AN INVESTIGATION OF THE EFFECT OF SURFACE FUNCTIONALISATION AND ELECTRICAL STIMULATION ON THE THROMBOGENICITY OF PCL-BASED MATERIALS

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Abstract

Thrombosis in small-diameter synthetic vascular grafts is a recurring issue that does not yet have an effective clinical solution, despite decades of development in anti-thrombotic materials. One of the most widely-accepted reasons for thrombosis in small-diameter constructs is the slow rate of blood flow, which accentuates the importance of material surface chemistry in preventing thrombosis in such synthetic graft designs. Material surface chemistry influences not only the biological processes occurring at the blood-material interface, such as protein adsorption and platelet activation, but it is also purported to influence the behaviour and thrombogenicity of endothelial cells seeded on the material surface. Hence, designing materials to prevent thrombosis requires a multi-angled approach to analyse both the material and cellular aspects of thrombogenicity. In addition, novel approaches to control thrombosis will have to be proposed.

In separate studies, this thesis examines the role of material surface chemistry in mediating cell thrombogenicity and the hypothesised the use of electrical stimulation to regulate thrombosis. PCL substrates were first functionalised for endothelial cell growth. Solvent-cast PCL (scPCL) was successfully functionalised with poly(glycidyl methacrylate) [P(GMA)] polymer brushes using surface-initiated atom transfer radical polymerisation (ATRP). The epoxy functional groups in P(GMA) then facilitated the conjugation of gelatin. The chemical intermediates at every reaction step were verified by Fourier Transform Infrared Spectroscopy (FTIR) and X-ray Photoelectron Spectroscopy (XPS). The amount of gelatin conjugated was found to correlate with increasing ATRP time, indicating a tunable reaction. Increased hydrophilicity and the presence of amine signatures in FTIR and XPS indicated the successful immobilisation of gelatin. Next, in order to develop a conductive vascular graft scaffold, electrospun PCL (ePCL) was coated with polypyrrole (PPy), a conducting polymer, through oxidative template polymerisation. As heparin is a well-established anti-thrombotic agent, it was used as a dopant of PPy. Thus, a novel PPy-heparin-coated ePCL (ePCL/PPyHEP) scaffold was fabricated. XPS and toluidine blue assay confirmed the doping of heparin in the PPy nanocoating.
Abstract
ePCL/PPyHEP scaffolds exhibited better bulk electrical conductivity and electrochemical properties than undoped PPy-coated ePCL (ePCL/PPy) and chloride-doped PPy-coated ePCL (ePCL/PPyCl) scaffolds. An increasing amount of heparin resulted in the increase of bulk conductivity. Finally, the synthesis of PPy-based nanoparticles as a conductive nanofiller on potential graft substrate material was investigated using heparin as a steric stabiliser during dispersion polymerisation. Sizing of the PPy nanoparticles was performed by dynamic light scattering (DLS) and it was found that heparin could be immobilised onto the nanoparticle surface. Heparin-immobilised PPy (PPy-HEP) nanoparticles exhibited better colloidal stability than conventional polyvinyl alcohol (PVA)-stabilised PPy nanoparticles. The PPy-HEP nanoparticles were utilised for the binding of vascular endothelial growth factor (VEGF).

Gelatin-functionalised scPCL substrates supported excellent endothelial cell (EC) coverage, and resulted in downregulated expression of thrombogenic markers von Willebrand Factor (vWF) and matrix metalloproteinase-2 (MMP-2). ECs on gelatin-functionalised scPCL substrates also demonstrated better nitric oxide production than control gelatin-coated coverslips, indicating the effect of other material properties in governing EC phenotype. The ePCL/PPyHEP scaffolds prolonged blood clotting responses when evaluated by human plasma, but promoted platelet activation that as marked by P-selectin upregulation. However, further application of 10 µA, 100 Hz AC sine current in customised cell array chambers reduced platelet activation. The AC current did not significantly affect the adsorption of fibrinogen onto all the ePCL/PPy and ePCL/PPyHEP scaffolds, but significantly reduced the adhesion of macrophages onto the scaffolds. PPy-HEP nanoparticles were demonstrated as conductive nanofillers in an insulative polymer matrix, and when conjugated with VEGF, demonstrated the retention of bioactivity and pro-angiogenic properties of VEGF by the Matrigel assay.

By integrating these multidisciplinary studies together, this thesis has shown the feasibility of designing a multifunctional graft material comprising of ATRP-functionalised PCL substrates dispersed with PPy-HEP nanoparticles that will function as VEGF carriers. Such
a hybrid material will also allow the transmission of an AC stimulation current to control thrombosis.
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### Abbreviations

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<th>Full Form</th>
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<tbody>
<tr>
<td>AC</td>
<td>Alternating Current</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulphate</td>
</tr>
<tr>
<td>APTMS</td>
<td>3-Aminopropyl Trimethoxysilane</td>
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<tr>
<td>ATR</td>
<td>Attenuated Total Reflectance</td>
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<tr>
<td>ATRP</td>
<td>Atom Transfer Radical Polymerisation</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic Acid</td>
</tr>
<tr>
<td>BIBBB</td>
<td>2-Bromo-isobutyryl Bromide</td>
</tr>
<tr>
<td>BrTMOS</td>
<td>2-Bromo-2-Methyl-N-3-((Triethoxysilyl)propyl)Propanamide</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular Disease</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
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<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
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<tr>
<td>ePCL</td>
<td>Electrospun Polycaprolactone</td>
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<tr>
<td>ePCL/PPyCl</td>
<td>Electrospun Polycaprolactone/Chloride-doped Polypyrrole</td>
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<tr>
<td>ePCL/PPyHEP</td>
<td>Electrospun Polycaprolactone/Heparin-doped Polypyrrole</td>
</tr>
<tr>
<td>ePTFE</td>
<td>Expanded Polytetrafluoroethylene</td>
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<tr>
<td>DAF-FM</td>
<td>4-Amino-5-Methylamino-2’,7’-Difluorofluorescein</td>
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<tr>
<td>DAPI</td>
<td>4,6-Diamidino-2’-Phenylindole</td>
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<tr>
<td>DC</td>
<td>Direct Current</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
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<tr>
<td>EC</td>
<td>Endothelial Cell</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<tr>
<td>FeCl₃</td>
<td>Iron (III) Chloride</td>
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<tr>
<td>FDA</td>
<td>Fluorescein Diacetate</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration (US)</td>
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<tr>
<td>FESEM</td>
<td>Field Emission Scanning Electron Microscopy</td>
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<tr>
<td>FGF-2</td>
<td>Fibroblast Growth Factor-2</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared Spectroscopy</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
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<td>GMA</td>
<td>Glycidyl Methacrylate</td>
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Abbreviations

GM-CSF  Granulocyte Macrophage-Colony Stimulating Factor
GPVI  Glycoprotein VI
GPIbα  Glycoprotein Ibα
HFP  1,1,1,3,3,3-Hexafluoro-2-Propanol
HUVEC  Human Umbilical Vein Endothelial Cell
ICAM-1  Intercellular Adhesion Molecule-1
MAPK  Mitogen-Activated Protein Kinase
MMP-2  Matrix Metalloproteinase-2
mRNA  Messenger Ribonucleic Acid
MSC  Mesenchymal Stem Cell
NO  Nitric Oxide
PBMC  Peripheral Blood Mononuclear Cell
PBS  Phosphate Buffered Saline
PCL  Poly-ε-Caprolactone
PCP  Poly(Pyrrole-co-Carboxy-Pyrrole)
PDI  Polydispersity Index
PDMS  Polymethylsiloxane
PEG  Polyethylene Glycol
PET  Polyethylene Terephthalate
PGA  Polyglycolic Acid
P(GMA)  Poly(Glycidyl Methacrylate)
PI3K  Phosphoinositide 3-Kinase
PLA  Polylactic Acid
PLCL  Poly(L-lactic-co-ε-Caprolactone)
PLGA  Poly(Lactic-co-Glycolic Acid)
PLRP  Platelet- and Leukocyte-Rich Plasma
PPy  Polypyrrole
PPy-HEP  Polypyrrole-Heparin
PPP  Platelet Poor Plasma
PRP  Platelet Rich Plasma
PRT  Plasma Recalcification Time
PSGL-1  P-Selectin Glycoprotein Ligand-1
PVA  Polyvinyl Alcohol
<table>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>scPCL</td>
<td>Solvent-casted Poly-ε-Caprolactone</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
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<tr>
<td>TEGV</td>
<td>Tissue-Engineered Vascular Grafts</td>
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<tr>
<td>tPA</td>
<td>Tissue Plasminogen Activator</td>
</tr>
<tr>
<td>TCP</td>
<td>Tissue Culture Plastic</td>
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<tr>
<td>TEA</td>
<td>Triethylamine</td>
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<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue Factor</td>
</tr>
<tr>
<td>TFPI</td>
<td>Tissue Factor Pathway Inhibitor</td>
</tr>
<tr>
<td>TM</td>
<td>Thrombomodulin</td>
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<tr>
<td>vWF</td>
<td>von Willebrand Factor</td>
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<tr>
<td>VCAM-1</td>
<td>Vascular Cell Adhesion Molecule-1</td>
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<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<tr>
<td>VEGFR</td>
<td>Vascular Endothelial Growth Factor Receptor</td>
</tr>
<tr>
<td>WCA</td>
<td>Water Contact Angle</td>
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<td>XPS</td>
<td>X-ray Photoelectron Spectroscopy</td>
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Chapter 1

Introduction

This chapter consists of the problem statement, the main hypothesis and sub-hypotheses for the research work. The objectives and scope of the research are also described, followed by novel findings and an overview of the dissertation. The rising incidence of cardiovascular disease necessitates the development of synthetic vascular grafts, but small diameter synthetic grafts have limited patency due to thrombogenicity. The first sub-hypothesis addressed the improvement of endothelialising synthetic material surfaces using a materials-mediated approach. The second sub-hypothesis was the use of a novel electrical stimulation method to control material thrombosis, enabled by development of a bioactive and conductive nanofiller. The third sub-hypothesis was on the novel use of heparin as a stabiliser in the synthesis of polypyrrole nanoparticles. This thesis elucidated the anti-thrombotic mechanisms of using gelatin-functionalised surfaces, and showed for the first time, decrease in platelet activity on polypyrrole-coated surfaces under AC stimulation. Heparin-immobilised nanoparticles successfully acted as both a conductive nanofiller, as well as an endothelialisation promoter.
1.1 Problem Statement and Hypotheses

Cardiovascular disease (CVD) is the leading cause of death globally with approximately 17.5 million deaths per year, and representing 31% of all global deaths [1]. Approximately 80% of all CVD deaths occur in low- and middle-income countries (defined as having an annual gross national income per capita of less than USD 10,066) [2]. In Singapore, CVD was attributed to 29.9% of all deaths in 2014 [3]. Out of the 17.5 million deaths attributable to CVD annually, 7.4 million deaths were due to ischaemic/coronary heart disease [1]. Coronary heart disease is the largest single cause of death in developed countries, and the biggest contributor to CVD burden in developing countries [1]. Coronary heart disease is almost always due to the atherosclerosis, which is the build-up of a plaque that results in stenosis (partial blockage or partial occlusion) and thrombosis of the coronary artery of the heart. Atherosclerotic plaque formation extends to peripheral (non-coronary) blood vessels, and the typical procedure to restore blood flow is the excision of the damaged vascular tissues, followed by the reconstruction of the vascular tissue using either autologous (native) or synthetic vascular grafts. As such, 600,000 vascular grafts are implanted annually worldwide [4]. As not every patient has readily-available disease-free autologous vascular tissue for grafting, ePTFE or Dacron® grafts can be used for repair of large vessels e.g. aorta, iliac artery. However, for small diameter vessels, which include the coronary artery, development of synthetic grafts has been difficult, mainly due to high risk of thrombosis for small diameter synthetic grafts (< 6 mm) [5]. One area of research in vascular grafts is focused on using biodegradable polymers such as polycaprolactone (PCL), with the view that biodegradable grafts are able to undergo in vivo remodelling after implantation to facilitate tissue integration [6]. The disappearance of the synthetic material after vascular tissue regeneration also minimises any potential long-term complications induced by the synthetic material. However, the various approaches undertaken to functionalise the surface of synthetic graft materials (including PCL) with anti-thrombotic agents or endothelial cell coverage have not shown clinical success for small diameter graft. Hence, several novel materials-mediated hypotheses to control thrombogenicity will be proposed and tested in this thesis.
Overarching hypothesis: It is hypothesised that the thrombogenicity of polycaprolactone (PCL)-based films and fibres can be reduced by endothelial cell (EC) coverage, using electrical stimulation to prevent platelet attachment and activation, or through the delivery of an angiogenic factor to improve endothelialisation. Each sub-hypothesis is detailed in the following paragraphs:

1) Gelatin attachment to PCL surface will be evaluated for improving EC coverage and reducing EC thrombogenicity \textit{ex vivo} to transplantation as a small-diameter graft.

One strategy that has been utilised widely to overcome the limitation of small-diameter synthetic vascular grafts involves endowing material surfaces with bioactive molecules via chemical functionalisation to improve endothelialisation of luminal graft surfaces [7]–[9]. The endothelialised material surface is an attractive strategy as a confluent endothelial monolayer is thought to prevent contact activation. ECs seeded on synthetic graft surfaces are increasingly recognised to be taking active roles in the regulation of thrombogenicity by influencing thrombotic pathways and inflammatory responses [10]. Yet, relatively few studies have examined the thrombotic phenotype of endothelial cells on biomaterials. In engineering a synthetic material for endothelialisation, an atom transfer radical polymerisation (ATRP)-based approach was used to functionalise a layer of bio-adhesive gelatin on the polycaprolactone (PCL), a biodegradable polyester. Endothelial coverage and its subsequent thrombogenicity on the functionalised PCL will be evaluated.

2) Fabrication of an electrically-conductive surface followed by the effect of electrical stimulation on thrombosis; such an approach will be used \textit{in situ} for small-diameter grafts.

In the context of material thrombogenicity, many studies have demonstrated the tailoring of biomaterial surfaces to reduce platelet attachment and activation [11], [12], or to prevent activation of thrombosis initiator proteins e.g. fibrinogen [13]. While the endothelialisation of synthetic grafts has demonstrated observable clinical benefits, one disadvantage of using endothelium-lined synthetic grafts is the long lead time in endothelial cell harvesting, expansion in culture and on graft surface, and the requirement for laboratory facilities [7], [9]. Thus the feasibility of using non-cellular
approaches, such as the use of physical electrical stimuli, was also evaluated in this thesis. While it is known that electrical fields can alter cell membrane potentials and subsequently influence intracellular signalling pathways [14], [15], the effects of electrical signals on other biological processes and blood components involved in thrombogenesis have not been explored. It is hypothesised that electrical stimuli applied through the conductive surface can reduce thrombosis by interfering with the activation of the platelets on the material surface. For electroactivity, polypyrrole (PPy) is selected as the conductive material to develop a novel heparin-doped PPy coating over PCL scaffolds.

3) The synthesis of VEGF-conjugated PPy nanoparticles will be explored to enhance endothelialisation rates in situ; such nanoparticles can be dispersed into an erodible PCL matrix to enhance electrical properties and for delivery of VEGF.

Paclitaxel-eluting grafts made of PCL has been studied in animal models, in which sustained release of the drug embedded in polymer matrix was enabled by the slow degradation of PCL over time [16]. As PPy, like most conducting polymers, is non-degradable, one proposed strategy to impart conductivity to PCL would be to use PPy nanoparticles as a conductive nanofiller dispersed within the PCL matrix. The synthesis of PPy nanoparticles can proceed by using heparin as a novel stabiliser in dispersion polymerisation. The synthesised PPy nanoparticles are hypothesised to be immobilised with heparin, and thus functions as a conductive nanofiller, as well as a nanocarrier for tethering VEGF.

1.2 Objective and Scope

The main objective of the thesis is to test the hypothesis that the thrombogenicity of PCL-based materials can be addressed through surface functionalisation with gelatin, electrical stimulation, and VEGF-conjugated nanocarriers. More specifically, each independent study undertaken in this thesis will expound the thrombogenic phenotype of the ECs on gelatin-functionalised PCL substrate, develop a PPy-coated PCL fibrous scaffold with haemocompatible properties to test the hypothesis that electrical stimulation can downregulate thrombotic responses and, to attempt the fabrication of PPy nanoparticles using heparin as a stabiliser. Additionally, these broad studies are
aimed at improving our understanding of the multiple players in thrombosis, from cellular behaviour to material properties and the effects of external physical stimuli. There have been relatively few studies of how materials and material surface chemistry can influence the thrombogenic status of ECs at the fundamental level, despite the wide acknowledgement that an endothelium on the surface of the graft is generally beneficial. Only with a more thorough understanding of how material, ECs, and blood components (platelets, clotting factors, leukocytes) interact with one another, then can better vascular grafts be designed (Figure 1.1). Thus, this thesis seeks to elucidate the novel understanding of thrombosis, and the feasibility of using these novel techniques to modulate thrombosis.

**Figure 1.1.** Venn diagram showing the interdependence of material properties, endothelial cells and blood components in regulating thrombosis.

The aims and scope of this thesis are as follows:
1. Develop gelatin-functionalised PCL substrates to evaluate EC proliferation and thrombogenicity.

Solvent-cast PCL substrates can be functionalised with gelatin via a dense layer of reactive polymer brushes through a surface-initiated polymerisation technique, followed by characterisation of the material properties of the gelatin-immobilised PCL substrates. Cellular and molecular studies will be conducted to evaluate EC proliferation and thrombogenicity. The surface functionalisation technique used will be limited to atom transfer radical polymerisation (ATRP) on PCL, and other biodegradable materials, direct grafting or surface modification methods will not be attempted. The scope of the studies conducted to evaluate EC thrombogenicity will not be exhaustive and be restricted to markers with important roles in the thrombogenicity.

2. Fabrication of conductive PPy-coated PCL scaffolds to evaluate the effects of electrical stimulation on thrombosis.

Template polymerisation of PPy on electrospun PCL fibrous scaffolds will first be performed using heparin as a dopant. The use of heparin dopant will be compared with another commonly-used anionic dopant. The successful dopant of heparin will be verified and the electrical properties of the PPy-coated PCL scaffolds are to be evaluated. Haemocompatibility studies will subsequently be performed, and in order to assess the thrombogenicity of the materials under electrical stimulation, platelet adhesion/activation and leukocyte adhesion onto the scaffolds will be evaluated. The electrical stimulation experiments will be performed in customised stimulation chambers, and restricted to one single low intensity AC waveform chosen for its advantages and demonstrated effects in the literature. DC stimulation and high frequency waveforms are not within the scope of this thesis.

3. To utilise heparin-stabilised PPy nanoparticles as conductive nanofillers and for the delivery of VEGF to improve endothelialisation.

The synthesis of PPy nanoparticles will be attempted using heparin as a steric stabiliser, and the fabricated nanoparticles will be characterised by sizing measurements. To test the hypothesis that heparin will be incorporated directly on the nanoparticle surface,
studies will be performed to validate the presence of heparin. The uptake efficiency and clearance of spherical nanoparticles of up to 100 nm by macrophages generally increases with size [17]. Hence, this thesis set out to achieve the fabrication of PPy nanoparticles with sizes of approximately 100 nm. The feasibility of using the nanoparticles as conductive nanofillers within a non-conductive polymeric matrix will be assessed, followed by the conjugation of VEGF onto the heparin-stabilised PPy nanoparticles and studies on VEGF angiogenic bioactivity. The bioactivity studies will be limited to cellular models of angiogenesis, while animal models are beyond the scope of this thesis.

1.3 Dissertation Overview

The thesis addresses the studies in 7 chapters:

Chapter 1 provides the rationale for developing materials to investigate cellular-mediated thrombogenicity and the need for new approaches to tackle thrombosis. The objective and scope of the thesis are also outlined.

Chapter 2 reviews the literature in the context of the thesis. The current state and challenges of vascular grafts, fabrication and surface modification process, biological and material regulation of thrombosis, conducting polymers, and the delivery of VEGF using biodegradable polymers are reviewed.

Chapter 3 describes the research methodology and experimental procedures, and also discusses the principles underlying the techniques employed in the studies.

Chapter 4 elaborates on fabrication, development and characterisation of all the bulk-and nano-materials utilised in the studies. This chapter is divided into 3 results subsections that each address a specific aim outlined in the introduction of the chapter.

Chapter 5 elaborates on endothelial cell behaviour on solvent-casted PCL substrates. Cell proliferation and expression of thrombogenic markers are reported and discussed.
Chapter 6 elaborates on using PPy-based materials for investigating the effects of heparin-doping and electrical stimulation on material thrombogenicity. The synthesis of PPy nanoparticles and evaluation of their suitability as conductive nanofillers and bioactivity of VEGF-conjugated PPy nanoparticles are reported and discussed.

Chapter 7 concludes the thesis and provides the outline and reconnaissance studies on future research continuing from the work accomplished in the thesis. The implications of the research are discussed.

1.4 Findings and Outcomes

The research carried out during the course of this thesis has led to several novel outcomes, including:

1. Functionalising solvent-cast PCL (scPCL) substrates with gelatin through surface-initiated atom transfer radical polymerisation (ATRP) of poly(glycidyl methacrylate) (PGMA) brushes and demonstrating the influence of materials surface chemistry on EC thrombogenicity. The scPCL-g-P(GMA)-c-gelatin substrates promoted the growth of a confluent EC monolayer, and resulted in less thrombotic activation of ECs. Interestingly, in spite of slightly better EC growth observed on comparable controls, ECs on gelatin-functionalised PCL substrates exhibited higher production of nitric oxide, indicating better overall anti-thrombotic regulation.

2. Achieving template polymerisation of PPy over electrospun PCL fibres (ePCL) with heparin doping. Anti-coagulative properties of heparin-PPy coated PCL scaffolds were demonstrated, and the scaffolds exhibited bulk conductivity comparable with conventional chloride-doped PPy coating, leading to a proposed model of heparin-templated PPy polymerisation on the PCL fibres.

3. Demonstrating that low intensity 100 Hz sinusoidal AC currents decreased the adhesion and activation of platelets, and decreased the adhesion of leukocytes on the scaffolds.

4. Discovering that using heparin as a stabiliser for dispersion polymerisation during PPy nanoparticle synthesis resulted in single-step immobilisation of heparin chains.
onto the nanoparticle surface, and stabilised the nanoparticle colloidal suspension in physiological solution. Synthesised nanoparticles imparted conductivity when dispersed into a non-conductive polymeric matrix.

5. Utilising the heparin immobilised on the PPy nanoparticle surface for VEGF conjugation and demonstrating the bioactivity of VEGF-conjugated PPy nanoparticles in an in vitro angiogenic assay.

References


Chapter 2

Literature Review

This chapter describes the literature relevant to the work conducted in the thesis. It provides the background and current knowledge regarding the materials development and hypotheses that are explored. Overall, this chapter is a review on the current state and challenges of vascular grafts, and the biological mechanisms of the thrombogenic processes. The understanding of the biology of thrombosis has aided in the selection of heparin-based materials for the work, and use of vascular endothelial growth factor to promote graft endothelialisation. The electrospinning fabrication method, surface-initiated ATRP process and the use of conducting polymers and electrical stimulation in tissue engineering are described. A review of clinical data suggested that thrombosis can still occur in spite of full endothelial coverage on the synthetic graft surface, and highlights the lack of understanding of the role materials surface chemistry can possibly play in the regulation of endothelial cell thrombotic states. The use of AC stimulation has achieved some successes in a few biological studies. Though its effects are not understood at the fundamental level, AC stimulation has not been attempted for the control of thrombosis.
2.1 Vascular Graft Materials


Despite decades of research and development of synthetic materials for vascular grafts, autologous vessels remain the gold standard for vascular tissue replacement. Only 2 classes of synthetic materials have been approved in synthetic vascular grafts, namely expanded polytetrafluoroethylene (ePTFE) and polyethylene terephthalate (PET or Dacron®) [1]. This is largely in part due to poor clinical performance and high thrombosis rates for synthetic materials, including ePTFE and Dacron®, in small-diameter grafts less than 6 mm in diameter [2]. Hence, although there is an unmet clinical need, to date, no small-diameter synthetic graft is available for use. Current approved vascular grafts intended for vessel replacement in arterial disease, haemodialysis access (e.g. arteriovenous graft) or vascular prosthetic devices either have bare polymer surfaces or heparin-modified surfaces (Table 2.1).

The evolution of modern vascular grafts can be traced back to the pioneering work of DeBakey on Dacron® grafts in 1955 [12]. Grafts made of other materials at that time e.g. nylon were prone to leakage and encountered progressive but rapid loss of tensile strength after implantation. By the 1960s, Dacron® grafts had widespread use, owing their success to tightly woven fibre architecture for aortic replacements (encountering high blood pressure) and porous knitted grafts for lower extremity bypass (encountering slow-flowing, more thrombotic environments) [12]. The first ePTFE graft was developed by Robert Gore in 1969, and had its first success in man by Campbell et al in 1976 [13]. Apart from incremental advances made in the fibre microarchitecture to improve haemocompatibility, and in suturing and sealing, significant advances have not been made. As seen in Table 2.1, the only bioactive modification used on approved vascular graft surfaces is heparin, a very well-established anticoagulant that has been in use for decades for treatment of thrombosis [14]. Heparin modification could proceed through a single(end)-point attachment of specific chemical moieties onto graft surfaces, multi-point attachment through amine/carboxyl cross-linking or ionic interactions (Table 2.1).
Table 2.1. Examples of vascular grafts in clinical development [3]–[11]

<table>
<thead>
<tr>
<th>Product name</th>
<th>Company/ Authors</th>
<th>Material</th>
<th>Bioactive Modification</th>
<th>Condition</th>
<th>Stage of Development</th>
</tr>
</thead>
<tbody>
<tr>
<td>GORE® Propaten® (with Carmeda® Bioactive Surface)</td>
<td>Gore Medical/ Medtronic</td>
<td>ePTFE</td>
<td>Heparin (Endpoint attachment)</td>
<td>PAD (BK) AVG</td>
<td>Approved Clinical Phase 4</td>
</tr>
<tr>
<td>GORE-TEX® Stretch</td>
<td>Gore Medical</td>
<td>ePTFE</td>
<td>-</td>
<td>AVG</td>
<td>Approved</td>
</tr>
<tr>
<td>GORE VIABAHN Endoprosthesis (with Carmeda® Bioactive Surface)</td>
<td>Gore Medical/ Medtronic</td>
<td>ePTFE</td>
<td>Heparin (Endpoint attachment)</td>
<td>PAD (BK) AVG</td>
<td>Approved</td>
</tr>
<tr>
<td>Advanta™ SST</td>
<td>Atrium</td>
<td>ePTFE</td>
<td>-</td>
<td>AVG</td>
<td>Approved</td>
</tr>
<tr>
<td>Vaslutek® Gelweave</td>
<td>Terumo</td>
<td>Dacron®</td>
<td>-</td>
<td>Aorta</td>
<td>Approved</td>
</tr>
<tr>
<td>Vaslutek® SealPTFE</td>
<td>Terumo</td>
<td>ePTFE</td>
<td>-</td>
<td>AVG</td>
<td>Approved</td>
</tr>
<tr>
<td>FlowLine Bipore</td>
<td>Jotec</td>
<td>ePTFE</td>
<td>Heparin (covalent and ionic)</td>
<td>PAD</td>
<td>European CE-approved only</td>
</tr>
<tr>
<td>GORE-TEX/Mediflex</td>
<td>Dohmen et al (2003)</td>
<td>4 mm ePTFE</td>
<td>Autologous ECs with fibrin glue</td>
<td>CAB</td>
<td>Success in pilot study (14 patients)</td>
</tr>
<tr>
<td>-</td>
<td>Deutsch et al (2009)</td>
<td>6 – 7 mm ePTFE</td>
<td>Autologous ECs with fibrin glue</td>
<td>PAD</td>
<td>High patency rates in 318 patients</td>
</tr>
<tr>
<td>-</td>
<td>Zhou et al (2013)</td>
<td>ePCL/ chitosan</td>
<td>Autologous ECs</td>
<td>-</td>
<td>Superior patency over unseeded grafts in canine model</td>
</tr>
<tr>
<td>Humacyte</td>
<td>PGA/ Decellularised ECM</td>
<td>Autologous ECs</td>
<td>AVG</td>
<td>PAD</td>
<td>Clinical phase 2 Clinical phase 1</td>
</tr>
</tbody>
</table>

AVG: Arteriovenous graft; HA: Haemodialysis access; PAD (BK): Peripheral arterial disease (below knee); PAD (SFA): Superior femoral artery; CAB: Coronary artery bypass; ECs: Endothelial cells

Since then, synthetic tissue-engineered vascular grafts (TEGVs) have been proposed for small-diameter vascular grafts, as it generally believed that the seeding of cells on the grafts will help to reduce thrombogenicity of the material by preventing the contact of the blood with the synthetic material (Table 2.1). Synthetic grafts in development today, unlike ePTFE or Dacron®, are bioresorbable polyesters, designed to degrade in the body after healing is complete. Polyesters under intensive research in this area are polyglycolic acid (PGA), poly-ε-caprolactone (PCL), polylactic acid (PLA), poly(L-
lactic-co-ε-caprolactone (PLCL), and polyhydroxybutyrate (PHB) [15]. These synthetic materials vary in mechanical properties (Table 2.2). Although they have advantages over biological scaffolds of natural extracellular matrix (ECM) origin in reproducibility, ease of manufacturing and less immunogenicity, they cannot fully mimic the native ECM and biological function of vascular tissues.

In 2001, an important milestone was reached in the field of TEGVs when Toshiharu Shin’oka performed the first human trial in history, using a polymeric scaffold of PLCL (50:50) reinforced over woven PGA fibres to improve the poor compliance match with vascular tissue and poor handling of PGA meshes [16]. The graft, designed to degrade within 8 weeks, was seeded with cells for 10 days after the cells were derived from the patient’s vein explant after a lengthy cell expansion period of 8 weeks. Nevertheless, the trial was successful with no complications after 7 months.

Table 2.2. Physical properties of polymers used in synthetic vascular graft research [15]

<table>
<thead>
<tr>
<th>Synthetic polymer</th>
<th>Elastic modulus (GPa)</th>
<th>Tensile strength (MPa)</th>
<th>Degradation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGA</td>
<td>8.4</td>
<td>890</td>
<td>2-3 weeks</td>
</tr>
<tr>
<td>PLA</td>
<td>8.5</td>
<td>900</td>
<td>6-12 months</td>
</tr>
<tr>
<td>PHB</td>
<td>-</td>
<td>43</td>
<td>&gt;6 weeks</td>
</tr>
<tr>
<td>PCL</td>
<td>0.3</td>
<td>50</td>
<td>&gt;12 months</td>
</tr>
<tr>
<td>PLCL (75:25)</td>
<td>4.8</td>
<td>500</td>
<td>8-10 weeks</td>
</tr>
<tr>
<td>PLCL (50:50)</td>
<td>0.9</td>
<td>12</td>
<td>4-6 weeks</td>
</tr>
</tbody>
</table>

Compared to synthetic materials, biological scaffolds are relatively new, and comprise either of decellularised xenograft tissues, or ECM components e.g. collagen or elastin. Decellularised tissues such as the small intestine submucosa are stripped of cellular components and maintain the biocompatibility and mechanical properties of native vascular tissues [17]. However, the main sources of decellularised tissues are xenogeneic in nature. No human trials have been performed on biological TEGVs yet, but such tissues have high immunogenicity and can elicit strong inflammatory responses and structural failure, as seen in human trials on tissue-engineered porcine heart valves [18].
Among the different TEGVs used for small diameter grafts, the implantation of cell-seeded scaffolds without the use of decellularisation and bioreactors shortens the procedure and lowers the costs related to the use of the bioreactor. To eliminate the need for cell seeding in cell expansion in culture, recent studies have been focused on off-the-shelf acellular scaffolds, simplifying the fabrication process and reducing the time and cost needed. These vascular grafts would require bioactive modifications to prevent thrombosis, and the to induce in vivo endothelialisation for the healing process.

2.1.2. Challenge for Vascular Grafts: Thrombosis

Despite having the potential to overcome existing challenges posed by autologous vascular tissue grafts (i.e. the lack of available vasculature tissues for harvest or demand shortfall), approval of polymeric synthetic grafts for clinical use has been hampered by the poor haemocompatibility of the materials, especially for small-diameter vascular grafts (< 6 mm). Preventing thrombosis and matching the mechanical properties of native vascular tissues are both imperative for ensuring long-term patency of the graft. The main failures of ePTFE grafts are related to neointimal hyperplasia (thickening of intima layer on the graft due to hyperproliferation of cells), calcification (accumulation of calcium deposits), thrombosis (clot formation), and foreign body reaction (immune reaction triggered by foreign implants) [19]. These complications are mainly triggered by events happening at the blood-material interface, mainly the protein adsorption on the material surface, followed by subsequent platelet attachment and activation [20], [21]. Thrombosis can be the cause of early (within 30 days after implantation) and midterm (3 months to 2 years after implantation) graft failures [19]. While larger endothelialised ePTFE grafts (diameter = 6-7 mm) have demonstrated some success in clinical trials for infrainguinal bypass, in which the presence of an endothelium 2-4 years after surgery, and 5-year patency rate of 68% for 6-mm grafts and 4-year patency rate of 83.7% for 7-mm grafts were observed [10], [22], [23], no long-term clinical study has been conducted on small-diameter TEVGs (Table 2.1). However, an ongoing study is proceeding on decellularised small-diameter TEVGs from Humacyte Inc after favourable results in large animal trials [24]. This graft requires a multi-step manufacturing process that utilises first the seeding of smooth muscle cells on a PGA scaffold to produce ECM. The grafts are subsequently decellularised and seeded with autologous endothelial cells (ECs) to prevent thrombosis.
2.1.3. Electrospun Polycaprolactone as a Potential Vascular Graft Material

Polycaprolactone (PCL) is a semi-crystalline polyester that has been approved for extensive biomedical uses due to its flexibility, and long-term degradation in vivo (Figure 2.2) [26]. PCL has found usage in bioresorbable surgical sutures, and it is currently undergoing clinical trials for load-bearing implants e.g. hip or bone prosthesis [27]–[29]. PCL has inherently poor affinity for most cell types, including ECs, due to its hydrophobicity. Therefore, research has focused on developing strategies to improve the cyto-compatibility of PCL by grafting on adhesion-promoting bioactive macromolecules through aminolysis [30], hydrolysis [31], [32], plasma treatment [33], or photo-induced grafting methods [34]. Subject to its initial molecular weight, PCL exhibits long term stability in vivo with a degradation period of up to 2 – 4 years [26]. PCL can also be modified to achieve faster degradation if needed, such as for drug delivery [35]. As a material already approved for use in certain medical devices by the U.S. Food and Drug Administration (FDA), and keeping in view of its long degradation time, PCL offers an attractive option as a scaffolding material for vascular grafts that may require a long endothelialisation period in vivo. The use of PCL for any TEVGs will necessitate surface modifications or the development of novel strategies to make PCL the material EC compatible and anti-thrombotic.
Since its re-emergence in polymer fibre production, electrospinning has surfaced as a simple and cost-effective technique to produce biomimetic nanofibrous structures for scaffold fabrication [28]. Electrospinning is a bottom-up fabrication technique that has found numerous biomedical applications in the preparation of both micro-scale and nano-scale polymer fibers [37]–[40]. In electrospinning the application of an electrical potential on the polymer solution generates electrostatic forces of the same polarity (usually positive charge) that causes mutual repulsion between polymer molecules in solution. The polymer solution was fed in using a conventional syringe and needle with a controlled flow rate. When the applied electric field is sufficiently large enough, a steady polymer jet stream in the form of a Taylor cone will be emitted from the needle tip towards a collector plate (that may be negatively charged to attract the polymer stream) (see Figure 2.3 for illustration). Factors such as the 1) type of solvent used, 2) solution concentration, 3) solution conductivity, and 4) solution viscosity will affect the formation and stability of the polymer jet formed [40], [41]. Ultimately, in order for jet formation to occur, the repulsive forces generated in the polymer solution have to be sufficiently large enough to overcome opposing forces such as the solution viscosity and the surface tension at the solution-air interface at the needle tip [41]. Solvent evaporation occurs while the polymer stream travels through the air, and the remaining polymer fibres are deposited on the collector.

