Asp-Gly-Glu- Ala [47,48]. This enhanced level of cell retention could reduce the need of initial high density cell seeding and may allow for single stage seeding protocol. Where there is a limited supply of autologous transplant tissue, efficient cellular attachment is critical.

Proliferation of ECs was measured over time using MTS assay (Fig. 49). These results showed that cells grown on UBM lumen grew at a statistically faster rate compared with fibrin coated ePTFE after day two (p≤ 0.05). Cells on UBM grew rapidly up till day six when thereafter growth rate levelled off as the cells reached confluency. Cells seeded on fibrin/ePTFE grew at a slower rate and only reached confluency at day nine. These results are in agreement with similar static studies on UBM sheets and fibrin/coated ePTFE in chapter two, section 2.3.4. The delayed proliferation of cells on fibrin/ePTFE could be explained by the dense matrix formed
by the polymerisation of fibrinogen by thrombin. Several studies have cited that this dense matrix could inhibit the migration and proliferation of cells [49,50]. As previously described UBM contains several components known to accelerate endothelial growth such as laminin and fibronectin and vascular endothelial growth factor [51]. As well as these, heparan sulphate contained in UBM is known to act as a reservoir for cellular growth factors leading to enhanced mitogenic activity upon seeding [52,53]. Faster endothelialisation would be clinically beneficial. Less culture time would lower the risk of bacterial contamination and could raise the possibility that this material could be suitable for single stage seeding.

In this study, human umbilical venous endothelial cells were examined as the major clinical trials have employed endothelial venous cells and several previous in vitro studies have used endothelial venous cells, specifically HUVECs. As seeding protocols advance, easier alternative and more plentiful sources of endothelial cells are being examined such as microvascular endothelial cells from adipose tissue or endothelial progenitor cells. In future studies the seeding of these cell types should also be examined.

In conclusion, we found we could reliably and repeatedly form a confluent, morphologically undisturbed endothelial lining on an extracellular vascular graft, which was the primary goal of the study. The seeding device used was a simple, reliable and safe. ECM grafts were shown to promote endothelialisation more rapidly when compared to fibrin coated ePTFE hybrids using the same seeding protocols. Cellular viability, phenotype and morphology were similar on both materials. Further studies will involve experimentation with differing cell densities and rotational speeds to determine the optimal protocol of endothelialisation of extracellular vascular grafts. Alternative cell types for example microvascular
endothelial cells, which would allow higher density seeding could be examined and may allow single stage seeding. ECM grafts exposed to shear stress will also give a more realistic view of their possible behaviour in vivo.

5.5 References


30. Pawlowski KJ, Rittgers SE, Schmidt SP, Bowlin GL. Endothelial cell


Conclusions and Future Research
The creation of a replacement artery has been the major goal of cardiovascular research for decades and will ultimately be achieved through tissue engineering [1,2]. Replicating the complex physical and biological properties of arteries with materials alone has proved unsatisfactory and the current consensus is that they need to be combined with vascular cells particularly for small diameter grafts. The most common approach is to line vascular grafts with a confluent layer of endothelial cells and this approach has had significant clinical success demonstrating long-term safety and efficacy in humans [3]. However existing synthetic materials have known limitations and are not ideal substrates for tissue engineering. Tissue engineers are continually searching for new materials to enhance biocompatibility and improve patency rates. Over the years it has become apparent that new graft materials must contain certain properties if they are to be successful and a list of design criteria has been drawn up [4-6]. Firstly a potential graft material must have suitable mechanical properties including high burst pressures, acceptable compliance, axial strength, ease of handling and suturability. The materials must be able to resist long-term haemodynamic forces, prevent aneurysm formation and adapt to changing systolic and diastolic pressures. All materials to be implanted in vivo have to be biocompatible. They must not trigger a harmful inflammatory or immune response. They should be non-thrombogenic and resist bacterial colonisation. Other properties include adequate solubility and accommodation of neovascularisation to sustain native cells and promote host cell invasion. Preferably the graft should act as a temporary scaffold that gradually and predictably degrades, being replaced by host cells, which produce their own ECM. To meet all these criteria in a single material is extremely challenging and in part explains why no new material have been accepted.
into wide spread clinical use since the introduction of ePTFE and Dacron in the 1970s.

