Biomimetic Poly(ester amide) Biomaterials for Vascular Tissue Engineering

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Chemical and Biochemical Engineering
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BIOMIMETIC POLY(ESTER AMIDE) BIOMATERIALS FOR VASCULAR TISSUE ENGINEERING

(Thesis format: Integrated Article)

by

Darryl Kenneth Knight

Graduate Program in Chemical and Biochemical Engineering

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

The focus of this research was to develop a biomimetic, degradable vascular scaffold that could be considered as part of a tissue-engineered vascular graft strategy. A family of degradable poly(ester amide)s (PEAs) derived from naturally occurring α-amino acids, aliphatic diols and diacids were synthesized to yield PEAs with glass transition temperatures below physiologic temperature ensuring their pliability. Tri-functional amino acids L-lysine or L-aspartic acid were incorporated into the polymer backbone yielding complementary functional handles for subsequent conjugation of growth factors. Higher molecular weight PEAs were obtained using an interfacial polycondensation technique compared with a solution polymerization approach.

Human coronary artery smooth muscle cells (HCASMCs) attached and proliferated on all two-dimensional (2D) PEA films. Well-spread cells were observed on the non-functional and aspartic acid functionalized PEAs up to 7 days, more so than on the corresponding lysine containing PEAs. The HCASMCs formed focal adhesions on all 2D PEA surfaces as illustrated by vinculin immunofluorescence, yet smooth muscle α-actin (SMαA) expression was not abundant, suggesting that the HCASMCs had adopted a synthetic phenotype. Uniform, nano-scale, fibrous PEA mats ranging in average fiber diameter from 130 to 294 nm were prepared by electrospinning. The increased surface charge attributed to the pendant carboxylic acid groups decreased the average fiber diameter. HCASMCs seeded on the three-dimensional fibrous mat revealed excellent cell attachment and spreading, but limited cell infiltration. In the aspartic acid containing functional PEA, the fibrous structure was lost post-processing due to a plasticizing effect facilitated by the aspartic acid monomer.

The dual effect of topography and TGF-β1 on HCASMC phenotype was investigated by Western blot analysis. While a modest increase in the expression of both smooth SMαA and calponin was observed on all 2D films at four days culture, the 3D topography alone increased both SMαA and calponin expression suggesting the cellular microenvironment can modulate SMC phenotype.

Finally, X-ray photoelectron spectroscopy and immunofluorescence demonstrated the successful conjugation of TGF-β1 to the pendant carboxylic acid groups suggesting that
functional PEAs can be used to generate degradable, biomimetic biomaterials for vascular tissue engineering.

**Keywords**

Functional poly(ester amide)s (PEAs), α-amino acids, cell culture, human coronary artery smooth muscle cells (HCASMCs), smooth muscle cell phenotype, focal adhesions, waveguide evanescent field fluorescence (WEFF) microscopy, Langmuir–Blodgett (LB) films, electrospinning, transforming growth factor-β1, Western blot analysis.
Co-Authorship Statement

The work contained within this thesis was a collaborative effort. Individual contributions are detailed below:

Chapter 1: Darryl Knight – author; Dr. Kibret Mequanint – chapter revision; Dr. Elizabeth Gillies – chapter revision.

Chapter 2: Darryl Knight – author; Dr. Kibret Mequanint – guidance and chapter revision; Dr. Elizabeth Gillies – guidance and chapter revision.

Chapter 3: Darryl Knight – experimental setup, data collection, author; Dr. Elizabeth Gillies – guidance and manuscript revision; Dr. Kibret Mequanint – guidance and manuscript revision.

Chapter 4: Darryl Knight – polymer synthesis, cell culture experimental setup – phase contrast and fluorescence microscopy imaging including immunostaining, cell viability and focal adhesion quantification, primary author; Rebecca Stutchbury – preparation of Langmuir-Blodgett films, area-pressure isotherm data collection, bright field and waveguide evanescent field fluorescence (WEFF) imaging and close contact counting; Daniel Imruck – grating fabrication, Langmuir-Blodgett film preparation, film analysis with Atomic Force Microscopy imaging and Dektak Surface Profilometry; Christopher Halfpap – waveguide fabrication; Shigang Lin – cell culture on waveguides; Dr. Uwe Langbein – manuscript revision; Dr. Elizabeth Gillies – guidance and manuscript revision; Dr. Silvia Mittler – guidance and manuscript revision; Dr. Kibret Mequanint – guidance and manuscript revision.

Chapter 5: Darryl Knight – experimental setup, data collection, author; Dr. Elizabeth Gillies – guidance and manuscript revision; Dr. Kibret Mequanint – guidance and manuscript revision.

Chapter 6: Darryl Knight – author; Dr. Kibret Mequanint – guidance and chapter revision; Dr. Elizabeth Gillies – guidance and chapter revision.
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<table>
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</thead>
<tbody>
<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>8-Ala-8</td>
<td>PEA derived from sebacic acid, L-alanine and 1,8-octanediol</td>
</tr>
<tr>
<td>8-Phe-4</td>
<td>PEA derived from sebacic acid, L-phenylalanine and 1,4-butanediol</td>
</tr>
<tr>
<td>8-Phe-8</td>
<td>PEA derived from sebacic acid, L-phenylalanine and 1,8-octanediol</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>Ala</td>
<td>L-alanine</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Asp</td>
<td>L-aspartic acid</td>
</tr>
<tr>
<td>BAEC</td>
<td>Bovine aortic endothelial cell</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BOC</td>
<td>t-Butyloxycarbonyl</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CABG</td>
<td>Coronary artery bypass graft</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CAG</td>
<td>Cysteine-alanine-glycine</td>
</tr>
<tr>
<td>CBZ</td>
<td>Carboxybenzyl</td>
</tr>
<tr>
<td>CCL2</td>
<td>C-C motif ligand 2</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
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<td>-------------</td>
</tr>
<tr>
<td>CXCL12</td>
<td>C-X-C motif ligand 12</td>
</tr>
<tr>
<td>D-PHI</td>
<td>Degradable polar/hydrophobic/ionic</td>
</tr>
<tr>
<td>DALY</td>
<td>Disability-adjusted life year</td>
</tr>
<tr>
<td>DAPI</td>
<td>4'-6-diamidino-2-phenylindole dihydrochloride</td>
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<tr>
<td>DCC</td>
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</tr>
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<td>DMA</td>
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<td>DMAP</td>
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<td>Dimethylolpropionic acid</td>
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<td>Deoxyribonucleic acid</td>
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<tr>
<td>DPTS</td>
<td>4-(Dimethylamino)pyridinium 4-toluenesulfonate</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDC</td>
<td>N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ELP</td>
<td>Elastin-like peptide</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>EPC</td>
<td>Endothelial progenitor cell</td>
</tr>
<tr>
<td>ePTFE</td>
<td>Expanded polytetrafluoroethylene</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FA</td>
<td>Focal adhesion</td>
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<td>Fibroblast growth factor-2</td>
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</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GPC</td>
<td>Gel permeation chromatography</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin &amp; eosin</td>
</tr>
<tr>
<td>hBM MNC</td>
<td>Human bone marrow mononuclear cell</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HCASMC</td>
<td>Human coronary artery smooth muscle cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>HMDS</td>
<td>Hexamethyldisilizane</td>
</tr>
<tr>
<td>HRMS</td>
<td>High resolution mass spectrometry</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>hTEV</td>
<td>Human tissue-engineered vessels</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iPS</td>
<td>Induced pluripotent stem</td>
</tr>
<tr>
<td>IRM</td>
<td>Interference reflection microscopy</td>
</tr>
<tr>
<td>ITA</td>
<td>Internal thoracic artery</td>
</tr>
<tr>
<td>IVC</td>
<td>Inferior vena cava</td>
</tr>
<tr>
<td>LB</td>
<td>Langmuir-Blodgett</td>
</tr>
<tr>
<td>Lys</td>
<td>L-Lysine</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MDM</td>
<td>Monocyte-derived macrophage</td>
</tr>
<tr>
<td>MES</td>
<td>4-morpholineethanesulfonic acid</td>
</tr>
<tr>
<td>Mn</td>
<td>Number average molecular weight</td>
</tr>
<tr>
<td>MPC</td>
<td>2-methacryloyloxyethylphosphorylcholine</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>$M_w$</td>
<td>Weight average molecular weight</td>
</tr>
<tr>
<td>NEt$_3$</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>NHS</td>
<td>$N$-hydroxysuccinimide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PAA</td>
<td>Poly(acrylic acid sodium salt)</td>
</tr>
<tr>
<td>PAH</td>
<td>Poly(allylamine hydrochloride)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphorylcholine</td>
</tr>
<tr>
<td>PCI</td>
<td>Percutaneous coronary intervention</td>
</tr>
<tr>
<td>PCL</td>
<td>Poly(caprolactone)</td>
</tr>
<tr>
<td>PCU</td>
<td>Poly(carbonate urethane)</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>Platelet-derived growth factor-BB</td>
</tr>
<tr>
<td>PDI</td>
<td>Polydispersity index</td>
</tr>
<tr>
<td>PEA</td>
<td>Poly(ester amide)</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEI</td>
<td>Poly(ethyleneimine)</td>
</tr>
<tr>
<td>PEO</td>
<td>Poly(ethylene oxide)</td>
</tr>
<tr>
<td>PET</td>
<td>Polyethylene terephthalate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PEUU</td>
<td>Poly(ester urethane)urea</td>
</tr>
<tr>
<td>PGA</td>
<td>Poly(glycolic acid)</td>
</tr>
<tr>
<td>PGS</td>
<td>Poly(glycerol sebacate)</td>
</tr>
<tr>
<td>Phe</td>
<td>L-Phenylalanine</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly(lactic acid)</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactide-co-glycolide)</td>
</tr>
<tr>
<td>PLLA</td>
<td>Poly(l-lactic acid)</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>POC</td>
<td>Poly(1,8-octanediol citrate)</td>
</tr>
<tr>
<td>PVAm</td>
<td>Poly(vinyl amine)</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-glycine-aspartic acid</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROP</td>
<td>Ring opening polymerization</td>
</tr>
<tr>
<td>S−</td>
<td>Serum-free</td>
</tr>
<tr>
<td>S+</td>
<td>Serum-containing</td>
</tr>
<tr>
<td>SCPL</td>
<td>Solvent casting and particulate leaching</td>
</tr>
<tr>
<td>SDF-1α</td>
<td>Stromal-cell derived factor-1α</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SF</td>
<td>Silk fibroin</td>
</tr>
<tr>
<td>SM MHC</td>
<td>Smooth muscle myosin heavy chain</td>
</tr>
<tr>
<td>SM1</td>
<td>Smooth muscle myosin heavy chain isoform 1</td>
</tr>
<tr>
<td>SM2</td>
<td>Smooth muscle myosin heavy chain isoform 2</td>
</tr>
<tr>
<td>SMαA</td>
<td>Smooth muscle α-actin</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>SMemb</td>
<td>Embryonic smooth muscle myosin heavy chain</td>
</tr>
<tr>
<td>SPRM</td>
<td>Surface plasmon resonance microscopy</td>
</tr>
<tr>
<td>SPU</td>
<td>Segmented poly(etherurethane)</td>
</tr>
<tr>
<td>t-Bu</td>
<td>tert-Butyl</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline-Tween</td>
</tr>
<tr>
<td>TCPS</td>
<td>Tissue culture polystyrene</td>
</tr>
<tr>
<td>Tₙ</td>
<td>Decomposition temperature</td>
</tr>
<tr>
<td>TEBV</td>
<td>Tissue-engineered blood vessels</td>
</tr>
<tr>
<td>TEMPO</td>
<td>2,2,6,6-tetramethylpiperidine-1-oxy</td>
</tr>
<tr>
<td>TEVG</td>
<td>Tissue-engineered vascular graft</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>T₉</td>
<td>Glass transition temperature</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>TGA</td>
<td>Thermogravimetric analysis</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TIRF</td>
<td>Total internal reflection fluorescence</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VE</td>
<td>Vascular endothelial</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
<tr>
<td>vWf</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>WEFF</td>
<td>Waveguide evanescent field fluorescence</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
</tr>
</tbody>
</table>
Chapter 1

1 Introduction

1.1 Overview

Despite improved outcomes from percutaneous coronary intervention, the lack of clinically available small diameter vascular grafts (< 6 mm) remains a significant concern for patients who lack a suitable autologous vessel for harvest due to vascular disease, mechanical mismatch or previous surgery. Although surface modifications of prosthetic grafts such as Dacron and Teflon to promote their haemocompatibility or in vivo endothelialization have improved patency rates, their inability to degrade hinder complete tissue regeneration, thus precluding their use in pediatric patients. Tissue engineering offers great potential in developing functional vascular tissues with mechanical and morphological properties of the native blood vessel.

Although there are several approaches to engineer vascular tissues, scaffold-guided tissue regeneration remains the strategy most frequently adopted, in part because the porous scaffold can be fabricated into the desired geometry of the engineered tissue. In these cases, vascular cells adhere and populate a porous scaffold, which serves as a temporary three-dimensional structure enabling the infiltrating cells to secrete and assemble their own extracellular matrix. The promotion of vascular cell attachment, proliferation and function can be induced through the usage of biomimetic materials, where a polymeric scaffold can be modified with cell signaling molecules. The immobilization of these bioactive molecules is specifically designed to elicit a given cellular response, which would then mediate appropriate tissue formation. Transforming growth factor-β1 is a multi-potent cytokine that can modulate both the inflammation process and smooth muscle cell phenotype, implicating its use in vascular tissue engineering.

Most polymeric biomaterials currently being investigated for vascular tissue engineering, lack the pendant functional groups necessary for biomolecule conjugation. Poly(ester amide)s (PEAs) are a class of polymers, which combine the beneficial properties of both polyesters and polyamides. The ester linkages of the PEAs facilitate hydrolytic
degradation, while the amide bonds confer mechanical strength through hydrogen bonding. The PEAs evaluated in this work are derived from α-amino acids, aliphatic diols and diacids. Functional monomers, including amino acids such as lysine and aspartic acid, can be incorporated during the polymerization to introduce functional handles for subsequent conjugation.

1.2 Research Outline

The objective of this study was to synthesize and characterize a family of functional poly(ester amide)s to screen them for potential use in vascular tissue engineering. PEAs based on L-alanine and L-phenylalanine were selected to impart suitable mechanical and thermal properties. L-lysine and L-aspartic acid were incorporated into the PEA backbone to provide pendant amine and carboxylic acid functional handles for subsequent TGF-β1 conjugation. The aliphatic diols 1,4-butanediol and 1,8-octanediol and diacid sebacic acid were selected to impart elasticity and hydrophobicity. The improved hydrophobicity facilitated processing of the PEAs in organic solvents. The selection of sebacic acid also permitted its polymerization at the interface of a heterogeneous mixture, which was compared with a conventional solution polycondensation. The PEAs were characterized with nuclear magnetic resonance (NMR) spectroscopy, Fourier transform infrared (FTIR) spectroscopy, gel permeation chromatography (GPC), thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC).

As potential vascular biomaterials, the PEAs were evaluated in terms of their ability to support human coronary artery smooth muscle cell attachment, viability and proliferation. Smooth muscle cell morphology, phenotype and ability to form focal adhesions were also investigated on two-dimensional films. In addition, the impact of nano-scale fibrous scaffolds obtained from electrospinning, on HCASMC attachment, morphology and infiltration was studied. The effect of chemical structure, architecture and exogenous addition of TGF-β1 on SMC contractile phenotype marker protein expression was also investigated. Finally, conjugation of TGF-β1 to the surface of a functional PEA was examined.
1.3 Thesis Outline

This thesis is divided into six chapters. A broad literature review of tissue-engineered vascular grafts is presented in Chapter 2. Following the review of current research, the motivation for this work is provided. The main research findings are presented in Chapters 3-5. Chapter 3 focuses on the syntheses and characterizations of non-functional and lysine-containing functional PEAs, along with their interactions with HCASMCs. Chapter 4 compares the use of waveguide evanescent field fluorescence with conventional immunostaining techniques for examining the interfacial area between HCASMCs and two-dimensional PEA films. Chapter 5 focuses on the introduction of aspartic acid into the functional PEAs, and its subsequent electrospinning into nano-scale fibers. Furthermore, conjugation of TGF-β1 to a functional PEA is also presented. Finally, a general discussion with conclusions outlining the strengths, limitations and future directions are presented in Chapter 6.

1.4 References


Chapter 2

2 Literature Review

2.1 Clinical Need for Vascular Grafts

Cardiovascular disease (CVD) is a significant cause of morbidity and mortality around the world with approximately 16.7 million people dying from CVD in 2002 alone.\textsuperscript{1} Despite decreased rates of CVD in Canada, CVD still claimed 68,342 lives in 2009, which accounted for almost 29 percent of all deaths in Canada.\textsuperscript{2} The United States of America (USA) has also recently observed a decreased rate in CVD; however, it still remains the biggest killer in the USA with a reported 599,413 deaths in 2009, accounting for almost 25 percent of all deaths.\textsuperscript{3}

Of CVDs, coronary artery disease (CAD) is the leading cause of death in the world, accounting for more than seven million deaths in 2002.\textsuperscript{1} CAD is a condition whereby the narrowing of the coronary arteries prevents sufficient blood flow to the heart. The narrowing of the coronary arteries through the deposition of cholesterol, lipids and calcium in a process known as atherosclerosis is the primary factor in coronary occlusion, as shown in Figure 2.1.\textsuperscript{4} There are two types of cardiac revascularization procedures currently available: percutaneous coronary intervention (PCI) and coronary artery bypass graft (CABG) surgery following coronary occlusion.\textsuperscript{4}

In PCI, a collapsed balloon attached to a balloon catheter is inserted into the narrowed blood vessel and is then inflated to compress the plaque against the arterial wall to improve or restore blood flow. The balloon is then deflated and withdrawn. A stent may also be inserted to stabilize the arterial wall and help maintain patency (opening) of the blood vessel.\textsuperscript{5} As PCI has become more effective and efficient and because PCI is less invasive than CABG surgery, it is generally preferred.\textsuperscript{6} Over the decade of 1998 through 2008, an age- and population-adjusted increase of 68.8% was observed in the number of PCIs performed in Canada. During that same span, there was an increase in revascularization procedures of 39%.\textsuperscript{7}

Although decreases in coronary revascularizations are currently observed in North America, the global burden of CAD will reach an estimated 82 million disability-adjusted life years (DALYs, or healthy years of life lost) by 2020, up from 47 million DALYs in 1990.\textsuperscript{8} The significant increase in DALYs is projected in part due to increased longevity, urbanization and lifestyle changes in developing and transitional countries.\textsuperscript{9}

In cases where the less invasive PCI is deemed inappropriate for long term revascularization due to advanced atherosclerosis, the more invasive CABG surgery is performed. In these cases, suitable bypass grafts are typically harvested from the patient themselves (known as autografts), but may also be obtained from a donor (allografts), a different species (xenografts) or obtained artificially.\textsuperscript{4}
2.2 Autologous Grafts

The use of natural vessel grafts, such as the saphenous vein, radial artery and internal thoracic artery have all been used as autologous bypass grafts.\textsuperscript{10} The saphenous vein is frequently the conduit of choice due to its technical ease of use in cases of multiple grafting procedures or those patients undergoing repeat surgeries.\textsuperscript{10} Despite the popularity of the saphenous vein bypass graft, it has shown limited long-term success with patency rates ranging from 57 to 61\% at 10 years\textsuperscript{10} due to intimal and medial hyperplasia (excessive cell growth in the tunica intima and media of the arterial wall).\textsuperscript{11-12} Hyperplasia typically occurs at the anastomoses (surgical connections of two blood vessels) in large part due to the change in haemodynamic environment. At the grafting site the vein graft is subject to increased pressures as well as shear and pulsatile forces of arterial blood flow. This results in a phenotypic switch of the smooth muscle cells from the contractile (vasoactive) to the synthetic phenotype.\textsuperscript{13}

To improve the mechanical properties of arterial vein grafts and subsequently reduce hyperplasia, researchers have focused on reinforcing vein grafts with external support devices. Zilla and colleagues have devised a constrictive nitinol (nickel-titanium alloy) wire mesh to limit intimal hyperplasia by controlling the dilatation of the vein grafts, hence the shear stress, by constricting the diameter of the blood vessel while concomitantly reducing the wall stress of the native artery. The authors suggested that the reduction in shear stress due to the dilatation of the vein graft would induce intimal hyperplasia and that reducing the diameter of the vein graft would maintain the shear stress of the native artery. The authors reported that the vein graft that was constricted by 50\% in a baboon model showed significantly suppressed intimal hyperplasia at both 6 and 12 weeks when compared to the unsupported vein grafts. Additionally, they demonstrated that only in the 50\% constricted vein grafts was endothelialization maintained.\textsuperscript{14} Although the authors demonstrated some pulse compliance in a subsequent knitted nitinol wire mesh study, wire breakage and a lower degree of surface endothelialization was observed.\textsuperscript{15}

Despite the decreased intimal hyperplasia observed with the supported vein grafts, the wire mesh supports are not biodegradable and thus risk future complications\textsuperscript{16} should the
wire supports fray, break or undergo radial narrowing from axial distension. Vijayan et al. examined a polyglactin biodegradable external sheath as support for porcine saphenous vein grafts to prevent any future risks from permanent stents. The authors adopted a loose-fitting macroporous polyglactin stent for bilateral saphenous vein into common carotid artery interposition grafting in Large White pigs. At one and six months, the stented vein grafts showed decreased neointimal and medial thickening compared to unstented controls; however, the use of relatively small saphenous veins for interposition grafts in large arteries may have created flow conditions not clinically observed in bypass graft surgery.

The radial artery is typically used as a free graft and has shown patency rates similar to or better than the saphenous vein; however, risk for graft occlusion was higher in small target-vessel diameter cases. Moreover, elevated levels of vasoconstrictors including endothelin-1 and angiotensin II have been reported during and following the use of the radial artery in CABG surgery necessitating pharmacological intervention.

The internal thoracic artery (ITA, also known as the internal mammary artery) has demonstrated greater long-term patency rates than the saphenous vein and radial artery due to its resistance to atherosclerosis and intimal hyperplasia, and is now considered the conduit of choice for coronary artery bypass graft surgery. Despite the increased patency rates of ITA grafting, several issues remain. Use of the right ITA is limited due to its lack of proximity to major coronary arterial sites, rendering its use non-standardized. Used as a free or composite graft, the right ITA has shown early graft occlusion, especially in cases of competitive flow, mild to moderate stenosis (narrowing of the blood vessel), and multiple anastomoses.

2.3 Synthetic Prostheses

Given that in coronary artery bypass surgery, conduit availability, repeat operations and graft failure are very real concerns of the autologous graft, synthetic materials as vascular grafts have been explored. Poly(ethylene terephthalate) (PET®, Dacron) and expanded polytetrafluoroethylene (ePTFE, Gore-Tex®) have demonstrated some degree of success as peripheral vascular grafts for larger vessel diameters (in excess of 6 mm) due to high
flow rates of blood past the luminal surface.\textsuperscript{28} In smaller diameter vessels, (3-6 mm), early graft occlusion is frequently encountered, resulting from thrombogenicity of the synthetic surface and anastomotic hyperplasia.\textsuperscript{29} Thrombus formation on the surface of a biomaterial is dictated by the Vroman effect of protein adsorption upon injury following implantation. Multiple factors including blood-material interactions, the extent of cellular necrosis and inflammatory response, the loss of basement membrane structures and subsequent provisional matrix formation are all contributing factors in the cascade of events of wound healing.\textsuperscript{30} In effort to mitigate this cascade of events, much research has been focused on the passivation of the surface of vascular prostheses through the grafting of materials to reduce the electrostatic and hydrophobic interactions at the blood-biomaterial interface, in particular the inhibition of platelet adhesion and activation.\textsuperscript{31} Natural and synthetic materials including poly(ethylene glycol) (PEG), dextran, pyrolytic carbon, phosphorylcholine, albumin and elastin mimetic protein polymers have been investigated to minimize thermodynamically favoured protein adsorption to the vascular graft surfaces.\textsuperscript{31}

### 2.3.1 Vascular Prostheses Passivation

Bureau and colleagues have recently examined the graft density of PEG on the surface of nonwoven PET fiber structures as non-thrombogenic surfaces. The authors activated the surface of the PET with poly(vinyl amine) (PVAm) yielding pendant amine groups which were conjugated to carboxylic acid terminated PEG oligomers via \textit{N}-(3-dimethylaminopropyl)-\textit{N}'-ethylcarbodiimide hydrochloride (EDC.HCl) / \textit{N}-hydroxysuccinimide (NHS) chemistry. The authors reported decreased platelet adhesion and activation with a 10\% PEG solution grafting to the PVAm modified PET fibers when compared to knitted Dacron; however, the results were not superior to previously investigated carbon-coated ePTFE.\textsuperscript{32} In a prospective randomized multicenter study, Kapfer \textit{et al.} demonstrated that in an extra-anatomical anterior tibial artery bypass model, carbon-impregnated ePTFE prostheses were not significantly better than standard ePTFE grafts in terms of graft patency or limb salvage.\textsuperscript{33} Similarly, Wang \textit{et al.} also used PVAm to develop a surfactant polymer to coat ePTFE vascular grafts to prevent platelet adhesion and activation. The authors conjugated both dextran and perfluoroundecanoyl
side chains to the PVAm backbone. The fluorocarbon enhanced adhesion to the hydrophobic PTFE even under dynamic conditions, while the dextran mimicked the polysaccharide-rich glycocalyx, generating a highly hydrated barrier, similar to the PEG-grafted PET fibers, inhibiting platelet adhesion and activation from both platelet rich plasma and whole blood. Despite the promising in vitro results, the data from the proposed future study in a porcine model were never reported.\(^3\)

The highly hydrated surface of erythrocytes is also responsible for their non-thrombogenicity. The presence of the polar phosphorylcholine (PC) heads of the lipid bilayer of the cell membrane are electrically neutral at physiologic pH while carrying both positive and negative charges. The zwitterionic nature of PC resulting in the hydrated surface has shown limited protein and cell adhesion in vitro and thus has been explored as a non-thrombogenic coating of synthetic prostheses.\(^3\) Yoneyama et al. demonstrated reduced thrombogenicity in an interpositional carotid rabbit model up to five days with a segmented poly(etherurethane) (SPU) / 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer blend coating of Dacron prostheses.\(^3\) Similarly, Chaikof and colleagues deposited a membrane-mimetic film onto the luminal surface of gelatin-impregnated ePTFE vascular prostheses and demonstrated reduced platelet adhesion after one hour in a baboon femoral arteriovenous shunt model when compared to uncoated ePTFE grafts. The authors also observed a reduction in fibrinogen adhesion in the membrane-mimetic film coated graft; however, this reduction was not significant.\(^3,3\)

Instead of preventing platelet adhesion and activation through highly hydrophilic surfaces, some researchers have adopted the approach of controlled protein adsorption to inhibit coagulation. Albumin induces less platelet adsorption than other plasma proteins such as fibrinogen.\(^3\) Despite albumin’s rapid adsorption to the surface of a synthetic material, more thermodynamically favoured proteins replace albumin on the surface.\(^3\) Given the susceptibility of albumin to be displaced by larger proteins including fibrinogen, the promotion of albumin immobilization on the surface of vascular prostheses has been investigated. Choi et al. evaluated polylactide coated ePTFE grafts as a mediator of protein adsorption. The authors observed a preferential adsorption of
albumin over fibrinogen on the surface of the coated ePTFE grafts compared to both unmodified and modified ePTFE grafts. The modified grafts were rendered more hydrophilic via chemical treatment, yielding an increase in surface energy. Despite the preferential adsorption of albumin, the polylactide surfaces induced greater blood cell adhesion than both control grafts suggesting a thrombogenic polylactide surface.\(^3\) In effort to mitigate the thrombogenicity of a secondary material, albumin was immobilized to the surface of knitted Dacron grafts through glutaraldehyde crosslinking. In this study, Kottke-Marchant et al. observed reduced platelet adhesion and aggregation, as well as reduced leukocyte adhesion with the crosslinked albumin-coated knitted Dacron grafts when compared to unmodified Dacron. In addition, the release of fibrinopeptide A, an indicator of fibrin formation, was also reduced in the albumin-coated knitted Dacron.\(^3\) Although promising *in vitro*, no difference was observed in clinical outcomes between the coated and uncoated grafts in arterial bypass grafts for aortoiliac disease.\(^{40-41}\)

Not unlike albumin, elastin has also demonstrated an inhibition of platelet aggregation;\(^{42-43}\) however, the mechanism is not fully understood. Despite the initial studies of the antagonistic effect of elastin on platelet aggregation, few studies have evaluated the extracellular matrix (ECM) protein for passivation of vascular prostheses. Woodhouse et al. have tested elastin-like peptides (ELPs) as coatings of vascular graft materials including PET (Mylar\(^\text{TM}\)), poly(tetrafluoroethylene/ethylene) copolymer (Tefzel\(^\text{TM}\)) and a poly(carbonate urethane) (Corethane\(^\text{TM}\)). The authors passively coated all three test materials with a recombinant human elastin peptide and demonstrated a reduction in platelet activation in all cases. Moreover, fibrin accretion and thrombus formation on the surface of a polyurethane catheter was retarded in a New Zealand white rabbit model.\(^{44}\) In a follow-up study, Woodhouse and colleagues reported platelet adhesion and fibrinogen adsorption on Mylar\(^\text{TM}\) coated with three different ELPs. The authors observed a reduction in fibrinogen adsorption in all ELP-coated films; however, only the longer polypeptide chains exhibited decreased platelet adhesion.\(^{45}\) In a related study, an elastin-mimetic triblock protein polymer was evaluated by Chaikof and colleagues as a non-thrombogenic coating of impregnated ePTFE vascular grafts. They too observed inhibition of platelet adhesion and fibrin deposition in a baboon arteriovenous shunt model.\(^{46}\)
2.3.2 Bioactive Vascular Prostheses

As active members of both anti-coagulant and anti-platelet mechanisms, endothelial cells are integral in regulating haemostasis. Therefore much research has been focused on mimicking the anti-thrombogenic features of the endothelial cell surface. Immobilization of anti-coagulant molecules thrombomodulin and heparin onto the surface of synthetic materials to directly inhibit thrombus formation has been studied. Mitigation of thrombus formation can be achieved through a number of coagulation or fibrinolytic pathways as illustrated in Figure 2.2.

To that end, the surfaces of ePTFE prostheses have been modified in numerous ways in order to minimize pro-coagulation or anti-fibrinolytic mechanisms or conversely promote anti-coagulation and pro-fibrinolytic pathways. Thrombomodulin, an endothelial cell surface glycoprotein, strongly binds with thrombin forming a complex that inhibits the pro-coagulant properties of thrombin. Thrombomodulin also activates the anticoagulant protease protein C, which further hinders thrombus formation. Li et al. initially demonstrated that a soluble recombinant human thrombomodulin dose dependently impeded α-thrombin-induced porcine arterial smooth muscle cell proliferation in vitro. The authors then attempted to graft thrombomodulin to ePTFE vascular prostheses with EDC; however, they failed to functionalize the surface of the prosthesis and thus may have simply crosslinked thrombomodulin within the graft. Despite this, they did observe an increase in bioactivity of thrombomodulin when compared to passively coated ePTFE grafts. In a follow-up study, the authors demonstrated a reduction in neointimal hyperplasia in immobilized recombinant human thrombomodulin-coated ePTFE stent grafts.

Heparin, a glycosaminoglycan that inhibits both thrombin and activated factors IX, X, XI and XII, which are involved in the conversion of prothrombin to thrombin has been extensively investigated. Along with its anti-coagulation properties, heparin has been shown to reduce smooth muscle cell proliferation and thus possesses the capability to inhibit intimal hyperplasia. Lin et al. demonstrated the reduction in neointimal hyperplasia at both the proximal and distal anastomoses on heparin-coated ePTFE grafts in a baboon arteriovenous shunt model as shown in Figure 2.3.
In addition to being a part of the anti-coagulation and anti-platelet mechanisms, endothelial cells also maintain haemostasis by providing a blood-compatible lining. In the absence of an adherent and coherent endothelium along the luminal surface of a synthetic graft, late stage thrombosis may still occur despite anti-coagulation therapy.
Consequently, research has also focused on improving endothelial cell growth, retention and confluency on the luminal surface of synthetic grafts. Due to electrostatic and hydrophobic interactions with ePTFE vascular grafts, incomplete endothelialization is typically encountered. Williams et al. have recently studied the effect of conjugated mouse laminin type 1 on endothelialization and neovascularization of ePTFE grafts as interpositional aortic grafts in rats. The presence of red cells, leukocytes, fibrin and platelets in the control grafts suggested a highly thrombogenic surface, while the laminin type 1-conjugated grafts exhibited a complete lining of cells indicative of an intact endothelium. The authors also observed neovascularization in the interstices of the ePTFE grafts in the laminin-conjugated grafts.

Figure 2.3: Neointimal hyperplasia at proximal (top) and distal (bottom) anastomoses. Collagens are blue; elastin is black; others are red. A, Anastomoses of untreated expandable ePTFE graft (control). B, Anastomoses of heparin-coated ePTFE graft (treated). L, Lumen; N, neointima; G, ePTFE graft material. (Verhoeff-Masson stain; original magnification: 40×). Reprinted from Lin, P. H.; Chen, C.; Bush, R. L.; Yao, Q.; Lumsden, A. B.; Hanson, S. R., Small-caliber heparin-coated ePTFE grafts reduce platelet deposition and neointimal hyperplasia in a baboon model. J. Vasc. Surg. 2004, 39 (6), 1322-1328, copyright (2004), with permission from Elsevier.
Alternatively, Kibbe and colleagues mechanically coated the luminal nodes and fibrils of ePTFE with poly(1,8-octanediol citrate) (POC) and implanted the grafts in a porcine carotid model. The authors demonstrated increased endothelial cell growth; however, confluency was only reached at 10 days. Moreover, the improved growth rate did not limit neointimal hyperplasia nor were the authors able to confirm that the neoendothelium was functional. Hoshi et al. took a further step by covalently immobilizing heparin to the shear thinned POC coated ePTFE vascular grafts. The authors demonstrated reduced whole blood clotting and platelet adhesion of the heparin immobilized POC-ePTFE grafts when compared to non-heparinized POC-ePTFE and ePTFE grafts. In addition, the heparin-conjugated grafts supported both endothelial cell and blood outgrowth endothelial cell (an endothelial progenitor cell (EPC)) adhesion as evidenced by expression of von Willebrand factor (vWF) and vascular endothelial (VE)-cadherin. vWF, synthesized and stored by endothelial cells, is integral in the pro-coagulation cascade as it aggregates platelets, while VE-cadherin is localized at intercellular boundaries of confluent endothelial cells and is instrumental in regulating endothelial permeability. The authors also claimed that smooth muscle cells cultured on the heparin-POC-ePTFE grafts showed increased expression of α-actin and decreased cell proliferation; however, the smooth muscle α-actin expression was only examined qualitatively. The cell proliferation data showed that the heparin-conjugated POC-ePTFE grafts resulted in a decreased smooth muscle cell proliferation rate only when compared to two-dimensional cell culture on tissue culture polystyrene (TCPS) and not when compared to POC-ePTFE grafts. Although this data does not suggest that intimal hyperplasia may be reduced due to decreased smooth muscle cell growth with the heparin immobilized POC-coated ePTFE grafts, Hoshi et al. did demonstrate the combined benefit of anti-coagulant therapeutic delivery and improved endothelialization.

In a study by Lu et al., the authors coated 6 mm diameter ePTFE vascular grafts with an anti-CD133 antibody functionalized heparin/collagen multilayer. Poly(ethyleneimine) (PEI) was electrostatically bound to the surface of the negatively charged ePTFE graft. Alternating immersion in heparin and collagen solutions generated a layer-by-layer self-assembled multilayered graft, which was then crosslinked with glutaraldehyde in the presence of anti-CD133 to immobilize the antibody to the surface of the vascular graft.
The authors chose to immobilize CD133 to the surface of the graft as CD133 is a cell surface antigen expressed on EPCs, which induced rapid endothelialization in a seven day porcine carotid artery transplantation model. In addition to the in situ endothelialization, the authors reported significantly decreased platelet adhesion in the heparin/collagen coated ePTFE grafts. Despite the promise of the reported work, future studies will need to address two pressing issues – appropriately sized ePTFE grafts (< 6 mm) and longer in vivo studies to ensure no late stage thrombosis occurs due to a loss of functional endothelium.55

Flameng and colleagues recently coated stem cell homing factor stromal-cell derived factor-1α (SDF-1α) on the surface of Gelsoft™ (Vascutek Ltd., Inchinnan, Scotland) and Polymaille C (Pérouse Laboratories, Ivry le Temple, France), which are both knitted polyester vascular grafts pre-coated with collagen. The authors examined SDF-1α (also known as CXCL12, C-X-C chemokine ligand 12), as it acts as a chemoattractant for hematopoietic stem cells (multipotent cells that can differentiate into all types of blood cells), while inducing the recruitment of EPCs. The authors observed an increase in endothelialization from 27 ± 4% to 48 ± 4% with the SDF-1α coating compared to vascular grafts alone in an ovine model. Concurrently, they also observed a decrease in intimal hyperplasia.61

2.4 Degradable Vascular Grafts

Although vascular prostheses are used effectively in large diameter vascular bypass graft surgery, particularly in the treatment of peripheral artery disease, there still remains no small diameter vascular graft with clinically acceptable outcomes. In addition, the inability of these biostable vascular prostheses to undergo in vivo remodeling following implantation would preclude their use in pediatric cardiac patients who would need grafts to grow with them. Despite this lack of growth potential, the research applied to the passivation and endothelialization of vascular prostheses could be employed to vascular grafts that could undergo remodeling in vivo. For the vascular graft to integrate with the host’s tissue, the graft itself must degrade following implantation, but in a manner adaptive to the needs of the host, ranging from pediatric to geriatric patients. Engineering of degradable vascular grafts currently under investigation include both synthetic and
biologically derived scaffolds, typically rendered biomimetic through functionalization and then potentially sodded with cells. These avenues of tissue engineering all seek to produce grafts promoting vascular cell migration and infiltration, possessing a non-thrombogenic luminal surface, while providing appropriate viscoelasticity and mechanical strength, especially during tissue regeneration.