Figure 2.2. Chemical structure of polycaprolactone
Formation of the polymer jet is initiated when applied positive charges concentrate at the tip of the solution, and when charge density at the critical voltage is able to overcome surface tension, the polymer pendant drop transits into a polymer jet.

Under optimized conditions for solvent choices, solution concentration, spinning distance and voltage, electrospinning of PCL has obtained fibres of the orders of 100 nm in diameter [26]. The controllable parameters of electrospinning have made the tuning of PCL nanofiber diameter and porosity of the fibre mesh possible [43]. A few groups have attempted to spin 3-dimensional scaffolds with large pore architecture for cell infiltration using a variety of methods, such using a solvent bath collector, or leaching of salt particles introduced during the spinning [44], [45]. Electrospinning is also a technique which can be scaled up quickly for mass production, making it an economical platform for production of PCL fibrous scaffolds which mimic the native nanofibrous extracellular matrix environment in tissues. Currently, while no electrospun PCL vascular devices exist on the market, an electrospun polyurethane vascular access graft, AVflo™, is available from Nicast.

2.1.4. Atom Transfer Radical Polymerisation (ATRP)

ATRP is a controlled radical polymerisation technique developed by the Matyjaszewski group at Carnegie Mellon in 1995 [46]. This polymerisation technique allows for the
addition of monomer subunits onto the radical terminal end of a growing polymer chain using readily-available alkyl halide initiators and copper-based catalysts. Being a radical process, ATRP can be applied for the synthesis of a wide range of chemical functional groups to create well-defined uniform polymers with low polydispersity [47]. The general mechanism of ATRP is presented in the schematic (Figure 2.4). A typical ATRP reaction involves first an alkyl halide initiator $\text{R-X}$ ($X=\text{Cl}$ or $\text{Br}$) reacting with a transition metal catalyst $\text{Mt}^{n}$ (e.g. Cu, Fe) complexed with a ligand $\text{L}$ to generate an oxidized transition metal-halide complex $\text{X-Mt}^{n+1}$ and the alkyl radical $\text{R}^\cdot$. The radical attacks available monomer $\text{M}$, transferring the radical to the alkyl-monomer complex end ($\text{R-M}^\cdot$). The radical complex is rapidly deactivated by reaction with the oxidized transition metal-halide complex to regenerate the original lower state catalyst $\text{Mt}^{n}$ and the oligomeric chain $\text{R-M-X}$. Chain propagation proceeds with the repetition of this sequence, until the monomer is depleted (Figure 2.4).

![Figure 2.4. Reaction schematic adapted from Matyjaszewski Polymer Group webpage at http://www.cmu.edu/maty/atrp-how/procedures-for-initiation-of-ATRP/normal-atrp.html.](image)

One strategy in the bio-functionalisation of biomaterial surfaces relies on the growth of intermediate polymer brushes with reactive ends to graft on bioactive molecules. Polymer brushes with reactive functional groups, such as methacrylic acid (MAA), have been used to create activation sites for subsequent grafting of bioactive molecules [48]. Surface grafting methods have the flexibility of introducing a wide variety of functional groups tailored to different groups of bioactive molecules for grafting. However, there are several limitations to polymer brush grafting, such as difficulty in achieving a dense layer of polymer brushes due to steric hindrance of reactive polymer groups adsorbed onto the polymer surfaces, or due to undesired covalent bond formation between reactive groups on the polymer brushes and the surface [49]. To
circumvent these limitations, ATRP has been developed to initiate polymerisation directly from the surface. One can simply adapt the ATRP process for the grafting of polymer chains onto material surfaces by immobilising a halide initiator onto the surface, and proceeding with polymerisation and growth of the polymer brush that remain tethered to the surface [50], [51]. Similar to ATRP in solution, this manner of surface modification is readily controllable by varying reaction conditions e.g. reaction time, and has been used to graft on dense “polymer brushes” of uniform and controllable length. As a result, ATRP of well-defined polymer brushes has been utilised to create antifouling, thermo-responsive and pH sensitive surfaces [52], [53].

ATRP-mediated surface grafting has been performed on PCL surfaces for the tethering of thermos-responsive poly(N-isopropyl acrylamide) [P(NIPAAm)] [54] and poly(2-dimethyl amino-ethyl methacrylate) [(P(DMAEMA)] [55] polymer brushes. In both studies, the polymer brushes were conjugated with bioactive collagen or gelatin to promote cell attachment and growth on the PCL surfaces.

2.2 Thrombogenic Processes

2.2.1. Biology of Thrombus Formation

The immediate consequence of haemo-incompatibility upon contact of the vascular graft material with blood is thrombosis, involving the activation of members of the coagulation cascade [56]. The thrombosis of biomaterials has parallels with thrombosis occurring in natural physiological settings, where it has a functional role in blood clotting during vessel injury (Figure 2.5). When vessel injury occurs, exposed proteins in the subendothelium i.e. collagen and von Willebrand factor (vWF) promoting platelet translocation and adhesion to the matrix [57], [58]. Upon adhesion to the matrix, receptors on the platelet surface are engaged by components in the subendothelial matrix e.g. collagen activates the integrin α2β1 and the immunoglobulin superfamily glycoprotein VI (GPVI) receptors [58], [59]. This results in the activation of platelets characterised by spreading and aggregation at the site of injury. Together with the formation of fibrin threads produced at the end of the coagulation cascade (reviewed in more detail in Section 2.2), activated platelets and fibrin formed a thrombus (clot) at the site of injury to effectively plug the gap in the vessel wall (haemostasis).
2.2.2. Thrombogenicity of Materials

Thrombosis on a vascular graft surface is a result of multiple physiological events happening at the blood-material interface. The coagulation cascade is designed to ensure rapid clotting of blood during vascular injury and involves multiple signaling molecules in the plasma, ECs and platelets. However, this same physiological pathway is responsible for thrombosis on synthetic material surfaces. The cascade is broadly classified into the intrinsic (contact activation) and extrinsic (cellular-mediated) pathways (Figure 2.6). While under physiological conditions, the activation of factor XII upon binding to collagen in the subendothelium is thought to be driving the classical intrinsic pathway, its role in the coagulation cascade is controversial [20], [60]. Although factor XII has been found to have adsorbed in moderate amounts on material surface (hence, contact activation) [61], individuals deficient in the factor has no impaired clotting responses [60]. However, contact activation is suggested to be important for thrombus stability, as demonstrated using factor XII-knockout mice [62]. The underlying mechanism for factor XII dependence in thrombus stability may yet to be elucidated.

Figure 2.5. The adhesion and activation of platelets during vascular injury. Graphics taken from website of Platelet Signalling lab, Monash University:
The blood coagulation cascade can be activated either through intrinsic (contact activation) or extrinsic (tissue factor) pathways that culminate in the formation of the fibrin clot. Factors that have been found to participate in material thrombosis through adsorption to material surfaces, and factors known to be targets of heparin inhibition are highlighted in blue.

The adsorption of plasma proteins, including other clotting factors, takes place immediately after blood contact. Apart from factor XII, factors XI, VII, thrombin, and fibrinogen are rapidly absorbed (Figure 2.6) [63]. Hydrophobic polymers have been reported to adsorb more protein than hydrophilic polymers, due to predominant hydrophobic-hydrophobic interactions between the protein and the polymer surface [64]. The initiation of the coagulation cascade eventually leads to the activation of thrombin, an enzyme that catalyses the formation of fibrin from fibrinogen [65]. As some of these major factors have been found to be absorbed onto material surfaces, the surface chemistry of the materials that result in the binding of these factors or other competitive plasma proteins (e.g. albumin) will influence the eventual coagulation response [64]. Recent experiments using quartz crystal microbalance (QCM) experiments have provided insights into the changes in strtrucnal conformation when hydrophilic plasma proteins (e.g. albumin) are adsorbed onto hydrophobic surfaces [64].

Platelets play an important role in thrombogenicity of materials. Physiologically within
the coagulation cascade, activated platelet membranes promote platelet receptor-mediated binding of factor VIII and its active form (factor VIIIa) to enhance the rate of factor X activation, leading to the assembly of a prothrombinase complex at the platelet membrane to activate thrombin [66] (Figure 2.6). On material surfaces, interactions between platelet receptors and adsorbed proteins (e.g. fibrinogen, thrombin, fibronectin, vitronectin, vWF) leads to platelet binding and activation [20]. Fibrinogen and vWF have been shown to be the key mediators in platelet adhesion under different wall shear rates, with fibrinogen mainly mediating platelet binding with integrin $\alpha_{IIb}\beta_3$ at low shear rates and vWF predominating at high shear rates through binding with glycoprotein Ibα (GPIbα) [67]. Fibronectin and vitronectin seem to play only adjunct, but not main roles in platelet adhesion [68]. It has been speculated and proven recently that the conformation of the adsorbed fibrinogen, as opposed to the amount adsorbed, is the critical determinant of platelet adhesion [69]. Intriguingly, the adsorption of fibrinogen onto hydrophobic material surfaces induces a conformation change that exposes 2 distinct active sites within fibrinogen for, one for platelet adhesion only and the other for platelet adhesion with activation [69]. In view of this, it is plausible that yet-to-be discovered hydrophobicity-dependent conformational change/denaturation exists for other adsorbed proteins, and have the potential to influence platelet activity in different ways.

2.2.3. Heparin Anti-thrombosis

Heparin, a highly-sulphated glycosaminoglycan (GAG) with large size heterogeneity (4 – 30 kDa), is widely used in the clinics as an anti-thrombotic drug for the prevention and treatment of postoperative arterial/vein thrombosis and peripheral/pulmonary embolism, and various types of coagulopathies (Figure 2.7) [70]. Isolated in 1918 and commercially produced from bovine lung or porcine intestine mucosa, heparin is one of the oldest drugs still widely in use today. Heparin is present in tissues such as lung, skin and intestine, and stored in mast cells in the blood for release upon vascular injury [71]. A less sulphated GAG, heparan sulphate, is ubiquitous to almost all tissue types and present in the ECM (Figure 2.7) [72]. In spite of its accidental discovery as an anti-thrombotic agent, the native biological function of heparin is not well-defined and is unlikely to be related to blood coagulation. Both heparin and heparan sulphate are proteoglycans attached to cell surface proteins, and are able to interact with various
proteins e.g. growth factors [71]. In line with the emerging evidence that glycans play active roles in molecular signalling and fundamental cellular processes, heparan sulphate has been proposed to be involved in fibroblast growth factor receptors (FGFR) activation, whereby heparan sulphate facilitates the binding of fibroblast growth factors (FGF) to FGFR [73]. As a drug, heparin manifests its anti-thrombotic effects through the targeting and inhibiting of multiple factors within the coagulation cascade (Figure 2.6).

![Figure 2.7](image_url)

**Figure 2.7.** Major and minor repeating units in heparin and heparan sulfate. $X = H$ or $\text{SO}_4^{2-}$; $Y = \text{Ac}, \text{SO}_4^{2-}$ or $H$. The differences in their functional groups are highlighted in yellow.

### 2.2.4. Materials Regulation of Endothelial Cell Thrombogenicity

One widely investigated approach to combat material thrombogenicity is to seed ECs onto the graft surface before implantation to prevent direct contact between the blood and material. First demonstrated by Herrring et al in 1978 [74], many pilot studies have indicated that a confluent layer of ECs on the luminal surface of the graft (6 – 7 mm in diameter) will decrease overall thrombosis and neointima hyperplasia rates, and improve long-term patency among patients [10], [22], [23]. Other studies have emphasised the importance of EC confluency and high-seeding density in order for any added benefit to long-term patency [75]. It has been suggested that ECs appear to exhibit a more thrombogenic profile at sub-confluent densities [76]. Hence, it is generally agreed that a material surface that is able to support optimal EC attachment and growth before implantation will able to minimise thrombogenicity and thus, improve long-term graft patency. However, histological evidence from failed vascular grafts from these clinical studies has cast doubts on any simplistic explanation regarding the thrombosis of vascular grafts *in vivo*. Although endothelialisation of grafts generally point to more favourable outcomes, questions were asked of how
endothelialised ePTFE grafts failed in the minority of patients (i.e. 31 % at 5 years, 39 % at 10 years) [10]. The prior coating of ePTFE grafts with fibrin glue had ensured 99 % of the grafts were covered with ECs throughout the entire graft luminal surface (87 % complete confluence, 12 % pre-confluence), and histological analysis of failed grafts did not find absence of an endothelium or thrombosis to be the root cause of failure [10]. Instead, failed grafts revealed highly inflammatory and significantly hyperplastic subendothelial areas to be the root cause of graft occlusion, rather than thrombosis. Peculiar to these patients, a thick layer of inflammatory immune and smooth muscle cells were present between the intact endothelium and the ePTFE surface, and in some areas large surface thrombi were attached to the endothelium where inflammatory was present [10]. This piece of clinical evidence thus led to suggestion that the endothelial layer, notwithstanding confluency, can turn pro-inflammatory and pro-thrombotic. In view of the clinical data, although thorough examination of the ePTFE surface on these failed grafts were not conducted, it can be postulated that in cases where the confluent endothelium had been activated, either patient-specific genetic reasons or inconsistencies in the material properties (fibrin glue, textile micro-topography etc.) could have resulted in the graft failures.

The above clinical example hints strongly that EC activation and phenotype can be regulated by material surface properties, and potentially influence clinical outcomes. In maintaining vascular homeostasis, non-activated ECs constitutively produce a range of regulatory factors, including nitric oxide (NO) to regulate vascular tone and prevent platelet aggregation, and fibrinolysis-promoting factors e.g. tissue plasminogen activator (tPA) to control clot formation [76]. Other influences of EC phenotype on thrombotic processes are apparent in the extrinsic clotting pathway, where upon activation, ECs upregulate expression of tissue factor (TF), [77], which acts as a co-factor for Factor VII activation in a calcium-dependent manner and leads to the assembly of the TF:FVIIa complex on the cell membrane (Figure 2.6) [78]. Upon activation, ECs also secrete factors that promote platelet and leukocyte adhesion, such as von Willebrand factor (vWF) [57] and pro-inflammatory molecules that promote leukocyte adhesion e.g. vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) [76].
2.3 Conducting Polymers and Electrical Stimulation in Tissue Engineering

2.3.1. Biological Effects of Electrical Fields

The application of electrical fields to cells has been studied for decades, and responses of excitable cell types are well-characterised in the literature. For instance, neuronal growth cones and nerve growth is known to be directed towards the cathodal end of an externally applied DC electric field with physiologically-relevant intensities (50 – 300 mV/mm) [79]. It remains contentious how cells can sense and respond to such small electrical fields, but the accepted consensus is that physiological responses are due to activation of voltage-gated ionic channels on the cell membrane to facilitate passive $\text{Ca}^{2+}$ entry into the cells, allowing calcium-driven molecular activity to happen [80]. Although not fully understood, the manipulation of $\text{Ca}^{2+}$ oscillations by DC stimulation has been reported to be able to promote osteodifferentiation of mesenchymal stem cells (MSCs) [81].

Galvanotactic responses (e.g. cell migration towards the cathode or anode of the electric field) is a well-known response of DC stimulation. The proposed theory has been that an applied DC field might move and redistribute charged membrane proteins and ion channels via electrophoresis across the fluid lipid bilayer, resulting in asymmetrical localization of various proteins that interact with the cytoskeleton and various cellular processes at the leading edge to promote directional movement (Figure 2.8) [82]. More recent studies have focused on the involvement of membrane-linked growth factor receptors e.g. the epidermal growth factor receptor (EGFR), which was observed to be polarised cathodally within minutes in an applied DC field (150-300 mV/mm) [83], [84]. Similar galvanotactic responses have also been observed for neural cells, adipose-derived stem cells and osteoblasts [85]–[87].

There has been little published work on the use of electrical fields in the area of thrombosis. One article described the use of electrical stimulation to prevent deep vein thrombosis four decades ago, but the idea entailed applying pulsed square waveforms to calf muscles to stimulate contractions and blood movement along soleal veins [88]. On the application of electrical stimulation directly to conductive materials, a 50 µA
DC current has been found to reduce bovine serum albumin and fibrinogen adsorption on polypyrrole [89], while another report demonstrated increased fibronectin adsorption to polypyrrole under 10 µA DC current and attributed it to the increase in neurite growth extension observed in nerve cells [90]. As both reports are contradictory, and did not offer explanations of the observations, these studies remain unconvincing for the electronic control of thrombosis.

![Diagram](image.png)

**Figure 2.8.** Polarisation of proteins involved in initiating motility and assembly of cytoskeleton at leading edge of the cell is required for galvanotactic responses in DC field.

In addition to DC fields, there have been studies harnessing the effects of alternating current (sinusoidal or pulsed) on cellular behaviour. Sinusoidal AC fields or currents are usually balanced in both direction phases (net DC current of zero), while pulsed current can either be monophasic (non charge-balanced) or biphasic (charge-balanced) (Figure 2.9) [91]. For monophasic and biphasic high intensity, short pulses are used in existing cardiac pacing devices and cardiac tissue engineering, while other high frequency modalities are being investigated [92]. Another area of intense research with high voltage stimulation pulses of micro-second pulse widths is in neuronal prostheses such as deep brain stimulation [93]. Sinusoidal or pulsed AC currents have been utilised by some research groups to enhance cellular behaviour relevant for tissue engineering e.g. cell proliferation or differentiation. Brighton et al observed increased cell proliferation, quantitated by DNA amount, and elevation of Ca$^{2+}$ signalling in MC3T3-E1 bone cells for 24 h after stimulation with 60 Hz, 2 mV/mm sinusoidal voltages [94]. The maturation of primary osteoblasts (bone-forming cells) and increased CM synthesis
has been demonstrated with the application of saw-tooth pulses of 16 Hz and 6 kV/m [95] The differentiation of stem cells has also been recently been demonstrated under AC electrical fields. Mesenchymal stem cells (MSCs) exposed to 10 Hz AC current fields of 40 µA for 6 hours a day demonstrated increased gene expression of osteogenic markers after 14 days in culture [96].

**Figure 2.9.** The various current waveforms used in cell stimulation experiments [91].

As gathered from the studies using AC stimulation that successfully modulated cellular behaviour, their protocols usually employ low-frequency but non-standardised AC fields which varies widely in the modality (voltage or current) and amplitude. Regardless of clinical success for stimulation devices or promising cellular modulating effects observed *in vitro*, the lack of understanding of the physiological effects of electrical stimuli at the molecular level constitutes a challenge to developing rational methods for AC stimulation in tissue engineering. However, on the flipside, this also allows investigators to pursue stimulation experiments with many degrees of freedom in selecting stimulation parameters.
2.3.2. Conducting Polymers and Biomedical Applications

Aromatic conducting polymers such as polypyrrole (PPy), polyaniline (PANI), polythiophene (PT) and poly(3,4-ethylenedioxythiophene) (PEDOT) have emerged as good candidates for biomedical and biosensing applications that require stable conductivity, electrical stability and ease of synthesis. The polymerisation of conducting polymers and concurrent doping can be performed through *in situ* chemical oxidation or using electrochemical polymerisation to generate a thin film on an electrode surface. For the coating of conducting polymer coating on a non-conductive material, *in situ* oxidative chemical polymerization has to be performed.

Conducting polymers derive their semi-conductive properties from the conjugated system of overlapping sp$^2$ orbitals. The delocalised electrons from these orbitals form a contiguous $\pi$-orbital electron cloud, but conductivity is limited by disorder of the electron cloud and electrostatic interactions [97]. Higher conductivity can be achieved by doping, which is the process of oxidising (p-doping) or reducing (n-doping) the neutral backbone with a counter anion or cation, respectively. In a p-doped conducting polymer e.g. PPy the anionic dopant introduces charge carriers (polaron, bipolaron) into the polymer backbone (Figure 2.10) [97]. The anionic dopant simultaneously neutralises and stabilises the polymer backbone with the aid of a co-cation to balance out charges. On application of a potential, the polymer backbone is destabilised and co-cations are exchanged into the aqueous medium, resulting in the passage of electrons through the conducting polymer.

2.3.3. Conducting Polymers with Bioactive Dopants

Utilising conducting polymers for biomedical and biosensing applications typically requires biofunctionalisation of the conducting polymer surface with bioactive macromolecules to direct a specific cell behaviour or a biosensing element (an enzyme or recognition receptor). The strategies to perform such immobilisation include surface chemical conjugation, adsorption or entrapment during the polymerisation process [97]. Dopants can be used as intermediate linkers to allow further modification of the conducting polymer surface. One possible way to utilise this strategy is the use of bioactive molecules that have net ionic charges for doping. Researchers have
incorporated large bioactive macromolecules as dopants in conducting polymers such that these macromolecules are entrapped within the polymer chain. These conducting polymer chains are balanced by counter-cations, which move out into the aqueous medium when a potential is applied (Figure 2.11) [98]. PPy has been used as an electrochemical actuator for the delivery of therapeutic compounds. Controlled release of the drug can be facilitated by changes in the redox state of PPy e.g. when the dopant is a negatively-charged drug such as dexamethasone [99] or positively-charged biotin conjugated to a streptavidin-linked bioactive molecule [100]. In the case of dexamethasone, the applied potential results in the flow of electrons into the PPy chains to compensate for the positive charges on the PPy backbone. The anion dopant is then released to maintain charge neutrality [99].

**Figure 2.10.** Oxidation (p-type doping) of heterocyclic conducting polymers. X = S, N or O. A radical cation introduced into the conjugated backbone after the loss of an electron results in the formation of a charged polaron (radical ion). Further oxidation of the same segment will cause the unpaired electron to be ejected, resulting in formation of a bipolaron (dication).

\[
\left[ \text{Pol}^+ \right] \left[ \text{MA}^- \right] \Leftrightarrow \left[ \text{Pol}^+ \right] \left[ \text{MA}^- \right] + n\left[ \text{C}^- \right] + n\left[ \text{e}^- \right]_{\text{aq}}
\]

**Neutral chains** \hspace{1cm} **Oxidized chains**

**Figure 2.11.** Oxidised conducting polymer chains \( \text{Pol}^+ \) (with polarons/bipolarons) entrapped with macroscopic anion \( \text{MA}^- \) and a co-cation \( C^+ \). The oxidised chains are formed when the cation is exchanged from the aqueous medium with the loss of electrons, while the macroscopic anion remains entrapped in the polymer backbone.
One such bioactive macromolecule used as a polyanionic dopant is heparin. Heparin has been used for the polymerisation of PPy through in situ chemical oxidation [101] or electrodeposition [102]–[105]. Garner et al has doped PPy with heparin for the attachment and proliferation of ECs [104]. Other studies have investigated the morphology, electrical and mechanical properties of heparin-doped PPy [101], [105], [106] and concluded that the PPy-heparin is a good candidate for tissue engineering owing to its highly charged and hence, strong electrostatic interactions with other macromolecules. However, one caveat for the use of large GAGs (including heparin/heparin sulphate) is the alteration of original surface topography of PPy and the decrease in conductivity [101], [107].

Though doped-PPy surfaces are known to be bioinert, they are not bio-degradable, and brittle nature of bulk PPy limits its practical use in tissue engineering. Several groups have attempted to minimise the amount of PPy on scaffolds by coating it onto biodegradable polyesters. The in situ chemical polymerisation uses the PLGA or PLA as fibre templates can be achieved by immersing pre-treated substrates in a polymerising solution with pyrrole monomers followed by the addition of the oxidising agent, or vapour phase deposition of monomers followed by oxidant treatment [108], [109]. While template polymerisation on such polyesters yields only slightly conductive hybrid materials, they exhibit good cyto compatibility. Direct electrical stimulation of cells on PPy-coated polyesters has demonstrated effects on cell behaviour. PPy doped with chondroitin sulfate, a GAG reportedly to be involved in osteogenic processes, was coated on PLA scaffolds to convey pulsed DC voltages to promote osteogenic differentiation in adipose stem cells [108]. PPy-coated electrospun PLGA meshes were used to impart DC voltages to neurons, inducing more and longer neurite formation [109].

2.4 Promoting Endothelialisation in with Growth Factors in Vascular Tissue Engineering

As highlighted in earlier sections, the endothelialisation of ePTFE vascular grafts have demonstrated improvements in their patency [10], [22], [23]. In addition to the bio-functionalisation of graft surfaces with cell-adhesive collagen or gelatin, protein growth factors such as vascular endothelial growth factor (VEGF) [110]–[112] and fibroblast
growth factor (FGF) [113] have been explored by various research groups to promote the formation of an EC monolayer. Physiologically, VEGF and FGF are mitogens that initiate EC proliferation and migration during the development of new tissues (e.g. in wound healing), and as a result, enable the formation of new vascular vessels through a process known as angiogenesis [114], [115]. For the purpose of creating vascularised scaffolds for tissue regeneration, various groups have induced angiogenic sprouting of ECs into these scaffolds by embedding or chemically-grafting VEGF or FGF within the scaffold matrices [116], [117]. Other studies have also explored the use of nanoparticulate systems for targeted or sustained delivery of VEGF to ischemic sites to promote vascular tissue formation, which typically requires the encapsulation of VEGF in a biodegradable polymer (e.g. poly(lactic-co-glycolic acid) (PLGA) [118], [119]. The angiogenic properties of these growth factors would also enable their usage in promoting the endothelialisation of vascular graft surfaces.

2.4.1. Involvement of Vascular Growth Factors in Angiogenesis

Vascular endothelial growth factor (VEGF) is a 45 kDa protein expressed by the human VEGFA gene and it is well known to be an important regulator of angiogenesis [114]. Alternative splicing of the gene leads to 4 active isoforms of VEGF (VEGF_{121}, VEGF_{165}, VEGF_{189}, VEGF_{206}) and they have 121, 165, 189 and 206 amino acids respectively [114]. With the exception of VEGF_{121}, the other major isoforms of VEGF each has a heparin-binding domain [114]. VEGFs initiate a multitude of EC functions such as cell proliferation, migration, differentiation, and survival upon binding to VEGF receptors (VEGFRs) VEGFR-1 and VEGFR-2 on the membrane [120]. Dimerisation of VEGFRs occur upon engagement of VEGF with VEGFRs, followed by the phosphorylation of the intracellular domains of the receptor. This event provides the signal to assemble signalling complexes at the cytoplasmic domains of VEGFR, leading to the activation of signal transduction pathways relevant to EC functions. For instance, VEGF activates EC proliferation via the mitogen activated protein kinase (MAPK)/ERK signal transduction pathway [114], and promotes EC survival through the phosphoinositide 3-kinase (PI3K)/Akt signalling cascade.

FGF members activate FGF receptors (FGFRs), which similar to VEGFRs, dimerise upon FGF binding to promote phosphorylation of intracellular domains [121]. The
subsequent assembly of signalling complexes activates similar signal transduction pathways that promote EC proliferation, survival, and migration. However, FGF has demonstrated effects on broader cell types, evidenced by promoting smooth muscle cell growth in vascular lesions [122]. For angiogenic applications, this can potentially lead to undesirable intima hyperplasia. Hence, this makes VEGF the more ideal candidate for vascular tissue engineering.

2.4.2. Immobilisation of VEGF to Promote Endothelialisation

The immobilisation of VEGF on vascular graft surfaces has been investigated as one approach to promote endothelialisation of the luminal graft surface [110]–[112]. Crombez et al. has determined, using a Boyden chamber assay, that VEGF immobilised on PTFE surfaces promoted the migration of ECs from the surrounding environment through the slow, localised release of VEGF [112]. This reiterated the well-defined role of VEGF as a chemoattractant for EC migration, an essential angiogenic function it has when inducing the formation of nascent vessels [114]. Another study by Shin et al has involved the immobilisation of VEGF onto polydopamine-functionalised poly(L-lactide-co-ε-caprolactone) (PLCL) substrates to improve EC proliferation [110]. However, both of these studies found no additional benefit of using VEGF for cell adhesion [110], [112]. This highlights the need to provide additional cell-adhesive signals (e.g. collagen or gelatin) on synthetic substrates that are inherently hydrophobic or non cell-adhesive. In the context of this thesis, as VEGF has a specific heparin-binding domain, one strategy to directly conjugate VEGF onto the material surface is via the functionalisation of the surface with heparin [116]. The doping of PPy with heparin during oxidative polymerisation would provide a convenient platform for conjugating VEGF onto heparin-doped PPy materials. As the binding affinity of VEGF to heparin is known to be very high with a dissociation constant $K_d$ of 80 nM [123], VEGF is unlikely to be released from any heparin-doped surfaces passively.

References


Literature Review

Chapter 2


Chapter 3

Experimental Methodology

This chapter describes in detail the experimental protocols and analysis methods employed to address the hypotheses and material development steps formulated for the thesis. These experiments are classified into material synthesis and modification, material characterisation, design of electrical stimulation setup, cellular studies, material thrombogenicity studies, and the preparation of vascular endothelial growth factor (VEGF)-conjugated nanoparticles. The principles underlying the characterisation techniques used are also discussed. The surface modification of polycaprolactone (PCL) using atom transfer radical polymerisation (ATRP) provided the means of functionalising PCL with a dense layer of gelatin, while template polymerisation of polypyrrole (PPy) onto PCL scaffolds conferred conductivity onto an otherwise non-conductive material. The synthesis of PPy nanoparticles offered an alternative method to introduce conductivity into a non-conductive matrix. The material characterisation techniques, mostly on surface chemistry analysis and determination of electrical properties, ensured desired material properties along each synthesis step. The cellular and biology studies were conducted to investigate the hypotheses this thesis had set out to investigate on the effects of materials- and electrical-control of cellular thrombogenicity and thrombotic processes.
3.1 Overview of Methodologies

The following sections in this chapter have been organised by the types of techniques performed i.e. material synthesis, material characterisation, design of customised stimulation setups, cellular studies, material thrombogenicity studies, and VEGF-loaded PPy nanoparticle studies. The overall strategy, as organised by the 3 main aims of the thesis outlined in Chapter 1 (Section 1.2), and summary of the methodologies formulated for these aims, are presented in the flow diagram below (Figure 3.1).

![Flow diagram of the main aims of the thesis, and the materials development approaches and characterisation studies that will be conducted to address these aims.](image-url)

**Figure 3.1.** Flow diagram of the main aims of the thesis, and the materials development approaches and characterisation studies that will be conducted to address these aims.
3.2 Material Synthesis and Surface Modification

3.2.1. Solvent Casting of PCL Substrates

The solvent casting technique is a simple method that enables preparation of relatively uniform polymeric substrates with an appropriate solvent. As PCL is soluble in a broad range of volatile organic solvents that can evaporate at room temperature, this technique was used for the preparation of flat PCL substrates. Another reason favouring the use of flat, solvent-cast PCL (scPCL) substrates is the relative ease of performing downstream surface modifications and subsequent biological experiments on flat surfaces. As these substrates are later intended for the investigating the effects of surface-mediated cues (i.e. surface chemistry or bio-functionalisation), flat and featureless substrates negated any possible physiological effects arising due to surface morphology (e.g. nano- or microscale features). Thus, scPCL substrates allowed for the evaluation of cellular and thrombogenic responses from a purely material surface approach.

For this approach, 5 g of PCL pellets were first dissolved in 40 ml of DCM to obtain a 12.5 w/v % PCL solution. The solution was then cast on a glass substrate using an automated film applicator (PA-2105, BYK Additives & Instruments, USA) and the solvent left to evaporate over 24 h. The resultant films of around 150 µm thick were then transferred to a vacuum oven for further drying for another 24 h. The films were then cut into disc-shaped substrates of 2 cm in diameter and washed thoroughly in absolute ethanol (Merck Millipore, USA) before use.

3.2.2. Surface Modification of scPCL with Gelatin via ATRP

scPCL substrates were functionalised with a dense layer of gelatin for investigations into surface cytocompatibility and thrombogenicity. The choice of method for grafting polymer chains onto the scPCL substrates in this study was ATRP. ATRP was used to grow intermediate epoxide-containing glycidyl methacrylate (GMA) polymer brushes, which were then conjugated with biofunctional macromolecules such as gelatin. A
schematic of steps involved in surface-initiated ATRP and gelatin conjugation is presented in Figure 3.2.

The modification of scPCL substrates first proceeded with the aminolysis reaction to introduce active free amine (-NH₂) groups. Aminolysis is a convenient and versatile method to introduce amine groups onto polyester surfaces, which can be furthered modified to yield other functional groups [1]. The pristine scPCL substrates were placed in a solution of 10% w/w 1,6-hexanediamine in isopropanol at 40 °C for 1 h (Figure 3.2a). The resulting scPCL-NH₂ films were then treated with large amounts of isopropanol and distilled water before being dried in a vacuum desiccator. This was followed by the immobilization of the ATRP initiator via a condensation reaction. 2-bromoisoobutryl bromide (BIBB) was reacted with free –NH₂ groups on the surface through a triethylamine (TEA)-catalyzed condensation reaction (Figure 3.2b). After degassing with nitrogen gas for 30 min on ice, scPCL-NH₂ films were placed in a round-bottom flask with 30 ml of anhydrous hexane solution containing 1.0 ml (7.2 mmol) of TEA. 7.2 mmol of BIBB was then added under stirring and the reaction was allowed to proceed at 0 °C for 2 h and then at room temperature for 12 h with gentle stirring to yield 2-bromoisoobutyl-immobilized scPCL (scPCL-Br) substrates.

With the objective of ultimately functionalising the scPCL scaffolds with biomolecules in mind, polymer chains with abundant reactive sites for the easy conjugation of biomolecules under moderate conditions was ideal. P(GMA) has epoxide functional groups that have been exploited in direct coupling to biomolecules through ring opening reactions with carboxylic and amine groups [2], [3]. For the purpose of creating a dense polymer brush network with multiple attachment sites for biomolecules, the growth of P(GMA) chains from the scPCL substrates via the ATRP process was performed. The reaction solution containing GMA (monomer), Cu(I)Br (metal catalyst), Cu(II)Br₂ (deactivator) and Bpy (ligand) with the molar feed ratio of 100 [GMA (3 ml)] : 1.0 [CuBr]: 0.2 [CuBr₂] : 2.0 [Bpy] in a methanol–water mixture (5:1, v/v) was prepared. The higher state transition metal Cu(II) was added in the reaction as the deactivator to allow good control of the reaction [4]. The scPCL-Br substrates were added into the reaction mixture at room temperature, and the reaction was allowed to proceed for either 1 h or 3 h to generate P(GMA)-grafted substrates with different P(GMA) graft density. The resultant substrates were termed scPCL-g-P(GMA)1 and scPCL-g-
P(GMA)2, respectively (Figure 3.2c). After the reaction, the substrates were washed with copious amounts of ethanol and deionized water (1:1, v/v) in alternate steps for 1 h to ensure removal of unreacted monomers, reaction catalysts, and physically-adsorbed polymers.

For the conjugation of gelatin to proceed, 3 mg/ml (0.3% w/v) of gelatin in phosphate buffered saline (PBS, pH 7.4) was reacted with the substrates under constant stirring for 24 h at 40 °C (Figure 3.2d). Following the coupling reaction, the reversibly-bound or physically-adsorbed gelatin was desorbed through washing in large amounts of PBS solution at room temperature for 24 h, followed by rinsing with deionized water. Depending on the ATRP reaction time and type of conjugated biomolecule, the resulting biofunctionalized scPCL substrates were termed scPCL-g-P(GMA)1-c-gelatin or scPCL-g-P(GMA)2-c-gelatin. The grafting yield of gelatin on the scPCL substrates was calculated in the following equation (equation 1)

\[ G_Y = \frac{W_a - W_b}{A} \]  

\( W_a \) = original weight of the scPCL
\( W_b \) = weight of scPCL substrate after GMA polymerisation
\( A \) = area of the substrate
Figure 3.2. Outline of the surface-initiated ATRP method (1 h or 3 h polymerization) to obtain P(GMA) chains of different length, followed by the coupling of gelatin or heparin to obtain gelatinized or heparinized PCL scaffolds.
3.2.3. Physical Adsorption of Gelatin on scPCL

The direct coating of gelatin onto polymeric surfaces of cell cultureware is a convenient method to improve cytocompatibility in cellular studies. However, the type of interaction between gelatin and treated polystyrene or polycarbonate (often with proprietary surface treatments) is different from interactions with scPCL scaffolds, as pristine PCL is inherently hydrophobic [5]. To perform comparisons between surfaces conjugated with a dense layer of gelatin grafted via ATRP against weak physically-adsorbed gelatin on the PCL surfaces, scPCL scaffolds were directly immersed in 3 mg/ml of gelatin (0.3% w/v) in phosphate buffered saline (PBS, pH 7.4) under constant stirring for 24 h at room temperature. The gelatin-adsorbed PCL films surfaces were then rinsed briefly with deionized water before drying in a vacuum oven at 37 °C.