As discussed in chapter one, ECM materials are at the forefront of biomaterial research and have been successfully used in a diverse range of medical applications [7]. As our understanding of these materials increases, it is becoming more apparent that they possess many properties that would make them ideal for use in vascular tissue engineering. Extensive research has been carried out on ECM materials, particularly SIS and UBM, which we used in our study and the results suggest the scaffolds fulfil many of the design criteria proposed for a novel vascular graft. In terms of mechanical characteristics, ECM has an axial strength greater than that of the canine carotid artery [8]. It has enhanced compliance, which more closely approximates that of an artery when compared to ePTFE or Dacron grafts [8]. The compliance of ECM grafts could reduce intimal hyperplasia and this has been born out in several studies [9,10]. Burst pressure studies on SIS show it to be easily capable of resisting the continuous haemodynamic stresses in vivo [8] and animal studies of up to five years have not shown aneurysmal formation or graft failure [11]. It should also be noted that it is possible to alter the mechanical properties of ECM materials through different methods of processing, sterilization and layering of the material as outlined in chapter one [12-14]. This could prove to be a means whereby the mechanical characteristics of the ECM graft could be tailored to the particular artery it replaces. An interesting property of ECM scaffolds is their ability to adapt and transform into the tissue it replaces. This has been proven in several animal studies [15-18]. In one such study, single layer SIS was implanted into the canine superior vena cava. At explantation SIS had remodelled into complex multilayered venous tissue, indistinguishable from the host vein [15]. This adaptive property of
ECM is known as “constructive remodelling” [7] and could prove of great benefit in vascular tissue engineering.

The use of xenogenic materials will always raise concern regarding eliciting an immune reaction from the host, this could prove particularly disastrous in the vasculature. However, ECM is remarkably homogenous across the species – bovine heart valves and porcine insulin have both been used successfully for decades. As well as this, the materials are rendered acellular, thus eradicating the gal epitope – the major cause of xenogenic graft immune reaction. The immune response to ECM implants has been exhaustively studied and the host’s reaction to the ECM repeatedly demonstrates an accommodative reaction rather than a harmful immune rejection [19]. The extensive use of ECM materials in medicine in a diverse range of specialties without reports of rejection supports their potential use in vascular applications. The ECM materials used in this study were not cross-linked. It has been shown cross-linkage can affect immune response to ECM materials. Indeed cross linkage can also have a negative influence on constructive remodeling. Badylak et al. compared the macrophage response to SIS and SIS cross-linked with carbodiimide in a rat model [49]. The native SIS demonstrated a mononuclear cell response predominantly of CD163+ macrophages. At the end of the study the unaltered SIS had remodeled into native host tissue. In contrast cross-linked SIS showed a predominantly CD80+ presence associated with mononuclear cells and multinucleate giant cells. The cross-linked implant healed with associated fibrosis and chronic inflammation.

As previously described the permeability of the material is an important consideration in vascular constructs. This is needed to accommodate neovascularization and in vivo endothelialisation. SIS has a porosity index of 52
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mL/min.cm², which is greater than that of ePTFE [20]. An interesting finding is that SIS has a dual porosity index; the abluminal layer is 10 times more porous when compared to its opposite side. This could be particularly advantageous in a vascular graft. While the luminal surface does not allow the leakage of blood, the greater porosity of the outer surface allows the ingrowth of capillaries and a means to create a neo-intima [21]. The other important considering in vascular graft design is a materials affinity for platelets in vivo which if high, could lead to thrombosis and graft failure. Although ECM is primarily composed of collagen, which has a high affinity for platelets, it has demonstrated a surprising lack of platelet attachment in several trials. In one study ePTFE, Dacron and SIS were implanted into the carotid and femoral vasculature of a dog. All materials demonstrated similarly low platelet aggregation [22]. Along with its low affinity for platelets, ECM has been shown to contain large quantities of heparin [21], which may potentially add to its antithrombotic properties.