2.4.1 Tissue Engineering of Vascular Grafts in Vivo

One approach in designing tissue-engineered vascular grafts (TEVG) is through the implantation of an ‘off-the-shelf’ acellular vascular scaffold. In vivo tissue engineering of vascular grafts necessitates the host’s cells to infiltrate and populate a non-thrombogenic three-dimensional porous matrix, while concomitantly preventing blood leakage through the graft. As stated, the scaffold must also remain compliant to preserve the necessary haemodynamic environment, yet provide sufficient burst pressure and suture retention strengths. Given the extensive list of prerequisites for a tissue-engineered vascular graft, numerous approaches have been explored.

Walpoth and colleagues recently evaluated a bilayered biodegradable electrospun polycaprolatone (PCL) scaffold as a vascular graft. The authors prepared the bilayered PCL graft by electrospinning two different concentrations of PCL to obtain a nanofibrous luminal surface, and a microfibrous adventitial side, as shown in Figure 2.4. The microfibrous matrix was designed to allow a dense infiltration of the smooth muscle cells, while the nanofibrous structure was designed to allow the endothelial cells to bridge the gaps to form a confluent monolayer to prevent blood leakage. In a four week bilateral porcine carotid artery model, the authors observed a marginal improvement in graft patency – 78% versus 67% for the PCL and ePTFE vascular grafts, respectively. An increase in neoendothelialization from 58% to 86% was also observed in the PCL grafts. Despite the increase in endothelialization, neointima and thrombus formation were not improved with the PCL electrospun vascular grafts. In a long-term follow-up study, the authors compared the patency of the bilayered PCL vascular graft against the ePTFE prosthesis in a Sprague-Dawley rat aorta replacement study. At a median of 16.5 months, the rats were euthanized, and the grafts were excised and examined. Graft patency in the PCL grafts was 100%, while only 67% of ePTFE grafts remained patent. Although there
was a reduction in calcification and a concomitant improvement in cell infiltration and graft compliance with the PCL grafts when compared to ePTFE grafts, the PCL graft compliance was markedly less than the native aorta.\textsuperscript{64}

Figure 2.4: Vascular graft constructs. Two scaffolds with different porosities were electrospun sequentially to form bilayered vascular grafts. The inside-barrier graft (A) has a high-porosity layer on the adventitial side (*) and a low-porosity layer on the luminal side (#, E). The outside-barrier graft (B) was constructed inversely. The transition between the two layers was continuous, with no delamination (D).\textsuperscript{62}


In effort to further improve the interaction of PCL with both endothelial and smooth muscle cells, recent work has focused on incorporating tripeptides to direct cell behaviour. The tripeptide arginine-glycine-aspartic acid (RGD), the cell adhesive motif of
fibronectin has been incorporated into a PCL-functionalized vascular graft. A naphthalene-conjugated hexapeptide phenylalanine-phenylalanine-glycine-arginine-glycine-aspartic acid (FFGRGD) self-assembled on the surface of the hydrophobic PCL yielding pendant RGD handles, which had previously demonstrated improved hydrophilicity, cell attachment and spreading. In an arteriovenous shunt model in female New Zealand White rabbits, Zheng et al. observed reduced platelet adhesion, improved patency and smooth muscle cell infiltration and greater endothelialization with the RGD-functionalized PCL grafts. Although the authors reported improved smooth muscle cell infiltration and vasoactivity, this did not result in a thicker smooth muscle tissue, nor was endothelialization complete even after a four-week implantation. In a similar study, the tripeptide cysteine-alanine-glycine (CAG) was electrospun with PCL fibers into a vascular graft and implanted in a Sprague-Dawley rat carotid artery model. Kuwabara et al. previously noted that the CAG tripeptide improved endothelialization, while it retarded smooth muscle cell proliferation. Upon excision at 1, 2 and 4 weeks, the authors reported improved endothelialization at all time points and virtually complete endothelialization had occurred within two weeks. The presence of vWF was demonstrated at one week, while the production of endothelial nitric oxide synthase (eNOS) was detected as early as one week demonstrating functional endothelium. The authors also demonstrated reduced smooth muscle α-actin expression at 6 weeks in the CAG-modified PCL graft suggesting a potential to reduce intimal hyperplasia; however, smooth muscle α-actin is a contractile phenotype marker protein and does not give a true representation of smooth muscle cell proliferation.

Although these studies investigated the ability of PCL-modified vascular grafts to either improve patency, endothelialization or smooth muscle cell infiltration, they did not fully examine neovascularization in vivo. Matsumura et al. had previously engineered a vascular graft consisting of polyglycolide knitted fibers and an ε-lactide and ε-caprolactone copolymer sponge reinforced with glycolide and ε-caprolactone copolymer monofilaments, but only recently applied the biodegradable scaffold as a cell-free graft in a canine inferior vena cava (IVC) model. Immunohistological studies revealed endothelialization (factor VIII-positive) and smooth muscle cell proliferation (smooth muscle α-actin) in the tissue-engineered vascular graft (TEVG) at one and 2.5 months
with elastic and collagenous fibers also observed at 24 months. The authors reported no significant differences in the hydroxyproline, elastin and calcium contents between the vascular graft and the native IVC up to 24 months follow-up. Moreover, the elastic modulus of the graft reached that of the native IVC at 2.5 months and continued to mirror the IVC up to 24 months following implantation. In a follow-up study, the authors examined their vascular graft in a canine pulmonary artery model. Again, they reported no significant differences in hydroxyproline and elastin content between the left pulmonary artery and the TEVG; however, significant calcification was observed with the TEVG at 12 months. Although the authors noted a lack of stenosis and thrombosis in the vasculature of any of the animals, the tubular scaffolds were 8 mm in diameter and may not replicate the haemodynamics of the small diameter blood vessel. In another study examining neovascularization, Wang and colleagues implanted a poly(glycerol sebacate) (PGS) vascular graft supported with an outer PCL sheath in the abdominal aorta of Lewis rats. The sheath was electrospun around the tubular PGS graft to strengthen the graft, allowing it to be sutured in place while preventing blood loss through the highly porous PGS network. The authors selected PGS because it is elastomeric, permitting effective transduction of mechanical stimulation, while degrading rapidly in vivo, hence minimizing the host response to a foreign body. At three months, the authors observed significant smooth muscle cell infiltration into the remodeled graft wall; however, smooth muscle α-actin expression was localized away from the luminal surface as shown in Figure 2.5. The authors also demonstrated significant ECM production at 90 days. Verhoeff’s, Masson’s trichrome and safranin O staining revealed the presence of elastin, collagen and glycosaminoglycans in the neoartery, nearing the ECM protein content of the native aorta, as shown in Figure 2.6. The considerable ECM protein content also had a significant impact on the mechanical properties. The neoartery was tough yet compliant with a burst pressure approaching that of the aorta and greater than that of the saphenous vein. Given the promising results, future studies with large animals more representative of the human vasculature are proposed.
Figure 2.5: Smooth muscle cell infiltration and organization at 14 days. (a) Smooth muscle cell distribution (SMαA, green) within the remodeled graft wall. The tissue was split longitudinally, half of which is shown. Native aorta is on the right; its border with the graft is indicated by the dashed line; scale bar, 500 μm. L, lumen. (b) Magnified view of the mid-graft shows distribution of both SMαA–positive (green) and SMαA–negative cells. Nuclei counterstained by DAPI (blue); scale bar, 250 μm. (c) Further magnification of the mid-graft to view the complicated smooth muscle cell distribution (SMαA, green); scale bar, 50 μm. (d) Distribution of endothelial cells (vWF, red) and smooth muscle cells (SMαA, green) in the graft wall. Immunofluorescent images merged with the bright-field image (darkened to not overwhelm the fluorescent images). Dark spots (*) in the bright-field image might be residual graft material. Scale bar, 100 μm. Reprinted by permission from Macmillan Publishers Ltd: [Nature Medicine] (Wu, W.; Allen, R. A.; Wang, Y. Nat. Med. 2012, 18 (7), 1148-1153, http://www.nature.com/nm/index.html), copyright (2012).
Another material garnering attention as a vascular biomaterial is the elastomeric poly(ester urethane)urea (PEUU) derived from PCL diol, 1,4-diisocyanatobutane and putrescine in part due to its mechanical properties, as it has a burst pressure strength and suture retention strength approaching that of the internal thoracic artery. Further to their original study, Vorp, Wagner and colleagues have covalently attached 2-methacryloyloxyethyl phosphorylcholine (MPC) to the luminal surface of electrospun PEUU conduits to impart anti-thrombogenicity. The surface of the PEUU was aminated via ammonia plasma treatment and then a phospholipid copolymer of MPC and methacrylic acid (PMA) was conjugated via EDC chemistry. The vascular grafts were then implanted in the abdominal aorta of rats and examined upon sacrifice at 4, 8, 12 and 24 weeks. The authors demonstrated a significant reduction in platelet adhesion in the PMA-conjugated PEUU grafts, with a corresponding patency rate of 92% compared to just 40% in the uncoated controls at eight weeks. In the occluded grafts, 90% were attributed to acute thrombosis, while the remaining 10% failed due to intimal hyperplasia. The PMA-immobilized PEUU grafts also yielded aligned collagen and elastin fibers, both necessary precursors in neovascularization. Moreover, the authors also demonstrated an
intact endothelium as evidenced by the continuity in vWF immunostaining between the native aorta and the electrospun PEUU graft. The authors also reported significant changes to the dynamic compliance and ultimate tensile strength of the functionalized PEUU grafts upon excision. An initial increase in the dynamic compliance and concomitant decrease in ultimate tensile strength was observed at 4 weeks and believed to be the result of acute degradation of the nodes of the electrospun fibers.71

Although the synthetic grafts may be easier to handle, suture, scale up and sterilize while yielding more reproducible properties, some researchers believe that the high modulus and tensile strength exhibited by fibroin, a component of spider silk, is equally viable as a vascular scaffold material. Catteneo et al. electrospun fibroin into 1.5 mm diameter tubular grafts that were implanted in the abdominal aorta of Lewis rats in end-to-end anastomoses. Upon explantation at seven days, the constructs were infiltrated with smooth muscle cells and lined with endothelial cells as evidenced by the immunostaining of smooth muscle α-actin and vWF, respectively. The authors also reported the presence of elastin at seven days, and that by volume density, was approximately 50% of native vessels. Despite the evidence of complete endothelialization, the graft was only 1.5 cm in length.72 In a similar study, Lovett et al. gel spun aqueous fibroin solutions to yield 2 cm silk tubular scaffolds and implanted them in the abdominal aorta of Sprague-Dawley rats. Silk fibroin grafts remained patent up to four weeks following implantation, while acute thrombosis in all PTFE constructs was observed in 24 hours. The recruitment of vascular cells from the adjacent native aorta resulted in a medial smooth muscle layer and complete endothelialization as confirmed by smooth muscle α-actin and factor VIII staining. Further to the in vivo studies, the haemocompatibility of the silk fibroin grafts were also studied in vitro. A reduction in thrombin and fibrinogen adsorption was observed compared to PTFE and although an increase in platelet adhesion was seen from incubation of platelet-rich plasma on silk fibroin films, the platelets were deemed non-activated by their non-spreading morphology.73

In a complementary study, Yagi et al. prepared three kinds of knitted double-raschel silk fiber grafts coated with crosslinked silk fibroin again with an internal diameter of 1.5 mm, but with a length of 7 cm. The silk grafts were also implanted in the abdominal aorta
of Sprague-Dawley rats. Upon excision at eight weeks, gross observation of the vascular constructs revealed patency of the most elastic and flexible silk fibroin tubular graft tested, attributed to a reduction in intimal hyperplasia. Immunohistological studies revealed smooth muscle cell infiltration resulting in smooth muscle tissue thickness similar to that of the native rat aorta; however, complete endothelialization was not achieved, in particular at the midpoint of the vascular graft following eight weeks implantation. Given that incomplete endothelialization was observed, along with growing smooth muscle tissue, longer studies are necessary to ensure intimal hyperplasia does not result in occlusion of the vascular grafts.

Despite some promising results from foreign biomaterials, other researchers believe that clinical success will incorporate all fundamental features of the vascular wall. Kumar et al. have recently incorporated both fibrillar collagen type 1 and an elastin-like polypeptide (ELP) as a vascular graft. Collagen fibrils were prepared from monomeric type 1 collagen extracted from rat tail tendon before the ELP was coated on the surface of the collagen and rolled to generate a luminal ELP layer on multiple fibrillar collagen layers. Initial studies revealed reduced platelet adhesion in the ELP-coated vascular graft when compared to the uncoated collagen graft control. Histologically, the two week rat aorta interposition study revealed little neointima formation, and continuous vWF staining, indicative of an intact endothelium. Despite the promising short-term in vivo data, the burst pressure strength and suture retention strength still remain a fraction of that of the internal thoracic artery. In a similar study by Niklasson and colleagues, the authors harvested human aortae and then decellularized the constructs to produce human tissue-engineered vessels (hTEV). The decellularized hTEVs exhibited burst strength pressures similar to the saphenous vein and twice those obtained from the fibrillar collagen obtained from rat tail tendon, yet still inferior to the native ITA. The grafts were implanted in an end-to-end anastomoses in the abdominal aorta of female nude mice. Upon explantation of the grafts at six weeks, there was evidence of neointima and neotissue formation on the decellularized grafts. Although cells did not fully infiltrate the decellularized graft, both elastin and smooth muscle cells were prevalent. In addition, vWF staining demonstrated the recruitment of endothelial cells to the luminal surface of the vascular graft. The authors acknowledge that larger animal studies are needed and
that these studies also need to examine the potential for late stage stenosis following neotissue or neointima formation.76

In a more complex approach, McClure et al. have recently prepared vascular grafts composed of PCL, elastin (ELAS), collagen (COL) and silk fibroin (SF) in effort to prepare a trilayered vascular graft mimicking the extracellular matrix of each individual component of the native artery. The subendothelial layer of the tunica intima is comprised of collagen type IV and elastin; the tunica media is composed of several dense layers of smooth muscle cells in a matrix of collagen types I and III, elastin and proteoglycans. The adventitial layer consists of fibroblasts embedded in randomly arranged collagen type I. Despite the presence of both collagen type IV and elastin in the subendothelial layer, the authors chose to electrospin the intima of the vascular graft from a PCL solution, due to recent studies illustrating rapid endothelialization of PCL vascular grafts. Because the percentage of ECM proteins of vascular tissue varies, different compositions of PCL:ELAS:COL, or PCL:ELAS:SF were co-electrospun prior to crosslinking with EDC or genipin to mimic both the tunica media and adventitia.77

2.4.2 Tissue Engineering of Vascular Grafts in Vitro

While some researchers have adopted the approach of in vivo tissue engineering, where cells are recruited into the biodegradable scaffold from adjacent tissue, others believe that seeded cells serve as the ‘building blocks of neotissue.’ Of these in vitro methods, there are two unique approaches to enhance tissue regeneration and remodeling: cell sheet self-assembly and cell sodding of biodegradable scaffolds. Vascular tissue maturation typically occurs in a bioreactor under perfusion, where the vascular constructs are exposed to the haemodynamic cyclic loading observed in vivo.

2.4.2.1 Cell Sheet Tissue Engineering

The first tissue-engineered vascular graft prepared wholly from cultured human cells was developed by L’Heureux et al. Vascular smooth muscle cells (SMCs) obtained from umbilical veins and fibroblasts from human skin were cultured for 30 days with ascorbic acid to produce a cohesive cellular sheet with an associated ECM. The SMC layers were first peeled from the culture dish and enclosed around a tubular support to produce
concentric sheet layers that mimic the media of the vessel. A secondary sheet of fibroblasts was then wrapped around the medial layers to emulate the adventitia. After an eight week maturation period in a bioreactor, the perforated tubular mandrel was removed and luminal endothelial cell seeding was performed. The authors reported expression of vWF and strong inhibition of platelet adhesion in vitro from a coherent endothelium. The three-layered organization of the TEVG included both collagen and elastin, yielding a burst strength of over 2000 mm Hg, which is similar to that of human vessels. In a seven day interpositional femoral artery graft in mongrel dogs, the authors reported patency in three of six animals, where occlusion from acute thrombosis resulted from non-endothelialized vascular grafts.78

Recent work using the cell sheet technology has focused on improved neovascularization through in vitro pre-vascularization. Okano and colleagues developed a five-layered construct where human umbilical vein endothelial cells (HUVECs) were seeded between myoblast sheets. Upon four days culture in vitro, capillary-like structures were partially formed and then implanted into dorsal subcutaneous tissue of nude rats. At one week post-implantation, the capillary-like structures joined the host’s blood vessels creating microvessels, which also contained red blood cells.79 In a more exhaustive study, Sekine et al. developed three-dimensional (3D) tissue with perfusable blood vessels in vitro. The authors isolated cardiac cells from the ventricles of Sprague-Dawley and Lewis neonatal rats.80 Cardiac cells were co-cultured with endothelial cells (ECs) and seeded on temperature-sensitive culture dishes for ease of cell sheet recovery as published in their previous study and shown in Figure 2.8.81

Upon stacking of the cardiac sheets, they were overlaid on a vascular bed from resected femoral tissue containing a connectable artery and vein as shown in Figure 2.8a. Media was supplied through the femoral artery (Figure 2.8b) and perfused through the vascular bed. The authors demonstrated that in the absence of ECs, no tubular structure was observed in the trilayered cell sheet, and that fibroblast growth factor-2 (FGF-2) was necessary to ensure media diffusion from the vascular bed to the cell sheet construct. In combination, the co-culture of ECs in the cardiac sheets enabled the formation of new blood vessels, while the FGF-2 facilitated the connection of these new blood vessels with
the vasculature of the underlying femoral tissue. The three-dimensional tissue-engineered construct remained viable in vitro via media supplied through the newly formed vessels. The functional tissue was then implanted in nude mice where the femoral artery and vein of the construct were reconnected to the carotid artery and the jugular vein, respectively. A cell sheet construct without a vascular bed and a vascular graft without blood vessel anastomoses served as controls. At two weeks, the rats were euthanized and tissue viability was significantly greater in the vascular graft with the blood vessel anastomoses. Moreover, immunostaining of CD31 and calponin highlighted a uniform distribution of blood vessels in the cell sheet construct.80

Figure 2.8: *In vitro* engineering of functional 3D tissue with perfusable blood vessels. (a) To engineer cell sheet constructs with perfusable blood vessels, EC co-cultured cardiac cell sheets are stacked, and then overlaid on a vascular bed *in vitro*. After appropriate perfusion using a bioreactor, the co-cultured ECs formed new blood vessels and connected with the blood vessels that originated from the vascular bed. Finally, the cell sheet constructs survive via the media supplied through the new vessels formed *in vitro*. (b) The engineered constructs are perfused in a custom-made bioreactor. The bioreactor is a one-pass system consisting of a delivery pump, a custom-made tissue culture chamber, pH transmitter, flow transmitter, pressure transmitter, CO₂ gas source, process controller and data acquisition system.®

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2.4.2.2 Cell Sodding of Biodegradable Scaffolds

As mentioned, the *in vitro* maturation of vascular tissue typically occurs in a bioreactor under perfusion; however, in cell sodding of biodegradable scaffolds, the vascular matrices are also exposed to the haemodynamic cyclic loading observed *in vivo*. Niklason *et al.* initially demonstrated that SMCs, when cultured dynamically for eight weeks on tubular degradable polyglycolic acid (PGA) meshes, yielded densely packed layers of SMCs. The engineered vessels exhibited rupture strengths in excess of 2000 mm Hg, exceeding the burst pressure of the saphenous vein. Subsequent studies have also revealed improved SMC proliferation and mechanical properties in dynamic culture conditions. In addition, the studies by Kim and colleagues revealed increased collagen and elastin deposition under dynamic culture in their collagen coated poly(L-lactide-co-ε-caprolactone) tubular grafts; however, upon continued dynamic culture, loss of seeded HUVECs was observed.

Although dynamic culture of SMCs has demonstrated improved cell proliferation, ECM production and cell orientation may be influenced by cell source, scaffold and bioreactor design. Wang *et al.* demonstrated increased collagen deposition from adipose stem cell derived SMCs in PGA meshes upon pulsatile flow, yet showed no effect on elastin synthesis. Three week dynamic cultures on elastomeric PGS tubular constructs resulted in increased levels of both insoluble collagen and elastin synthesis, which the authors attributed to improved mechanical transduction of the cyclic radial distension from the bioreactor to the SMCs. Kaplan and colleagues observed SMC orientation parallel to fluid flow on a silk-based scaffolds, while both Lee and Wang and Ratcliffe reported SMC orientation perpendicular to fluid flow on synthetic polymer-based scaffolds, similar to native vessels. Despite the conflicting data, realizing that mechanical properties mimicking those of native blood vessels can be imparted on to a tubular scaffold through the dynamic loading of a perfusion bioreactor, facilitates the development of a TEVG.

Although evidence of improved cell infiltration and orientation into vascular scaffolds during *in vitro* maturation further reinforced the adage that seeded cells served as the building blocks of neotissue, this behavior has not fully replicated *in vivo*. In 2008, Roh
et al. developed a small (< 1 mm) diameter vascular graft for use in a mouse model. The scaffolds were prepared by rolling non-woven polyester sheets of either PGA or poly(l-lactic acid) (PLLA) around either 21 or 22 gauge needles and then sealing them with a copolymer solution of poly(ε-caprolactone-co-L-lactide) (P(CL/LA)). The scaffolds had excellent mechanical properties with burst pressures in excess of the venous burst pressures and greater suture retention strength than both venous and arterial blood vessels. The intrinsic elasticity was measured as a function of Young’s modulus, and despite the vascular scaffolds being stiffer than physiologic vessels, the scaffolds were more elastic than ePTFE prosthetic grafts.⁹₀

In a follow-up study, the PGA-P(CL/LA) scaffolds were seeded with human bone marrow mononuclear cells (hBM-MNCs) and implanted as IVC interposition grafts in immunodeficient mice. The authors investigated the premise that stem cells in the hBM-MNC population differentiate into vascular SMCs and ECs of the neovessel. All hBM-MNC seeded vascular grafts remained patent throughout the 24 week study. Upon study completion, mature vasculature was also evident, including the presence of a confluent endothelium and a sub-endothelium SMC medial layer. Despite the degradation of the scaffold and its replacement with collagen fibrils, no elastin was detected in the TEVGs. Upon excision of the implants throughout the course of the 24 week study, the authors tested the grafts for hBM-MNC surface antigens. After 1 week post-implantation, Roh et al. confirmed the absence of human ribonucleic acid (RNA) from the seeded hBM-MNCs in the TEVGs using quantitative real time polymerase chain reaction (qPCR). Although the hBM-MNCs were not retained or incorporated into the TEVGs as differentiated cells, the presence of mouse SMCs and ECs following mouse monocyte recruitment suggested that the TEVGs appear to undergo inflammation-induced vascular remodeling. Despite the ultimate loss of hBM-MNCs, their secretion of the cytokine CCL2 (C-C motif ligand 2 and previously known as monocyte chemoattractant protein-1 (MCP-1)) increased monocyte recruitment suggesting that the hBM-MNCs were adopting a paracrine mechanism in neovessel development.⁹¹ Paracrine signaling is a cell-cell communication where secreted paracrine factors (signaling molecules) trigger a change in behaviour or differentiation of nearby cells.
The postulation that seeded cells participate in neovessel formation via paracrine signaling was further evaluated. Breuer and his colleagues tested the effect of BM-MNCs on the TEVGs implanted in macrophage-depleted mice. The authors demonstrated that macrophages (differentiated monocytes) are critical for TEVG patency, as the complete inhibition of macrophage infiltration prevented neotissue formation. Conversely, excessive macrophage infiltration was observed in the unseeded grafts, which resulted in neointimal hyperplasia, and ultimately a stenotic or occluded graft. In fact, early stenosis occurred in 80% of unseeded TEVGs, but only 20% of seeded TEVGs in an IVC interposition graft. Consequently, patency was improved in the cell-seeded grafts when compared to unseeded controls.92

In spite of hBM-MNC loss upon implantation of the TEVG in immunodeficient mice, the patency of all grafts at 24 weeks with a well-developed vasculature including a coherent endothelium and SMC medial layer suggested that the neovessel was comprised entirely of host cells. To ensure that xenograft transplantation rejection was not responsible for the loss of hBM-MNCs in the immunodeficient mice, syngeneic (genetically identical) BM-MNCs were also seeded on TEVGs and implanted in mice. Again the authors observed almost complete replacement of the BM-MNCs with macrophages, with only 0.02% of the originally seeded cells still present at 14 days. Moreover the BM-MNCs were not a significant source of either the ECs or SMCs of the neovessel. The vascular cells forming 93% of proximal neotissue were in fact recruited from the adjacent vessel wall.93

Although seeded cells may not be incorporated into neotissue, extensive research demonstrates the improved patency of TEVGs seeded with cells. Ultimately patent TEVGs will need to be lined with autologous ECs and supported with a medial layer of vasoactive SMCs; however, due to their slow propagation in vitro, focus has shifted to more rapidly proliferating stem cells or the incorporation of progenitor cells. Much controversy has surrounded the use of embryonic stem cells, but with the ability to harvest adult mesenchymal stem cells (MSCs) from BM-MNCs, adipose tissue and skeletal muscle tissue, significant research in stem cell-based tissue engineering has emerged.94 In addition to the MSCs, the reprogramming of somatic cells to induced
pluripotent stem (iPS) cells has further expanded the possible use of stem cells in tissue engineering.\textsuperscript{95}

Cho \textit{et al.} were the first to examine differentiated BM-MNCs in a TEVG in a canine carotid artery model. Both SM\textalpha A/smooth muscle myosin heavy chain (SM MHC)-positive cells and vWF/CD31-positive cells from the mononuclear cell fraction were cultured for three weeks \textit{in vitro} to obtain enough cells for seeding. First, SM\textalpha A/SM MHC-positive cells were uniformly seeded onto small diameter decellularized canine carotid arteries, followed two hours later by the vWF/CD31-positive cells, which were seeded onto the luminal surface of the scaffolds and cultured for one week prior to implantation in an end-to-end anastomoses of the carotid artery. Explanted grafts showed elements of an intact endothelium with well-defined medial and adventitial layers. Moreover, the TEVGs remained patent up to eight weeks post-implantation, while unseeded grafts occluded within two weeks. The explanted grafts also revealed the presence of the fluorescently labeled BM-MNCs up to eight weeks, indicating that some of the BM-MNCs actively participated in the vascular tissue regeneration.\textsuperscript{96} The incorporation of the BM-MNCs into the remodeled vascular tissue contradicts the observations of Breuer and colleagues; however, the behaviour of mesenchymal stem cells is directly related to their perivascular environment\textsuperscript{97} and may differ between humans and dogs. In a follow-up study, the authors seeded the canine BM-MNCs onto a poly(lactide-co-\epsilon-caprolactone) scaffold supported by PGA fibers. Excision of the TEVGs at eight weeks in a canine abdominal aorta model revealed that the TEVG underwent significant remodeling with a structure similar to that of the native aorta as shown in Figure 2.\textsuperscript{98}

Notwithstanding the \textit{in vivo} remodeling following the TEVG implantation, the time needed for differentiation of the BM-MNCs would hinder its use as an off-the-shelf vascular graft. The seeding of non-differentiated BM-MNCs on to a biodegradable vascular scaffold was first reported by Matsumura \textit{et al.} The BM-MNCs were also seeded on a poly(lactide-co-\epsilon-caprolactone) scaffold, but supported with PLLA fibers and then implanted in the IVC of beagles. The explanted grafts were analyzed immunohistochemically at three hours and again at 2, 4 and 8 weeks post-implantation.
Stenosis was not observed in any of the TEVGs while patency was maintained up to two years following implantation. Immunohistochemistry revealed that seeded BM-MNCs initially expressed endothelial cell lineage markers including CD34, CD31, Flk-1, and Tie-2. Following proliferation and differentiation of the BM-MNCs, expression of EC markers CD146, factor VIII and CD31, as well as SMC markers SMαA, embryonic smooth muscle myosin heavy chain (SMemb), and smooth muscle myosin heavy chain isoforms SM1 and SM2 were observed implying the differentiation of the BM-MNCs into mature vascular cells \textit{in vivo}.\textsuperscript{99} Despite the promising remodeling \textit{in vivo}, the inner diameter of the degradable grafts selected for these studies were 10 and 8 mm, respectively, approximately twice the diameter of any healthy male coronary artery.\textsuperscript{100}

Figure 2.9: Histological and immunohistochemical analyses of TEVGs excised eight weeks after implantation. (A) H&E staining of retrieved TEVGs showed regeneration of vascular tissues (100×). (B) Masson’s trichrome staining indicated collagen regeneration (100×). (C) Cells on the luminal sides of explanted TEVGs stained positively for vWF, indicating endothelium regeneration (400×). (D) Immunohistochemical staining for SMαA (400×) showed regeneration of SM tissue. (E) H&E and (F) Masson’s trichrome staining and immunohistochemical staining of (G) vWF and (H) SMαA of native abdominal aortas. The scale bars in (A), (B), (E), and (F) represent 200 µm. The scale bars in (C), (D), (G) and (H) represent 50 µm.\textsuperscript{98} Copyright © 2007 Wiley Periodicals, Inc.
More recently Wu et al. compared four types of scaffolds (inner diameter of 5 mm) to evaluate their ability to direct BM-MNC differentiation \textit{in vitro} and promote vascular tissue formation. Rat BM-MNCs were seeded on PLGA, PGS, platelet-poor plasma-coated PGS and plasma-coated PGS supplemented with platelets. The authors examined the impact of platelet adhesion on differentiation of bone marrow mononuclear cells as platelets have been identified in recruiting circulating EPCs through paracrine release of stem cell-derived factor-\(\alpha\) and vascular endothelial growth factor. The expression of calponin-I and SM\(\alpha\)A and deposition of collagen and elastin from cells cultured on PGS scaffolds suggested smooth muscle differentiation. Although increased cell proliferation was observed with platelet supplementation, and hence ECM secretion, the authors did not report the impact of platelets on the differentiation of stem cells into mature endothelial cells. vWF immunohistochemistry revealed the presence of the blood glycoprotein in platelets,\textsuperscript{101} but its absence at the luminal surface, as expected in ECs, perhaps suggests that the platelets did not actively recruit the EPCs from BM-MNCs, or that the BM-MNCs preferentially differentiated into SMCs.

Although Wu et al. did not report the presence of ECs or their markers, bone marrow MSCs, a small portion of the mononuclear cell fraction, have been successfully differentiated into ECs \textit{in vitro}.\textsuperscript{102-103} The controlled \textit{in vitro} differentiation of bone marrow MSCs to smooth muscle-like and endothelial-like cells can be achieved through supplement manipulation of the culture media.\textsuperscript{104} Decellularized ovine carotid artery grafts were seeded with the autologous cells and implanted as interposition grafts. The seeded grafts remained patent for up to five months, while the unseeded controls occluded within two weeks. The presence of an endothelium and smooth muscle within the TEVG was observed at both 2 and 5 months as demonstrated through immunohistochemical staining of vWF and SM\(\alpha\)A, respectively. MSCs labeled with PKH26, a cell membrane bound fluorescent dye, was detected in the luminal side of the tunica media at two months, but not at five months, suggesting repopulation of the vascular graft.\textsuperscript{104} Similar observations of improved patency have been reported with bone marrow MSC- and EPC-seeded PLLA-PGA scaffolds in a mouse carotid artery model. The authors also labeled the bone marrow MSCs with green fluorescent protein (GFP) and noted a reduction in GFP expression at 35 days when compared to seven days,
further supporting the premise of graft repopulation with host cells. Hashi et al. seeded bone marrow MSCs onto PLLA scaffolds and implanted them into the common carotid artery of rats for 60 days. Well-distributed SMCs within the TEVG indicated strong MSC infiltration and differentiation. Neointimal thickening was only observed in the unseeded controls. Staining for CD31 revealed not only luminal coverage of the TEVG with endothelial cells, but their presence in the outer layer of the graft suggested microvessel formation in the surrounding tissue. The authors also reported that explanted grafts at 60 days showed significant collagen deposition; however, only the MSC-seeded grafts exhibited a sub-luminal elastic lamina layer. Finally, the authors also showed that as early as seven days, most cells were again recruited from the host due to the absence of human antigen nuclear mitotic apparatus staining in the seeded grafts.

The accumulating evidence that cells sodded on vascular grafts significantly improve patency rates when compared to unseeded grafts, yet fail to integrate in neotissue formation, further validates the postulation that seeded cells supplement the host’s innate healing process through paracrine signaling. Specifically, Breuer and colleagues believe that upon implantation of the bone marrow mononuclear cell-seeded tissue engineering graft, the BM-MNCs secrete chemokines such as CCL2, which is known to attract circulating monocytes. Following the differentiation of monocytes to macrophages, platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) are released, promoting infiltration of adjacent ECs and SMCs, which together with the macrophages and fibroblasts begin secreting the extracellular matrix. During scaffold degradation, ECM remodeling continues with a concomitant exodus of macrophages yielding a completely autologous neovessel as illustrated in Figure 2.10.
Figure 2.10. Proposed mechanism of vascular transformation of hBM-MNC-seeded biodegradable scaffolds. Early pulse of CCL2 (MCP-1) secreted from seeded hBM-MNCs enhances early monocyte recruitment to the scaffold. Infiltrating monocytes release multiple angiogenic cytokines, which recruit SMCs and ECs to the scaffold predominantly from mature vascular cells in adjacent vessel segments.91 Copyright © 2010 National Academy of Sciences.

2.5 Mediating the Inflammation and Wound Healing Response

The recent change in the tissue engineering paradigm from passivation of the biomaterial surface to mediating the host inflammatory response has also been studied by Labow and
Santerre. A porous and elastomeric degradable polar/hydrophobic/ionic (D-PHI) polyurethane scaffold was ultimately developed to investigate its modulation of the inflammatory-wound healing response through its direction of monocyte behaviour. To replicate physiological conditions, the translation of biomechanical stimuli to vascular SMCs was initially studied. Human coronary artery smooth muscle cells (HCASMCs) were seeded on D-PHI scaffolds and subjected to four weeks of cyclic mechanical strain. Cell morphology, infiltration and contractile phenotype marker proteins were studied in addition to tensile properties. The dynamic culture of SMCs for four weeks resulted in greater cell proliferation and infiltration within the scaffold when compared to static cultures. Tensile testing also revealed greater elastic modulus and yield strength in the four week dynamic cell culture study, implying an increase in ECM deposition. In addition to the increase in protein synthesis, the expression of contractile proteins: smooth muscle α-actin, calponin, and SM MHC suggested that the HCASMCs still maintained their contractile nature throughout the duration of the study. Given that D-PHI polyurethane scaffolds did not result in dedifferentiation of the HCASMCs, the authors then investigated the influence of this material on monocyte activation and differentiation. Monocyte culture was studied, as implantation of the biomaterial initially results in adsorption of plasma proteins and then recruitment and adhesion of monocytes to the foreign body. Upon adhesion to the surface, monocytes differentiate into monocyte-derived macrophages (MDMs) and then secrete cytokines, which ultimately guide the inflammation and wound healing mechanisms. The cytokine release profile from MDMs that direct effective tissue regeneration is debatable, as anti-inflammatory cytokines are not necessarily pro-wound healing and vice versa as shown in Figure 2.11. Interleukin-1β (IL-1β) is both pro-inflammatory and pro-wound healing, as it activates inflammatory cells (lymphocytes and monocytes) and wound healing cells (fibroblasts). Conversely IL-10 is known to down-regulate the activity of these cells, while concomitantly suppressing further cytokine production. The impact of the D-PHI polyurethane on MDM cell expression of pro- versus anti-inflammatory cytokines was investigated over 72 hours and compared to tissue culture polystyrene (TCPS). Adherent monocytes to D-PHI polyurethane films secreted less pro-inflammatory cytokines TNF-α and IL-1β at 2, 24 and 72 hours when compared to TCPS. A corresponding increase in
the anti-inflammatory cytokine IL-10 from monocytes cultured on D-PHI was observed at 72 hours suggesting IL-10 may have down-regulated monocyte activity and or cytokine production. Taken together, this data indicates that D-PHI may cause less inflammation than TCPS.\textsuperscript{111} In a follow-up study, the authors compared the effect of D-PHI architecture on monocyte activation. Up to seven days, monocytes were more activated on 2D films, as evidenced by their differentiation to MDMs and subsequent up-regulation of tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and unchanging IL-10 levels when compared to D-PHI polyurethane scaffolds.

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<th>Wound Healing</th>
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<td>IL-1ra</td>
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<td>TGF (\beta)</td>
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**Figure 2.11. Cytokine classification based on their roles in the foreign body reaction.** Adapteed from Brodbeck \textit{et al.}\textsuperscript{112} (copyright © 2002 Wiley Periodicals, Inc.) and Schutte \textit{et al.}\textsuperscript{113} (copyright © 2008 Wiley Periodicals, Inc.)

Over the same course, the 3D scaffolds exhibited a decreasing secretion of TNF-\(\alpha\) and significant increase in IL-10 concentration further suggesting a mitigated inflammatory response in \textit{vivo}.\textsuperscript{114} In a preliminary \textit{in vivo} study, unseeded D-PHI polyurethane scaffolds implanted in a subcutaneous dorsal model in mice exhibited a muted inflammatory response up to six weeks. Stable levels of TNF-\(\alpha\) over the course of the six weeks was observed, while a concomitant increase in IL-10 was observed. Moreover, increased levels of IL-13 and CCL2 were detected, while there was a decline in IL-6.\textsuperscript{115}
Although CCL2 is classified as pro-inflammatory, as previous stated, CCL2 was necessary in neovessel formation in a mouse IVC interposition graft model.\textsuperscript{116}

Although the D-PHI polyurethane scaffold has demonstrated good mechanical properties and a mitigated inflammatory response \textit{in vitro} and \textit{in vivo}, the authors only examined the inherent inflammatory response of the scaffold. The localized delivery of cytokines from the D-PHI polyurethane scaffold could have further augmented the wound healing process and subsequent neovessel formation.