3.2.4. Electrospinning of ePCL Nanofibrous Scaffolds

Due to its simplicity and ease of operation, electrospinning is chosen as the technique to generate electrospun PCL (ePCL) scaffolds with different fibre diameters ranging from the sub-micron to nano-scale in size. Nanofibrous scaffolds are more ideal than flat substrates for the evaluation of vascular and cardiac graft as nanofibers not only mimic the natural microenvironment of vascular and cardiac extracellular matrix (ECM), but also such fibrous scaffolds also accommodates the flexibility and ductility of prosthetic vascular graft constructs [6]–[9].

In this thesis, a 6 wt % PCL was dissolved in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP). The solutions were loaded into 5 ml syringe attached to a 22G needle (inner tip diameter of 0.4 mm). A syringe pump (Harvard Apparatus, USA) was used to feed the polymer solution and electrospinning of the PCL solution was conducted using horizontal setup using a high-voltage power supply (Gamma High-Voltage Research, USA) at 11 kV. An aluminium foil was used as a collector and the tip-to-collector distance was set at 8 cm, while the feed rate was fixed at 3 ml/h. 3 ml of the solution was electrospun and the fibrous scaffolds collected were dried in a vacuum oven overnight and cut into required sizes in subsequent experiments.
3.2.5. Template Polymerisation of ePCL Scaffolds with Doped PPy

As reviewed in Chapter 2, Section 2.3, although PPy possesses excellent cytocompatibility and conductivity, it is generally regarded as bioinert and non-degradable. Hence, it will be of interest to minimise the amount of PPy by synthesising a thin coating of PPy onto other degradable polymeric materials for support. In situ template polymerisation initiated through the addition of oxidising agents e.g. ferric chloride ($\text{FeCl}_3$) or ammonium persulfate (APS) have been performed on a variety of polyester fibers such as poly(lactic-co-glycolic acid) (PLGA) and poly(lactic acid) (PLA) [10]–[12]. This method of chemical oxidative polymerisation typically involves immersing the pre-treated nanofibrous scaffolds in a polymerising solution with pyrrole monomers followed by the addition of the oxidising agent, or impregnating the scaffolds with the oxidising agent before depositing monomers in vapour phase over the scaffold surface [10], [11]. However, one drawback of this method is the contamination of the oxidant on the substrate [13].

The biofunctionalisation of PPy with bioactive molecules can be performed via surface chemical conjugation, adsorption or entrapment during the polymerisation [14]. Dopants can be used as intermediate linkers to allow further modification of the conducting polymer surface. For example, heparin has been grafted onto PPy surfaces via poly(ethylene glycol) methacrylate (PEGMA) linkers that have been copolymerised with PPy [15]. To reduce the number of steps needed for immobilising heparin onto PPy surfaces, synthesis strategies were designed to explore the direct doping of heparin into PPy whilst polymerising PPy onto the ePCL template. Independently, heparin has been used as a polyanionic dopant for the polymerization of PPy through chemical oxidation [16] or electrodeposition [17]–[19]. These bulk PPy-heparin substrates, however, have limited practical biomedical usage as graft materials due to the semicrystalline and brittle behaviour of PPy.

The inspiration for the proposed methodology used in this thesis came from a paper published by Shi et al. on the use of heparin as a template to grow PPy nanowires [20]. Though not completely understood, the authors postulated that $-\text{SO}_3^-$ and $-\text{COO}^-$ functional groups on heparin could have reacted with pyrrole monomers to form chain-shaped intermediate compounds (Figure 3.3)
While the authors have achieved growth of one-dimensional PPy nano-structures from an electrode using heparin, the role of heparin did not extend beyond its use as a molecular template. The anti-coagulant intended role of heparin in this thesis, however, depends on the manner by which heparin, being a highly-sulphated long chain polymeric GAG, can be incorporated into PPy chains, and how the 3 separate components comprising of the ePCL template, pyrrole monomers, and heparin would interact with one another during oxidative polymerisation. To carry out this approach, ePCL scaffolds were to be immersed in a solution of pyrrole monomers and heparin before the addition of the oxidising agent APS. Reported starting pyrrole concentration for templated synthesis of PPy with other dopants varied from 0.03 – 0.3 M [10], the pyrrole concentration in this thesis was kept low at 15 mM to slow the polymerisation of PPy. The heparin:pyrrole weight ratios of 0.2, 0.5, 1 and 3 were investigated. The polymerisation was carried out at low temperatures (0 – 4 °C) as literature sources report such temperatures lead to higher extents of polymerisation and better electrical conductivities [13]. To offer comparisons between heparin-doping and other commonly-used dopants, camphor-sulfonic acid (CSA), another SO$_3^-$-containing anionic acid, and Cl$^-$ were also used as dopants.

**Figure 3.3.** Heparin-assisted growth of PPy nanowire from surface of indium tin oxide (ITO) electrode. Schematic diagram adapted from [20].
Hence, the polymerisation of PPy using the ePCL nanofibrous scaffolds as the assisting template was designed to be a one-pot synthesis method to achieve 2 steps simultaneously:

1) Confer electroactivity to a biodegradable fibrous material already used widely in biomedical applications
2) Confer specific bioactive properties e.g. thromboresistance through the doping of biomolecules such as heparin

The outcome of this approach would be an inherently thromboresistant nanofibrous material that is also suitable for investigating electricity-mediated effects on material surface thrombogenicity.

To perform template polymerisation of PPy onto the ePCL, ePCL scaffolds were first cut into disc-shaped substrates 2-cm in diameter before the oxidative polymerisation reaction was conducted in situ (in solution). Pyrrole alone (15 mM), pyrrole with hydrochloric acid (1 M), or pyrrole with heparin (heparin/pyrrole wt. ratios 0.2, 0.5, 1, 3), was dissolved in deionised water. The ePCL scaffolds were immersed in the solutions and ultrasonicated for 10 s (250 W, 5 s pulses) on ice. The ePCL scaffolds were then incubated in the polymerisation mixture on ice for 1 h before APS (15 mM) was added dropwise under vigorous stirring. The polymerisation reactions on the ePCL scaffolds proceeded for either 4 h or 16 h at 4 °C. After polymerisation, the coated ePCL scaffolds were rinsed with large amounts of deionised water under stirring, and followed by 10 s ultrasonication (250 W, 5 s pulses) on ice to remove adsorbed/unreacted pyrrole monomers from the surface. The prepared ePCL-PPy scaffolds were left in a vacuum oven to dry at 37 °C.

3.2.6. Synthesis of VEGF-loaded PPy Nanoparticles

As VEGF is an angiogenic factor and a specific growth promoter for endothelial cells, the delivery of VEGF from vascular graft/stent surfaces has been studied as an approach for promoting graft/stent endothelialisation [21], [22] or to facilitate vascularisation or re-endothelialisation of ischaemic tissues [23], [24]. To further explore the use of PPy as a conductive material for vascular graft applications, the synthesis of VEGF-loaded PPy nanoparticles was carried out. PPy nanostructures or nanocomposites are already broadly applied as coatings for solar cells [25], [26] and for use as resistive gas sensors.
[27]–[29]. In this thesis, the approach undertaken was to incorporate heparin, which is also a direct binding partner of VEGF (Figure 3.4) [30], [31], into PPy nanoparticles, and concurrently use heparin as the steric stabilizer to aid in the synthesis of PPy nanoparticles. VEGF-loaded PPy nanoparticles would then be dispersed throughout the biodegradable polymeric substrate to confer conductivity and to promote endothelialisation on the substrate surface. As opposed to having an inert layer of PPy coating on the polymeric substrate, PPy nanoparticles can be cleared by immune cells such as macrophages and be removed from the site of tissue regeneration as the substrate degrades.

**Figure 3.4.** The heparin-binding domain of VEGF is located at the C-terminus of the protein. Adapted from [http://www.slideshare.net/Ouniyeh/therapeutic-antibodies-3humanization](http://www.slideshare.net/Ouniyeh/therapeutic-antibodies-3humanization)

Colloidal dispersions of PPy nanoparticles in sub-micrometer sizes are usually synthesised by oxidative emulsion polymerisation or dispersion polymerisation techniques [32], [33]. Dispersion polymerization requires the presence of polymeric stabilizers such as polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP) [33]–[35]. In aqueous dispersion polymerisation of PPy, the pyrrole monomer is completely miscible with the aqueous reaction medium, but the synthesised polymer (PPy) is non-soluble and is thus precipitated. Large polymeric stabilisers have the effect of preventing particle agglomeration and macroscopic precipitation during PPy chain growth. Using this method, the size and PPy nanoparticles has been controlled by tuning the concentration of the polymeric stabiliser used; as increasing amounts of PVA or
PVP chains provide increasing steric effects, smaller nanoparticle size could be achieved when higher concentrations of PVA or PVP were used [33], [35].

Though unfractionated heparin has a long polymeric structure with a size distribution of 17,000 – 19,000 Da, which is lower than that of PVA (~125,000 Da) or PVP (~40,000 Da), it is hypothesised that it will provide sufficient steric effects during dispersion polymerisation of PPy nanoparticles. FeCl$_3$ was used as the oxidant and the [FeCl$_3$/[pyrrole]] ratio of 2.3 was used in the reaction. This ratio was employed as theoretically, for a 1 electron oxidant such as Fe$^{3+}$, 2 ions (or 2 electrons) are accepted for oxidation of each pyrrole unit, with the remaining 0.3 electrons being used for 30% oxidative doping of the neutral PPy product into a conducting form which carries a positive charge on every third pyrrole unit [36]. As previous studies using heparin as the anionic dopant most likely resulted in entrapment of heparin within polypyrrole chains [16]–[19], this thesis sets out to investigate if heparin can be incorporated into the polypyrrole nanoparticles during synthesis. One-pot synthesis and heparin immobilisation on PPy nanoparticles would be attainable if the hypothesis holds true.

The delivery of VEGF has entailed the loading of VEGF into a biodegradable polymer matrix, e.g. poly(lactic-co-glycolic acid) (PLGA), during synthesis. The main drawback of using such loading/encapsulation methods is that they frequently require the use of organic solvents during fabrication, and such solvents cause the irreversible denaturation and loss of bioactivity for VEGF. The low VEGF loading yield during nanoparticle synthesis is one challenge that currently needs addressing [37]. One approach is through the complexation of VEGF with a natural binding partner on the nanoparticle surface (e.g. heparin on PPy) in physiological conditions, which can negate most of the denaturing conditions during synthesis and the protein loading process.

To first synthesis heparin-immobilised PPy nanoparticles, 230 mM of FeCl$_3$ solution was added with 2.5 w/v %, 5 w/v %, 7.5 w/v % or 10 w/v % heparin (Dongying Tiangong Biochemical, China) in deionized H$_2$O and stirred for 1 h at room temperature. Following that, 100 mM of pyrrole solution (Sigma-Aldrich, USA) was added to the mixture solution in a drop-wise manner and further stirred for 4 h for the synthesis of heparin-stabilized PPy. The resultant heparin-stabilized PPy nanoparticles were then
pelleted and washed twice using deionized H$_2$O by centrifuging at 30,000 × g for 30 min each time. The re-suspended heparin-stabilized PPy powder was left in a glass petri dish for drying under vacuum. For PVA-stabilized PPy nanoparticles, the exact synthesis method was carried out with 2 wt % PVA instead of heparin.

For the loading of VEGF onto heparin-stabilised PPy nanoparticles, 500 µg of PPy-HEP10 (stabilized with 10 w/v % heparin during synthesis) and PPy (stabilised with PVA) nanoparticles was each dispersed in 1 ml of PBS and incubated with 500 ng of recombinant human VEGF 165 (R&D Systems, USA) at 4 °C overnight with slow orbital shaking. The nanoparticles were collected by centrifuging at 20,000 × g for 5 min. The VEGF loading efficiency was studied using the bicinchoninic acid (BCA) protein assay. The details of procedure are outlined in Section 3.7.1.
3.3 Material Characterisation

A variety of bulk and surface characterisation methods encompassing the characterisation of the mechanical, chemical functionalities, electrochemical and the biochemical properties of the fabricated materials were conducted. The rationale for the selection and working principle behind each of the characterisation method is presented in the following sections.

3.3.1. ATR-FTIR

Fourier transform infrared spectrometer (FTIR) is a spectroscopic technique used commonly for the analysis of material surface chemistry. It is based on the principle that most molecular entities absorb electromagnetic waves in the infra-red region, and that the photon energies absorbed in the IR range, while not large enough to induce electronic transition, can induce vibrational excitation (bending and stretching) of molecular covalent bonds and transitions in bond energy levels. The specific transition in each energy level corresponds to the associated IR frequency or wavelength [38]. As covalent bonds in organic molecules are labile and undergo a wide range of vibrational motions, organic molecules and polymers exhibit strong IR absorption (Table 3.1). The resultant absorption spectrum of the molecule or polymer therefore forms a unique signature with which it can be identified. IR absorption requires a change in the dipole moment of the molecule; hence, the symmetric stretching of bonds in molecules such as O\textsubscript{2} and N\textsubscript{2} do not result in an IR signal [38]. Peaks in 4000 – 1500 cm\textsuperscript{-1} region arises from the masses of the vibrating atoms and bond stretching, and hence, can give identify to the functional groups present. Peaks in the 1500 – 400 cm\textsuperscript{-1} region are usually present due to complex deformations in the bond (Figure 3.5)
Figure 3.5. Molecular bond vibrations are classified according to stretching and bending motions. Stretching motions can be symmetric or asymmetric, while bending motions, also called deformations, can either be in-plane (scissoring, rocking) or out-of-plane (wagging, twisting).

Table 3.1. Characteristic IR absorptions of some major functional groups [39]

<table>
<thead>
<tr>
<th>Major functional group</th>
<th>Absorption frequency (cm⁻¹)</th>
<th>Intensity of absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>C–H (alkanes)</td>
<td>2800 – 3000</td>
<td>Medium to strong</td>
</tr>
<tr>
<td>C=C (alkenes)</td>
<td>1620 – 1680</td>
<td>Medium</td>
</tr>
<tr>
<td>=C–H (alkenes)</td>
<td>3000 – 3150</td>
<td>Medium</td>
</tr>
<tr>
<td>O–H (alcohols)</td>
<td>3200 – 3570</td>
<td>Strong, broad</td>
</tr>
<tr>
<td>C–O (alcohols)</td>
<td>1050 – 1150</td>
<td>Strong</td>
</tr>
<tr>
<td>C=O (aldehydes)</td>
<td>1725 – 1740</td>
<td>Strong</td>
</tr>
<tr>
<td>C=O (carboxylic acids)</td>
<td>1700 – 1725</td>
<td>Strong, very broad</td>
</tr>
<tr>
<td>N–H (amines)</td>
<td>3380 – 3400</td>
<td>Medium</td>
</tr>
<tr>
<td>C–N (amines)</td>
<td>1150 – 1210</td>
<td>Medium</td>
</tr>
</tbody>
</table>
As different unique functional groups are introduced onto the polymer substrates with each successive modification step, FTIR can be used to verify the presence of these groups and the successful completion of each reaction step was performed on pristine PCL, PCL functionalised by P(GMA) [PCL-g-P(GMA)] and gelatin-conjugated PCL substrates [PCL-g-P(GMA)-c-gelatin]. For instance, the presence of epoxide groups in GMA will give characteristic bond vibrations of C–O–C that allows distinction from PCL. Once successfully conjugated with gelatin, the peaks associated with amine groups e.g. N–H can confirm the presence of gelatin.

As opposed to using FTIR in the transmission mode, sampling in the attenuated total reflectance (ATR) mode (where the IR beam is directed and reflected from the internal surface of the crystal to generate an evanescent wave that penetrates into the sample) allows for the analysis of thick, opaque samples, such as in the case of scPCL substrates. In this thesis, the infrared absorption spectra of the pristine and modified scPCL surfaces were obtained using an ATR-FTIR spectrometer (Frontier™ IR/NIR, Perkin-Elmer Inc, USA). The scPCL surfaces were pressed on the germanium (Ge) ATR crystal and the spectra were accumulated over 32 scans at 4 cm$^{-1}$ resolution in the range of 600 to 4000 cm$^{-1}$. The spectra of NaOH-treated electrospun PCL scaffolds were obtained in a similar manner to verify the presence of O-H groups, which is an indication of the successful hydrolysis of PCL.

For the collection of the infrared spectrum from synthesised PPy nanoparticles, the traditional transmission mode in FTIR was utilised. PPy nanoparticles were mixed with potassium bromide (KBr) powder, pressed into a pellet and mounted on the pellet holder, following which the IR spectra of the PPy in KBr matrix was obtained from a Ge crystal with an incident angle of 45° and sampling area of 2 mm. The spectra of the PPy nanoparticles were accumulated over 32 scans at resolution of 4 cm$^{-1}$ in the range of 600 to 4000 cm$^{-1}$. Each spectrum was corrected with the background spectrum obtained with a KBr only pellet.

### 3.3.2. XPS

X-ray photoelectron spectroscopy (XPS) is a true surface analysis technique that performs quantitative elemental analysis. Using low energy, monochromatic x-rays to
irradiate the sample, electrons bound to the orbitals in the atoms will be ejected from the surface of the sample. The binding energy of the electron can be inferred by subtracting the kinetic energy of the electron reaching the detector by the excitation energy [40]. Plotting the intensity of electrons detected against the binding energy will produce the XPS spectrum, which will be characteristic for each element. This makes XPS a highly quantitative method for measurements of elemental composition. As high resolution analysis of binding energies can be performed in XPS, the electrons from specific orbitals of an atom (e.g. 1s, 2s, 2p, 3s etc) can be identified after deconvolution of the spectral peaks and careful curve-fitting using developed algorithms (Table 3.2). Therefore, this also makes XPS a suitable technique for resolving organic functional groups (Table 3.2).

### Table 3.2. Examples of C 1s and N 1s binding energies in some functional groups [41]

<table>
<thead>
<tr>
<th>Functional group</th>
<th>Atomic orbital</th>
<th>Binding energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C–C, C–H</td>
<td>C 1s</td>
<td>285.0</td>
</tr>
<tr>
<td>C–N</td>
<td>C 1s</td>
<td>286.0</td>
</tr>
<tr>
<td>C–O–H, C–O–C</td>
<td>C 1s</td>
<td>286.5</td>
</tr>
<tr>
<td>C–Cl</td>
<td>C 1s</td>
<td>286.5</td>
</tr>
<tr>
<td>C–F</td>
<td>C 1s</td>
<td>287.8</td>
</tr>
<tr>
<td>C=O</td>
<td>C 1s</td>
<td>288.0</td>
</tr>
<tr>
<td>H$_3$N$^+$–H</td>
<td>N 1s</td>
<td>401.7</td>
</tr>
<tr>
<td>N–Ti</td>
<td>N 1s</td>
<td>397.2</td>
</tr>
<tr>
<td>N≡C</td>
<td>N 1s</td>
<td>399.4</td>
</tr>
<tr>
<td>O=N–O</td>
<td>N 1s</td>
<td>403.9</td>
</tr>
<tr>
<td>O=N$^+$–O$_2$</td>
<td>N 1s</td>
<td>407.2</td>
</tr>
</tbody>
</table>

Unlike near-surface techniques such as FTIR or energy-dispersive X-ray spectroscopy (EDX), XPS is considered a true surface technique as the photoelectron peaks are collected only from the material surface. These peaks arise from emitted electrons with a mean free path i.e. that has not undergo an inelastic collision. Electrons that have encountered such a collision loses energy and contribute to weaker and broader peaks in the spectrum known as “loss peaks”. Electrons emitted from a higher depth in the sample encounter more inelastic collisions, even higher energy loss and therefore
constitute the background of the spectrum. The electron path lengths for Al X-ray source is 1.4 – 2 nm, and most XPS photoelectron peaks are obtained from the top 10 nm of the sample, giving XPS unprecedented surface sensitivity [40].

XPS analysis performed on scPCL substrates that were modified in ATRP steps were complementary to FTIR analysis. Regarded as a more quantitative method than FTIR, the measured elemental peaks of the sample yield useful information about the chemical composition of the sample. For instance, ratios of [Br]/[C] after the immobilisation of the ATRP initiator on the scPCL substrate can give an indication of how the immobilisation reaction has proceeded. Likewise, analysing the [N]/[C] ratio after conjugation of gelatin on the scPCL-g-P(GMA) brushes will give an inference to the amount of gelatin conjugated to the surface. Using defined curve-fitting parameters on the C 1s spectral peak, the different components of the C 1s peaks can be deconvoluted into sub-peaks that represent the different binding energies of C in different chemical functional groups (e.g. C–N, C–H, –O=C etc). This allows for the verification of the results obtained in FTIR.

For the measurements, an AXIS HSi spectrometer (Kratos Analytical, UK) with a monochromatized Al Kα X-ray source (1486.6 eV photons) was employed at a constant dwell time of 100 ms and a pass energy of 40 eV. All the wide scans (range of 0 – 1100 eV) and the core-level spectra were obtained using an anode voltage of 15 kV and an anode current of 10 mA at 90°. The core-level spectra were referenced to the C 1s hydrocarbon peak at 284.6 eV to compensate for the surface charging effect. After the XPS spectra were obtained, curve-fitting was performed using XPSpeak 4.1. The full width at half-maximum (FWHM) of the Gaussian peaks was maintained constant for all components in a particular spectrum range. The peak area ratios for the various elements were corrected by experimentally-determined instrumental sensitivity factors. XPS analysis was also performed on the PPy-coated ePCL scaffolds. Owing to its surface sensitivity, it was proposed that if a uniform coating layer of PPy on ePCL can be achieved, chemical analysis can be performed solely on the PPy layer, confirming the presence of the dopant, as well as giving a hint of the PPy/dopant ratios.

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3.3.3. Water Contact Angle Measurements

The static water contact angles of the scPCL scaffolds were collected to determine initial hydrophilicity/wettability of the surface, and they were collected after each subsequent modification step in ATRP to verify the presence of grafted groups on the PCL. Measurements were performed using the sessile drop method with 3 μl water droplets on a FTÅ 200 contact angle goniometer (First Ten Angstroms Inc.) equipped with an automated dosing system and contact angle determination software. The mean contact angle was calculated from four substrates, with the value of each substrate obtained by averaging the contact angles for at least three surface locations.

3.3.4. Tensile Testing

As the PPy-coated ePCL scaffolds are intended for vascular graft applications, developing a graft with adequate mechanical strength to withstand physiological blood pressure is paramount. Tensile testing will provide insights into the ultimate tensile strength (UTS), as well as the elastic moduli of the scaffolds and allow for comparisons with native vascular tissues. Native femoral arteries have an UTS of 1–2 MPa [42]. The average elastic moduli of vascular tissues vary for different studies depending on age of donors and testing protocol: 1.54 MPa for femoral arteries [43], 3.11 MPa for femoral veins [43], or 6.8 MPa for saphenous veins [44]. As PCL is selected to be the matrix material of the scaffolds partly for its mechanical strength and ductility, it is hypothesised that the scaffolds will be able to provide adequate strength and match the elastic moduli of native vascular tissues. The PPy-coated PCL scaffolds were cut into dumbbell-shaped samples in accordance to ASTM D638 standards and stretched using a 10 N load cell (Instron 5567, Instron) to obtain stress-stain curves, from which the Young’s modulus and tensile strength could be found.

3.3.5. Electrical Properties

The conductivity of PPy-coated ePCL scaffolds and pressed PPy or PEG/PPy pellets were measured using an automated probe station (CMT-SR2000N, Advanced Instrument Technology, South Korea). The standard van der Pauw method is frequently used in 4-point probe measurement systems for the measurement of bulk resistivity
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(Figure 3.6). The 4-point probe method is developed for measuring homogenous materials e.g. semiconductor thin films. Nonetheless, due to ease of measurement, many groups have extended the 4-point probe method to electroactive fibrous scaffolds, as it provides a rough indication of scaffold conductivity [45], [46]. The bulk resistivity values were averaged from 3 scaffolds (each 2 cm in diameter) at 6 different locations on each scaffold. The material conductivity (S/cm²) is taken to be the reciprocal of resistivity (ρ).

\[ \rho = \frac{\pi}{\ln(2)} t \left( \frac{V}{I} \right) \]

(Figure 3.6. The 4-point probe method where the probes are equidistant to one another. The bulk resistivity (ρ) is calculated using the van der Pauw method: I is the current passed through the material sample between the 2 outer probes, V is the measured voltage drop between the 2 inner probes, and t is the thickness of the sample.

3.3.6. Cyclic voltammetry

Cyclic voltammetry is an analytical technique widely used for studying the electrochemical behaviour of conductive materials in solutions [47]. In a typical 3-electrode configuration for the characterisation of electroactive materials, the material under study is utilised as the working electrode, the reference electrode is a standard electrode with a known half reaction potential (e.g. Ag/AgCl), and a counter electrode is an inert material (Figure 3.7a). When a potential is applied to the working electrode (material under test), the current flow to the counter electrode in the solution is measured, as well as the voltage across the reference electrode and working electrode. In cyclic measurements, the voltage (working electrode vs reference) is ramped up and down linearly at a constant rate, and the resulting current flow can be presented in a
cyclic voltammogram (Figure 3.7b). When the potential applied is sufficient to drive the transition of an electroactive material to the oxidised or reduced state, the spike in the current produced, called the peak current, will be proportional to the amount of electroactive species present. The peak anodic current \( i_{pa} \) and peak cathodic current \( i_{pc} \) corresponds to the oxidation and reduction of the material, respectively. This current has a faradaic component (i.e. the transfer of electrons between the electrodes and the ionic species in the solution) and a non-faradaic component (transient potential changes resulting from capacitive charging or discharging of the electric double layer i.e. adsorption/desorption of charged species on the material). As such, cyclic voltammetry is a useful analytical tool that provides insights into the material redox behaviour. For instance, the electroactivity of the material in physiological solutions (e.g. PBS) can be confirmed with the presence of redox peaks.

\[ \text{Faradaic Current} \quad \text{Capacitive Current} \]

To characterise the redox behaviour of the ePCL/PPy and ePCL/doped PPy fibrous scaffolds, cyclic voltammetric measurements were performed in a standard three-electrode setup using the Autolab potentiostat (ECO Chemie, The Netherlands). During
each measurement, the scaffold (20 mm in diameter) was as the working electrode, a Pt wire as the counter electrode and an Ag/AgCl electrode as the reference electrode. The measurements were carried out in 1 × phosphate buffer saline (PBS) solution at room temperature. The cyclic voltammograms were recorded at the applied potential from -0.8V to +0.8V at a scan rate of 3 mV/s.

3.3.7. Toluidine Blue assay

After the polymerisation of heparin-doped PPy onto the ePCL fibrous scaffolds or the synthesis of PPy nanoparticles using heparin as the stabiliser, the amount of heparin in the PPy nanocoating or immobilised on the PPy nanoparticles, if present, can be quantititated using the Toluidine Blue assay, respectively [48]. Toluidine Blue is a basic metachromatic dye that has found wide applications as biological stains. Due to its high affinity and selectivity for acidic functional groups e.g. sulfates, carboxylates and phosphates in tissues, [49] it has been developed for the quantitation of heparin, which is rich in sulphates [48]. Colorimetric methods that utilise the precipitation of the heparin-Toluidine Blue complex (either in solution or on heparin-bound surfaces), followed by organic phase extraction, measure the leftover Toluidine Blue reagent through its 631 nm absorbance.

The Toluidine Blue solution was prepared by dissolving Toluidine Blue reagent (2.5 mg) and NaCl (0.2 wt %) in HCl solution (0.01 M, 50 ml). Standard heparin solutions were made by dissolving known amounts of heparin sodium salt in sodium chloride solution (0.2% w/v, 2 ml), which was then added to Toluidine Blue solution (3 ml) and mixed well. Test samples were immersed in sodium chloride solution (0.2% w/v, 2 ml) added to Toluidine Blue solution (3 ml) for 30 min. Subsequently, n-hexane (3 ml) was added to each heparin standard sample or test sample and mixed vigorously. The TB-heparin complex was separated out from the suspension into the organic phase while unreacted TB remained in the aqueous phase. The organic phase in each sample was carefully removed and the aqueous phase (100 ml) was transferred to a 96-well plate, and the absorbance at 631 nm was measured. A standard curve showing a linear relationship between heparin content and absorbance value was obtained. The absorbance readings obtained for the PCL test samples were correlated to the standard curve to calculate the absolute amounts of heparin incorporated or immobilized onto
the ePCL fibres. As the hybrid ePCL/heparin-doped PPy scaffolds proposed in this thesis afford selectivity only to heparin (due to the presence of sulfates and carboxylates in heparin), the Toludine Blue assay is suitable for quantitation of immobilised heparin on material or nanomaterial surfaces.

### 3.3.8. Dynamic Light Scattering and Zeta Potential Measurements

The size measurements of the PPy nanoparticles are carried out using dynamic light scattering (DLS). DLS measures the random Brownian motion of particles in suspension, and uses the Stokes-Einstein equation (equation 2) to calculate the hydrodynamic diameter (which includes the electric double layer) and size distribution of the particles. As such, this technique is suitable for the analysis of spherical particles comprising any material, and measurements can be obtained without prior knowledge of the material physical properties e.g. molecular weight, refractive index. The polydispersity index (PDI) of the nanoparticles is calculated from the standard deviation of the size distribution equation (equation 3) [50].

\[
d_H = \frac{kT}{6\pi\eta D} \quad \text{(2)}
\]

\(d_H\) = hydrodynamic diameter
\(k\) = Boltzmann’s constant
\(T\) = absolute temperature
\(\eta\) = viscosity
\(D\) = diffusion coefficient

\[
PDI = \left(\frac{\sigma}{d}\right)^2 \quad \text{(3)}
\]

\(PDI\) = polydispersity index
\(\sigma\) = standard deviation
\(d\) = mean diameter

Zeta potential pertains to the electric potential at the particle-solution interfacial double layer and it is a general indication of colloidal stability of the particles in dispersion. High zeta potential measurements (either positively- or negatively-charged) imply
strong repulsion forces between individual particles and that they are stable in suspension and likely to resist aggregation. Low zeta potential measurements is a reflection that the electrostatic attraction between the particles could be stronger than repulsive forces and will cause particle aggregation and flocculation.

For sizing and zeta potential measurements, heparin-stabilised PPy nanoparticles were re-dispersed in deionized H$_2$O by mechanical stirring overnight at 4 °C. PVA-stabilised nanoparticles could not be re-dispersed by stirring alone and had to be further ultrasonicated at 50 W for 5 min (Q500, Qsonica, USA). The nanoparticle size, size distribution and PDI were recorded using the Zetasizer Nano ZS (Malvern Instruments, UK). Zeta potential readings for the nanoparticles in 1 × PBS were obtained by first re-dispersing heparin-stabilised PPy nanoparticles (stirring) and PVA-stabilized PPy nanoparticles (ultrasonication) at 100 µg/ml in PBS buffer, followed by measurements in the Zetasizer Nano ZS using disposable folded capillary cells.

3.3.9. Scanning Electron Microscopy and Field Emission Scanning Electron Microscopy

Scanning electron microscopy (SEM) of the ePCL and ePCL/PPy scaffolds were taken for observation of fibre morphology using low-energy secondary electron imaging at 5 – 10 kV (JSM-6360, JEOL Ltd, Japan). For the imaging of PPy nanoparticles, the higher magnification required necessitated the use of the field-emission scanning electron microscope (FE-SEM) at 10 – 15 kV (JSM-6340F, JEOL Ltd, Japan). For the imaging of both the scaffolds and nanoparticles, the samples were coated with gold for 20 s using a sputter coater.

3.4 Design of Customised Setups for Electrical Stimulation

This thesis proposed the exploration of using electrical stimulation for investigating potential modulation in thrombogenic responses. Initially, the option of deploying a stimulation electrode board (C-Dish™) was considered. However the C-Dish™ stimulation board was designed to be used in conjunction with the C-Pace EP Cell Stimulator (IonOptix, USA). Although the stimulation board fits multi-well culture
plates, it allows only for single-channel stimulation, and current biological cell stimulators are limited to DC and pulsed/square voltages (monophasic and biphasic). And due to this limitation, alternative waveforms such as sinusoidal waves are seldom explored in cell stimulation. Creecy et al., however, did demonstrate mesenchymal stem cell (MSC) osteodifferentiation with low frequency sine waves (10 Hz) of micro-ampere amplitudes [51]. In order to generate the required AC sine waves, their alternating electrical current setup consists of a function generator instead of a conventional cell stimulator. This approach was adopted for the AC stimulation of PCL/doped PPy scaffolds in thrombogenic studies in this thesis. In addition to the function generator, a resistor was connected in series with the cell chamber, to produce a sufficient voltage drop for a micro-ampere current, which can be determined by using an oscilloscope (Figure 3.8).

**Figure 3.8.** AC stimulation of the PCL/doped PPy scaffolds was performed in a multi-array cell chamber connected to a function generator. An oscilloscope in parallel to a 4.7 kΩ resistor measures the signal waveform and the voltage drop across the resistor. The peak-to-peak current flowing through the system can be calculated from \( I_{p-p} = \frac{V_{p-p}}{R} \), where \( V_{p-p} \) is the measured peak-to-peak voltage across the resistor and \( R = 4.7 \) kΩ.
3.4.1. Multi-array Cell Chamber

Customised setups in laboratory for electrical stimulation fall generally into 2 main categories: setups constructed from petri dishes or cell chambers with a conductive element (e.g. graphite or platinum wires) directly in culture medium (Figure 3.9a) [52], or setups with agar/salt bridges to indirectly conduct ionic flow from electrodes to an isolated cell chamber (Figure 3.9b) [53]. Biomimetic systems designed to recapitulate the extracellular environment of target cell types, such as in providing bioactive surfaces or 3-dimensional scaffolds, are also used in conjunction with electrical stimulation [54], [55]. While such setups may suffice in single-waveform stimulation investigations, they do not allow output of multiple stimulation channels in a single experiment. For example, the agar/salt bridge configuration allows for the isolation of cells undergoing stimulation from the harmful electrolytic products of the electrodes, but it maintains a large profile in the cell culture incubator due to its use of saline chambers and salt bridge connectors. Sample replicates are also not possible with the use of these customized setups.

![Figure 3.9](image)

**Figure 3.9.** General schemes of commonly-employed experimental setups for electrical stimulation experiments. (a) Cells on coverslip between electrodes tissue culture dish with culture medium. (b) Cell chamber isolated from electrodes and electrical path connected to cells via agar salt connectors.

In order to facilitate the electrical stimulation experiments, a 6-chamber array was designed using SolidWorks (Dassault Systèmes, France) (Figure 3.10). The chamber array was fabricated using polymer by 3D printing, and the cell chambers receive
independent electrical signals from parallel outputs spilt from a single channel on the waveform generator. The fabrication of the multiarray cell chamber would be a crucial step for the investigation of electrical stimulation on thrombotic responses.

Figure 3.10. Design of cell chamber in SolidWorks. (a) 6-chamber multi-array for use with material substrate. (b) Individual cell chamber with material holder, and (c) top view with dimensions of cell chamber and holder.

3.5 Cellular Studies and Molecular Assays

This thesis hypothesised the use of material surface chemistry to induce an anti-thrombotic phenotype in the endothelial cells seeded on the material surface. Endothelial cell compatibility was first assessed on the functionalised scPCL scaffolds through proliferation assays and cell viability staining. To study the endothelial cell thrombotic responses mediated by the material substrates, the production of nitric oxide, and expression of thrombosis-associated markers by endothelial cells were investigated.