The importance of sterility cannot be over stated with regard to vascular surgery. To date, ECM scaffolds have demonstrated remarkable resistance to infection and in over 250 animal implants; no cases of infection have been reported [23]. ECM vascular grafts performed better than Dacron materials when directly inoculated with bacteria prior to implantation, demonstrating enhanced resistance to colonization [24]. ECM also proved superior at resisting infection when compared to ePTFE vascular grafts following deliberate contamination with Staphylococcus aureus in a dog model [25]. The mechanisms behind this antimicrobial ability are probably multifactorial as outline in chapter one. Briefly, the scaffolds degrade quickly thus limiting attachment sites for pathogens and the time available to colonize the material. ECM scaffolds support rapid neovascularization as stated in chapter one,
which allows for a rapid immune response. Lastly, it has been proven that peptide by-products of ECM degradation are themselves antimicrobial [26].

The above in-vitro studies documenting ECM favourable properties for potential use as a vascular graft have been replicated in several animal trials. Badylak et al. implanted large diameter (10mm) SIS grafts in the aorta of 12 dogs [16]. 11 grafts remained patent up to 52 weeks. No graft displayed evidence of infection, aneurysm formation or intimal hyperplasia. At explantation, histological examination showed a complete endothelium at 28 days. A middle layer of organized collagen fibres and an outer layer composed of smooth muscle cells. This study demonstrated that ECM grafts could remodel into complex multi-layered host tissue, in this case; an artery. The same group examined the ECM graft material following one or two months implanted in the canine aorta [17]. They found the material had transformed and gained the physical and mechanical characteristics of the aortic tissue it had replaced. This is one of the characteristics an ideal graft should possess however no synthetic graft is able to achieve. Badylak et al. also examined SIS grafts in the canine carotid artery and compared them to ePTFE grafts [27]. The study showed that SIS grafts demonstrated superior healing properties with SIS showing a histological appearance similar to the native artery. These results have also been replicated in primate models [7]. The use of ECM scaffolds in venous and valve replacement has also been encouraging [28,29]. Despite the success of ECM grafts in large diameter arteries in animal studies, the results in replacing small diameter arteries have been less successful [18]. Even though constructive remodelling did occur in small diameter ECM grafts, the lower flow rates where thought to contribute to the lower patency rates and the current consensus is that these grafts will require endothelialisation prior to implantation [7].
This thesis examined the endothelialisation of ECM grafts (specifically UBM and SIS) and directly compared them to existing synthetic graft materials ePTFE, Dacron and fibrin-coated ePTFE. In chapter two we examined the static seeding of all five materials. There was a significant increase in cellular attachment to all biologic scaffolds when compared to synthetic grafts ePTFE and Dacron ($p \leq 0.001$) at every time point examined. ECM analogues and fibrin/ePTFE materials proved equally as efficacious at attaching cells at every time point ($p \leq 0.001$) and there was no difference between SIS and UBM ($p \leq 0.001$). The attachment of cells onto ECM scaffolds and fibrin/hybrid were visually shown on SEM analysis. Cell developed a larger, flatter morphology with multi point contact to the substrate. This type of morphology is known to correlate with high levels of attachment and hence minimising cell loss secondary to blood flow [30,31]. The excellent adherent properties of fibrin have been documented in numerous animal [32,33] and human trials [34]. The components of both SIS and UBM have been studied extensively [35-37]. Both Materials have been shown to contain adhesion proteins such as fibronectin, collagen type I and laminin [38]. As well as this, ECM contains growth factors such as FGF-2, TGF-β and VEGF, which may play a synergic role in promoting cellular attachment [39,40]. The enhanced cell adhesion on ECM and fibrin/ePTFE could lessen the amount of cells lost at implantation and reduce the initial cell density required. Future studies evaluating adhesion would involve subjecting the cells to shear stresses. This would give a more information regarding the efficacy of cellular attachment and give an indication of how the cells would behave in vivo. Following this, animal trials involving the placement of small diameter endothelialised ECM grafts would allow for the
examination of the long term functioning of these grafts and constructive remodelling.