The chemokine CXCL12 (C-X-C motif, ligand 12) was bound to the surface of heparin, which was covalently conjugated to an electrospun tubular scaffold comprised of PLA and PCL. CXCL12-bound scaffolds resulted in the recruitment of EPCs to the luminal surface of the graft, which accelerated endothelialization in male Sprague-Dawley rats. Moreover, CXCL12 also resulted in the recruitment of smooth muscle progenitor cells, which were differentiated into SMCs, ultimately improving the elastic modulus and ultimate tensile strength of the graft.\textsuperscript{117} The delivery of recombinant human platelet-derived growth factor-BB (PDGF-BB), which stimulates wound healing has also been incorporated into PLGA microspheres embedded in PLA nanofibrous scaffolds. Immunohistochemistry revealed greater neovascularization in the PDGF-BB delivered scaffolds. Quantitative real-time qPCR demonstrated enhanced gene expression of the CXC chemokine members CXCL1, CXCL2 and CXCL5 also in Sprague-Dawley rat model. These chemokines are active in angiogenesis, inflammation and wound healing, suggesting their implication in the improved angiogenesis \textit{in vivo}.\textsuperscript{118} The co-delivery of cytokines CCL2 and VEGF from alginate microparticles in a collagen/fibronectin gel implanted subcutaneously in mice led to improved functional vessel formation. Despite the delivery of the potent monocyte/macrophage chemoattractant CCL2, the authors observed neither continuous recruitment of monocytes/macrophages, nor their shift to the pro-inflammatory M1 macrophage phenotype.\textsuperscript{119} The combination of growth factors in neovascularization has been examined through the controlled release of VEGF, PDGF and transforming growth factor-\textit{β1} (TGF-\textit{β1}) to promote vasculogenesis and angiogenesis. Alginate crosslinked scaffolds containing 0.1\% alginate-sulfate used to bind the growth factors were implanted subcutaneously in rats. The incorporation of
alginate-sulfate retarded the release of the growth factors, allowing for a controlled release of VEGF, PDGF-BB and TGF-β1 based on their equilibrium binding constants. Explanted scaffolds revealed significant blood vessel formation at one month with the triple growth factor delivery. However, at three months, significant blood vessel regression was observed except in the case of the delayed release of VEGF, PDGF-BB and TGF-β1 from the alginate-sulfate scaffolds.120

Although VEGF and PDGF are critical to both vasculogenesis and angiogenesis, transforming growth factor-β (TGF-β) is the most recognized mediator of immune regulation. TGF-β is a cytokine that initiates inflammation through its recruitment of monocytes, neutrophils and lymphocytes, while aiding in the adhesion and activation of these leukocytes, yet can inhibit them once activated through the reduction in expression of cytokine receptors or increasing expression of IL-1 receptor antagonist (IL-1ra).121 As such, TGF-β does not have an intrinsic action, but functions as a switch so that cells have the ability to respond appropriately to changes in their environment.122 This ability to transmit biological information in vivo has been exploited in tissue regeneration following implantation of a TEVG. Further implicating the use of TGF-β1 in vascular tissue engineering is that TGF-β1 also regulates SMC proliferation, differentiation and up-regulation of contractile phenotype marker proteins,123-125 as illustrated in Figure 2.12.126
2.5.1 Vascular Tissue Engineering with TGF-β1

Due to the inherent properties of TGF-β1, its incorporation into the design of tissue-engineered vascular constructs has been explored. Tranquillo and colleagues demonstrated that the exogenous addition of TGF-β1 resulted in improved SMC proliferation and protein synthesis from neonatal rat aortic smooth muscle cells in fibrin gel constructs. Significant collagen was observed at two weeks, while elastin fibers were visualized at five weeks. The synthesis of both collagen and elastin enhanced both the ultimate tensile strength and the tensile tangent modulus of the fibrin constructs over the course of the five week study. The increased deposition of extracellular matrix proteins from rat SMCs was also previously reported from a TGF-β1-tethered PEG hydrogel.
The conjugation of TGF-β1 has also been studied by Andreadis and colleagues who immobilized TGF-β1 in fibrin hydrogels using a Factor XIII domain. A fusion protein between TGF-β1 and the α₂-plasminogen inhibitor I leader sequence was prepared. The α₂-plasminogen inhibitor I leader sequence was enzymatically conjugated to fibrin during polymerization by factor XIII, effectively immobilizing the TGF-β1 within the hydrogel. Following degradation of the fibrin hydrogel, release of the TGF-β1 from the fusion protein was induced through the incorporation of a plasmin cleavage site. MSCs were embedded in the fibrin hydrogel and after two weeks culture, the fibrin gel contracted to 5% of its original volume. The contractility of the constructs was measured in response to receptor and non-receptor mediated vasoconstrictors with the immobilized TGF-β1 twice as contractile as the exogenous addition of native TGF-β1, suggesting the manner of TGF-β1 presentation in three-dimensional constructs has a significant impact on the ability of SMCs to contract.129

In a follow-up study, the authors examined the synergistic effect of TGF-β1 immobilization within fibrin scaffolds and cyclic mechanical stimulation on differentiation of human neonatal fibroblasts. No differences were observed in the early stage contractile marker protein SMαA; however, the late stage marker SM MHC was significantly up-regulated in the pulsed TGF-β1-conjugated fibrin gels, demonstrating a synergistic effect. Well-distributed and circumferentially aligned SMCs in the vascular wall were also reported. The increase in both the ultimate tensile strength and Young’s modulus were attributed to increased collagen synthesis induced through the cyclic distension. However, the increase in elastin deposition, unlike collagen, was attributed to the conjugated TGF-β1. The prolonged release of the conjugated TGF-β1 may have stimulated elastin synthesis through a couple of mechanisms, including the stabilization of elastin mRNA or the activation of lysyl oxidase, which activates desmosine in the crosslinking of soluble tropoelastin fibers.130 The impact of TGF-β1 on elastin synthesis was further studied by Kothapalli et al. The authors demonstrated a moderate increase in collagen and matrix elastin synthesis from the exogenous addition of TGF-β1 in rat aortic SMCs. A small but significant up-regulation of elastin mRNA was again observed supporting the results in the Liang et al. study. To ascertain the mechanism by which the elastin was up-regulated, the effect of TGF-β1 on the crosslinking of elastin was also
evaluated. Neither tropoelastin nor desmosine levels were affected by exogenous TGF-β1 addition; however, TGF-β1 had conflicting effects on lysyl oxidase. A modest reduction in activity was noted with TGF-β1, yet it resulted in a significant increase in lysyl oxidase protein expression. Despite the moderate reduction in overall lysyl oxidase activity, the significant increase in lysyl oxidase protein expression may have facilitated tropoelastin crosslinking via enhanced catalysis of desmosine.\textsuperscript{131}

Recently, our group has studied the effect of topography and exogenous TGF-β1 on elastogenesis from HCASMCs cultured on a biostable poly(carbonate urethane) (PCU). Contrary to the reported results of increased elastogenesis in two-dimensional cultures with rat aortic SMCs, no elastin was observed from the HCASMCs seeded on 2D films of the PCU although elastin gene expression was detected. Moreover, the addition of TGF-β1 did not enhance this gene expression in 2D cultures. Three-dimensional studies revealed not only an increase in elastin gene expression, but its translation into matrix elastin, which was further enhanced by the exogenous addition of TGF-β1. Immunoblotting of contractile phenotype marker proteins SMαA and calponin also revealed their up-regulation in 2D culture; however, the onset of their up-regulation in 3D was delayed, with significance only reached at 14 days.\textsuperscript{132} The lack of observed elastin in 2D culture supplemented with TGF-β1 contradicts the study by Kothapalli \textit{et al.}, though rat aortic SMCs were used in their study, which may not translate to HCASMCs. The use of tissue-appropriate autologous vascular cells (or progenitor cells) may offer a better representation of their true behaviour in a clinical setting.\textsuperscript{133} The increased elastogenesis in 3D culture, yet delayed onset in the up-regulation of contractile protein expression could suggest that TGF-β1 may initially stabilize the elastin mRNA, promoting its synthesis prior to enhancing HCASM differentiation to the vasoactive contractile phenotype, both of which are critical in tissue engineering of a vascular graft.\textsuperscript{132}

### 2.6 Motivation

Given that TGF-β1 can modulate the inflammatory response, while concomitantly induce extracellular matrix deposition including elastin from HCASMCs, resulting in improved contractile protein expression, suggests that a TGF-β1 incorporated vascular graft could mediate inflammation, promote wound healing and ultimately long-term patency. The
motivation of this study was to develop a functional, biodegradable, porous scaffold that could not only support human vascular cell adhesion and proliferation, but also the incorporation of TGF-β1 for consideration as a biomimetic scaffold for in vitro vascular tissue engineering.

2.7 Vascular Scaffold Design

Additional considerations in the development of a vascular scaffold include retaining cell function and differentiation upon cell adhesion to the surface of the biomaterial. The scaffold should be highly porous with significant pore interconnectivity to allow cell infiltration, nutrient and waste diffusion and subsequent vascularization. As the tissue construct matures, the scaffold should degrade in a controlled rate mirroring that of ECM production.\(^8\)\(^9\),\(^1\)\(^3\)\(^4\) Obviously, the scaffold and the degradation products thereof, must neither elicit a chronic inflammatory response, nor result in dedifferentiation of the vascular cells.\(^1\)\(^3\)\(^5\) This biocompatibility is a function of the scaffold’s chemical structure, morphology and manner of processing. Although the ease of processability is an important aspect in scaffold design, toxicity arising from residual monomers, stabilizers, initiators, crosslinking agents or solvents can limit scaffold biocompatibility.\(^1\)\(^3\)\(^4\) Furthermore, the scaffold must be readily sterilizable to be used in a clinical setting. Control of these factors is critical in designing a successful TEVG.

The delivery of TGF-β1 from a vascular scaffold could be achieved in a number of ways. In the simplest form, the growth factor could be passively adsorbed to the surface of the scaffold; however, in the haemodynamic environment, rapid loss of a physically adsorbed growth factor would likely occur, limiting its efficacy. Thus, controlled release of TGF-β1 via an osmotic pressure mechanism\(^1\)\(^3\)\(^6\) or through a slowly degrading polymer matrix, whether embedded or covalently attached, may offer the most promise in achieving a TGF-β1 incorporated vascular scaffold. In the bioconjugation approach, ensuring that the biological activity is not compromised during attachment is paramount. As Andreadis and colleagues demonstrated, TGF-β1 can be immobilized during fibrin polymerization through a fusion TGF-β1 protein,\(^1\)\(^2\)\(^9\) but this approach is highly specific, potentially limiting its versatility. Secondly, plasma surface treatment can be used to generate functional groups on the surface of a synthetic graft, whereby the growth factor could be
covalently attached to the surface using conventional peptide synthesis chemistry. However the use of plasma surface treatment can result in a rough topography, random pore size, non-uniform pore interconnectivity or loss of scaffold morphology. Using NH₃, O₂ and H₂O species in plasma can penetrate microporous polymer membranes, effectively modifying the surface at depths of 50–500 μm, which could potentially affect the bulk properties of the synthetic graft. Alternatively, the conjugation of TGF-β1 to a biodegradable polymer with pendant functional groups could overcome potential issues with the use of plasma surface treatment.

2.8 Functional Degradable Polymers

Of all the degradable biomaterials examined as vascular scaffolds and grafts, few contain pendant functional groups, with the vast majority of them being natural polymers, excluding the synthetic polymers which underwent plasma surface treatment. Despite the presence of several different functional handles, bioconjugation to natural polymers is problematic due to immunogenicity, batch-to-batch variability and structural complexity. The only synthetic polymer with pendant functional groups was the D-PHI polyurethane scaffold due to the methacrylic acid monomers co-polymerized during the curing process. Despite the incorporation of the pendant carboxylic acid groups, to our knowledge, the conjugation of a biomolecule to the polymer’s surface was never reported.

A common route to generate functionalizable biodegradable polymers is through the ring opening polymerization (ROP) of functional monomers. The ROP of 6-hydroxynon-8-enoic acid lactone to yield pendant allyl groups or cyclic ketones such as 2-oxepane-1,5-dione, which can be readily reacted with hydrazines to yield pendant hydroxyl or amine groups, have been previously reported. Although synthetic monomers have been used in polyester co-polymerizations, significant research has focused on the incorporation of functional α-amino acids yielding a variety of pendant functional groups. Amino acids (and poly(amino acid)s) have been used to generate random, block, alternating or graft co-polymers, as illustrated in Figure 2.13. The incorporation of functional monomers into polyesters has been thoroughly scrutinized due to their ease of syntheses and tunability. Modulation of their molecular weight or co-polymer
composition provides a degree of control on their mechanical properties and hydrolytic degradation. Despite the versatility in manipulating these properties, vascular scaffolds based on the PGA, PLLA or PLGA tend to be stiffer (high elastic modulus)\textsuperscript{134} than native arteries, which has been shown to decrease elastin synthesis in vascular grafts.\textsuperscript{146}

**Figure 2.13:** Schematic representation of different macromolecular structures of α-amino acid-containing degradable polymers.\textsuperscript{143} Reprinted with permission from (Sun, H.; Meng, F.; Dias, A. A.; Hendriks, M.; Feijen, J.; Zhong, Z. *Biomacromolecules* 2011, 12 (6), 1937-1955). Copyright (2011) American Chemical Society.
Although not a polyester, poly(trimethylene carbonate) is another material that has been functionalized with allylglycine,\textsuperscript{147} lysine,\textsuperscript{147} and glutamic acid,\textsuperscript{148} while separately being investigated for use in vascular tissue engineering.\textsuperscript{85,149-150} Although preliminary results of the base polymer are promising, only ring opening polymerization of trimethylene carbonate is being investigated; therefore, its functionalization would result in a block copolymer based on amino acid incorporation or alternatively, necessitate the incorporation of synthetic monomers, which may not offer the same biochemical cues as amino acids upon implantation.\textsuperscript{143}

2.9 Poly(ester amide)s (PEAs)

Although polyesters offer the ability to tune certain parameters, making them ideal for several biomedical applications including sutures,\textsuperscript{134} bone pins\textsuperscript{151} and drug delivery vehicles, their high elastic modulus\textsuperscript{134,146} and generation of acidic by-products\textsuperscript{151} renders them poor choices as vascular scaffold materials. Poly(ester amide)s (PEAs), a related class of polymers, composed of both ester and amide linkages along the polymer backbone, emulate polyesters in that they also offer a degree of control over both the mechanical and thermal properties as well as their rates of degradation.\textsuperscript{152} The PEAs studied in this work are derived from an amino acid, a diol and diacid, as shown in Chart 2.1, and provide a significant degree of control over the polymer’s final properties.

\begin{center}
\textbf{Chart 2.1: Schematic of $\alpha$-amino acid based poly(ester amide)s.}
\end{center}

Along with the tunability of the PEA properties, incorporation of amide linkages along the polymer backbone promote its enzymatic degradation.\textsuperscript{153-155} The susceptibility of the PEA to enzymatic degradation should enhance its surface degradation, limiting the possibility of a large accumulation of acidic species in the local tissue. Moreover, the by-products following degradation of the PEA will include amino acids, which are found physiologically, limiting their potential toxicity. Finally, monomers with pendant functional groups, including those based on amino acids such as lysine and aspartic acid
can be incorporated during the polymerization to provide functional, biodegradable polymers.

The incorporation of functional groups along the backbone of PEAs using α-amino acids has been previously reported. Jokhadze et al. protected the carboxylic acid group of lysine as a benzyl ester, and used the α,ε-amino groups as the diamine in a solution polycondensation. After the polymerization, the benzyl ester was selectively cleaved via hydrogenolysis, yielding pendant carboxylic acid groups, which they further functionalized with 4-amino-TEMPO to mimic nitric oxide to demonstrate the reactivity of the side group. In a follow-up study, DeFife et al. used the TEMPO-functionalized PEA to assess leukocyte adhesion and activation and platelet depletion from whole blood. While the TEMPO-functionalized PEA neither depleted leukocytes nor platelets from blood, it also muted the inflammatory response through a down-regulation of pro-inflammatory cytokines IL-6 and IL-1β from monocytes. A concomitant up-regulation in the anti-inflammatory cytokine IL-1ra, a natural inhibitor of IL-1β, which competitively binds the IL-1 receptor, was also observed. Guan et al. conducted a similar study to that of Jokhadze et al. where again a benzyl ester was hydrogenated to yield the pendant carboxylic acid; however, in their study, Guan et al. used dimethylolpropionic acid (DMPA) to yield the PEA based on glycine with the functional handle. This study was the first to report a functional interfacial polycondensation of poly(ester amide)s.

Chu and colleagues have gone a step further and incorporated both D,L-allylglycine and L-arginine as the functional amino acid into a solution polymerized PEA. The introduction of the double bond allowed the authors to introduce pendant amine, carboxylic acid and sulfonate groups through the reaction of the double bond with functional thiol molecules. Moreover, the double bond could also be used in photo or thermal crosslinking potentially yielding a PEA elastomer. The incorporation of the L-arginine was devised so that the cationic PEAs could then be fabricated into nanoparticles, used as protein delivery vehicles or to improve cell attachment. Release of bovine serum albumin (BSA) from arginine-based PEA nanoparticles demonstrated a burst effect with over 30% of the model compound being released into phosphate buffered saline (PBS) within two days with little BSA being released over the following
eight days. The authors demonstrated bovine aortic endothelial cell attachment and proliferation on the surface of the arginine-based PEA up to four days culture. The authors also reported a reduction in inflammatory response due to a decrease in TNF-α release from mouse J774 macrophages when compared to PCL controls; however, no statistical analyses were conducted.\textsuperscript{161}

Not only have the $\alpha$-amino acids been varied to generate different PEAs, so too have the diols and diacids. Unsaturated 2-butene-1,4-diol and fumaric acid has been incorporated into PEAs to generate a double bond along the polymer backbone\textsuperscript{162} and subsequently reacted with thiols,\textsuperscript{163-164} amines\textsuperscript{164} or PEG diacrylate to generate hydrogels.\textsuperscript{165} Newly synthesized epoxy-based PEAs using cis- and trans-epoxysuccinic acids have been reported and subsequently functionalized with both electrophilic and nucleophilic model compounds. Both chemical (1,6-diaminohexane) and free radical (benzoyl peroxide) crosslinking following the reaction of the oxirane with either an acrylate or methacrylate group were also reported.\textsuperscript{166}

### 2.10 Objectives

Even though functional poly(ester amide)s have been examined in the biomedical field as drug delivery vehicles,\textsuperscript{167} non-viral gene delivery vehicles,\textsuperscript{168-170} stimuli-responsive degradable polymers\textsuperscript{171} and as coatings on stents,\textsuperscript{158} to our knowledge, biomimetic poly(ester amide)s as vascular scaffolds have not been investigated. Despite the use of these functional PEAs in the biomedical field, one challenge that has limited the use of PEAs in vascular tissue engineering is the relatively low molecular weights of the polymers achieved through solution polycondensation.\textsuperscript{172-174} The polycondensation between two reactive monomers at the interface of an aqueous and organic layer is an attractive alternative to polycondensation done in solution, as it is faster and less influenced by impurities since they tend to remain in the bulk phase, resulting in high molecular weight polymers.\textsuperscript{175} In an interfacial polymerization, selection of the monomers is paramount, necessitating the use of a hydrophobic acid chloride to prevent premature hydrolysis. Given the hydrophobic nature and elastomeric attributes of sebacic acid in vascular scaffolds,\textsuperscript{146,176} sebacoyl chloride was selected as the acid chloride in this study.
Although both solution\textsuperscript{153,157,160,162-163,169,174,177-179} and interfacial\textsuperscript{180-191} polymerizations of PEAs with sebacoyl chloride have been well documented including the functional interfacial polycondensation that generated pendant carboxylic acid groups,\textsuperscript{159} prior to this study there were no published accounts comparing the two polymerization techniques. In addition, the functional interfacial polycondensation of PEAs yielding a pendant amine group for future conjugation had not been previously reported.

The ultimate goal of this study was to synthesize a biodegradable, porous, functional poly(ester amide) scaffold that would facilitate the conjugation of bioactive TGF-\(\beta\)1 to evaluate its potential use for \textit{in vitro} vascular tissue engineering. Specifically, targeted objectives included:

- Synthesize a family of poly(ester amide)s based on natural \(\alpha\)-amino acids, aliphatic diols and sebacoyl chloride via both solution and interfacial polycondensation:
  - L-alanine and L-phenylalanine to impart suitable mechanical and thermal properties; L-lysine and L-aspartic acid to introduce amine and carboxylic acid functional handles for TGF-\(\beta\)1 conjugation
  - 1,4-butanediol, 1,8-octanediol and sebacoyl chloride to impart elasticity and hydrophobicity to improve processability in organic solvents

- Evaluation of human coronary artery smooth muscle cell attachment, morphology, focal adhesion and proliferation on synthesized PEAs on two-dimensional films

- Electrospinning of PEAs to generate a three-dimensional scaffold and investigate HCASMC attachment, morphology and infiltration

- Investigation of poly(ester amide) chemical structure, topography and exogenous addition of TGF-\(\beta\)1 on HCASMC phenotype expression via Western blot analysis

- Conjugation of bioactive TGF-\(\beta\)1 to the surface of a functional PEA
The evaluation of these PEAs also included aspects such as ease of synthesis and cost. Given that new polymers were synthesized, detailed characterization of the PEAs is also reported.

2.11 References


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Chapter 3

3 Strategies in Functional Poly(ester amide) Syntheses to Study Human Coronary Smooth Muscle Cell Interactions*

The chapter initially compares two polymerization approaches for the syntheses of α-amino acid based poly(ester amide)s (PEAs). The subsequent effect of these PEAs on human coronary artery smooth muscle cell attachment, morphology, viability and propensity to form focal adhesions was also examined.

3.1 Abstract

The design of new generation cardiovascular biomaterials focuses on biomimetic properties that are capable of eliciting specific cellular responses and directing new tissue formation. Synthetic poly(ester amide)s (PEAs) containing α-amino acid residues have the potential to elicit favourable cellular responses. Furthermore, they are biodegradable owing to the incorporation of naturally occurring amino acids. In this study, a family of PEAs was synthesized from selected α-amino acids using both solution and interfacial polymerization approaches to optimize their properties for vascular tissue engineering applications. By careful selection of the monomers and the polymerization approach, high molecular weight PEAs with low glass transition temperatures were obtained. Human coronary artery smooth muscle cells (HCASMCs) cultured directly on bare PEA films attached and spread well up to 7 days of culture. Moreover, cell viability was significantly enhanced on all non-functional PEAs compared with tissue culture polystyrene controls. The trifluoroacetic acid salt of the lysine-containing functional PEAs was found to retard cell growth, but still supported cell viability up to 5 days of culture. Immunostaining of HCASMCs revealed strong vinculin expression suggesting that the HCASMCs initiated cellular processes for focal adhesion contacts with all PEA

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surfaces. Conversely, smooth muscle α-actin expression was not abundant on the PEA surfaces suggesting a proliferative smooth muscle cell phenotype. Altogether, our results indicate that these PEAs are promising materials for vascular tissue engineering scaffolds.

**Keywords:** vascular tissue engineering, poly(ester amide)s, interfacial polycondensation, human coronary artery smooth muscle cells, vinculin, smooth muscle α-actin.

### 3.2 Introduction

Vascular tissue engineering has emerged as a viable strategy for the design of biologically and mechanically responsive vascular constructs for therapeutic uses and pharmacologic studies.\(^1\)\(^-\)\(^2\) With the exception of a few cases\(^1,\)\(^3\) biodegradable scaffolding biomaterials are required for engineering three-dimensional vascular tissues.\(^4\) In this regard, both naturally occurring (such as collagen) and synthetic biodegradable or biostable biomaterials have been extensively studied.\(^5\)\(^-\)\(^6\) Because naturally occurring materials afford limited control over their final properties, many vascular tissue engineering studies in the past were conducted using poly(lactic acid) (PLA), poly(glycolic acid) (PGA), or copolymers of PLA and PGA.\(^7\) Despite encouraging results, these biomaterials produce acidic products during degradation that can induce undesired phenotype modulation in seeded vascular smooth muscle cells (VSMCs).\(^8\)\(^-\)\(^9\) Poly(trimethylene carbonate) which degrades into non-acidic byproducts has been considered for vascular tissue engineering,\(^10\)\(^-\)\(^11\) but its effect on cultured VSMCs is not fully understood. Biomaterials to be used in vascular tissue engineering must therefore be able to regulate vascular cell phenotype, in addition to cell adhesion, proliferation and migration. Despite considerable progress towards the synthesis of new generation biodegradable biomaterials for vascular tissue engineering, regulation of HCASMC phenotype remains a challenge. Thus, we propose the use of biodegradable α-amino acid-based poly(ester amide)s as an alternative biomaterial which may regulate HCASMC growth and phenotype.

Poly(ester amide)s (PEAs) are a class of synthetic polymers bearing both ester and amide repeat units. As these repeat units along the polymer chain can be introduced using a
variety of materials and methods, the name PEA is generic rather than specific. Due to
the susceptibility of PEAs to both hydrolytic and enzymatic degradation, a wide range of
PEAs with varying degradation rates are reported in the literature\textsuperscript{12-18} and reviewed
elsewhere.\textsuperscript{19-20} Although the ester linkages afford cleavage via hydrolysis, the
hydrophobicity of the PEAs will promote enzyme adsorption, thus enhancing its surface
erosion,\textsuperscript{21} which may limit a large accumulation of degradation products in the local
tissue.\textsuperscript{22} The chemical compositions of the amino acid-based PEAs are analogous to
peptides and proteins. The selection of monomers based on $\alpha$-amino acids with side
chains offer pendant functional groups\textsuperscript{14,23-26} for which cell signaling molecules can be
conjugated, potentially accelerating favourable cell-material interactions. Finally, the by-
products following degradation of these PEAs will include amino acids, which are found
physiologically, limiting their potential systemic toxicity.\textsuperscript{27}

Although the syntheses and characterization of PEAs derived from dicarboxylic acids,
diols and $\alpha$-amino acids have been reported,\textsuperscript{12-18} studies on their potential use as
biomaterials are only a recent endeavor. For example, Liu and coworkers reported the use
of arginine-based PEAs as a non-viral gene delivery vehicle with a high binding capacity
toward plasmid DNA.\textsuperscript{28} PEAs have also been examined for the sustained release of
antibiotics and paclitaxel.\textsuperscript{29-30} Li and Chu studied the release profile of a nitroxyl radical
model compound, 4-amino-2,2,6,6-tetramethylpiperidine-1-oxy (4-amino-TEMPO)
loaded into PEA fibers based on l-phenylalanine,\textsuperscript{31} while others reported the conjugation
of 4-amino-TEMPO to a carboxylic acid functionalized PEA as an anti-inflammatory
stent coating.\textsuperscript{25} Furthermore, Reinhart-King and coworkers synthesized PEAs from l-
phenylalanine and l-lysine in an attempt to investigate the effect of polymer charge
(neutral, negative or positive) on cellular response.\textsuperscript{32} Although there is no clear evidence
that endothelial cells formed a monolayer (due to the lack of specific immunostaining
such as VE-cadherin or $\alpha$-catenin), their study suggested that endothelial cell attachment,
spreading, and growth were favoured on positive and neutral PEA substrates when
seeded at high density. However, in this latter study, the examination of vascular
endothelial cells on the surface of the PEA would suggest that the material is being
considered as a synthetic vascular graft. Because these PEAs are readily biodegradable,\textsuperscript{14}
the in vivo integrity of the grafts in the presence of hemodynamic forces could potentially limit such application.

Despite the aforementioned studies highlighting the use of PEAs as biomaterials, there are limited studies conducted on their potential use as in vitro vascular tissue engineering scaffolds. Recently our group has synthesized both non-functional and functional PEAs from α-amino acids. In these studies, most of the PEAs synthesized had either too low a molecular weight, which were not considered useful biomaterials or were too brittle with high glass transition temperatures which equally could not be considered as vascular scaffold materials. Increasing the molecular weight increases polymeric chain entanglements, allowing long range segmental motion of the chains, while reducing the rate of viscous deformation. This enhanced viscoelasticity is needed in vascular tissue engineering as it mimics the natural extracellular matrix (collagen and elastin) so that seeded cells can adapt to their microenvironment. Collectively, published data suggest that the molecular weight of PEAs may not impart the viscoelasticity required of a vascular tissue engineering scaffold material, thus warranting further optimization of the synthetic approach. Therefore the objectives of the present study were: (i) to investigate the effect of the polymerization method on the molecular weight of the PEAs, (ii) to investigate human coronary artery smooth muscle cell (HCASMC) adhesion, spreading and viability on the synthesized PEAs. The rationale for using HCASMCs is based on our long-term objective of fabricating a human vascular tissue model with clinical relevance.

3.3 Experimental

3.3.1 Materials
Di-p-toluenesulfonic acid salt monomers 1 and 2 were prepared as previously reported (Scheme 3.1). Di-p-toluenesulfonic acid salt monomer 3 was a new monomer prepared by the same procedure as monomers 1 and 2 (Scheme 3.1). Di-p-nitrophenyl sebacate (4) was also prepared as previously reported (Chart 3.1). Bis-N-ε-t-BOC-L-lysine ester (5) was prepared as previously reported by our group (Chart 3.1). Solvents were purchased from Caledon Labs (Georgetown, ON). All other chemicals were purchased from Sigma Aldrich (Milwaukee, WI). Unless noted otherwise, all chemicals
were used as received. Anhydrous tetrahydrofuran (THF) was obtained from a solvent purification system. *N*,*N*-dimethylacetamide (DMA) and triethylamine (NEt₃) were distilled from calcium hydride (CaH₂) and dichloromethane (CH₂Cl₂, DCM) was distilled from P₂O₅. Flash chromatography was performed using silica gel 60 with a particle size range of 0.063-0.200 mm (EMD Chemicals Inc. Gibbstown, NJ). Dialysis was performed against *N*,*N*-dimethylformamide (DMF) with Spectra/Por 6 dialysis tubing (Spectrum Laboratories, Inc., Rancho Domínguez, CA), molecular weight cutoff of 25 kDa.

### 3.3.2 Methods

¹H (400 MHz) and ¹³C (100 MHz) nuclear magnetic resonance (NMR) spectra were obtained on a Varian Inova 400 spectrometer (Varian Canada Inc., Mississauga, ON). Chemical shifts are reported in parts per million (ppm) and are calibrated against residual solvent signals of chloroform (CDCl₃, δ 7.27 ppm, 77.00 ppm) or dimethyl sulfoxide (DMSO, δ 2.50 ppm). All coupling constants (J) are reported in Hertz (Hz). Fourier transform infrared (FTIR) spectra were obtained using a Bruker Tensor 27 (Bruker Corporation, Milton, ON) from KBr disks or as films from DCM on NaCl plates. Absorption frequencies of the functional groups are reported in wavenumbers (cm⁻¹) and assigned as published by Pavia et al.³⁷ Low-resolution mass spectrometry was achieved using a Finnigan MAT 8400 mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) with a mass range (m/z) up to 8,400. High-resolution mass spectrometry was achieved using a Micromass LCT (electrospray time-of-flight (ES+)) mass spectrometer (Waters Corporation, Milford, MA) with a mass range (m/z) up to 10,000. Gel permeation chromatography data were obtained using a Waters 2695 Separations Module equipped with a Waters 2414 Refractive Index Detector (Waters Limited, Mississauga, ON) and two PLgel 5 µm mixed-D (300 mm × 7.5 mm) columns connected in series (Varian Canada Inc., Mississauga, ON). Samples (5 mg/mL) dissolved in the eluent, which comprised of 10 mM LiBr and 1 % (v/v) NEt₃ in DMF at 85°C were injected (100 µL) at a flow rate of 1 mL/min and calibrated against polystyrene standards. Molecular weights are reported in grams/mole (g/mol). Thermogravimetric analyses were performed on a SDT Q600 (TA Instruments – Waters LLC, New Castle, DE) under dry nitrogen at a heating rate of 20°C/min up to 600°C. Differential scanning calorimetry was performed
on a DSC Q20 (TA Instruments – Waters LLC, New Castle, DE) at a heating rate of 10°C/min from -50 to 200°C. All samples prepared ranged from 2 to 5 mg, and glass transition temperatures (T_g's) were obtained from the second heating cycle.

3.3.3 PEA Nomenclature

The polymers are labeled by the number of methylene groups contributed by the diacid, the three letter amino acid designation, the number of methylene groups in the diol and finally by the method of preparation – solution (Sol) or interfacial (Int) polycondensation – for example 8-Ala-8-Sol. Lys(BOC) represents the incorporation of the lysine monomer 5 as a co-monomer at a mole ratio of 10% relative to the di-p-toluenesulfonic acid salt monomer 1, 2, or 3 - for example 8-Ala-8-Lys(BOC)-4-Sol. The 4 represents the number of methylene groups in the butanediol moiety of 5.

3.3.4 Syntheses of Monomers

3.3.4.1 Di-p-Toluenesulfonic Acid Salt Monomer, 1

Syntheses of all di-p-toluenesulfonic acid salt monomers were modified from Guo et al.36 A suspension of L-alanine (5.0 g, 56 mmol, 2.2 equiv.), p-toluenesulfonic acid•H2O (11 g, 61 mmol, 2.4 equiv.) in toluene (100 mL) was refluxed at 140°C with stirring in a flask equipped with a Dean-Stark trap for 2 h to remove the residual water. To this solution, 1,8-octanediol (3.7 g, 26 mmol, 1.0 equiv.) was added and heated at reflux for 48 h. The resulting material was filtered and recrystallized from isopropanol (30 mL) twice following its filtration in hot isopropanol, to provide monomer 1. Yield: 75%.

1H NMR (400 MHz, DMSO-d6): δ 8.26 (br s, 6H, -NH3+ TsO-), 7.48 (d, 4H, J = 8.2, Ar-H meta to CH3), 7.12 (d, 4H, J = 7.2, Ar-H ortho to CH3), 4.20-4.08 (m, 6H, -C(O)O-CH2-, -CαH), 2.29 (s, 6H, Ar-C6H3), 1.64-1.57 (m, 4H, -C(O)O-CH2-CH2-(C6H2)4-), 1.38 (d, 6H, J = 7.2, -CαH-CH3), 1.35-1.25 (m, 8H, -C(O)O-CH2-CH2-(CH2)4-). 13C NMR (100 MHz, DMSO-d6): δ 170.0, 145.2, 138.0, 128.2, 125.5, 65.6, 48.0, 28.5, 27.9, 25.1, 20.8, 15.8. FTIR (KBr pellet, cm⁻¹): 3062 (N-H stretch, primary amine salt; C-H stretch, aromatic), 2929 (C-H stretch, aliphatic), 2854 (C-H stretch, aliphatic), 1751 (C=O stretch, ester), 1602 (C-N stretch, primary amine salt; C=C stretch, aromatic), 1534 (C-N stretch,
primary amine salt), 1498 (C=C stretch, aromatic), 1400 (CH$_3$ bend, aliphatic), 1332 (CH$_2$ wag, aliphatic), 1246 (S=O stretch, SO$_3$ salt), 1155 (C-O stretch, ester). HRMS (m/z): calc for C$_{14}$H$_{29}$N$_2$O$_4^+$, 289.2127; found 289.2130, [M+H]$^+$.  

### 3.3.4.2 Di-$p$-Toluenesulfonic Acid Salt Monomer, 2

A suspension of L-phenylalanine (10 g, 61 mmol, 2.2 equiv.), $p$-toluenesulfonic acid•H$_2$O (11 g, 66 mmol, 2.4 equiv.) in toluene (100 mL) was refluxed at 140°C with stirring in a flask equipped with a Dean-Stark trap for 2 h to remove the residual water. To this solution, 1,4-butanediol (2.5 mL, 28 mmol, 1.0 equiv.) was added and heated at reflux for 48 h. The product was filtered and recrystallized twice from water (300 mL) following its hot filtration, to provide monomer 2. Yield: 55%.

$^1$H NMR (400 MHz, DMSO-d$_6$): $\delta$ 8.41 (br s, 6H, -NH$_3^+$ TsO$^-$), 7.50-7.47 (m, 4H, Ar-h meta to CH$_3$), 7.36-7.21 (m, 10H, Ph), 7.13-7.10 (m, 4H, Ar-H ortho to CH$_3$), 4.30 (dd, 2H, J$_1$ = 7.7, J$_2$ = 6.2, -C$_a$H)$_2$, 4.00-3.97 (m, 4H, -C(O)O-C$_2$H$_2$-), 3.17-2.99 (m, 4H, -C$_a$H-$C$H$_2$-Ph), 2.29 (s, 6H, Ar-C$_3$H$_3$), 1.39-1.28 (m, 4H, -C(O)O-CH$_2$-CH$_2$-). $^{13}$C NMR (100 MHz, DMSO-d$_6$): $\delta$ 169.0, 145.2, 138.0, 134.6, 129.3, 128.6, 128.2, 127.3, 125.5, 64.9, 53.3, 36.2, 24.1, 20.8. FTIR (KBr pellet, cm$^{-1}$): 3103 (N-H stretch, primary amine salt), 3023 (C-H stretch, aromatic), 2935 (C-H stretch, aliphatic), 1737 (C=O stretch, ester), 1523 (C-N stretch, primary amine salt), 1499 (C=C stretch, aromatic), 1456 (CH$_2$ wag, aliphatic), 1239 (S=O stretch, SO$_3$ salt), 1196 (C-O stretch, ester). HRMS (m/z): calc for C$_{22}$H$_{29}$N$_2$O$_4^+$, 385.2127; found 385.2120, [M+H]$^+$.  

### 3.3.4.3 Di-$p$-Toluenesulfonic Acid Salt Monomer, 3

A suspension of L-phenylalanine (10 g, 61 mmol, 2.2 equiv.), $p$-toluenesulfonic acid•H$_2$O (11 g, 61 mmol, 2.4 equiv.) in toluene (100 mL) was refluxed at 140°C with stirring in a flask equipped with a Dean-Stark trap for 2 h to remove the residual water. To this solution, 1,8-octanediol (4.0 g, 28 mmol, 1.0 equiv.) was added and heated at reflux for 48 h. The resulting product was filtered and recrystallized from water (2 L) to provide monomer 3. Yield: 77%.  