3.5.1. Endothelial Cell Seeding on Material Substrates

Endothelial cells were seeded onto the functionalised scPCL scaffolds for subsequent studies on endothelial cytocompatibility and thrombotic behaviour. Human umbilical vein endothelial cells (HUVECs) are the ubiquitous endothelial cell type used in many biomaterial studies as they are non-transformed primary cells, and they recapitulate the functional phenotypes exhibited by endothelial cells in vivo, including the chemotactic responses and sprouting behaviour towards growth factors [56].
HUVECs obtained from American Type Culture Collection (ATCC Number CRL-1730TM) were expanded on tissue culture plastic (TCP) flask in endothelial cell-specific EndoGRO-LS™ Complete Medium (with 2% serum) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin under conditions of 5% CO2 and 95% humidity. The cells were subcultured upon reaching 80% confluency by detaching them from the TCP flask using 0.25% trypsin-EDTA (Life Technologies, USA) after washing twice with sterile phosphate buffered saline (PBS). The medium was replaced every 48 h and as HUVECs are primary cells with finite doublings, only early-passage cells (passages 4-8) were used for the experiments. For cell culture on gelatin-physisorbed PCL surfaces and glass coverslips, the glass coverslips and PCL were coated with 0.3% gelatin in water (v/w) for 24 h.

3.5.2. Cell Proliferation Assay on Material Substrates

Cell proliferation on material substrates gives an indication of the cytocompatibility of the material under assessment. The enumeration of cell numbers on material substrates 24 h post-seeding reflects how well the substrates support the initial attachment of the cell of interest. Subsequent quantitation of cell numbers at specified time points allows for proliferation analysis over a period of time e.g. 7 days. Cell division and proliferation on material substrates is a general indicator of cell survival and health on the substrates, and is regarded as a measurable parameter of cytocompatibility. In this thesis, the alamarBlue® colorimetric assay (Life Technologies, USA), based on the resazurin dye, was selected as the method for quantitation of living cell numbers. Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) is commonly used for the assessment of mitochondrial metabolic activity, and its working principle is based on its reduction to highly-fluorescent resorufin in the presence of NADH dehydrogenase within the mitochondria of living cells (Figure 3.11). The amount of resorufin produced is hence associated to the level of biochemical metabolic activity in living cells under assay, which in turn is correlated to the cell numbers. One advantage of using resazurin-based dyes for proliferation analysis, as compared to other colorimetric assays such as the MTT assay, is that both resazurin and its reduced product resorufin are non-toxic, water-soluble and fully cell membrane-permeable. The assay, unlike the MTT assay, is not an endpoint assay and is thus suitable for continuous monitoring of the same batch.
of cells in culture over several time points. As the alamarBlue® assay fluorescence readout has a strong linear correlation with living cell numbers, the assay has been widely used to evaluate cell proliferation on synthetic biomaterial scaffolds [57].

![Figure 3.11](image)

**Figure 3.11.** The working principle of the alamarBlue® assay. The reduction of weakly-fluorescent resazurin dye requires the presence of NADH, and catalysed by NADH dehydrogenase, in living cells. The reduced form of the dye, resorufin, is highly-fluorescent (Ex/Em 570/585 nm).

For the cell attachment and proliferation assay, the material substrates were cut into circular discs of 15 mm in diameter for use in 24-well cell culture plates. Before conducting the assay, the material substrates were first sterilised with 70% ethanol for 2 h, immersed in 1 x PBS for 2 h and conditioned in culture medium for 2 h. HUVECs were seeded on each surface placed in 24-well plates at the density of 2.0 x 10^4 cells/well (∼0.8 x 10^4 cells/cm^2). The assay was performed 24 h after seeding to obtain initial cell attachment numbers, and subsequently at days 3, 5 and 7 for proliferation analysis. During the assay, each surface was incubated in 5% alamarBlue® in the colorless EndoGRO-LSTM medium for 4 h, following which, the reagent was removed and its fluorescence measured at 570/585 nm using the Varioskan Flash microplate reader (ThermoFisher Scientific, USA). The fluorescence readings were normalised with blank well readings of the reagent incubated without cells. A standard curve correlating fluorescence readings to known cell numbers plated in 24-well cell culture plates was obtained, and the standard curve was used to calculate the number of cells on the material substrates. After each assay, the cells on the surfaces could be
maintained in fresh culture medium after washing away the residual reagent with 1 x PBS, thus allowing continuous monitoring of the cells on the same surface over the course of the 7 days.

### 3.5.3. Cell Viability Staining

To visualise the coverage of viable cells on material substrates, LIVE/DEAD® cell viability assay (Life Technologies, USA) was performed. The assay consists of 2 probes, calcein AM and ethidium homodimer-1 (EthD-1), designed for use in fluorescence detection and respectively stain for living and dead cells. Calcein AM is a cell membrane-permeable probe that is converted into highly-fluorescent calcein by intracellular esterase activity within live cells. The green-fluorescent calcein is retained within the viable cells and has an excitation/emission peak of 494/517 nm. EthD-1, on the other hand, enters cells with damaged membranes and upon binding to nucleic acids, undergoes enhancement of fluorescence. As the membrane of living cells is impermeable to EthD-1, its red fluorescence (Ex/Em 528/617 nm) is an indication of dead cells.

To assess the extent of coverage by viable endothelial cells on the material substrates, 4 mM of calcein AM dissolved in anhydrous dimethyl sulfoxide (DMSO) and 2 mM of EthD-1 (DMSO/H₂O, 1:4 v/v) were added at the ratio of 1:1000 v/v to the colourless EndoGro-LS medium to make the staining solution. The cell-seeded material substrates were first washed thrice with 1 x PBS, followed by staining using 0.1 ml of staining solution. After incubation at 37°C for 30 min, the samples were visualised by a Nikon Image Ti fluorescence microscope to acquire fluorescent images using NIS-Elements Br software (Nikon Instruments).

### 3.5.4. Fluorescent Cell Imaging by Nuclear Staining

The presence of cell nuclei on material substrates is an alternate method to affirm the presence of cells on material substrates. Cell nuclei can be visualised with 4’,6’-Diamidino-2-phenylindole (DAPI), a nucleic acid binding fluorescence compound that binds preferentially to the A-T minor grooves in DNA. Before performing the staining, the substrates were first washed using 1 x PBS and fixed with paraformaldehyde (4%,
w/v) in PBS for 20 min at room temperature. 200 μl of DAPI with a concentration of 5 μg/ml in PBS was added to each surface in a 24-well plate for 30 min at room temperature. The cells were then washed and mounted between glass slides. Fluorescent images of HUVEC nuclei were captured under an excitation of 380 nm using the Nikon Image Ti fluorescence microscope and the NIS-Elements Br software (Nikon Instruments, Japan).

3.5.5. Quantitation of Nitric Oxide Production

The measurement of nitric oxide production by endothelial cells on material substrates is an assay to assess their functional behaviour on the substrates. In physiological settings, nitric oxide is produced by endothelial cells to regulate vascular homeostasis, promote vasodilation, and also has important anti-thrombogenic roles in inhibiting platelet and leukocyte adhesion [58]. Thus, this thesis has used the nitric oxide production by the HUVECs on the material substrates as one of the indications for thrombogenicity that is mediated by material surface properties. Also, it is desirable to demonstrate that endothelial cells on the synthetic material substrates are not inhibited in their native physiological function of regulating vascular homeostasis.

Prior to the measurement of nitric oxide production by the HUVECs, the cells were serum-starved overnight. 4-Amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM diacetate) (Life Technologies, USA), a reagent that is non-fluorescent until it reacts with nitric oxide to form a fluorescent benzotriazole, was added to fresh serum-free medium at a final concentration of 10 μM and subsequently incubated at 37 °C for 1 h. The HUVECs were treated with trypsin to detach them from the material substrates and counted before loading into capillary cells for fluorescence to be measured at the excitation/emission peaks of 495/515 nm using the GloMax® 20/20 luminometer (Promega, USA). The readings were then normalized to the cell numbers and reported as arbitrary fluorescence units.

3.5.6. Gene and Protein Expression of vWF and MMP-2 by Endothelial Cells

To further analyse the endothelial cell behaviour on material substrates, genetic and protein markers that serve as an indication of endothelial cell activation can be assessed.
Two such markers, namely von Willebrand factor (vWF) and matrix metalloproteinase-2 (MMP-2), were analysed. vWF is present in the subendothelial matrix and has a role in the clotting response by promoting platelet adhesion to the matrix during vascular injury [59]. Endothelial cells, too, secrete vWF constitutively and upregulate the production of vWF in response to external events such as vascular injury [60]. MMP-2, a member of the MMP class of proteinases, is known to participate in the degradation and remodeling of the endothelial extracellular matrix when endothelial cells are activated by angiogenic factors to migrate [61].

Gene level expression of vWF and MMP-2 was measured using quantitative polymerase chain reaction (qPCR), whereby the relative amounts of vWF and MMP-2 mRNA transcripts can be quantitated. Total RNA was first extracted from the HUVECs after 7 days in culture using Illuma RNAspin mini (GE Healthcare, USA) following the supplier's protocol. 200 ng of total RNA was reverse transcribed into cDNA with oligo-dT primers using RevertAid™ H Minus M-MuLV reverse transcriptase. The RNA was removed by RNase H digestion prior to qPCR. qPCR was performed with the KAPA™ SYBR qPCR Universal master mix (Kapa Biosystems, USA), using the SYBR green probe that binds to double-stranded DNA amplicons for fluorescence detection. Primers of specific target genes were designed to generate PCR amplification products of 100 to 250 base pairs. The primers used were [vWF-forward: 5’- AGCCTTGTGAAACTGAAGCAT-3’, vWF-reverse: 5’- GCCCTGGTTGCCATTGTAATTC-3’; MMP-2-forward: 5’- GATACCCCTTTGACGGTAAGGA-3’, MMP-2-reverse: 5’- CCTTCTCCCAAGGTCCATAGC-3’]. Melt curve analysis was conducted after the reaction to ensure specificity during amplification. The mRNA levels of the vWF and MMP-2 were normalized to the housekeeping ribosomal protein L27 (rpl27).

For the immunoblot detection of the vWF protein, the HUVECs on material substrates were lysed using protein lysis buffer (0.1% sodium dodecyl sulfate, 0.5% triton X-100, and 0.5% sodium deoxycholate dissolved in pH 7.4 PBS) and resolved using denaturing. The protein was denatured by adding Laemmli sample buffer and boiling at 95°C for 5 min. The protein samples were then resolved using a denaturing 10% polyacrylamide gel in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For immunoblotting, the protein bands were electrotransferred onto a nitrocellulose
membrane. After blocking with 5% non-fat milk in Tris Buffered Saline with 0.1% Tween (TBS-T), the membrane was stained using a mouse anti-human vWF antibody (Dako, Clone F8/86, Denmark) at 1:5,000 in TBS-T and subsequently with goat anti-mouse HRP-conjugated antibody (Santa Cruz Biotechnology, USA) at 1:10,000 in TBS-T. The vWF was then visualized using chemiluminescence.

3.5.7. Surface Expression of Tissue Factor and Thrombomodulin by Flow Cytometry

Apart from the functional and activation status of the endothelial cells on the material substrates, the expression of markers directly involved in the coagulation cascade reviewed in Chapter 2, Section 2.2 would be an important indication of endothelial cell thrombotic/anti-thrombotic phenotype. Hence, the expression of 2 thrombosis-related surface molecules, tissue factor and thrombomodulin, on endothelial cells were also analysed using flow cytometry. Thrombomodulin is a co-factor of thrombin, but partakes in the negative feedback of the coagulation cascade by catalysing the formation of activated protein C, an anticoagulant factor [62]. The complex formation between thrombin and thrombomodulin also inhibits the action of thrombin on fibrinogen by blocking fibrinogen binding sites on thrombin [62]. In this thesis, the surface expression of tissue factor and thrombomodulin was performed using flow cytometry.

HUVECs were detached from the material substrates and washed with 1 x PBS before incubation with tissue factor (R&D Systems, USA) or thrombomodulin (BD Biosciences, USA) mouse IgG anti-human antibodies (1.5 µg/ml in 2% BSA in PBS) for 1 h on ice. HUVECs incubated with mouse IgG isotype control antibody, which has no known recognition site in mammalian cells, were used as negative controls. The HUVECs were washed thrice with PBS, after which 2 µg/ml of goat Alexa Fluor-488-conjugated anti-mouse IgG (H+L) antibody (Life Technologies, USA) was added to the cells for 40 min incubation on ice. The cells were washed thrice with PBS before the surface expression of tissue factor and thrombomodulin was determined using the Accurri C6 Flow Cytometer (Becton, Dickinson and Company, USA). The data was
analysed and histogram plotting was conducted using the FlowJo software (FlowJo, LLC, USA).

### 3.6 Material Thrombogenicity Studies

The goal of fabricating functionalised scPCL substrates or electroactive ePCL/PPy hybrid scaffolds was to enable investigation into the control of material thrombogenicity via surface chemistry or electrical stimulation, respectively. Upon contact with isolated blood components (i.e. red blood cell fraction, platelets, and leukocytes), the haemocompatibility of the material, platelet adhesion and activation, and leukocyte adhesion, were studied.

#### 3.6.1. Isolation of Blood Components

Whole blood from human volunteers can be separated into different fractions using Ficoll-paque, a highly-branchied hydrophilic polysaccharide commonly used for this purpose. Human blood was first collected in tubes containing sodium citrate (in 3.8 % w/v sodium citrate at a volume ratio of 1:9) from healthy volunteers under protocols approved by the Nanyang Technological University Institutional Review Board (IRB-2014-08-002, 26th September 2014). 6 ml of citrated anti-coagulated whole blood was then layered carefully onto 3 ml of Ficoll-Paque™ PLUS (GE Healthcare, USA) in a 15 ml falcon tube and centrifuged at 400 g for 30 min at 20 °C according to manufacturer’s instructions. After centrifugation, the blood was visibly separated into a platelet-rich plasma (PRP) layer, a ‘buffy coat’ consisting of peripheral blood mononuclear cells (PBMCs) and a red blood cell fraction at the bottom. Each fraction was carefully aspirated for use in subsequent experiments. Polymorphonuclear leukocytes (i.e. neutrophils, basophils and eosinophils) have different densities from mononuclear leukocytes (i.e. monocytes) and were thus not present in the ‘buffy coat’. Additionally, to obtain platelet-poor plasma (PPP), PRP was centrifuged at 2,000 g for 15 min. Figure 3.12 summarises the isolation procedure.
Figure 3.12. Isolation of blood components from whole blood. (a) Citrated blood layered onto Ficoll-Paque™ was subsequently centrifuged at 400 g for 30 min to obtain the PRP, PBMC and red blood cell fractions. (b) PPP can be obtained by pelleting platelets in PRP through high-speed centrifugation at 2,000 g for 15 min.

3.6.2. Haemocompatibility Assessment

One important assessment of haemocompatibility for material substrates is its haemolytic potential towards red blood cells. Haemolysis in red blood cells can be determined by the release of haemoglobin. After Ficoll-Paque™ separation, the packed red blood cell fraction was resuspended in 1 x PBS to the original volume and further diluted with 1 × PBS (50 volumes) to obtain the red blood cell suspension for use in the haemolysis assay. The material substrates were cut into discs and incubated with red blood cell suspension (500 µl) in 24-well plate at 37 °C for 3 h. A reference haemoglobin control was created by incubating the RBC suspension with Triton-X100 (1% w/v) to fully lyse the red blood cells. As background haemolysis does occur to a little extent, the red blood cell suspension in blank wells served as the negative control.
After 3 h incubation, the free haemoglobin released by lysed red blood cells was collected by centrifuging at 4,000 rpm for 15 min and collecting the supernatant. Using the method of Harboe, the absorbance of the haemoglobin in the supernatant can be collected at 415 nm, with Allen baseline corrections at 380 nm and 450 nm [63]. 200 µl of the supernatant of each sample was in a 96-well plate for readings to be taken with a microplate reader (SpectraMax M2, Molecular Devices, USA). The corrected absorbance (Ab) of the free haemoglobin was calculated using equation (4)

\[
\text{Ab}_{\text{free hemoglobin}} = 2 \times A_{415} - (A_{380} + A_{450}) \quad \text{(4)}
\]

where \(A_{415}, A_{380}\) and \(A_{450}\) are the corrected absorbance readings of the sample at 415 nm, 380 nm and 450 nm, respectively, after normalisation with absorbance readings of the blank controls. The haemolysis percentage was calculated by dividing the free haemoglobin of the test sample by the total amount of free hemoglobin released in the reference control (100% lysis), as determined in equation (5).

\[
\% \text{ hemolysis} = \frac{\text{Ab}_{\text{free hemoglobin}} \text{ of test sample}}{\text{Ab}_{\text{free hemoglobin}} \text{ of positive control}} \times 100\% \quad \text{(5)}
\]

### 3.6.3. Plasma Recalcification Times

The sodium citrate added to the whole blood performs its anti-coagulative function by chelating calcium ions in the plasma. Recalcification of citrated plasma leads to restoration of clotting factor functions, and re-initiate clot formation. In this thesis, this procedure was performed with the SpectraMax M2 microplate reader for the continuous monitoring of clot initiation. 400 µl of PPP was first incubated with the material substrates placed in 24-well plates for 10 min at 37°C. 100 µl of PPP was then transferred to a clear-bottom 96-well plate. 100 µl of 0.025 M calcium chloride, pre-warmed to 37°C, was then added and immediately recorded for absorbance at 400 nm for 60 min at 30 s intervals using the SpectraMax M2. A steep rise in the absorbance curve over time for each substrate was taken to be the PRT.
3.6.4. P-Selectin Platelet Activation Assay of functionalised scPCL Surfaces

The P-selectin assay conducted on platelets attached to functionalised scPCL surfaces is an enzyme-linked immunosorbent assay (ELISA) with a colorimetric substrate. 100 μl of fresh human PRP was first incubated with the modified scPCL surfaces placed in a 24-well plate at 37 °C for 120 min. The PRP was then removed and the materials were washed with PBS thrice, each with a 5 min incubation, to remove unattached platelets. Primary antibody staining was conducted with 40 μl of anti-CD62p antibodies (MCA796GA, AbD Serotec, USA) added to each surface at 1:100 dilution and incubated for 60 min at 37 °C. After washing for three times with PBS, secondary antibody staining was performed on each material substrate by incubating with 40 μl of house radish peroxidase (HRP)-conjugated anti-mouse polyclonal antibody (KPL, Inc, USA) at 1:100 for 60 min at 37 °C. After washing thrice with PBS, they were reacted with 150 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) chromogenic solution, the enzymatic substrate of HRP, for 10 – 30 min. TMB-diimine formed was visible as blue colour. When the blue color development was deemed sufficient, the reaction was stopped by adding 100 μl of 1 M H₂SO₄. This changes the reagent to yellow in colour and the absorbance was measured using SpectraMax M2 microplate reader at 450 nm. In this assay, a higher level of P-selectin, which is indicative of the amount of activated platelets on the material substrate, would produce higher absorbance readings at 450 nm.

3.6.5. Fibrinogen Adsorption under AC Stimulation

Fibrinogen adsorption onto the ePCL/PPy and ePCL/heparin-doped PPy scaffolds under AC stimulation was performed. 22 x 22 mm pieces of ePCL/PPy, ePCL/heparin-doped PPy or ePCL scaffolds were placed in the cell chambers of the multiarray, designed and fabricated by 3D printing as outlined in Section 3.4.1. After setting up the electrical stimulation system as described in Section 3.4 (Figure 3.8), an AC sinusoidal voltage waveform that registered a 100 Hz, 10 μA peak-to-peak current was supplied from the waveform function generator to the PPy-coated ePCL scaffolds. After verifying the flow of the 100 Hz, 10 μA sinusoidal current, 1 ml of fibrinogen (2 mg/ml in PBS solution) was added each chamber under stimulation for 2 h at 37 °C.
After stimulation, the scaffolds were removed from the chamber and rinsed 3 x in fresh PBS. 1 ml of 2% wt sodium dodecyl sulphate (SDS) was added to each scaffold for 2 h with shaking to desorb the fibrinogen from the scaffold [64], after which the microbicinchoninic acid assay (BCA) was performed to quantitate the amount of fibrinogen that was initially adsorbed onto the scaffolds (microBCA assay kit, ThermoFisher Scientific). The microBCA assay detects the presence of peptide (amide) bonds in proteins through a 2-step reaction. Firstly, the Cu$^{2+}$ ions in the solution are reduced to Cu$^{+}$ ions by the amide bonds. The reduced Cu$^{+}$ ions are then chelated by BCA to form a BCA-Cu complex that has strong absorbance at 562 nm. The intensity of 562 nm absorbance is thus proportional to the amount of peptide bonds in the solution. The assay was performed in accordance to the manufacturer’s instructions and protein standards used were 0, 0.05, 0.1, 0.15 and, 0.2 mg/ml of fibrinogen in SDS (2 wt % in distilled H$_2$O).

3.6.6. P-Selectin Platelet Activation Assay under AC Stimulation

The P-selectin ELISA assay conducted for ePCL/PPy and ePCL/heparin-doped PPy scaffolds was performed to investigate the effects of AC stimulation on platelet activation on the scaffolds. After preparation of the scaffolds and setting up of the electrical stimulation system as described in Section 3.6.5, a 100 Hz, 10 µA peak-to-peak sinusoidal AC current was similarly supplied from the waveform function generator. After affirming the flow of the 10 µA sinusoidal current, diluted PRP (1 ml, with 9 volumes of Tyrode’s solution) was pipetted into each cell chamber. Non-stimulated samples were placed in each chamber and held down by the material holder without platinum wires in a similar manner. The PRP was incubated with the scaffolds under stimulation for 2 h at 37 °C. After stimulation, the PRP was removed and the scaffolds were transferred to 6–well plates and subsequently washed in 1 ml of PBS on a shaker (15 min × 3 times) to remove unattached platelets. The activation status of the platelets adhered to the scaffolds was assessed using the P-selectin (CD62P) assay similar to that of the assay carried out for the scPCL substrates in Section 3.6.4. One advantage of measuring P-selectin levels as opposed to absolute counts of platelets on
the surfaces is that it is a quantitative and definitive marker of platelet activation, unlike morphological observations of the platelets in SEM, which can be subjective.

3.6.7. Adhesion of Platelets and Leukocytes under AC Stimulation

Platelet and leukocyte adhesion on the scaffolds is a consequence of inflammation responses. To perform this study, isolated PBMCs in the ‘buffy coat’ fraction were resuspended in the PRP to obtain a platelet- and leukocyte-rich plasma (PLRP). PLRP was diluted with 10 volumes of Tyrode’s solution and 1 ml of the diluted PLRP was added to PCL/PPy or PCL/heparin-doped PPy scaffolds under 100 Hz, 10 µA sinusoidal AC current simulation for 2 h at 37 °C, as described in Section 3.6.5. Unstimulated scaffolds were prepared and incubated with PLRP in a same manner. After stimulation, the scaffolds were washed with 1 ml of PBS thrice to remove unattached platelets and leukocytes (15 min × 3 times on a shaker) and fixed with 2.5 % w/v glutaraldehyde for 1 h at 4 °C. The leukocytes and platelets on the scaffolds were then dehydrated in the following ethanol gradient series: 25% ethanol for 5 min, 50% ethanol for 5 min, 75% ethanol for 5 min, 100% ethanol for 10 min, 1:1 ethanol/hexamethyldisilazane (HMDS) for 15 min, before critical-point drying with HMDS overnight. The scaffolds were then gold-sputtered for 20 s in a sputter coater before imaging with a scanning electron microscope (JSM6360, JEOL Ltd, Japan).

3.7 VEGF Loading and Bioactivity of VEGF-conjugated PPy-heparin Nanoparticles

3.7.1. Study of VEGF Loading Efficiency

VEGF loading was performed in physiological conditions for prepared PPy and heparin-stabilised PPy nanoparticles as described in Section 3.2.6 after verifying that the synthesis resulted in the doping of heparin on the PPy nanoparticles through the Toluidine Blue assay (Section 3.3.7), 500 µg of PPy-HEP10 (stabilised with 10 w/v % heparin during synthesis) and PPy (stabilized with PVA) nanoparticles were each dispersed in 1 ml of PBS and incubated with 500 ng of recombinant human VEGF 165...
(R&D Systems) at 4 °C overnight with slow orbital shaking. The nanoparticles were pelleted by centrifuging at 20,000 × g for 5 min.

To determine the loading efficiency of VEGF on the nanoparticles, the unbound VEGF in PBS buffer was quantitated using the QuantiPro BCA assay kit (Sigma Aldrich, USA). This assay has a working principle similar to that of the BCA assay performed in Section 3.6.5. In this assay, 100 µl of the unbound VEGF in PBS (supernatant after centrifuging) was added with 100 µl of BCA reagent prepared according to manufacturer’s instructions in a 96-well flat-bottom plate. The reaction was allowed to proceed at 37 °C for 1 h. 100 µL of VEGF solutions at concentrations of 0, 100, 500 and 1000 ng/ml in PBS were used as reference standards for the assay. Absorbance readings were taken at 562 nm using the SpectraMax M2 microplate reader. The % VEGF binding efficiency of the PPy-HEP10 and PPy was calculated by subtracting the unbound VEGF from the initial amount of VEGF incubated with the nanoparticles.

3.7.2. Assessment of PPy-HEP10-VEGF Bioactivity via Matrigel Angiogenesis Assay

While the BCA assay can quantitate the amount of VEGF loaded on the PPy and PPy-HEP10 nanoparticles, the actual bioactivity of the VEGF conjugated onto the nanoparticles was confirmed using a functional angiogenesis assay. The binding of VEGF to VEGF receptors on endothelial cells results in the activation of mitogen-activated protein kinase (MAPK) signaling pathways that promote the angiogenic response in endothelial cells [65]. In this thesis, the angiogenic response is assessed using the Matrigel tube formation assay. Matrigel is a soft gelatinous substrate comprising of a mixture of basement membrane-derived proteins. This tube formation assay is commonly employed as an in vitro model of vasculature formation or angiogenesis to assess the activity of angiogenesis-promoting or angiogenesis-inhibiting agents [66] (Figure 3.13).
Figure 3.13. Matrigel angiogenic assay procedure. Endothelial cells are dispersed and seeded onto Matrigel matrix overlays. Network of endothelial cells in tube-like structures is observed on Matrigel in the presence of angiogenic agents.

HUVECs (Merck Millipore, USA) were cultured in EndoGro-LS complete medium (Merck Millipore, USA) and used at passage 4. Matrigel matrix overlays (BD Biosciences, USA) were prepared in 24-well plates by thawing the Matrigel overnight at 4 °C and adding 300 µL of Matrigel solution to each well for gelation to occur at 37 °C. The HUVECs were detached by trypsinisation, enumerated and 4 × 10⁴ cells were re-suspended in 2 ml of EndoGro-LS complete medium supplemented with 50 µg/ml of VEGF-loaded nanoparticles. The HUVEC-nanoparticle cell suspension was then added to the Matrigel overlays in the 24-well plate, and the plate was incubated for 24 h in the cell culture incubator at 37 °C to allow tube formation. The cells on Matrigel were then stained with 5 µg/ml of fluorescein diacetate (FDA) to visualise live endothelial tubule networks. Fluorescence images of 4 different wells in each treatment group were acquired using a fluorescence microscope (AxioObserver, Carl Zeiss, Germany). Analysis carried out to quantitate the number of tubule branch points and total tubule length was conducted using ImageJ.

3.8 Statistical Analysis

Quantitative data was reported as mean ± standard deviation. Single-factor analysis of variance testing (ANOVA) was conducted for multiple independent sample groups and \( p < 0.05 \) was considered to be statistically significant and amenable to Tukey HSD posthoc analysis. When smaller sample sizes (\( n < 10 \)) were present, the non-parametric
Kruskal-Wallis ANOVA was used. The Mann-Whitney test was conducted between individual sample groups when Kruskal-Wallis had determined significant differences among the tested sample groups. In all the tests, significant difference was determined with a confidence level of 95% ($p<0.05$).

References


[50] “Nanocomposix’s guide to dynamic light scattering measurement and analysis.”


Chapter 4

Development of Surface-modified Polycaprolactone and Polypyrrole Nanomaterials

This chapter describes the results from the development of the materials and nanomaterials utilised in the thesis. Polycaprolactone (PCL) substrates have been functionalised with intermediate glycidyl methacrylate brushes via atom transfer radical polymerisation (ATRP), and the successfully conjugation of gelatin was verified. A haemocompatible, anti-coagulant surface was fabricated by coating electrospun PCL scaffolds with polypyrrole doped with heparin through oxidative polymerisation. The presence and activity of the heparin were verified, along with the good conductivity of the heparin-doped polypyrrole coating. For use as a conductive nanofiller, size-controllable synthesis of PPy nanoparticles was achieved using heparin as a steric stabiliser. Heparin was found to be directly immobilised onto the nanoparticle surface, and that enabled the direct conjugation of vascular endothelial growth factor under physiological conditions.
4.1 Introduction

As reviewed in Chapter 2, due to the major challenges facing the use of synthetic materials, expanded polytetrafluroethylene (ePTFE) and Dacron® remain the only approved synthetic alternatives to autologous grafts for vascular replacement procedure [1]. ePTFE and Dacron® afford the mechanical strength needed to withstand vascular burst pressure, but carry high risk of thrombosis when used in small-diameter vascular graft prosthesis (< 6 mm) [2]. In order to ensure long-term patency and structural integrity, the ideal vascular graft should be mechanically sound, be able to maintain its anti-thrombogenicity even during slow blood flow (such as the case during small diameter grafts), and yet exhibit good endothelial cytocompatibility and haemocompatibility. In this thesis, we seek to address the issues of material endothelial cytocompatibility, material thrombogenicity and poor vascularisation on material surfaces respectively through surface-functionalised PCL, electrical stimuli and nanocarrier-based delivery of vascular endothelial growth factor (VEGF) (see research objectives in Chapter 1 and overview of methodologies in Section 3.1).

In this chapter, we explore the development of bioactive and electroactive materials based on polycaprolactone (PCL) and polypyrrole (PPy) to address the stated needs of vascular tissue engineering. PCL is a moderate-to-high strength bioresorbable polymer which constitutes the material in FDA-approved tissue engineering constructs [3], and due to its mechanical strength and non-toxicity, fibrous PCL scaffolds have recently been assessed for suitability in a rat model of aortic graft replacement [4]. PPy is a conjugated polymer that has found many uses in neural tissue engineering and drug release applications [5]–[9]. The strategy undertaken for the development of the materials is summarised in the 3 parallel aims encompassing the cellular, anti-thrombotic material, and drug delivery aspects of vascular tissue engineering:

**Aim 1: Cytocompatible material.** Investigating PCL as the material of choice for vascular grafts, and modifying PCL via ATRP and gelatin grafting to make it conducive for endothelial cell support.

**Aim 2: Anti-thrombotic material.** Incorporating both electroactivity and anti-thrombogenicity onto electrospun PCL fibres by synthesising a heparin-doped PPy
nanocoating on the PCL fibres. Heparin is a known anti-thrombotic agent, and electrical stimulation will be investigated as a means to control material thrombogenicity.

Aim 3: Conductive nanofiller and VEGF anchor. PPy to function as a conductive nanofiller and structural support of VEGF. Heparin is investigated for use as a stabiliser to control the size of PPy nanoparticles, and the immobilisation of heparin on the PPy nanoparticle surface will ease the conjugation of VEGF under physiological conditions.

4.2 Experimental Methods

The solvent casting of PCL substrates and gelatin functionalisation of the solvent-casted PCL (scPCL) substrates via intermediate glycidyl methacrylate (GMA) polymer brushes using ATRP are detailed in Section 3.2.1 and Section 3.2.2, respectively. The physical adsorption of gelatin onto the scPCL substrates are described in Section 3.2.3. Surface chemistry characterisation techniques attenuated total reflection-Fourier transform infrared spectrometer (ATR-FTIR) and X-ray photoelectron spectroscopy (XPS) were detailed in Section 3.3.1 and Section 3.3.2, respectively. Observations of the morphology of the scPCL substrates were performed using the scanning electron microscope (SEM) (Section 3.3.9).

The fabrication of electrospun PCL (ePCL) scaffolds was conducted by the electrospinning of 6 wt % PCL in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) as described in Section 3.2.4. Subsequent polymerisation of polypyrrole (PPy), chloride-doped PPy, and heparin-doped PPy over the ePCL fibres can be referred to in Section 3.2.5. XPS characterisation was performed on the ePCL/PPy meshes as described in Section 3.3.2. For the characterisation of the physical properties of the ePCL meshes, water contact angles were measured (Section 3.3.3) and the tensile testing of the ePCL and ePCL/PPy meshes was performed (Section 3.3.4). The electrical properties of the ePCL/PPy meshes, namely bulk conductivity measurements performed with a 4-point probe, were characterised as detailed in Section 3.3.5. Electrochemical characterisation through cyclic voltammetry (C-V) measurements was taken in Section 3.3.6. Toluidine blue assay was performed to verify the presence of heparin on the ePCL/heparin-doped PPy meshes (Section 3.3.7). Observations of the morphology of the meshes were performed using SEM (Section 3.3.9).
The heparin-stabilised synthesis of PPy nanoparticles and conjugation of vascular endothelial growth factor (VEGF) were performed using the protocol described in Section 3.2.6. The size of the synthesised PPy nanoparticles and their zeta potentials were measured using dynamic light scattering (DLS) as detailed in Section 3.3.8. Morphological observation of the PPy nanoparticles was performed using the field emission scanning electron microscope (FE-SEM) (Section 3.3.9).

4.3 Surface Functionalisation of scPCL Substrates with Gelatin via ATRP

(Aim 1)

In the first aim of this chapter, scPCL substrates were functionalised using the surface-initiated ATRP method to covalently graft a high density of intermediate GMA polymer chains onto the scPCL surface. As P(GMA) consists of highly-reactive epoxide groups, this method introduced copious binding sites for macromolecules such as gelatin (refer to Chapter 3, Figure 3.2 for reaction scheme) [10], [11]. Analysis of surface chemistry was carried out using ATR-FTIR and XPS at the end of each intermediate step to verify the presence of expected intermediate functional groups on the PCL substrate surface before proceeding.

4.3.1. Chemical Surface Analysis of P(GMA)-grafted scPCL Substrates

Prior to the growth of polymer brushes on scPCL via ATRP, the ATRP initiator, alkyl halide 2-bromoisobutyryl bromide (BIBB), has to be immobilised on the scPCL substrate surface. BIBB is an alkyl bromide with dual bromine terminal ends, making a condensation reaction possible with amine-containing molecules (Figure 3.2b). As PCL has an abundance of ester groups (-COO-), an aminolysis reaction with 1,6-hexanediamine, whereby one terminal amine can react with the ester group of PCL through a nucleophilic attack to form an amide covalent bond, can proceed while leaving the other amine terminal free (Figure 3.2a). Such aminolysis reactions have been utilised as a convenient chemical technique to introduce amine groups onto polyesters [10], [12], [13], and previous work by us have established that the optimal aminolysis reaction time is 1 h, when measured free amine density reached its peak on the scPCL surface [10]. Hence, in this study the aminolysis of scPCL substrates was kept to 1 h. Subsequently, the ATR-FTIR spectra of pristine scPCL and aminolysed
scPCL (scPCL-NH\textsubscript{2}) were collected. The characteristic carbonyl (C=O) stretching vibration at 1726 cm\textsuperscript{-1} is one of the strongest IR absorption bands and was also subsequently present in pristine and all functionalised scPCL substrates. The common (C-H) absorption bands present in aliphatic polymers resulting from both asymmetrical stretching and symmetrical stretching can be observed in the spectrum the scPCL substrate at 2947 and 2865 cm\textsuperscript{-1}, respectively (Figure 4.1). XPS scans of the scPCL substrates also revealed the presence of three curved-fitted peak components of the C 1s core-level spectra at C-H (284.6 eV), C-O (286.2 eV), and O=C-O (288.7 eV) (Figure 4.3b).