Apart from firm adherence, endothelial cells need to be able to proliferate quickly on a particular substrate. This speeds up production time, lessening expense whilst also minimising the risk of infection. It also lowers the initial cell density required and could allow the use of alternative cell sources such as EPCs. Rapid endothelialisation may also make single stage seeding a possibility. In chapter two cell proliferation over nine days was measured and compared on the biological and the synthetic scaffolds. To summarize the results; from day three onward cellular proliferation was significantly faster on biological scaffolds compared to the synthetic counterparts. This was to be expected, particularly following the poor cellular attachment results on ePTFE and Dacron. Of note, UBM and SIS showed no difference in proliferation during the study at any time point measured (p ≤ 0.001). The interesting finding was from day three, both ECM scaffolds out performed fibrin/ePTFE grafts (p ≤ 0.001). ECM materials are known to be rich in substances that are mitogenic to endothelial cells. Growth hormones such as VEGF and functional proteins such as laminin and fibronectin all accelerate endothelial proliferation [41]. More recent research into the composition of UBM has shown it to contain the proteins obscurin, lumican and gelatines, which are known to promote cell adhesion and enhance proliferation [37]. Thus from our results, ECM scaffolds appear to be superior to current fibrin/ePTFE in promoting endothelialisation. In a clinical setting with limited time and finite availability of cells, more efficacious endothelialisation will be an important factor in the ultimate clinical outcome of the graft. An important point to note is that the ECM grafts were uncoated in this study. Fibrin coating adds a step in the production of fibrin/ePTFE
grafts increasing costs. Concerns regarding incomplete coating and embolization of the glue persists [42].

Further study in this area should explore the effect of culturing of endothelial cells on ECM materials. As the cells grow they release matrix metalloproteinases (MMPs), which degrade ECM, while at the same time cells produce their own ECM. It would be interesting to examine the changes in the physical properties of the scaffold during endothelialisation and also if ECM has an affect on MMP production. The co-culture of vascular smooth muscle cells (SMC) within in the scaffold would also be an avenue for further research. SMCs are important in regulating the calibre of the artery wall during the cardiac cycle and also confer compliance to an artery. Niklason et al. seeded SMCs into a polyglycolic acid graft and noted increased compliance approaching that of a native artery [43]. The co-culture of ECs with SMCs on ECM scaffolds may confer increased biocompatibility of the construct.

As research into tissue engineered vascular grafts progresses, more and more emphasis is placed on mechanical preconditioning to improve the functionality of grafts. Indeed it is the current consensus that endothelialised vascular graft will require preconditioning to functionalise the cells so that they will to be able to cope with the physical demands of the haemodynamic forces they are exposed to [44]. The are four principle forces found inside an artery include shear stress, luminal pressure, longitudinal and circumferential stretching of the vessel wall. All of these forces are known to influence the behaviour of vascular cells [45-47] however it is not known which forces or combination of forces are required to optimise the functioning of the endothelium. Studies involving preconditioning of vascular grafts attempt to recreate the in vivo environment as
much as possible employing complex bioreactors, which are prone to contamination and require expert technicians to operate. Specialist laboratories would be needed adding to the difficulty and expense of using them in widespread clinical practice.

We examined a bioreactor for vascular graft materials, which would subject cells to mechanical stretch only using subatmospheric pressures. Chapter three detailed the construction and validation of the bioreactor. Briefly the bioreactor consisted of a three-part biochamber constructed from sterilisable polyetherimide. Subatmospheric pressure is applied to the endothelialised vascular graft material with a VAC pump via a Granufoam dressing. The subatmospheric pressure and the Granufoam VAC dressing create a mechanical stretch on the UBM and populating cells. Following the application subatmospheric pressure, surface markings were visible on the seeded UBM graft, which corresponded to the VAC GranuFoam dressing. This indicated that subatmospheric pressure was being applied and the construct was in contact with the dressing. Cell viability was not affected by the bioreactor any of the subatmospheric pressures tested (75-200mmHg) (p=0.832). Contamination was not observed during any of the experiments. Hence, we constructed and validated a simple yet novel bioreactor for vascular graft materials. It was constructed from available and mostly reusable materials, making it cost effective. The vacuum was created using a VAC unit and dressing. This is already licensed and used worldwide for the treatment of chronic and refractory wounds. The bioreactor is easy to operate requiring minimal training and requires only basic laboratory equipment. It could also be easily adapted for larger scale production using materials of differing lengths and widths.
Chapter four outlines the effect of applying subatmospheric pressures on endothelialised UBM constructs specially looking at morphology and proliferation. Under subatmospheric pressures, cells demonstrated a flatter more cobblestone morphology. This shape is indicative of well-attached healthy proliferative cells capable of resisting shear stress forces found in vivo. Cells grown in static culture for the same period of time had more of a rounded morphology with less contact points to the substrate. Thus it appears that cells exposed to subatmospheric pressures adapted to UBM at a faster rate than those grown at static pressures. Proliferation studies showed that cells at all subatmospheric pressures (75, 125 & 200mmHg) grew at a faster rate at each time point measured. Hence, our results demonstrated that cells subjected to subatmospheric pressures proliferative faster than the control. Future studies could look at longer incubation periods, continuous versus intermittent pressure and it’s effect on other cellular behaviour such as migration and ECM production.