\(^1\)H NMR (400 MHz, DMSO-d6): \(\delta\) 8.40 (br s, 6H, -NH\(^3\) TsO'), 7.48 (d, 4H, J = 8.2, Ar-H meta to CH\(_3\)) 7.36-7.22 (m, 10H, Ph), 7.11 (d, 4H, J = 7.6, Ar-H ortho to CH\(_3\)) 4.30 (dd, 2H, J\(_1\) = 7.6, J\(_2\) = 6.3, -C\(_a\)H), 4.05-4.02 (m, 4H, -C(O)O-CH\(_2\)-), 3.17-2.99 (m, 4H, \(-\text{C}^{\alpha}\text{H}-\text{C}^{\beta}\text{H}-\text{Ph}\)), 2.29 (s, 6H, Ar-C\(_3\)), 1.48-1.38 (m, 4H, -C(O)O-CH\(_2\)-CH\(_2\)-CH\(_2\)-) 1.20-1.07 (m, 8H, -C(O)O-CH\(_2\)-CH\(_2\)-CH\(_2\)-CH\(_2\)-). \(^{13}\)C NMR (100 MHz, DMSO-d6): \(\delta\) 169.1, 145.3, 137.9, 134.6, 129.3, 128.5, 128.1, 127.2, 125.5, 65.5, 53.3, 36.2, 28.4, 27.7, 25.0, 20.8. FTIR (KBr pellet, cm\(^{-1}\)): 3181 (N-H stretch, primary amine salt), 3030 (C-H stretch, aromatic), 2920 (C-H stretch, aliphatic), 2856 (C-H stretch, aliphatic), 1733 (C=O stretch, ester), 1601 (C-N stretch, primary amine salt; C=C stretch, aromatic), 1525 (C-N stretch, primary amine salt), 1496 (C=C stretch, aromatic), 1459 (CH\(_2\) wag, aliphatic), 1233 (S=O stretch, SO\(_3\) salt), 1167 (C-O stretch, ester). HRMS (m/z): calc for C\(_{26}\)H\(_{37}\)N\(_2\)O\(_4\)^+, 441.2753; found 441.2755, [M+H]^+.

### 3.3.4.4 Synthesis of Di-p-Nitrophenyl Sebacate Monomer, 4

The synthesis of di-p-nitrophenyl sebacate was adapted from Katsarava et al.\(^{21}\) A solution of p-nitrophenol (8.3 g, 60 mmol, 2.0 equiv.) and triethylamine (8.4 mL, 60 mmol, 2.0 equiv.) in dry THF (100 mL) was cooled to below 0°C using a dry ice/acetone bath. Sebacoyl chloride (6.4 mL, 30 mmol, 1.0 equiv.) in dry THF (50 mL) was added dropwise over a period of 30 min. The reaction mixture was then allowed to reach room temperature and stirred overnight. The resulting solution was concentrated under rotary evaporation and then precipitated in cold water (150 mL) yielding monomer 4, which was recrystallized from ethyl acetate (250 mL) following its hot filtration. Yield: 92%.

\(^1\)H NMR (400 MHz, DMSO-d\(_6\)): \(\delta\) 8.31-8.27 (m, 4H, Ar-H), 7.45-7.41 (m, 4H, Ar-H), 2.64 (t, 4H, J = 7.4, -O(CO)-CH\(_2\)-), 1.70-1.62 (m, 4H, -O(CO)-CH\(_2\)-CH\(_2\)-CH\(_2\)-), 1.43-1.30 (m, 8H, -O(CO)-CH\(_2\)-CH\(_2\)-CH\(_2\)-CH\(_2\)-). \(^{13}\)C NMR (100 MHz, DMSO-d\(_6\)): \(\delta\) 171.2, 155.4, 144.9, 125.2, 123.1, 33.4, 28.5, 28.3, 24.1. FTIR (KBr pellet, cm\(^{-1}\)): 3118 (C-H stretch, aromatic), 3088 (C-H stretch, aromatic), 2937 (C-H stretch, aliphatic), 2847 (C-H stretch, aliphatic), 1754 (C=O stretch, ester), 1620 (C=C stretch, aromatic), 1593 (C-H stretch, aromatic, para-substituted), 1536 (N=O stretch, Ar-NO\(_2\)) 1489 (C-H stretch, aromatic,
para-substituted), 1346 (N=O stretch, Ar-NO₂), 1201 (CH₂ wag, aliphatic), 1129 (C=O stretch, ester). HRMS (m/z): calc for C₂₂H₂₅N₂O₈⁺, 445.1611; found 445.1596, [M+H]⁺.

3.3.4.5 Synthesis of Bis-N-α-Carboxybenzyl (CBZ)-N-ε-tert-Butyloxycarbonyl (BOC)-l-Lysine Diester Monomer, 5

Synthesis of bis-N-α-CBZ-N-ε-t-BOC-L-lysine diester was prepared as previously reported.²⁴ N-α-CBZ-N-ε-t-BOC-L-lysine (2.0 g, 5.2 mmol, 3.1 equiv.) was dissolved in 5 mL of anhydrous dichloromethane. To this solution was added 1,4-butanediol (0.15 mL, 1.7 mmol, 1.0 equiv.), N,N’-dicyclohexylcarbodiimide (DCC) (1.1 g, 5.3 mmol, 3.1 equiv.), 4-(dimethylamino)pyridine (DMAP) (0.049 g, 0.40 mmol, 0.24 equiv.), and 4-(dimethylamino)pyridinium 4-toluenesulfonate (DPTS) (0.10 g, 0.36 mmol, 0.21 equiv.). The resulting solution was stirred under a nitrogen atmosphere at 25°C and was monitored by thin layer chromatography (70:30 ethyl acetate:hexanes) until completion (approximately 1.5 h). The reaction mixture was then filtered to remove dicyclohexylurea (DCU) byproduct and the solvent was removed under reduced pressure. The product was purified by column chromatography using 50:50 ethyl acetate:hexanes as an eluent to yield 5 as a viscous oil. Yield: 89%.

¹H NMR (400 MHz, CDCl₃): δ 7.36-7.26 (m, 10H, Ph), 5.79-5.50 (m, 2H, O-C(O)-NH-), 5.08 (m, 4H, Ph-CH₂-O-C(O)-), 4.79-4.61 (m, 2H, -NH-BOC), 4.37-4.26 (m, 2H, -C₆H), 4.22-4.01 (m, 4H, -C(O)O-CH₂-), 3.13-2.94 (m, 4H, -CH₂-NH-BOC), 1.88-1.54 (m, 8H, -C₆H-CH₂-, -CH₂-CH₂-NH-BOC), 1.54-1.27 (m, 26H, -C₆H₂-CH₂-, BOC CH₃). ¹³C NMR (100 MHz, CDCl₃): δ 172.4, 156.0, 156.0, 136.2, 128.4, 128.1, 128.0, 67.0, 66.9, 64.6, 53.7, 39.9, 31.9, 29.5, 28.3, 25.0, 22.3.

3.3.4.6 Synthesis of Bis-N-ε-tert-BOC-l-Lysine Diester Monomer, 6

The hydrogenation of bis-N-α-CBZ-N-ε-t-BOC-l-lysine diester with Pd/C catalyst (35 mg, 10 wt%) was achieved as reported.²⁴ Pd/C was added to a solution of protected monomer 5 (340 mg, 0.42 mmol, 1.0 equiv.) in ethanol (5 mL). The resulting solution was stirred under a hydrogen atmosphere and monitored with thin layer chromatography (70:30 ethyl acetate:hexanes) until completion (at approximately 2 h). The reaction
mixture was filtered through celite and the solvent was removed in vacuo providing monomer 6. Yield: 77%.

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 4.64 (br m, 2H, -NH-BOC), 4.20-4.09 (m, 4H, -C(O)O-CH$_2$-), 3.43 (dd, 2H, J = 7.0 and 5.5, -C$_{\alpha}$H), 3.12 (m, 4H, -CH$_2$-NH-BOC), 1.80-1.31 (m, 38H, -C(O)O-CH$_2$-CH$_2$, NH$_2$-C$_{\alpha}$H-, -C$_{\alpha}$H-CH$_2$-CH$_2$-CH$_2$-, BOC CH$_3$). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 175.9, 155.9, 78.9, 64.2, 54.3, 40.2, 34.5, 29.7, 28.3, 25.2, 22.8.

3.3.5 General Procedure for Solution Polymerization of the Non-Functional Poly(ester amide)s

Solution polycondensation of the non-functional poly(ester amide)s was performed as previously reported (Scheme 3.2). The di-p-nitrophenyl sebacate monomer 4 (1.0 equiv.) and the di-p-toluenesulfonic acid salt monomer (1.0 equiv.) were combined in a round bottom flask, evacuated and purged with nitrogen. Distilled DMA (4.0 mL) was added to these monomers and the resulting mixture was heated to 60°C. Triethylamine (1.0 mL, 7.3 mmol, 2.2 equiv.) was then added dropwise to the solution and the temperature was raised to 70°C. The reaction mixture was maintained at this temperature for 48 h. The solution was then precipitated in cold ethyl acetate (125 mL) and collected. Residual impurities were first removed by washing with ethyl acetate followed by its Soxhlet extraction with ethyl acetate for 48 h to provide pure polymer.

3.3.5.1 8-Ala-8-Sol

This polymer was prepared by the general procedure described above, from monomers 1, 4. Yield: 1.2 g, 67%. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 6.17 (d, 2H, J = 7.4, -NH-CO-), 4.59 (m, 2H, -C$_{\alpha}$H), 4.18-4.08 (m, 4H, -C(O)O-CH$_2$-), 2.23-2.17 (m, 4H, -NH-C(O)-CH$_2$-), 1.69-1.58 (m, 8H, -C(O)O-CH$_2$-CH$_2$-, -NH-C(O)-CH$_2$-CH$_2$-), 1.42-1.27 (m, 22H, -C$_{\alpha}$H-CH$_3$, -C(O)O-CH$_2$-(CH$_2$)$_4$-, -NH-C(O)-CH$_2$-CH$_2$-(CH$_2$)$_4$-). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 173.3 (-C(O)O-), 172.6 (-NH-CO-), 65.4 (-C(O)O-CH$_2$-), 47.8 (-C$_{\alpha}$H), 36.3 (-NH-CO-CH$_2$-, 29.0 (-C(O)O-CH$_2$-CH$_2$-), 28.4 (-C(O)O-CH$_2$-CH$_2$-(CH$_2$)$_4$-), 25.6 (-NH-CO-CH$_2$-CH$_2$-), 25.4 (-NH-CO-CH$_2$-CH$_2$-(CH$_2$)$_4$-), 18.5 (-C$_{\alpha}$H-CH$_3$). FTIR (KBr pellet, cm$^{-1}$): 3311 (N-H stretch, amide), 3085 (Fermi resonance overtone of the 1560 cm$^{-1}$ band), 2930 (C-H stretch, aliphatic), 2854 (C-H stretch, aliphatic), 1736 (C=O stretch,
ester), 1655 (C=O stretch, amide I), 1560 (N-H bend, C-N-stretch, amide II), 1459 (CH₂ wag, aliphatic), 1219 (C-O stretch, ester). GPC: $M_n = 36,600$, $M_w = 51,400$, PDI = 1.41. DSC: $T_g = 13.5^\circ$C.

### 3.3.5.2 8-Phe-4-Sol

This polymer was prepared by the general procedure described above, from monomers 2 and 4. Yield: 2.4 g, 85%. Spectral data agreed with those previously reported.¹⁸ GPC: $M_n = 53,500$, $M_w = 103000$, PDI = 1.93. DSC: $T_g = 38.5^\circ$C.

### 3.3.5.3 8-Phe-8-Sol

This polymer was prepared by the general procedure described above, from monomers 3 and 4. Yield: 0.71 g, 61%. ¹H NMR (400 MHz, CDCl₃): $\delta$ 7.31-7.08 (m, 10H, Ph), 5.96 (d, 2H, J = 7.6, -NH-C(O)-), 4.89 (m, 2H, -CαH), 4.16-4.04 (m, 4H, -C(=O)O-C₂H₅), 3.18-3.05 (m, 4H, -CαH-CH₃), 2.20-2.13 (m, 4H, -NH-C(O)-CH₂-), 1.67-1.51 (m, 8H, -C(O)O-CH₂-CH₂-, -NH-C(O)-CH₂-CH₂-), 1.35-1.20 (m, 16H, -C(O)O-CH₂-C₂H₅, -NH-C(O)-CH₂-CH₂-(CH₂)₄, -NH-C(O)-CH₂-CH₂-(CH₂)₄). ¹³C NMR (100 MHz, CDCl₃): $\delta$ 172.5 (-C(=O)O), 171.8 (-NH-C(O)-), 135.9 (Ar-C), 129.2 (Ar-C), 128.4 (Ar-C), 127.0 (Ar-C), 65.5 (-C(O)O-CH₂-), 52.9 (-CαH), 38.0 (-CαH-CH₂-Ph), 36.4 (-NH-C(O)-CH₂-), 29.0 (-C(O)O-CH₂-CH₂-), 28.3 (-C(O)O-CH₂-CH₂-(CH₂)₄), 25.7 (-NH-C(O)-CH₂-CH₂-), 25.4 (-NH-C(O)-CH₂-CH₂-(CH₂)₄). FTIR (KBr pellet, cm⁻¹): 3305 (N-H stretch, amide), 3065 (C-H stretch, aromatic), 3030 (C-H stretch, aromatic), 2928 (C-H stretch, aliphatic), 2853 (C-H stretch, aliphatic), 1736 (C=O stretch, ester), 1649 (C=O stretch, amide I), 1543 (N-H bend, C-N stretch, amide II), 1498 (C=C stretch, aromatic), 1455 (CH₂ wag, aliphatic), 1181 (C-O stretch, ester). GPC: $M_n = 44400$, $M_w = 63300$, PDI = 1.43. DSC: $T_g = 25.7^\circ$C.

### 3.3.6 General Procedure for Interfacial Polymerization of the Non-Functional Poly(ester amide)s

Sebacoyl chloride (5.0 mmol, 1.0 equiv.) was dissolved in anhydrous dichloromethane (30 mL), and this solution was added dropwise over 30 min to an aqueous solution (30 mL) containing di-p-toluenesulfonic acid salt monomer 1, 2 or 3 (5.0 mmol, 1.0 equiv.) and sodium carbonate (10 mmol, 2.0 equiv.) and allowed to react for 12 h (Scheme 2).
Upon completion of the reaction, the solvent was removed in vacuo. The polymer was then washed with excess water prior to purification via Soxhlet extraction with ethyl acetate for 48 h and dried in vacuo.

3.3.6.1 8-Ala-8-Int

This polymer was prepared by the general procedure described above using monomer 1 and sebacoyl chloride. Yield: 1.6 g, 68%. Spectral data agreed with those reported above for 8-Ala-8-Sol. GPC: $M_n = 45,100$, $M_w = 62,500$, PDI = 1.38. DSC: $T_g = 13.5^\circ C$.

3.3.6.2 8-Phe-4-Int

This polymer was prepared by the general procedure described above using monomer 2 and sebacoyl chloride. Yield: 2.1 g, 78%. Spectral data agreed with those previously reported. GPC: $M_n = 63,600$, $M_w = 168,000$, PDI = 2.64. DSC: $T_g = 38.8^\circ C$.

3.3.6.3 8-Phe-8-Int

This polymer was prepared by the general procedure described above using monomer 3 and sebacoyl chloride. Yield: 1.8 g, 60%. Spectral data agreed with those reported above for 8-Phe-8-Sol. GPC: $M_n = 65,600$, $M_w = 89,100$, PDI = 1.36. DSC: $T_g = 24.2^\circ C$.

3.3.7 General Procedure for Solution Polymerization of the Functional Poly(ester amide)s

The solution polymerized functional PEAs were synthesized similarly to the base poly(ester amide)s. Di-p-nitrophenyl sebacate monomer 4 (2.6 mmol, 1.0 equiv.), the di-p-toluenesulfonic acid salt monomer 1, 2 or 3 (2.4 mmol, 0.9 equiv.) and bis-\(N\-\epsilon\-t\)-BOC-L-lysine diester monomer, 6 (0.26 mmol, 0.1 equiv.) were combined in a round bottom flask, evacuated and purged with nitrogen. Distilled DMA (5.0 mL) was added to these monomers and the resulting mixture was heated to 60°C. Distilled triethylamine (5.7 mmol, 2.2 equiv.) was then added dropwise to the solution and the temperature was raised to 70°C. The reaction mixture was maintained at this temperature for 48 h. The solution was then precipitated in cold ethyl acetate (100 mL) and collected. The crude product was dissolved in DMF and dialyzed for at least 8 h twice. The purified PEA was concentrated on the rotary evaporator, precipitated in water (5 mL) and lyophilized for 24
h. Finally, the polymer was precipitated in cold ethyl acetate (50 mL) from chloroform (5 mL), collected and dried in vacuo.

3.3.7.1 8-Ala-8-Lys(BOC)-4-Sol

This polymer was prepared by the general procedure described above using monomers 1, 4 and 6. Yield: 0.43 g, 34%. \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta 6.39-6.11\) (br m, 2H, -C(O)-NH-C\(_\alpha\)H-(CH\(_2\))\(_4\)-NH-BOC, -C(O)-NH-C\(_\alpha\)H-CH\(_3\)), 4.78 (br m, 0.2H, -NH-BOC), 4.65-4.52 (m, 2H, -C\(_\alpha\)H-CH\(_3\), -C\(_\alpha\)H-(CH\(_2\))\(_4\)-NH-BOC), 4.23-4.08 (m, 4H, -C(O)O-CH\(_2\)-), 3.15-3.05 (m, 0.4H, -NH-C\(_\alpha\)H-CH\(_2\)-Ph), 2.36-2.11 (m, 4H, -NH-C(O)-CH\(_2\)-), 1.90-1.70 (br m, 0.8H, -C\(_\alpha\)H-CH\(_2\)-CH\(_2\)-CH\(_2\)-CH\(_2\)-NH-BOC), 1.70-1.54 (m, 8H, -C(O)O-CH\(_2\)-CH\(_2\)-, -NH-C(O)-CH\(_2\)-CH\(_2\)-), 1.44 (br s, 1.8H, BOC CH\(_3\)), 1.40 (d, 5.4H, J = 7.0, -C\(_\alpha\)H-CH\(_3\)), 1.38-1.27 (m, 15.6H, -NH-C(O)-CH\(_2\)-, -C(O)O-CH\(_2\)-CH\(_2\)-, -NH-C(O)-CH\(_2\)-CH\(_2\)-, -NH-C(O)-CH\(_2\)-CH\(_2\)-NH-BOC). FTIR (KBr pellet, cm\(^{-1}\)): 3307 (N-H stretch, amide), 3069 (Fermi resonance overtone of the 1560 cm\(^{-1}\) band), 2931 (C-H stretch, aliphatic), 2856 (C-H stretch, aliphatic), 1743 (C=O stretch, ester), 1649 (C=O stretch, amide I), 1543 (N-H bend, C-N-stretch, amide II), 1457 (CH\(_2\) wag, aliphatic). GPC: \(M_n = 19,000\), \(M_w = 28,700\), PDI = 1.51. DSC: \(T_g = 15.1^\circ\)C.

3.3.7.2 8-Phe-4-Lys(BOC)-4-Sol

This polymer was prepared by the general procedure described above using monomers 2, 4 and 6. Yield: 0.79 g, 52%. \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta 7.33-7.08\) (m, 9H, Ph), 6.28 (br m, 0.2H, -C(O)-NH-C\(_\alpha\)H-(CH\(_2\))\(_4\)-NH-BOC), 6.01 (d, 1.8H, J = 7.8, C(O)-NH-C\(_\alpha\)H-CH\(_2\)-Ph), 4.92-4.83 (m, 1.8H, -NH-C\(_\alpha\)H-CH\(_2\)-Ph), 4.74 (br m, 0.2H, -NH-BOC), 4.57 (br m, 0.2H, -NH-C\(_\alpha\)H-(CH\(_2\))\(_4\)-NH-BOC), 4.21-3.98 (m, 4H, -C(O)O-CH\(_2\)-), 3.18-3.04 (m, 4H, C\(_\alpha\)H-CH\(_2\)-Ph, C\(_\alpha\)H-CH\(_2\)-CH\(_2\)-CH\(_2\)-CH\(_2\)-NH-BOC), 2.25-2.09 (m, 4H, -NH-C(O)-CH\(_2\)-), 1.90-1.68 (m, 0.8H, -C\(_\alpha\)H-CH\(_2\)-CH\(_2\)-CH\(_2\)-NH-BOC), 1.68-1.47 (m, 8H, -C(O)O-CH\(_2\)-CH\(_2\)-, -NH-C(O)-CH\(_2\)-CH\(_2\)-), 1.44 (br s, 1.8H, BOC CH\(_3\)), 1.40-1.15 (m, 8.4H, -C\(_\alpha\)H-CH\(_2\)-CH\(_2\)-CH\(_2\)-NH-BOC, -NH-C(O)-CH\(_2\)-CH\(_2\)-), FTIR (KBr pellet, cm\(^{-1}\)): 3309 (N-H stretch, amide), 3064 (C-H stretch, aromatic), 3030 (C-H stretch, aromatic), 2928 (C-H stretch, aliphatic), 2854 (C-H stretch, aliphatic), 1745 (C=O stretch, aromatic).
stretch, ester), 1647 (C=O stretch, amide I), 1542 (N-H bend, C-N stretch, amide II),
1496 (C=C stretch, aromatic), 1455 (CH2, wagging vibration) 1176 (C-O stretch, ester).
GPC: $M_n = 48,800$, $M_w = 73,900$, PDI = 1.52. DSC: $T_g = 37.6^\circ C$.

3.3.7.3 8-Phe-8-Lys(BOC)-4-Sol

This polymer was prepared by the general procedure described above using monomers 3,
4 and 6. Yield: 0.76 g, 41%. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.32-7.08 (m, 9H, Ph), 6.27 (br m, 0.2H, -C(O)-NH-C$_6$H$_4$-CH$_2$-Ph), 4.89 (m, 1.8H, -C$_6$H$_5$-CH$_2$-Ph), 4.75 (br m, 0.2H, -NH-BOC), 4.57 (br m, 0.2H, -C$_6$H$_5$-(CH$_2$)$_4$-NH-BOC), 4.16-4.04 (m, 4H, -C(O)O-CH$_2$-Ph), 3.18-3.05 (m, 4H, -C$_6$H$_5$-NH-BOC), 2.36-2.09 (m, 4H, -NH-C(O)-CH$_2$-), 1.90-1.70 (m, 0.8H, -C$_6$H$_5$-CH$_2$-CH$_2$-CH$_2$-NH-BOC), 1.70-1.47 (m, 8H, -C(O)O-CH$_2$-CH$_2$-NH-BOC), 1.44 (br s, 1H, BOC CH$_3$), 1.38-1.18 (m, 15.6H, -C$_6$H$_5$-CH$_2$-CH$_2$-CH$_2$-NH-BOC, -C(O)O-CH$_2$-CH$_2$-(CH$_2$)$_4$, NH-C(O)-CH$_2$-CH$_2$-(CH$_2$)$_4$-). FTIR (KBr pellet, cm$^{-1}$):
3302 (N-H stretch, amide), 3065 (C-H stretch, aromatic), 3030 (C-H stretch, aromatic), 2929 (C-H stretch, aliphatic), 2853 (C-H stretch, aliphatic), 1738 (C=O stretch, ester), 1649 (C=O stretch, amide I), 1543 (N-H bend, C-N stretch, amide II), 1498 (C=C stretch, aromatic), 1455 (CH$_2$, wagging vibration) 1178 (C-O stretch, ester). GPC: $M_n = 38,100$, $M_w = 49,800$, PDI = 1.31. DSC: $T_g = 23.4^\circ C$.

3.3.8 General Procedure for Interfacial Polymerization of the Functional Poly(ester amide)s

The di-p-toluenesulfonic acid salt monomer 1, 2 or 3 (1.9 mmol, 0.9 equiv.) and sodium carbonate (4.3 mmol, 2.0 equiv.) were dissolved in distilled water (30 mL). Bis-N-$\varepsilon$-t-BOC-L-lysine diester monomer 6 (0.21 mmol, 0.1 equiv.) was dissolved in dichloromethane (15 mL) and added to the aqueous phase and allowed to mix for 30 min. Sebacoyl chloride (2.1 mmol, 1.0 equiv.) diluted in anhydrous dichloromethane (15 mL), was added dropwise over 30 min to the biphasic solution and was allowed to react for 24 h. Upon completion of the reaction, solvent was removed in vacuo. The functional PEA was dissolved in DMF permitting filtration of the insoluble salts. The filtrate was then dialyzed against DMF for at least 8 h twice. The purified PEA was concentrated on the
rotary evaporator, precipitated in water (5 mL) and lyophilized for 24 h. Finally, the polymer was precipitated in cold ethyl acetate (50 mL) from chloroform (5 mL), collected and dried in vacuo.

3.3.8.1 8-Ala-8-Lys(BOC)-4-Int

This polymer was prepared by the general procedure described above using monomers 1, 6 and sebacoyl chloride. Yield: 0.40 g, 40%. Spectral data agreed with those reported above for 8-Ala-8-Lys(BOC)-4-Sol. GPC: $M_n = 29,500$, $M_w = 47,600$, PDI = 1.62. DSC: $T_g = 14.5^\circ$C.

3.3.8.2 8-Phe-4-Lys(BOC)-4-Int

This polymer was prepared by the general procedure described above using monomers 2, 6 and sebacoyl chloride. Yield: 0.84 g, 69%. Spectral data agreed with those reported above for 8-Phe-4-Lys(BOC)-4-Sol. GPC: $M_n = 48800$, $M_w = 73900$, PDI = 1.52. DSC: $T_g = 37.6^\circ$C.

3.3.8.3 8-Phe-8-Lys(BOC)-4-Int

This polymer was prepared by the general procedure described above using monomers 3, 6 and sebacoyl chloride. Yield: 0.84 g, 57%. Spectral data agreed with those reported above for 8-Ala-8-Lys(BOC)-4-Sol. GPC: $M_n = 21,200$, $M_w = 45,700$, PDI = 2.16. DSC: $T_g = 22.9^\circ$C.

3.3.9 General Deprotection Procedure

Each BOC-protected functional PEA (100 mg) was dissolved in 1:1 trifluoroacetic acid (TFA):CH$_2$Cl$_2$ (2 mL) mixture and reacted for 2 h. The solvents were subsequently removed in vacuo, and then the product was precipitated in cold ethyl ether (50 mL) from dichloromethane (5 mL). The precipitate was collected and again dried in vacuo, yielding the functional PEA with pendant amine groups as their TFA salts.
3.3.9.1 8-Ala-8-Lys(TFA)-4

Yield: 75%. $^1$H NMR (400 MHz, DMSO-d$_6$): $\delta$ 8.15 (d, 2H, J = 7.0, -C(O)-NH-C$_a$H-CH$_3$, -C(O)-NH-C$_a$H-(CH$_2$)$_3$-NH$_3^+$TFA$^-$), 7.65 (br m, 0.6H, -NH$_3^+$TFA$^-$), 4.42-4.34 (m, 0.2H, -C$_a$H-(CH$_2$)$_4$-NH$_3^+$TFA$^-$), 4.20 (m, 1.8H, -C$_a$H-CH$_3$), 4.10-3.92 (m, 4H, -C(O)O-CH$_2$-), 2.81-2.71 (br m, 0.4H, -C$_a$H-CH$_2$-CH$_2$-CH$_2$-CH$_2$-NH$_3^+$TFA$^-$), 2.21-1.96 (m, 4H, -CH$_2$-C(O)-NH-C$_a$H-(CH$_2$)$_4$-NH$_3^+$TFA$^-$), 1.68-1.59 (m, 0.8H, -C$_a$H-CH$_2$-CH$_2$-CH$_2$-NH$_3^+$TFA$^-$), 1.59-1.38 (m, 8H, -C(O)-CH$_2$-CH$_2$-CH$_2$-CH$_2$-NH$_3^+$TFA$^-$), 1.38-1.12 (m, 21H, -C$_a$H-CH$_2$-CH$_2$-CH$_2$-NH$_3^+$TFA$^-$, -C$_a$H-CH$_3$). FTIR (KBr pellet, cm$^{-1}$): 3315 (N-H stretch, amide), 3076 (Fermi resonance overtone of the 1560 cm$^{-1}$ band), 2931 (C-H stretch, aliphatic), 2856 (C-H stretch, aliphatic), 1742 (C=O stretch, ester), 1648 (C=O stretch, amide I), 1543 (N-H bend, C-N stretch, amide II), 1458 (CH$_2$ wag, aliphatic).

GPC: M$_n$ = 17,000, M$_w$ = 30,000, PDI = 1.76. DSC: T$_g$ = 15.4°C.

3.3.9.2 8-Phe-4-Lys(TFA)-4

Yield: 65%. $^1$H NMR (400 MHz, DMSO-d$_6$): $\delta$ 8.23 (d, 1.8H, J = 8.2, -C(O)-NH-C$_a$H-CH$_2$-Ph), 8.14 (d, 0.2H, J = 5.5, -C(O)-NH-C$_a$H-(CH$_2$)$_4$-NH$_3^+$TFA$^-$), 7.63 (br m, 0.6H, -NH$_3^+$TFA$^-$), 7.33-7.12 (m, 9H, Ph), 4.51-4.39 (m, 1.8H, -C$_a$H-CH$_2$-Ph), 4.19 (br m, 0.2H, -C$_a$H-(CH$_2$)$_4$-NH$_3^+$TFA$^-$), 4.09-3.88 (m, 4H, -C(O)O-CH$_2$-), 3.06-2.81 (m, 3.6H, -C$_a$H-Ph), 2.75 (br m, 0.4H, -C$_a$H-CH$_2$-CH$_2$-CH$_2$-CH$_2$-NH$_3^+$TFA$^-$), 2.21-2.07 (m 0.8H, -C$_a$H-CH$_2$-CH$_2$-CH$_2$-NH$_3^+$TFA$^-$, -CH$_2$-C(O)-NH-C$_a$H-(CH$_2$)$_4$-NH$_3^+$TFA$^-$), 2.03 (t, 3.6H, J = 7.0, -CH$_2$-C(O)-NH-C$_a$H-CH$_2$-Ph), 1.60 (br m, 0.4H, -C$_a$H-CH$_2$-CH$_2$-CH$_2$-CH$_2$-NH$_3^+$TFA$^-$), 1.56-1.28 (m, 8H, -C(O)O-CH$_2$-CH$_2$-CH$_2$-CH$_2$-NH$_3^+$TFA$^-$, -NH-C(O)-CH$_2$-CH$_2$-(CH$_2$)$_4$-NH$_3^+$TFA$^-$), 1.28-1.00 (m, 8.4H, -C$_a$H-CH$_2$-CH$_2$-CH$_2$-CH$_2$-NH$_3^+$TFA$^-$, -C$_a$H-CH$_2$-CH$_2$-CH$_2$-NH$_3^+$TFA$^-$, -NH-C(O)-CH$_2$-CH$_2$-(CH$_2$)$_4$-NH$_3^+$TFA$^-$, -NH-C(O)-CH$_2$-CH$_2$-(CH$_2$)$_4$-NH$_3^+$TFA$^-$, -NH-C(O)-CH$_2$-CH$_2$-(CH$_2$)$_4$-NH$_3^+$TFA$^-$). FTIR (KBr pellet, cm$^{-1}$): 3317 (N-H stretch, amide), 3064 (C-H stretch, aromatic), 3030 (C-H stretch, aromatic), 2928 (C-H stretch, aliphatic), 2855 (C-H stretch, aliphatic), 1736 (C=O stretch, ester), 1655 (C=O stretch, amide I), 1543 (N-H bend, C-N stretch, amide II), 1496 (C=C stretch, aromatic), 1458 (CH$_2$ wag, aliphatic). GPC: M$_n$ = 27,600, M$_w$ = 63,500, PDI = 2.30. DSC: T$_g$ = 43.5°C.
3.3.9.3 8-Phe-8-Lys(TFA)-4

Yield: 62%. $^1$H NMR (400 MHz, DMSO-d$_6$): $\delta$ 8.27-8.10 (m, 2H, -C(O)-NH$_2$H-CH$_2$-Ph, -C(O)-NH$_2$H-(CH$_2$)$_4$-NH$_3^+$TFA$^-$), 8.00 (br m, 0.6H, -NH$_3^+$TFA$^-$), 7.29-7.13 (m, 9H, Ph), 4.44 (m, 1.8H, -C$_6$H$_6$-CH$_2$-Ph), 4.17 (br m, 0.2H, -C$_6$H$_6$-(CH$_2$)$_4$-NH$_3^+$TFA$^-$), 4.07-3.89 (m, 4H, -C(O)O-CH$_2$-), 3.05-2.81 (m, 3.6H, -C$_6$H$_6$-CH$_2$-Ph), 2.74 (br m, 0.4H, -C$_6$H$_6$-CH$_2$-CH$_2$-CH$_2$-NH$_3^+$TFA$^-$), 2.28-2.13 (m 0.8H, -C$_6$H$_6$-CH$_2$-CH$_2$-CH$_2$-CH$_2$-NH$_3^+$TFA$^-$, -CH$_2$-C(O)-NH$_2$H-(CH$_2$)$_4$-NH$_3^+$TFA$^-$), 2.13-1.93 (m, 4H, -CH$_2$-C(O)-NH$_2$H-CH$_2$-CH$_2$-CH$_2$-NH$_3^+$TFA$^-$, -CH$_2$-C(O)-NH$_2$H-CH$_2$-Ph), 1.59 (br m, 0.4H, -C$_6$H$_6$-CH$_2$-CH$_2$-CH$_2$-CH$_2$-NH$_3^+$TFA$^-$), 1.56-1.29 (m, 8H, -C(O)O-CH$_2$-CH$_2$-CH$_2$-NH$_3^+$TFA$^-$, -C(O)O-CH$_2$-CH$_2$-(CH$_2$)$_4$-, -NH-C(O)-CH$_2$-CH$_2$-(CH$_2$)$_4$-, FTIR (KBr pellet, cm$^{-1}$): 3308 (N-H stretch, amide), 3064 (C-H stretch, aromatic), 3030 (C-H stretch, aromatic), 2929 (C-H stretch, aliphatic), 2854 (C-H stretch, aliphatic), 1741 (C=O stretch, ester), 1648 (C=O stretch, amide I), 1543 (N-H bend, C-N stretch, amide II), 1497 (C=C stretch, aromatic), 1455 (CH$_2$, wagging vibration) 1179 (C-O stretch, ester). GPC: $M_n$ = 41,600, $M_w$ =70,500, PDI = 1.70. DSC: $T_g$ = 24.5°C.

3.3.10 Cell Culture and Immunofluorescence Microscopy

Polymer films were obtained by twice dip-coating 12 mm diameter glass coverslips into 1% (wt) solutions of the PEAs in DMF and were dried at 60°C under reduced pressure overnight. One glass coverslip was placed in each well of a 24-well culture plate. The films were immersed in 70% ethanol for 30 min and then exposed to UV irradiation for 1 h, prior to conditioning overnight in Hank’s Balanced Salt Solution (HBSS, 0.5 mL; Invitrogen Canada Inc., Burlington, ON). Natural, human fibronectin (2 µg/cm$^2$; Santa Cruz Biotechnology Inc., Santa Cruz, CA) in HBSS was adsorbed to glass coverslips for 1 h and the resulting surfaces served as positive controls. The human coronary artery smooth muscle cells (HCASMCs; Lonza Walkersville Inc., Walkersville, MD) were seeded directly on the surface of the PEA films in 24-well culture plates and were cultured for 1 and 7 days before fixation and immunostaining. All experiments were conducted in triplicate.
Human coronary artery smooth muscle cells were grown in smooth muscle cell basal medium (SmBM; Lonza Walkersville Inc., Walkersville, MD) supplemented with smooth muscle growth medium-2 (SmGM-2 SingleQuots; Lonza Walkersville Inc., Walkersville, MD) and used between passages 5 and 9. Cultures were maintained at 37°C in a humidified incubator containing 5% CO₂.

For immunostaining studies, cells were washed with pre-warmed PBS immediately prior to fixing at ambient temperature for 10 min in 4% formaldehyde solution (1 mL; EMD Chemicals, Gibbstown, NJ) in divalent cation-free PBS. Following 3 washes in PBS, HCASMCs were permeabilized with 0.1% Triton X-100 (0.5 mL; VWR International, Mississauga, ON) in PBS for 5 min and again washed 3 times with PBS. The HCASMCs were incubated with 1% bovine serum albumin (BSA) in PBS (0.5 mL; Sigma-Aldrich, Oakville, ON) for 30 min at ambient temperature prior to their incubation with either monoclonal anti-vinculin (SPM227, 1:50 dilution; Abcam, Cambridge, MA) for 1 h or monoclonal anti-α-actin (clone 1A4, 1:50 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Following 3 washes in PBS, the HCASMCs were then incubated in the dark for 1 h at ambient temperature with Alexa Fluor® 488-conjugated goat anti-mouse IgG (1:300 dilution; Invitrogen Canada Inc., Burlington, ON). Following 3 washes in PBS, the HCASMCs were further incubated in the dark at ambient temperature with Alexa Fluor® 568-conjugated phalloidin (1:50 dilution; Invitrogen Canada Inc., Burlington, ON) for 20 min in a 1% BSA/PBS solution followed by another 3 washes with PBS. The HCASMCs were then counterstained with 4’-6-diamidino-2-phenylindole dihydrochloride (DAPI, 300 nM in PBS, 0.5 mL; Invitrogen Canada Inc., Burlington, ON) for 5 min to label the nuclei and again washed 3 times with PBS. Coverslips were mounted on microscope slides with SHURMount™ – Toluene Based Liquid Mounting Media (Triangle Biomedical Sciences, Inc., Durham, NC). Samples were analyzed with a Zeiss LSM 5 Duo confocal microscope with 9 laser lines and appropriate filters (Carl Zeiss Canada Ltd., Toronto, ON).

3.3.11 Cell Toxicity and Proliferation Studies Using MTT Assays

Colorimetric assays of the metabolic activity of viable cells were used to quantify cell toxicity and proliferation and were performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-
diphenyltetrazolium bromide (MTT) (Vybrant® MTT Cell Proliferation Assay Kit, Invitrogen Canada Inc., Burlington, ON, Canada). At predetermined time points, fresh culture media was added (100 µL) followed by MTT (10 µL, Component A) and incubated for 4 h. MTT salts are reduced to water insoluble formazan salts by metabolically active cells. Sodium dodecyl sulfate (SDS, 100 µL, Component B) was then added and thoroughly mixed and incubated for a further 18 h to solubilize the formazan, which was quantified using a BioTek EL307C multiplate reader (BioTek Instruments, Inc., Winooski, VT) at 570 nm (maximum absorbance).

3.3.12 Statistical analysis

MTT assay data are given as the mean ± standard deviation for three independent experiments conducted in triplicate. Differences between two groups were compared using a student’s unpaired t test with GraphPad Prism 4, where values of p < 0.01 were considered statistically significant.