After 1 h of aminolysis reaction, a weak broad band at around 3335 cm\textsuperscript{-1} and a small peak appearing at 1550 cm\textsuperscript{-1}, attributable to the stretching vibrations of (N–H) and (C=O) in amide I, and the bending vibrations of (N–H) in amide II of the aminolysed scPCL surface [10], can be observed (Figure 4.1). Though weak, the presence of these bands indicates that amide functional groups (-O=C-NH-) were successfully introduced on the scPCL surface, and that the scPCL substrates have been successfully aminolysed. In addition, the wide scans conducted in XPS revealed the presence of an additional N 1 s signal with the binding energy (BE) of ~400 eV on the scPCL-NH\textsubscript{2} surface (Figure 4.3a) when compared to the XPS spectra of pristine scPCL. Also, the curve-fitted C 1s core level spectrum of scPCL-NH\textsubscript{2} substrate showed the presence of an addition peak attributable to (C-N) bond (Figure 4.3c) that was previously not present in the scPCL substrate (Figure 4.3b), further confirming the introduction of amine groups onto the scPCL substrates. The [N]/[C] ratio, as determined from sensitivity factor-corrected C 1s and N 1s spectral area, was found to be around 0.043 (Figure 4.3c).
Figure 4.1. ATR-FTIR spectra of pristine scPCL, scPCL-NH₂, and scPCL-g-P(GMA)₂.

\[
\text{scPCL-C}^\text{CONH-CH₂-NH-CO(CH₃)C-Br} + \text{Cu(I)} \rightarrow \text{scPCL-C}^\text{CONH-CH₂-NH-CO(CH₃)C-} \cdot + \text{Cu(II)-Br}
\]

\[
\text{scPCL-C}^\text{CONH-CH₂-NH-CO(CH₃)C-GMA} \cdot \rightarrow \text{scPCL-C}^\text{CONH-CH₂-NH-CO(CH₃)C-GMA-Br} + \text{Cu(I)}
\]

Figure 4.2. The growth of GMA polymer brushes on bromine-immobilised scPCL surfaces.

The grafting of polymer brushes onto the scPCL substrate proceeded via surface-initiated ATRP (Figure 4.2). After the immobilisation of the bromine initiator on the scPCL surface, the ATRP is initiated by the Cu (I) catalyst, which catalyses the formation of a PCL-alkyl radical and a Cu(II)-Br transition complex. The radical attacks an available GMA monomer, thereby transferring the radical onto scPCL-alkyl-GMA. Swift deactivation of this intermediate occurs when it reacts with Cu(II)-Br to form scPCL-alkyl-GMA-Br with the regeneration of the Cu(I) catalyst. The process repeats...
itself, adding a free GMA monomer to the growing chain until all GMA is consumed or when reaction is stopped (Figure 4.2). For the polymerisation of GMA to proceed at an optimal rate, the density and uniformity of the initiator has been found to be crucial [14]. As aforementioned a condensation reaction between BIBB and the aminolysed scPCL surface results in the immobilisation of a Br terminal end on the scPCL surface. In the XPS spectrum, the successful immobilisation of the BIBB initiator was marked by the appearance of three additional signals at the binding energies of 70, 189, and 256 eV in the wide scan spectra, corresponding to Br 3d, Br 3p, and Br 3s, respectively (Figure 4.3a). The [Br]/[C] ratio, as determined from the Br 3d peak area and C 1s core-level spectra area, was found to be about $3.17 \times 10^{-2}$ (Figure 4.3d, d’), which is comparable to a previous study using BIBB as ATRP initiator [15].
Figure 4.3. XPS surface analysis of pristine scPCL and functionalised scPCL surfaces. (a) Wide scan spectra of scPCL, scPCL-NH$_2$, and scPCL-Br. The C 1s core-level spectra and curve-fitted components of (b) pristine scPCL, (c) scPCL-NH$_2$, and (d) scPCL-Br are shown. (c’) The N 1s core-level spectrum of scPCL-NH$_2$ and (d’) deconvoluted peaks of the Br 3d core-level spectrum of scPCL-Br are presented in smaller insets. (e) The wide scan and (f) C 1s core-level spectra of the scPCL-g-P(GMA) after 3 h ATRP reaction with GMA.
After the successful immobilisation of the Br initiator, the GMA grafting was commenced using the GMA monomer, Cu(I)Br catalyst, Cu(II)Br₂ deactivator and 2,2’-Bipyridine (Bpy) ligand. The molar ratio of GMA:CuBr:CuBr₂:Bpy was controlled at 100:1.0:0.2:2.0 in the 1 h or 3 h reaction to produce scPCL-g-P(GMA)₁ and scPCL-g-P(GMA)₂ surfaces, respectively. ATR-FTIR analysis of the GMA-grafted scPCL substrates revealed the characteristic peaks of epoxide groups at 903, 846, and 756 cm⁻¹ (Figure 4.1) [16]. As XPS collects surface chemistry data from only the top surface, the disappearance of the amide-related peaks found in scPCL-NH₂ also hints at the extensive coverage of the scPCL surface with thick GMA polymer brushes. The chemical composition of the grafted P(GMA) brushes was also analysed using XPS. A relatively higher O 1s peak in the wide scan spectrum and larger C-O (286.2 eV) component in the C 1s core-level spectrum observed for P(GMA)-grafted-scPCL surfaces, when compared to pristine scPCL, is a result of the abundance of C-O bonds in the GMA (Figure 4.3e, f). During the chain growth in ATRP, the scPCL-P(GMA)· radical reacts with the Cu(II)-Br intermediate complex to yield a Br-terminated chain (Figure 3.1, Figure 4.2). This accounts for the presence of the Br 3d and Br 3p signals in the wide scan spectrum of scPCL-g-P(GMA) (Figure 4.3e). The decrease in [Br]/[C] ratio of 4.72 x 10⁻³ after 3 h of ATRP, as compared to the [Br]/[C] ratio of 3.17 x 10⁻² in PCL-Br, was brought about by the P(GMA) chain growth. The calculated area ratio of (C-H):(C-O):(O=C-O) is approximately 3.1:3.0:1.0, which is close to the theoretical value of the GMA unit structure of 3:3:1 (Figure 4.3f).

Calculations of grafting yield (GY) via weight measurements can be used to determine the kinetics of polymer chain growth in ATRP [17], [18]. A linear increase in GY of the P(GMA) chains as a function of polymerization time could be observed (Figure 4.4). We have thus demonstrated a well-defined first-order P(GMA) grafting reaction that can be controlled by varying the reaction time. In this study, the ATRP polymer chain extension could proceed *ad libitum*. ATRP is an equilibrium process of the activation/deactivation of a propagating radical. Premature chain termination in ATRP usually causes excessively-high radical concentration, resulting in large reverse reaction kinetics (Figure 4.2) [19]. The addition of the higher state transition metal Cu(II) has allowed good control of the reaction [20]. However, the molecular weight and molecular weight distribution of the surface-grafted GMA could not be determined.
with sufficient accuracy without precise cleavage of the grafted P(GMA) chains from the scPCL substrates.

**Figure 4.4.** The grafting yield is the gain in weight per unit area of the scPCL substrate after 1 h or 3 h of ATRP.

4.3.2. Conjugation of Gelatin on P(GMA)-grafted scPCL Substrates

The conjugation of gelatin onto the P(GMA)-grafted scPCL substrates was carried out at 40 °C in physiological buffer PBS. In general, mild reaction conditions are favoured for reactions involving biological molecules (i.e. proteins or peptides). In addition to denaturation of protein native conformation, peptide backbone deamidation and acid-catalysed peptide backbone cleavage can occur in low pH [21], [22] and high temperature [23]. As PCL is a semi-crystalline polymer with a relatively low melting temperature ($T_m$) of around 60 °C, it is well-suited for such epoxide conjugation reactions [3].

After the 24 h gelatin conjugation reaction, ATR-FTIR and XPS were performed to evaluate the scPCL substrates. The 3 addition absorption bands in the FTIR spectrum that were previously absent in the spectra of the GMA-grafted scPCL surfaces, namely 3295 cm$^{-1}$, 1642 cm$^{-1}$, and 1543 cm$^{-1}$, are characteristic bands common in proteins (Figure 4.5). The broad band at 3200 – 3500 cm$^{-1}$ corresponds to the IR absorption by
the (O-H) bond within the carboxyl (-COOH-) group in proteins. The absorption peak associated with (N-H)/(C=O) stretching vibrations in amide I at 3295 cm\(^{-1}\) was previously present in scPCL-NH\(_2\) surfaces at 3335 cm\(^{-1}\) as a weak band (Figure 4.1). With the abundance of amide (-O=C-NH-) functional groups in gelatin, this absorption band reappeared as a broad, strong band in gelatin-conjugated PCL surfaces. Likewise, the absorption band at 1543 cm\(^{-1}\) due to amide II (N-H) bending vibrations, previously manifested as a small peak in scPCL-NH\(_2\), is present as a large band. Another characteristic amide I band at 1642 cm\(^{-1}\), usually the most intense absorption band in proteins, is associated mainly with (C=O) stretching vibrations within the amide functional groups (O=C-NH) [24]. Hence, the appearance of these characteristic absorption bands in the FTIR spectrum suggests that the scPCL substrates were successfully functionalised with gelatin.

In the XPS spectrum of scPCL-g-P(GMA)2-c-gelatin surfaces, the appearance of a strong N 1s signal in the wide scan spectrum of the gelatin-conjugated substrates was evident (Figure 4.6a). Additional peak components in the curve-fitted C 1s core-level spectrum are attributable to the (C-N) and (O=C-NH) bonding at 285.5 eV and 288.2 eV, respectively (Figure 4.6b). When determined from sensitivity factor-corrected N 1s and C 1s core-level spectral area, the [N]/[C] ratio can be used to assess the relative amount of immobilized gelatin. The [N]/[C] ratios of the functionalised substrates prepared with different reaction times, increased from 0.169 in scPCL-g-P(GMA)1-c-gelatin to 0.202 in scPCL-g-P(GMA)2-c-gelatin, indicating that the P(GMA) brushes from 3 h of ATRP provided more attachment sites for gelatin conjugation. Hence, the XPS data provides further evidence that the amount of the immobilised gelatin is tunable through the ATRP reaction time, which varies the amount of P(GMA) brushes grafted on the scPCL substrates.
Figure 4.5. ATR-FTIR spectra of pristine scPCL, scPCL-\textit{g}-P(GMA)$_2$, and scPCL-\textit{g}-P(GMA)$_2$-c-gelatin.

Figure 4.6. XPS surface analysis of scPCL-\textit{g}-P(GMA)$_2$-c-gelatin surfaces. (a) Wide scan spectrum and the (b) C 1s core-level spectrum and its curve-fitted components.
4.3.3. Physical Adsorption of Gelatin onto scPCL Substrates

While the chemical conjugation of gelatin onto P(GMA)-grafted scPCL surfaces is mediated through stable covalent bonding, studies were also conducted to investigate if physical adsorption of gelatin onto non-functionalised scPCL surfaces was able to produce stable coatings of gelatin. Physisorption is the common technique used for preparation of gelatin-coated substrates for cell culture (e.g. on glass coverslips and tissue culture plastic). Rouxhet et al. has studied the adsorption of collagen onto PCL surfaces over 2 h and detected the presence of the protein [25]. However, the stability and retention of the adsorbed protein on scPCL over time in cell culture medium is not known. This will be crucial for the use of scPCL substrates for vascular engineering.

Hence, the adsorption of gelatin on the scPCL substrate was performed using the same concentration used in ATRP-mediated conjugation. The substrates were subsequently immersed in endothelial cell culture medium for 7 days. ATR-FTIR spectrum of the scPCL substrate after physisorption of gelatin revealed the small amide-associated bands at 1543 cm\(^{-1}\) (N-H bending vibrations) and 1650 cm\(^{-1}\) (C=O stretching vibrations) (Figure 4.7). These bands are much smaller than that observed in scPCL-g-P(GMA)-c-gelatin substrates (Figure 4.5). Following the day 7 immersion in cell culture medium, these bands disappeared, suggesting that the gelatin had desorbed in the cell culture medium (Figure 4.7). Taken together, the physisorption of gelatin on the scPCL substrates led to comparatively little gelatin detected on the substrate surface, and the gelatin was not retained on the surface in cell culture medium.
4.3.4. Static Water Contact Angles of Functionalised PCL Substrates

Static water contact angles (WCAs) of the functionalised scPCL substrates were taken using the sessile drop method, and used as an indicator of surface hydrophilicity (Table 4.1). The WCA of the pristine scPCL surface expectedly exhibited a hydrophobic character with a WCA of $93 \pm 2^\circ$. The presence of amine groups after aminolysis reaction led to a decrease in WCA to $66 \pm 3^\circ$, indicating a more hydrophilic scPCL surface. Upon grafting of P(GMA) brushes, the average WCAs of the scPCL-g-P(GMA)1 and scPCL-g-P(GMA)2 surfaces were found to be unchanged at $62 \pm 4^\circ$ and $61 \pm 5^\circ$, respectively. Due to the presence of hydrophilic epoxide groups, similar WCA readings of P(GMA)-grafted surfaces have been reported [26].

A substantial decrease in WCA was observed after the conjugation of gelatin, a hydrolysed form of collagen that is a highly hydrophilic biomolecule. The conjugation of gelatin onto scPCL-g-P(GMA)1 surfaces resulted in a WCA as low as $37 \pm 5^\circ$, and
with a higher density of grafted P(GMA) polymer brushes, the WCA of scPCL-g-P(GMA)2-c-gelatin decreased even further to \( 35 \pm 4 ^\circ \). The open, unfolded secondary structure of gelatin with abundant hydrogen bonds exposed is thought to be the main contributing factor in the strong hydrophilic nature of gelatin \([27]\). Gelatin is comprised of only a small fraction of hydrophobic amino acids e.g. valine (Val), leucine (Leu), tryptophan (Trp) \([27]\). The hydroxyl groups (-OH) generated in the ring-opening reaction of epoxide groups by the coupling of gelatin further contributed to the decrease in hydrophilicity for the gelatin-functionalised scPCL surfaces. WCA readings of physically adsorbed of gelatin onto pristine scPCL surfaces after 24 h were also taken. The WCA of \( 57 \pm 4 ^\circ \) is a marked decrease from the WCA of scPCL, but demonstrated that the density of gelatin on the scPCL surface did not reach the levels at that of chemically conjugated PCL surfaces. This is consistent with FTIR data obtained after 24 h adsorption of gelatin onto pristine scPCL surfaces (Figure 4.5). Comparatively, gelatin coating of glass coverslips for 24 h yielded very hydrophilic surfaces \( (24 \pm 1 ^\circ) \), which reflect substantial gelatin physisorption and unfolding of gelatin on the glass surface.

\[
\text{Table 4.1. Static water contact angle values for functionalised scPCL substrates.}
\]

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Reaction time (h)</th>
<th>Water contact angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin-coated glass</td>
<td>24</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>Pristine scPCL</td>
<td>-</td>
<td>93 ± 2</td>
</tr>
<tr>
<td>scPCL-NH₂</td>
<td>1</td>
<td>66 ± 3</td>
</tr>
<tr>
<td>scPCL-g-P(GMA)1</td>
<td>1</td>
<td>62 ± 4</td>
</tr>
<tr>
<td>scPCL-g-P(GMA)2</td>
<td>3</td>
<td>61 ± 5</td>
</tr>
<tr>
<td>scPCL-g-P(GMA)1-c-gelatin</td>
<td>24</td>
<td>37 ± 5</td>
</tr>
<tr>
<td>scPCL-g-P(GMA)2-c-gelatin</td>
<td>24</td>
<td>35 ± 4</td>
</tr>
<tr>
<td>Physisorbed gelatin on scPCL</td>
<td>24</td>
<td>57 ± 4</td>
</tr>
</tbody>
</table>

4.4 Template Polymerisation of Electroactive PPy onto ePCL Scaffolds (Aim 2)

In the second part of material development in this chapter, a nanofibrous PCL scaffold with both electrical and anti-thrombotic properties was developed. Electrospinning of the solution of 6 wt % PCL dissolved in HFIP was performed to produce fibrous ePCL scaffolds. Electrospinning of 2, 4, and 8 wt % PCL in HFIP was also performed
To confer electroactivity onto the ePCL scaffolds, the PPy was coated on the ePCL fibres via the process of template polymerisation (*Chapter 3, Section 3.2.5*). After immersing the ePCL scaffolds in the aqueous reaction buffer comprising of pyrrole monomers with heparin (at 1:1 wt. ratio to pyrrole) or 1 M HCl (Cl\(^-\)) dopants, and using ammonium persulfate (APS) as the oxidising agent, the coating of PPy onto the PCL template could first be detected by a colour change of the ePCL scaffolds (Figure 4.8).

![Figure 4.8. Colour change observed after 4 h template polymerisation of PPy on pristine ePCL (left) from white to the black of the PPy-coated ePCL (right).](image)

4.4.1. Scanning Electron Microscopy of Polypyrrole-coated ePCL Scaffolds

After the observation of colour change on the ePCL scaffolds after template polymerisation of PPy, scanning electron microscopy (SEM) was conducted to examine the morphology of the PPy coating on the fibres. SEM imaging first shows the typical micro-architecture of randomly-oriented ePCL fibres before template polymerisation (Figure 4.9a). After 4 h of polymerisation with PPy, the presence of non-uniform aggregates can be observed on undoped PPy-coated fibres (ePCL/PPy) and 1 M chloride (Cl\(^-\)) doped PPy-coated ePCL fibres (ePCL/PPyCl) (Figure 4.9b, d). On the contrary, heparin doping (1:1 wt. ratio) of PPy resulted in a smooth, uniform coating over the ePCL fibres (ePCL/PPyHEP) (Figure 4.9c). With increasing concentration of PCL in HFIP solution, an increase in the average diameter of the pristine ePCL fibres could be observed in SEM (*Appendix I, Figures A1 and A2*).
Figure 4.9. SEM images of (a) pristine ePCL, (b) ePCL/PPy, (c) ePCL/PPyHEP, and (d) ePCL/PPyCl scaffolds. (c’) Delamination of PPy coating performed by stretching shows thickness of coating is approximately 260 nm. (d’) Close-up magnification of Cl-doped PPy aggregates on ePCL fibres. Scale bar = 10 µm, unless stated otherwise.

It can be postulated that the different morphological outcomes of PPy is influenced by the different polymerisation conditions and the type of dopant used. The formation of PPy aggregates on polyester fibrous templates after oxidative polymerisation has also been reported in a previous study, whereby high reactant concentrations resulted in the observation of aggregates on poly(lactic-co-glycolic acid) (PLGA) fibres [28]. Shi et al. has studied the oxidative polymerisation of pyrrole within a polymer matrix and found that the continuous network of PPy within the polymer matrix is formed first by PPy aggregates and then the continued growth by the aggregates into a percolating network [29]. These studies likely provide an explanation for the observation of aggregates on ePCL/PPy and ePCL/PPyCl fibres in the current study. As the scaffolds were washed thoroughly by ultrasonication after polymerisation to remove unbound PPy, these aggregates were known to be tightly bound to the PCL. This could have
formed due to PPy growth occurring in clusters along the ePCL fibre, which was preceded by interaction clusters between pyrrole monomers and the regions on the fibre.

A distinctive smooth appearance of heparin-doped PPy coating is unique among the different doped PPy coatings performed in this study. In agreement with this study, Shi et al. has obtained similarly smooth PPy nanowires in situ using heparin as the molecular template [30]. The use of heparin as a novel long-chain molecular template for the growth of nanowires was the goal of their study, but in this study, the aim was to also utilize heparin for its anti-thrombotic properties in subsequent studies. XPS analysis and electrical properties of ePCL/PPyHEP scaffolds, together with the other PPy-coated ePCL scaffolds, were measured and detailed in the following sections to yield further insights into heparin-doping mechanism.

4.4.2. XPS Analysis of PPy-coated ePCL Scaffolds

X-ray photoelectron spectroscopy (XPS) was performed to verify the presence of heparin and Cl on the ePCL/PPyHEP and ePCL/PPyCl scaffolds, respectively. In the wide scan spectrum of ePCL/PPyHEP, the presence of S 2s and S 2p peaks corresponding to the sulphur-containing moieties of heparin was observed [31] (Figure 4.10). In the C 1s core-level spectra, curve-fitted peak components attributed to C-O and C=O bonds were common in all the scaffolds. However, these carbonyl bonds may have a different origin in the different scaffolds as XPS is a surface technique. C-O and C=O signals from ePCL/PPy and ePCL/PPyCl could be due to detection of exposed PCL, evident from morphological appearance of discrete PPy aggregates (Figure 4.9b), while in ePCL/PPyHEP, they could have originated from the heparin in the uniform PPy coating (Figure 4.9c). The peak associated with the C-N bond was present in ePCL/PPy, whilst a higher peak intensity was obtained for overlapping C-N/C-S peaks in ePCL/PPyHEP, due to the presence of heparin [31] (Figure 4.10). In addition, the curve-fitted C 1s core-level spectrum of ePCL/PPyHEP revealed a peak component at 289.2 eV as a result of carboxylic moieties (O-C=O) present in heparin [32]. Further analysis of the S 2p core-level spectrum of ePCL/PPyHEP sulfoxide saw the deconvolution of the curve fitted peak into OSO\(_3\) and NSO\(_3\) components [33]. The Cl 2p core-level spectrum of ePCL/PPyCl could be deconvoluted into peak components of Cl 2p\(_{3/2}\) and Cl 2p\(_{1/2}\) [34].
Figure 4.10. XPS surface analysis of ePCL/PPy, ePCL/PPyHEP and ePCL/PPyCl. Wide scan spectra and the C 1s core-level spectra and their curve-fitted peak components are shown. Core-level spectra of the S 2p and Cl 2p peaks were obtained for ePCL/PPyHEP and ePCL/PPyCl, respectively.

4.4.3. Electrical Properties of PPy-coated ePCL Scaffolds

The bulk electrical conductivities of the PPy-coated ePCL scaffolds was obtained using 4-point probe measurements (Section 3.3.5), and the electrochemical behaviour of the scaffolds in physiological fluid was determined in cyclic voltammetry (Section 3.3.6). For the characterisation of electrical properties, ePCL scaffolds that have undergone 4 h and 16 h template polymerisation of PPy were investigated. Also, ePCL/PPyHEP polymerised with different heparin dopant amounts (at heparin:pyrrole wt. ratios of 0.2, 0.5, 1, and 3) were performed. It was found that all ePCL/PPyHEP scaffolds exhibited significantly higher conductivities than ePCL/PPy scaffolds (4 h polymerization: AVG. 1.0 x 10^{-3} S/cm; 16 h polymerization: AVG. 5.5 x 10^{-4} S/cm) (Figure 4.11). In general,
the conductivities of ePCL/PPyHEP scaffolds increased with the amount of heparin used (Figure 4.11). The conductivities of the scaffolds after 4 h polymerisation using heparin-doped PPy range from av. $2.3 \times 10^{-3}$ S/cm for ePCL/PPyHEP0.2 to av. $1.9 \times 10^{-2}$ S/cm for ePCL/PPyHEP3. 16 h polymerisation reactions yielded ePCL/PPyHEP scaffolds with conductivities between av. $1.4 \times 10^{-3}$ S/cm (ePCL/PPyHEP0.2) and av. $2.2 \times 10^{-2}$ S/cm (ePCL/PPyHEP3). The highest electrical conductivity observed in the study was for the ePCL/PPyCl scaffold from 4 h PPy polymerisation at $2.4 \times 10^{-2}$ S/cm.

It is evident from the data that while increasing the heparin:pyrrole wt. ratios had an impact on the electrical conductivity of the scaffold, the polymerisation time did not influence the scaffold electrical properties with any apparent trend.

![Graph showing bulk conductivities of PPy-coated ePCL scaffolds](image)

**Figure 4.11.** Bulk electrical conductivities of the PPy-coated ePCL fibrous scaffolds after 4 h and 16 h polymerisation with undoped PPy (ePCL/PPy), PPy doped with heparin at various heparin:pyrrole wt. ratios (ePCL/PPy-HEP0.2 – HEP3), and chloride (ePCL/PPyCl), as measured using a 4-point probe station. *$p < 0.05$; **$p < 0.001$.

The reported bulk conductivities of the hybrid ePCL/PPyCl and ePCL/PPyHEP scaffolds lie in the semi-conducting range. Measured scaffold bulk conductivities are
Development of PCL and PPy Nanomaterials

in the similar range or higher than other reported hybrid polymer/PPy systems. Kai et al. has fabricated hybrid gelatin/PPy nanofibrous scaffolds by electrospinning gelatin dispersed with PPy (up to 30% wt. ratio), and found the average conductivity of the scaffolds to be $3.7 \times 10^{-4}$ S/cm, which is 2 orders of magnitude lower than the ePCL/PPy-HEP1 scaffolds [35]. A poly(L-lactide)/PPy composite membrane that entailed coating of PPy onto poly(L-lactide) membranes exhibited bulk conductivity in the range of $10^{-2}$ S/cm [36], similar to that our scaffolds. It is interesting that despite the different morphology of the PPy coating between the ePCL/PPyHEP and ePCL/PPyCl scaffolds (Figure 4.9), they exhibit similar conductivity. This can only be explained by the presence of a continuous, percolating networking of PPy coating on the ePCL/PPyCl fibres alongside with the PPy aggregates observed. When comparing both ePCL/PPy and ePCL/PPyCl scaffolds, although both scaffolds have PPy aggregates, the much higher conductivity in the anionic doped state, where the PPy molecular backbone is comprised of charged polarons/bipolarons and displays a lower electronic bandgap, is well known (reviewed in Chapter 2, Section 2.3.2) [37], [38]. In addition to 4-point probe measurements, 2-point I-V curves obtained for scaffolds are also in agreement with the bulk conductivity data (Appendix II, Figure A3)

The electrochemical behaviour of the PPy-coated ePCL scaffolds in physiological buffer was investigated using a 3-electrode cyclic voltammetry (C-V) configuration. The PPy-coated ePCL scaffolds were used as the working electrodes immersed in 1x phosphate buffered saline (PBS) solutions. Cyclic voltammograms were collected and analysed for the presence of redox peaks that are indicative of electroactivity. Both anodic (oxidation) and cathodic (reduction) current peaks could be observed for ePCL/PPyHEP scaffolds fabricated from 4 h and 16 h polymerization reactions (Figure 4.12). Redox current peaks were also detected in ePCL/PPyCl scaffolds, but only for scaffolds fabricated through 4 h polymerisation (Figure 4.12). On the other hand, redox activity was largely absent in undoped PPy-coated ePCL/PPy scaffolds (Figure 4.12). These findings recapitulate the mechanism driving the redox reactions of doped PPy. In PPy doped with Cl⁻ ions after 4 h of polymerisation, the flow of electrons into the PPy at the cathodic potential (-0.46 V) balanced out the positive charge on the PPy backbone and displaced the Cl⁻ ions into the PBS solution (Figure 4.12a). When the polarity was reversed and the anodic potential was on the PPy (0.05 V), the outflow of electrons resulted in PPy regaining the positive charge on its backbone and caused the
re-doping of PPy with Cl⁻ and other anions (e.g. PO₄³⁻) from the solution. The loss of redox activity in ePCL/PPyCl after 16 h polymerisation is suspected to be due to the over-oxidation of PPy due to prolonged oxidative reaction time with the use of APS, a strong oxidising agent. Over-oxidation of PPy leads to the irreversible formation of carboxyl groups on the pyrrole ring, interrupting the -NH₂- positive charge and hence, loss of redox activity [39]. Further supporting this notion of over-oxidation was the decrease in electrical conductivity observed for ePCL/PPyCl scaffolds polymerised for 16 h when compared to scaffolds polymerised for 4 h (Figure 4.11) [40].

![Figure 4.12](image)

**Figure 4.12.** Cyclic voltammograms of scaffolds fabricated with (a) 4 h polymerisation and (b) 16 h polymerisation reactions obtained at scan rates of 20 mVs⁻¹ from -0.8 V to 0.4 V in 1x phosphate buffer saline (PBS). The anodic and cathodic peak currents and potentials are shown in tables below the corresponding voltammograms.

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Epa /V</th>
<th>ipa /μA.cm⁻²</th>
<th>Epc /V</th>
<th>ipc /μA.cm⁻²</th>
<th>ipc/iap</th>
<th>Scaffold</th>
<th>Epa /V</th>
<th>ipa /μA.cm⁻²</th>
<th>Epc /V</th>
<th>ipc /μA.cm⁻²</th>
<th>ipc/iap</th>
</tr>
</thead>
<tbody>
<tr>
<td>ePCL/PPyHEP</td>
<td>-0.11</td>
<td>2.95</td>
<td>-0.58</td>
<td>-2.41</td>
<td>1.22</td>
<td>ePCL/PPyHEP</td>
<td>-0.09</td>
<td>1.79</td>
<td>-0.54</td>
<td>-1.43</td>
<td>1.25</td>
</tr>
<tr>
<td>ePCL/PPyCl</td>
<td>0.05</td>
<td>3.47</td>
<td>-0.46</td>
<td>-2.27</td>
<td>1.53</td>
<td>ePCL/PPyCl</td>
<td>0.05</td>
<td>3.47</td>
<td>-0.46</td>
<td>-2.27</td>
<td>1.53</td>
</tr>
</tbody>
</table>

In the case of ePCL/PPy-HEP, the large polyanionic dopant is integrated with the PPy backbone. Thus, redox activity was largely due to the movement of cations from PBS (e.g. K⁺, Na⁺) into the PPy/heparin backbone during reduction at cathodic potentials (-0.58 for 4 h scaffolds / -0.54 V for 16 h scaffolds) and the discharge of cations at anodic potentials (-0.11 for 4 h scaffolds / -0.09 V for 16 h scaffolds). Unlike the chloride-doped PPy, heparin-doped PPy did not appear to be over-oxidised in the 16 h reaction, and was able to retain electroactivity (Figure 4.12b). When comparing ePCL/PPyHEP with ePCL/PPyCl, the peak currents are similar in magnitude, but the lower cathodic and anodic potentials for ePCL/PPyHEP indicate that the heparin-doped PPy requires
a larger reducing potential. The ratio of the anodic peak current/cathodic peak current \((i_{pa}/i_{pc})\) is slightly more than 1 (~1.2 for ePCL/PPyHEP and ~1.5 for ePCL/PPyCl), indicating that the redox reactions for these scaffolds are quasi-reversible, with ePCL/PPyHEP scaffolds exhibiting better characteristics of reversible diffusion controlled redox activity.

### 4.4.4. Mechanical Properties of PPy-Coated ePCL

The mechanical properties of the PPy-coated ePCL scaffolds were investigated. The tensile behavior for the uncoated ePCL and all PPy-coated ePCL meshes was fairly similar, with stress-strain curves that represent the typical elastic deformation of randomly-oriented PCL fibres (Figure 4.13a) [41]. The extension of the scaffolds occurred in 2 stages, as characterised by the initial sharp increase in the slope that is reported as the Young’s modulus in this study (Figure 4.13b), followed by a smaller rate of increase as a function of strain, not unlike the profile of metals undergoing strain hardening. But in the case of electrospun fibres, the initial increase is due to the simultaneous processes of randomly-aligned fibres rearranging themselves and being stretched during uniaxial tensile test. The decrease in the slope in the 2nd stage is caused by the gradual fracturing of fibres simultaneously during rearrangement/strain, and it persists until failure at break [42]. The Young’s moduli of ePCL/PPyHEP and ePCL/PPyCl are on average, 6.8 MPa and 6.6 MPa, respectively, and significantly improved over pristine ePCL scaffolds \((p < 0.05)\) (Figure 4.13b). However, PPy-coated ePCL scaffolds exhibited lower failure strains as compared to ePCL scaffolds (Figure 4.13a). In comparison, reported values of the average elastic moduli of vascular tissues are: 1.54, 3.11, and 6.8 MPa for femoral arteries [43], femoral veins [43], and saphenous veins [44], respectively. Therefore, this study indicates that their stiffness of our PPy-coated ePCL scaffolds is similar to certain vascular tissues.

One other important criterion is for the tensile strength of the vascular graft material to be comparable to that of the native vascular tissues. Overall, the tensile strengths measured for our scaffolds range from 4.5 – 5.7 MPa (Figure 4.13b), which makes them well-suited for vascular graft applications after further development. Reports in the literature have suggested that a tensile strength of above 260 kPa is required for a vascular graft to withstand \textit{in vivo} blood pressure [45].
Figure 4.13. Mechanical properties of ePCL and PPy-coated ePCL/PPy scaffolds. (a) Average stress-strain curves and (b) Young’s moduli and ultimate tensile strength of ePCL and ePCL coated with undoped PPy, PPyHEP and PPyCl after 4 h polymerisation reaction (*p < 0.05).

4.4.5. Quantification of Heparin on ePCL/PPyHEP Scaffolds

As a polyanionic macromolecular chain, heparin has been proposed to be entrapped in between PPy chains and unable to diffuse in and out of the PPy coating like small anion dopants can during redox state changes [46]. This study attempted to quantitate the amount of heparin present in the PPy coating on the ePCL fibres using the Toludine blue assay and XPS elemental composition analysis. XPS analyses the surface chemistry the material (collecting data from the underlying 10 nm). On the other hand, the Toluidine blue assay detects only exposed heparin on the PPy coating that it can
bind to, which is the physiologically-relevant heparin that clotting factors can have access when the coating functions as an anti-thrombosis surface.

Heparin was measurable in all ePCL/PPyHEP scaffolds with the Toluidine blue assay (Table 4.2). However, changes in heparin:pyrrole wt. ratios during polymerisation did not result in significant differences in the amount of heparin exposed on the PPy-coated ePCL fibre surface. Elemental composition analysis performed during XPS on these PPy-coated ePCL fibres, on the other hand, showed an increasing S/N peak ratio as heparin:pyrrole wt. ratio increases. As N is present in every repeat unit of PPy, and in every alternate repeat unit of heparin, and S is present only in heparin, this suggests that more heparin was doped into the PPy coating (Table 4.2).

**Table 4.2.** Amount of measurable heparin by Toluidine blue on ePCL/PPy meshes and XPS elemental composition of meshes

<table>
<thead>
<tr>
<th>Sample</th>
<th>Immobilised heparin on surface (µg/cm²) a</th>
<th>C 1s</th>
<th>N 1s</th>
<th>O 1s</th>
<th>S 2p</th>
<th>Cl 2p</th>
<th>S/N ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>ePCL/PPy</td>
<td>-</td>
<td>72.55</td>
<td>11.55</td>
<td>15.90</td>
<td>N.D. b</td>
<td>N.D.</td>
<td>-</td>
</tr>
<tr>
<td>ePCL/PPyCl</td>
<td>-</td>
<td>76.36</td>
<td>7.79</td>
<td>15.62</td>
<td>N.D.</td>
<td>0.24</td>
<td>-</td>
</tr>
<tr>
<td>ePCL/PPyHEP0.2</td>
<td>7.9 ± 0.9</td>
<td>72.55</td>
<td>6.71</td>
<td>19.19</td>
<td>1.55</td>
<td>N.D.</td>
<td>0.231</td>
</tr>
<tr>
<td>ePCL/PPyHEP0.5</td>
<td>7.9 ± 0.3</td>
<td>70.26</td>
<td>7.53</td>
<td>20.42</td>
<td>1.79</td>
<td>N.D.</td>
<td>0.238</td>
</tr>
<tr>
<td>ePCL/PPyHEP1</td>
<td>7.4 ± 1.4</td>
<td>68.41</td>
<td>7.96</td>
<td>21.69</td>
<td>1.94</td>
<td>N.D.</td>
<td>0.244</td>
</tr>
</tbody>
</table>

a Determined by Toluidine blue assay. Expressed as mean ± S.D
b Not detected

**4.4.6. Proposed Mechanism of Heparin Doping and Assisted PPy Coating on ePCL Fibres**

Integrating the data gathered from XPS surface chemistry, Toluidine blue assay and electrical conductivity measurements, the mechanism by which the heparin assisted the formation of the uniform PPy coating in ePCL/PPyHEP scaffolds was proposed (Figure
While undoped PPy and PPyCl formed from the growth of pyrrole monomers adsorbed onto discrete regions of the PCL fibre surface and culminated in the particulate appearance observable in SEM, the PPyHEP coating was formed differently. The interactions of the linear polyanionic heparin chains with the PCL carbonyl groups through H-bonding, alongside with the interactions between pyrrole monomers and the heparin chain, set the stage for alternate layers of polypyrrole and heparin to be formed during polymerisation. When the ratio of heparin dopant to pyrrole was increased, more heparin chains became entrapped within the PPy coating, as reflected in XPS S/N ratios. The amount of accessible heparin on the surface measured by Toluidine blue, more a function of the surface area of the PPy coating, remained unchanged. The heparin/pyrrole wt. ratio of 3 appears to result in the most electrically conductive PPy coating (Figure 4.11), as under these conditions, the molar ratio of each heparin unit:pyrrole monomer is estimated to be around 1:1 (MW of heparin monosaccharide unit: ~190 – 220 g/mol, MW of pyrrole: 67 g/mol). This may have resulted in the packing of long, continuous, and linear PPy networks within the PPyHEP coating that turned out to be the most conductive. When lesser amount of heparin is used (e.g. heparin/pyrrole wt. ratio of 0.2), insufficient templating prevents the pyrrole monomers from forming such well-aligned, networks during growth. Hence, the conductivity of the coating decreases (Figure 4.14).