Our final chapter examined the seeding of cells onto 6mm vascular grafts. We compared the endothelialisation of UBM with fibrin-coated ePTFE replicating the materials and methods of endothelialisation as much as possible as those used in clinical trials [48]. Our results mirrored those found whilst culturing cells on graft sheets in chapter two. Both materials demonstrated excellent cell attachment properties with cells attaching strongly and rapidly to each material. SEM imaging demonstrated the typical cobblestone morphology of a healthy well-attached endothelium. ECM scaffolds provided a superior surface for rapid endothelialisation with cells reaching confluency faster. This finding was similar to those obtained seeding sheets of graft material. Additional studies will need to examine the endothelialisation of longer grafts; our experiments only looked at 10cm
grafts. In vivo animal studies will need to be carried out to determine cell retention, long-term graft patency and the effects on the physical properties of endothelialised ECM grafts.

In conclusion, endothelialisation of vascular grafts enhances patency rates significantly. Limiting factors to this technology include prolonged cell culture times and poor cell retention upon implantation. Our experiments have shown ECM biological scaffolds provide a superior substrate for endothelialisation when compared to synthetic grafts and fibrin coated ePTFE grafts. Additional mechanical preconditioning in the form of simple mechanical stretching enhanced further growth. ECM combined with optimal seeding technology and mechanical preconditioning may allow for a single stage seeding and advancement of tissue engineered vascular grafts.

6.1 References


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the resistance to infection of intestinal submucosa arterial autografts versus

Antimicrobial activity associated with extracellular matrices. Tissue Eng


Publications
7.1 International Conference Presentations


### 7.2 National Conference Presentations


7. Coakley DN, Shaikh FM, Callanan A, Kavanagh EG, Burke PE, Grace PA, McGloughlin TM Effects of negative pressure on human dermal fibroblasts


7.3 Poster Presentations

1. Comparing the Endothelialisation of Synthetic and Biological Vascular Grafts under Static and Shear Stress. **Coakley DN.** EUROPEAN SOCIETY FOR VASCULAR SURGERY XXIII ANNUAL MEETING. Oslo, Norway. September 2009.

7.4 Invited Presentations

1. **Coakley DN.** Taking time out to do research: opportunities and pitfalls. 2\textsuperscript{nd} Annual Research Forum. UL Medical School. University of Limerick. January 2009.

7.5 Published Articles


Appendices

Published and Submitted Articles
In vitro evaluation of acellular porcine urinary bladder extracellular matrix – A potential scaffold in tissue engineered skin

Daniel N. Coakley, Faisal M. Shaikha, Kathleen O'Sullivan, Eamon G. Kavanagh, Pierce A. Grace, Tim M. McGloughlin

http://dx.doi.org/10.1016/j.wndm.2015.11.004
Appendix 2

Comparing the endothelialisation of extracellular matrix bioscaffolds with coated synthetic vascular graft materials
D.N. Coakley, F.M. Shaikh, K. O'Sullivan, E.G. Kavanagh, P.A. Grace, S.R. Walsh, T.M. McGloughlin
http://dx.doi.org/10.1016/j.ijsu.2015.11.008
Appendix 3

Irish Journal of Medical Science
March 2010, Volume 179, Issue 1, pp 1-2
Regenerative medicine, tissue engineering and vascular surgery: twenty first century clinical challenges
F. M. Shaikh, D. N. Coakley, M. T. Walsh, T. M. McGloughlin, P. A. Grace
http://dx.doi.org/10.1007/s11845-009-0457-7
Appendix 4

Evaluation of xenogenic extracellular matrices as adjuvant scaffolds for the treatment of stress urinary incontinence

Niall F. Davis, Daniel N. Coakley, Anthony Callanan, Hugh D. Flood & Tim M. McGloughlin

http://dx.doi.org/10.1007/s00192-013-2147-2
Appendix 5