3.4 Results and Discussion

3.4.1 Monomer Syntheses

The rationale for synthesizing a family of poly(ester amide)s based on the hydrophobic α-amino acids L-alanine and L-phenylalanine was to impart suitable thermal and mechanical properties. PEAs based on these α-amino acids are semi-crystalline, which may result in improved mechanical properties. Alanine-based PEAs also exhibit high degradation temperatures, which would facilitate their processing in the melt. Moreover, the hydrophobic nature of L-alanine (Ala) and L-phenylalanine (Phe) in combination with long aliphatic diols would further enhance the processability of these PEAs due to their improved solubility in organic solvents such as chloroform. Four different monomers would potentially be available from the selection of α-amino acids – L-alanine and L-phenylalanine, and diols – 1,4-butanediol and 1,8-octanediol. Characterization of di-p-toluenesulfonic acid salts of bis-L-alanine and -L-phenylalanine esters (monomers 1 and 2) are included within this report and agree with previously reported studies. The bis-L-alanine ester derived from 1,4-butanediol was very low yielding and subsequently, its polycondensation was not pursued. The previously unreported acid catalyzed
condensation of L-phenylalanine and 1,8-octanediol was successfully performed to provide monomer 3. Di-p-nitrophenyl sebacate monomer 4 and the bis-N-ε-t-BOC-L-lysine diester monomer 6 were synthesized in good yields as previously reported (Chart 3.1).21,24

Scheme 3.1: Syntheses of di-p-toluenesulfonic acid salt monomers 1-3.

![Scheme 3.1: Syntheses of di-p-toluenesulfonic acid salt monomers 1-3.](image)


3.4.2 Comparison of Solution and Interfacial Polycondensation of Non-Functionalized Poly(ester amide)s

Although solution polymerization to prepare PEAs has been carried out,21 interfacial polymerization is generally attractive since it is faster, less influenced by impurities, and yield higher molecular weights than solution polycondensation reactions.40 Despite these benefits, the hydrolysis of the diacid chloride could lead to early termination of the interfacial polymerization.40 This hydrolysis can be minimized through the selection of a hydrophobic diacid chloride, preventing its undesirable interaction with water, ensuring its reaction only upon encountering an amine group. In this study, the selection of the hydrophobic sebacoyl chloride enabled the syntheses of PEAs from both polycondensation approaches, permitting a direct comparative study to examine the effect of polymerization technique on the properties of the PEAs for vascular tissue engineering applications.
As shown in Scheme 3.2(A), the solution polymerizations were performed by combining di-p-toluenesulfonic acid salt monomer 1, 2 or 3 with 4 in N,N-dimethylacetamide (DMA) and reacting at 70°C for 48 h. The interfacial polymerizations, shown in Scheme 2(B), were performed by adding a solution of sebacoyl chloride in CH₂Cl₂ to a solution of the di-p-toluenesulfonic acid salt monomer 1, 2 or 3 in an aqueous Na₂CO₃ solution and allowing the reaction mixture to stir for 12 h. The structure of each non-functionalized PEA pair was confirmed through its ¹H and ¹³C NMR and FTIR spectra. The effectiveness of both the solution and interfacial polycondensation approaches in generating highly pure PEAs can be observed through the identical spectral output of 8-Ala-8, 8-Phe-4 and 8-Phe-8 from both approaches (Figure 3.1, Figure 3.2 and Figure 3.3).

<table>
<thead>
<tr>
<th>Chemical Structure</th>
<th>Reaction Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scheme 3.2(A)</td>
<td>NEt₃, DMA, 70°C, 48 h</td>
</tr>
<tr>
<td>Scheme 3.2(B)</td>
<td>Na₂CO₃/H₂O, CH₂Cl₂, 12 h</td>
</tr>
</tbody>
</table>

Scheme 3.2: Comparison of the interfacial and solution polymerization approaches.

Upon successful elucidation of the chemical structure of each PEA pair, the thermal properties were examined. Differential scanning calorimetry (DSC) and thermogravimetric analyses (TGA) were conducted on the PEAs to evaluate their glass transition and decomposition temperatures and are shown in (Table 3.1). As illustrated, the glass transition temperature of 8-Ala-8 is around 13°C, while those PEAs containing L-phenylalanine with its bulkier side chain have higher Tgs as expected. The length of the diol also has a significant impact on the glass transition temperature, as the Tg increased from 25°C in the 8-Phe-8 case to around 39°C when 1,4-butanediol was incorporated, which is consistent with published data.¹⁴ Although the Tg in the 8-Phe-4 case is slightly above physiologic conditions, a lower glass transition temperature is anticipated due to the plasticizing effect of culture media. In vascular tissue engineering, a scaffold which
deforms under physiologically-relevant forces is better suited to mimic the natural ECM composition,\textsuperscript{41} thus a polymer with a glass transition temperature below or near that of physiologic conditions is preferred. Furthermore, given the low $T_g$ values, three-dimensional (3D) scaffolds fabricated from the current PEAs are likely to be more pliable, allowing cells to interact favorably. In fact, 3D scaffold pliability has been reported to mimic physiologic focal adhesion contacts when compared with rigid substrata.\textsuperscript{42-44} In addition to having suitable glass transition temperatures, all PEAs had a decomposition temperature exceeding 300°C, indicating that these materials could be easily processed in the melt without degradation. To demonstrate that such processing is feasible, the molecular weight of 8-Phe-4 before and after melt pressing was measured. The results indicate that the molecular weight did not change due to melt pressing.

Figure 3.1: Overlayed $^1$H NMR spectra of 8-Ala-8 synthesized from solution and interfacial polycondensations.
Figure 3.2: Overlayed $^1$H NMR spectra of 8-Phe-4 synthesized from solution and interfacial polycondensations.

Figure 3.3: Overlayed $^1$H NMR spectra of 8-Phe-8 synthesized from solution and interfacial polycondensations.
Table 3.1: Comparison of solution and interfacial polycondensation of non-functionalized PEAs.

<table>
<thead>
<tr>
<th>PEA</th>
<th>Yield (%)</th>
<th>$M_n$ (g/mol)</th>
<th>$M_w$ (g/mol)</th>
<th>PDI</th>
<th>$T_g$ (°C)</th>
<th>$T_d$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-Ala-8-Sol</td>
<td>67</td>
<td>36,600</td>
<td>51,400</td>
<td>1.40</td>
<td>13.5</td>
<td>336</td>
</tr>
<tr>
<td>8-Ala-8-Int</td>
<td>68</td>
<td>45,100</td>
<td>62,500</td>
<td>1.39</td>
<td>11.8</td>
<td>311</td>
</tr>
<tr>
<td>8-Phe-4-Sol</td>
<td>85</td>
<td>53,500</td>
<td>103,000</td>
<td>1.93</td>
<td>38.5</td>
<td>334</td>
</tr>
<tr>
<td>8-Phe-4-Int</td>
<td>78</td>
<td>63,600</td>
<td>168,000</td>
<td>2.64</td>
<td>38.8</td>
<td>320</td>
</tr>
<tr>
<td>8-Phe-8-Sol</td>
<td>61</td>
<td>44,400</td>
<td>63,300</td>
<td>1.43</td>
<td>25.7</td>
<td>343</td>
</tr>
<tr>
<td>8-Phe-8-Int</td>
<td>60</td>
<td>71,800</td>
<td>111,000</td>
<td>1.55</td>
<td>24.5</td>
<td>336</td>
</tr>
</tbody>
</table>

For each non-functional PEA pair examined, the interfacial polymerization approach produced polymers with higher molecular weights. In interfacial polymerization, any impurities from the di-$p$-toluenesulfonic acid salt monomer syntheses remained in the bulk aqueous phase, ensuring the effective polymer chain propagation at the interface.\

From a synthetic vantage point, 8-Phe-4 offers the highest molecular weight and yield; however, the polydispersity index (PDI) is higher than either of the other two PEAs. Furthermore, the PDI of 8-Phe-4-Int is over 2.6, which is considerably higher than its solution polymerized counterpart. The high PDI observed may in part be due to the comparatively high weight average molecular weight broadening the distribution of polymer chains. Since solution polymerization was carried out for 48 h at 60°C, thermolysis may have occurred which, in turn, could reduce the molecular weights of the polymers. In order to rule out this possibility, the molecular weight of 8-Phe-4-Sol was determined after 24 h of polymerization and found to be 36,300 Da. The molecular weight after 48 h of polymerization was 53,500 Da indicating that thermolysis did not occur.

3.4.3 Comparison of Solution and Interfacial Polycondensation of Functionalized Poly(ester amide)s

The solution polycondensation of the functional PEAs containing L-lysine was previously reported in our group and the same procedure was used here to prepare the functional PEAs: 8-Ala-8-Lys(BOC)-4-Sol, 8-Phe-4-Lys(BOC)-4-Sol, and 8-Phe-8-Lys(BOC)-4-Sol. To date, no study has been conducted to assess the incorporation of pendant amine groups in an interfacial polycondensation of PEAs. Therefore, we investigated the effect of the polymerization method on the synthesis of functional PEAs. The challenge in this
approach was that unlike the di-$p$-toluenesulfonic acid salt monomers, the protected lysine monomer 6 was not readily water soluble, preventing its dissolution in the aqueous phase. Furthermore, the addition of the BOC-protected lysine monomer into the organic phase with the sebacoyl chloride would have resulted in polymerization of these two monomers. Consequently, the di-$p$-toluenesulfonic acid salt monomer was dissolved in water, while monomer 6 was dissolved in dichloromethane and mixed for 30 min resulting in a biphasic mixture. Following the addition of sebacoyl chloride, the reaction was allowed to proceed for 24 h, which yielded functional PEAs of higher molecular weight than at shorter reaction times. The base stable BOC protecting group could be readily removed by treatment of the polymers with 1:1 trifluoroacetic acid (TFA):CH$_2$Cl$_2$ to provide the polymers 8-Ala-8-Lys(TFA)-4-Int, 8-Phe-4-Lys(TFA)-4-Int, and 8-Phe-8-Lys(TFA)-4-Int.

The structures of the protected and deprotected functional PEAs were again confirmed with $^1$H NMR and FTIR spectroscopy, while $^{13}$C NMR spectroscopy was not a sufficiently sensitive technique to clearly detect the relatively low abundance of lysine moieties in the high molecular weight polymers. Representative $^1$H NMR spectra of 8-Ala-8-Lys(BOC)-4 and the corresponding deprotected polymer 8-Ala-8-Lys(TFA)-4 are shown in Figure 3.4(A) and (B) respectively. Overlaid NMR spectra of the protected and deprotected functional 8-Phe-4- and 8-Phe-8-based PEAs are shown in Figure 3.5(A) and (B) and Figure 3.6(A) and (B). By comparison with the spectrum of 8-Phe-4 (Figure 3.2), all the major peaks associated with the PEA backbone are also observed in the functional PEAs. However, additional peaks associated with the lysine monomer are clearly observed. For example, the peak at 6.74 ppm (labeled as 20) can be attributed to the proton on the nitrogen atom adjacent to the BOC group. The relative integration of these peaks and those of the PEA backbone were used to assess the incorporation of the lysine monomer and the results are given in Table 3.2. The solution and interfacial methods led to very similar lysine incorporations. Following the cleavage of the BOC group, the presence of the broad peak in the $^1$H NMR spectrum at 7.65 ppm arises due to the protons of the protonated amine salt (labeled as 28).
Figure 3.4: Overlayed $^1$H NMR spectra of 8-Ala-8-based functional PEAs. Note that the unlabeled peaks above correspond to the peaks labeled in Figure 3.1.

Figure 3.5: Overlayed $^1$H NMR spectra of 8-Phe-4-based functional PEAs. Note that the unlabeled peaks above correspond to the peaks labeled in Figure 3.2.
Figure 3.6: Overlayed $^1$H NMR spectra of 8-Phe-8-based functional PEAs. Note that the unlabeled peaks above correspond to the peaks labeled in Figure 3.3.

As with the non-functional PEAs, the thermal properties of the functional PEAs were investigated. Thermogravimetric analyses were conducted to determine whether the incorporation of the lysine had an impact on the thermal decomposition of the PEAs. Representative thermographs are shown in Figure 3.7, Figure 3.8 and Figure 3.9. The introduction of the BOC-protected lysine monomer in 8-Phe-4 for example, results in decomposition around 250°C (Figure 3.8), which may be attributed to the thermal cleavage of the BOC group. In the deprotected case, thermal degradation occurs closer to 220°C. In comparing these two cases, the weight loss is approximately the same indicating that this loss could in fact be due to the TFA salt. Thus the incorporation of the lysine monomer has little impact on the thermal degradation of the PEA backbone.
Figure 3.7: Overlayed TGA thermographs of 8-Ala-8-based functional PEAs.

Figure 3.8: Overlayed TGA thermographs of 8-Phe-4-based functional PEAs.
As observed with the thermogravimetric analyses, the glass transition temperatures of the functional PEAs were also influenced by the incorporation of the protected and deprotected lysine residues, as shown in Figure 3.10, Figure 3.11 and Figure 3.12. Again, using 8-Phe-4 as an example, the glass transition temperature decreased from 39 to 38°C with 10 mol% BOC-protected lysine incorporated, indicating the additional free volume created by the functional handle mitigated the bulkiness of the BOC group. Upon cleavage of the BOC group, yielding the TFA salt, the T_g increased to 44°C, suggesting potential intra- and intermolecular hydrogen bonding further inhibiting rotation of the functional handles.

**Figure 3.9: Overlaid TGA thermographs of 8-Phe-8-based functional PEAs.**
Figure 3.10: Overlayed DSC traces of 8-Ala-8-based functional PEAs.

Figure 3.11: Overlayed DSC traces of 8-Phe-4-based functional PEAs.
In addition to having an effect on the thermal properties of the PEAs, the incorporation of lysine also impacted the molecular weights of the functional PEAs. As shown in Figure 3.13, Figure 3.14 and Figure 3.15 and tabulated in Table 3.2, the molecular weights of the functional PEAs are lower than their corresponding non-functional PEAs, which may be attributed to the fact that the BOC-protected lysine monomer did not react as readily as the other monomers. However, the molecular weights of those PEAs synthesized from an interfacial polycondensation were again all higher than their solution counterparts, as observed in the non-functional PEAs. Also observed from Table 3.2 is that upon cleavage of the BOC protecting group with TFA, there is a reduction in the molecular weight of the functional PEAs, presumably from cleavage of the PEA backbone. In our studies, a reduction in $M_w$ between 23% and 48% was observed. Molecular weight reduction in functional PEAs following protecting group removal has been previously reported.\textsuperscript{26,45} In Deng \textit{et al.},\textsuperscript{26} when a comparable L-lysine content to our study was incorporated into the PEAs, the authors observed over a 70% reduction in molecular weight following deprotection of the carboxybenzyl (CBZ) groups, yielding molecular weights under 20.
kDa. These authors also observed greater cleavage with increasing L-lysine content. More recently, other approaches including the use of unsaturated PEA backbones or allyl glycine monomers followed by thiol-ene chemistry have been used to introduce pendant functional handles to PEAs. While these approaches circumvent the need for protection and deprotection of functional amino acids, the coupling of organic thiols results in loss of biomimicry of the PEA. Also, the high cost of the allylglycine monomer would make this approach costly to scale up. By careful selection of the monomers and employing interfacial polymerization, the present study provides a means to prepare both non-functional and functional PEAs of high purities and molecular weights, with low glass transition temperatures and thermal processability. All of these properties are desirable in a vascular biomaterial.

Table 3.2: Comparison of solution and interfacial polycondensation of functionalized PEAs.

<table>
<thead>
<tr>
<th>PEA†</th>
<th>Yield (%)</th>
<th>Subst. (%)</th>
<th>(M_n) (g/mol)</th>
<th>(M_w) (g/mol)</th>
<th>PDI</th>
<th>(T_g) (°C)</th>
<th>(T_d) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-Ala-8-Lys(BOC)-4-Sol</td>
<td>34</td>
<td>11</td>
<td>19,000</td>
<td>28,700</td>
<td>1.51</td>
<td>15.1</td>
<td>231</td>
</tr>
<tr>
<td>8-Ala-8-Lys(BOC)-4-Int</td>
<td>40</td>
<td>12</td>
<td>29,500</td>
<td>47,600</td>
<td>1.61</td>
<td>15.8</td>
<td>252</td>
</tr>
<tr>
<td>8-Ala-8-Lys(TFA)-4-Int</td>
<td>75</td>
<td>10</td>
<td>17,000</td>
<td>30,000</td>
<td>1.76</td>
<td>15.4</td>
<td>182</td>
</tr>
<tr>
<td>8-Phe-4-Lys(BOC)-4-Sol</td>
<td>52</td>
<td>13</td>
<td>48,800</td>
<td>73,900</td>
<td>1.51</td>
<td>39.8</td>
<td>247</td>
</tr>
<tr>
<td>8-Phe-4-Lys(BOC)-4-Int</td>
<td>57</td>
<td>13</td>
<td>73,400</td>
<td>122,000</td>
<td>1.66</td>
<td>37.9</td>
<td>252</td>
</tr>
<tr>
<td>8-Phe-4-Lys(TFA)-4-Int</td>
<td>65</td>
<td>13</td>
<td>27,600</td>
<td>63,500</td>
<td>2.30</td>
<td>43.5</td>
<td>219</td>
</tr>
<tr>
<td>8-Phe-8-Lys(BOC)-4-Sol</td>
<td>41</td>
<td>10</td>
<td>38,100</td>
<td>49,800</td>
<td>1.31</td>
<td>23.4</td>
<td>277</td>
</tr>
<tr>
<td>8-Phe-8-Lys(BOC)-4-Int</td>
<td>57</td>
<td>11</td>
<td>52,400</td>
<td>91,200</td>
<td>1.74</td>
<td>24.2</td>
<td>295</td>
</tr>
<tr>
<td>8-Phe-8-Lys(TFA)-4-Int</td>
<td>62</td>
<td>13</td>
<td>41,600</td>
<td>70,500</td>
<td>1.70</td>
<td>24.5</td>
<td>215</td>
</tr>
</tbody>
</table>

† As the interfacial method proved to be more effective in providing higher molecular weights, only those PEAs prepared interfacially were deprotected and characterized.
Figure 3.13: Overlapped GPC chromatographs of 8-Ala-8-based functional PEAs.

Figure 3.14: Overlapped GPC chromatographs of 8-Phe-4-based functional PEAs.
3.4.4 Vascular Smooth Muscle Cell Attachment and Proliferation

To fully assess these PEAs as potential materials for vascular tissue engineering, their interactions with human coronary artery smooth muscle cells (HCASMCs) were studied. Firstly, insight into which PEA would best support HCASMC attachment and spreading was sought from the cellular interactions with the bare non-functional PEAs. Because interfacial polycondensation resulted in higher molecular weight for both the non-functional and functional PEAs required of vascular tissue engineering applications, only data from those PEAs produced from an interfacial polymerization were included in the remaining study. Initially, a time-course analysis of HCASMC attachment and spreading was studied using phase contrast microscopy up to one week of culture to assess whether the bare PEAs could support HCASMCs (Figure 3.16). Cell spreading was observed on all PEAs and were similar to that of fibronectin (FN)-treated surfaces at 24 hours (panels I and M). Given that HCASMCs were sparsely seeded (2,000 cells/cm²), these images illustrate that HCASMC attachment and spreading is directly on the surface of PEAs rather than on the surface of another cell. Furthermore, the surfaces were not treated with...
extracellular matrix (ECM) protein indicating acceptable HCASMC interactions with the PEAs themselves. HCASMCs remained well spread up to 7 days of culture. The phase contrast micrographs for the functional PEAs, namely 8-Phe-4-Lys(TFA)-4-Int and 8-Phe-4-Lys(TFA)-4-Int appeared to be somewhat diffuse due to the rough surface texture of these PEAs, but individual spread cells are clearly visible.

Figure 3.16: Time-course phase contrast micrographs of HCASMCs seeded at 2,000 cells/cm² at 24 hours and 7 days on glass coverslips (A, E), FN-coated coverslips (I, M), non-functional PEAs: 8-Ala-8-Int (B, F), 8-Phe-4-Int (C, G) and 8-Phe-8-Int (D, H). The phase contrast micrographs of the corresponding lysine-functionalized PEAs: 8-Ala-8-Lys(TFA)-4-Int, 8-Phe-4-Lys(TFA)-4-Int and 8-Phe-8-Lys(TFA)-4-Int are shown in panels (J, N), (K, O), and (L, P) at 24 hours and 7 days respectively. HCASMCs were uniformly spread on all PEA surfaces. Scale bar = 200 µm.
The cell attachment and spreading data observed in the phase contrast images of HCASMC were confirmed by quantifying cell viability. The optical density results of the MTT assay on the PEAs are provided in Figure 3.17. For the non-functionalized PEAs (Figure 3.17A), cellular growth and survival on each PEA film was significantly greater at each time point than on the tissue culture polystyrene (TCPS), which served as the control. Given that TCPS is a well established cell culture substrate, it is interesting to note that the bare PEAs showed increased cell viability even at 1 day of culture.

Moreover, there was no delayed toxicity as cell viability continued to increase up to 5 days of culture, suggesting the potential use of these PEAs for long-term cell cultures needed in vascular tissue engineering strategies. In fact, a statistically significant (p < 0.01) increase in cell viability was observed between day 3 and day 5 cultures on both 8-Phe-4-Int and 8-Phe-8-Int indicating these PEAs also support HCASMC proliferation.

Figure 3.17: HCASMC viability as determined by MTT assay. Cells were seeded at 20,000 cells/cm² on TCPS (control) and non-functional (A) and functionalized (B) PEAs and measured following 1, 3 and 5 days of culture. Data are expressed as mean ± standard deviation for three independent experiments conducted in triplicate. Statistical significance, denoted by *, is observed between all non-functional PEAs and TCPS at each day (p < 0.01).
Cell viability was also carried out for the corresponding lysine-functionalized PEAs. As the data in Figure 3.17B indicate, at day 1, cell viability on PEAs based on phenylalanine (8-Phe-4-Lys(TFA)-4-Int and 8-Phe-8-Lys(TFA)-4-Int) was significantly higher than both the TCPS and the alanine-based PEA (p < 0.001). This result is consistent with the non-functional counterparts (Figure 3.17A). For both day 3 and 5 cultures, 8-Ala-8-Lys(TFA)-4-Int was significantly lower than the phenylalanine-based functional PEAs. At day 5, cell proliferation on all functional PEAs was significantly lower than TCPS indicating that for HCASMCs seeded on functionalized PEAs, metabolic events do not seem to lead to robust cell growth. Statistical analysis (p < 0.01) of cell viability on each functional PEA as a function of culture time, however, showed significant cell growth suggesting that cells were growing on the functional PEAs, but that the rate is lower than on TCPS. Given that the functionalized PEAs were positively charged, we anticipated comparable cell viability results to TCPS which is also positively charged. This, however, was not the case presumably due to the effect of the TFA salt, which has been reported to retard cell growth and viability.47 Collectively, these results are promising as they suggest that the current PEAs can support HCASMC attachment, spreading and proliferation without modification with cell adhesive proteins. Although the current cell work is focused on understanding the interactions of the HCASMCs, our long-term goal is to utilize the functionalized PEAs for conjugating biomolecules that regulate cell growth, differentiation, and signaling pathways which may ultimately determine the success of engineered vascular tissues. Previous work in our group has shown that the pendant amine groups of PEAs containing lysine can be conjugated to molecules using standard reagents.23-24

Although HCASMC viability data on degradable PEAs is not available in the literature, bovine aortic endothelial cell (BAEC) and fibroblast growth on different PEAs was recently reported.32,48 The data, however, showed that BAEC growth was slower than the control32 whereas in the other study, it was similar to the control material used.48 In another study, enhanced cell viability of gelatin-coated PEAs was also reported,26 but unlike our study, cells were not exposed directly to the PEA surface making direct comparison difficult.
Our data (Figure 3.16 and Figure 3.17) showed that without surface modification, HCASMC attachment and proliferation on the PEAs was encouraging. In order to gain information on the cytoskeletal organization, staining for filamentous actin (F-actin), vinculin and smooth muscle α-actin cell marker protein expression was examined. F-actin serves as the building block necessary for cell motility, thus its qualitative intercellular abundance and organization on all PEAs was examined. As shown in Figure 3.18, HCASMCs adhered to the current PEAs with abundant F-actin expression regardless of the PEA chemical composition. At 24 h culture, all cells seeded on PEAs had spread similar to the FN-coated coverslip positive control (Figure 3.18 I). However, at day 7, cells on the positive control surface appeared to be better aligned (Figure 3.18 M) while the cells on the functionalized PEAs remained randomly oriented.

The adhesion of cells to a biomaterial is the first event in tissue engineering. Subsequent cellular events such as differentiation and ECM secretion depend on this critical event. Cells seeded on materials that cannot present specific ligand to integrins adhere by non-specific adhesive forces. Vinculin is a cytoskeletal protein associated with cell-cell and cell-matrix adhesions by anchoring F-actin to the membrane. To gain information on the level of focal contacts, we labeled cells with antibodies to vinculin. The results shown in Figure 3.19 (A-D; I-L) suggest that cells started to form focal attachment sites (shown by the arrowhead as the red tip in the periphery of the cells).

In addition to cell attachment, spreading and viability, vascular tissue engineering requires that cells be given a more specific level of instruction so that tissue fabrication is successful. In this regard, regulation of vascular smooth muscle cell (VSMC) phenotype is important. VSMCs are not terminally differentiated and are able to modulate their phenotype in response to changing environmental cues. During tissue generation and maturation, a synthetic VSMC phenotype is beneficial for cellular proliferation and ECM secretion, which is achieved through the rapid up-regulation of genes required for proliferation including matrix metalloproteinases and l-caldesmon, while suppressing the expression of genes that define the contractile phenotype. Conversely, in a contractile phenotype, VSMCs have a relatively low proliferative activity and produce only small amounts of ECM proteins while robustly expressing contractile cytoskeletal marker
proteins such as smooth muscle α-actin (SMαA) and contract in response to electrical, chemical and mechanical stimuli. To obtain preliminary information on the state HCASMC differentiation, we stained 7 day cultures for SMαA. The immunofluorescence images illustrates that the expression of SMα-actin on all PEAgs was not abundant (Figure 3.19 E-H; M-P) suggesting a proliferative-type smooth muscle cell phenotype. Taken together, these data suggest the current PEAs support HCASMC attachment, spreading, proliferation, vinculin expression and a proliferative-type smooth muscle cell phenotype that warrants further investigation of these functional PEAs for vascular tissue engineering applications.

Figure 3.18: Confocal microscopy images of HCASMCs seeded at 2,000 cells/cm² at 24 hours and 7 days on non-functional (B-D, F-H) and lysine-functionalized (J-L, N-P) PEAs and labeled for F-actin (green) and nuclei (blue). At longer culture times, cells on the FN-coated control appeared to be better aligned (M); whereas, on the functionalized PEAs, cells were randomly oriented. Scale bar = 50 μm.
Figure 3.19: Immunofluorescence microscopy images of HCASMCs after 24 hours and 7 days culture on non-functional and lysine-containing functional PEAs. HCASMCs were stained for F-actin (green), vinculin (A-D and I-L; shown by the arrowhead; red), smooth muscle α-actin (E-H and M-P; red) and nuclei (blue). Scale bar = 50 μm.

3.5 Conclusions

A series biodegradable of PEAs was synthesized from the α-amino acids L-alanine, L-phenylalanine and L-lysine. To prepare high molecular weight PEAs that can be used for vascular tissue engineering applications, both solution and interfacial polymerization methods were investigated. All PEAs prepared by interfacial polycondensation gave higher molecular weights compared with their solution polymerization counterparts. The synthesized PEAs had glass transition temperatures ranging from 10 to 40°C, indicating
their pliability under physiologic conditions. HCASMC attachment and spreading was observed on all PEAs up to 7 days of culture, while cell viability was significantly higher on all non-functional PEAs than TCPS. The trifluoroacetic acid salt of the lysine-containing functional PEAs supported HCASMC, but retarded cell growth when compared to TCPS. Immunostaining of cells illustrated strong vinculin expression on all surfaces; however, smooth muscle $\alpha$-actin expression was not abundant suggesting a proliferative smooth muscle cell phenotype. Collectively, this study shows that all PEAs are good candidates as vascular biomaterials as they support HCASMC attachment, spreading and proliferation. Specifically, 8-Phe-4-Lys(TFA)-4-Int and 8-Phe-8-Lys(TFA)-4-Int are more attractive since the pendant functional groups enable the conjugation of molecules that regulate cell growth, differentiation, and signaling pathways. Future work will focus on both mechanical properties and detailed cellular responses to 3D scaffolds of these functional PEAs.

3.6 References


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Chapter 4

4 Focal Contact Formation of Vascular Smooth Muscle Cells on Langmuir-Blodgett and Solvent Cast Films of Biodegradable Poly(ester amide)s†

This chapter examines the adhesion of human coronary artery smooth muscle cells on two-dimensional PEA films. Ultrathin films prepared by Langmuir-Blodgett technology were prepared on waveguides, which enabled the observation of close and focal contacts between the smooth muscle cells and the PEA surface using waveguide evanescent field fluorescence (WEFF) microscopy. The data was then compared to conventional immunostaining of focal adhesions.

4.1 Abstract

The ability of biomaterials to support the adhesion of cells is a necessary condition for their use in scaffold-guided tissue engineering. Waveguide evanescent field fluorescence (WEFF) microscopy is a relatively new microscopic technique that allows the number of cell adhesions to a waveguide surface be measured by imaging the interfacial contact region between the cells and their substratum. In this work, the adhesion of human coronary artery smooth muscle cells (HCASMCs) to ultrathin films (20 nm) of poly(ester amide)s (PEAs) prepared by Langmuir-Blodgett (LB) technology on waveguides was investigated and compared with conventional vinculin immunostaining on solvent cast PEA films. Cell culture was conducted both in the presence and absence of serum to evaluate the effect of non-specific protein adsorption that mediates cell adhesion. WEFF microscopy analyses revealed that the cationic PEA enhanced the number of attachment sites compared with the control waveguides regardless of the culture medium. Although differences in cell adhesions between different PEAs were suggested by the results, no statistically significant differences were found. Similar results were observed with

presently and previously reported vinculin immunostaining studies, further validating the use of WEFF microscopy to quantify cell adhesions. Moreover, the focal adhesions of the HCASMCs to the PEA surfaces indicate these PEAs can promote integrin signaling, which is vital in cell survival, migration and proliferation and ultimately in scaffold-guided vascular tissue engineering.

**Key words:** Waveguide evanescent field fluorescence (WEFF) microscopy, Langmuir-Blodgett (LB) films, poly(ester amide)s (PEAs), human coronary artery smooth muscle cells (HCASMCs), vinculin immunostaining, focal adhesions.

### 4.2 Introduction

With the aim to successfully engineer scaffold-guided tissues, there is an ever-increasing interest in the interactions of cells with both synthetic and natural biomaterials. When cells are seeded onto a biomaterial, both non-specific and receptor-mediated cell interactions between various molecules on the cell membrane and chemical groups on the biomaterial determine the fate of cell adhesion, spreading, proliferation and differentiation. In view of this, the study of the contact regions between a cell and its substratum is of considerable interest as its investigation provides useful information about the cytocompatibility of the substratum and the affinity of cells towards that particular surface. While information concerning these interactions is often inferred from enzyme-linked immunosorbent assays (ELISA) and immunostaining of focal adhesion molecules, different microscopic techniques such as total internal reflection fluorescence (TIRF), surface plasmon resonance microscopy (SPRM), interference reflection microscopy (IRM) and fluorescence interference-contrast (FLIC) microscopy have also been used.

Recently, waveguide evanescent field fluorescence (WEFF) microscopy was developed with the capability to image cell-substratum contact regions with certain advantages. In WEFF microscopy, waveguides are implemented as microscopy substrates. The generation of an evanescent field of up to ~70 nm in depth from the waveguide surface enables the excitation of fluorescent dyes located in the cells within this range only. Due to evanescent field decay beyond ~70 nm, the remainder of the cell is not illuminated and...
thus does not contribute to the image. It has been established that WEFF and TIRF microscopy can deliver comparable information.\textsuperscript{13} However, the key advantages of WEFF microscopy are that biomaterials can be easily coated on to the surface of the waveguide and that the user can freely choose the field of view and the magnification during an experiment.\textsuperscript{12,14} As WEFF microscopy restricts the distance above the waveguide to \~70 nm, it necessitates the fabrication of ultrathin films of the material being tested for cell adhesion.

The objectives of the present work were two-fold: (i) to prepare Langmuir-Blodgett (LB) films from three different poly(ester amide)s (PEAs) on waveguide substrates and, (ii) to describe the use of WEFF microscopy for investigating human coronary artery smooth muscle cell (HCASMC) adhesions on LB PEA films to compare it with a conventional immunostaining approach. The class of biodegradable PEAs used in this study is of significant interest for the development of a wide range of biomedical applications as the incorporation of naturally occurring $\alpha$-amino acids enables the syntheses of functional biomimetic materials. Recently, our group has been investigating the potential of PEAs to serve as vascular tissue engineering scaffolds.\textsuperscript{16-17} The favourable interaction of HCASMCs with these materials will be a necessary condition in enabling the attachment, proliferation, and differentiation of cultured cells. While a number of classes of PEAs have been previously reported,\textsuperscript{18-20} the particular PEAs of interest in this work are composed of $\alpha$-amino acids, diols and dicarboxylic acids. As their backbones contain both ester and amide linkages they can undergo both hydrolytic and enzymatic degradation where suitable monomer selection can provide non-toxic metabolic intermediates upon degradation. Another attractive feature of these PEAs is that their biological and mechanical properties can be readily tuned by the crystallinity, hydrophobicity and chemical functionality of their constituent monomers.\textsuperscript{21} In the current study, Langmuir-Blodgett (LB) technology was used to prepare ultrathin films of three different PEAs on waveguide substrates and the average number of cell adhesions (focal and close contacts) formed by HCASMCs were determined as a function of the polymer structure and cell culture conditions. The data obtained from WEFF microscopy are compared with quantitative data obtained by immunostaining for vinculin, which is a
focal adhesion component localized to adhesive plaques. The attachment, spreading and morphology of HCASMCs on PEA films were also evaluated.

4.3 Experimental

4.3.1 Waveguide Evanescent Field Fluorescence (WEFF) Microscopy

The WEFF microscope which is described elsewhere\textsuperscript{14} consists of an inverted microscope (Carl Zeiss, Oberkochen, Germany), where the specimen was placed on top of the waveguide. A HeNe laser ($\lambda = 543$ nm) was coupled into a waveguide mode by a grating located on the waveguide. In order to block the undesired excitation wavelength, a long pass filter with a cut-off wavelength of $\lambda_{\text{cut-off}} = 560$ nm was fitted between the objective and the camera. The camera was connected to the computer on which the images were processed with Image-Pro 6 software. Bright field microscopy images of the sample were captured with the same field of view as the WEFF microscopy images to enable counting of cells.

4.3.2 Waveguide Fabrication

Step-index waveguides were fabricated from SG11 glass slides (Schott, Grünenplan, Germany). The glass slides were first sonicated in a 2 \% Hellmanex\textsuperscript{®} solution (Hellma, Müllheim, Germany) in deionized (DI) water for 15 min and then rinsed followed by a further sonication with DI water. The rinse and sonication steps were repeated with anhydrous ethanol. Following this cleaning procedure, the glass slides were placed in molten AgNO$_3$ (EMD Chemicals, Gibbstown, NJ) in a tube furnace (Yokogawa Meters and Instruments Corporation, Tokyo, Japan). This enabled ion exchange to occur at the interface of the glass slide and molten salt. After ion exchange, the glass slides were removed from the melt, cooled with nitrogen gas under ambient conditions, thoroughly rinsed and sonicated for 30 min in Milli-Q water.\textsuperscript{13} A waveguide mode coupling grating was fabricated by a holographic laser set-up in photoresist.\textsuperscript{22} Multimode waveguides with thicknesses of 500-600 nm and gratings with periodicities ($\Lambda$) in the order of 530-550 nm and 600-650 nm were achieved.
4.3.3 Preparation of Ultrathin Langmuir-Blodgett (LB) Poly(ester amide) (PEA) Films for Cell Culture

The syntheses and characterization of the PEAs are described elsewhere.\textsuperscript{17} PEAs (Scheme 4.1) were dissolved in chloroform at a concentration of 1.0 mg/mL. The solutions were sonicated to promote dissolution and then filtered through a 0.2 µm syringe filter. The LB film preparation parameters were identical for all PEAs - LB trough: KSV 3000 (KSV Nima, Espoo, Finland); spread volume: 200 µL; compression rate: 5 mm/min; film lift speed: 2 mm/min; and surface pressure (\(\pi\)) at deposition: 2 mN/m. The substrates used for film transfer were the waveguides, which were first cleaned with 70% ethanol followed by a rinse with milliQ water. The waveguides were mounted in the water phase of the trough before spreading the polymer solution. The polymer solution was then carefully spread onto the milliQ water subphase with a Hamilton microliter syringe. After solvent evaporation, the barriers were moved to achieve a surface pressure of 2 mN/m. The LB transfer took place with the waveguide substrate oriented perpendicular to the barrier direction, to allow for stretching of the aggregates.

4.3.4 LB Film Characterization

LB films prepared from PEAs were viewed under dark-field light microscopy (Axioskope-2 MAT, Carl Zeiss, Oberkochen, Germany) to examine the homogeneity of the films before cell seeding and culturing. Film thicknesses were determined by interference between light reflecting from the surface and light traveling through the film using variable angle spectroscopic ellipsometer (M2000V, J.A. Woollam, Lincoln, NE). For comparative purposes film thicknesses were also measured by Dektak 3 surface profilometer and Atomic Force Microscopy (AFM) (both from Veeco Instruments, Santa Barbara, CA).

4.3.5 Cell Culture

Primary HCASMCs were purchased from Lonza Walkersville, Inc. (Walkersville, MD) and used between passages 5 and 9. HCASMCs cultured in serum were grown in smooth muscle cell basal medium (SmBM, Lonza Walkersville, Inc., Walkersville, MD)
supplemented with smooth muscle cell growth medium-2 (SmGM-2, SingleQuots; Lonza Walkersville Inc., Walkersville, MD). HCASMCs cultured in the absence of serum were grown in BioWhittaker PC-1™ base medium (Lonza Walkersville, Inc., Walkersville, MD) supplemented with PC-1™ sterile supplement (Lonza Walkersville Inc., Walkersville, MD) and L-glutamine (2 mM, Invitrogen Canada Inc., Burlington, ON). All cultures were maintained at 37°C in a humidified incubator containing 5% CO₂. Culture media was replaced at day 1 and every other day thereafter.