![Figure 4.14. The proposed mechanism of template polymerisation of PPyHEP onto ePCL fibres.](image)
4.5 Facile Synthesis of Heparin-Stabilised PPy Nanoparticles for Effective VEGF Conjugation (Aim 3)

The third aspect of material development for vascular tissue engineering entails the delivery of the angiogenic factor VEGF. Owing to its cytocompatibility [7], [36], [47], synthesis in aqueous solvents, and ease of doping with biological agents during polymerisation [48]–[50], PPy was studied as the material for a nanoparticulate system to deliver VEGF. PPy particles in sub-micrometer sizes can be synthesised via dispersion polymerization in the presence of polymeric stabilisers such as polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP) [51]–[53]. Using this method, the size and dispersity of the PPy nanoparticles can be determined by tuning the concentration of the stabiliser used; owing to increasing steric effects, a higher concentration of PVA or PVP used had a decreasing effect on nanoparticle size. As heparin is a long chain polymer, the possibility of using heparin as the stabiliser during PPy nanoparticle synthesis was investigated.

4.5.1. Size-Controllable Synthesis of Heparin-Stabilised PPy Nanoparticles

Synthesis of PPy nanoparticles was carried out from pyrrole monomers with varying concentrations of heparin and using FeCl$_3$ as the oxidative polymerising agent. When the heparin concentration was increased from 2.5 – 10 w/v %, the diameter of the resulting PPy nanoparticles was reduced from 275 to 120 nm (Figure 4.15). For comparison purposes, PVA-stabilised PPy nanoparticles were also synthesised. Using 2 wt % PVA in solution, PPy nanoparticles with a uniform diameter of ~70 nm were obtained (Figure 4.15a). The concentration of heparin used as stabiliser had an effect on the dispersity of the PPy nanoparticles, which is evident in the particle size distribution and polydispersity index (PDI) obtained using dynamic light scattering (DLS) measurements (Figure 4.15b). While the use of 5 – 10 w/v % heparin during synthesis generated monodisperse size distributions in the resulting PPy nanoparticles with PDIs of ~0.15, PPy nanoparticles synthesised in 2.5 w/v % heparin in solution had a bimodal distribution with a high PDI value of 0.413 (Figure 4.15b). The concentration of heparin stabiliser used in synthesis provided us with a direct means of controlling the size and dispersity of the resulting PPy nanoparticles. We also performed heparin-stabilised synthesis of PPy nanoparticles using APS, a stronger oxidant, and obtained a
similar trend of decreasing nanoparticle sizes when heparin concentration was increased (Figure 4.16). The size of PPy nanoparticles as a function of heparin concentration used as stabiliser in both FeCl$_3$- and APS-oxidised reactions show non-linear correlations. As we found heparin at 10 w/v % in water to be at its solubility limit, this was the maximum concentration at which we can use heparin as stabiliser.

**Figure 4.15.** Effect of heparin concentration on size and morphology of PPy nanoparticles. (a) The average diameter of the PPy nanoparticles, measured using DLS, as a function of heparin stabiliser concentration during FeCl$_3$-activated synthesis. PVA-stabilised PPy nanoparticles were synthesized for comparison. (b) The corresponding size distribution histograms and polydispersity index (PDI) are shown for nanoparticles synthesised in 2.5 w/v % heparin, 10 w/v % heparin and 2 wt % PVA.
4.5.2. Morphology and Electrical Properties of Heparin-Stabilised PPy Nanoparticles

FESEM was performed to visualise the morphology of the synthesised PPy nanoparticles. Our results revealed that discrete and spherical PPy nanoparticles could be obtained when heparin was used as the dispersing agent/stabiliser (Figure 4.17). The observed nanoparticles exhibited sizes that were in agreement with the DLS data. PPy nanoparticles synthesised via APS-initiated oxidation also presented with a spherical morphology, not unlike that of nanoparticles synthesised using FeCl₃ (Figure 4.18).

The proposed theory by Hong et al. for the formation of spherical PPy nanoparticles with the use of FeCl₃ as the oxidising agent describes the prerequisite formation of an initial iron cation/polymer complex which acts as the forming agent for their eventual spherical morphology when synthesized [52]. In this study, however, the spherical morphology was also evident without the presence of iron cations during APS-oxidised polymerisation. As the study differs in using heparin instead of PVA as the stabiliser, one logical explanation could be that the heparin, being highly-charged and repulsive, can alone provide the driving force for spherical organisation of the pyrrole monomers in solution. Also, as heparin was shown to be an effective, yet unusual dopant for the PPy coating over the PCL fibres in Section 4.4, there was also a possibility that heparin...
could be doped (i.e. directly immobilised onto the PPy nanoparticle surface) during synthesis.

**Figure 4.17.** FESEM micrographs of PPy nanoparticles synthesised using FeCl₃ oxidative polymerisation taken using field emission scanning electron microscopy (FESEM). The PPy nanoparticles were respectively synthesised in (a) 2.5 w/v %, (b) 5 w/v %, (c) 7.5 w/v% and (d) 10 w/v % heparin solution.

**Figure 4.18.** Representative FESEM images of PPy nanoparticles synthesised using APS as the oxidative agent in (a) 2.5 w/v % heparin and (b) 10 w/v % heparin solutions.
When the electrical conductivity of these heparin-stabilised nanoparticles were analysed using a 4-point probe measurement station, the nanoparticles exhibited sheet resistances between 6.0 to 19.3 \( \Omega \text{ sq}^{-1} \), and conductivity readings ranging from 1.4 to 2.3 \( \text{S cm}^{-1} \) (Table 4.3). The measured conductivity values are in agreement with that of average values of PPy nanoparticles synthesized using PVA [52].

Table 4.3. Electrical properties of heparin-stabilised PPy nanoparticles.

<table>
<thead>
<tr>
<th>% w/v heparin</th>
<th>2.5</th>
<th>5</th>
<th>7.5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheet resistance (Ohm/sq)(^a)</td>
<td>19.3 ± 3.6</td>
<td>8.9 ± 4.4</td>
<td>6.0 ± 1.0</td>
<td>9.1 ± 3.2</td>
</tr>
<tr>
<td>Conductivity (S/cm)(^b)</td>
<td>1.37 ± 0.9</td>
<td>2.3 ± 0.2</td>
<td>1.9 ± 0.6</td>
<td>2.0 ± 0.4</td>
</tr>
</tbody>
</table>

\(^a\) Nanoparticles in powder form were pressed into a pellet using a hydraulic press.

\(^b\) Bulk resistivity (\( \Omega \text{ cm}^{-1} \)) was obtained using the same instrument. Conductivity is calculated as a reciprocal of bulk resistivity.

4.5.3. Determination of Heparin Immobilisation on PPy Nanoparticles and Effect on Colloidal Stability

Next, the possibility that heparin chains can be directly immobilised onto the PPy nanoparticle surface was investigated. Toluidine blue assay performed on PPy nanoparticles synthesised via the FeCl\(_3\) oxidation elucidated the fact that heparin was indeed present on the PPy nanoparticles. With increasing heparin concentration at the point of synthesis, more heparin was consequentially incorporated and detected on the synthesised PPy nanoparticles (Figure 4.19a). This can be explained by higher total particle surface area for spontaneous heparin incorporation when smaller nanoparticles were produced. Furthermore, the retention of heparin following thorough washing of the resulting nanoparticles after synthesis, suggested that the heparin stabiliser was not weakly adsorbed onto the particle surface. As heparin is a densely charged macromolecule that interacts readily with polar solutions, the immobilisation of heparin aided in the re-dispersion of washed and dried heparin-immobilised PPy nanoparticles (referred to as PPy-HEP).
It was found that all heparin-immobilised PPy nanoparticles (synthesised in 2.5 – 10 w/v % heparin solutions) could be dispersed in 1 x PBS by stirring. Also, average zeta potential values of between -52.1 mV and -55.9 mV (Figure 4.19b) indicated that the colloidal stability of the PPy-HEP nanoparticles was conferred by electrostatic repulsion between heparin immobilised on different particles in PBS. On the other hand, the adsorbed PVA onto PPy nanoparticle surface could have detached after washing (referred to as PPy nanoparticles). The lack of electrostatic stabilisation therefore resulted in a low average zeta potential value of -4.7 mV. The high zeta potential of the nanoparticles in PBS (pH 7.4) at physiological ionic concentration is indicative of good colloidal stability resulting from their excellent dispersion, which could be achieved with ease by mechanical stirring. This difference in the colloidal stability of PPy-HEP and PPy nanoparticles in PBS was evident at 24 h post dispersion, where sedimentation had occurred in PPy, but not in PPy-HEP nanoparticles (Figure 4.19c). As zeta potentials correlate with surface charges, the highly-negative zeta potentials in PPy-HEP nanoparticles also suggest that heparin was directly immobilised on the PPy nanoparticle surface during synthesis.

Figure 4.19. Physical properties of heparin-stabilised PPy nanoparticles. (a) The amount of immobilised heparin per unit mass of PPy nanoparticles synthesised. (b) Zeta potential measurements of PPy nanoparticles in PBS solution, and (c) nanoparticle dispersions in PBS that were left standing for 24 h.
The single-step synthesis and immobilisation of heparin onto PPy nanoparticles is unique among polymer-stabilised synthesis of PPy nanoparticles. Although there are many studies on the use of water-soluble polymers such as PVA as stabilisers in aqueous-based PPy synthesis, these studies did not demonstrate that the PVA adsorbed on the PPy nanoparticles during synthesis can be retained on the particle surface after repeated washing in aqueous solutions [52]–[54]. In fact, the retention of the polymeric stabiliser is usually regarded as undesirable as it may affect electrical and other material properties. In another study on gold nanoparticles, the loss of PVA ligands from the gold nanoparticle surface during water extraction was reported by Lopez-Sanchez et al. [55].

4.5.4. Thermogravimetric Analysis (TGA) of Heparin-Stabilised PPy Nanoparticles

TGA analysis on the heparin-stabilised PPy nanoparticles was carried out in an attempt to study their thermal properties and to give insights into the proportion of their constituent materials (i.e. polypyrrole and heparin). From the TGA curves, it can be observed that full TGA runs of up to 850 °C at 15 °C/min did not result in the full decomposition of either PPy alone (synthesised in PVA, and washed thoroughly to remove stabilising ligands), or heparin. However, the differences in thermal profiles between PPy and heparin-immobilized PPy are evident in the TGA curves (Figure 4.20). While the thermal degradation of pristine PPy (PVA stabilised) was gradual from onset, all heparin-immobilised PPy nanoparticles exhibited a major thermal degradation event at ~240 °C that is similar to that of pure heparin. The residual weight of the samples at 850 °C was 49.4 % for pure PPy, and ranged from 44.9 % (PPy-HEP2.5) to 43.6 % (PPy-HEP10) for heparin-immobilised PPy nanoparticles, and 19.9 % for pure heparin. The decrease in residual weight from pristine PPy is due to the incorporation of heparin on the PPy nanoparticles, owing to the lower thermal stability of heparin. The slight differences in residual weights between the PPy-HEP nanoparticles also pointed at the increasing amounts of heparin immobilised on the PPy nanoparticle surfaces as more heparin was used in the synthesis.
4.5.5. Proposed Mechanism of Single-Step Synthesis and Immobilisation of Heparin

In the novel use of heparin as the stabiliser for PPy synthesis, the inherent hydrophobicity of the pyrrole was apparently sufficient to drive attraction between pyrrole monomers in the observed “hydrophobic effect,” that resulted in the formation of spherical nanoparticles. This led to the proposal of the mechanism behind the single-step synthesis and heparin immobilisation (Figure 4.21). In both modes of synthesis (FeCl₃ or APS), heparin provides steric stabilisation against excessive coalescence mechanisms, while the oxidant in the aqueous phase acts at the phase boundary to initiate polymerisation. Regardless of the presence of iron cations, the heparin chains, due to abundance of highly-charged sulphated functional groups and hydrogen bonds, could interact with pyrrole monomers in solution to form a spherical nano-emulsion, thus functioning as nano-templates on which polymer chain growth proceeded along.
Figure 4.21. Proposed mechanism of the single-step synthesis of polypyrrole-heparin (PPy-HEP) nanoparticles. When pyrrole monomer is added slowly to a solution of heparin and FeCl₃, the heparin functions as a stabiliser, since the hydrophobic effect draws the pyrrole monomers into a hydrophobic nano-emulsion. Simultaneously, the Fe³⁺ oxidant initiates the polymerization of pyrrole, resulting in a polypyrrole nanoparticle with heparin stably immobilised onto its surface.

4.5.6. PPy Nanoparticles as Conductive Nanofiller

The heparin-immobilised PPy nanoparticles were utilised as conductive nanofiller to impart conductivity to non-conductive matrices. Synthesised PPy-HEP2.5 and PPy-HEP10 powder were mixed by grinding and blending with polyethylene glycol (PEG) before pressing into pellets using a hydraulic bench press. PEG was used as the insulative polymer matrix as opposed to PCL due to availability of PEG in powdered format. Expectedly, the bulk conductivity of the pellet increased when wt % of the PPy-HEP nanoparticles in the PEG/PCL pellet increases from 5 to 15 wt %, as higher amount of PPy within the insulative matrix increases the percolation of conductive channels within the PEG matrix (Table 4.4). The bulk conductivity conferred by PPy-
HEP2.5 and PPy-HEP10 nanoparticles were compared, and it is evident that the smaller-sized PPy-HEP10 nanoparticles resulted in a better percolating network than PPy-HEP2.5 nanoparticles. When 15 wt % of the pellet consists of PPy, the bulk conductivity of PPy-HEP10/PEG was av. $1.4 \times 10^{-2}$ S/cm, almost a 400-fold increase over the bulk conductivity of PPy-HEP2.5/PEG (av. $4.4 \times 10^{-4}$ S/cm). This was not due to the intrinsic conductivity of the PPy-HEP nanoparticle itself, as pure pellets comprising of PPy-HEP nanoparticles synthesised with the range of heparin concentration used in the study exhibited similar bulk conductivity values from 1.37 to 2.0 S/cm on average (Table 4.5). Numerous studies have been performed on similar binary powder mixtures of conductive and insulative material. The percolation theory of electrical conductivity holds that smaller particles have a better chance at forming continuous conductive networks within the insulative matrix [56], [57], and our results corroborates this theory. Overall, this study has demonstrated the feasibility of using PPy nanoparticles dispersed within a non-conductive polymer to impart electrical conductivity.

**Table 4.4.** Bulk conductivity of pressed PEG/PPy pellet composite

<table>
<thead>
<tr>
<th>Composite</th>
<th>PPy wt %</th>
<th>5</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPy-HEP2.5/PEG</td>
<td></td>
<td>($8.6 \pm 7.6) \times 10^{-7}$</td>
<td>($2.4 \pm 3.0) \times 10^{-5}$</td>
<td>($4.4 \pm 0.7) \times 10^{-4}$</td>
</tr>
<tr>
<td>PPy-HEP10/PEG</td>
<td></td>
<td>($1.7 \pm 0.4) \times 10^{-3}$</td>
<td>($5.4 \pm 2.0) \times 10^{-5}$</td>
<td>($1.4 \pm 0.9) \times 10^{-2}$</td>
</tr>
</tbody>
</table>

**Table 4.5.** Bulk conductivity of pressed PEG/PPy pellet composite

<table>
<thead>
<tr>
<th>% w/v heparin</th>
<th>2.5</th>
<th>5</th>
<th>7.5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk conductivity (S/cm)</td>
<td>$1.37 \pm 0.9$</td>
<td>$2.3 \pm 0.2$</td>
<td>$1.9 \pm 0.6$</td>
<td>$2.0 \pm 0.4$</td>
</tr>
</tbody>
</table>

### 4.5.7. VEGF loading on heparin-immobilised PPy nanoparticles

In order to test the hypothesis that the immobilised heparin on the particle surfaces enables them to be a suitable delivery vehicle of VEGF, the loading efficiency of VEGF onto PPy-HEP10 nanoparticles was compared to that of control PPy nanoparticles. PPy-
HEP10 nanoparticles were used for the study, as they exhibited the highest amount of immobilised heparin per unit mass (Figure 4.22). The conjugation of the nanoparticles with VEGF was conducted in physiological PBS buffer. Following incubation, the amount of VEGF left in the buffer was quantitated to calculate the amount of VEGF conjugated onto the nanoparticles. The average loading efficiencies of PPy-HEP2.5, PPy-HEP5 and PPy-HEP7.5 nanoparticles were found to be 61 – 63 %, and significantly higher at 92 % for PPy-HEP10 (Figure 4.22). Non-specific adsorption of VEGF onto control PPy nanoparticles resulted in 26 % loading, significantly lower than any of the heparin-immobilized PPy nanoparticles.

![Figure 4.22. Loading efficiencies of VEGF to heparin-stabilised PPy and PPy nanoparticles in physiological PBS solution after overnight incubation. *p < 0.05](image)

This binding mechanism is thought to be primarily driven by interfacial hydrophobic protein-polymer interactions, and unlike the weak adsorption of VEGF onto non-heparin immobilised control PPy nanoparticles, is non-reversible [58].

### 4.6 Conclusion

In summary, the results in this chapter have demonstrated the successful development of PCL and PPy-based materials for the aims outlined for vascular tissue engineering at the beginning of this chapter. In order for PCL to be utilised as a vascular graft
material for the support of endothelial cells, scPCL was functionalised via ATRP to grow intermediate P(GMA) brushes that afford dense epoxide reactive sites for the conjugation of gelatin. Surface chemical analysis at each intermediate step, and after gelatin conjugation verified the presence of GMA and gelatin, respectively. From static water contact angle measurements, a marked improvement in hydrophilicity was observed upon gelatin grafting. Next, in a separate study, the PCL modification was taken in another direction to confer electroactive and anti-thrombotic properties. Fibrous PCL scaffolds, which mimic a vascular graft material, was fabricated by electrospinning. Using the ePCL as template for the polymerisation of PPy, a heparin-doped PPy layer was coated onto the ePCL fibres. The morphological differences in the heparin-doped PPy coating and other PPy-coated ePCL scaffolds were investigated, and surface chemistry analysis and Toluidine blue assay verified the presence of heparin in the PPy coating over ePCL fibres. The electrical conductivity of ePCL/PPyHEP scaffolds were found to be on par with that of PPy doped with conventional Cl⁻ dopant. Hence, these scaffolds are deemed suitable for investigations into heparin-mediated anti-thrombosis and electrical control of material thrombogenicity in subsequent studies. In order to further develop PPy into a nanomaterial, heparin-stabilised synthesis of PPy nanoparticles was performed. It was subsequently found that heparin was directly immobilised onto the PPy nanoparticle surface and could promote the conjugation of VEGF onto the nanoparticle surface in physiological conditions. Their potential for use as conductive nanofillers was also demonstrated.

In order to meet the challenges of vascular tissue engineering, the development of the materials has been performed with several novel techniques. Heparin-assisted coating of PPy onto a fibrous polymer template is a novel way to utilise heparin for template polymerisation. The use of heparin as a stabiliser for PPy nanoparticle synthesis, and its direct immobilisation onto the nanoparticle surface was also demonstrated for the first time. Both uses of heparin at the nanoscale level could only be possible due to its unique highly-charged properties, and seemingly interesting interactions with PPy that makes it to be a capable nano-templating agent for the growth of PPy.
References


Chapter 5

Endothelial Cell Thrombogenicity on Gelatin-Functionalised scPCL

This chapter describes the data from the cellular and molecular studies on the endothelial cells cultured on the functionalised solvent-casted polycaprolactone (scPCL) substrates. Endothelial cells were found to adopt a more thrombotic phenotype on PCL substrates functionalised with glycidyl methacrylate (GMA) brushes. Gelatin conjugation on the substrates reduced cell thrombogenicity, induced better nitric oxide production from endothelial cells, and surprisingly did not increase platelet activation on the substrate. This chapter had demonstrated how, in spite of cell confluency, surface chemistry can influence endothelial cell thrombotic behaviour, underlining its importance in the selection of materials for vascular graft applications.
5.1 Introduction

The seeding of endothelial cells on synthetic materials is generally known to reduce material thrombogenicity (reviewed in Chapter 2). At the fundamental level, a layer of endothelial cell (EC) coverage functions as a physical barrier to prevent the contact of clotting factors and platelets with synthetic surfaces, thus inhibiting or delaying contact activation and platelet activation [1]. Next, thrombosis can also be regulated by the endothelial cells. In their native state, endothelial cells produce low levels of nitric oxide (NO), an anti-thrombotic molecule that prevents platelet aggregation [2], [3]. Cell surface molecules e.g. thrombomodulin expressed on the EC surface inhibits the coagulation cascade by interacting with thrombin, an important coagulation promoter, and modulates its function to activate the anti-coagulant cascade [4], [5]. Thus, a complex interplay exists between endothelial cells, clotting factors and platelets to determine the thrombogenicity of the microenvironment. Many studies have acknowledged the importance of ECs on the luminal graft surface, and many research efforts have been directed at functionalising synthetic materials to support endothelialisation, such that tissue-engineered vascular grafts (TEVGs) with a living endothelium can be developed for vascular tissue replacement [6], [7]. Studies have shown that ECs at sub-confluent densities are more thrombogenic [1]. In vascular stents, delayed or absent surface endothelialisation has resulted in late thrombosis and adverse clinical outcomes [8]. It has also been demonstrated in animal models that the patency of small caliber synthetic grafts can be improved by endothelialisation of the luminal graft before implantation [9], [10].

While most thrombogenicity studies have focused on the material aspect, developing strategies to prevent the activation of the coagulation cascade or platelet attachment, fewer studies have examined the influence of the material on the thrombotic phenotypes of the endothelial cells. The thrombogenicity of the ECs can be assessed by studying the expression of abovementioned EC markers. In this study the endothelialisation of the scPCL substrates functionalised with gelatin via ATRP will first be investigated, followed by the thrombogenic profiles of the attached ECs on the surfaces. These comparisons will provide insights into the feasibility of using gelatin-functionalised PCL as a material for vascular tissue engineering applications.
5.2 Experimental Methods

The procedure for seeding ECs onto functionalised scPCL substrates is detailed in Section 3.5.1. The cell proliferation was calculated by correlating to metabolic activity quantitated using alamarBlue® reagent described in Section 3.5.2. The extent of endothelial coverage and cell viability on the substrates were analysed using DAPI nuclear stain (Section 3.5.4) and the LIVE/DEAD® cell viability assay (Section 3.5.3). Nitric oxide production by the ECs on the functionalised scPCL was quantitated using DAF-FM (Section 3.5.5). The expression of thrombogenicity-related genes von Willebrand factor (vWF) and matrix metalloproteinase-2 (MMP-2) in the ECs were quantitated using qPCR with designed primer sequences, as described in Section 3.5.6. The SDS-PAGE and immunoblotting protocols for the quantitation of vWF and MMP-2 proteins are also detailed in Section 3.5.6. The surface expression of surface adhesion molecules tissue factor and thrombomodulin were analysed in flow cytometry (Section 3.5.7).

To characterise the activation of the platelets on the functionalised scPCL substrates, a P-selectin ELISA was performed on platelets attached on the surfaces as described in Section 3.6.4.

5.3 Endothelial Cell Compatibility on Functionalised scPCL Substrates

To assess the endothelial cytocompatibility of the scPCL substrates, endothelial cells (ECs) were seeded onto the substrates. Cell proliferation and endothelial coverage on the substrates were measured for 7 days to obtain general endothelial compatibility on surfaces when compared to gelatin-coated glass controls. Endothelial cell function was assessed by measuring the amount of nitric oxide (NO) produced by the cells on the substrates.

5.3.1 Endothelialisation of Functionalised scPCL Substrates

The capacity for endothelial cell support on the functionalised scPCL surfaces was quantitatively evaluated by measuring cell proliferation on the substrates using the alamarBlue® assay. The poor endothelial cell numbers observed on pristine PCL
substrates at day 7 is not surprising as PCL by itself lacks bioactive sites and is inherently hydrophobic (Figure 5.1a). This is in agreement with previous studies, too, that observed poor endothelial compatibility on flat PCL surfaces [11], [12]. Overall EC growth remained poor upon grafting of scPCL with P(GMA) brushes through ATRP, despite the observed decrease in static water contact angle and improvements in the hydrophilicity of P(GMA)-grafted scPCL over pristine scPCL substrates (Table 4.1). scPCL surfaces physisorbed with gelatin initially improved the attachment of ECs on day 1 but the ECs stopped proliferating or dividing from day 5 onwards (Figure 5.1a, b). On the other hand, the gelatin-conjugated scPCL surfaces, scPCL-g-P(GMA)1-c-gelatin and scPCL-g-P(GMA)2-c-gelatin, exhibited significant improvements in EC growth. Total cell numbers on scPCL-g-P(GMA)1-c-gelatin and scPCL-g-P(GMA)2-c-gelatin substrates did not differ significantly (Figure 5.1), in spite of a denser P(GMA) polymer brush layer on scPCL-g-P(GMA)2-c-gelatin substrates.

In order to determine if the higher cell numbers at day 7 for scPCL-g-P(GMA)-c-gelatin substrates were due to obvious differences in initial cell attachment, as can be inferred from cell numbers on day 1 (Figure 5.1), or due to differences in the doubling frequency, the population doublings of the ECs were analysed. It became apparent that only ECs on scPCL-g-P(GMA)2-c-gelatin substrate had significantly higher cumulative doublings on day 7 than other substrates (Figure 5.1b). Unsurprisingly, the ECs on the scPCL-g-P(GMA)2 substrate exhibited the least cumulative population doublings over the 7 days (Figure 5.1b). Cumulative doublings on day 7 did not differ significantly between pristine scPCL and the other substrates, suggesting that it was the differences in the initial attachment which played a major factor in the observed final cell numbers on the surfaces (Figure 5.1a). The positive control used in this study was the gelatin-coated glass coverslips that yielded better EC numbers and population doublings than any of the functionalised scPCL substrates (Figure 5.1a, b). Gelatin-coated surfaces (e.g. tissue culture plastic or glass) are commonly used to increase cell adhesiveness of surfaces. Gelatin adsorption on glass (hydrophilic silica), with a measured water contact angle of $57 \pm 6^\circ$, occurs through electrostatic interactions [13].
Figure 5.1. Proliferation of ECs on the functionalised scPCL substrates. (a) Cell numbers over 7 days determined using the alamarBlue® assay. (b) Cumulative population doublings of ECs calculated from proliferation data. (*p < 0.05)

The EC coverage on the functionalised scPCL substrates was visualised using DAPI nuclear staining and LIVE/DEAD® viability staining, consisting of calcein-AM (green fluorescence) for the detection of viable cells, and EthD-1 (red fluorescence) for dead cells, on day 7. The extent of endothelialisation on the different surfaces visualised by both DAPI staining (Figure 5.2) and LIVE/DEAD® staining (Figure 5.3) was in agreement with our EC proliferation data. As the LIVE/DEAD® staining constitutes an
uptake of the dye into the cytoplasm, it also allows for the visualisation of the morphology of the ECs on the functionalised scPCL substrates. ECs on sPCL-g-\(P(GMA)\) substrates were not characterised by typical “cobblestone” morphology, but incompatibility of these surfaces drove the cells into adopting unusually large and irregular shapes (Figure 5.3). Conversely, full surface coverage of ECs on sPCL-g-P(GMA)-c-gelatin substrates was observed, providing evidence that ATRP-mediated functionalisation of PCL material can potentially be used as graft material for TEVGs. In parallel with the cell proliferation data, gelatin-coated glass coverslip was the best surface for endothelialisation (Figure 5.1). In general for adherent cell types, the effects of gelatin on cell proliferation lies in the amphiphilic cell-adhesive RGD motifs within gelatin/collagen that bind to integrin receptors on the cell membrane during cell adhesion, leading to the activation of phosphoinositide 3-kinase (PI3K) and downstream activation of genes involved in cell survival and cell cycle control [14].

Taken together, the conjugation of gelatin on flat scPCL surfaces promoted rapid EC attachment and endothelialisation when compared to the scPCL or P(GMA)-grafted scPCL surfaces. Though the physisorption of gelatin onto pristine PCL surfaces promoted good EC adhesion initially, continuous proliferation of the ECs over 7 days was not observed. In the previous chapter, the loss of physisorbed gelatin from pristine PCL surfaces over 7 days in culture has been demonstrated (Figure 4.7), and this could explain why EC proliferation was not sustained over time in culture.
Figure 5.2. Representative images of DAPI-stained ECs on functionalised scPCL substrates and gelatin-coated coverslips. Scale bar: 20 µM.

Figure 5.3. Representative images of ECs stained with LIVE/DEAD® viability assay on functionalised scPCL substrates and gelatin-coated coverslips. Scale bar: 20 µM.
5.3.2. Nitric Oxide Production by Endothelial Cells

This study aims to address the influence of material chemistry on the functional behaviour of ECs. Thus, nitric oxide (NO) production, a physiological process by which ECs mediate vascular homeostasis and anti-thrombogenicity, was measured. NO secreted by ECs exerts important anti-thrombogenic roles by preventing platelet and leukocyte adhesion to the endothelium [15]. An increased production of NO by ECs on sdcPCL-g-P(GMA)1-c-gelatin and scPCL-g-P(GMA)2-c-gelatin substrates over pristine scPCL was observed (Figure 5.4). NO production was significantly higher by ECs on the scPCL-g-P(GMA)2-c-gelatin surface, suggesting that gelatin-functionalised scPCL substrates have a role in influencing EC phenotype, and if developed as the synthetic substrate for TEVGs, ensures proper vascular tone regulation by the endothelium.

Studies in the literature have hinted that the NO production and cell growth appear to be linked mechanistically. Arnal et al. first discovered the link when proliferating aortic endothelial cells expressed significantly higher levels of endothelial nitric oxide synthase (eNOS) and produced more NO than growth-arrested endothelial cells [16]. eNOS is the NOS isoform specific for endothelial cells. It was subsequently discovered that eNOS can directly be phosphorylated and activated by the serine/threonine protein kinase Akt at Ser1179 [17]. Being a downstream effector of PI3K, Akt is well-known to have multiple roles in cell cycle progression, and is studied extensively in both tumour cell progression [18] and normal cell proliferation [19]. The PI3K/Akt signalling axis has multiple phosphorylation targets and activates genes involved in cell survival and cell cycle progression e.g. the transition between G1 to S and G2 to M phases in the cell cycle [18]. This could offer an explanation for the higher levels of NO produced by ECs on gelatin-functionalised scPCL substrates, being more proliferative than ECs on other scPCL surfaces.

Interestingly, the production of NO by ECs on gelatin-coated glass coverslips was significantly lower than the functionalised scPCL substrates. This is despite the good proliferation and endothelialisation observed on the glass coverslips. While the mechanism underlying the relatively impaired response in NO production is unclear, the vast difference in the stiffness of the substrate (Tensile modulus of bulk PCL: ~350...
MPa [20], Glass: 72.4 GPa [21]) led us to believe that apart from surface chemistry, substrate stiffness may play a role in the regulation of NO. Indeed, it has been found that the culture of valvular interstitial cells (VICs) on stiff tissue culture polystyrene and poly(ethylene glycol)-based hydrogels elicited different cellular fates via cellular mechano-sensing pathways that signal through PI3K/Akt [22]. VICs significantly upregulate PI3K and Akt when cultured on the stiffer tissue culture polystyrene [22]. It can be postulated that this mechanism may also underlie the differential nitric oxide production by ECs we observed on functionalised PCL substrates and glass coverslips. Importantly, it highlights the fact that apart from material chemistry, other physical properties of the substrate e.g. stiffness may influence physiological outcomes.

![Figure 5.4](image.png)

**Figure 5.4.** Production of NO by ECs functionalised scPCL surfaces. Statistical significance was determined between ECs on pristine scPCL and functionalised scPCL surfaces (*p < 0.05).

### 5.3.3. Expression of Thrombosis-Related Cell Adhesion Molecules

To further investigate how the functionalisation of scPCL can influence the thrombotic phenotype of the ECs, qPCR and immunoblotting were performed to quantitate the expression of pro-thrombogenic factors that indicate the activation of ECs to a pro-thrombotic phenotype. Downregulation of mRNA levels was observed for vWF in the ECs on both scPCL-g-P(GMA)1-c-gelatin and scPCL-g-P(GMA)2-c-gelatin surfaces in comparison to ECs on pristine scPCL substrates and gelatin-coated glass coverslips.
Endothelial Cell Thrombogenicity on Gelatin-PCL  

Figure 5.5a. scPCL-g-P(GMA)2-c-gelatin substrates induced a 2-fold upregulation of vWF mRNA level in ECs when compared to scPCL substrates (Figure 5.5a). In native settings, vWF is present in the subendothelial matrix and plays an important role in the coagulation response by promoting platelet adhesion during vascular injury and when the subendothelial matrix is exposed. In ECs, vWF is released constitutively at low levels, but is stored in Weibel-Palade bodies and secreted in response to EC activation during vascular injury [23]. The dysfunctional regulation of vWF has been implicated in certain pathological conditions, such as deep vein thrombosis [24], [25]. TNF-α activated ECs, used as positive controls, expectedly resulted in a significant upregulation of vWF expression (Figure 5.5a). Consistent with the qPCR data, the protein expression of vWF was lower in ECs on scPCL-g-P(GMA)-c-gelatin substrates than on P(GMA)-grafted scPCL substrates (Figure 5.5c). The protein expression of vWF in ECs on gelatin-coated glass coverslips is barely detectable, which is slightly different from the qPCR data. This is not uncommon as it is generally known that due to post-transcriptional and post-translational regulation of gene expression, the final expressed protein product may not reflect the mRNA level completely. Wissink et al. has found that low cell density can induce vWF expression in ECs on collagen-grafted surfaces, and this may correlate to our observed data that substrates with less EC coverage on day 7 after seeding (pristine scPCL, P(GMA)-grafted scPCL) induced a higher expression of vWF than substrates with higher EC coverage (gelatin-functionalised scPCL) [26].

The expression of MMP-2 was also assessed. MMP-2 is a protease known to be involved the degradation and remodeling of the endothelial extracellular matrix, and is also involved in platelet activation and aggregation [18], [19]. qPCR data for MMP-2 revealed mRNA levels to be lowest on scPCL-g-P(GMA)2-c-gelatin substrate (Figure 5.5b). Similar to the trend observed in the qPCR data for vWF, ECs on PCL-g-P(GMA)-c-gelatin surfaces generally exhibited lower mRNA expression than pristine scPCL and P(GMA)-grafted scPCL substrates. Activation with the TNF-α cytokine strongly upregulated the expression of MMP-2 expectedly. As MMP-2 belongs to a group of MMPs that are secreted to regulate matrix proteolytic and remodeling activity in response to triggers for invasion [29], it is upregulated in ECs exhibiting an activated state. These results suggest that the P(GMA)-grafted scPCL surfaces led to an increase
in EC activation and pro-thrombogenicity that is ameliorated by the conjugation of gelatin onto the substrates.

Figure 5.5. Relative mRNA expression of (a) vWF and (b) MMP-2 in the ECs cultured on functionalised scPCL substrates measured in qPCR. Gene expression is normalised to ECs on scPCL when compared to expression by ECs on scPCL (*p < 0.05, n=3). (c) Immunoblot of vWF protein expressed by ECs on functionalised scPCL. β-tubulin was used as loading control.