4.3.6 Cell Culture for WEFF Microscopic Analysis

Waveguides were immersed in 70% ethanol for 30 min and were allowed to dry for 30 min under germicidal UV light. HCASMCs were then seeded directly onto the surface of the PEA LB films or control waveguides at a density of 10,000 cells/waveguide and cultured up to 80% confluency (typically for 3 or 4 days). The cells were fixed at ambient temperature for 10 min in 4% formaldehyde (0.5 mL; EMD Chemicals, Gibbstown, NJ) in phosphate-buffered saline (PBS), followed by 2 washes in PBS. The cell membrane labeling solution was prepared by adding 5 µL of the lipophilic dye solution – 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate in ethanol (Vybrant® DiI cell-labeling solution, Invitrogen Canada Inc., Burlington, ON) to 1 mL of normal growth medium. The labeling solution (100 µL) was added and gently agitated to cover the cell-seeded waveguides and then incubated at 37°C for 20 min. The staining medium was then drained off and replaced with fresh media and incubated for a further 10 min. The cells were washed three times with normal growth medium prior to microscopic analysis.

4.3.7 Fluorescence Microscopy

Polymer films were obtained by twice dip-coating 12 mm diameter glass coverslips into 1% (wt) solutions of the PEAs in DMF and were dried at 60°C under reduced pressure overnight. One glass coverslip was placed in each well of a 24-well culture plate. The films were sterilized with 70% ethanol for 30 min and then exposed to germicidal UV for 1 h, prior to conditioning overnight in Hank’s Balanced Salt Solution (HBSS, 0.5 mL; Invitrogen Canada Inc., Burlington, ON). For examining HCASMC morphology, the HCASMCs were seeded directly on the surface of the dip-coated PEA films at a density
of 15,000 cells/cm² and cultured in the presence of serum for 24 h prior to fixation and staining as described below. For vinculin immunostaining studies, natural, human fibronectin (FN, 2 µg/cm²; Santa Cruz Biotechnology Inc., Santa Cruz, CA) in HBSS was adsorbed to glass coverslips for 1 h and the resulting surfaces served as positive controls. All cells were seeded directly on the surface of the PEA or FN coated coverslips at a density of 2,000 cells/cm² and cultured in the presence or absence of serum for 24 h (serum) or 4 days (serum-free). Cells were washed with pre-warmed PBS before fixation with a 4% formaldehyde solution (1 mL; EMD Chemicals, Gibbstown, NJ) in divalent cation-free PBS at ambient temperature for 10 min. Following 3 washes in PBS, HCASMCs were permeabilized with 0.1% Triton X-100 (0.5 mL; VWR International, Mississauga, ON) in PBS for 5 min and again washed 3 times with PBS. The HCASMCs were incubated with 1% bovine serum albumin (BSA) in PBS (0.5 mL; Sigma-Aldrich, Oakville, ON) for 30 min at ambient temperature prior to their incubation with monoclonal anti-vinculin (SPM227, 1:50 dilution; Abcam, Cambridge, MA) for 1 h. Following 3 washes in PBS, the HCASMCs were then incubated in the dark for 1 h at ambient temperature with Alexa Fluor® 488-conjugated goat anti-mouse IgG (1:300 dilution; Invitrogen Canada Inc., Burlington, ON). Following 3 washes in PBS, the HCASMCs were further incubated in the dark at ambient temperature with Alexa Fluor® 568-conjugated phalloidin (1:50 dilution; Invitrogen Canada Inc., Burlington, ON) for 20 min in a 1% BSA/PBS solution followed by another 3 washes with PBS. The HCASMCs were then counterstained with 4’-6-diamidino-2-phenylindole dihydrochloride (DAPI, 300 nM in PBS, 0.5 mL; Invitrogen Canada Inc., Burlington, ON) for 10 min to label the nuclei and again washed 3 times with PBS. The coverslips were mounted on microscope slides with ProLong® Gold antifade reagent (Invitrogen Canada Inc., Burlington, ON), dried overnight at room temperature in the dark, prior to sealing with nail enamel. Samples were analyzed with a Zeiss LSM 5 Duo confocal microscope with 9 laser lines and appropriate filters (Carl Zeiss, Oberkochen, Germany).

4.3.8 Statistical Analyses

The statistical data are given as the mean ± standard error for experiments conducted at least in triplicate. Differences between two groups were compared using a two-tailed
unpaired student’s t-test with GraphPad Prism 4, where values of $p < 0.05$ were considered statistically significant.

### 4.4 Results and Discussion

While a diverse array of PEAs have been previously reported by our group\cite{16-17,21,23} and others\cite{24-28} and recently reviewed,\cite{29} The PEAs depicted in Scheme 4.1 were selected for this investigation. This was in part due to their solubility in chloroform, a solvent compatible with LB technology; whereas, many previously synthesized PEAs are only soluble in water-miscible solvents such as $N,N$-dimethylformamide (DMF).\cite{30} In addition, these PEAs are currently under investigation in our group as viable materials for the development of tissue engineering scaffolds. The PEAs all contain sebacic acid and L-phenylalanine. 8-Phe-4-Sol contains 1,4-butanediol, while 8-Phe-8-Int contains 1,8-octanediol and is therefore slightly more hydrophobic. In 8-Phe-8-Lys(TFA)-4-Int, 10 mol% of the L-phenylalanine was randomly substituted with L-lysine. This PEA was of interest because the cationic amines of the lysine moieties could be used to promote cell adhesion\cite{31} or to conjugate biomolecules that regulate cell growth, differentiation and other signaling pathways.\cite{32}

![Scheme 4.1: Chemical structures of the selected PEAs.](image-url)
The molecular weight data of the specific batches of PEAs used in this study are provided in Table 4.1.

Table 4.1: Molecular weight and polydispersity indices of the selected PEAs.

<table>
<thead>
<tr>
<th>PEA</th>
<th>Number Average Molecular Weight (Mn, g/mol)</th>
<th>Weight Average Molecular Weight (Mw, g/mol)</th>
<th>Polydispersity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-Phe-4-Sol</td>
<td>53,500</td>
<td>103,000</td>
<td>1.92</td>
</tr>
<tr>
<td>8-Phe-8-Int</td>
<td>65,600</td>
<td>89,100</td>
<td>1.36</td>
</tr>
<tr>
<td>8-Phe-8-Lys(TFA)-4-Int</td>
<td>20,700</td>
<td>44,700</td>
<td>2.15</td>
</tr>
</tbody>
</table>

Given that the evanescent field decays beyond 70 nm, the polymer film thickness needed to be less than 20 nm to observe cell adhesion to the various PEA surfaces. This film thickness could not be achieved by solvent casting. Alternatively, spin coating was attempted, but also proved unsuccessful because the gratings on the waveguides were undesirably covered by the PEA films. The above difficulties were addressed by using LB technology to obtain ultrathin PEA films (Table 4.2). Polymers are used in LB technology despite their long chains, inherent polydispersity, high viscosity, and strong intra- and intermolecular interactions. For example, intra- and intermolecular hydrogen bonding of the amide groups of these PEAs could potentially lead to aggregate formation. As aggregates deposited onto the waveguide at low pressure can result in the formation of a non-closed and non-uniform film, each PEA solution was transferred to the waveguide perpendicular to the barrier direction. This technique facilitated stretching of the aggregates minimizing inhomogeneities in the PEA film transfer, which otherwise could result in uncoated areas on the waveguide. Despite this approach to minimize inhomogeneities in preparing LB films of the PEAs, those prepared from DMF resulted in aggregation and inhomogeneous distribution over the substrate as evidenced by the clustering of bright spots in Figure 4.1B. This is attributed to DMF’s slow evaporation and miscibility with the subphase. However, PEA LB films obtained from a chloroform solution were more evenly distributed on the subphase and the films formed more homogeneously as the scattering spot distribution shows (Figure 4.1C). Unlike typical LB monolayers, when the PEAs were compressed by the barriers they did not collapse, even when the barriers were near complete closure. This is evident in the isotherm as depicted for 8-Phe-4-Sol in Figure 4.1D, which has no steep decline or peak corresponding to a
collapse of the solid phase at small mean molecular areas. Similar isotherms were obtained with 8-Phe-8-Int and 8-Phe-8-Lys(TFA)-4-Int as seen in Figure 4.1(E) and (F). Further compression led to an undesirable thick smear between the barriers of the LB trough resulting from stacked PEA chains. Consistent with the present study, such stacked and non-collapsing films at increased compression (corresponding to small mean molecular areas) were also reported for collagen.34

Figure 4.1: Dark-field microscopy images of a bare waveguide (A), LB film of 8-Phe-4-Sol obtained using DMF (B), LB film of 8-Phe-4-Sol obtained using chloroform (C). Surface pressure isotherms for 8-Phe-4-Sol (D), 8-Phe-8-Int (E) and 8-Phe-8-Lys(TFA)-4-Int (F) from a solution in chloroform (D). Scale bar applicable to A, B and C represents 200 µm.

Not surprisingly, these PEA LB films did not perform like classic LB films. Aggregates were visible on the subphase which may have been the result of strong intra- and intermolecular interactions (Figure 4.1B and C). These aggregates were floating into and upon each other, and the increase in the surface pressure during compression of the barriers was caused by an increase in packing density of the aggregates. An increase in the thickness of the film must be accompanied by this pressure increase. Therefore, when preparing LB films with these PEAs, the layer formed on the subphase is not a monolayer, but most likely a multilayer (Table 4.2).
Table 4.2. Film thicknesses of 8-Phe-4-Sol from different LB film preparation conditions.

<table>
<thead>
<tr>
<th>LB Film Preparation Conditions</th>
<th>Film Thickness</th>
<th>Roughness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spread volume (µL)</td>
<td>Concentration (mg/mL)</td>
<td>Surface Pressure (π, mN/m)</td>
</tr>
<tr>
<td>300</td>
<td>1.0</td>
<td>5</td>
</tr>
<tr>
<td>300</td>
<td>1.0</td>
<td>10</td>
</tr>
<tr>
<td>300</td>
<td>0.5</td>
<td>10</td>
</tr>
<tr>
<td>200</td>
<td>1.0</td>
<td>10</td>
</tr>
</tbody>
</table>

Previously non-classic LB behavior with aggregate formation was observed for collagen due to strong intermolecular interactions. Since the spacer diol chain between the adjacent amide bonds is short for 8-Phe-4-Sol (Scheme 4.1) compared with 8-Phe-8-Int and 8-Phe-8-Lys(TFA)-4-Int, the formation of a multilayer rather than a monolayer is likely for 8-Phe-4-Sol. This increased density of amide bonds due to the short chain diol chain could yield more hydrogen bonding and an increase in film viscosity. In contrast, 8-Phe-8-Int and 8-Phe-8-Lys(TFA)-4-Int have a longer diol chain and therefore have fewer possibilities for intra- and intermolecular hydrogen bonding resulting in less viscous films. In addition to this longer diol chain, 8-Phe-8-Lys(TFA)-4-Int has an L-lysine side chain that allows free rotation, resulting in weaker intermolecular bonding. As presented in Table 4.2, film thicknesses of LB films of 8-Phe-4-Sol were less than 20 nm when the spread volume was maintained at 300 µL. A surface pressure change from 5 mN/m to 10 mN/m and a PEA concentration change from 0.5 mg/mL to 1 mg/mL did not have any appreciable effect on the film thicknesses. Although film thickness data varied between ellipsometry and AFM measurements for a spread volume of 200 µL, it appeared that a higher film thickness resulted from a lower spread volume, at least on the basis of the ellipsometry data.

To examine and quantify HCASMC adhesion to these PEAs, two approaches were adopted – WEFF microscopy to observe cell adhesion to the substratum and vinculin immunostaining to illustrate possible integrin-mediated HCASMC focal adhesion. For WEFF microscopy analyses, cells were grown on LB films until 80% confluent (typically 3-4 days). The LB technology for film formation was necessary because the total thickness of the PEA film and the cell membrane should not exceed the penetration depth
of the evanescent field of the manufactured waveguides, which decays within roughly 70 nm. By staining the cell membranes with the fluorescent dye, DiI, the areas of the cells that are closest to the surface of the waveguide, are the most strongly excited and fluorescent, while those areas outside the evanescent field do not fluoresce. In illuminating only this interfacial area, the number of attachment sites per HCASMC could then be determined by counting the bright spots appearing in the WEFF microscopy images using ImageJ software (National Institutes of Health, Bethesda, MD) and dividing it by the number of cells present. Figure 4.2 shows representative images of HCASMCs cultured on the control waveguide and PEA LB films taken with bright field and WEFF microscopy. In the bright field images (left), individual cells are well-spread. The white spots in the corresponding WEFF images (right) show the sites of cell attachment to the PEA LB films. The number of cells on the control waveguide (Figure 4.2A and B) appeared to be lower than those on the PEA LB films suggesting that the PEAs provided a better environment for HCASMC attachment and spreading. The WEFF image of the control waveguide (Figure 4.2B) does not exhibit any epi-fluorescence because of the absence of the PEA film. The PEA films located in the area of the highest evanescent fields of the waveguide are the primary source of scattered excitation photons (compare with Figure 4.1C). These scattered photons can reach all areas of the cell, including those outside the evanescent field and excite fluorescence photons. While the WEFF images of the PEA-coated waveguides illustrate predominantly the evanescent information and hence the contact points of the cell, (Figure 4.2D, F, H), it could also show a nebulous background outlining the cell body due to this epi-fluorescence. Epi-fluorescence which is also a common observation in TIRF can be removed from WEFF images by choosing smaller integration times, but the cell outline information will be lacking.13

An analysis of the number of adhesions that the HCASMCs formed on both the control waveguide and PEA LB films was first conducted in the presence of serum in the culture media. As shown in Figure 4.3, few cell adhesion sites on the control waveguide were observed. Although the waveguide was not expected to be an ideal substrate for cell culture, the adsorption of proteins on a surface is often considered the first step towards the adhesion of cells on that surface.3 In particular, the serum used in cell culture contains
many cell adhesive proteins including fibronectin and vitronectin, which may promote the formation of differential focal adhesions. Therefore, the role of protein adsorption in the establishment of adhesions of HCASMCs on the waveguides was also evaluated by culturing cells in the absence of serum. On the control waveguides, this resulted in an average of 0.9 attachment sites per cell. This result was not statistically significant (p > 0.05) from the serum-containing (S+) media study of 2.1 attachment sites per cell indicating protein adsorption did not play a significant role in HCASMC adhesion to the control waveguides. Moreover, the number of attachment sites in both S+ and S- control waveguides appeared to be lower than those obtained from immunostaining studies reported in the literature (at least 5-10 focal adhesions per cell) for various cell types on different materials. In generating a waveguide on the surface of a glass slide, low polarizability ions (sodium) are exchanged for high polarizability ions (silver) to enhance the refractive index on the surface. Given that the waveguide surfaces are silver ion-exchanged, reduced attachment sites are attributed to residual silver ions which are known to reduce cell adhesion. However, the presence of the LB PEA films may have masked the detrimental effects of the residual silver ions on cell adhesion. In addition to focal adhesions, there is a potential for cells to also form non-focal contacts such as close contacts. Although close contacts typically range from 20 to 50 nm from the substratum, the ventral cell membrane of the HCASMCs may remain equidistant from the surface, as previously reported with retinal pigment epithelial cells, inhibiting effective isolation and segregation of individual close contacts, arbitrarily underestimating the number of cell attachment sites. We did not distinguish between focal contacts and close contacts as the distances of the individual contacts were not investigated in this study.
Figure 4.2: Bright field and corresponding WEFF microscopy images of HCASMCs seeded on the control waveguide (A and B) and LB films of 8-Phe-4-Sol (C and D), 8-Phe-8-Int (E and F) and 8-Phe-8-Lys(TFA)-4-Int (G and H). HCASMCs were cultured until 80% confluent, fixed and stained using DiI prior to imaging. Scale bars represent 200 µm.

Not only did the presence of serum not influence the number of attachment sites in the control waveguide, this behavior was also seen in all three PEA LB films as no statistical significance was observed among them (p > 0.05, Figure 4.3). In the S⁺ culture, 8-Phe-4-Sol exhibited 15.3 ± 6 adhesions per cell, which was high but not significantly different from the control waveguide. 8-Phe-8-Int exhibited 27.2 ± 9 adhesions per cell suggesting that the increased hydrophobicity imparted by the longer chain diol created a more favourable environment for cell attachment, potentially due to increased protein adsorption. However, due to the relatively high standard error associated with the measurements, these results were not statistically different. 8-Phe-8-Lys(TFA)-4-Int containing the lysine moiety exhibited a similar number (28.7 ± 8) of cell adhesions to 8-
Phe-8-Int, the same polymer lacking the incorporation of the lysine residues. While the presence of cationic lysine residues was expected to result in increased cell adhesion based on previous reports, the WEFF microscopy results suggest that the number of lysine moieties incorporated into these polymers may not have been sufficient to significantly increase the number of cell adhesion sites. However, the number of adhesion sites of the HCASMCs on 8-Phe-8-Lys(TFA)-4-Int in both S− and S+ culture was significantly higher than the control waveguides (p < 0.05). This result may indicate the cationic nature of the lysine residues of 8-Phe-8-Lys(TFA)-4-Int is engaged in non-specific electrostatic binding with the negatively charged glycocalyx layer on the cell surface in the absence of serum. In S+ culture, the cationic amine groups of 8-Phe-8-Lys(TFA)-4-Int may also bind electrostatically with cell adhesive proteins such as fibronectin or vitronectin, which carry net negative charges at physiologic conditions as their isoelectric points are 5.6-6.1 and 4.8-5.0 respectively, thereby also enhancing cell adhesion. Ultimately, the number of attachment sites observed in the comparative serum study, suggests that for all substrata, protein adsorption did not play a major role in the establishment of cell adhesions.

Figure 4.3: Average number of attachment sites per cell on control waveguides and on PEA LB films cultured in serum-containing (S+) or serum-free (S−) media. * denotes statistical significance (p < 0.05).
After evaluating the potential use of WEFF microscopy for determining the number of cell attachment sites on PEA LB films, we compared the data with vinculin immunostaining, which is a well-established method for quantifying focal adhesions. To achieve this, HCASMCs were cultured on solvent cast PEAs films. Initially, a time-course analysis was conducted using phase contrast microscopy to ensure HCASMC attachment and spreading on the solvent cast PEA films, prior to their immunostaining (Figure 4.4). In order to emulate the expected cell density on the waveguides after 3 or 4 days culture, cells were seeded at 15,000 cells/cm² on the solvent cast PEA films. Four hours after seeding, most cells adhered to the surface (including the glass coverslip control), but remained spherical in shape with little evidence of spreading (Figure 4.4A, D, G, J). However, after 8 hours, cell spreading was observed on all materials as judged by the flat morphology and larger contact areas occupied by individual cells (Figure 4.4B, E, H, K). Given that the surfaces were not coated with cell adhesive proteins, the observed spreading is likely attributed to the surface adsorption of serum proteins from the culture media. To confirm this, we cultured cells in the absence of serum and evaluated cell spreading. The data (Figure 4.5 and Figure 4.6) suggest that cell attachment and spreading on the PEAs is retarded when compared with those cultured in the presence of serum as cell spreading only became evident at 24 hours of culture, presumably due to cell-secreted adhesive proteins. Although this was not entirely unexpected, it suggests that these PEAs can support attachment of HCASMCs even in the absence of serum. Complete HCASMC spreading and their cell-cell contact formation is seen following 24 hours of S⁺ culture as presented by the confocal microscopy images (Figure 4.4C, F, I, L). It is noted that favourable cell-substratum adhesion is required for subsequent cellular responses such as proliferation and differentiation.
Figure 4.4: Phase contrast and confocal microscopy images of HCASMCs cultured on PEA films. Cells were seeded at 15,000 cells/cm² and cultured for up to 24 hours in serum-containing (S⁺) media. Phase contrast images were taken at 4 and 8 hours; whereas, the confocal microscopy images were taken after 24 hours of culture. HCASMC F-actin (green) and nuclei (blue) are shown in the confocal images (C, F, I, L). Phase contrast scale bar represents 200 µm, while confocal microscopy image scale bars represent 50 µm. Glass coverslips served as controls.
Figure 4.5: Phase contrast microscopy images of HCASMCs cultured on PEA films. Cells were seeded at 2,000 cells/cm² and cultured for up to 24 hours in serum-containing (S⁺) media. Scale bar represents 200 μm. Glass coverslips served as controls.

Given that HCASMCs were well-spread on all PEA films after 24 hours, we then examined whether these cells could form focal contacts also known as focal adhesions (FAs), which are specialized sites of adhesion between the cell and the substrate. In addition to anchoring cells, focal adhesion proteins are involved in integrin signaling that activates various intracellular signaling pathways that direct cell viability, proliferation and differentiation. Although there are many proteins involved in FAs, vinculin is the most studied and well understood actin-binding protein⁴,⁴³ therefore, we immunostained for vinculin and quantified the number of HCASMC focal contacts.
Figure 4.6: Phase contrast microscopy images of HCASMCs cultured on PEA films. Cells were seeded at 2,000 cells/cm² and cultured for up to 24 hours in serum-free (S⁻) media. Scale bar represents 200 μm. Glass coverslips served as controls.

As shown in Figure 4.7, HCASMCs expressed vinculin in both S⁻ and S⁺ culture media. However, the vinculin appeared to be concentrated at the cell periphery for the S⁻ cultures with the exception of the fibronectin-coated control cultures (Figure 4.7A, E, I, M). The rationale for using fibronectin was due to its cell adhesive RGD peptide motif, which served as a positive control. In the presence of serum, the vinculin was distributed uniformly throughout the cell body (Figure 4.7C, G, K, O). For the fibronectin-treated positive control cultures, the distribution of vinculin seems to be independent of serum (Figure 4.7Q, S). Because vinculin is localized on the cytoplasmic face of integrin-mediated cell–extracellular matrix junctions, HCASMCs cultured on these fibronectin coated controls appear to be making more focal contact areas than on the PEA surfaces.
Figure 4.7: Confocal microscopy images of vinculin immunostained HCASMCs on glass coverslips (A-D), PEA films (E-P) and FN-coated coverslips (Q-T) in the absence or presence of serum. Black and white images show vinculin immunostaining only, while fluorescence labeling of HCASMC F-actin (green), vinculin (red) and nuclei (blue) are all shown in the composite images. Scale bar represents 50 um.
In order to gain further insight into these cultures, the number of FAs per cell and per unit area of cell on the PEA films were determined (Figure 4.8A, B), where the number of focal contacts and cell area were both calculated using ImageJ software. In the S+ cultures, there was a significant increase in FAs in the fibronectin-coated control cultures compared with both 8-Phe-4-Sol and 8-Phe-8-Int, but not 8-Phe-8-Lys(TFA)-4-Int likely due to the scatter in data that introduced a relatively large error in this case. This scatter in the data is in part attributed to those cells fixed in the process of migration as they tend to form extensive close contacts, which under-express vinculin. This data, however, is consistent with the immunostaining findings, where HCASMCs formed more FAs with the fibronectin-coated controls (Figure 4.7S, T). Although the fibronectin-coated control in the S+ culture exhibited an increased number of focal adhesions, there was no statistical significance in the number of FAs per cell on all other materials tested regardless of culture conditions (p > 0.05). Similarly the number of focal adhesions per unit area on all the materials cultured in serum is significantly lower than the fibronectin coated controls (p < 0.05). However, fibronectin had no significant effect on the number of focal adhesions per unit area in the absence of serum (p > 0.05). In comparison to the WEFF data presented in Figure 4.3 where 8-Phe-8-Lys(TFA)-4-Int showed a statistically significant increase in the number of attachment sites per cell, the immunostaining data (Figure 4.8) did not show significance between the control and 8-Phe-8-Lys(TFA)-4-Int. This difference is attributed to the two different control surfaces used in the respective experiments. For reasons mentioned earlier, the WEFF control experiments were done on silver ion-exchanged glass slides needed to form a waveguide; whereas, the immunostaining experiments were conducted using conventional cell culture glass coverslips.

Overall, an average of 35–64 FAs per HCASMC cultured on PEA films calculated from vinculin immunostaining are consistent with the WEFF microscopy data. Although the WEFF microscopy data shown in Figure 4.3 (average attachment sites of 15–29 per cell) are slightly lower than the vinculin immunostaining data shown in Figure 4.8 (average focal adhesions of 35–64 per cell), there is no statistical significance among any of the PEAs tested in either serum-containing or serum-free culture media (p > 0.05), which may be the result of adhesive proteins present in the serum-free media facilitating cell
adhesion. In addition, these results compare favorably with literature data.\textsuperscript{45-48} For example, for fibroblasts, an average of 20–30 FAs per cell is reported,\textsuperscript{35-36} whereas, for myoblasts an average of 70 FAs per cell is reported.\textsuperscript{37,49} It also appears that FAs per cell is dependent on the culture substrates used where stiff surfaces had higher FAs per cell than flexible substrates. For example, fibroblasts cultured on graphene and carbon nanotubes had more than 100 FAs per cell;\textsuperscript{50} whereas, the same cells cultured on a standard culture dish had 20 FAs per cell while lung fibroblasts seeded on silicone rubber had 5 FAs per cell.\textsuperscript{45} Finally, a decrease in the FAs per cell from 20–5 is reported for HT-1080 cells cultured in 3D surfaces.\textsuperscript{47} In view of these reported values, our data on solvent cast PEA films are within the expected range. Furthermore, given that HCASMCs were making focal adhesions on these PEAs, it suggests these materials can promote integrin signaling, which is critical to cell migration and differentiation.

**Figure 4.8:** Number of focal adhesions per HCASMC (A) and per unit area (µm\textsuperscript{2}) (B) on the PEA films in serum-containing (S\textsuperscript{+}) and serum-free (S\textsuperscript{-}) media. The * denotes statistical significance (p < 0.05).

### 4.5 Conclusions

In this study, we investigated the adhesion of HCASMCs to PEA films using WEFF microscopy and compared it with a conventional vinculin immunostaining technique. The
WEFF microscopy work required the preparation of ultrathin films of the PEAs on waveguide surfaces by LB technology, an aspect that was challenging due to the hydrogen bonding capabilities of the PEAs resulting in their propensity to aggregate. Despite this challenge, 20 nm thick PEA films were successfully prepared. The WEFF results indicated that all PEAs deposited on the waveguides enhanced HCASMC adhesion compared with the uncoated control waveguides. However, due to the relatively high standard error in the WEFF data, only the cationic PEA was significantly higher than the control waveguide. The quantification of vinculin immunostaining supported the WEFF microscopy data, as there was no statistical difference in the number of focal adhesions per cell or per unit area among the PEAs tested regardless of culture conditions. Only the fibronectin-coated controls in serum-containing media exhibited a significantly higher number of focal adhesions per cell and per unit area. The presence of these focal adhesions implies that all these PEAs promote integrin-mediated signaling, a crucial step in cell migration and proliferation. Taken together, our results demonstrate the promise of PEA surfaces for the attachment of HCASMCs and suggest that a diverse range of PEAs can potentially be used for vascular tissue engineering applications.

4.6 References


5 Synthesis and Evaluation of a Biomimetic Aspartic Acid-Derived Poly(ester amide) for Vascular Tissue Engineering

This chapter examines the syntheses of functional PEAs containing L-aspartic acid to generate pendant carboxylic acid groups. The subsequent effect of these PEAs on HCASMC attachment, morphology, viability and focal adhesion formation was also examined. A base and functional PEA were electrospun into a three-dimensional fibrous scaffold and their subsequent impact on HCASMC morphology, infiltration and phenotype marker protein expression was analyzed. Finally, transforming growth factor-β1 was successfully conjugated to the surface of the functional PEA demonstrating the ability to attach signaling molecules that can direct cell function.

5.1 Abstract

Functionalization of polymeric biomaterials permits the conjugation of cell signaling molecules capable of directing cell function. Poly(ester amide)s (PEAs) containing pendant carboxylic acid groups were successfully synthesized by incorporating L-aspartic acid into the PEA backbone through an interfacial polycondensation approach. Human coronary artery smooth muscle cell (HCASMC) attachment, spreading and proliferation was observed on two-dimensional PEA films. Vinculin expression at the cell periphery suggested the HCASMCs formed focal adhesions on the functional PEAs, while the absence of smooth muscle α-actin expression implied the cells adopted a proliferative phenotype. The PEAs were further electrospun to yield nano-scale three-dimensional scaffolds with average fiber diameters ranging from 130–294 nm. Immunoblotting studies revealed an increase in smooth muscle α-actin and calponin expression from HCASMCs cultured on 3D scaffolds when compared to 2D films. X-ray photoelectron spectroscopy and immunofluorescence demonstrated the conjugation of transforming growth factor-β1 (TGF-β1) to the surface of the functional PEA through the pendant carboxylic acid groups. Taken together, this study demonstrates that the aspartic acid containing PEAs are viable biomaterials for vascular tissue engineering.
5.2 Introduction

Successful tissue engineering strategies rely on the design of appropriate biomaterials that mimic the natural extracellular matrix. Cells seeded on a degradable 3D scaffold can either be implanted directly to a host or matured in a bioreactor prior to implantation. As part of the biomaterials toolbox, the use of functionalized materials that can modulate cell function is attractive. Degradable, functional polymers that permit the immobilization of growth factors or other signaling molecules may facilitate the development of new biomimetic biomaterials. Functional polymers with well-defined surface chemistry can be achieved through the introduction of tri-functional monomers during polymerization. Although synthetic monomers have been used in these co-polymerizations, significant research has focused on the incorporation of functional α-amino acids, yielding a variety of pendant functional groups. The co-polymerization of functional α-amino acids provides polymers with the combined properties of synthetic polymers and naturally occurring polypeptides. Poly(ester amide)s (PEAs), a class of biodegradable polymers consisting of ester and amide linkages along the polymer backbone, can be synthesized from naturally occurring α-amino acids. PEAs derived from α-amino acids have been investigated for drug delivery, non-viral gene delivery, stimuli-induced degradation, coatings for drug-eluting stents and vascular tissue engineering.

The most appropriate amino acid choices for introducing functional handles for subsequent conjugation of biomolecules are L-lysine, L-glutamic acid and L-aspartic acid. The incorporation of L-lysine into functional PEAs has been previously reported, to which model compounds N-acetyl L-valine and 2-[2-(2-methoxyethoxy)ethoxy]acetic acid were conjugated, while also promoting vascular smooth muscle cell attachment, proliferation and focal adhesion formation. The introduction of L-aspartic acid into the PEA is complementary and will present a negative charge to cells owing to the pendant carboxylate at neutral pH, in comparison to the cationically charged pendant amine groups in the L-lysine functional PEA. However, data on the synthesis of suitable
L-aspartic acid containing PEA biomaterials is limited. For instance, water-soluble polydepsipeptides incorporating aspartic acid were synthesised by ring opening polymerization of cyclodepsipeptides and tin 2-ethylhexanoate catalyst at elevated temperatures, which produced low molecular weight polymers with little practical utility. More recently, Li and co-workers have synthesized hyperbranched PEAs from α-amino acids including L-aspartic acid at elevated temperatures in the presence of catalyst after converting the functional groups to a methyl ester limiting the availability of the pendant functionality. The incorporation of L-aspartic acid into PEAs comprising amino acids, dicarboxylic acids and diols through a homogeneous polycondensation has also been previously reported. However, the solution polymerization approach provided relatively low molecular weight polymers not suitable for biomaterials. It should be reiterated that polymeric biomaterials designed for tissue engineering applications must have sufficiently high molecular weight to be processed into films and scaffolds to have the necessary initial mechanical and dimensional stability for use in a cell culture environment.

Although there are several strategies to fabricate or regenerate vascular tissues, scaffold-guided tissue engineering remains the most frequently encountered approach. Thus far, PEAs containing α-amino acids have been demonstrated to support vascular smooth muscle cells (VSMC) attachment, proliferation and focal adhesions. In vascular tissue engineering, transforming growth factor β1 (TGF-β1) is a target biomolecule as it has been reported to modulate smooth muscle cell proliferation, differentiation and up-regulation of the quiescent contractile phenotype. The ability to deliver TGF-β1 easily and locally from a biodegradable PEA scaffold could aid in vascular tissue regeneration. The immobilization of TGF-β1 within a degradable scaffold may also promote prolonged signaling beneficial in elastogenesis and adoption of quiescent smooth muscle cells following controlled release of the cytokine. As the functional PEAs containing L-aspartic acid are anticipated to support VSMC adhesion and proliferation, TGF-β1 may further promote extracellular matrix production and the contractile VSMC phenotype. In view of the above, a synergistic effect in the development of a tissue-engineered vascular graft was sought.
Several key developments in the application of PEAs for vascular tissue engineering are herein described. PEAs containing l-aspartic acid were synthesized, characterized and evaluated through the in vitro culture of HCASMCs. Nano-scale electrospun fibrous mats were prepared from PEAs and the effect of PEA fibrous topography and exogenous TGF-β1 on contractile phenotype marker protein expression of smooth muscle α-actin (SMαA) and calponin by HCASMCs were investigated. Finally, TGF-β1 was successfully conjugated onto the surface of PEAs containing functional l-aspartic acid.

5.3 Materials and Methods

5.3.1 Materials

Monomers 2 and 3 and bis-l-aspartic acid-β-(tert-butyl ester) diester 7 were prepared as previously reported (Chart 5.1). Solvents were purchased from Caledon Labs (Georgetown, ON). All other chemicals were purchased from Sigma Aldrich (Milwaukee, WI). Unless noted otherwise, all chemicals were used as received. DCM was distilled from CaH2 and dried over molecular sieves 4A. Flash chromatography was performed using silica gel 60 with a particle size range of 40-63 µm (SiliCycle Inc, Quebec City, QC). Dialysis was performed against DMF with Spectra/Por 6 dialysis tubing (Spectrum Laboratories, Inc., Rancho Dominguez, CA), molecular weight cutoff of 25 kDa.

![Chart 5.1: Structures of monomers 2, 3 and 7.](image)

5.3.2 Methods

1H (400 MHz) NMR spectra were obtained on a Varian Inova 400 spectrometer (Varian Canada Inc., Mississauga, ON). Chemical shifts are reported in parts per million (ppm)
and are calibrated against residual solvent signals of chloroform (CDCl₃, δ 7.27 ppm) or dimethyl sulfoxide (DMSO-d₆, δ 2.50 ppm). All coupling constants (J) are reported in Hertz (Hz). FTIR spectra were obtained using a Bruker Tensor 27 (Bruker Corporation, Milton, ON) from KBr disks. Absorption frequencies of the functional groups are reported in wavenumbers (cm⁻¹) and assigned as published by Pavia et al.²⁷ GPC data were obtained using a Waters 2695 Separations Module equipped with a Waters 2414 Refractive Index Detector (Waters Limited, Mississauga, ON) and two PLgel 5 µm mixed-D (300 mm x 7.5 mm) columns connected in series (Varian Canada Inc., Mississauga, ON). Samples (5 mg/mL) dissolved in the eluent, which comprised of 10 mM LiBr and 1 % (v/v) NEt₃ in DMF at 85°C were injected (100 µL) at a flow rate of 1 mL/min and calibrated against polystyrene standards. Molecular weights are reported in kilograms/mole (kg/mol). TGA was performed on a SDT Q600 (TA Instruments – Waters LLC, New Castle, DE) under dry nitrogen at a heating rate of 20°C/min up to 600°C. DSC was performed on a DSC Q20 (TA Instruments – Waters LLC, New Castle, DE) at a heating rate of 10°C/min from -50 to 200°C. All samples prepared ranged from 2 to 5 mg, and glass transition temperatures (Tgs) were obtained from the second heating cycle.

5.3.3 PEA Nomenclature

The polymers are labeled by the number of methylene groups contributed by the diacid, the three letter amino acid designation, and finally by the number of methylene groups in the diol – for example 8-Phe-8. Asp(O-t-Bu) represents the incorporation of the aspartic acid monomer 7 as a co-monomer at a mole ratio of 10% relative to the di-p-toluenesulfonic acid salt monomer 2 or 3 - for example 8-Phe-8-Asp(O-t-Bu)-4. The 4 represents the number of methylene groups in the butanediol moiety of 7.

5.3.4 General Procedure for Interfacial Polymerization of the Functional Poly(ester amide)s

The di-p-toluenesulfonic acid salt monomer 2 or 3 (0.9 equiv.) and sodium carbonate (2.0 equiv.) were dissolved in distilled water (30 mL). Bis-L-aspartic acid β-(t-butyl ester) diester, 7 (0.1 equiv.) was dissolved in DCM (15 mL) and added to the aqueous phase
and allowed to mix for 30 min. Sebacoyl chloride (1.0 equiv.) diluted in anhydrous DCM (15 mL), was added dropwise over 20 min to the biphasic solution and was allowed to react for 24 h. Upon completion of the reaction, solvent was removed in vacuo. The functional PEA was dissolved in DMF permitting filtration of the insoluble salts. The filtrate was then dialyzed against DMF for at least 8 h twice. The solvent was again removed in vacuo, reconstituted in chloroform (5 mL) and precipitated in cold ethyl acetate (50 mL) and finally collected and dried in vacuo.

5.3.4.1 8-Phe-4-Asp(O-t-Bu)-4

This polymer was prepared by the general procedure described above using monomers 2 (3.5 g, 4.9 mmol), 7 (0.23 g, 0.54 mmol) and sebacoyl chloride (1.2 mL, 5.4 mmol).

Yield: 1.38 g, 46%. 1H NMR (400 MHz, CDCl3): δ 7.31-7.09 (m, 9H, Ph), 6.51 (d, 0.2H, J = 8.2, -C(O)-NH-CαH-CH2-C(O)O-t-Bu), 6.01 (d, 1.8H, J = 7.6, -C(O)-NH-CαH-CH2-Ph), 4.91-4.84 (m, 1.8H, -CαH-CH2-Ph), 4.83-4.77 (m, 0.2H, -CαH-CH2-C(O)O-t-Bu), 4.21-3.99 (m, 4H, -C(O)O-CH2-), 3.16-3.04 (m, 3.6H, -CαH-CH2-Ph), 2.91 (dd, 0.2H, J1 = 17.3, J2 = 4.4, -CαH-CHaHb-C(O)O-t-Bu), 2.76 (dd, 0.2H, J1 = 17.0, J2 = 4.7, -CαH-CHaHb-C(O)O-t-Bu), 2.25-2.11 (m, 4H, -CH2-C(O)-NH-CαH-CH2-C(O)O-t-Bu, -CH2-C(O)-NH-CαH-CH2-Ph), 1.74-1.51 (m, 8H, -CαH-CHaHb-C(O)O-t-Bu), 1.43 (br s, 1.8H, t-Bu CH3), 1.36-1.20 (m, 8H, -(CH2)4-CH2-CH2-C(O)-NH-). FTIR (KBr pellet, cm⁻¹): 3307 (N-H stretch, amide), 3064 (C-H stretch, aromatic), 3030 (C-H stretch, aromatic), 2931 (C-H stretch, aliphatic), 2856 (C-H stretch, aliphatic), 1744 (C=O stretch, ester), 1647 (C=O stretch, amide I), 1543 (N-H bend, C-N stretch, amide II), 1498 (C=C stretch, aromatic), 1455 (CH2, wagging vibration) 1180 (C-O stretch, ester).