Coagulation-related factors tissue factor (TF) and thrombomodulin (TM) are commonly-characterised surface molecules expressed on ECs. TF, a key member of the extrinsic coagulation pathway, is a transmembrane receptor for clotting factor VII/VIIa (Chapter 2, Figure 2.6) [30]. In normal physiological settings, TF is expressed by cells surrounding the vascular tissue and only comes into contact with clotting factors circulating in the blood during vascular injury [30]. Studies have indicated that ECs have non-detectable or low expression of TF in culture, but is highly upregulated upon activation by TNF-α [31]. TF packaged in microvesicles are found to be released by endothelial cells to promote drive thrombosis [32]. Conversely, TM inactivates the coagulation cascade by binding to thrombin to form a protein C-activating
TM/thrombin complex and promoting fibrinolysis [5]. Activated protein C is known to be inhibitory for the coagulation pathways by inactivating clotting factors [5]. Acting as a guard against unwarranted initiation of thrombosis, TM is constitutively expressed on the surface of ECs in homeostatic conditions but downregulated upon EC activation by pro-inflammatory cytokines to allow initiation of coagulation [33].

As the expression of TM and TF of ECs reflects their pro-coagulation phenotypes on synthetic materials, we conducted flow cytometry analysis of TM and TF. TM expression remained constitutively high on all the substrates (Figure 5.6a). TF was found to be lowly expressed by ECs on all the functionalised scPCL surfaces (Figure 5.6b), but not expressed in ECs on gelatin-coated coverslips. The TM/TF expression profile can be studied in parallel, in addition to other thrombogenic factors, to obtain a more complete picture of overall thrombogenicity of the ECs on the surfaces [1]. From the flow cytometry data, although the different functionalised scPCL surfaces did upregulate TF a little, which may lead to activation of clotting factors to some extent, these effects may be ameliorated by the maintained high levels of TM, which inhibit these factors. Based on these findings, it can be suggested that the overall outcomes of the EC influence on the coagulation responses would not differ very much from other material surfaces that maintained high TM/low TF expression profiles in non-activated ECs [1].
Figure 5.6. Histograms obtained from flow cytometry analysis of (a) thrombomodulin (TM) and (b) tissue factor (TF) for the cultured ECs on functionalised scPCL surfaces. Dotted grey histograms represent the baseline intensity from isotype control staining while black histograms represent expression levels of surface molecules.

5.4 Thrombogenicity of Functionalised scPCL Substrates

The study of the thrombotic states of ECs on the functionalised scPCL substrates is followed by the assessment of platelet adhesion on the bare scPCL surfaces. Thrombus formation entails the activation of platelets that adhere to the subendothelial matrix of
Endothelial Cell Thrombogenicity on Gelatin-PCL

an injured vessel wall. Thrombosis can be initiated by the myriad of proteins present in the subendothelial matrix, and some them include the family of collagen (types I–IV), which are known to be able to interact directly with platelets [34], [35]. This interaction is a result of collagen receptors that have been identified on platelets, most notably integrin $\alpha_2\beta_1$ and the immunoglobulin superfamily glycoprotein VI (GPVI) [34], [35]. As gelatin is essentially hydrolysed collagen, this may of concern for our gelatin-functionalised scPCL substrates. Any non-endothelialised area of the material could potentially promote the activation of platelets, which will in turn initiate the coagulation cascade. Hence, it was imperative to evaluate platelet activation on our functionalised scPCL surfaces through quantitation of P-selectin (CD62p). P-selectin is a cell adhesion molecule expressed on the surfaces of both ECs and platelets [36]. Through its ligand P-selectin glycoprotein ligand-1 (PSGL-1), it is known to facilitate the interactions between platelets and activated ECs or leukocytes; P-selectin on activated platelets mediate platelet aggregation and activation during thrombosis [36]. In this study, platelets from platelet-rich plasma (PRP) were exposed to functionalised scPCL substrates and P-selectin expression on the platelets that are attached to the surfaces was measured using an ELISA-like luminescence assay to evaluate platelet activation. Interestingly, the data shows that the P-selectin expression of the platelets on scPCL-g-P(GMA)1-c-gelatin and scPCL-g-P(GMA)2-c-gelatin surfaces was not upregulated when compared to platelets on pristine scPCL surfaces (Figure 5.7). However, P-selectin expression was significantly higher on PCL-g-P(GMA)1 and PCL-g-P(GMA)2 surfaces, indicating P(GMA)-grafted substrates without the covering the gelatin proved to be a more thrombogenic surface (Figure 5.7). That the platelets on gelatin-functionalised substrates were not activated can be explained by studies in literature describing GPIV as the primary receptor for platelet-collagen adhesion and that GPIV has shown binding activity with fibrillar, but not denatured collagen (gelatin) [37]. Hence, our data has shown that gelatin functionalisation of scPCL substrates did not contribute additional thrombogenic risk to the scPCL when assessed by platelet activation on the substrates.
Figure 5.7. Platelet activation on functionalised scPCL substrates. The activation of adherent platelets was determined by P-selectin levels (quantitated in luminescent units in assay) after incubation with PRP for 2 h (*p < 0.05).

5.5 Conclusion

The functionalisation of scPCL substrates using surface-initiated ATRP provided us with means of grafting the PCL surface with gelatin-conjugated P(GMA) polymer chains to improve endothelialisation. The layer of gelatin functionalised via ATRP onto the scPCL substrates promoted EC attachment and the substrate grafted with the higher amount of P(GMA) for gelatin conjugation exhibited slightly better overall EC doublings. Being integrin ligands, gelatin and the gelatin-derived RGD sequence are well-utilised for cell adhesion and growth on substrates. In addition, cross-linked gelatin matrices are pro-angiogenic and supported the formation of endothelial tubules in vitro [38]. The P(GMA)-grafted surfaces induced the activation of endothelial cells on the surface, promoting a pro-thrombogenic phenotype that is characterised by increased expression of vWF and MMP-2 and increased platelet activation, but others have also observed the pro-inflammatory effects of bisphenol A-glycidyl methacrylate, a GMA-containing compound used in dental composites. Studies have found that it promotes the production of pro-inflammatory cytokines such as TNF-α in macrophages [39], [40]. Such studies can give us a hint that P(GMA) chains may favour pro-inflammatory conditions in cells which lead to thrombogenicity. The non-specific
reactivity of epoxide groups towards proteins in general may also compromise the normal signalling functions of membrane surface proteins and result in toxicity.

In this chapter, the conjugation of gelatin on the P(GMA)-functionalised substrates decreased the expression of thrombogenic markers vWF and MMP-2, and promoted the production of nitric oxide from endothelial cells. TF and TM expression was similar in all functionalised scPCL substrates, regardless of the presence of gelatin. It was also observed that the gelatin-coated coverslips used in the experiments have led to downregulated NO production in ECs, which was proposed to be partly due to other non-surface chemistry properties e.g. substrate stiffness. Based on this study, a model of the underlying mechanisms involved in the regulation of EC thrombogenic phenotypes by material surface chemistry is proposed (Figure 5.8). EC thrombogenicity, like the expression of most cellular phenotypes, is likely to be much more complicated, involving multiple factors, redundancy and crosstalk between different signaling pathways e.g. treatment with TNF-α overrides all influence of surface chemistry. Nevertheless, this is a simplified model that attempts to partly capture the important signalling pathways controlled by biomaterial surfaces. This study has also demonstrated how the alteration of material surface chemistry can have beneficial or deleterious effects on endothelial cell attachment, physiology and thrombogenicity. Through the functionalisation of scPCL substrates via ATRP and gelatin conjugation, a PCL-based material for pre-seeded TEVGs has been successfully developed.
Figure 5.8. Proposed model on the influence of materials on endothelial cell phenotypes. Question marks denote speculative or hitherto not understood mechanisms.

References


Chapter 6

Improving Haemocompatibility and Thrombogenicity with Heparin-Doped PPy Nanomaterials

This chapter describes the work involved to improve the haemocompatibility and thrombogenicity using heparin nanomaterials-based control hypothesis that electrical stimulation Heparin doping in polypyrrole (PPy)-coated electrospun polycaprolactone (ePCL) scaffolds exhibited excellent haemocompatibility, but increased platelet activation. The application of low-amplitude AC sine current ameliorated thrombogenicity. Leukocyte adhesion was greatly reduced under stimulation. PPy nanoparticles were successfully synthesised using heparin as a stabiliser. Heparin was found to be immobilised on the nanoparticle surface, and facilitated the conjugation of vascular endothelial growth factor.
6.1 Introduction

Material haemocompatibility and thrombogenicity is of utmost importance to synthetic materials designed for vascular tissue engineering applications. The previous chapter has demonstrated how material surface chemistry, specifically using functionalised PCL, can influence EC activation and thrombotic phenotypes. In this chapter, heparin-doped PPy nanomaterials, fabricated as a nanocoating on ePCL scaffolds (Chapter 4, Section 4.4) and as spherical nanoparticles (Chapter 4, Section 4.5), was utilised to improve the haemocompatibility and thrombogenicity of the base material upon contact with blood. Heparin is a polysaccharide used clinically as an anticoagulant, and it functions by inhibiting factor Xa and thrombin in the coagulation cascade (Chapter 2, Figure 2.6) [1], [2]. In spite of the detection of heparin on both types of our nanomaterials, this chapter will evaluate whether the amount of heparin doped into the PPy nanocoating is sufficient for its anticoagulant role. Heparin also offers us a convenient method to immobilise vascular endothelial growth factor (VEGF) onto the PPy nanoparticle surface, due of the natural affinity of VEGF with heparin [3], [4]. One study in this chapter will evaluate the feasibility of using VEGF-conjugated PPy nanoparticles for the delivery of VEGF. The bioactivity of the VEGF upon conjugation to PPy nanoparticles will be directly addressed by using the Matrigel tube formation assay, an in vitro model of angiogenesis.

As the electrical conductivity of PPy was retained with heparin-doping, the PPy-coated ePCL scaffolds were administered with electrical stimuli in the form of AC currents to investigate their effects on the thrombogenic processes at the surface of the scaffolds. As reviewed in Chapter 2, Section 2.3, alternating current (AC) or pulse/square waves have not been studied as extensively for non-excitable cell types e.g. non-neuron, cardiac or muscle cells. Thrombogenic and inflammatory processes on surfaces frequently involve the interactions between the surface with the proteins, platelets and leukocytes in blood. Protein adsorption [5], [6] and platelet/leukocyte attachment [5], [7], [8] are crucial processes that occur at the blood-material interface for the initiation of the coagulation cascade. Based on emerging evidence in the literature that non-directionally AC currents can guide physiological processes e.g. stem cell differentiation [9], and coupled with the fact that AC stimuli are charge-balanced and leave no residual charges at the site of stimulation, the influence of 100 Hz sinusoidal
waves (also employed by Creecy et al. [9]) on protein adsorption, platelet/leukocyte attachment and platelet activation was investigated.

### 6.2 Experimental Methods

Blood components used in the material thrombogenicity studies were isolated in accordance to protocol listed in Section 3.6.1. Section 3.6.2 describes the assessment of the haemolytic potential of PPy-coated ePCL scaffolds. Plasma recalcification times on the scaffolds were obtained using procedures listed in Section 3.6.3. The measurement of fibrinogen adsorption under AC stimulation was described in Section 3.6.5. To measure platelet activation, P-selectin expression on the platelets was performed as described in Section 3.6.6. Visualisation of attached platelets and leukocytes during AC stimulation was performed using SEM after preparation steps outlined in Section 3.6.7.

For the heparin-doped PPy nanoparticles, the efficiency of VEGF loading was performed using the bicinchoninic acid (BCA) assay as described in Section 3.7.1. The assessment of the bioactivity of the VEGF conjugated onto the nanoparticles was performed through the Matrigel angiogenesis tube formation assay (Section 3.7.2).

### 6.3 Assessment of Haemocompatibility of PPy-Coated ePCL Scaffolds

The inherent material haemocompatibility of the PPy-coated ePCL scaffolds can be determined by conducting the red blood cell (RBC) haemolysis assay and assessment of the coagulation response on the scaffolds. In this study, the coagulation response of the scaffolds were tested by the measuring the plasma recalcification times (PRT) of platelet-poor plasma (PPP) on the scaffolds. The RBCs and citrated PPP required for the haemolysis assay and PRT, respectively, were obtained after separation of whole blood donated by healthy human volunteers using Ficoll-paque. Endothelial cell compatibility of the scaffolds was also of interest and assessed simply by culturing ECs onto the scaffolds and staining for cell viability.
6.3.1. Red Blood Cell Haemolysis Assay on Scaffolds

According to ISO 10993-4 (Biologic evaluation of medical devices: Selection of tests for interactions with blood), haemolysis testing is an important assessment for biomaterial haemocompatibility, which assesses the amount of haemoglobin leaked from RBCs due to loss of RBC membrane integrity [10]. The rupture of red blood cells and its release of intracellular contents can occur for a variety of reasons e.g. infection with haemolytic microorganisms [11], [12], in the presence of certain chemical compounds [13], or damage from shear forces during processing [14]. The red blood cell membrane comprises the typical lipid bilayer found in other cell types. Although the exact mechanism behind material-induced haemolysis is not well understood, it is found that certain anionic polymers e.g. poly(ethylene-co-acrylic acid) or hydrophobic functional groups are able to cause membrane penetration and disruption [15].

Through haemolysis testing, it was evident that uncoated ePCL scaffolds exhibited a rather high haemolytic potential with the percentage of haemolysis at 22.1 ± 4.0 % after 3 h incubation of the RBCs on the scaffolds in static conditions (Figure 6.1). Conversely, all the PPy-coated scaffolds had significantly lowered rates of haemolysis at between 1 – 4%, with no statistical significance between scaffolds with different dopants (Figure 6.1). The RBC alone assessed the background haemolytic rate in the PBS solution, and it was found it be negligible. Incubation of the RBCs with 1% Triton-X100 ensured total lysis of the RBCs and the amount of haemoglobin released served as the positive control (100% lysis) by which all our samples were normalised against. The most ideal assessment for haemolysis would be to perform testing using dynamic flow models, where blood flow and shear stress can be simulated. While static conditions cannot account for haemolytic events due to mechanical forces, they allow the decoupling of mechanical-induced haemolysis from the inherent haemolytic properties of the materials upon contact. Hence, the results demonstrated a reduction in the inherent haemolysis induced upon contact with native ePCL fibres when a nanocoating of PPy was present.
Figure 6.1. Haemocompatibility of PPy-coated ePCL scaffolds. The percentage of red blood cell (RBC) haemolysis after incubation of diluted RBC fractions of whole human blood on the different scaffolds. **p < 0.01

6.3.2. Coagulation Response of Scaffolds

In this study, the anti-coagulative functions of the heparin doped onto the ePCL/PPyHEP scaffolds were validated. For assessment of the coagulation response, plasma recalcification times (PRT) were taken. PRT is the time taken for clot formation in recalcified blood (after re-addition of calcium). Calcium is required for clot initiation for the function of various clotting factors within the coagulation cascade, including factors Va, VIIa, and XIII [16]. Typically used in haematology to determine the presence of anti-coagulants in the blood or defects in the intrinsic coagulation cascade, the PRT for blood-contacting materials is measured after bringing the material surface into contact with citrated plasma, and the time taken for clot initiation after the addition of calcium is interpreted as one important measurement of haemocompatibility of the scaffold [17], [18].

In this study, the measurement of PRT utilizes absorbance readings taken using a microplate reader, and the PRT is taken at the beginning of a spike in the kinetic absorbance profile (Figure 6.2). It was demonstrated that heparin on the ePCL/PPyHEP scaffolds was sufficient enough to inactivate clotting factors present in the PPP such
that clot initiation was not detected even after 30 min (Figure 6.2). On the other hand, PPP incubated with uncoated ePCL took an average of 9 min 30 s for clot formation. ePCL/PPyCl and ePCL/PPy scaffolds took an average of 5 min and 8 min 30 s, respectively.

![Plasma recalcification times of PPP on scaffolds after the addition of calcium chloride. Representative OD405 profiles are shown, and clot formation times presented in the table were taken at the beginning of each spike in the OD405 profiles.]

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>ePCL</th>
<th>ePCL/PPy</th>
<th>ePCL/PPyHEP</th>
<th>ePCL/PPyCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clot initiation time</td>
<td>9 min 30 s (± 40 s)</td>
<td>8 min 30 s (± 32 s)</td>
<td>&gt; 30 min</td>
<td>5 min (± 20 s)</td>
</tr>
</tbody>
</table>

**Figure 6.2.** Plasma recalcification times of PPP on scaffolds after the addition of calcium chloride. Representative OD405 profiles are shown, and clot formation times presented in the table were taken at the beginning of each spike in the OD405 profiles.

### 6.3.3. Endothelial Cell Compatibility of Scaffolds

As the impetus for the development of PPy-coated ePCL scaffolds was to conceptualise an acellular scaffold that offers investigation into alternative methods of controlling thrombogenicity, the scaffolds were not functionalised with cell-adhesive bioactive molecules to support endothelialisation. Nevertheless, the cyto-compatibility of scaffolds was assessed by high-density seeding of ECs (2.5 x 10⁴ cells/cm²) onto the scaffold surface, followed by viability staining with fluorescein diacetate (FDA). ePCL scaffolds, being a hydrophobic material with no bioactive properties, was marked by very poor cell attachment at 24 h post-seeding (Figure 6.3a). The ePCL/PPy scaffolds exhibited comparatively better, but still poor coverage of ECs (Figure 6.3b). By qualitative assessment, the EC attachment on ePCL/PPyHEP scaffolds was better
(Figure 6.3c), which can be proposed to be due to the binding of growth factors (e.g. fibroblast growth factor 2, fibronectin) in the culture medium to heparin [19], thus concentrating adhesion-promoting factors onto the scaffold surface. However, both types of PPy-coated scaffolds demonstrated comparatively poor EC adhesion as compared to control tissue culture plastic (TCP) (Figure 6.3d). Interestingly, the hollow ring-like structures were observed for the ECs on the ePCL/PPyHEP scaffolds and the TCP surface. Endothelial ring-like structures with hollow lumens are often observed for ECs seeded in 3-dimensional hydrogels and on Matrigel surfaces, and as they resemble capillary lumens, their presence is affirmative of a pro-angiogenic environment [20], [21]. The formation of these ring-like lumen structures on planar non-gel type scaffolds or TCP has not been reported. One can suggest that these structures are formed mainly in the event of high-density seeding when ECs get confluent within 24 h. Taken together, the cyto-compatibility of PPy-coated ePCL scaffolds remained poor, but viability staining suggested that these scaffolds did not result in overt toxicity for ECs and could even induce lumen formation to some extent.

Figure 6.3. ECs seeded on (a) ePCL, (b) ePCL/PPy, (c) ePCL/PPyHEP, and (d) tissue culture plastic at high densities (2.5 x 10⁴ cells/cm²). Staining with viability dye FDA was performed 24 h post-seeding. White arrows point to the lumen-like structures and representative structures are traced in white dotted lines.
6.4 Use of AC Stimulation for Control of Thrombogenicity and Inflammation

After establishing the haemocompatibility of the PPy-coated ePCL scaffolds and demonstrating anti-coagulative effects of ePCL/PPyHEP scaffolds, subsequent blood contact studies on the effects of electrical stimulation were conducted on the ePCL/PPyHEP scaffolds, and using ePCL/PPy scaffolds for comparison. Uncoated ePCL scaffolds were also used as non-stimulated controls. As biological effects had been observed with the application of electric fields previously [22]–[27], investigations of the effect of electrical stimulation on fibrinogen adsorption, platelet attachment and activation, and leukocyte attachment onto the PPy-coated ePCL scaffolds were conducted using a customized 6-chamber electrical stimulation array we designed using SolidWorks and fabricated using 3D printing technology (Chapter 3, Section 3.4). The scaffolds, cut into 22 mm x 22 mm pieces, were held in place by material holders with platinum wires and clipped with crocodile clips connected to a dual-channel waveform generator (Figure 6.4).

![Figure 6.4. Setup of the AC stimulation study on PPy-coated ePCL scaffolds. Shown here are 2 chambers connected to each output for a dual-channel waveform generator, and 2 other chambers used for unstimulated scaffolds. Non-conductive ePCL scaffolds were also included in the setup.](image)
6.4.1. Influence of Electrical Stimulation on Protein Adsorption

The immobilisation of plasma proteins onto implanted blood-contacting materials is the first step and key event in thrombosis (Chapter 2, section 2.2). Clotting factors of the coagulation cascade, thrombin and fibrinogen have all been found to be able to adsorb onto material surfaces, which eventually catalyses the formation of crosslinked fibrin clots (Figure 2.6). Some studies have attempted to design materials that resist the adsorption of fibrinogen, the key precursor protein of fibrin, by grafting on poly(ethylene glycol) (PEG) or using fibrinolytic enzymes [28]–[30]. Fibrinogen adsorption on surfaces has also been found to potentiate immune responses by increasing monocyte attachment and macrophage activation [31]. The engagement of leukocyte integrin αMβ2 to fibrinogen is critical for the onset of inflammatory responses [32]. As reviewed in Chapter 2, section 2.3.1, the concept of using electrical stimulation in modulating thrombosis is not new, but only few studies have explored the idea, specifically on the effects of constant DC current on plasma proteins [33], [34]. None have, however, investigated the use of charge-balanced AC waveforms on protein adsorption. This thesis attempts to elucidate differences in protein adsorption on ePCL and PPy-coated ePCL scaffolds and the effects, if any, of applied low-intensity AC waveforms on fibrinogen adsorption.

![Graph showing fibrinogen adsorption](image)

**Figure 6.5.** The amount of fibrinogen adsorbed on ePCL/PPy and ePCL/PPyHEP scaffolds with and without 10 µA, 100 Hz AC (sine waves) stimulation was measured using the BCA assay. * $p < 0.05$ when compared against ePCL.
When 2 mg of fibrinogen was incubated on the ePCL and PPy-coated ePCL scaffolds in the absence of AC stimuli, ~8 µg of fibrinogen was adsorbed per cm² of pristine ePCL scaffolds (Figure 6.5). Significantly-less fibrinogen was found to be adsorbed onto ePCL/PPy and ePCL/PPyHEP scaffolds (Figure 6.5). When 10 µA, 100 Hz AC sine waves were applied to the scaffolds during adsorption, there was a slight increase in the amount of fibrinogen adsorbed for both scaffolds (Figure 6.5). However, this increase was not found to be statistically significant. Thus, from our data it seems that fibrinogen adsorption was not influenced to a significant degree by low-intensity sinusoidal AC stimulation. Instead, the presence of the PPy coating did reduce the adsorption of fibrinogen onto the ePCL scaffolds. Among the several functions that fibrinogen is recognised to be involved in during thrombosis, one important role of adsorbed fibrinogen is to promote platelet adhesion. Recent studies have found evidence that the conformation of the adsorbed fibrinogen, rather than the amount, is the key determinant in facilitating platelet adhesion [29]. Hence, although we did not find significant differences in fibrinogen adsorption between stimulated and non-stimulated scaffolds, the possibility that a conformational change in fibrinogen could have occurred will likely influence platelet adhesion and activation. This will be studied in the next section.

6.4.2. Influence of Electrical Stimulation on Activated Platelets on Substrates

During the formation of the fibrin clot, platelet adhesion and activation on the material substrates occur simultaneously. As reviewed in Chapter 2, section 2.2, platelet activation occurs after adhesion onto material surfaces, and is calcium-dependent and requires the activation of GPIIb/IIIa integrin receptors [8]. Another integrin receptor, GPIb, mediates the binding of platelets to vWF present in the subendothelium, or possibly to vWF expressed on the surfaces of endothelial cells [35]. In this study, the amount of P-Selectin (CD62P), a cell adhesion molecule upregulated on activated platelets, on the substrates was measured. P-Selectin aids in the recruitment of leukocytes through binding to PSGL-1, a P-selectin ligand expressed on leukocyte surfaces [36]. The P-selectin assay is henceforth used as a semi-quantitative measurement of the amount of activated platelets attached to the PPy-coated ePCL scaffolds.
P-selectin expression on ePCL/PPy scaffolds after 2 h incubation with platelet-rich plasma (PRP) was similar to that of uncoated ePCL scaffolds, and levels remained unchanged with the application of 10 μA, 100 Hz AC sine waves (Figure 6.6). High levels of P-selectin expression were detected on unstimulated ePCL/PPyHEP scaffolds. However, when the PRP was incubated with these scaffolds under AC electrical stimulation, there was a significant decrease in the detection of P-Selectin on the ePCL/PPyHEP scaffolds (Figure 6.6). This heparin-mediated activation of platelets was ameliorated sharply to a level lower than that of platelets on ePCL/PPy and uncoated ePCL scaffolds. This can either indicate a decrease in platelet attachment, which naturally brings about a drop in P-selectin levels, or a decrease in activated platelets without changing the number of attached platelets. This would not be apparent until the scaffolds are examined in SEM.

The fact that heparin-doped PPy nanocoating was activating platelets in the absence of electrical stimuli has been recapitulated in studies that show the induction of the platelet integrin αIIbβ3 complex by heparin [37]. A recent study by Gao et al. that showed the direct binding of the polysaccharide to both heparin in solution and immobilised heparin [38] could prompt a reassessment of the clinical administration of heparin for anti-thrombotic purposes as it becomes apparent that heparin may be inadvertently activating platelets, even as it is inhibiting clotting factors and fibrin formation concomitantly. In this regard, our method of using electrical signals to modulate activation responses could offer an improved protocol for heparin usage.
Figure 6.6. Amount of activated platelets on the ePCL/PPy and ePCL/PPyHEP scaffolds with and without 10 µA, 100 Hz AC (sine waves) stimulation. An ELISA-based P-selectin luminescence assay was conducted on the platelets attached to the scaffolds 2 h after incubation. P-selectin expression by the platelets on uncoated ePCL surfaces is also shown (dashed line).

6.4.3. Influence of Electrical Stimulation on Leukocyte Adhesion

Leukocytes are crucial mediators of both the thrombotic and inflammatory responses. Blood contact onto surface results in protein adsorption that not only mediates platelet adhesion and activation, but also the attachment of leukocytes [39]. A well-studied process called the foreign body reaction (FBR) following platelet activation involves the release of chemoattractants and interleukins that can direct monocytes to synthetic surfaces (‘foreign bodies’) and induce them to differentiate into macrophages on the material surface [40]. Another well-known mechanism that governs leukocyte recruitment starts with the adsorption of members of the complement system, a group of proteins that recognise, opsonize and clear invading microorganisms or foreign materials, onto the material surface [39], [41]. Other studies have also implicated leukocytes directly in the coagulation cascade. Tissue factor (TF) is long known to be produced by leukocytes after activation by complement member C5 [42]. As abovementioned in Section 6.4.2, leukocyte-platelet aggregation after platelet activation is mediated by the binding of fibrinogen to GPIIb/IIIa receptors on the platelets and integrin αMβ2 (Mac-1) receptors on leukocytes [39].
In this study, the ePCL and PPy-coated ePCL scaffolds were incubated with PRP spiked with leukocytes obtained from the same donor blood. The resulting plasma is termed platelet- and leukocyte-rich plasma (PLRP). Under SEM, extensive leukocyte coverage onto the uncoated ePCL scaffolds was observed (Figure 6.7a). The large and flat appearance of the cells is characteristic of macrophages [43], [44]. Lymphocytes, which are smaller cells with a rounded morphology and often recruited in response to chemokines secreted by monocytes [43], were also observed attached to the fibrous scaffolds (Figure 6.7). These cells are typically present at the site of injury during the chronic inflammation phase [40] and typically interact predominantly with adherent macrophages instead of the material surface itself [43], [45]. On ePCL/PPy scaffolds, extensive coverage of macrophages, too, was observed on the unstimulated scaffolds (Figure 6.8a). Both the ePCL and ePCL/PPy scaffolds hence induced a strong FBR characterised by extensive coverage of the material with immune cells. Platelets interacting with macrophages, exhibiting activated morphology, were also observed on both surfaces (Figure 6.7, 6.8a). However, it was observed that with the application of 10 µA, 100 Hz AC sinusoidal currents, leukocyte attachment to the surface is almost totally abrogated (Figure 6.8b). Macrophages, the first leukocytes to respond to foreign materials, were absent from scaffolds stimulated with AC current whilst incubating with PLRP. The few lymphocytes observed did not seem to be adhering to the scaffold surface, but look trapped in between the fibres (Figure 6.8b).

When the unstimulated ePCL/PPyHEP scaffolds were observed under SEM, the thrombogenic and inflammatory response to the scaffold was comparatively much reduced as compared to the other scaffolds (Figure 6.9). No extensive macrophage adhesion could be observed on the scaffolds (Figure 6.9a). Instead, adherent platelets are visible in the SEM images (Figure 6.9a’). This supports the previous observation that higher P-selectin levels were detected on ePCL/PPyHEP scaffolds (Figure 6.6). Also in agreement with P-selectin data, stimulation of the scaffolds with 10 µA, 100 Hz sinusoidal currents prevented the attachment of platelets (Figure 6.8b). The absence of leukocyte adhesion to the ePCL/PPyHEP scaffolds is of significance as it suggests that inherently the inflammatory responses towards these scaffolds have been modulated by surface chemistry. Indeed, a study of leukocyte adhesion onto different chemically functionalised surfaces have found hydrophilic COOH and OH groups to
be the least adhesive for leukocytes when compared to hydrophobic CH2 groups [46].
This suggests that our heparin-doped scaffolds, being highly charged, could have an immediate anti-inflammatory effect.

**Figure 6.7.** Leukocyte attachment on uncoated ePCL scaffolds. (a) The coverage of macrophages (red arrows), lymphocytes (yellow arrows) and platelets (white arrows) on scaffold surface and (b) close-up magnification of leukocytes.

**Figure 6.8.** Leukocyte attachment on (a) unstimulated ePCL/PPy and (b) ePCL/PPy scaffolds stimulated with 10 μA, 100 Hz AC sinusoidal currents. Macrophages (red arrows), lymphocytes (yellow arrows) and platelets (white arrows) are present.
Figure 6.9. Platelet attachment observed on (a) unstimulated ePCL/PPyHEP and (a’) close-up of attached platelets (white arrows). (b) ePCL/PPyHEP scaffolds stimulated with 10 µA, 100 Hz AC sinusoidal currents.

Taken together, there was a general decrease in thrombogenic and inflammatory response when low intensity, sinusoidal AC stimulation was administered to the PPy-coated ePCL scaffolds. However, the mechanism behind this phenomenon, as with the other studies published on low intensity and low frequency AC stimulation, remains unclear. As AC currents are non-directional, they are theoretically not able to electrically polarise or bias the scaffolds, and 100 Hz is far below the resonance frequencies of proteins or nucleic acids to exert any effect. Further studies have to be conducted on the physical and molecular mechanisms underlying the weak cellular-material interactions in this case.
Conjugation of VEGF on Heparin-stabilised PPy Nanoparticles

The immobilisation of heparin onto the PPy nanoparticles provided active sites whereby VEGF can be conjugated to the nanoparticles with ease (Chapter 4, Section 4.5). As reviewed in Chapter 2, Section 2.4, VEGF is a 45 kDa protein expressed by the human VEGFA gene and it is well known to be an important promoter of angiogenesis [47]. The predominant isoform used in this study (VEGF_{165}) has a heparin-binding domain [47]. Native heparin-like molecules on the cell surface are proposed to be involved in the binding complex between VEGF and VEGF receptors (VEGFR) and the addition of heparin to in vitro endothelial cell cultures is able to potentiate this binding [4], [48]. Our study exploited the natural affinity of VEGF for heparin immobilised onto the PPy nanoparticles.

6.5.1. Matrigel Angiogenic Assay on VEGF-conjugated PPy-Heparin Nanoparticles

In this study, the bioactivity of VEGF on the PPy nanoparticles was assessed through a biological functional Matrigel tube formation assay, a rapid and quantitative in vitro assay commonly employed to investigate angiogenic mechanisms [21]. With pro-angiogenic agents, formation of endothelial tubules resembling the putative unit of capillaries can be observed on Matrigel within a few hours of endothelial cell seeding, and the assay is typically continued for 24 h before the cells undergo apoptosis [20].

VEGF-conjugated PPy-HEP10 nanoparticles were then administered to endothelial cells at the concentration of 50 µg/ml to evaluate Matrigel tube formation. Endothelial tubules comprising of single ECs in a ring-like structure surrounding a hollow lumen were observed 24 h after the administration of VEGF-conjugated PPy-HEP10 nanoparticles, whereas PPy nanoparticles adsorbed with VEGF did not exhibit formation of complete endothelial tubule networks (Figure 6.10). From the quantitative measurements of the average number of branching points (Figure 6.11a) and average total tubule length (Figure 6.11b), it is evident that the endothelial cells treated with VEGF-loaded PPy-HEP10 nanoparticles had a much stronger angiogenic phenotype than cells treated with VEGF-adsorbed PPy. The unloaded PPy-HEP10 and PPy nanoparticles used as blank controls in this experiment did not result in angiogenic
behavior in the endothelial cells. Overall, this experiment not only demonstrated the efficacy for the PPy-HEP10 to work as a delivery vehicle for VEGF, but also showed that the angiogenic bioactivity of VEGF on the PPy-HEP10 nanoparticles was retained.

**Figure 6.10.** Assessment of the bioactivity of VEGF-conjugated PPy-HEP10 nanoparticles using Matrigel tube formation assay. Viability staining (green fluorescence) and brightfield images of endothelial tubule network on Matrigel matrix 24 h after treatment with VEGF-conjugated PPy-HEP10 nanoparticles and VEGF-adsorbed PPy nanoparticles.

**Figure 6.11.** Quantitation of tube formation on Matrigel after nanoparticle treatment. (a) Number of branch points and (b) total tube length quantitated from fluorescence images of endothelial tubules (*p < 0.05).
The demonstrated bioactivity of VEGF in our study is partly due to the fact that our conjugation method for VEGF did not involve covalent crosslinking or the use of harsh reaction conditions. As VEGF conjugation proceeded only after the synthesis of the nanoparticles and took place in physiological buffer, it prevented denaturation of the growth factor. As the natural binding affinity between VEGF and heparin on the PPy nanoparticle surface makes any release of VEGF from the nanoparticles under physiological unlikely, it has to be assumed that whole VEGF-conjugated nanoparticles performed the engagement and activation of the VEGFR. One obvious problem to this hypothesis would be the steric hindrance of the VEGFR binding site from the comparatively large nanoparticle. However, the VEGF binding might be facilitated if it is tethered to the end of long heparin chains extending from the nanoparticle surface (Figure 6.12). That the heparin-binding domain of VEGF lies near its C-terminal, while its VEGFR-binding domain is distinct at near its N-terminal lends support to this hypothesis [49]. In fact, physiologically, heparan sulfate proteoglycans on the cell surface and ECM are co-receptors for VEGF binding, and VEGF-VEGFR interactions are possibly stabilised and potentiated by these heparin-like GAGs [50], [51].

Figure 6.12. Proposed model of PPy-HEP10-VEGF binding to VEGF receptors on the EC membrane.
The binding of the VEGF ligand (immobilised on the nanoparticles in this study) to the extracellular domain of VEGFR is followed by receptor dimerisation and subsequent phosphorylation of specific tyrosine residues located in the intracellular domains of the receptor, which then results in the assembly of signaling complexes that activate the cell survival and migration signalling members e.g. PI3K/Akt and p38MAPK in endothelial cells for angiogenic behavior [51].

6.6 Conclusion

In this chapter, the haemocompatibility and thrombogenicity of PPy-coated ePCL scaffolds were first assessed. In vivo, thrombus formation on a vascular graft material is initiated by the adsorption of pro-thrombogenic proteins such as fibrinogen, and largely dependent on the attachment and activation of platelets, which is marked by the expression of P-selectin (CD62p) on platelet surfaces [52]. Activated leukocytes, monocytes and macrophages especially [40], come into play when the complement system is activated or when chemoattractants are released by activated platelets. In turn, they produce other inflammatory mediators such as cytokines and interleukins which result in a chronic inflammatory state, further accelerating thrombus formation and leading to granulation (formation of connective tissue) and graft occlusion [40]. The ePCL/PPyHEP scaffolds were thus studied for any modulatory effects on the host thrombogenic and inflammatory responses.

As evidenced from the studies, the higher amount adsorption of fibrinogen onto pristine ePCL scaffolds over PPy-coated ePCL scaffolds did not result in higher amounts of activated platelets on the scaffolds, whereby activated platelet amount was similar between non-stimulated ePCL and ePCL/PPy and much higher on ePCL/PPyHEP scaffolds. This is likely to be due to several factors: the conformation of the adsorbed fibrinogen is known to be more important than its amount. There may be other important protein factors involved in platelet adhesion and activation that were not assessed (e.g. fibronectin, clotting factor XII). The platelet-activating effects of ePCL/PPyHEP scaffolds are likely to be independent of protein adsorption as it is known that negatively-charged anticoagulants (e.g. heparin) have direct platelet-activating effects through inducing GPIIb-IIIa complex (integrin αIIbβ3) signalling on platelets [37]. It can be posited that several different events could have led to the
reduction in platelet activation and leukocyte adhesion when AC stimulation was applied to the scaffolds. Fibrinogen adsorption is generally known to be lower for more hydrophilic surfaces [53], but this was not observed in this study (Figure 6.5). Under AC stimulation, while fibrinogen adsorption was not greatly affected by the electrical signal, the low-intensity AC current could be modulating protein activity of other critical enzymes involved in the coagulation cascade. Studies have highlighted the influence of substrate surface chemistry on the activity of thrombin, the enzyme that converts adsorbed fibrinogen to fibrin, revealing that the same amount of fibrinogen adsorbed on surfaces may yield significantly different eventual amount of insoluble fibrin [53]. A recent study performed using circular dichroism spectroscopy with a modified quartz cell concluded unequivocally the conformation of adsorbed fibrinogen to be an important mediator for platelet adhesion [29]. Though no substantial evidence exists in the literature for the effects of AC stimulation on protein structure or function, at least one study has described the denaturation of sarcoplasmic proteins under AC stimulation [54]. The significant reduction in leukocyte adhesion for ePCL/PPy scaffolds under AC stimulation is an important finding that could be due to 1) direct modulating of leukocyte signalling pathways, leading to reduced expression of leukocyte binding receptors or the foreign body response, or 2) reduction in the adsorption of leukocyte-binding inflammatory factors from the plasma e.g. complement proteins [52]. As shown in an earlier study, although ePCL/PPyHEP scaffolds exhibited good electrochemical properties in physiological buffer (Chapter 4, Figure 4.12a), it is unlikely that the material redox activity is a cause for these bio-modulatory observations, as the demonstrated anodic/cathodic (oxidative/reductive) potentials were measured at -0.11 V/-0.58 V. As the electrical signals applied in this study were charge-balanced, low-intensity sinusoidal currents (10 µA), the voltage waveforms required to obtain them were < 0.05 V<sub>p-p</sub> in magnitude, and thus insufficient to result in significant changes to the material surface chemistry. Taken together, it remains plausible that AC stimulation was modulating certain physiological processes at the blood-material interface. As with the findings of Creecy et al. on the effects of AC stimulation on osteoblast phenotypes [9], the underlying biological mechanisms are little understood and require further investigation.

The studies in this chapter also explored the conjugation of VEGF on heparin-immobilised conductive PPy nanoparticles. VEGF-bound PPy-HEP10 nanoparticles
were found to be able to promote angiogenic activity, characterised by increased tube length and number of branch points. A model of VEGF immobilisation on PPy nanoparticles, based on the assumption of unhindered binding of VEGF to VEGF receptors, was proposed. One advantage of using VEGF-conjugated nanoparticles as opposed to free VEGF is that proteins immobilized to solid matrices often retain higher stability and functional activity, owing to structural integrity over longer periods of time as they better avoid unfolding and aggregation, which are typical problems for soluble proteins in free form at low concentrations [55], [56].

References


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Chapter 7

Conclusion and Future Work

This chapter describes the implications of the research findings and concludes with studies to further evaluate the feasibility of using the substrates for endothelialisation and control of thrombogenicity. A high throughput stimulation device that allows for experimentation of more electrical waveforms is also proposed for future development. It is hypothesised that certain voltage-gated membrane receptors are involved in the biological responses towards electrical stimulation. Overall, the findings have potential implications in several areas: the clinical usage of rapidly-endothelialised grafts, possible new anti-thrombotic and anti-inflammatory drug targets from the AC stimulation studies, and the use of VEGF-conjugated PPy nanoparticles for treatment of ischaemic diseases.
7.1 Overview of Research Findings

The objectives and scope of this thesis were set against the backdrop of the increasing incidence of CVD, atherosclerosis and need for less-thrombotic synthetic vascular graft replacements. By this premise, the three sub-hypotheses outlined in Chapter 1 were designed to address the overarching problem of vascular graft thrombogenicity (in small diameter grafts) from three different angles. The first goal sought the understanding of EC growth and thrombogenicity that is mediated by material surface chemistry through a survey of EC growth and thrombogenic biomarkers; the second goal investigated a novel physical approach in the form of AC stimulation on a conductive, haemocompatible substrate for the control of material thrombogenicity; the third goal engineered conductive nanoparticles that are capable as conductive nanofiller, as well as a growth factor carrier. One common thread running through the second and the third goal was the use of heparin for its biological, as well as structural properties. For each of these sub-hypothesis, smaller objectives were created, and these formed the backbone of the studies described in the thesis. The summaries of the findings for each sub-hypothesis are detailed below:

1) Evaluation of gelatin attachment to PCL surface for improving EC coverage and reducing EC thrombogenicity ex vivo to transplantation as a small-diameter graft.

PCL was chosen to be the base graft material, owing to biocompatibility, slow degradability, and tensile strength. To test the first sub-hypothesis, PCL has to be functionalised for support EC adhesion and growth. A tunable surface-initiated grafting strategy via ATRP was selected as it offers the flexibility to create PCL surfaces with different material chemistries, which resulted in increased cytocompatibility, primarily as a result of better attachment, on the gelatin-conjugated surfaces. The ECs on gelatin-conjugated PCL substrates also showed decreased thrombogenicity, as assessed by the expression of thrombogenic markers vWF and MMP-2, and promoted the production of nitric oxide from endothelial cells. To conclude for this study, gelatin attachment via ATRP is a feasible method for improving the ex vivo endothelialisation and anti-thrombogenicity of the PCL material.
2) Fabrication of an electrically-conductive surface followed by the effect of electrical stimulation on thrombosis; such an approach will be used \textit{in situ} for small-diameter grafts.

The second sub-hypothesis of the thesis was motivated by studies that utilised an old concept i.e. bioelectricity for new purposes. Although galvanotactic DC fields have been known for decades, AC stimulation was recently demonstrated to have effects in certain physiological areas. For the AC signals to traverse the material, PPy was selected as the conductive coating material on electrospun PCL scaffolds. Though only few studies have utilised heparin as a dopant for PPy, it was an obvious choice in this thesis, as it is the only FDA-approved anti-thrombotic coating in clinical use. The study demonstrated template polymerisation of heparin-doped PPy on the PCL fibres, effectively rendering PCL simultaneously electroactive and anti-thrombotic. Increasing the proportion of heparin doped did not have an effect on the accessible heparin on the surface of the scaffolds, but did improve the bulk conductivity of the scaffolds. This was proposed to be due to the better templating and better-aligned PPy chains when heparin was present in equimolar ratios. Subsequent studies on thrombosis regulation demonstrated impaired clot formation on heparin-doped PPy-coated PCL scaffolds, and a decrease in amount of activated platelets adhered to the scaffolds when electrical stimulation was conducted. A reduction in macrophage binding to the PPy-coated scaffolds also highlighted potentially downregulated foreign body response that can be achieved with AC stimulation. To conclude, we successfully obtained conductive heparin-doped scaffolds using our technique of template polymerisation, and through the application of AC electrical stimulation, thrombosis can potentially be reduced. More studies will be needed to validate these findings using physiologically-relevant models.

3) The synthesis of VEGF-conjugated PPy nanoparticles will be explored to enhance endothelialisation rates \textit{in situ}; such nanoparticles can be dispersed into an erodible PCL matrix to enhance electrical properties and for delivery of VEGF.

The third sub-hypothesis of the thesis was formulated to address the delivery of VEGF from an erodible PCL matrix in order to improve endothelialisation, while attempting to maintain the conductivity of a PCL-PPy hybrid material. As PPy is bio-inert and non-degradable, the proposed idea was using VEGF-conjugated PPy nanoparticles that can
function as a conductive nanofiller within a PCL matrix, and that can be removed by the immune system as they get released by a degrading polymer matrix. Using heparin as a stabiliser during dispersion polymerisation, the immobilisation of heparin on the PPy nanoparticle surface was found to have occurred in a dose-dependent manner. Heparin-doped PPy was synthesised, and demonstrated loading of VEGF under physiological conditions, via the well-known interaction between heparin and VEGF. The VEGF-loaded PPy nanoparticles were found to retain bioactivity when angiogenic assays utilising Matrigel tube formation were carried out.

The overarching hypothesis for this thesis was stated to be: The thrombogenicity of PCL-based materials can be reduced using EC coverage, electrical stimulation, or delivery of an angiogenic factor. Overall, the findings from testing each sub-hypothesis allow us to reach the conclusion that this hypothesis is supported.

### 7.2 Implications of Research Findings

The promise of growing a tissue-engineered vascular graft is still some time from becoming a reality, as the first clinical trials are still underway for acellular, decellularised constructs [1]. Therefore, synthetic vascular grafts still constitute the best option for vascular graft replacements currently, when clinical data, costs and shelf-life are taken into account. The most encumbering issue for synthetic vascular grafts today is the thrombogenicity of small-diameter grafts, which many is the focus of many research groups, and also that of this thesis. From the conclusions reached in this thesis, the following sections will detail the implications that the research findings have on the area of synthetic vascular graft research.

#### 7.2.1. Rapid Endothelialisation and Importance of Materials Chemistry on Small-diameter Endothelialised Vascular Grafts

The functionalisation of vascular grafts with gelatin via the ATRP method promoted rapid EC growth and coverage. In a clinical setting, this can shorten the *ex vivo* culture time and the amount of autologous ECs that needs to be harvested for graft seeding. As demonstrated, the amount of gelatin needed is tunable through the reaction time and the chain length of the P(GMA) intermediate; but the benefits of more gelatin on the PCL
substrates needs to be re-examined. While comparisons to current graft materials used in clinical trials (ePTFE and fibrin glue) have not been performed, the reduction in EC thrombogenicity is promising.

The thrombosis of blood-contacting surfaces can be predicted by revisiting “Virchow’s triad” of hypercoagulability, endothelial injury (thrombogenicity), and stasis of blood flow [2]. Modern cellular evidence has uncovered that the release of NO from ECs to be dependent on shear stress generated by blood flow [2]. Nowhere else is the importance of stasis in thrombosis more exemplified by the small-diameter grafts that typically encounters slow blood flow. In larger grafts, high flow rates can overcome any potential thrombogenic processes occurring. In smaller endothelialised grafts, where molecular processes e.g. protein adsorption, cell-material interactions can almost be unperturbed, material surface chemistry plays the dominant role as proposed in Chapter 1, Figure 1.1. The experiments in this thesis were conducted in static conditions that best simulate stasis of flow and accentuates the investigation on the role of material surface chemistry on the thrombogenicity of the endothelialised grafts.

This thesis has demonstrated differential regulation of thrombogenic responses by material surface chemistry. EC behaviour and thrombogenicity on materials has not matched the advances made in materials development. Often, biological compatibility assessment of a new material focuses on haemocompatibility and blood-material interactions, but stops at endothelial proliferation and surface coverage with regards to cytocompatibility [3]–[5]. Together with some others in the field [6], the research here has highlighted the importance of conducting detailed EC physiological and thrombogenicity studies on novel materials used in vascular applications, even for surfaces that seemingly promote good endothelial cytocompatibility. One relevant observation is that although the gelatin-coated (physisorbed) coverslips appeared to be better at promoting EC attachment and proliferation than scPCL substrates immobilised with gelatin via ATRP, they were significantly lower in NO production. Although the reason is unclear, it is postulated that it may be connected to the central PI3K signaling pathways (Chapter 5, Figure 5.8). This is also possibility that the way gelatin is tethered to surface (strong adsorption to glass surfaces vs. chemical conjugation to dense polymer brushes) has resulted in different conformation, and hence, differences in the
engagement of integrin receptors. Further studies would have to be conducted. Nevertheless, as there is now a trend to conduct surface functionalisation on materials to promote endothelialisation, the choice of grafting chemistry, should be carefully evaluated. The assessment should be conducted beyond its capacity to promote endothelialisation, and for broader implications such as its influence on EC thrombogenicity.

7.2.2. Anti-thrombotic and Anti-inflammatory Responses to Electrical Stimulation

As demonstrated in Chapter 6, Section 6.4, the application of AC stimulation through conductive materials led to reduction of the amount of adherent activated platelets and macrophages. As passing AC stimulation through a material do not cause a modulation of its surface charge, it implies that the AC stimulation is exerting biological effects by influencing intrinsic cellular molecular pathways. Electrotactic (migration) responses of DC electric fields have been known for decades, and they have been observed in epithelial cells, endothelial cells, stem cells, and lymphocytes [7]–[10]. Although it is unclear how DC fields generate electrotactic effects in these studies, it was observed that members of the PI3K/MAPK pathways seem to be involved in the sensing of DC field polarity. This hypothesis is supported by a seminal paper by Zhao et al, in which epithelial cells with gene level knockouts for members of the PI3K signalling cascade demonstrated diminished migratory responses to DC fields [8]. Other theories have proposed that physiological DC electric field moves charged receptor molecules across the fluid lipid bilayer cell membrane to induce the asymmetry required for motility [11]. The biological mechanisms governing the cellular responses in AC stimulation is less clear, but hints in the literature point to intensity-dependent and frequency-dependent effects observed in various biological outcomes that involve 10 – 40 Hz AC stimulation e.g. differentiation of mesenchymal stem cells [12] and angiogenesis [13]. However, the exact molecular pathways or cellular factor that is involved in such responses are unclear. Identifying the protein or signalling pathways involved in the responses we have observed in this thesis will have practical clinical implications for this research – including the identification of new “druggable” molecular targets for controlling graft-induced thrombosis or foreign body responses. This proposed study is detailed in Section 7.3.2.
7.2.3. Heparin as Bio-active Dopant for Nanotechnology

In addressing two of the sub-hypotheses, heparin has been used for its structural properties, firstly as a molecular template for the tight molecular alignment of PPy during coating on ePCL fibres, and secondly as a nanoparticle-forming stabiliser during synthesis of PPy nanoparticles. Heparin was present in sufficient quantities on the ePCL fibres to prevent blood clotting. To synthesise spherical PPy nanoparticles in aqueous solution without the use of emulsifiers or nanotemplates, heparin was used as the dispersant and steric stabilising agent. The stable immobilisation of poly-anionic heparin chains onto the PPy nanoparticle surface was postulated to be similar to the mechanistic doping of PPy coating on ePCL fibres. The broader implications of this phenomenon could be applied to synthesis strategies of cationic particulate system that requires dispersion polymerisation. Conventional steric stabilisers used during aqueous-based nanoparticle synthesis such as PVA are likely removed from the nanoparticle surface after washing, such as from gold nanoparticles during water extraction as reported by Lopez-Sanchez et al. [14]. The stable immobilisation of heparin, on the other hand, affords researchers a strategy to explore downstream biomedical applications of the nanoparticles.

The negative charges and linearity of the heparin polymer makes it an excellent choice in other material synthesis strategies, such as for layer-by-layer assembly with another cationic polymer. For the applications, the high-affinity binding of many other protein factors to heparin (e.g. EGF, GM-CSF, FGF-2) also makes heparin an option for vascular drug eluting/delivery systems. One caveat for the use of heparin for blood-contacting materials is the evidence that heparin may cause unintended activation of platelets [15], which was also observed for our non-stimulated ePCL/PPyHEP scaffolds. This can be resolved by changing the type of heparin from unfractionated heparin, which was used in this thesis, to low-molecular weight heparin (LMWH). Similar to heparin, LMWH is able to inhibit thrombin, and though it inhibits a lesser spectrum of blood clotting factors than unfractionated heparin, there is clinical evidence that it induces comparatively lesser platelet activation [16].
7.2.4. PPy Nanoparticles for Ischaemic Diseases

This thesis has proposed using PPy nanoparticles as an anchor for VEGF within the polymeric matrix to improve endothelialisation. A wider implication of synthesising VEGF-conjugated PPy nanoparticles would be exploring them as potential VEGF-delivery vehicles for treatment of ischaemic disorders [17], [18]. Among the various angiogenic growth factors, vascular endothelial growth factor (VEGF) is the most important and most widely studied for therapeutic angiogenesis, as it is one of first factors to be expressed during the initiation of the angiogenic response [19]. VEGF has been found to be effective in treating various preclinical models of ischemia, such as in the ischemic brain [20], myocardial ischaemia [21], and ischaemic hind limbs [22], [23]. However, despite the successes in animal studies, VEGF has not demonstrated efficacy in human clinical trials. Reasons for this lack of efficacy have been attributed to the short circulation half-life of free VEGF and the need for repeated administration of VEGF to sustain elevated blood concentration levels to allow for any appreciable benefits [24]. The potential use of the VEGF-conjugated PPy nanoparticles as a therapeutic agent would have to be preceded by more cellular toxicity studies, followed by assessment of the nanoparticle circulation time in animal models and the prolonged activity of the VEGF-conjugated nanoparticle in the blood.

7.2.5. Multifunctional Vascular Graft in Controlling Thrombosis

This thesis describes the first example of using an externally-applied electrical current for the modulation of thrombotic and inflammatory responses. As detailed in Section 7.2.2, the this finding can possibly lead to a discovery of voltage-regulated receptors that are involved in these processes, which then constitute the new drug targets against thrombosis and inflammation. However, as noted, this requires much more research in fundamental biological mechanisms that govern these processes. An alternative strategy would be to instead directly utilise the electrical fields on a functional vascular graft.

As a novel physical approach, one critical development before getting electrical stimulation to be deployable for an actual anti-thrombotic application is the advancement in stimulator design. Implantable heart pacemakers have been used for
decades, and Medtronic recently introduced a pacemaker that measures only 15 mm in length (size of a large vitamin capsule) – Micra™ Transcatheter Pacing System (TPS) – for clinical trials. TPS has a purported battery life of 10 years. A working vascular graft stimulator will have to be smaller than that, considering that the defined size of a small-diameter graft is < 6 mm. However, as research in complementary metal-oxide semiconductor (CMOS) integrated circuit (IC)-based stimulators continue to progress, one can hope of the acceptance of vascular graft-stimulators in future. Other relevant technological advances are the advent of battery-free, wireless powered neurostimulators [25], [26] that can further reduce its size to a true system-on-chip device, and microfabricated microelectromechanical systems (MEMS) for \textit{in vivo} biosensing and drug delivery [27]–[30].

When the findings of the studies are integrated together, one can envision a multi-functional graft material that incorporates multiple strategies to tackle thrombosis (Figure 7.1). It should be noted that this thesis does not seek to already develop a clinically-ready vascular graft ready for pre-clinical models. It should also be recognised that stimulators for control of thrombosis, even if clinically-available, is unlikely to be accepted by patients at this point. Nevertheless, the concept is presented here as a scientifically feasible device.

\textbf{Figure 7.1.} A multi-functional graft material enabled by the research carried out in this thesis.
7.2.6. Implications for Current Clinical Use of Electrical Stimulation

Electrical stimulation in the clinical use (i.e. electrotherapy) has been in existence as the adjuvant therapy for neurological disorders [31], [32], neuromuscular disorders [33], and wound healing [34], [35]. Most cells are electrically active due to the electrical potential that exists across the cell membrane. These membrane potentials are generated from the action of voltage-gate ion receptors/channels. Excitable cells (e.g. neurons and cardiac myocytes) have large membrane potentials, termed action potentials, that they utilise for the transmission of signals. FDA-approved functional electrical stimulation devices (FES) deliver various forms of electrical signals, depending on the required treatment protocol, to target tissues via electrodes. The signals can range from DC, AC, square, or pulsatile waveforms in the voltage or current mode [33], [36], [37]. The nascent, but emerging, field of “electroceuticals” relies on the stimulation of the vagus nerve to release neurotransmitters that are envisioned to have purported therapeutic effects on various disorders, including chronic pain and systemic inflammatory disorders [38].

Among the different forms of electrotherapies in clinical use, the findings of this thesis will have the most relevance for wound healing. The intricate links between wound healing and inflammation have been established by studies demonstrating that chronic (non-healing or very slow healing) wounds have a prolonged and unresolved inflammatory phase, and the successful repair of the wound requires resolution of these inflammatory responses [39]. As chronic wounds are a major complication of diabetes, but yet have a poor prognosis with current medical options, electrical stimulation has been approved (in the United States) as a treatment modality for wounds that have failed standard therapies [36]. The field has seen an evolution of devices from large discrete stimulator units with electrode pads to miniaturised portable electronic devices that are bundled with wound dressings (e.g. RecoveryRx®, BioElectronics). In the context of our findings that AC stimulation can downregulate macrophage adherence to material surfaces, it is hypothesised that AC stimulation can also regulate the invading macrophages and neutrophils that are characteristic of non-healing wounds. One possible future development can be the investigation of proprietary electrical signals that are able to promote both epithelial cell migration and reduced inflammation for chronic wound healing.
7.3 Further Studies and Future Research Direction

This section proposes studies that can be carried out to fill in gaps in the current work and to expand on the studies conducted in this thesis. In order to further reinforce the conclusions that were reached during the testing of each sub-hypothesis, the following studies can be conducted. Some reconnaissance data on the feasibility on these studies will be described.

7.3.1 Gelatin Attachment to Promote EC Growth and Reduce Thrombogenicity

Endothelial cell biomarkers
To complement the findings made on endothelial cell thrombotic states and their propensity to induce thrombosis on gelatin-functionalised PCL substrates, gene or protein expression of the prothrombotic plasminogen activator inhibitor-1 (PAI-1) [40] and anti-thrombotic tissue plasminogen activator (tPA) [41] by ECs can be studied. PAI-1 and tPA have antagonistic functions and the regulation of the tPA/PAI-1 balance in the blood plasma by ECs is one of the ways ECs can influence thrombotic processes [40]. tPA belongs to the class of plasminogen activators that promotes fibrinolytic activity, and PAI-1 is an important inhibitor of plasminogen activators. One can also measure the secretion of prostacyclin (PGI$_2$) and tissue factor pathway inhibitor (TFPI) by ECs. PGI$_2$ belongs to a family of lipid molecules known as eicosanoids, and it is known to inhibit platelet adhesion and activation [42]. TFPI negatively regulates the tissue factor (extrinsic pathway) of the coagulation cascade by inhibiting the tissue factor/factor VIIa complex and factor Xa [43]. As in this thesis, quantitative PCR and immunoblotting (Western blot) can be conducted to measure gene level and protein expression by the ECs, respectively.

In addition to thrombotic markers, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) are inflammatory markers that mediate EC-leukocyte interactions [44]. Like TF and TM, they are plasma membrane surface molecules, and hence, their expression can be quantitated using flow cytometry.

Evaluation in animal models
In vitro culture models such as seeding ECs on PCL substrates have allowed us to obtain reproducible data on EC behaviour and phenotype in a convenient manner, but they hardly address the dynamic environment of the blood vessel. In fact, one of the concerns with endothelialised grafts in earlier studies was the detachment of cells under physiological shear stress due to blood flow [45]. Rabbit models have been used to assess the patency of small-diameter synthetic vascular grafts [46], [47]. A rotational seeding device will be necessary to perform EC seeding on the graft’s tubular structure. After implantation, the endothelialised vascular grafts can be explanted at predetermined time points for evaluation of efficacy (overall patency, integrity of EC layer, and signs of thrombotic occlusions).

While the re-endothelialisation of PCL-based vascular grafts have been assessed in animal models [46], [48], no animal studies have been implanted with vascular prostheses prepared by surface-initiated polymerization, including ATRP. Long-term in vivo assessment of ATRP-functionalised PCL grafts is essential to establish chronic inflammatory responses and toxicology. This will be a crucial step towards potential clinical usage. Of relevance are the findings that bare scPCL-P(GMA) substrates have induced high expression of thrombogenic markers (MMP-2 and vWF) in ECs, highlighting potential pitfalls in using graft surfaces that are not thoroughly covered with gelatin. As controlled polymerisation techniques are now gaining traction in the surface functionalisation of materials for vascular grafts, careful evaluation of the polymer intermediates used should be conducted in preclinical animal models.

7.3.2. Effect of Electrical Stimulation on Thrombosis of PPy-coated PCL Scaffolds

Protein adsorption and conformational change under AC stimulation
As highlighted in Chapter 6, there could be plasma proteins other than fibrinogen playing more important roles in the thrombotic responses on PPy-coated ePCL scaffolds. The amount of fibrinogen adsorbed on the scaffolds was studied under AC stimulation in this thesis, but as mentioned in Section 6.4.1, the conformation of fibrinogen is the more important factor that determines platelet adhesion and activation. Techniques for studying the conformation of adsorbed proteins on polymer surfaces include ATR-FTIR [49] or through the use of monoclonal antibodies that are able to
discriminate and bind conformational epitopes [50]. The observation of any conformational change under AC stimulation will provide first insights into the electrical manipulation of fibrinogen structure, and hence, biological function in the context of clot formation. This work can then be extended to other important factors in the coagulation cascade, such as thrombin, and the clotting factors X and XII (Chapter 2, Figure 2.6). The work performed in this thesis employed the spiking of PBS with known amounts of the protein for adsorption studies. For a more physiologically-relevant in vitro model, these studies can be performed with blood plasma or whole blood, and specificity and sensitivity of the experiments can be improved by using enzyme-linked immunosorbent assays (ELISAs).

**Frequency-dividing stimulator device**

Further studies on using AC stimulation would be greatly facilitated by a high throughput stimulator that can provide variable waveforms from a single channel. In collaboration with the School of Electrical and Electronic Engineering, a stimulation circuit board was fabricated for integration with the cell chamber array designed in Chapter 3, Section 3.4. The stimulator receives is powered by a 3.3 V DC voltage and receives the signal waveform through signal input pins (Figure 7.2a). The first prototype of the device is able to perform a simple $f/2$ frequency division of square waveforms from the input signal (Figure 7.2b), but this will be the first step towards a multichannel, programmable cell stimulator.
Figure 7.2. Design and function of the frequency-dividing stimulator. (a) DC bias connectors supply the power to the ICs on the circuit board, while the stimulation signal is supplied to the Pi input pin. Frequency-divided output pins PO1 - PO4 transmit the processed signals (b) Demonstration of the frequency-dividing function using a 1 MHz input signal, and recorded signals at PO2 (f/4) and PO3 (f/8).

Underlying biological mechanisms in responses towards AC stimulation

As explained in Section 7.2.2., the identification of cellular factors involved in the responses to AC stimulation investigated further to elucidate biological mechanisms underlying the phenomenon. In DC stimulation, the activation of voltage-gated Na⁺ receptors on the cell membrane [51] and redistribution/localisation of voltage-gated Ca²⁺ intracellular receptors [52] were some of the proposed theories to explain the migration of various cell types. As reviewed in Chapter 2 (Section 2.3.1), electrical signals are believed to be transduced through Ca²⁺/calmodulin signalling, probably through the activation of voltage-gated calcium channels on the membrane. As direct
stimuli are proposed to exert biophysical effects mainly only on the cell membrane (due to its high electrical resistance) [53], it is plausible that in our case the electrical signals were transduced from the conductive material onto the cell membranes when activated platelets and macrophages attempted to adhere to ePCL/PPy fibres. As with DC stimulation, electrically-responsive voltage-gated receptors or channels are suspected to be modulated by the electrical signals, eventually leading to inhibition of platelet and macrophage attachment. Voltage-gated K\(^+\) receptors such as K\(_{1.3}\) are ubiquitous and exist on both macrophage and platelet membranes [41], [42]. They are known to have roles in immune cell division upon activation [56], and hence they will be studied to for their involvement in platelet and macrophage attachment on foreign materials. The studies can proceed by first knocking down the expression of the voltage-gated potassium channel members before conducting the AC stimulation to measure thrombogenic and inflammatory responses. If feasible, receptor knock-out mice models can be generated to validate in vitro findings. The identification of the receptors responsible for the downregulation of thrombogenic and foreign body responses can potentially lead to the use of receptor inhibitors on biomaterial surfaces for improved graft patency.

7.3.3. Synthesis of VEGF-conjugated PPy Nanoparticles to Enhance Endothelialisation

Migration studies for ECs
The bioactivity of the VEGF-conjugated PPy nanoparticles was assessed with the Matrigel tube formation assay in Section 6.5.1. To complement this data, another functional assay, EC migration, can be conducted to assess VEGF bioactivity. EC migration is a chemotactic response dependent on VEGF and other growth factors. A simple ‘scratch wound’ created in a confluent EC monolayer, followed by the administration of VEGF-conjugated PPy nanoparticles and noting the time for ‘wound’ closure will be indicative of cell migration responses due to the bioactivity of VEGF [57].

Evaluation of PPy nanoparticle cytotoxicity in vitro and in vivo
Although PPy is acknowledged to be bio-inert, and cyto-compatibility was demonstrated on PPy-coated PCL fibres in Section 6.3.3, nanomaterials have differing
cyto-compatibility from bulk materials due to the possible uptake of the nanomaterials by cellular phagocytic or endocytic mechanisms. The long-term biological effects of nanoparticles need further assessment, and there have been reports of inflammatory responses induced by PPy nanoparticles phagocytosed by macrophages [58]. The cytotoxicity of nanomaterials is known to be dependent on the type of material, the cell type, administered dosage, and size. As this sub-hypothesis is the use of PPy nanoparticles within erodible PCL grafts that can eventually be released into the circulation during PCL degradation, it is pertinent that cytotoxicity assays are conducted on the VEGF-PPy nanoparticles using blood cell types (e.g. monocyte cell line THP-1, red blood cells, lymphocytes). In general, cytotoxicity assays that measure cell membrane integrity (e.g. lactate dehydrogenase release or uptake of calcein AM) are most commonly-used. Other assays that discriminate types of cell death (e.g. apoptosis or necrosis) include Annexin V staining (for apoptotic cells) and propidium iodide (for total dead cells including necrotic cells).

In vivo experiments assess the systemic toxicity of nanomaterials and are often conducted in the early stages before further development for biomedical applications. Similar to traditional drug compounds/macromolecules, the pharmacokinetic principles of adsorption, distribution, metabolism and excretion (ADME) apply to nanomaterials. The PPy nanoparticles with highest VEGF loading in this study was sized at about 100 nm (Section 4.5.1). While it has been recognised that nanoparticles of this size are too large for renal excretion, Fu et al. has found silica nanoparticles of 110 nm to be excreted in the faeces and urine of mice when administered intravenously [59]. The mechanism is unclear but kidney histology did not find any abnormalities to the kidney glomerulus microstructure of the mice. To examine the systemic effects of the PPy nanoparticles, similar mice models can be employed, and major organs harvested to perform histopathological analysis and TEM imaging to study the accumulation of the PPy nanoparticles in the body.

Synthesis of biodegradable polypyrrole
Although PPy is bio-inert, it is not known to be biodegradable. While the motivation of synthesising PPy nanoparticles for use as nanofiller is for removal of nanoparticles by the immune system when the polymer matrix degrades, it would be more ideal to create conductive PPy-based co-polymers that are biodegradable. Recently, a PPy-PCL block
copolymer has been synthesised by the Schmidt group [60]. The biodegradation of this copolymer relies on the hydrolysis of ester linkages to facilitate its degradation to pyrrole oligomers (Figure 7.3a). However, as the conjugated $\pi$ bonding is not continuous along the copolymer, electrical conductivity can only be due to inter-chain electron transport (Figure 7.3b).

![Figure 7.3. The proposed design of a degradable conducting polymer and mode of electron transport. (a) Polypyrrole-polycaprolactone (PPy-PCL) block copolymer is degradable due to the presence of ester links. (b) Electron transport in the copolymer takes place between tightly-packed PPy moieties in adjacent chains.](image)

An alternative strategy is to introduce hydrophilic functional groups into PPy to increase solubility in aqueous solutions. As solvation of polymer chains imply solvent access and chain disentanglement, this may result in the degradation of the bulk material. Preliminary studies were performed on the synthesis of water-soluble polypyrrole by co-polymerising pyrrole monomers with 1-pyrrole-3-carboxylic acid monomers (Figure 7.4a). FTIR confirmed the presence of carboxylic groups in the resulting poly(pyrrole-co-carboxy-pyrrole) (PCP) (Figure 7.4b).
Figure 7.4. The synthesis and verification of carboxyl functional group. (a) Co-polymerisation of pyrrole and 1-pyrrole-3-carboxylic acid in APS at 50:50 molar ratio. (b) The presence of absorbance groups specific to the carboxyl functional group is detected in FTIR spectroscopy for the resulting polymer.

References

Conclusion and Future Work

Chapter 7


Conclusion and Future Work


Conclusion and Future Work


List of Publications

Publications as First Author


* Co-first authors

Publications as Co-author

8. Yuan, S., **Xiong, G.**, He, F., Jiang, W., Liang, B., and Choong, C. Multifunctional REDV-conjugated zwitterionic polycarboxybetalaine–polycaprolactone hybrid


**Book Chapters**


**Conference Publications and Presentations**


5. Xiong, G., Yuan, S., Tan, C.K., Tan, N.S., Roguin, A., Teoh, S.H. and Choong, C. The regulation of endothelial cell thrombogenicity by surface functionalization
using ATRP. *International Conference of Young Researchers on Advanced Materials (ICYRAM), Singapore*, 2012.

**List of Technology Disclosures**

Appendix

Appendix I: Electrospinning of ePCL Fibres

The fabrication of ePCL fibres was carried out by electrospinning ePCL solutions in varying concentrations of 2 – 8 wt % in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) (corresponding to 3 – 14 w/v %). As more difficulties may be encountered in electrospinning solutions at lower concentrations due to insufficient viscosity, the electrospinning of 2 wt % ePCL was first conducted in order to determine the minimum critical voltage. The formation of a stable polymer jet and fibre formation on the collector was observed at 8 kV when the tip-to-collector distance was set at 13 cm, and when the solution feed rate was 1.5 mL/h. This voltage was determined to be the minimum critical voltage for the specific for electrospinning of ePCL in HFIP.

Figure A1. SEM images of ePCL fibres electrospun from (a) 2 wt %, (b) 4 wt %, (c) 6 wt % and (d) 8 wt % PCL in HFIP.
From the SEM images, there was an apparent correlation between fibre thickness and PCL solution concentration (Figure A1). Low polymer concentration and the resulting slower rate of evaporation gave rise to large regions of fibre melding when electrospinning was carried out from the 2 wt % PCL solution (Figure A1a). In contrary, the material obtained from electrospinning of 4, 6, and 8 wt % PCL solutions exhibited a more uniform fibrous morphology with a certain amount of jointed fibres (Figure A1b, c, d). When quantitated in ImageJ, the average diameter of PCL fibres was found to increase with increasing concentrations of the PCL in HFIP solution. Notably, PCL fibres electrospun from 2 and 4 wt % solutions were able to attain diameters of submicron thickness, while for 6 and 8 wt % PCL solutions, fibre diameters were recorded to be from 1 - 1.8 µm (Figure A2).

Appendix II: I-V Measurements of ePCL/PPy Scaffolds

The fundamental electrical behaviour of electroactive scaffolds can be determined via a 2-point current-voltage (I-V) measurement using a parameter analyzer with source measure units (SMUs) connected to probes. For the measurement of the I-V characteristics of the ePCL/PPy and ePCL/doped PPy scaffolds, the Keithley 4200-SCS probe station setup was used (Keithley Instruments, USA). The 2 probes were set 1 cm
apart and manipulated onto the sample (circular discs cut to 2 cm in diameter) at random positions. The voltage was swept from -5 V to 5V in steps of 0.02 V and current was collected simultaneously. The measurements were taken from 3 scaffolds of each treatment group and 3 I-V readings were collected from each scaffold. I-V curves with linear (ohmic) characteristics were obtained with all PPy-coated ePCL scaffolds. In agreement with related conductivity measurements in Chapter 4, Figure 4.11, the resistances of ePCL/PPyHEP1 and ePCL/PPyCl are lower than that of pristine ePCL/PPy scaffolds for both 4 h and 16 h polymerisation reactions (Figure A3).

**Figure A3.** Representative I-V curves of ePCL/PPy, ePCL/PPyHEP1, and ePCL/PPyCl scaffolds fabricated via (a) 4 h and (b) 16 h template polymerisation reactions.