GPC: Mₙ = 34.7 kg/mol, Mₙ = 74.4 kg/mol, PDI = 2.15. DSC: Tg = 36.5°C. TGA: Td = 217°C.

5.3.4.2 8-Phe-8-Asp(O-t-Bu)-4

This polymer was prepared by the general procedure described above using monomers 3 (1.6 g, 2.0 mmol), 7 (0.095 g, 0.22 mmol) and sebacoyl chloride (0.47 mL, 2.2 mmol).

Yield: 0.62 g, 46%. 1H NMR (400 MHz, CDCl3): δ 7.32-7.08 (m, 9H, Ph), 6.50 (d, 0.2H, J = 8.6, -C(O)-NH-CαH-CH2-C(O)O-t-Bu), 5.96 (d, 1.8H, J = 8.2, -C(O)-NH-CαH-CH2-
Ph), 4.93-4.85 (m, 1.8H, -C₆H₄-CH₂-Ph), 4.83-4.77 (m, 0.2H, -C₆H₄-CH₂-C(O)-O-t-Bu),
4.22-4.03 (m, 4H, -C(O)-CH₂-C(H₂)₄-, -CH₂-CH₂-C(O)-NH-), 3.18-3.05 (m, 3.6H, -C₆H₄-CH₂-Ph), 2.91 (dd, 0.2H, J₁ = 17.0, J₂ = 4.5, -C₆H₄-CH₂-Hb-C(O)-O-t-Bu), 2.76 (dd, 0.2H, J₁ = 17.2, J₂ = 4.7, -C₆H₄-CH₂-Hb-C(O)-O-t-Bu), 2.25-2.10 (m, 4H, -CH₂-C(O)-NH-C₆H₄-CH₂-C(O)-O-t-Bu, -CH₂-C(O)-NH-C₆H₄-CH₂-Ph), 1.74-1.50 (m, 8H, -C(O)-CH₂-CH₂-(CH₂)₄-, -(CH₂)₄-CH₂-CH₂-C(O)-NH-), 1.46-1.41 (m, 1.8H, t-Bu CH₃), 1.37-1.20 (m, 15.2H, -C(O)-CH₂-CH₂-(CH₂)₄-, -(CH₂)₄-CH₂-CH₂-C(O)-NH-). FTIR (KBr pellet, cm⁻¹): 3307 (N-H stretch, amide), 3064 (C-H stretch, aromatic), 3030 (C-H stretch, aromatic), 2929 (C-H stretch, aliphatic), 2853 (C-H stretch, aliphatic), 1737 (C=O stretch, ester), 1649 (C=O stretch, amide I), 1542 (N-H bend, C-N stretch, amide II), 1498 (C=C stretch, aromatic), 1455 (CH₂, wagging vibration) 1181 (C=O stretch, ester). GPC: Mₙ = 30.8 kg/mol, Mₘ = 62.4 kg/mol, PDI = 2.02. DSC: T₉ = 21.8°C. TGA: Tₐ = 253°C.

5.3.5 General Deprotection Procedure

Each t-Bu-protected functional PEA (0.1–0.2 g) was dissolved in 1:1 trifluoroacetic acid (TFA):CH₂Cl₂ (2 mL) and reacted for 2 h. The solvents were subsequently removed and the desired product was precipitated in cold ethyl acetate (50 mL) from chloroform (5 mL). The precipitate was collected and dried in vacuo, yielding the functional PEA with pendant carboxylic acid groups.

5.3.5.1 8-Phe-4-Asp(OH)-4

This polymer was prepared by the general deprotection procedure described above. Yield: 0.057 g, 46%. ¹H NMR (400 MHz, DMSO-d₆): δ 8.15 (d, 1.8H, J = 8.2, -C(O)-NH-C₆H₄-CH₂-Ph), 7.98 (d, 0.2H, J = 7.6, -C(O)-NH-C₆H₄-CH₂-C(O)OH), 7.40-7.04 (m, 9H, Ph), 4.60-4.53 (m, 0.2H, -C₆H₄-CH₂-C(O)OH), 4.52-4.42 (m, 1.8H, -C₆H₄-CH₂-Ph), 4.11-3.89 (m, 4H, -C(O)-O-CH₂-Ph), 3.05-2.84 (m, 3.6H, -C₆H₄-CH₂-Ph), 2.70-2.55 (m, 0.4H, -C₆H₄-CH₂-C(O)OH), 2.12-1.97 (m, 4H, -CH₂-C(O)-NH-C₆H₄-CH₂-C(O)OH, -CH₂-C(O)-NH-C₆H₄-CH₂-Ph), 1.62-1.31 (m, 8H, -C(O)-O-CH₂-CH₂-, -CH₂-CH₂-C(O)-NH-), 1.26-1.05 (m, 8H, -(CH₂)₄-CH₂-CH₂-C(O)-NH-). FTIR (KBr pellet, cm⁻¹): 3313 (N-H stretch, amide), 3064 (C-H stretch, aromatic), 3030 (C-H stretch, aromatic), 2928 (C-H stretch, aliphatic), 2855 (C-H stretch, aliphatic), 1742 (C=O stretch, ester), 1640 (C=O
stretch, amide I), 1542 (N-H bend, C-N stretch, amide II), 1498 (C=C stretch, aromatic), 1455 (CH₂, wagging vibration) 1180 (C-O stretch, ester). DSC: T<sub>g</sub> = 39.8°C. TGA: T<sub>d</sub> = 282°C.

5.3.5.2 8-Phe-8-Asp(OH)-4

This polymer was prepared by the general deprotection procedure described above. Yield: 0.10 g, 56%. <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>): δ 8.14 (d, 1.8H, J = 7.6, -C(O)-NH-<br>\(\text{C}_\text{H}-\text{CH}_2-\text{Ph}\), 8.08-8.02 (m, 0.2H, -C(O)-NH-C<sub>6</sub>H-CH₂-C(O)OH), 7.40-7.03 (m, 9H, Ph), 4.60-4.52 (br m, 0.2H, -C<sub>6</sub>H-CH₂-C(O)OH), 4.52-4.40 (m, 1.8H, -C<sub>6</sub>H-CH₂-Ph), 4.12-3.89 (m, 4H, -C(O)O-CH₂-), 3.05-2.82 (m, 3.6H, -C<sub>6</sub>H-CH₂-Ph), 2.60-2.47 (m, 0.4H, -C<sub>6</sub>H-CH₂-CH₂-C(O)OH), 2.14-1.96 (m, 4H, -CH₂-C(O)-NH-C<sub>6</sub>H-CH₂-C(O)OH, -CH₂-C(O)-NH-C<sub>6</sub>H-CH₂-Ph), 1.62-1.33 (m, 8H, -C(O)O-CH₂-CH₂-, -CH₂-CH₂-C(O)-NH-), 1.33-1.03 (m, 15.2H, -C(O)O-CH₂-CH₂-(CH₂)₄-, -(CH₂)₄-CH₂-CH₂-C(O)-NH-). FTIR (KBr pellet, cm<sup>-1</sup>): 3308 (N-H stretch, amide), 3064 (C-H stretch, aromatic), 3030 (C-H stretch, aromatic), 2928 (C-H stretch, aliphatic), 2854 (C-H stretch, aliphatic), 1740 (C=O stretch, ester), 1651 (C=O stretch, amide I), 1541 (N-H bend, C-N stretch, amide II), 1497 (C=C stretch, aromatic), 1455 (CH₂, wagging vibration) 1179 (C-O stretch, ester). DSC: T<sub>g</sub> = 26.3°C. TGA: T<sub>d</sub> = 241°C.

5.3.6 Electrospinning

Three-dimensional fibrous PEA mats were achieved by preparing various concentrations of PEA solutions in a co-solvent mixture of chloroform (CHCl₃):dimethyl sulfoxide (DMSO) or DMF. The viscous solution was transferred to a glass syringe (0.5 mL) equipped with a stainless steel 22 gauge needle, which was connected to a high voltage (20 kV) supply. The flowrate was maintained at 0.1 mL/h with a syringe pump. The fibers were collected on aluminum foil on either a static collector or on a rotating mandrel (1000 rpm) 8 cm from the needle tip.

5.3.7 Scanning Electron Microscopy (SEM)

The electrospun fiber mats were visualized using scanning electron microscopy (S-2600 or 3400S, Hitachi, Ltd., Tokyo, Japan). The mats were mounted on carbon-taped
aluminum stubs and sputtered with gold (Quorum Emitech K550X, Quorum Technologies Ltd, Kent, UK) or a gold-palladium mixture (HummerVI Sputter Coater, Anatech USA, Union City, CA) at a current of 15 mA for 90 or 300 sec, respectively. Secondary electron detection was achieved at voltages between 5 and 20 kV at various magnifications. From these micrographs, fiber diameters were measured using ImageJ software (NIH, Bethesda, MD).

5.3.8 Two-Dimensional Cell Culture and Immunofluorescence Microscopy

Two-dimensional polymer films were obtained by twice dip-coating 12 mm diameter glass coverslips into 1% (wt) solutions of the PEAs in DMF and were dried at 60°C under reduced pressure overnight. One glass coverslip was placed in each well of a 24-well culture plate. The films were immersed in 70% ethanol for 30 min and then exposed to UV irradiation for 1 h, prior to conditioning overnight in Hank’s Balanced Salt Solution (HBSS; Invitrogen Canada Inc., Burlington, ON). Natural, human fibronectin (2 µg/cm²; Santa Cruz Biotechnology Inc., Santa Cruz, CA) in HBSS was adsorbed to glass coverslips for 1 h and the resulting surfaces served as positive controls. Human coronary artery smooth muscle cells (HCASMCs; Lonza Walkersville Inc., Walkersville, MD) at a density of 2,000 cells/cm² were seeded directly on the surface of the PEA films in 24-well culture plates and were cultured for 1 and 7 days before fixation and immunostaining.

Human coronary artery smooth muscle cells were grown in smooth muscle cell basal medium (Medium 231; Life Technologies Inc., Burlington, ON) supplemented with smooth muscle growth supplement (SMGS, 25 mL, 20X; Life Technologies Inc., Burlington, ON) and 10,000 Units/mL Penicillin/Streptomycin (5 mL, 100X, Life Technologies Inc., Burlington, ON). HCASMCs were used between passages 5 and 9. Cultures were maintained at 37°C in a humidified incubator containing 5% CO₂.

For immunostaining studies, cells were washed with pre-warmed PBS immediately prior to fixing at ambient temperature for 15 min in 4% formaldehyde solution (EMD Chemicals, Gibbstown, NJ) in divalent cation-free PBS. HCASMCs were then
permeabilized with 0.1% Triton X-100 (VWR International, Mississauga, ON) for 5 min followed by their incubation with 1% bovine serum albumin (BSA) (Sigma-Aldrich, Oakville, ON) for 30 min. Primary antibody incubation with either mouse monoclonal anti-vinculin (1:50 dilution, SPM227; Abcam, Cambridge, MA) or monoclonal anti-α-actin (1:50 dilution, clone 1A4; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was conducted for 1 h prior to the secondary antibody incubation in the dark for 1 h at ambient temperature with Alexa Fluor® 488-conjugated goat anti-mouse IgG (1:300 dilution; Invitrogen Canada Inc., Burlington, ON). The HCASMCs were further incubated in the dark at ambient temperature with Alexa Fluor® 568-conjugated phalloidin (1:50 dilution; Life Technologies Inc., Burlington, ON) for 20 min in a 1% BSA/PBS solution. The HCASMCs were then counterstained with 4′-6-diamidino-2-phenylindole dihydrochloride (DAPI, 300 nM in PBS; Life Technologies Inc., Burlington, ON) for 10 min to label the nuclei. Coverslips were mounted on microscope slides with ProLong® Antifade Kit (Life Technologies Inc., Burlington, ON) and sealed with nail enamel upon drying. Samples were analyzed with a Zeiss LSM 5 Duo confocal microscope with 9 laser lines and appropriate filters (Carl Zeiss Canada Ltd., Toronto, ON).

5.3.9 Cell Toxicity and Proliferation Studies Using MTT Assays

Colorimetric assays of the metabolic activity of viable cells were used to quantify cell toxicity and proliferation and were performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Vybrant® MTT Cell Proliferation Assay Kit, Invitrogen Canada Inc., Burlington, ON). At predetermined time points, fresh culture media was added (100 µL) followed by MTT (10 µL, Component A) and incubated for 4 h. Sodium dodecyl sulfate (SDS, 100 µL, Component B) was then added and thoroughly mixed and incubated for a further 18 h to solubilize the formazan, which was quantified using a Biochrom Asys UVM340 Microplate Reader (Biochrom Ltd., Cambridge, U.K.) at 570 nm (maximum absorbance).
### 5.3.10 Three-Dimensional Cell Culture, Scanning Electron and Laser Scanning Confocal Microscopy

Three-dimensional polymer scaffolds were cut from electrospun fibrous mats. The scaffolds were also immersed in 70% ethanol for 30 min and then exposed to UV irradiation for 1 h, prior to conditioning overnight in HBSS. HCASMCs were seeded directly on the surface of the PEA scaffolds, at a density of 30,000 cells/scaffold and cultured up to 4 days prior to fixation with formaldehyde solution for 15 min. HCASMC morphology was preserved prior to SEM analysis by washing the cells with PBS and deionized water three times each prior to sequential ethanol dehydration. Samples were dehydrated in an increasing series of ethanol concentrations (10, 30, 50, 70, 90, 95 and 100%) for 5 min each followed by drying with an increasing series of hexamethyldisilazane (HMDS, Sigma-Aldrich, Oakville, ON) concentrations in ethanol (50, 75 and 100%) for 10 min each. Finally, the cells were air dried prior to sputtering and imaging. The three-dimensional Z-stacks of the confocal microscopy images were obtained in 2–5 μm slices. Despite not immunostaining, images were also collected in the green channel to obtain a background contribution from the polymer to be subtracted from the autofluorescence observed in the other channels. The image subtraction was conducted in Image-Pro Plus software (Media Cybernetics, Inc., Rockville, MD). The resulting 2D images were then re-assembled into a composite 3D image using Imaris software (Bitplane Inc., South Windsor, CT).

### 5.3.11 Western Blot Analysis

For human coronary artery smooth muscle cell phenotype marker protein analysis via Western blot, HCASMCs were seeded at a density of 2.5 to 5.0 x 10^5 cells/film or scaffold. Protein lysates obtained from a radioimmunoprecipitation assay (RIPA, 50–100 μL) buffer (bicinconinic acid (BCA) protein assay compatible) supplemented with ethylenediaminetetraacetic acid, disodium salt dihydrate (EDTA, 5 mM), phenylmethylsulfonyl fluoride (PMSF, 1 mM) and a protease inhibitor mixture (1X, Roche Complete Mini EDTA-Free Protease Inhibitor Cocktail, Cedarlane Labs, Burlington, ON) were quantified with Pierce BCA Protein Assay (Thermo Fisher Scientific Inc., Rockford, IL). The protein lysates were separated by sodium dodecyl
sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12% bis/acrylamide gel, 160 V, 1 h) using Towbin’s buffer supplemented with 0.1% SDS. The mini-gel was subsequently wet transferred to a nitrocellulose blotting membrane (BioTrace™ NT, Pall Corporation, Pensacola, FL) in a cooled chamber at 70 V for 2 h in Towbin’s buffer supplemented with 0.02% SDS and 20% (v/v) methanol. The transfer efficiency was confirmed by gel staining with Coomassie Brilliant Blue (R-250) solution (Bio-Rad Laboratories (Canada, Ltd., Mississauga, ON) and reversible Ponceau S staining of the nitrocellulose membrane. For immunoblotting of calponin, α-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), blocking was achieved with 5% nonfat dry milk (Blotting Grade Blocker, Bio-Rad Laboratories (Canada) Ltd., Mississauga, ON) in Tris-buffered saline-Tween-20 (TBST, Tris.HCl: 20 mM, pH 7.6; NaCl: 137 mM; Tween-20: 0.1%) for 1 h, followed by incubation overnight at 4°C with either rabbit polyclonal calponin 1/2/3 antibody (1:250 dilution, FL-297; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse monoclonal anti-α-actin, (1:250 dilution, clone 1A4; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or mouse monoclonal anti-GAPDH (1:1000 dilution, clone 6C5, EMD Millipore Corporation, Temecula, CA) in 5% nonfat dry milk in TBST. Membranes were then washed with TBST (3 x 5 min) and incubated for 1 h in a horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000 dilution; Thermo Fisher Scientific, Rockford, IL) in TBST. Membranes were then washed and then incubated for 5 min in SuperSignal® West Pico Chemiluminescent substrate (Thermo Fisher Scientific, Rockford, IL) and examined with a Molecular Imager® ChemiDoc™ XRS+ system (Bio-Rad Laboratories (Canada) Ltd., Mississauga, ON). Re-probing of the nitrocellulose membrane was achieved following stripping with mild stripping buffer (10 mL, 200 mM glycine, 0.1% SDS, 1% Tween-20, pH 2.2) under gentle agitation at 37°C for 30 min.

5.3.12 Transforming Growth Factor-β1 Conjugation

Protein carrier-free human platelet-derived TGF-β1 (R&D Systems Inc., Minneapolis, MN) reconstituted in 4 mM HCl was conjugated to the surface of 8-Phe-4-Asp(OH)-4 electrospun mats using a conventional peptide conjugation technique. The electrospun mat was immersed in 4-morpholineethanesulfonic acid (MES, Sigma-Aldrich, Oakville,
ON) buffer (0.1 M, pH 5.0, 4 mL) for 15 min, which contained N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide (EDC, 2 mM; Sigma-Aldrich, Oakville, ON) and N-hydroxysulfosuccinimide sodium salt (sulfo-NHS, 5 mM; VWR International, Mississauga, ON) to activate the pendant carboxylic acid groups of the 8-Phe-4-Asp(OH)-4. After 15 min, the activation with EDC was halted with the addition of 2-mercaptoethanol (2.8 µL, 10 mM; Caledon Laboratories Ltd., Georgetown, ON) for 5 min. The MES buffer was removed and replaced by phosphate-buffered saline (10 mM, pH 8.5, 4 mL) before the reconstituted TGF-β1 was added to provide a final concentration of 3 ng/mL and allowed to react for 2 h. The conjugated mats were washed three times with 1X PBS (pH 7.4) and then Milli-Q (18.2 MΩ-cm) water for 5 min each. Adsorbed samples served as controls, where the activation step with EDC and sulfo-NHS was omitted. For X-ray photoelectron spectroscopy (XPS) analysis, conjugated and adsorbed mats were dried overnight under reduced pressure at room temperature. For laser scanning confocal microscopy images, the conjugated and adsorbed samples were blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich, Oakville, ON) in 1X PBS for 1 h, followed by primary antibody incubation overnight at 4°C with rabbit TGF-β receptor 1 antibody (1:50 dilution, 250 µL; Cell Signaling Technology, Danvers, MA) in 1% BSA in 1X PBS. Secondary antibody incubation was conducted in the dark for 1 h at ambient temperature with Alexa Fluor® 488-conjugated donkey anti-rabbit IgG (1:300 dilution; Invitrogen Canada Inc., Burlington, ON) prior to three washes with 1X PBS for 5 min each. Finally, the samples were mounted with ProLong Gold (Invitrogen Canada Inc., Burlington, ON), dried overnight and then sealed with nail enamel. Samples were analyzed with a Zeiss LSM 5 Duo confocal microscope with nine laser lines and appropriate filters (Carl Zeiss Canada, Toronto, ON).

5.3.13 X-Ray Photoelectron Spectroscopy

XPS data were obtained using a Kratos Axis Ultra spectrometer (Kratos Analytical Ltd, UK, Manchester, UK) with integrated Magnetic Immersion Lens and Charge Neutralization System with Spherical Mirror Analyser using a monochromatic Al Kα X-ray source (15 mA, 14 kV). The Kratos Charge Neutralization System was used on all samples, which were analyzed to a depth of 5-7 nm, with detection limits ranging from
0.1 to 0.5 atomic percent. Survey scan and high resolution analyses were carried out on an analysis area of 300–700 µm at a pass energy of 160 and 20 eV, respectively. Spectra have been charge corrected to the main line of the C 1s spectrum (adventitious carbon) set to 284.8 eV. X-ray photoelectron spectra were analyzed using CasaXPS version 2.3.14 software.

5.3.14 Statistical Analysis

MTT assay and Western blot data are given as the mean ± standard error for experiments conducted in triplicate. Differences between groups were compared using a two-way ANOVA analysis with a Bonferroni post-test. The average fiber diameter data is also provided as the mean ± standard error, calculated from 30 randomly selected fibers from each of three separate SEM images. Differences between groups were compared with a one-way ANOVA analysis with a Bonferroni post-test. All statistical analyses were conducted with GraphPad Prism, where values of p < 0.05 were considered statistically significant.

5.4 Results and Discussion

5.4.1 Interfacial Polymerization of L-Aspartic Acid Containing Poly(ester amide)s

Functional poly(ester amide)s containing L-aspartic acid were incorporated into both 8-Phe-4 and 8-Phe-8 to provide pendant carboxylic acid groups to complement the previously reported pendant amine functional PEAs. The incorporation of complementary functional groups facilitates their conjugation to biomolecules using conventional peptide coupling approaches. The functional PEAs were synthesized via the interfacial polymerization approach previously developed, where the β-carboxylate group remained tert-butyl protected during the polycondensation. Following the polymerization, the protecting groups were cleaved using TFA. Confirmation of the incorporation of aspartic acid into these PEAs was achieved using $^1$H NMR spectroscopy, as shown in Figure 5.1 and Figure 5.2. Due to the low abundance of aspartic acid moieties, $^{13}$C NMR spectroscopy was not sensitive enough for their detection. In Figure 5.1, two sets of peaks clearly illustrate the presence of the aspartic acid. The small peaks
at 4.5 and 2.6 ppm represent the protons on the α- and β-carbons respectively, denoted as 15 and 16 in the protected PEA and 20 and 21 in deprotected 8-Phe-4-based functional PEAs. These peaks are also visible in the 1H NMR spectra of the 8-Phe-8 functional PEAs shown in Figure 5.2, although more overlapped by the DMSO peak at 2.5 ppm.

**Figure 5.1:** Overlayed 1H NMR spectra of 8-Phe-4-Asp(O-t-Bu)-4 and 8-Phe-4-Asp(OH)-4 functional PEAs. Note that the unlabeled peaks above correspond to the peaks labeled in Figure 3.2.
Figure 5.2: Overlayed $^1$H NMR spectra of 8-Phe-8-Asp(O-t-Bu)-4 and 8-Phe-8-Asp(OH)-4 functional PEAs. Note that the unlabeled peaks above correspond to the peaks labeled in Figure 3.3.

Upon successful syntheses of the PEAs containing aspartic acid, their thermal properties were studied via both thermogravimetric analyses (TGA, Figure 5.3 and Figure 5.4) and differential scanning calorimetry (DSC, Figure 5.5 and Figure 5.6); the data is provided in Table 5.1. As shown in Figure 5.3 and Figure 5.4, the inclusion of the aspartic acid induced a decrease in the onset of decomposition. In the case of the 8-Phe-4-Asp(O-t-Bu)-4, a 1% mass loss was observed at 217°C, yet the deprotected 8-Phe-4-Asp(OH)-4 demonstrated an onset of degradation at 282°C, suggesting the initial mass loss was the result of thermolysis of the tert-butyl groups.28 In the 8-Phe-8-Asp(O-t-Bu)-4 case, a significant reduction in the onset of degradation was also observed, again due to loss of the tert-butyl protecting group. However, in comparing the two deprotected PEAs, 8-Phe-8-Asp(OH)-4 resulted in a lower decomposition temperature (241°C) than the corresponding 8-Phe-4-Asp(OH)-4 (282°C), which was attributed to decreased hydrogen bonding between polymer chains due to the increased chain length of the diol.
Figure 5.3: Overlayed TGA thermographs of aspartic acid containing 8-Phe-4-based PEA.

Figure 5.4: Overlayed TGA thermographs of aspartic acid containing 8-Phe-8-based PEA.
The incorporation of the protected functional groups also resulted in a decrease in the glass transition temperature, $T_g$ (Figure 5.5 and Figure 5.6), which was attributed to an increase in free volume associated with the pendant tert-butyl groups. The inclusion of the Asp(O-t-Bu)-4 monomer into 8-Phe-4 resulted in a decrease in $T_g$ from 38.8°C to 36.5°C. The deprotection of the tert-butyl group then resulted in a subsequent increase in the $T_g$ to 39.8°C. Similar behaviour was observed with the 8-Phe-8, where the $T_g$ decreased to 21.8°C from 24.5°C with 10 mol% Asp(O-t-Bu)-4. Subsequent cleavage of the protecting group again resulted in an increase in the $T_g$ to 26.3°C. Both polymers exhibited increased glass transition temperatures such that it was greater than the base polymer suggesting improved hydrogen bonding between polymer chains with the free carboxylic acid groups.

![Figure 5.5: Overlayed DSC traces of aspartic acid containing 8-Phe-4-based PEAs.](image)
As seen in Figure 5.7 and Figure 5.8, the incorporation of the protected aspartic acid monomer delayed the elution of the polymer chains indicative of lower molecular weight polymers for both 8-Phe-4 and 8-Phe-8 PEAs. There was an approximately two-fold decrease in both the number average ($M_n$) and weight average ($M_w$) molecular weight (Table 5.1) when 10 mol% of the Asp(O-t-Bu)-4 monomer was incorporated into the polymer. The reduction in molecular weight suggests that the aspartic acid functional monomer does not polymerize as well as the phenylalanine-based diester monomer.

Interestingly, the protected functional PEAs exhibited similar PDIs to one another, while the 8-Phe-4 and 8-Phe-8 had a PDI value of 2.6 and 1.6 respectively. The decreased PDI in the 8-Phe-4-Asp(O-t-Bu)-4 case may be attributed to the increased solubility in DMF and thus its removal during dialysis. The increased polydispersity of 8-Phe-8-Asp(O-t-Bu)-4 may be the result of the variability in the reactivity of the base and functional monomer. Molecular weight data was not obtained for the deprotected PEAs, as the pendant carboxylic acid groups would interact with the GPC columns.
Figure 5.7: Overlayed GPC chromatographs of 8-Phe-4 and 8-Phe-4-Asp(O-t-Bu)-4.

Figure 5.8: Overlayed GPC thermographs of 8-Phe-8 and 8-Phe-8-Asp(O-t-Bu)-4.
Table 5.1: Molecular weights and polydispersity indices of functional PEAs.

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<th>PEA</th>
<th>Yield (%)</th>
<th>$M_n$ (kg/mol)</th>
<th>$M_w$ (kg/mol)</th>
<th>PDI</th>
<th>$T_g$ (°C)</th>
<th>$T_d$ (°C)</th>
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</table>

5.4.2 Vascular Smooth Muscle Cell Proliferation, Morphology and Focal Adhesion Formation on Two-Dimensional Films

Upon successful syntheses of the PEAs containing aspartic acid, their evaluation with human coronary artery smooth muscle cells was sought to ensure their suitability as vascular biomaterials. Initially, the SMCs were cultured on 2D films for 7 days to assess cell attachment and metabolic activity. As illustrated in Figure 5.9, over the course of the week long study, metabolic activity was enhanced as indicated by the cumulative increase in absorbance. The cell viability studies were conducted in conjunction with the PEAs containing lysine to compare the effects of the pendant carboxylic acid versus amine functional groups. At 7 days, more metabolically active HCASMCs were present on 8-Phe-4-Asp(OH)-4 than either the base polymer or its cationic counterpart, which were very similar to one another. To ascertain whether the reduction in metabolic activity was attributed to cell proliferation, the data was also normalized to day 1, shown in Figure 5.10(A). Once normalized to the number of metabolically active cells at day 1, it became apparent that the PEAs are not retarding cell growth, and thus the decreased cell metabolism in 8-Phe-4 and 8-Phe-4-Lys(TFA)-4 at 7 days was a consequence of reduced initial cell attachment to these materials. Similar behaviour with 8-Phe-4 and cationic functionalized PEAs was previously reported with bovine aortic endothelial cells (BAECs), but in their study, significantly fewer cells were metabolically active in their pendant carboxylic acid functional PEA and those which were viable formed clusters on the surface of the PEA, suggesting an inhospitable surface. Moreover, the metabolic activity of the BAECs was lower than on glass and appeared to stagnate at 72 hours in the carboxylic acid functionalized PEA. In Figure 5.9(B), SMC metabolic activity on 8-Phe-8-Asp(OH)-4 was only significantly greater than the cationic 8-Phe-8-Lys(TFA)-4 at
all time points. The increased cell activity is again attributed to the decreased cell attachment observed at day 1 as illustrated by the normalized data shown in Figure 5.10(B).

Figure 5.9: HCASMC metabolic activity as determined by MTT assay. Cells were seeded at 20,000 cells/cm² on 2D 8-Phe-4 (A) and 8-Phe-8 (B) functionalized PEA films and measured following 1, 3, 5 and 7 days of culture. Data are expressed as mean ± standard error for experiments conducted in triplicate. Statistical significance (p < 0.05) is denoted by *.

Given the ability of the PEAs containing aspartic acid to support HCASMC activity up to 7 days, the cell-material interactions were further investigated. In particular, the capability of the HCASMCs to form focal adhesions with the substrate was studied because it is a critical event in cell adhesion, migration and signal transduction. Integrins are transmembrane proteins with surface receptors that bind specific ECM ligands. Binding with a surface receptor induces a change in the intracellular component, effectively transmitting information from the ECM to the cell. This signal transduction allows the cells to respond to their environment. The ability of HCASMCs to communicate with the substrate is conditional on the cell being able to form focal adhesive plaques, which link the transmembrane integrins with F-actin, the microfilamentous cytoskeleton of the cells. Vinculin is one of a number proteins involved in anchoring F-actin to integrin adhesive molecules and thus its presence was
investigated to confirm integrin-mediated communication between the HCASMCs and the extracellular matrix.

**Figure 5.10:** HCASMC metabolic activity as determined by MTT assay. Cells were seeded at 20,000 cells/cm² on 2D 8-Phe-4 (A) and 8-Phe-8 (B) functionalized PEA films and measured following 1, 3, 5 and 7 days of culture. Data are normalized to day 1 and expressed as mean ± standard error for experiments conducted in triplicate. Statistical significance (p < 0.05) is denoted by *.

Figure 5.11 shows laser scanning confocal microscopy images of the HCASMCs cultured for 24 hours and 7 days on 2D PEAs containing aspartic acid. At 24 hours, the HCASMCs were stained for F-actin (green), vinculin (red) and nuclei (blue) as shown in panels (A)–(D). The 8-Phe-4-Asp(OH)-4, 8-Phe-8-Asp(OH)-4 and control glass coverslip (panels A–C) showed vinculin expression at the peripheries of the cells as highlighted by the white arrows. In the fibronectin-coated coverslip (D), vinculin expression was observed throughout the cell indicative of a more favourable substrate, which was anticipated due to the presence of the cell adhesive RGD motif.
Figure 5.11: Immunofluorescence microscopy images of HCASMCs after 24 h and 7 days culture on 2D 8-Phe-4-Asp(OH)-4 and 8-Phe-8-Asp(OH)-4 PEA films. Control coverslips with and without fibronectin were used as controls. HCASMCs were stained for F-actin (green), vinculin (A – D; red, shown by arrowheads), smooth muscle α-actin (E – H, red), and nuclei (blue). Scale bar = 50 μm.

In addition to supporting focal adhesion, an understanding of the effect of the functional PEAs on VSMC phenotype is critical as the smooth muscle cells can adopt one of two phenotypes. In the synthetic phenotype, the cells proliferate and secrete much of the extracellular matrix, a necessary first step in tissue maturation and remodeling. In the contractile phenotype, the cells contract in response to chemical, electrical and mechanical stimuli necessary in vasoactive blood vessels. Modulation of their phenotype is therefore paramount to the success of any tissue-engineered vascular graft. To examine the smooth muscle cell phenotype, the cells were stained for smooth muscle α-actin (SMαA, red), a known marker protein of the contractile phenotype. After 7 days of
culture, the immunofluorescence images (panels E–H) illustrate robust expression of F-actin (green), but very little expression of SMαA (red), suggesting the HCASMCS were well-spread on the surface and primarily adopted a synthetic phenotype in both the PEAs containing aspartic acid and the controls.

5.4.3 Three-Dimensional Electrospun Fibrous Mats

Although the HCASMCs proliferate and form focal adhesions on two-dimensional films of the functional PEAs, the use of a functional PEA for vascular tissue engineering necessitates its fabrication into a three-dimensional scaffold to mimic the extracellular matrix. Collagen and elastin fibers, ranging in diameter from tens to hundreds of nanometers, serve as the primary structural proteins of the ECM. The tissue specific fibril arrangement of these proteins can also influence cell function. In fact, nano-scale topography resulted in differential changes in cytoskeletal organization, focal contact formation and subsequent motility, differentiation and gene expression between cell types. This nano-topography-dependent signal transduction may also occur independently of integrin-mediated signaling, thus dictating a nano-scale fibrillar scaffold.

Electrospinning is a facile and versatile approach to generate nano-fibrous structures that can mimic the fibrillar structure of the extracellular matrix. The high porosity and surface area to volume ratio obtained from randomly oriented fibers are also ideal for cell infiltration, nutrient and waste transport and subsequent vascularization. Few studies have investigated electrospun PEAs, and to date, none have electrospun a functional PEA that can be further modified. Initial electrospinning studies were conducted with 8-Phe-4, as it provided higher molecular weights, which was expected to generate more uniform fiber diameters. 8-Phe-4 polymers having two different molecular weight were tested to evaluate the impact of PEA molecular weight on average fiber diameter.

The PEAs were electrospun from a 9:1 (wt:wt) co-solvent mixture of CHCl₃:DMF, at varying concentrations to generate uniform fibers of nano-scale size as seen in Figure 5.12. The lower molecular weight 8-Phe-4 (Mₙ = 48.4 kg/mol) generated a slightly larger average fiber diameter, yet a narrower distribution of fiber diameters (panels A–C) when compared to the higher molecular weight 8-Phe-4 (Mₙ = 70.3 kg/mol, panels D–F) as the
average fiber diameters were 294 ± 7 and 274 ± 14 nm, respectively. The statistically equivalent average fiber diameters suggest that uniform nano-scaled fibers can be obtained reproducibly from 8-Phe-4 PEAs of different molecular weights provided that the viscosities of the solutions are comparable.

Figure 5.12: Scanning electron microscopy images of electrospun fibrous mats from a solution of 8-Phe-4 or 8-Phe-4-Asp(OH)-4 in a co-solvent mixture of 9:1 (wt:wt) CHCl₃:DMF. 8-Phe-4 was electrospun at a concentration of 9.2 wt% for a \( M_n = 48.4 \) kg/mol (panels A–C); and 5 wt% for a \( M_n = 70.3 \) kg/mol (panels D–F), while 8-Phe-4-Asp(OH)-4 (panel G–I) was electrospun from a 7.5 wt% solution. Scale bars represent 50, 10 and 5 \( \mu \)m, respectively.

Uniform, bead-free fibers of 8-Phe-4 have been reported previously; however, in the study by Li and Chu, the smallest average fiber diameter from a 2:1 (vol:vol) co-solvent mixture of CHCl₃:DMF obtained was 640 nm. The larger average fiber diameter achieved in their study is attributed to their relatively low molecular weight (\( M_n = 13.6 \) kg/mol).
kg/mol) and subsequent need for a high polymer concentration (39% wt/vol). In the 8-Phe-4-Asp(OH)-4 case, an average fiber diameter of 130 ± 5 nm (panels G–I) was obtained. The reduced average fiber diameter could be a result of the increased charge density from the pendant carboxylic acid groups. As little as 0.1% poly(acrylic acid sodium salt) (PAA) and poly(allylamine hydrochloride) (PAH) has shown to significantly decrease the average fiber diameter of poly(ethylene oxide) (PEO) fibers. Moreover, the addition of these polyelectrolytes reduced the concentration of the PEO needed to form fibers, further reducing the fiber diameter. The molecular weight of the protected 8-Phe-4-Asp(O-t-Bu)-4 was 34.7 kg/mol, which based on the 8-Phe-4 data, would suggest needing a higher concentration than the 9.2 wt% (48.8 kg/mol 8-Phe-4) to maintain the same viscosity. Given that uniform fibers were obtained at a concentration of 7.5 wt%, it is believed that the increasing charge density from 8-Phe-4-Asp(OH)-4 resulted in a increase in whipping instability upon ejection from the needle tip, thus resulting in a decreased fiber diameter.

5.4.4 Vascular Smooth Muscle Cell Morphology on Three-Dimensional Scaffolds

The successful electrospinning of both 8-Phe-4 and 8-Phe-4-Asp(OH)-4 enabled the study of their interaction with HCASMCs. Initially, a time series analysis was conducted to assess the attachment and morphology of the HCASMCs on the surface of the electrospun mats. As can be seen from Figure 5.13(A), at 4 h, the HCASMCs are well-spread on the surface of the 8-Phe-4 electrospun mat. At 8 h (panel C), the fibers begin to flatten and fuse together, while at 24 h (panel E), the appearance of a secondary layer becomes apparent, presumably the extracellular matrix secreted by the SMCs. At 4 days, the fibers are no longer detected, as the fibers are completely covered by the secretion of the ECM (panel G). In the 8-Phe-4-Asp(OH)-4 case, fibers were not detected as early as 4 h (panel B). Although the cells attached and spread on the surface of 8-Phe-4-Asp(OH)-4, including up to 4 days of culture (panel H), the lack of a fibrous mat suggested an interaction of the HCASMCs with a two-dimensional substrate.
Figure 5.13: Scanning electron microscopy images of HCASMCs cultured up to 4 days on fibrous 8-Phe-4 and 8-Phe-4-Asp(OH)-4 electrospun mats. Scale bar represents 50 µm.

The loss of electrospun fibers was further investigated to ascertain whether the cells resulted in the loss of the 3D porous scaffold. Individual stages in the culture of the cells on the material were investigated and are shown in Figure 5.14. As illustrated, the fibrous mat was retained following long-term storage at 4°C (panel A), but following sterilization with 70% ethanol and UV irradiation, the fibers had fused together to form a film-like structure (panel B). The addition of media (panel C) or culture of cells for 4 h (panel D) had no supplemental impact on the surface of the 8-Phe-4-Asp(OH)-4. However, when the fibers were exposed to the fixation media and the dehydration and drying steps necessary for SEM imaging, again changes in the surface could be observed, in particular following the addition of ethanol and hexamethyldisilazane (HMDS). Given the loss of the 3D porous network, yet lack of solubility of 8-Phe-4-Asp(OH)-4 during sterilization
and post-culture image preparation, a plasticizing effect facilitated by the 10 mol% Asp(OH)-4 monomer is proposed.

Figure 5.14: Scanning electron microscopy images of 8-Phe-4-Asp(OH)-4 at individual stages of cell culture following – storage at 4°C (A); sterilization with 70% ethanol and UV irradiation (B); sterilization and exposure to culture media (C); 4 hour culture with HCASMCs (D); fixation with 4% formaldehyde (E); and dehydration and drying with ethanol and hexamethyldisilazane, respectively (F). Scale bar represents 50 µm.

Despite the inability of the HCASMCs to infiltrate the 8-Phe-4-Asp(OH)-4 scaffold due to loss of the 3D network prior to seeding, the porous structure of the 8-Phe-4 electrospun mat was retained, and thus cell infiltration was assessed using confocal microscopy. Focus stacking (also known as Z-stacking) was employed to provide a greater depth of field to include all parts of the cells within the field of view. In Figure 5.15(A), the HCASMCs appeared stretched with abundant F-actin expression and could be detected to a depth of 45 µm. Upon rotation of the stacked image though, cell penetration was shown to be non-uniform (panel B). In an orthogonal view and using the fluorescence of the 8-Phe-4 (shown in red), the variability in the cell depth was attributed to the surface roughness of the electrospun mat as cells were only observed on the surface of the fibrous scaffold and not within it (panel C). The diminished migration of VSMCs into porous
scaffolds following static seeding has been previously reported. Dynamic seeding using spinner flasks or vacuum seeding apparatuses have shown improved smooth muscle cell and fibroblast penetration and distribution in PGA and PCL matrices, respectively.\textsuperscript{40-41} Despite the lack of cell infiltration from the passive seeding technique though, the porous 8-Phe-4 structure would still enable nutrient or growth cues to be delivered three-dimensionally.\textsuperscript{33}

![Figure 5.15: Confocal microscopy images of HCASMCs seeded at a density of 30,000 cells/scaffold and cultured for 4 days on 8-Phe-4 electrospun mats. HCASMCs were stained for F-actin (shown in green) and nuclei (blue) with fluorescence of the 8-Phe-4 scaffold (shown in red). Composite Z-stack image taken up to a depth of 45 μm on 8-Phe-4 (A); rotated to show depths of cells (B); and an orthogonal view to demonstrate cells sitting on surface of the electrospun mat (red) (C). Scale bar = 100 μm.]

5.4.5 Western Blot Analysis

The impact of the three-dimensional delivery of nutrients or signaling molecules was investigated via Western blot analysis. The loss of the 3D network of the 8-Phe-4-Asp(OH)-4 prior to HCASMC seeding precluded its examination in the immunoblotting studies. Therefore, the cells were cultured on 2D films of 8-Phe-4 and 8-Phe-4-Asp(OH)-4 and 3D scaffolds of 8-Phe-4 for 4 (Figure 5.16) and 7 days (Figure 5.17) to compare the effect the functional amino acid in 2D cultures and the impact of 3D architecture on SMC contractile phenotype marker proteins. The exogenous addition of transforming growth factor-β1 (TGF-β1) was also tested to assess whether TGF-β1 could up-regulate contractile marker protein expression from HCASMCs cultured on the surfaces of the PEAs as previously observed with the biostable poly(carbonate urethane).\textsuperscript{25}
Figure 5.16: Western blot data following HCASMC culture for 4 days. HCASMC contractile phenotype marker proteins smooth muscle α-actin (SMαA) and calponin were analyzed from 8-Phe-4 (2D films and 3D electrospun mats), 8-Phe-4-Asp(OH)-4 (2D films) and normalized to tissue culture polystyrene (TCPS). Protein expression was also evaluated following exogenous addition of TGF-β1.

The addition of TGF-β1 in all 2D films resulted in a modest increase in the expression of the contractile phenotype marker proteins SMαA and calponin at four days as shown in Figure 5.16. In the absence of TGF-β1, the 3D structure alone suggested an increasing trend in contractile phenotype marker protein expression. Despite these trends in the immunoblotting data, the results are not significant due to the inherent error in the data, in part because of the variability in primary cell culture where primary cells are harvested from multiple donors. Although studies have been conducted with TGF-β1 at concentrations as little as 1 ng/mL, an exogenous concentration of 3 ng/mL was selected for this study based on previous work with HCASMCs. In those studies, the TGF-β1 was
reconstituted with carrier molecules bovine serum albumin (BSA) and trehalose, while in this study, the TGF-β1 was reconstituted in diluted hydrochloric acid in the absence of carrier molecules as their presence would have interfered with the subsequent conjugation to the functional PEA. The lack of carrier molecules may have affected the reconstitution of the growth factor resulting in sub-optimal delivery of the TGF-β1.

Moreover, the potential presence of TGF-β1 in the fetal bovine serum of the media may have limited the efficacy of additional growth factor.

Figure 5.17: Western blot data following HCASMC culture for 7 days. HCASMC contractile phenotype marker proteins smooth muscle α-actin (SMαA) and calponin were analyzed from 8-Phe-4 (2D films and 3D electrospun mats), 8-Phe-4-Asp(OH)-4 (2D films) and normalized to tissue culture polystyrene (TCPS). Protein expression was also evaluated following exogenous addition of TGF-β1.
At seven days (Figure 5.17), little change was observed with the addition of TGF-β1. In only the 8-Phe-4-Asp(OH)-4 was there an increasing trend in contractile phenotype marker protein expression following the addition of TGF-β1, but it too did not reach statistical significance. As observed in the Western blot data at four days, enhanced contractile phenotype marker protein expression was observed between the 8-Phe-4 films and electrospun mats, implying that despite the lack of cell infiltration, the porous scaffold may allow the cells to interact with its environment three-dimensionally. Although architecture alone suggested an enhanced expression of SMαA and calponin, the exogenous addition of TGF-β1 had no effect on the contractile phenotype marker protein expression on 3D scaffolds at 7 days. Again, the lack of effect is attributed to the inherent variability in the data, potential sub-optimal delivery of the growth factor, its saturation due to TGF-β1’s potential presence in the fetal bovine serum or combinations thereof.

5.4.6 TGF-β1 Conjugation to 8-Phe-4-Asp(OH)-4

The loss of the 3D network of the 8-Phe-4-Asp(OH)-4 electrospun mat did not preclude it from being functionalized with TGF-β1. A functional PEA with a pendant carboxylic acid group was selected for conjugation in part due to the structure of the TGF-β1. The primary structure revealed an equivalent number of pendant amine and carboxylate groups at physiologic pH43 and thus did not favour the reaction of one complementary group over the other. The bioactivity of the growth factor was believed to be part of the octapeptide sequence at the C-terminus of the growth factor as its cleavage resulted in a loss of bioactivity.43 The quaternary structure of TGF-β1 suggests the C- and N- termini are in close proximity, as shown in Figure 5.18;44 therefore, conjugation to amino acids in the heart of the primary structure was sought. Given the flexibility in conjugating to TGF-β1, the PEA having pendant carboxylic acid moieties was selected due to its ease in activation using a simple EDC/sulfo-NHS conjugation strategy and subsequent reaction with a deprotonated amine group of the TGF-β1, as shown in Scheme 5.1.
Figure 5.18: Ribbon diagram representing the Cα backbone coordinates of the solution structure of the TGF-β1 homodimer. Regular elements of secondary structure are identified, along with the N and C termini of the two monomers. Reprinted with permission from (Hinck, A. P.; Archer, S. J.; Qian, S. W. et al. Biochemistry 1996, 35 (26), 8517-8534). Copyright (1996) American Chemical Society. Protein chains are coloured from the N-terminus to the C-terminus using a rainbow (spectral) colour gradient.

Scheme 5.1: Conjugation of TGF-β1 to 8-Phe-4-Asp(OH)-4 using EDC/sulfo-NHS peptide coupling.
Two approaches were explored to confirm the conjugation of TGF-β1 to 8-Phe-4-Asp(OH)-4. Initially, the scaffolds were characterized by X-ray photoelectron spectroscopy (XPS) to reveal the elemental analysis up to a depth of 5–7 nm from the surface. The spectra, shown in Figure 5.19, and their corresponding elemental analyses provided in Table 5.2, illustrate the adsorbed and covalently conjugated TGF-β1 to the surface of 8-Phe-4-Asp(OH)-4 scaffolds. The scaffolds were treated identically except that in the adsorption study, the activation with EDC/sulfo-NHS was omitted.

![Figure 5.19: Wide-scan X-ray photoelectron spectra of TGF-β1 adsorbed and conjugated to the surface of 8-Phe-4-Asp(OH)-4.](image)

The XPS data revealed both an increase in the sulfur (S 2p) and nitrogen (N 1s) content between the adsorbed and conjugated scaffolds suggesting an increase in TGF-β1 following conjugation. The increase in sulfur content is attributed to the presence of the cysteine amino acid, as no sulfur is present in the functional PEA. This atomic composition is comparable to a previous finding where fibronectin was conjugated to 3D
scaffolds and showed a reaction time-dependent sulfur content of 0.2-0.6%. However, unlike fibronectin which has 62 cysteine residues per subunit, there are only seven cysteine residues in TGF-β1, suggesting that its conjugation in the present study was more effective than the fibronectin conjugation. The enhanced nitrogen content is ascribed to the significant number of peptide bonds from the growth factor. The presence of silicon was attributed to silicone grease contamination, which also impacted the atomic percentages of oxygen (O 1s) and carbon (C 1s) rendering their comparisons difficult.

Table 5.2: Elemental analysis from X-ray photoelectron spectra of TGF-β1 adsorbed and conjugated to the surface of 8-Phe-4-Asp(OH)-4.

<table>
<thead>
<tr>
<th>Element</th>
<th>Binding Energy (eV)</th>
<th>Adsorbed (Atomic %)</th>
<th>Conjugated (Atomic %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na 1s</td>
<td>1067.65</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>F 1s</td>
<td>686.15</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>O 1s</td>
<td>528.65</td>
<td>14.7</td>
<td>13.9</td>
</tr>
<tr>
<td>N 1s</td>
<td>396.35</td>
<td>2.2</td>
<td>2.9</td>
</tr>
<tr>
<td>C 1s</td>
<td>281.55</td>
<td>77.8</td>
<td>78.8</td>
</tr>
<tr>
<td>S 2p</td>
<td>164.65</td>
<td>0.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Si 2p</td>
<td>98.15</td>
<td>5.0</td>
<td>2.8</td>
</tr>
</tbody>
</table>

The increase in TGF-β1 at the surface of 8-Phe-4-Asp(OH)-4 following covalent attachment was further evaluated with immunofluorescence as illustrated in Figure 5.20. The functional PEA alone (panel A) showed little fluorescence, while the TGF-β1 adsorption study showed stronger fluorescence (panel B). The smooth surface in the adsorbed case appears similar to the film-like structure upon exposure to aqueous media (see Figure 5.14). The covalently conjugated surface was rougher and exhibited higher fluorescence than the adsorbed TGF-β1 suggesting the successful coordination of the fluorescently labeled secondary antibody. The inherent fluorescence of the adsorbed study is attributed to the entrapment of the secondary antibody as the surface was plasticized in buffer, which would also occur in the TGF-β1-conjugated study, and thus the difference between the adsorbed and conjugated studies would provide some indication of the true fluorescence contribution from the TGF-β1 grafted functional PEAs.
Figure 5.20: Immunofluorescence images of 8-Phe-4-Asp(OH)-4 alone (A); TGF-β1 adsorbed to surface of 8-Phe-4-Asp(OH)-4 (B); and TGF-β1-conjugated to surface of 8-Phe-4-Asp(OH)-4 (C). Scale bar represents 50 µm.

5.5 Conclusions

Functional poly(ester amide)s containing pendant carboxylic acid groups were successfully synthesized by incorporating a protected version of the naturally occurring α-amino acid L-aspartic acid into the PEA backbone through a facile interfacial polycondensation approach. The molecular weights of the functional PEAs containing 10 mol% functional monomer were lower than the corresponding base polymers suggesting that the functional monomer did not polymerize as effectively as the base monomer. The protected functional PEAs had glass transition temperatures lower than the base polymer suggesting improved free volume attributed to the tert-butyl group, while the deprotected functional PEAs exhibited a $T_g$ greater than the base polymer implying increased hydrogen bonding of the pendant carboxylic acid groups.

Human coronary artery smooth muscle cell attachment and spreading was observed on two-dimensional PEA films with robust F-actin expression throughout the cells, while vinculin expression was primarily seen at the periphery of the HCASMCs. Vinculin expression suggested the cells were forming focal adhesions with the substrate, while the lack of smooth muscle α-actin expressed at seven days implied a proliferative phenotype.

Metabolic activity on 2D films increased throughout the seven day culture, with the aspartic acid containing PEAs supporting as many or more metabolically active cells as the corresponding base and cationic PEAs.
Nano-scale three-dimensional scaffolds were successfully electrospun from low polymer concentrations in a co-solvent mixture of CHCl3:DMF of 9:1, where the increasing charge density from 8-Phe-4-Asp(OH)-4 resulted in a decreased average fiber diameter. HCASMC culture on these 3D scaffolds illustrated cell spreading on the surface of electrospun mats as early as four hours, but with little cell infiltration likely due to static seeding.

Immunoblotting studies suggested an increasing trend in smooth muscle α-actin and calponin expression from HCASMCs cultured on 2D PEA films with the exogenous addition of TGF-β1 at four days. At seven days, increasing contractile phenotype marker protein expression was observed between 2D films and 3D scaffolds suggesting architecture alone promotes the HCASMC contractile phenotype. Despite the trends in the immunoblotting data, significance was not achieved. X-ray photoelectron spectroscopy and immunofluorescence demonstrated the conjugation of transforming growth factor-β1 to the surface of 8-Phe-4-Asp(OH)-4. Collectively, this study shows that the aspartic acid containing PEAs are good candidates as vascular biomaterials because they support HCASMC attachment, spreading, proliferation and can be electrospun into 3D scaffolds. The pendant carboxylic acid groups also enabled the conjugation of TGF-β1 to the surface, warranting further investigation into these functional PEAs as biomimetic vascular scaffolds.

5.6 References


Chapter 6

6 General Discussion and Conclusions

This chapter provides a summary of the research and discusses the strengths and limitations of this work and future directions. Significance of the research will also be discussed.

6.1 Summary

The focus of the research was to develop a biomimetic degradable vascular scaffold that would support human coronary artery smooth muscle cell attachment, spreading, proliferation and phenotype modulation that could ultimately be considered as part of an in vitro tissue-engineered blood vessel strategy. To that end, a family of degradable PEAs was initially synthesized to screen them for vascular tissue engineering applications. The rationale for investigating PEAs stemmed from the fact that they combine the advantageous properties of both polyesters and polyamides – the tunability in the degradation rate via the ester groups and mechanical strength via the hydrogen bonding of the amide groups. The ester and amide linkages promote both hydrolytic and enzymatic degradation, which should ensure a surface degradation mechanism, whereby the individual constituents would control the rate of degradation. In addition, the careful selection of the monomers would enable the generation of polymers with suitable thermal properties.

The PEAs used in this study were derived from naturally occurring α-amino acids, aliphatic diols and diacids. The incorporation of a long chain aliphatic diacid (sebacic acid) was selected for two reasons. First, the long range segmental motion of the polymer chains yielded PEAs with glass transition temperatures at or below physiologic temperature ensuring a degree of pliability. Secondly, the hydrophobicity of the monomer enabled its usage in an interfacial polycondensation reaction, conventionally used in polyamide syntheses. The acid-catalyzed condensation of L-alanine or L-phenylalanine with the aliphatic diols 1,4-butanediol or 1,8-octanediol were selected to evaluate their impact on the thermal and mechanical properties. It was anticipated that the
PEAs based on these monomers would generate polymers with relatively low melting temperatures, which would facilitate their processing in the melt. Additionally, PEAs based on L-alanine have demonstrated lower Tgs than those PEAs incorporating the bulkier benzyl group of L-phenylalanine; however, these PEAs also tend to be more semi-crystalline, which may not offer the elasticity needed of a vascular scaffold. As such, a family of PEAs based on these monomers was synthesized to ascertain the most suitable PEA for vascular tissue engineering.

In addition, the synthetic approach adopted in this work permitted the introduction of trifunctional amino acids such as L-lysine and L-aspartic acid into the polymer backbone yielding complementary functional handles for conjugation of biomolecules that can modulate host response or direct cell function. Although an acid-catalyzed condensation of the functional amino acids could not be adopted due to the acid labile protecting groups necessary for selective polymerization of the monomer, a facile condensation approach was achieved using dicyclohexylcarbodiimide as a coupling agent that could be used for both orthogonally protected functional amino acids.

As mentioned, the selection of the aliphatic diacid sebacic acid enabled the polymerization to occur at the interface of a heterogeneous mixture. Specifically, the acid chloride of sebacic acid (sebacoyl chloride) was selected and added dropwise to a solution containing the amine in the presence of base to generate the amide group in a reaction known as the Schotten-Baumann reaction. In the solution polymerization, sebacoyl chloride was not used in the presence of base as the reaction would potentially result in unwanted side reactions. Consequently, the acid chloride was converted to a more stable activated ester to ensure selective polycondensation with the diamine monomers. Although the polymerization approaches were slightly different, each method yielded identical polymer structures, permitting a direct comparison. The PEAs derived from each polymerization approach were compared in terms of purity, yield and molecular weight. Equally pure PEAs could be obtained from both approaches as evidenced by identical $^1$H and $^{13}$C NMR and FTIR spectra. Characterization of the polymer molecular weights obtained from GPC revealed that all PEAs synthesized interfacially were higher molecular weight. The enhanced polymerization was in part
attributed to the improved reactivity of the acid chloride when compared to the activated ester.\textsuperscript{10} Secondly, any impurities from the monomer syntheses would likely remain in the bulk phase and have less impact on polymer chain propagation at the interface than in solution.\textsuperscript{8} Of the PEAs tested, 8-Phe-4 yielded the highest weight average molecular weights with 8-Ala-8 yielding the lowest. The molecular weight trend was also maintained with the functional PEAs, even though the functional PEAs did not polymerize as readily. Of the functional monomers, the lysine-based PEAs generated greater weight average molecular weights when compared to their corresponding aspartic acid containing PEAs.

The thermal properties were measured using DSC and TGA. As expected, all PEAs had glass transition temperatures ranging from 11.8 to 43.5°C with 8-Ala-8 providing the lowest $T_g$ and 8-Phe-4 exhibiting the highest $T_g$. Despite the $T_g$ of 8-Phe-4 being greater than physiologic temperature, it is anticipated that in a hydrated state during \textit{in vitro} tissue maturation in a bioreactor, the free volume of the polymer chains would be increased through solvation, thus resulting in a lower $T_g$,\textsuperscript{11-12} and providing a degree of pliability. A comparison to 8-Phe-8 revealed the direct impact of the amino acid and diol chain length on the $T_g$. Compared to 8-Ala-8, the bulky benzyl group of 8-Phe-8 restricted polymer chain motion increasing the $T_g$ to approximately 25°C. In contrast to 8-Phe-4, the longer diol limited hydrogen bonding between chains, facilitating long-range segmental motion, hence giving a lower $T_g$ than 8-Phe-4.\textsuperscript{5} Incorporation of the functional amino acids generally resulted in an increase in $T_g$ attributed to improved hydrogen bonding of the charged functional groups following removal of the protecting groups.

The increased $T_g$ was observed despite the replacement of the bulky benzyl groups of L-phenylalanine with the aliphatic functional handles of L-lysine or L-aspartic acid.

The TGA data revealed that all non-functional PEAs yielded decomposition temperatures in excess of 300°C, well above their melting ranges, thus ensuring these PEAs could be processed in the melt. The incorporation of lysine into the PEA backbone had little effect on the decomposition of the polymer itself. The mass loss attributed to the thermal cleavage of the BOC protecting group was also observed in the TFA salts (albeit at lower temperatures). The derivative weight demonstrated no change thereafter implying the
lysine monomer did alter the bulk decomposition of the PEA. In the aspartic acid containing PEAs, again thermal cleavage of the tert-butyl protecting group was observed; however, differences in the decomposition of the base and the deprotected aspartic acid containing PEAs suggest that the introduction of aspartic acid does ultimately reduce the decomposition temperature of the functional PEA. Despite the effect of aspartic acid, these functional PEAs still exhibited decomposition temperatures well in excess of 200°C, suggesting they still could be processed in the melt.

The consideration of these PEAs for vascular tissue engineering depends not only on the polymer’s chemical and thermal properties, but on their ability to support vascular cell attachment, proliferation and function. HCASMC attachment and morphology, investigated using light and fluorescence microscopy, demonstrated that the cells attached and spread well on 2D films of all base PEAs up to 7 days of culture. Abundant expression of filamentous actin (F-actin), a cytoskeletal protein necessary in cell motility, was also observed in the laser scanning confocal microscopy images. In addition to cell morphology, the MTT assay data revealed increased metabolic activity of the cells on the surface of the 2D films up to 5 days.

Although the pendant functional groups were ultimately designed for the incorporation of signaling molecules, the impact of surface charge on cell behaviour was also studied due to the varied response of cells cultured on different surface functionalized materials. While hydrophilic surfaces tend to promote cell adhesion, the surface charge appears to have a greater impact on the surface adhesion of proteins, which can influence cell adhesion, morphology, proliferation and differentiation. The lysine-containing functional PEAs supported HCASMC proliferation, but generally yielded lower metabolic activity at 7 days than the corresponding base and functional PEA containing aspartic acid, which was attributed to an initial reduction in metabolic activity, due to the trifluoroacetate anion. HCASMCs cultured on 2D lysine-containing functional PEAs appeared less stretched with a corresponding reduction in F-actin expression than their non-functional counterparts. The aspartic acid containing functional PEAs did not inhibit either SMC adhesion or metabolic activity. Moreover, the HCASMCs spread well and expressed abundant F-actin on the functional PEAs containing aspartic acid, similar to
the base PEAs. These results are promising and contradict a previous report, where bovine aortic endothelial cells formed clusters on the surface of a functional PEA with pendant carboxylic acid groups, while concomitantly reducing metabolic activity.\textsuperscript{15}

Given the encouraging cell attachment, spreading and metabolic activity data on the 2D films, in particular the base and the functional PEAs containing aspartic acid, the ability of HCASMCs to form focal adhesions with the substrate was sought. Focal adhesions enable the transmission of information from the ECM to the cell, allowing the cells to respond to its surroundings.\textsuperscript{16} Vinculin, a protein involved in anchoring F-actin to the cell membrane of focal adhesions was expressed by HCASMCs on all 2D PEA films as illustrated by the immunofluorescence studies. The integrin-mediated focal adhesions were primarily formed at the peripheries of the cells on all PEA films.

Although the HCASMCs formed focal adhesions with the substrate, given that protein adsorption is typically the first event in cell adhesion to that particular surface,\textsuperscript{17} cell culture was also conducted in the absence of serum to ascertain the direct interaction between the HCASMCs and the PEAs. The formation of focal adhesions with the underlying PEAs was examined and compared using two separate techniques: waveguide evanescent field fluorescence (WEFF) and immunofluorescence. The use of WEFF microscopy for analyzing cell-substratum contact regions is a relatively new endeavor, whereby an evanescent field can be generated up to 70 nm from a waveguide, resulting in excitation of fluorescent molecules within that evanescent field. The examination of the interfacial contact region between HCASMCs and the polymer substrate necessitated the fabrication of ultrathin PEA films. Neither solvent casting nor spin-coating afforded suitable films, and therefore Langmuir-Blodgett technology was adopted to prepare the films. Despite the long polymeric chains and propensity to hydrogen bond, closed and uniform PEA films could be obtained, with film thicknesses ranging from 11 to 21 nm.

In the WEFF microscopy studies, no differences in the number of cell adhesions (which included both close contacts and focal adhesions) were observed in serum-containing or serum-free culture; however, a statistically significant increase in cell adhesions between the 8-Phe-8-Lys(TFA)-4 and bare waveguide was observed in both culture conditions.
The immunofluorescence studies also revealed a similar number of focal adhesions per cell and per unit area in both culture conditions. It was anticipated that the lysine-containing functional PEA may promote cell adhesion to the polymer surface in the absence of serum through electrostatic interactions between the cationic pendant amine groups and the negatively charged glycocalyses of the cells. In the presence of serum, the cationic surface was expected to interact with adhesive serum proteins fibronectin and vitronectin, which are both negatively charged at physiologic pH. However, only in the case of serum-based media and fibronectin-coating, was an increase in the number of focal adhesions observed. This suggested that the fibronectin may have facilitated the adsorption of a secondary protein from the serum that ultimately improved HCASMC focal adhesion formation.

Even though focal adhesions were formed with all PEA surfaces in the presence or absence of serum, smooth muscle α-actin expression was not abundant, suggesting that the HCASMCs had adopted a synthetic phenotype, typical of 2D SMC culture. Modulation of the SMC phenotype has been shown through 3D architecture, signaling molecules including TGF-β1, mechanical stimulation and co-culture with other cells, suggesting that the quiescent contractile phenotype of SMCs can be realized in vascular tissue engineering using one or more of these strategies.

The 3D network not only modulates SMC phenotype, but the cellular microenvironment also influences cytoskeletal organization, focal adhesions, cell motility, differentiation and gene expression. This signal transduction from the nano-scale extracellular matrix can act independently of integrin-signaling, implying that extracellular matrix mimicry is a vital tool in the development of vascular tissue. Nano-scale fibrous PEA mats were achieved by electrospinning PEA solutions in a co-solvent mixture of 9:1 (wt:wt) CHCl₃. As higher molecular weight polymers are expected to generate more uniform fibers, 8-Phe-4 was initially selected for electrospinning. Statistically equivalent average fiber diameters (294 ± 7 and 274 ± 14 nm) were achieved from two different 8-Phe-4 molecular weights (Mₐ = 48.4 kg/mol and 70.3 kg/mol, respectively) when comparable viscosities were used. These results compare favourably with a previous study where the smallest 8-Phe-4 average fiber diameter from a 2:1 (vol:vol) co-solvent mixture of
CHCl₃:DMF was 640 nm. The larger average fiber diameter is attributed to their relatively low molecular weight ($M_n = 13.6$ kg/mol) and consequently high polymer concentration (39% wt/vol)²⁴ compared with polymer concentrations of 9.2 and 5 wt% in this study.

Although non-functional PEAs have been electrospun in previous studies,³,²⁵ to date, there are no reported studies of a successfully electrospun functional PEA. The 8-Phe-4-Asp(OH)-4 PEA was selected for electrospinning due to its facilitation of HCASMC attachment, spreading and proliferation compared to the functional PEAs containing lysine. Uniform, bead-free fibers with an average fiber diameter of 130 ± 5 nm were obtained. The smaller average fiber diameter is attributed to the increased charge density from the pendant carboxylic acid groups, increasing the whipping instability of the jet, reducing the fiber diameter.²³,²⁶ The increase in whipping instability also enabled uniform fibers to be generated from lower polymer concentrations further reducing the average fiber diameter.²⁷

The generation of nano-scale fibrous mats enabled the interaction between HCASMCs and the PEAs to be studied in a 3D context. A time-course analysis revealed that the cells attached and spread well on the surface of the 8-Phe-4 electrospun mats as early as 4 hours. By 24 hours, the cells began to cover the electrospun mat with their own ECM and at 4 days, fibers could no longer be detected. In the 8-Phe-4-Asp(OH)-4 case, HCASMCs attached and spread; however, fibers were not detectable. The loss of the fibrillar structure was attributed to a plasticizing effect due to the increased hydrophilicity of the aspartic acid monomers, potentially due to the hydration of the pendant carboxylic acid groups.

Although the fusion of the 8-Phe-4-Asp(OH)-4 fibers precluded the investigation of cell infiltration into these scaffolds, the porous structure of the 8-Phe-4 electrospun mats was retained allowing cell penetration to be studied using LSCM. The fluorescence contributed by the material illustrated that the HCASMCs were in fact sitting on the surface, and did not infiltrate the porous electrospun mat, confirming the SEM data. The inability to infiltrate porous scaffolds has been previously observed with static culturing
techniques. Although the cells did not migrate into the electrospun mat, the porosity of the scaffold would still allow nutrients or signaling molecules to be delivered three-dimensionally.\textsuperscript{28}

The 3D delivery of signaling molecules was assessed using Western blot analysis, where TGF-\(\beta\)1 was added exogenously to 8-Phe-4 electrospun mats and compared with 2D controls to assess the growth factor’s impact on the regulation of the SMC phenotype. Although a modest increase in the expression of both smooth muscle \(\alpha\)-actin and calponin was observed on all 2D films at 4 days, the results were not statistically significant. The architecture alone suggested an effect on SMC phenotype with an increasing trend in both SM\(\alpha\)A and calponin expression in the 3D scaffolds compared to 2D films at 4 and 7 days, reinforcing the concept that the cellular microenvironment can modulate the SMC contractile phenotype.

To date, most studies demonstrating the beneficial effects of TGF-\(\beta\)1 have used an exogenous addition of TGF-\(\beta\)1, but improved elastin synthesis has been demonstrated with controlled delivery of TGF-\(\beta\)1 from a fibrin gel.\textsuperscript{29} For sustained delivery as part of an \textit{in vitro} matured vascular graft construct, the cytokine must be immobilized to the surface or embedded within the scaffold. The structure of the growth factor revealed a degree in flexibility in conjugating to TGF-\(\beta\)1, thus the free carboxylic acid containing PEA was selected due to its ease in activation using a simple EDC/sulfo-NHS conjugation strategy. Subsequently, the activated intermediate was reacted with a deprotonated amine group of the TGF-\(\beta\)1, affixing the growth factor to the surface of the functional PEA, as determined by X-ray photoelectron spectroscopy and immunofluorescence. The XPS data showed an increase in both N 1s and S 2p binding energies following covalent attachment of the TGF-\(\beta\)1, attributed to the peptide linkages and cysteine residues, respectively. The immunofluorescence studies revealed increased fluorescence following the conjugation of TGF-\(\beta\)1 to the surface of 8-Phe-4-Asp(OH)-4 suggesting the successful grafting of the growth factor to the functional PEA.
6.2 Strengths and Limitations

Firstly, this study demonstrated the versatility in the synthetic approach, where substituting monomers could achieve polymers with differing properties without having to adopt new synthetic routes. The base monomers were generated through a simple acid-catalyzed condensation reaction,\(^5\) while the functional monomers were achieved through a dicyclohexylcarbodiimide coupling reaction of orthogonally protected amino acids.\(^7\) In all cases, polymerization could be achieved through a solution or interfacial polycondensation of the selected monomers followed by cleavage of the pendant protecting group.\(^30\)

Another strength of this work lies in the positive interaction of these PEAs with human coronary artery smooth muscle cells. To best evaluate the real interaction between the material and cells, HCASMCs were seeded directly on the surfaces of all PEAs. Surfaces were not pre-treated nor coated with any cell adhesive proteins or peptides. Vinculin immunostaining of the HCASMCs showed that cells expressed vinculin at the periphery of the cell implying integrin-mediated signaling with all 2D PEA surfaces\(^30\) and that it was independent of serum.\(^31\) Although fluorescence microscopy may have demonstrated that the HCASMCs did not spread as well on the lysine-containing functional PEAs, compared to the base\(^30\) and functional PEAs containing aspartic acid, this was attributed to the trifluoroacetate anion,\(^14\) which appeared to inhibit cell attachment. Despite the reduction in cell adhesion, metabolic activity increased throughout the culture period, suggesting all PEAs served as a favourable surface for HCASMC proliferation. The selection of tissue-specific human vascular cells may also serve to better model the interactions between the cells and PEAs,\(^32\) as demonstrated by the poor cell spreading and muted metabolic activity of bovine aortic endothelial cells on a functional PEA with pendant carboxylic acid moieties.\(^15\)

Given that the nano-scale fibrillar arrangement of extracellular matrix proteins can dictate cell function,\(^28\) the PEAs were electrospun to achieve a non-woven fibrous mat. Although PEAs have also been electrospun from alanine\(^25\) and phenylalanine,\(^24\) smaller average fiber diameters were achieved in this study, in part, due to the higher molecular weight, and subsequent reduction in the minimum concentration needed for electrospinning. In
fact, over a two-fold decrease in average fiber diameter was achieved with 8-Phe-4 when compared to the previously reported study.\textsuperscript{24} In addition, a functional PEA was electrospun for the first time, where the surface charge of the pendant carboxylic acid group increased the whipping instability of the jet resulting in a further two-fold decrease in average fiber diameter.\textsuperscript{26}

In addition to the promising interactions with HCASMCs, ultrathin films (11–21 nm in thickness) of the PEAs could also be prepared through Langmuir-Blodgett technology, despite the long polymeric chains, which can undergo significant hydrogen bonding. The generation of ultrathin films enabled the examination of the interfacial region between the cell surface and the PEA film using waveguide evanescent field fluorescence (WEFF) microscopy, a relatively new technique to investigate the contact region between the cells and their substratum.\textsuperscript{31}

Although positive interactions between the PEAs and HCASMCs were reported, much of the work to date has focused on 2D films; however, cells are known to behave differently in a 3D context versus 2D culture.\textsuperscript{13} The initial cell culture work with 8-Phe-4 electrospun mats has limited cell infiltration either due to impenetrable pore sizes or static seeding or a combination thereof. The 8-Phe-4-Asp(OH)-4 could be electrospun, but upon exposure to culture conditions, fiber fusion occurred, resulting in loss of the 3D network. Despite the lack of cell infiltration, there did appear to be an impact on HCASMC behaviour when cultured on 3D scaffolds, as evidenced by the increasing expression of contractile phenotype marker proteins SM\(\alpha\)A and calponin compared to 2D films. This data did not reach statistical significance, and may be the result of a number of contributing factors. Primary cell culture is known to induce significant variability as a result of harvesting cells from multiple donors. Moreover, the exogenous addition of TGF-\(\beta\)1 did not result in statistically significant increases in contractile phenotype marker protein expression either, suggesting that carrier-free TGF-\(\beta\)1 may have been delivered at a sub-optimal concentration or that the fetal bovine serum of the media contained TGF-\(\beta\)1, limiting the efficacy of the additional exogenous TGF-\(\beta\)1.
Although the selection of HCASMCs may serve to better model the interactions of the cells with the PEAs for \textit{in vitro} vascular tissue engineering, their low proliferation rate and difficulty to harvest may preclude them from being used as part of a vascular construct strategy.\textsuperscript{33} The ability of stem cells to self-renew and proliferate rapidly could facilitate the maturation of an engineered vascular tissue \textit{in vitro}, provided their differentiation can be controlled. In addition, harvesting adult stem cells from bone marrow, adipose tissue, skeletal muscle or more recently through the reprogramming of somatic cells to induced pluripotent stem cells would also provide a source of readily available autologous cells needed for \textit{in vitro} vascular tissue engineering.\textsuperscript{33-34}

\subsection*{6.3 Future Directions}

Future work should focus on the interactions of HCASMCs on 3D scaffolds, to better represent the \textit{in vivo} microenvironment. Improved cell infiltration into the porous scaffolds through dynamic seeding techniques such as vacuum or rotational seeding should be pursued.\textsuperscript{35-36} In conjunction with dynamic seeding, increased pore size through the use of sacrificial electrospun fibers,\textsuperscript{37} ice crystals,\textsuperscript{38} leachable porogens (salts, sugars or polymers)\textsuperscript{39} or other techniques\textsuperscript{40} should be considered.

The plasticizing effect of the aspartic acid in the 8-Phe-4-Asp(OH)-4 fibers may suggest that a more hydrophobic polymer would be more suitable. In this case, the incorporation of aspartic acid into 8-Phe-8 may limit this plasticizing effect and should be explored. Alternatively, the non-functional 8-Phe-4 resisted fiber fusion, suggesting that a lower percentage of aspartic acid in this PEA may also limit the plasticizing effect. The reduction in functional groups though, may limit the ability to conjugate signaling molecules such as TGF-\(\beta\)1.

The retention of the 3D network of a cytokine-conjugated scaffold would enable the impact of soluble versus conjugated TGF-\(\beta\)1 on both contractile phenotype marker protein expression and elastogenesis. To understand the full extent of these parameters, a long-term culture in a bioreactor would be necessary.
Although this study has not addressed the mechanical properties of these PEAs, preliminary uniaxial tensile testing has been conducted with non-functional PEAs. The Young’s modulus, yield strength, ultimate tensile strength and strain at break have all been investigated at 23°C for 8-Ala-8, 8-Phe-4 and 8-Phe-8 (data to be published shortly). To supplement the current study, mechanical properties of the functional PEAs should also be conducted, but the mechanical testing should be conducted at 37°C to better represent physiologic temperature. It is anticipated that the critical strain at the yield point of PEAs is expected to remain unchanged; however, both the Young’s modulus and yield strength are expected to decrease at 37°C. Given that the glass transition temperature impacts the physical properties of the polymers and that hydrated samples reduce the T_g, a further reduction in the Young’s modulus and ultimate tensile strength can be expected. With the ultimate goal of an in vitro tissue-engineered vascular graft, the mechanical properties of the matured vascular construct would also need to be addressed, with burst strength, suture retention and compliance all needing to be further evaluated.

6.4 Significance

This study serves as the foundation for using functional poly(ester amide)s for vascular tissue engineering applications, whether it be as in vitro tissue-engineered vascular grafts, coating for stents or therapeutic angiogenesis, but does not preclude it from being investigated as controlled drug delivery vehicles through its incorporation into micelles or for stimuli-triggered degradation. The facile and versatile approach to synthesizing these functional degradable polymers may yet lead to other biomedical applications where significant control of the polymer’s physicochemical, thermal and mechanical properties are of utmost importance.

6.5 References


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